High Expression of ErbB Family Members and Their Ligands in Lung Adenocarcinomas That Are Sensitive to Inhibition of Epidermal Growth Factor Receptor


Departments of 1Thoracic/Head and Neck Medical Oncology, 2Imaging Physics, and 3Pathology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas; 4UPRESEA 3493, CHU Saint-Antoine, Universite Paris VI, Paris, France; 5Department of Genetics, Lineberger Cancer Center, University of North Carolina, Chapel Hill, North Carolina; and 6Harold Simmons Cancer Center, University of Texas Southwestern Medical Center, Dallas, Texas

Abstract
Recent findings in tumor biopsies from lung adenocarcinoma patients suggest that somatic mutations in the genes encoding epidermal growth factor receptor (EGFR) and Kirsten ras (KRAS) confer sensitivity and resistance, respectively, to EGFR inhibition. Here, we provide evidence that these genetic mutations are not sufficient to modulate the biological response of lung adenocarcinoma cells to EGFR inhibition. We found high expression of ErbB family members, ErbB ligands, or both in three models that were sensitive to EGFR inhibition, including alveolar epithelial neoplastic lesions in mice that develop lung adenocarcinoma by oncogenic KRAS, human lung adenocarcinoma cell lines, and tumor biopsies from lung adenocarcinoma patients. Thus, lung adenocarcinoma cells that depend on EGFR for survival constitutively activate the receptor through a combination of genetic mutations and overexpression of EGFR dimeric partners and their ligands. (Cancer Res 2005; 65(24): 11478-85)

Introduction
Somatic mutations in epidermal growth factor receptor (EGFR), the gene encoding the EGFR, have been found in the tumors of 10% to 40% of patients with non–small cell lung cancer (NSCLC; refs. 1–4). These mutations activate the EGFR tyrosine kinase and are associated with adenocarcinoma histology, female gender, and a nonsmoking history. Lung cancer patients with EGFR mutations frequently experience rapid and sustained shrinkage of primary and metastatic disease after treatment with the EGFR tyrosine kinase inhibitors (TKI) gefitinib or erlotinib (1–4). In a phase III trial, treatment with erlotinib conferred a survival benefit (5), but the proportion of patients who experienced this benefit exceeded the expected frequency of EGFR mutations. In addition, a small proportion of patients whose tumors shrank in response to EGFR TKIs had no evidence of EGFR mutations (1–5). Together, these findings suggest that factors other than EGFR mutations confer sensitivity to EGFR inhibition.

EGFR forms homodimers and heterodimers with the other ErbB family members ErbB2, ErbB3, and ErbB4 (6). These dimeric complexes have distinct ligand binding and signaling activities (7). For example, ErbB3 is unique in that it lacks a functional domain. Despite this deficiency, ErbB3 undergoes transphosphorylation in complex with other ErbBs and activates downstream kinases, such as phosphatidylinositol 3-kinase (PI3K), in response to ligand binding. Recent studies have implicated ErbB3 in the sensitivity of NSCLC cell lines to EGFR inhibition (8, 9). Together, these findings suggest that aberrant expression of ErbB family members contributes to the responsiveness of NSCLC cells to EGFR inhibition.

A recent report (10) linked de novo resistance to treatment with EGFR TKIs in lung adenocarcinoma patients with somatic mutations in Kirsten ras (KRAS). A growing body of evidence indicates that KRAS mutations are important in the development of lung adenocarcinoma. They occur in 30% to 50% of lung adenocarcinomas (11, 12) and are mutually exclusive from mutations in EGFR (13). Mice that express mutant KRAS develop lung adenocarcinoma rapidly and with high penetrance (14–17). In this study, we investigated the role of KRAS mutations in the resistance of lung adenocarcinoma to EGFR TKIs. We examined KrasL41 mice, which develop lung adenocarcinoma through somatic activation of a KRAS allele carrying an activating mutation in codon 12 (G12D; ref. 17). Alveolar epithelial cells in this mouse model recapitulate the series of morphologic stages through which human atypical alveolar hyperplasia (AAH) evolves into adenocarcinoma. Our findings suggest that the presence of KRAS mutations is not sufficient to confer resistance to EGFR inhibition and reveal a novel mechanism of response to EGFR inhibition that is potentially relevant to lung cancer patients.

Materials and Methods

Animal experiments. Animal experiments were compliant with the guidelines of The University of Texas M.D. Anderson Cancer Center. The KrasL41 mice were provided by Dr. Tyler Jacks (Massachusetts Institute of Technology, Cambridge, MA), and gefitinib was provided by AstraZeneca (Wilmington, DE). Four-month-old KrasL41 mice were randomly allocated to treatment with vehicle (PBS with 0.05% Tween 80 in PBS by oral gavage daily), low-dose gefitinib (100 mg/kg/d), or high-dose gefitinib (250 mg/kg/d). Treatment was given for 28 days with a 4-day intermission at 14 days to minimize dermatologic toxicity. Mice treated with high-dose
gefitinib or vehicle were imaged by micro–computed tomography (micro-CT) at the beginning and completion of treatment to examine changes in lesion size as described previously (18). Mice were killed at the completion of therapy. At that time, an investigator who was blinded to the identity of the treatment groups counted lesions visible on lung pleural surfaces. One lung from each mouse was then frozen for protein extraction, and the other was formalin fixed for immunohistochemical analysis.

Cell lines. The LKR-13 and LKR-10 cell lines (provided by Dr. Tyler Jacks) were derived by serial passage of minced lung adenocarcinoma tissues from two tumors isolated from separate lobes of the same KrasLAI mouse. These cells and the human NSCLC cell lines (H1299, HCC2827, H3525, H1819, H4006, and HCC2279) were passaged in RPMI 1640 supplemented with 10% fetal bovine serum on standard Falcon (Bedford, MA) plasticware. Immortalized human bronchial epithelial cells (HBEC) and KRAS/HBECs were cultured with keratinocyte serum-free medium containing bovine pituitary extract and recombinant EGF (Gibco). KRAS/HBECs were derived by stably infecting parental HBECs with the retroviral vector pBabe-hyg-KRAS2-V12. H1299 cells were transfected with a vector (pcDNA3.1) expressing wild-type or mutant (ΔT46-750) EGF, and single-cell subclones were selected in G418. Expression of the exogenous (wild-type or mutant) EGF mRNA was confirmed in single-cell subclones by real-time PCR (RT-PCR) analysis of total cellular RNA (1 μg) using primers spanning exons 18 and 19 and by direct sequencing of the PCR products. PCR products of the ΔT46-750 EGF transfectants lacked 15 bp corresponding to the deletion mutation. EGF-transfected H1299 cells were subsequently transfected with a vector expressing ErbB3, and single-cell subclones of the double transfectants (EGF and ErbB3) were selected in G418 and hygromycin. All cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

Antibodies. For Western blotting and immunohistochemical analyses, we used antibodies specific for EGF (NeoMarkers, Fremont, CA), ErbB2 and ErbB3 (Santa Cruz Biotechnology, Santa Cruz, CA), β-actin (Sigma, St. Louis, MO), and Tyr1370-phosphorylated ErbB3 (pErbB3). Ser772-phosphorylated AKT (pAkt), actin, and AKT (Cell Signaling Technologies, Beverly, MA).

RNA analysis. Total RNA was prepared from whole mouse lungs and cell lines using a RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Quantitative RT-PCR (Q-PCR) was done using 1 μg of each RNA sample, which was reverse transcribed and used in a PCR reaction to measure the transcript levels of each gene. Levels were normalized to those of β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in each sample. Gene expression was assayed using Assays-On-Demand (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol using a Stratagene (La Jolla, CA) MX3000P RT-PCR machine.

Cell proliferation. Cell proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) colorimetric dye reduction method. Cells were seeded at a density of 1 × 10⁵ to 5 × 10⁵ per well (10 replicate wells per assay condition) in 0.1 mL medium, treated for 5 days with different dosages of gefitinib, and subjected to MTT assays.

Immunoblot analysis. Whole lung tissues were homogenized using a Tissue Homogenizer in Reinaudal Iysis buffer [200 mMm/L Tris (pH 7.4), 137 mM/L NaCl, 2 mM/L EDTA, 10% glycerol, 1% Triton X-100, 10 mM/L phenylmethylsulfonyl fluoride, 100 mM/L phosphatase inhibitors, and phosphatase inhibitor cocktail]. Cell lines were washed twice with ice-cold PBS and scraped in radioimmunoprecipitation assay buffer [50 mM/L Tris-HCl (pH 7.4), 150 mM/L NaCl, 0.1% Tween-20, 0.1% sodium deoxycholate, 1% NP40, 1 mL/L phenylmethylsulfonyl fluoride, 1 mL/L sodium orthovanadate, 1 mL/L NaF, and protease inhibitor cocktail]. Lysates were cleared by centrifugation, and protein concentrations were measured with a protein assay kit (Bio-Rad, Hercules, CA). Equal amounts of protein were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were then immunoblotted overnight at 4°C with primary antibodies in TBS containing 5% nonfat dry milk. Antibody binding was detected with an enhanced chemiluminescence kit (Amersham, Piscataway, NJ) according to the manufacturer's directions.

Mutational analysis of tumor samples and cell lines. Approximately 10³ tumor cells were microdissected from sequential 8-μm-thick tissue sections that were stained with H&E, fixed in formalin, and embedded in paraffin. DNA was extracted from tumor samples and cell lines using 25 μL Pico Pure DNA extraction solution (Arcturus, Mountain View, CA) containing protease K and incubated at 65°C for 24 hours. Exons 18 to 21 of EGF and exon 12 of KRAS were amplified by PCR using intron-based primers as described previously (13). All PCR products were directly sequenced using the Applied Biosystems PRISM dye terminator cycle sequencing method (Perkin-Elmer, Wellesley, CA). All sequence variants were confirmed by independent PCR amplifications from at least two independent microdissections and were sequenced in both directions.

Tissue microarrays. Tissue microarrays were constructed with cores from formalin-fixed, paraffin-embedded blocks, and triplicate core samples were included for each tumor. To examine changes in the expression of ErbB3 and pAkt with malignant progression, an array was constructed with specimens of mouse normal lung tissue (n = 30), AAH (n = 40), adenoma (n = 206), and adenocarcinoma (n = 11) according to the histologic criteria established (17). To examine changes in the expression of ErbB3 and pAkt resulting from gefitinib treatment, an array was constructed from all lesions identified by histologic analysis from the mice treated with gefitinib or vehicle. Each lesion was sampled with a single core 1 mm in diameter, as the lesions were too small to permit multiple cores to be obtained.

For human studies, we constructed an array using tumor samples from patients before oral administration of gefitinib (250 mg/d) at The University of Texas M.D. Anderson Cancer Center. Only patients for whom pertinent clinical data were available were included. Response to treatment was measured with the Response Evaluation Criteria in Solid Tumors, and patients with more than one lesion were categorized according to the lesion that achieved the best response. Stabilization of disease was defined as an absence of change in lesion size for at least 6 months following treatment initiation.

Immunohistochemistry. For immunohistochemical analyses, 4-μm sections were deparaffinized, rehydrated, and washed with PBS as described previously (18). Antigens were retrieved with 0.01 mol/L citrate buffer (pH 6; DakoCytomation) for 30 minutes in a steamer. Samples were blocked for endogenous activity with 3% hydrogen peroxide/PBS, avidin/biotin solution (Zymed, Carlsbad, CA), Dako serum-free protein block (DakoCytomation, Carpinteria, CA) before incubation with the primary antibodies overnight at 4°C. Standard avidin/biotin immunoperoxidase methods, with diaminobenzidine as the chromogen, were used for detection. As negative controls for the specificity of the immunostaining results, we pretested the samples with blocking peptides. ErbB3-blocking peptides were the 17 COOH-terminal amino acids.

Staining was quantified by two investigators (M.W. and L.L.W.) who were blinded to the identity of the treatment groups. Staining in each sample was quantified from a single tissue section using a combined score based on staining intensity × extension. Staining intensity was graded as undetectable (0), weak (1), medium (2), or strong (3). Staining extension was graded as the percentage of positive cells per square at ×20 magnification. The tumor score was defined as the highest of the three core samples, and results were presented as mean (SE).

Statistical analysis. The immunostaining scores, patient age, tumor numbers, and tumor volumes were considered continuous variables and were compared using the Kruskal-Wallis test followed by the Mann-Whitney nonparametric test with Bonferroni correction. P = 0.05 was considered significant for two pair-wise comparisons, 0.017 for three pair-wise comparisons, and 0.012 for four pair-wise comparisons. Fisher's exact test was used to compare categorical variables, and P = 0.05 was considered significant. Data were processed with StatView and Survival Tools software version 5.0 (Abacus Concepts, Berkeley, CA).

Results

Epidermal growth factor receptor inhibition suppresses the expansion of alveolar neoplasia in KrasLAI mice. We treated KrasLAI mice with the TKI gefitinib. Mice were treated daily by
gavage for 28 days with low-dose gefitinib (100 mg/kg; 23 mice), high-dose gefitinib (250 mg/kg; 21 mice), or vehicle only (47 mice) beginning at 4 months of age. After treatment, we counted the lesions on lung pleural surfaces at the time of autopsy. In addition, mice treated with high-dose gefitinib or vehicle were imaged by micro-CT at the beginning and completion of treatment to examine changes in lesion size (examples are shown in Fig. 1A). Gefitinib treatment was well tolerated; mice experienced facial dermatitis but showed no evidence of weight loss or other overt toxicities. We observed a reduction in the number of lung lesions in mice treated with low-dose or high-dose gefitinib, and lesion size decreased in the high-dose group (Fig. 1B).

**Alveolar neoplastic lesions in KrasLA1 mice overexpress ErbB3 and ErbB ligands.** We hypothesized that the sensitivity of alveolar neoplastic lesions in KrasLA1 mice to gefitinib is a consequence of aberrant expression of ErbB family members, ErbB ligands, or both. We examined the expression of ErbB family members in lung tissues at 5 months, when early epithelial changes (AAH and adenomas but no adenocarcinomas) were present. Q-PCR analysis was done on RNA prepared from whole lung tissues. Relative to their wild-type littermates, KrasLA1 mice expressed higher levels of c-ErbB3 and lower levels of ErbB1 (EGFR) and ErbB2 (HER-2/neu; Fig. 2A). Because the lung mRNA in KrasLA1 mice represented that of both normal and transformed cells, we next used immunohistochemical analysis to examine expression specifically in epithelial precursors of lung adenocarcinoma. A tissue microarray was constructed with punch biopsy samples of lung tissues from KrasLA1 mice (normal lung, AAH, adenoma, and adenocarcinoma). We observed staining for ErbB3 protein in the epithelial precursors but not in the adjacent normal alveolar epithelium (Fig. 2B). The staining increased with histologic progression from AAH to adenocarcinoma, indicating that the increase in ErbB3 expression was linked to malignant progression.

We then used Q-PCR to examine the expression of ErbB ligands in the same lung RNA samples analyzed for expression of ErbB family members. The ligands epiregulin, epigen, and amphiregulin were expressed at 23.0-, 7.5-, and 5.0-fold higher levels, respectively, in KrasLA1 mice than in their wild-type littermates, whereas β-cellulin, epidermal growth factor, diphtheria toxin receptor, transforming growth factor-α, neuregulin-1, and neuregulin-2 were expressed at levels similar to those in the wild-type mice (Fig. 2A). Epiregulin binds to ErbB2/ErbB3 and ErbB2/ErbB4 heterodimers with a moderate affinity and to ErbB1 homodimers and ErbB1/ErbB2 heterodimers with weak affinity, whereas amphiregulin binds selectively to ErbB1 (19). The ErbB-binding specificity of epigen has not yet been reported.

Because ErbB3 can activate PI3K, we examined the Ser473 pAKT, a downstream mediator of PI3K, as a marker of ErbB3 activation in the lungs of KrasLA1 mice. We did immunohistochemical analysis of epithelial precursor lesions using the same tissue microarray used to examine ErbB3 expression. The degree of staining was undetectable in normal alveolar epithelium and increased with malignant progression (Fig. 2B and C). These data are consistent with the conclusion that AKT phosphorylation increased as a consequence of increased ErbB3 expression, but it is possible that AKT phosphorylation increased through direct interactions of mutant KRAS with PI3K, Ras-independent mechanisms, or both.

**Epidermal growth factor receptor inhibition decreased expression of ErbB3 and ErbB ligands.** We examined the effect of gefitinib treatment on the expression of ErbB family members...
and ErbB ligands in lung tissues of Kras<sup>LA1</sup> mice. ErbB3 RNA levels decreased markedly in mice treated with gefitinib (Fig. 3A). Western blotting showed that gefitinib treatment was associated with a decrease in Tyr<sub>1289</sub> pErbB3, total ErbB3, and, to a lesser extent, pAKT, but total EGFR expression did not change (Fig. 3B). Amphiregulin and epiregulin RNA expression also decreased, whereas the expression of epigen and the other ligands did not measurably change (Fig. 3A).

We next investigated the effect of gefitinib treatment on ErbB3 expression and AKT phosphorylation in epithelial precursor lesions by immunohistochemical analysis. A tissue microarray was constructed with punch biopsy samples of lung tissues (normal lung, AAH, and adenoma) from the mice treated in the above gefitinib experiment. Immunohistochemical quantitation of ErbB3 (B) and pAKT (C) in normal tissue (NI), AAH, adenoma (Ad), and adenocarcinoma (ADC). Representative staining patterns for ErbB3 (<20 magnification) and pAKT (<10 magnification). B, the specificity of ErbB3 staining was examined in a lung adenocarcinoma derived from a Kras<sup>LA1</sup> mouse in the presence (+) and absence (-) of blocking peptides.

High expression of ErbB3 and ErbB ligands in sensitive non–small cell lung cancer cell lines. We investigated the expression of ErbB3 and ErbB ligands in human lung adenocarcinoma cell lines that have been characterized for somatic mutations in \(EGFR\) and sensitivity to gefitinib (Table 1; ref. 8). With respect to gefitinib sensitivity, cell lines were classified into one of three categories: highly sensitive (HCC827, H3255, and H4006), moderately sensitive (H1819 and HCC2279), and resistant (H1299).

Relative to the gefitinib-resistant H1299 cells, the highly sensitive cell lines HCC827, H3255, and H4006 expressed high basal levels of EGFR, ErbB2, and ErbB3 and the ligands amphiregulin and epiregulin (Fig. 4A). Epiregulin levels were particularly enhanced in the highly sensitive cells (up to 4.5 \(\times\) 10<sup>5</sup>-fold). Gefitinib treatment decreased expression of pErbB3 and pAKT in the highly sensitive cell lines (HCC827, H3255, and H4006) and in one (H1819) but not the other (HCC2279) moderately sensitive cell line or in the gefitinib-resistant cell line H1299 (Fig. 4B). All of the cell lines in which pErbB3 and pAKT expression decreased had activating \(EGFR\) mutations.
mutations with the exception of H1819, which expressed high basal ErbB2 and ErbB3 (Fig. 4A; ref. 8). HCC2279 cells, which has an EGFR mutation and is moderately sensitive to gefitinib, expressed no detectable ErbB2 or ErbB3 and low AKT1 and AKT2 (Fig. 4A and B). We analyzed EGFR in HCC2279 cells and found no evidence of a T790M mutation, which was reported recently to confer resistance to EGFR inhibition (20). Thus, gefitinib sensitivity in human NSCLC cells correlated with the presence of EGFR mutations and high basal expression of ErbB family members and ErbB ligands, which decreased in response to EGFR inhibition. The high expression of ErbB family members and their ligands in gefitinib-sensitive NSCLC cells was consistent with our findings in KrasLA1 mice (Fig. 3).

Introduction of KRAS mutations is not sufficient to confer resistance. In contrast to KrasLA1 mice that were sensitive to gefitinib, patients with KRAS mutant lung adenocarcinomas were reported to be gefitinib resistant (10). Therefore, we directly tested the role of mutant KRAS in HBECs that had been immortalized by the introduction of genes encoding cyclin-dependent kinase-4 and human telomerase reverse transcriptase (21). KRAS/HBECs, which have been transfected with a retroviral vector expressing mutant KRAS, acquire enhanced anchorage-independent growth and increased saturation density.7 We found that HBECs and KRAS/HBECs were both moderately sensitive to the antiproliferative effect of gefitinib treatment (IC50, 0.4 μmol/L). These findings indicate that the introduction of mutant KRAS was not sufficient to confer resistance to EGFR inhibition.

High expression of ErbB3 is associated with gefitinib sensitivity. Given that high expression of ErbB3 was associated with sensitivity to EGFR inhibition in KrasLA1 mice and human lung adenocarcinoma cells, we hypothesized that patients with high intratumoral ErbB3 levels in their pretreatment biopsy samples would be more likely to respond to gefitinib than patients with lower levels. We studied tumor samples from 42 patients with lung adenocarcinoma who had been treated with gefitinib and from whom there was sufficient tissue and clinical data available for analysis. Patient demographics are summarized in Supplementary Table S1. Patients were classified into three response categories, including objective responders (4 patients with complete or partial response), stable disease for at least 6 months (4 patients), and progressive disease (34 patients).

We did mutational analysis of EGFR (exons 18-21) and KRAS (codons 12, 13, and 61) on all samples, with the exception of 11 patients with progressive disease from whom there was inadequate tissue for DNA analysis (Table 2). We observed KRAS

| Table 1. EGFR mutational status and gefitinib sensitivity of NSCLC cell lines |

<table>
<thead>
<tr>
<th>Cells</th>
<th>EGFR Mutations</th>
<th>Gefitinib IC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCC827</td>
<td>Δ746-750</td>
<td>16.0 μmol/L</td>
</tr>
<tr>
<td>HCC2279</td>
<td>Δ746-750</td>
<td>27.0 μmol/L</td>
</tr>
<tr>
<td>H3255</td>
<td>L858R</td>
<td>9.0 μmol/L</td>
</tr>
<tr>
<td>H1819</td>
<td>Wild-type</td>
<td>4.7 μmol/L</td>
</tr>
<tr>
<td>H1299</td>
<td>Wild-type</td>
<td>38.0 μmol/L</td>
</tr>
<tr>
<td>H4006</td>
<td>Δ746-750, S752V</td>
<td>30 μmol/L</td>
</tr>
</tbody>
</table>

NOTE: IC50 for NSCLC cell lines treated with gefitinib for 5 days. EGFR mutational status is indicated for each cell line.

and B). We analyzed EGFR in HCC2279 cells and found no evidence of a T790M mutation, which was reported recently to confer resistance to EGFR inhibition (20). Thus, gefitinib sensitivity in human NSCLC cells correlated with the presence of EGFR mutations and high basal expression of ErbB family members and ErbB ligands, which decreased in response to EGFR inhibition. The high expression of ErbB family members and their ligands in gefitinib-sensitive NSCLC cells was consistent with our findings in KrasLA1 mice (Fig. 3).

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We did mutational analysis of EGFR (exons 18-21) and KRAS (codons 12, 13, and 61) on all samples, with the exception of 11 patients with progressive disease from whom there was inadequate tissue for DNA analysis (Table 2). We observed KRAS
mutations in tumors from patients with progressive disease [6 of 23 (26%) and stable disease [1 of 4 (25%)]] but not in responding tumors. We observed EGFR mutations in responding tumors [3 of 4 (75%)] and those from patients with progressive disease [2 of 23 (8%)] but not in those from patients with stable disease. Immunohistochemical analysis was done to quantify ErbB3 expression in biopsy samples using conditions that specifically detected ErbB3 (Fig. 5A). ErbB3 expression was higher in tumors from patients who achieved an objective response or stabilization of disease than in those with progressive disease (Fig. 5B). Thus, in this group of patients, EGFR mutational analysis did not identify one of the four patients who achieved an objective response or any of those whose disease stabilized. In contrast, ErbB3 expression clearly segregated patients with progressive disease from those who achieved tumor response or disease stabilization after treatment.

Discussion

The findings presented here provide the first evidence that lung adenocarcinoma cells that depend on EGFR for survival constitutively activate the receptor through a combination of genetic mutations and overexpression of EGFR dimeric partners and their ligands. In Kras\textsuperscript{LA1} mice, high expression of ErbB3, amphiregulin, and epiregulin in the lung was dependent on EGFR activation. These findings suggest the presence of an autocrine loop in which EGFR activates expression of ErbB3 and ErbB ligands, which in turn activate EGFR. However, the introduction of mutant EGFR into H1299 cells was not sufficient to increase the expression of ErbB3, amphiregulin, or epiregulin, suggesting that factors other than EGFR activation contributed to the high expression of these proteins in NSCLC cells with somatically acquired EGFR mutations.

Consistent with our findings in Kras\textsuperscript{LA1} mice, human tumor cells with KRAS mutations express high levels of ErbB ligands (22–24). The levels of ErbB3 mRNA are significantly higher in human lung adenocarcinomas than in normal tissues \((P < 0.001;\text{ ref. 25}).\) However, high ErbB3 expression in human lung adenocarcinoma cells does not correlate with the presence of KRAS mutations (25). Thus, lung adenocarcinomas arising in humans and Kras\textsuperscript{LA1} mice differ with respect to the role of KRAS mutations in the regulation of ErbB3 expression. Nevertheless, the observation that both mouse and human lung adenocarcinomas have high expression of ErbB3 underscores its importance in tumorigenesis. Moreover, ErbB3 is required to drive breast epithelial cell proliferation, and overexpression of ErbB3 leads to the development of lung adenocarcinomas in mice and is associated with poor prognosis in patients with NSCLC (26–28).

Several of our findings suggest that EGFR mutations are neither sufficient nor required for sensitivity to EGFR inhibition and that other ErbBs and ErbB ligands have an important role in this sensitivity. First, gefitinib-sensitive NSCLC cells had high expression of ErbB2, ErbB3, and ErbB ligands. Second, pharmacologic inhibition of EGFR in Kras\textsuperscript{LA1} mice decreased the expression of ErbB2, ErbB3, and ErbB ligands. Third, HCC2279 cells, which express undetectable levels of ErbB2 and ErbB3, were only moderately sensitive to gefitinib despite having an activating mutation in EGFR; in addition, they did not have the T790M EGFR mutation, which confers resistance to EGFR inhibition (20). Fourth, H1819 cells, which have no EGFR mutations and are moderately sensitive to

| Table 2. EGFR and KRAS mutational status of tumors from lung cancer patients treated with gefitinib |
|----------------------------------|--------|--------|--------|
| Category                         | No. (%) patients |                  |                  |
|                                  | Progressive disease | Stable disease | Objective response |
|                                  | \(n = 23\) | \(n = 4\) | \(n = 4\) |
| KRAS                             | 6 (26) | 1 (25) | 0 (0) |
| G12T                             | 5      | 1      | 0      |
| G12D                             | 1      | 0      | 0      |
| EGFR                             | 2 (9)  | 0 (0)  | 3 (75) |
| \(\Delta746-750\)                | 1      | 0      | 3      |
| Leu\textsuperscript{690}Phe      | 1      | 0      | 0      |

NOTE: Mutational analysis of KRAS (codons 12, 13, and 61) and EGFR (exons 18-21) was done on tumor DNA as described in Materials and Methods. Patients were grouped into three categories based on response to EGFR inhibitor therapy: progressive disease, stable disease, and objective response. Specific mutations detected in tumors are listed.
EGFR inhibition (8), expressed high levels of EGFR, ErbB2, ErbB3, and ErbB ligands. However, stable cotransfection of H1299 cells with ErbB3 and mutant (Δ746-750) EGFR was not sufficient to confer gefitinib sensitivity (data not shown). Of note, gefitinib treatment did not extinguish pAKT expression in H1299 cells (Fig. 4A). These negative data make an important point: cells must depend on EGFR and its downstream pathways for their survival to be sensitive to EGFR inhibition. H1299 cells maintain their survival through EGFR-independent mechanisms and thus remain gefitinib resistant despite the introduction of mutant EGFR and ErbB3.

**KRAS** mutations in NSCLC occur most commonly in smokers, who are typically resistant to the antitumor effects of EGFR TKIs. Consistent with these findings, **KRAS** mutations have been linked with de novo resistance to treatment with EGFR TKIs (10). Similarly, we observed **KRAS** mutations in tumors from patients who were resistant to EGFR inhibition. However, KrasLA1 mice were sensitive to EGFR inhibition, and introduction of mutant **KRAS** into HBECs did not measurably inhibit their sensitivity to gefitinib. In addition, we observed **KRAS** mutations in one patient treated with gefitinib who experienced prolonged stabilization of disease. Together, these findings indicate that the presence of mutant **KRAS** is not sufficient to confer resistance to EGFR inhibition in lung adenocarcinoma cells.

**EGFR** mutations have been found in 30% to 60% of never smokers (13). In the present study, **EGFR** mutations were more common in never smokers and former smokers than in current smokers (P = 0.002; Supplementary Table S1). Never smokers and former smokers account for the vast majority of patients who benefit from treatment with EGFR TKIs (1–5). However, these studies identified patients with wild-type **EGFR** who have also benefited from treatment with EGFR TKIs. Similarly, in the present study, we observed wild-type **EGFR** in one patient who achieved an objective response and in the four patients who had stable disease. A large clinical trial of lung cancer patients treated with erlotinib suggested that stabilization of disease is clinically meaningful, as the survival benefit exceeded the observed rate of tumor response (5). These findings indicate that factors other than **EGFR** mutations contribute to sensitivity to EGFR inhibition. We found that responding patients and those with stable disease could be segregated from patients with progressive disease based on intratumoral ErbB3 levels. Although these observations are preliminary and should be tested in a larger population of patients with lung adenocarcinoma, our data point to ErbB3 and ErbB ligands as potentially important mediators of EGFR inhibition in patients with lung adenocarcinoma. Analysis of the expression of these molecules may complement **EGFR** mutational analysis as a predictive marker of sensitivity to EGFR TKIs. Finally, although the current clinical application of EGFR inhibition is targeted at late-stage cancers, the response of the early neoplastic disease in KrasLA1 mice to EGFR inhibition raises the possibility that TKIs may be used to treat early-stage lung cancers in humans.

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