Aberrant Epidermal Growth Factor Receptor Signaling and Enhanced Sensitivity to EGFR Inhibitors in Lung Cancer

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Abstract

Epidermal growth factor receptor (EGFR) is occasionally amplified and/or mutated in non–small cell lung cancer (NSCLC) and can be coexpressed with other members of the HER receptor family to form functional heterodimers. We therefore investigated lung cancer cell lines for alterations in EGFR gene copy number, enhanced expression of EGFR and other HER family members, and EGFR coding sequence mutations and correlated these findings with response to treatment with the EGFR inhibitors and the kinetics of ligand-induced signaling. We show here that somatic deletions in the tyrosine kinase domain of EGFR were associated with increased EGFR gene copy number in NSCLC. Treatment with the specific EGFR tyrosine kinase inhibitors (TKI) gefitinib or erlotinib or the EGFR inhibitory antibody cetuximab induced apoptosis of HCC827, a NSCLC cell line with EGFR gene amplification and an exon 19 deletion. H1819, a NSCLC cell line that expresses high levels of EGFR, ErbB2, and ErbB3 but has wild-type EGFR, showed intermediate sensitivity to TKIs. In both cell lines, ligand-induced receptor tyrosine phosphorylation was delayed and prolonged and AKT was constitutively activated. Thus, in addition to EGFR mutations, other factors in NSCLC cells, such as high expression of ErbB family members, may constitutively activate AKT and sensitize cells to EGFR inhibitors. (Cancer Res 2005; 65(1): 226-35)

Introduction

Epidermal growth factor (EGF) receptor (EGFR) expression is readily detectable in ~50% of non–small cell lung cancers (NSCLC; refs. 1, 2), and drugs that inhibit this receptor are under investigation in patients with NSCLC (3). Gefitinib, a p.o. available tyrosine kinase inhibitor (TKI) selective for this receptor, was recently approved for use as second-line therapy for advanced NSCLC based on an objective response rate of ~10% in nonrandomized trials (4, 5). This response rate is less than that of first-line cytotoxic chemotherapy for NSCLC and is similar to that of other second-line chemotherapies. A striking difference between gefitinib and chemotherapy, however, is that the likelihood of response to gefitinib is approximately the same regardless of the number of previous chemoradiotherapy regimens; in contrast, response rates to chemotherapy decrease substantially with each chemotherapy regimen used. In addition, some of the observed responses to gefitinib are dramatic and sustained even in tumors that are completely refractory to cytotoxic chemotherapy, suggesting a unique molecular mechanism of response to gefitinib that is unrelated to mechanisms of response to cytotoxic chemotherapy and still not well understood.

The EGFR gene is amplified and overexpressed in a variety of epithelial cancers, including NSCLC (6). More commonly, EGFR protein levels are increased in NSCLC cells through enhanced gene transcription (1). In breast cancer, intratumoral levels of ErbB2 before treatment predict response to treatment with the trastuzumab (Herceptin; ref. 7). In these tumors, high levels of ErbB2 correlate with the presence of ErbB2 gene amplification (7). Although initial immunohistochemical studies indicated that pretreatment levels of intratumoral EGFR do not predict response to gefitinib treatment in NSCLC, there is a close correlation of gefitinib response with the presence of coding sequence alterations in the tyrosine kinase domain of EGFR (8, 9). However, data from these studies and others have identified patients whose tumors did not show EGFR mutations who nonetheless benefited from gefitinib, suggesting that additional genetic or biochemical factors contribute to gefitinib response in NSCLC (10).

Given the evidence that EGFR is amplified and mutated in NSCLC, we investigated whether alterations in copy number or coding sequence correlated with gefitinib response in patients with NSCLC. We identified an amplified EGFR gene locus and an in-frame deletion in the EGFR kinase domain in the tumors of three patients who had experienced dramatic responses to treatment with gefitinib. In examining NSCLC cell lines, we found that the presence of EGFR coding sequence alterations was associated with increased gene copy number, and both EGFR gene mutations and high expression of ErbB family members were associated with altered receptor biochemical properties and enhanced sensitivity of NSCLC cells to EGFR TKIs. Particularly striking in gefitinib-sensitive cells was AKT phosphorylation that was independent of exogenous EGF but inhibited by gefitinib, which was associated with prominent apoptotic cell death. Thus, beyond the clear contribution of EGFR gene mutations, high expression of ErbB family members, through gene amplification or other mechanisms, with dysregulation of AKT signaling may also contribute to gefitinib sensitivity in NSCLC patients.

Note: J. Amann and S. Kalyankrishna contributed equally to this work.

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Materials and Methods

Reagents. cDNA was purchased from the pharmacy at Vanderbilt-Ingram Cancer Center (Nashville, TN). Geltinin and erlotinib were gifts from AstraZeneca (Wilmington, DE) and OSI Pharmaceuticals (Melville, NY), respectively. Rabbit polyclonal antibodies against pY1068-EGFR, pT202/Y204-ERK, pS473-akt, total AKT, pY239/Y240-Shc, and total Shc and extracellular signal-regulated kinase (ERK) were purchased from Cell Signaling Technology (Beverly, MA); total EGFR, ErbB2, ErbB3, and ErK1 were from Santa Cruz Biotechnology (Santa Cruz, CA); and pY1148-EGFR and pY845-EGFR were from Biosource International (Camarillo, CA). The anti-EGFR antibody used for immunoprecipitations and Western blotting for total EGFR was a mouse monoclonal antibody obtained from BD Transduction Laboratories (Lexington, KY).

Nomenclature. Two systems for numbering the amino acids of the EGFR were developed. One method numbers each of the amino acids from the N-terminus from which the protein is translated yielding a protein of 1,211 amino acids long. The more familiar method used by those who study functional EGFR consider the mature and functional protein to be L186 amino acids after processing. All of the antibodies that have been developed for the tyrosine-phosphorylated residues in the cytoplasmic tail use this nomenclature. In attempt to be clear and unambiguous and avoid renumbering all of the known autophosphorylation and Src phosphorylation sites, we have chosen to use the nomenclature for the mature and processed protein.

Cloning of the Mutant EGFR. The mutant EGFR, EGFR Δ722-726, was made by PCR using site-directed mutagenesis by overlap extension (11). Primers used to generate the PCR products were 5’-AATTTCTGAGATCCGACCTCCGAGACC-3’, 5’-AATGAAAGTCCTGCTCAAATATT-3’, 5’-GCTCTACAATTGCCAGTTA-3’, 5’-GTTAAATTGCTGCATATACAAAATCTCAGAGAAAGGG-3’, and 5’-CTCCTTGCTGCTGAGATTTGTATGACCGGAGATTGTAAC-3’. For the first round of PCR, wild-type EGFR in pcDNA3.1 (template) was amplified as follows: 95°C (5 minutes) and 30 cycles of 95°C (1 minute), 55°C (1 minute), and 72°C (1 minute) (followed by extension at 72°C (10 minutes). For the second round, the overlapping products from the first round of PCR were amplified as follows: 95°C (5 minutes) and 30 cycles of 95°C (1 minute), 55°C (1 minute), and 72°C (4 minutes) followed by extension at 72°C (10 minutes). The resulting PCR product containing the mutation was digested with BstXI to release a fragment containing the mutation and subcloned into a BstXI-digested pcDNA3.1/wild-type EGFR construct. Resulting clones were sequenced to verify the presence of the mutation and that no PCR generated mutations were introduced.

DNA Isolation and PCR. DNA was isolated from paraffin-embedded tissues according to the protocol of the Puregene DNA purification kit for such tissues (Genta Systems, Plymouth, MN). Briefly, paraffin was removed by xylene washes and the tissue was dehydrated with ethanol. Cell lysis buffer was added and cells were mechanically disrupted with a microcentrifuge tube pestle. The resulting lysate was treated with proteinase K overnight at 55°C followed by RNase digestion. Protein was precipitated and removed by centrifugation. DNA was precipitated with isopropanol in the presence of glycogen, washed in 70% ethanol, air dried, and resuspended in DNA hydration solution. DNA was isolated from cell pellets of 106 cells by adding 200 μL of a solution containing 4 μg/mL sodium pyruvate, 2 mmol/L glutamine, penicillin (100 units/mL), and streptomycin (100 μg/mL). The 293T cells were grown in DMEM supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, penicillin (100 units/mL), and streptomycin (100 μg/mL) and were grown at 37°C in a 5% CO2 atmosphere of 5% CO2. Western Blot Analysis. For ligand stimulation, NSCLC cells were serum starved overnight. After treatment with EGF for the appropriate periods of time, cells were washed twice with ice-cold PBS, lysed by sonication in radioimmunoprecipitation assay buffer, and stored at −80°C. Protein concentrations were estimated with a Protein Assay kit (Bio-Rad, Hercules, CA), and equal amounts of protein were subjected to SDS-PAGE followed by Western blot analysis with the indicated antibodies and detected by electrochemiluminescence (Amersham Biosciences, Piscataway, NJ). CHO cells were seeded on 10-cm culture dishes the day before transfection at a density of 2 × 104 cells per plate. Cells were transfected with FuGene 6 (Roche Diagnostics, Indianapolis, IN) according to the manufacturer’s instructions with 10 μg DNA and 30 μL FuGene 6 per plate. Medium was replaced 24 hours after transfection followed by serum starvation for 40 to 48 hours after transfection. Cells were starved for 20 to 40 minutes and after transfection.
24 hours before stimulation with 100 ng/mL recombinant human EGF (R&D Systems, Minneapolis, MN) for various periods. Cells were then washed, lysed, sonicated on ice, and centrifuged at 14,000 rpm at 4°C to clarify. Protein was quantified with a DC Protein Assay kit (Bio-Rad) in which 150 ng of lysozyme, boiled in the presence of SDS-PAGE sample buffer, were run on a Criterion gel (Bio-Rad) followed by transfer onto nitrocellulose. Immunoprecipitations were carried out on 1.5 mg protein lysozyme by adding 5 μg EGFR monoclonal antibody per lysate and incubating at 4°C, with rocking, for 4 to 6 hours. Protein G-Sepharose (Sigma, St. Louis, MO) and 30 μL of a 50% slurry, blocked with bovine serum albumin, were added to the lysate and incubated for 1 hour at 4°C, with rocking. Beads containing immune complexes were washed thrice with lysis buffer followed by addition of SDS-PAGE sample buffer and boiling for 4 minutes. After separation by SDS-PAGE, proteins were transferred to nitrocellulose, subjected to Western blotting, and visualized by chemiluminescence (Pierce Biotechnology, Rockford, IL).

For 293T cell transfections, plates were plated on 10-cm culture plates at a density of 2.4 × 10^6 the day before transfection. Cells were transfected with Polyfect (Qiagen) according to the manufacturer’s instructions for 293T cells. Serum starvation was initiated 24 hours before induction and continued for 36 to 42 hours. The appropriate concentrations of gefitinib or erlotinib were added to the culture medium 2 hours before induction with 100 ng/mL EGF for 15 minutes. Cetuximab was added 3 hours before induction. Harvests and transfers were done as described above, except that 100 μg of protein were loaded onto the SDS-PAGE gels.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assays. Cells were seeded at a density of 1,000 to 5,000 cells per well in 96-well plates and allowed to attach for 24 hours. Cells were then treated with EGFR inhibitors in the presence of 10% serum for 6 days, at the end of which 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (0.05 mg in PBS) was added to each well. After an incubation of 4 hours, absorbance was read at 570 nm/mL. Values for control cells were considered as 100% viability.

Hoechst 33342 Staining. Control and treated cells were washed gently with PBS, fixed with 4% formaldehyde in PBS at room temperature, washed again with PBS, and incubated with bisbenzimide (Hoechst 33342, 10 μg/mL, Sigma) in PBS for 15 minutes at room temperature. Nuclei were analyzed for chromatin condensation using a fluorescence microscope with an UV filter at a magnification of ×20.

Cell Cycle Analysis. Subconfluent cells in 100-mm dishes were treated with EGFR inhibitors and the supernatants were collected and centrifuged. Adherent cells were trypsinized and pooled with cells from the supernatant. Cell pellets were washed with PBS containing bovine serum albumin, fixed using 70% methanol, and stored at −20°C. The fixed cells were subsequently washed twice with PBS and stained with propidium iodide and RNase A (Phoenix Flow Systems, San Diego, CA), and DNA content was analyzed by flow cytometry. Analyses of 3,000 to 10,000 events were done with a FACScan flow cytometer (Becton Dickinson, San Jose, CA) equipped with a 488-nm argon ion laser and two software packages: CellQuest 3.1 (Becton Dickinson) and ModFit LT 2.0 (Verity Software House, Topsham, ME). Live gating of the forward and orthogonal scatter channels was used to exclude debris and to selectively acquire cell events. DNA area was used to determine the percentage of propidium iodine–stained cells in the sub-G1 fraction (i.e., apoptotic cells).

Results

Cloning of an EGFR Gene Mutation from NSCLC. Tumor cells were isolated from a male, never-smoking patient with bilateral adenocarcinoma. This patient had experienced disease progression through four chemotherapeutic regimens before being given gefitinib as single-agent therapy, which produced rapid and nearly complete tumor shrinkage and remarkable improvement in symptoms. After 18 months, while still taking gefitinib, the patient experienced tumor progression and subsequently died. Sequencing of the kinase domain of the EGFR gene in DNA and RNA isolated from tissue samples taken after treatment as well as from a pre-gefitinib biopsy sample revealed a mutation in exon 19 in both tissue samples (Fig. 1A). The mutation consisted of a 15-bp in-frame deletion corresponding to nucleotides 2,235 to 2,249 and resulted in a 5–amino acid deletion [amino acids 722-726 (Δ722-726 mutant)] in the mature protein. DNA was isolated from pretreatment tumor samples of two additional female patients with NSCLC who experienced objective tumor response to gefitinib treatment. One of these patients (patient 2 in Fig. 1A) was a never smoker who also presented with bronchioalveolar carcinoma and had undergone five rounds of chemotherapy before treatment with gefitinib. Patient 3, an ex-smoker (>10 years), was diagnosed with large cell carcinoma of the lung and had received one round of chemotherapy before being treated with gefitinib. These patients relapsed at 8 and 18 months, respectively, while still on gefitinib therapy. Sequence analysis of the EGFR kinase domains in the pretreatment samples revealed similar 15-bp in-frame deletions (Fig. 1A).

We examined EGFR gene copy number in the lung tumors of the three patients by FISH analysis using probes for EGFR and, as a control, the centromere of chromosome 7. The EGFR probe detected 6.4, 5.0, and 9.1 copies per cell in tumors from the three patients, and the centromeric probe detected 3.5, 3.8, and 6.8 copies per cell, respectively. Thus, the tumors with EGFR mutations also had increased EGFR gene copy number, and EGFR copy number was increased through a mechanism involving both intrachromosomal gene amplification and chromosomal aneuploidy.

Effects of Ligand on Phosphorylation of EGFR and Downstream Mediators. After binding its ligand, EGFR homodimerizes or heterodimerizes with other ErbB family members, undergoes tyrosine autophosphorylation, and initiates signaling directly through the recruitment of adaptor proteins and intracellular kinases (16). To investigate the effects of the mutation on EGFR function, we cloned and transfected wild-type and mutant receptors into CHO cells, which express low levels of ErbB. Examination of ligand-induced EGFR tyrosine phosphorylation using an anti-phosphotyrosine antibody recognizing all phosphorylated tyrosines revealed that phosphorylation of the mutant receptor was severely impaired when equal amounts of wild-type and mutant protein were compared (Fig. 1B). We also examined the kinetics of ligand-induced EGFR phosphorylation on Tyr^1068 and Tyr^1180, which are autophosphorylated, and Tyr^845, which is phosphorylated by Src (Fig. 2A). The wild-type receptor was phosphorylated rapidly at these sites, peaking at 10 minutes; by comparison, basal phosphorylation of the mutant receptor was lower at Tyr^845 and Tyr^1180, whereas phosphorylation at Tyr^1180 was approximately equal to wild-type receptor. Ligand-induced phosphorylation of the mutant was delayed at all three sites (peaking at ~2 hours).

We next investigated the kinetics with which ligand-induced EGFR activation induced phosphorylation of downstream mediators (Fig. 2B). With the wild-type receptor, Shc phosphorylation peaked between 10 and 30 minutes and declined to barely detectable levels by 6 hours. With the mutant receptor, Shc phosphorylation persisted for longer periods and was still very strong 6 hours post-EGF treatment. In the mutant transfectants, total Shc levels increased with time, which may have contributed to the persistence of Shc phosphorylation. With wild-type EGFR, ERK phosphorylation peaked at 10 minutes and then declined, whereas phospho-ERK levels persisted for longer periods after activation of the mutant EGFR.
Effects of EGFR Inhibitors on Phosphorylation of EGFR and Downstream Mediators. The presence of mutations in most lung tumors that respond to gefitinib suggests that the mutation confers either a unique dependence of the tumor on the EGFR or an increased sensitivity to inhibition by TKIs. To test the sensitivity of the mutant receptor to TKIs, we expressed the wild-type and mutant receptors in 293T cells, which express low levels of other ErbB family members. The transfectants were then treated with TKIs (gefitinib or erlotinib), pulsed with EGF, and subjected to Western analysis. In the absence of EGF, basal phosphorylation at Tyr1148, Tyr1068, and Tyr845 was lower in the mutant receptor than in the wild-type receptor (Fig. 3A), which is consistent with differences in total EGFR phosphorylation (Fig. 1B). After adjusting exposure times to account for differences in basal phosphorylation, IC50 values for gefitinib and erlotinib were ~5 to 20 nmol/L for the wild-type receptor and 1 to 5 nmol/L for the mutant receptor at all three phosphorylation sites (Fig. 3B and D), supporting a 4- to 5-fold increased sensitivity of the mutant receptor to TKIs. However, concerning ERK and Shc phosphorylation, TKI IC50 values were similar for the wild-type and mutant receptors (Fig. 3C), raising the possibility that other upstream activators maintain the phosphorylation of ERK and Shc in these cells.

EGFR Gene Amplification and Mutation in NSCLC Cell Lines. After finding that EGFR mutation was associated with gene amplification in NSCLC biopsy samples, we investigated whether this association held true in NSCLC cell lines. For this purpose, we screened eight NSCLC cell lines representing the major histologic subtypes of NSCLC for the presence of EGFR gene amplification or mutation in exons 19, 20, and 21, the exons most frequently altered in tumors (refs. 8, 9; Table 1). Seven of the cell lines had wild-type EGFR gene coding sequences. The eighth cell line, HCC827, established from a 39-year old Caucasian, female, non-smoker had greatly increased EGFR gene copy numbers (>20 copies by FISH and 36 copies by comparative genomic hybridization arrays) and a mutation in the EGFR coding sequence—a deletion in exon 19 with a resulting amino acid sequence identical to that of our index patient (Fig. 1A). Similarly, FISH analysis of H3255, a cell line mutation in exons 19, 20, and 21, the exons most frequently altered in tumors (refs. 8, 9; Table 1). Seven of the cell lines had wild-type EGFR gene coding sequences. The eighth cell line, HCC827, established from a 39-year old Caucasian, female, non-smoker had greatly increased EGFR gene copy numbers (>20 copies by FISH and 36 copies by comparative genomic hybridization arrays) and a mutation in the EGFR coding sequence—a deletion in exon 19 with a resulting amino acid sequence identical to that of our index patient (Fig. 1A). Similarly, FISH analysis of H3255, a cell line
shown to have a point mutation in exon 21 (9), was also amplified with >20 copies of the \( \text{EGFR} \) locus (data not shown). H1819, Calu-1, and H1299 cell lines had smaller increases in \( \text{EGFR} \) copy number but no evidence of \( \text{EGFR} \) gene mutations in exons 19, 20, or 21 (all 28 exons were sequenced in H1819 cells). Thus, \( \text{EGFR} \) mutation was associated with high levels of \( \text{EGFR} \) gene amplification in NSCLC cell lines.

**ErbB Expression in NSCLC Cell Lines.** We next examined the expression of ErbB family members in these eight NSCLC cell lines (Fig. 4A). \( \text{EGFR} \) protein levels were highest in H1819 and HCC827 cells. ErbB2 expression was highest in H1819 cells, which had \( \text{ErbB2} \) gene amplification (8.0 copies per cell by comparative genomic hybridization array), lower in HCC827 cells (which had no evidence of \( \text{ErbB2} \) gene amplification), and undetectable in the other cell lines. ErbB3 was detected in HCC827, H1819, A549, and H226B cells, which had no evidence of \( \text{ErbB3} \) gene amplification. Thus, NSCLC cell lines with increased \( \text{EGFR} \) gene copy number expressed high levels of \( \text{EGFR} \) and other ErbB family members in part through genetic amplification.

**Delayed and Prolonged Phosphorylation of EGFR in NSCLC Cell Lines.** After finding that \( \text{EGFR} \) mutation conferred altered phosphorylation of receptor tyrosines in the transfected cells, we compared the levels of phosphorylated \( \text{EGFR} \) (Y1068) in NSCLC cell lines (Fig. 4A). Of the eight cell lines examined under growth conditions, phosphorylation of Y1068 was significantly higher in HCC827 and H1819 cells compared with the other six lines. Although total \( \text{EGFR} \) levels were also increased in these cell lines, the increase in Y1068 phosphorylation was proportionally greater.

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**Figure 3.** Phosphorylation of the mutated EGFR is more sensitive to the inhibitors gefitinib and erlotinib, whereas the levels of phosphorylated ERK and Shc are unaffected by increasing dose of inhibitor. Wild-type or \( \Delta \text{722-726} \) EGFR was expressed in 293T cells. Cells were serum starved and treated with increasing doses of gefitinib or erlotinib 2 hours before induction with EGF (100 ng/mL). Total lysate (100 µg) was separated by SDS-PAGE followed by immunoblot with indicated antibodies. A, dose curve for gefitinib showing immunoblot for pY1068. As with expression in CHO cells (Fig. 2A), there is disparity between phosphorylation of the wild-type and mutant receptor after EGF treatment. B, comparison of wild-type and mutant receptor phosphorylation at indicated tyrosines with increasing dose of gefitinib. Exposure times of the blots were equivalent for wild-type and mutant transfected cells, except where noted. C, dose curves for erlotinib showing phosphorylation of wild-type and mutant receptor at the indicated tyrosines.
than the increase in total EGFR. H1819 cells also had the highest levels of ErbB2 and ErbB3, which is notable given the presence of multiple phosphatidylinositol 3'-kinase–binding sites on ErbB3.

We next examined the effects of ligand deprivation and ligand stimulation on receptor phosphorylation in these cells. Multiple phosphorylation sites were examined, including Y1068, Y1045, and Y845, with similar results. Therefore, Y1068 was used to represent ligand-induced phosphorylation activity of the EGFR. In addition, all of the resistant cell lines, which showed similar phosphorylation kinetics, will be represented by H1299 in the following experiments. Serum deprivation reduced phosphorylation at Y1068 to undetectable levels in all resistant cell lines and to low, yet detectable, levels in H1819 and HCC827 cells. (Fig. 4B; data not shown). This indicates that EGFR is constitutively phosphorylated in a moderately sensitive cell line (H1819; IC₅₀ 4.7 μmol/L) and a very sensitive cell line (HCC827; IC₅₀ 0.016 μmol/L). EGFR treatment increased phosphorylation at Y1068 in all eight cell lines (Fig. 4B; data not shown). In resistant cells with wild-type EGFR, represented by phosphorylation at Y1068 in all eight cell lines (Fig. 4A; data not shown). This decreased turnover of receptor may also account for a portion of the higher phosphorylation levels seen in the two sensitive cell lines at later time points.

The phosphorylation of AKT, known to participate in an important survival pathway, was also examined in these cell lines (Fig. 4B). The sensitive cell lines, H1819 and HCC827, showed slight (2-fold maximum) and no induction with ligand, respectively, and were constitutively phosphorylated over the entire time course. The resistant cell line, H1299, on the other hand, was inducible by EGFR stimulation and declined to basal levels by 24 hours. Thus, a protein known to be involved in an antiapoptotic pathway is constitutively active in cell lines that show either moderate or high sensitivity to gefitinib.

### Sensitivity of NSCLC Cell Lines to EGFR Inhibitors.

Given the findings presented here and elsewhere that gefitinib is effective against NSCLC tumors that have *EGFR* gene mutations (8, 9), we investigated whether the antitumor effect of this drug is related to the presence of *EGFR* gene mutation, expression levels of ErbB family members, or both. We also examined the efficacy of erlotinib and cetuximab, an inhibitory antibody against the EGFR ligand-binding domain. We used MTT assays to examine the effect of these agents on the proliferation of NSCLC cells (Fig. 5A; Table 1). Most cell lines with wild-type *EGFR* were relatively resistant to gefitinib, with IC₅₀ values of 17.2 to 41.0 μmol/L. However, H1819 cells were moderately sensitive (IC₅₀ 4.7 μmol/L) and HCC827 cells were highly sensitive (IC₅₀ 10 nmol/L; Table 1). Like gefitinib, erlotinib inhibited the growth of H1819 and HCC827 cells (IC₅₀ 5.0 and 0.010 μmol/L, respectively) but not that of H1299 cells (IC₅₀ 50.0 μmol/L). Interestingly, cetuximab potently inhibited the growth of HCC827 cells (IC₅₀ 7.0 nmol/L) but not H1819 cells (IC₅₀ >1.0 μmol/L) or H1299 cells (IC₅₀ >1.0 μmol/L; Fig. 5A). Thus, HCC827 cells were uniquely sensitive to agents that target either the EGFR kinase domain or the extracellular domain. Further, the modest sensitivity of H1819 cells to TKIs supports the hypothesis that factors other than *EGFR* gene mutations contribute to the antitumor efficacy of these agents in additional subsets of NSCLC.

We examined HCC827 cells for evidence of apoptosis after treating with EGFR inhibitors (Fig. 5B). Flow cytometry of HCC827 cells treated with gefitinib, erlotinib, or cetuximab showed a hypodiploid peak, representing an increase in the sub-G₁ population. Western analysis revealed reductions in the levels of procaspase-3 and caspase-3 in HCC827 cells treated with gefitinib, erlotinib, or cetuximab.
poly(ADP-ribose)polymerase (Fig. 5B), providing evidence of caspase activation and proteolysis of a caspase-3 substrate. Further, staining of gefitinib-treated HCC827 cells with Hoechst 33342 showed nuclear fragmentation and membrane blebbing (Fig. 5C). Together, these findings indicate that HCC827 cells undergo dramatic apoptosis after treatment with EGFR inhibitors.

Finally, we investigated changes in EGFR-dependent signaling following treatment with EGF and EGFR inhibitors. Under serum-starved conditions, AKT is constitutively phosphorylated at high levels and EGF treatment does not further increase phosphorylation in the sensitive cell lines, but a high level of induction was observed in all examined resistant cell lines (Figs. 4B and 6A-C; data not shown). Treatment with gefitinib (Fig. 6A), erlotinib (Fig. 6B), or cetuximab (Fig. 6C) inhibited basal and EGF-induced phosphorylation of ERK and AKT in HCC827 cells. EGFR is inhibited by cetuximab in H1819, but phospho-AKT and phospho-ERK are not (Fig. 6C), correlating with its failure to respond to this agent. Cetuximab treatment decreased EGFR phosphorylation less prominently in HCC827 cells than in H1819 and H1299 cells. Among these cell lines, HCC827 was the most sensitive with respect to TKI-mediated phosphorylation inhibition of ERK and AKT. Thus, the IC50 of the TKIs and cetuximab correlated more closely with the inhibition of ERK and AKT phosphorylation in the three cell types than with receptor autophosphorylation. Interestingly, H1819 and H1299 cells,
the TKI IC₅₀ values for which differed by a factor of 10, showed similar sensitivities to the TKI-induced inhibition of EGFR phosphorylation, indicating that EGFR phosphorylation was uncoupled from the regulation of cell growth and viability in H1299 cells.

DISCUSSION

We identified deletions in the EGFR tyrosine kinase domain from three of three tumors from patients with NSCLC. The EGFR genetic alterations, tumor histologies, and smoking histories of these patients were similar to previous reports of patients with NSCLC that responded to gefitinib treatment, with the exception that one patient was diagnosed with large cell carcinoma (8, 9). We also identified EGFR mutations in HCC827 cells and two other NSCLC cell lines not reported previously,7 involving in-frame deletions and point mutations similar to the spectrum of changes in NSCLCs with EGFR mutations described previously (8, 9). To date, these EGFR gene mutations have been found less often in NSCLC in people who were smoking at diagnosis, suggesting that the genetic basis of lung tumorigenesis in such individuals is distinct from that

![Figure 6](https://example.com/figure6.png)

Figure 6. Inhibition of EGF-induced phosphorylation of EGFR, ERK, and AKT by gefitinib, erlotinib, and cetuximab. HCC827, H1819, or H1299 cells were pretreated with (A) gefitinib for 2 hours, (B) erlotinib for 3 hours, or (C) cetuximab (Cetux) for 3 hours and treated with EGF for 60 minutes (for HCC827 and H1819) and 5 minutes (for H1299; to coincide with the peak timing of EGFR activation in these cells). Lysates were probed for phosphorylated and total proteins by Western blot analysis. Western analysis for actin levels revealed equal loading of the wells (data not shown).
of people who either had never smoked or had quit smoking. The frequency of NSCLC among former smokers has increased over the past decade, with former smokers now accounting for >50% of all NSCLC cases. Thus, elucidating the molecular basis of lung cancer in this subgroup has tremendous public health implications.

We found that EGFR mutations were associated with an increased number of EGFR gene copies (three of three tumors and four of four cell lines), raising the possibility that the process of genetic instability that caused EGFR gene amplification also facilitated the development of coding sequence mutations or amplified their effect. In the four cell lines containing mutations, including HCC827 and H3255, reported previously to have the L858R point mutation, FISH analysis indicated that the EGFR locus was highly amplified by intrachromosomal duplication and contained anywhere from 12 to >20 EGFR loci (Table 1; data not shown; ref. 9). Seven cell lines that contained wild-type EGFR were also examined by FISH, and although many were aneuploid, none contained >5 EGFR loci on average (Table 1) and, more importantly, none showed evidence of intrachromosomal duplication. These data combined with the patient data showing amplification of the EGFR locus increase the likelihood that EGFR mutational events may depend on the specific process driving genetic instability in NSCLC.

The mutant receptor also displayed altered biochemical properties. Our transfection studies in CHO and 293T cells revealed that the overall phosphorylation level of the mutant receptor was lower than that of the wild-type receptor. This is in agreement with the studies of Pao et al., who also used 293T cells for transfections, but differ from the other two studies where Cos7 and mouse mammary epithelial cells were used (8, 17, 18). Monitoring the levels of phosphorylation of specific tyrosines in the mutant transfected cells revealed large differences between mutant and wild-type receptor. For example, in CHO cells, the phosphorylation of Y1148 was at or near wild-type levels, whereas Y1068 and Y845 were much reduced when compared with wild-type (Fig. 2A). Sordella et al. also noted differences in tyrosine phosphorylation; however, the specific tyrosine phosphorylation in the mutant stable transfectants was usually higher. This is most likely due to higher levels of wild-type EGFR or other ErbB family members. Basal tyrosine phosphorylation in HCC827 (this study) and H3255 (9) NSCLC cells was also increased relative to that of cells with wild-type receptor. This discrepancy between transfected CHO and 293T cells and NSCLC cell lines containing a mutation is most likely a consequence of the low levels of endogenous ErbB present in CHO and 293T cells, which limit the dimeric partners with which the transfected EGFR could interact. HCC827 cells and H1819 cells expressed high levels of ErbB2 and ErbB3, which can dimerize with EGFR and potentially enhance its phosphorylation. In fact, overexpression of ErbB2 has been shown to promote constitutive phosphorylation of EGFR and to delay and prolong the phosphorylation of the receptor (19). Importantly, the use of the C225 mouse monoclonal antibody was unable to eliminate constitutive phosphorylation of the EGFR, implicating overexpression of ErbB2, and not EGF binding, as the most likely reason for the enhanced signaling through the EGFR. These results recapitulate exactly what is seen in the H1819 cell line including the inability of cetuximab to eliminate constitutive phosphorylation of the EGFR. In that study (19), it was determined that the reason for the prolonged signaling was due to altered trafficking and thus decreased down-regulation of the receptor. It will be interesting to see if in future studies the mutations associated with the enhanced sensitivity to gefitinib and erlotinib also affect trafficking of the EGFR.

Additional factors may contribute to the changes in the kinetics of phosphorylation, including interaction of the receptor with phosphatases. Previous studies, using time courses of 30 to 180 minutes after ligand stimulation, have shown peak phosphorylation of mutant EGFR by 2 to 3 hours in transfected cells without diminution of signal (8, 17, 18). This suggests that interaction of the mutated receptor with phosphatases may be altered. We have monitored phosphorylation of the receptor for up to 24 hours after ligand stimulation and noted a peak of 2 to 3 hours followed by decreased phosphorylation at 4 to 6 hours post-stimulation, indicating the mutated receptor can still interact with and be dephosphorylated by phosphatases. Importantly, the same kinetics is seen in the H1819 cells, which do not have a mutated receptor. This result argues that mutation is not altering the association of the receptor with phosphatases and that some other mechanism of receptor down-regulation is being affected.

Downstream targets of the mutated receptors have also been examined in one study using a 30-minute time course post-ligand stimulation (17). It was determined that ERK1/2 phosphorylation in stably transfected cells was no different whether cells contained mutant or wild-type receptor. In our studies, we also do not see a change in the phosphorylation pattern of ERK1/2 by 30 minutes, but by 2 hours there is a higher level of phosphorylated ERK1/2 in the cells transfected with mutant receptor when compared with lysates from cells transfected with wild-type receptor. Because the ERK1/2 signaling pathway is an important proliferation pathway, prolonged phosphorylation of this protein may have profound effects on the growth characteristics of cells that contain the mutation as is seen in the cell lines harboring the mutation that are grown in the presence of EGF (17). She, a direct target of the EGFR and upstream activator of the Ras/mitogen-activated protein kinase pathway, as expected, is also phosphorylated for a prolonged period of time. Thus, our results are consistent with an up-regulated proliferation pathway involving the Ras oncogene and mitogen-activated protein kinase signaling.

Lastly and perhaps most importantly, Akt was constitutively phosphorylated in HCC827 and H1819 cells (characterized by persistent Akt phosphorylation in the serum-starved state and lack of response to ligand stimulation), but both cell types retained phospho-Akt sensitivity to TKIs. Our findings differed from those of a recent report in which EGF increased Akt phosphorylation in NSCLC cells with mutant EGFR (17). The biological basis for this difference is not clear but may be related to the different cell lines used in the two studies, which had different EGFR mutations and may vary in the expression levels of ErbB family members (particularly ErbB3), which can directly or indirectly activate phosphatidylinositol 3’-kinase.

Treatment with EGFR inhibitors also revealed biochemical differences between mutant and wild-type receptor. Our mutant receptor was more sensitive than the wild-type to TKI-induced inhibition of EGFR phosphorylation, a finding consistent with previous reports (8, 9). However, the resistance of NSCLC cells with wild-type EGFR to growth inhibition by TKIs was not simply a consequence of reduced EGFR sensitivity to drug. Relative to H1299 cells, HCC827 cells showed a 2,500-fold increased sensitivity to TKI-induced inhibition of growth but only a 10-fold increased sensitivity to EGFR phosphorylation, raising the possibility that the mechanism of
resistance to TKIs in NSCLC cells without 
EGFR mutations is downstream of EGFR. If proven true, this hypothesis has clinical consequences; increasing the dose of TKIs given to patients with NSCLC without EGFR mutations will not be clinically effective. Other features of the tumor such as increased EGFR copy number, coexpression of other ErbBs, or specific aspects of receptor signaling may better predict relative sensitivity in tumors with wild-type receptor. TKI-induced inhibition of AKT and ERK phosphorylation correlated closely with the IC_{50} values of the TKIs in these cells, supporting recent (17) and previous (20, 21) reports that AKT is a crucial mediator of EGFR-induced cell survival and clinical response.

It is interesting to note that the tumor from patient 1 in our study who developed clinical resistance to gefitinib continued to express high levels of the mutant EGFR protein (data not shown), implying the acquisition of mechanisms to bypass the apoptotic response. Like the authors of previous reports (8, 9), we observed a striking association of EGFR coding region alterations with enhanced sensitivity to treatment with TKIs. HCC827 cells underwent apoptosis in response to treatment with TKIs, demonstrating that EGFR activation was required for the survival of these cells. Furthermore, HCC827 cells also underwent apoptosis in response to cetuximab treatment, indicating that the apoptotic response was not simply a consequence of enhanced affinity of mutant EGFR for TKIs; instead, it reflects a dependence of the cells on EGFR activity for survival. Moreover, H1819 cells were also sensitive to treatment with TKIs, albeit to a lesser extent than HCC827 cells, supporting the hypothesis that factors other than EGFR mutations, such as expression levels of other ErbBs, also sensitized NSCLC cells to TKIs. It is paradoxical that cetuximab did not reduce the phosphorylation of AKT in H1819 cells and result in cell death like the TKIs. It is likely that overexpression of ErbB2, which makes cells with wild-type receptor sensitive to the TKIs, is the reason why the H1819 cells are refractory to the treatment with cetuximab. The constitutively active EGFR/ErbB2 heterodimer most likely remains at the surface for a longer period of time than the EGFR homodimer (18) and it is at the cell surface where signaling through phosphatidylinositol 3′-kinase seems to occur (22). This interpretation of the cetuximab finding again reinforces the importance of AKT signaling on cell survival.

These findings have potential clinical applications. EGFR gene mutations have not been detected in patients with NSCLC who experience stable disease after erlotinib treatment (23). However, erlotinib treatment prolonged the survival of such patients. Thus, we hypothesize that, in response to treatment with TKIs, most NSCLCs with EGFR gene mutations will undergo tumor shrinkage, whereas tumors that have no EGFR mutations but express high levels of other ErbB family members (through gene amplification or other mechanisms) may respond or exhibit stability of disease, conferring clinically meaningful benefit. In addition, these studies suggest that coadministration of gefitinib or erlotinib with specific ErbB2 inhibitors may prove useful in NSCLC where both EGFR and ErbB2 are overexpressed.

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