Autocrine Production of Amphiregulin Predicts Sensitivity to Both Gefitinib and Cetuximab in EGFR Wild-type Cancers

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Abstract Purpose: Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors, gefitinib and erlotinib, lead to significant tumor regressions in 10% to 15% of non-small cell lung cancer (NSCLC) patients with EGFR activating mutations. However, 30% to 40% of NSCLC patients, majority of whom are EGFR wild-type, develop stable disease following EGFR tyrosine kinase inhibitor therapy. EGFR-directed antibodies (cetuximab) are effective treatments for head and neck squamous cell carcinomas, which seldom contain EGFR mutations. The determinant(s) of efficacy of EGFR-targeted therapies in EGFR wild-type cancers is not well defined.

Experimental Design: We examined the relationship of EGFR ligands, EGF, transforming growth factor-α, and amphiregulin and the efficacy of gefitinib and cetuximab in EGFR wild-type NSCLC (n = 10) and head and neck squamous cell carcinoma (n = 4) cell lines. We compared amphiregulin expression using immunohistochemistry in EGFR wild-type NSCLC patients (n = 24) that developed either stable or progressive disease following erlotinib or gefitinib treatment.

Results: Cell lines which produced ≥20 pmol/L amphiregulin, as detected by an ELISA, were significantly more likely to be growth inhibited by both gefitinib and cetuximab than those that produced minimal or no amphiregulin. In these cell lines, both cetuximab and gefitinib led to cell cycle arrest at the G1-S boundary and was associated with preferential inhibition of extracellular signal-regulated kinase 1/2 but not Akt signaling. Amphiregulin expression was significantly higher in NSCLC patients that developed stable disease compared with those that developed disease progression following gefitinib or erlotinib treatment.

Conclusions: Amphiregulin expression may help select EGFR wild-type patients who are likely to develop stable disease from EGFR-targeted therapies.

Aberrant overexpression of the epidermal growth factor receptor (EGFR) has been detected by immunohistochemistry in many malignancies including non-small cell lung cancer (NSCLC) and head and neck squamous cell carcinoma (HNSCC; ref. 1–3). Some, but not all, studies have shown that EGFR overexpression is associated with a poor prognosis in both NSCLC and HNSCC (2, 3). EGFR can be activated by EGF, transforming growth factor-α (TGF-α), amphiregulin, betacelululin, heparin-binding EGF, or epiregulin. These ligands bind to the extracellular region of the EGFR and induce a conformational change in EGFR leading to dimerization and activation of EGFR signaling (reviewed in ref. 4). In some cancers, EGFR ligands are locally secreted by the cancer cells and activate EGFR in an autocrine fashion. Coexpression of both EGFR ligands and EGFR has been associated with a poor prognosis in both NSCLC and HNSCC (1, 3). An alternative method of EGFR activation includes somatic mutations in the tyrosine kinase domain (5). These have been most extensively described in patients with NSCLC who have never smoked cigarettes but are rare in other malignancies including HNSCC (6, 7). In the presence of an EGFR mutation, the receptor is constitutively active in a ligand-independent manner and is sufficient to lead to transformation in vitro and to cancer formation when expressed in the alveolar epithelium of mice (5, 8). Increased EGFR copy number assessed by fluorescence in situ hybridization has also been detected in NSCLC and HNSCC and is associated with a poor prognosis in both cancers (9, 10).

Inhibitors of EGFR have been clinically evaluated and are effective therapeutic strategies in both NSCLC and HNSCC (11, 12). Two main classes of EGFR inhibitors are currently in clinical use: small-molecule EGFR tyrosine kinase inhibitors (TKI), which compete for ATP binding in the TKI domain, and monoclonal antibodies, which interfere with ligand binding in...
The goal of this study was to identify potential biomarkers associated with efficacy of gefitinib and cetuximab in EGFR wild-type cancers. Our studies focused on ligand-mediated activation of EGFR and identified autocrine production of amphiregulin as an important biomarker associated with growth inhibition by cetuximab and gefitinib. In amphiregulin producing cells, both gefitinib and cetuximab led to cell cycle arrest and preferential inhibition of ERK1/2 signaling in both NSCLC and HNSCC cells. High amphiregulin expression, as detected by immunohistochemistry, was also associated with stable disease in NSCLC patients treated with gefitinib or erlotinib. Our findings suggest that amphiregulin expression could potentially be used as a biomarker to select EGFR wild-type patients who may clinically benefit from EGFR-targeted therapies.

In this study, we evaluated the mechanisms leading to stable disease in NSCLC and HNSCC cell lines and tumors with wild-type EGFR following gefitinib or cetuximab treatment. We examined ligand-mediated activation of EGFR and determined the effect of amphiregulin-mediated autocrine activation of EGFR on the development of cell cycle arrest in vitro and stable disease in patients with NSCLC treated with gefitinib or erlotinib.

**Materials and Methods**

**Cell culture and reagents.** The 14 NSCLC cell lines (PC9, HCC4006, H3255, Calu-3, H1648, H1437, HCC15, HCC193, H9C5, H661, H2126, H1666, and H358) and 4 HNSCC cell lines (HN11, HN12, HN13, and HN28) have been characterized previously (16, 23, 26, 29, 30). All cells, except H3255, H1666, and H1648, were cultured in RPMI 1640 (Sigma) supplemented with 5% or 10% fetal bovine serum, 100 units/mL streptomycin, and 1 mmol/L sodium pyruvate. The H3255, H1666, and H1648 cells were cultured in ACL-4 medium (Invitrogen) supplemented with 5% fetal bovine serum, 100 units/mL streptomycin, and 1 mmol/L sodium pyruvate.

Gefitinib was obtained from commercial sources and purified through an ethyl acetate extraction. The resulting product was verified by liquid chromatography and mass spectrometry as described previously (31). CI-1040 was purchased from American Custom Chemical. Stock solutions of both drugs (10 mmol/L) were prepared in DMSO and stored at -20°C. Cetuximab (2 mg/mL) was purchased from the pharmacy at Dana-Farber Cancer Institute and stored at 4°C. The drugs were diluted in fresh medium before use.

**Antibodies and Western blotting.** Cells were seeded at a density of 1 × 10⁶ per plate and allowed to grow overnight in medium containing 5% to 10% FBS. The medium was then replaced with RPMI 1640 containing 0.1% FBS for 24 h following which gefitinib or cetuximab were added to the medium. The cells were incubated for another 3 h, washed with PBS, and lysed in buffer containing 25 mmol/L Tris (pH 8.3), 192 mmol/L glycine, 0.1% SDS, and 1 mmol/L phenylmethylsulfonyl fluoride as described previously (26). For studies evaluating the effects of gefitinib or cetuximab on phospho-EGFR, we used lysis buffer containing 1% SDS [50 mmol/L], Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1% SDS, 50 mmol/L NaF, 1 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 25 μg/mL leupeptin, and 25 μg/mL aprotinin as described in our prior studies (26). Immunoblotting was done according to the antibody manufacturer’s recommendations. Antibody binding was detected using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

**Cell proliferation and growth assays.** In colorectal cancer, cetuximab appears most effective (partial responses and stable disease) in tumors that also coexpress amphiregulin mRNA (27). Furthermore, increased EGFR copy number may be associated with therapeutic benefit in patients with colorectal cancer treated with cetuximab (28).
recommended conditions. For all studies, cells were incubated for 6 days and viability assessed by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl) 2H-tetrazolium, inner salt assay. All experimental points were set up in 6 to 12 wells and all experiments were repeated at least three times.

**Detection of ligands.** EGFR, amphiregulin, TGF-α, and heregulin were measured in cell culture medium using an ELISA were done according to the manufacturer's recommended procedures (Quantikine; R&D Systems) and as described previously (36). All samples were run in triplicate. Color intensity was measured at 450 nm using a spectrophotometric plate reader. Growth factor concentrations were determined by comparison with standard curves.

**Flow cytometry.** We used fluorescence-activated cell sorting to analyze cell cycle distribution following drug treatment and to detect cell surface expression of amphiregulin. The cell cycle distribution analyses following drug treatment were done as described previously (23, 26). Cell surface detection of amphiregulin was done as described in ref. 37. Cells were grown in 0.1% serum-containing medium for 24 h, harvested, and resuspended in PBS at 0.5 × 10^6/mL. The anti-amphiregulin or control antibody (diluted in 3% bovine serum albumin/PBS at 1:100 ratio) was added for 45 min, following which the cells were washed and exposed to an anti-goat secondary antibody at a 1:200 dilution for 45 min. The cells were washed in PBS and analyzed using FACScan as described previously (26). All experiments were repeated three times.

**EGFR and KRAS sequencing.** All cell lines in this study were analyzed for EGFR and KRAS mutations. DNA was extracted by standard techniques, and exons 18 to 21 of EGFR and exon 1 of KRAS were sequenced as previously published (23, 26). NSCLC patient specimens were sequenced for EGFR and KRAS using direct sequencing and/or a heteroduplex-based analysis as described previously (16, 38). The EGFR and KRAS primers and sequencing conditions are available on request.

**EGFR copy number detection.** Quantitative genomic PCR was used to determine EGFR copy number as described previously (23). DNA was prepared from each of the cell lines using Qiagen genomic tips. All quantitative PCRs were carried out in an ABI 7700 thermal cycler using TaqMan Universal PCR Master Mix (Applied Biosystems). The amount of template calculated using the EGRF probe was divided by the average amount of template calculated from seven reference genes distributed throughout the genome (CD20, 11q13; FANC, 16q24; GART, 21q22; MAPK4, 18q12q21; TSN, 2q21; VEST1, 8q13; and FLT3, 13q12) to generate the relative EGFR copy number.

**Real-time reverse transcription-PCR analysis.** Total RNA was isolated from cultured NSCLC and HNSCC cell lines using Trizol (Life Technologies) according to the manufacturer's specifications. Total RNA (1 μg) was primed with oligo(dT)\(_{20}\) (Invitrogen), and cDNAs were synthesized with Transcriptor Reverse Transcripase (Roche Applied Sciences) according to manufacturer's specifications. For endogenous control selection, equal volumes of cDNAs were analyzed using the TaqMan Human Endogenous Control Plate (Applied Biosystems) according to manufacturer's specifications. Human phosphoglycerate kinase 1 was selected as endogenous control as its level of expression showed least SD (0.79) across all samples (data not shown). The cDNAs were then used for real-time PCR using TaqMan chemistries for human phosphoglycerate kinase 1 (part 4333765) and amphiregulin (assay ID HS00155832_m1). Levels of amphiregulin expression relative to HCC4006 were determined using the ddCt method and ABI 7500 Fast System SDS Software.

**Immunohistochemical analyses.** Cultured cells from cell lines (HN11 and HN28) were pelleted by centrifugation, embedded in a fibrinogen matrix, fixed in formalin, and processed and embedded as a histologic section. Sections (5 μm) of paraffin-embedded tissue (either whole sections or embedded cell pellets) were cut onto PLUS slides and deparaffinized for 10 min at room temperature in Hemo-De (Scientific Research). The sections or embedded cell pellets were stained with anti-amphiregulin antibody (AF262; R&D Systems) and diluted 1:50 in TBS. Slides were then rinsed briefly in TBS and washed in 500 mL PBS with 10 mL of 1% BRIJ for 10 min. Slides were then incubated with 25 μL biotinylated rabbit anti-goat antibody (Vector) in 75 μL normal rabbit serum/5 mL TBS for 30 min, rinsed and washed in TBS/BRIJ as above, incubated with avidin-biotin complex (Vector) for 30 min, rinsed and washed in TBS/BRIJ as above, incubated with diaminobenzidine (DAKO) for 5 min, rinsed in tap water, and counterstained with Mayer's hematoxylin, dried, and coverslipped according to standard protocols.

Stained slides were interpreted by a pathologist (N.I.L.) who was blinded to treatment outcome at the time of review. Granular cytoplasmic staining was interpreted as positive. Artifactual nucleolar staining was seen in some tissues and was not interpreted as positive. Cancer cells were scored for intensity (scale 0-4+) and percentage of cells staining (0-11% in 5% increments); a scoring index was calculated by multiplying the modal intensity by the percentage of cells staining to give a product ranging from 0 to 400 as described previously (39).

**NSCLC patients.** Tumor specimens from EGFR wild-type NSCLC patients (n = 24) treated with either erlotinib (n = 18) or gefitinib (n = 6) were obtained as part of a phase II clinical trial of erlotinib for chemotherapy-naive patients ≥70 with advanced NSCLC or as part of the gefitinib expanded access program included in our prior studies and have been published previously (24, 38, 40). Only NSCLC patients that had sufficient tumor material for EGFR genotyping, who were EGFR wild-type, had stable or progressive disease as their best clinical response to therapy as defined by RECIST, and had sufficient tumor material for immunohistochemical analyses were included in this study. Based on these requirements, we identified 24 NSCLC patient specimens that met all of these criteria. All patients provided written informed consent and the study was approved by the Dana-Farber Cancer Institute institutional review board. Eighteen of the patients were chemotherapy-naive and treated on a phase II clinical trial of erlotinib (24), whereas 6 had received either 1 (n = 3) or 2 (n = 3) prior chemotherapy regimens and were treated on the gefitinib expanded access clinical trial (40). All patients were followed and treated according to protocol defined criteria. Best clinical response to treatment was determined using Response Evaluation Criteria in Solid Tumors (41). Duration of stable disease was calculated from the date of enrollment to the date of progression. All patients were evaluable for response and none discontinued treatment due to treatment-related toxicity.

**Statistical analyses.** Statistical analyses were done using StatView version 5.01 (SAS Institute). P < 0.05 was considered to be statistically significant. All statistical tests were two-sided.

**Results**

**NSCLC and HNSCC cell lines produce varying amounts of EGFR ligands.** We first analyzed the concentrations of EGFR ligands amphiregulin, EGF and TGF-α, in the cell culture medium of 14 NSCLC and 4 HNSCC cell lines using an ELISA. Four NSCLC cell lines contained EGFR mutations (E746_A750del in PC-9 and HCC827 cells, L747_E749del in HCC4006 cells, and L858R in H3255 cells), whereas the remaining cells were EGFR wild-type. All except H358 were also KRAS wild-type. Although amphiregulin concentrations varied, it was detected in the majority of EGFR wild-type cell lines, whereas the 4 EGFR mutant cell lines produced undetectable (n = 1) of very low (n = 3) levels of amphiregulin (Fig. 1A). This is consistent with prior observations that show that EGF
mutations lead to constitutive activation of the receptor in a ligand-independent fashion (5). Only 2 cell lines, H1666 and Calu-3, produced any detectable amount of TGF-α, whereas it was undetected in the remaining cell lines. We have shown previously that H1666 produces TGF-α (23). EGFR production was detected (Fig. 1A) at very low levels in only 6 cell lines (HN11, H1666, H112, H358, HN13, and H661). We did not detect TGF-α or EGFR in the supernatant of any of the EGFR mutant cell lines (Fig. 1A). We also evaluated for the presence of heregulin in the cell culture medium, but only 2 cell lines (HN12 and HCC95) produced any significant amounts of heregulin (Supplementary Fig. S1). We thus focused our studies on amphiregulin.

Exogenous amphiregulin can stimulate the proliferation of murine keratinocytes or the GEO colon cancer cell line (42, 43). The observed stimulation in cell proliferation is concentration dependent and occurs most effectively when amphiregulin concentrations are ≥15 to 20 pmol/L (42, 43). Based on these findings, we chose 20 pmol/L as the minimum biologically significant concentration of amphiregulin. We divided the EGFR wild-type cell lines into two categories: a high amphiregulin group (n = 7; HN11, H1666, H112, H358, Calu3, HN13, and H1648), in which we detected ≥20 pmol/L amphiregulin, and a low amphiregulin group (n = 7; H1437, HCC15, HCC193, HCC95, H661, HN28, and H2126), in which we detected <20 pmol/L amphiregulin in the cell culture medium. We next used quantitative PCR to examine for any differences in amphiregulin mRNA produced by these cell lines (Fig. 1B). We compared the level of amphiregulin expression in the different cell lines with that in HCC4006 as this cell line produced almost undetectable levels of amphiregulin as detected by ELISA (Fig. 1A). We detected significantly higher levels (P < 0.05, paired t test) of amphiregulin mRNA in the high amphiregulin-producing cell lines defined by ELISA compared with the low amphiregulin group (Fig. 1B). However, there was not a perfect correlation as in some cell lines (e.g., HCC827 or H2126) we detected significant production of amphiregulin mRNA but not the protein by ELISA (Fig. 1A and B). These differences could result from the production of a pro-ligand that is produced but not cleaved and thus not secreted into the cell culture medium.

Gefitinib and cetuximab effectively inhibit the growth of NSCLC and HNSCC cell lines that produce amphiregulin.

We next studied the effects of gefitinib and cetuximab on the growth of 9 NSCLC and 5 HNSCC cancer cell lines in vitro using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl) 2H-tetrazolium, inner salt assay (data not shown). In contrast, in the low amphiregulin group, cetuximab and gefitinib lead to either no significant increases or only minor changes in the G1-S

![Fig.1. Production of EGFR ligands in EGFR mutant and wild-type cancer cell lines. A: detection of EGFR ligands by ELISA. The EGFR ligands amphiregulin, TGF-α, and EGF were detected by ELISA (See Materials and Methods) from EGFR mutant (n = 4) or wild-type (n = 14) cell lines. Amphiregulin production is detected in a significant portion of EGFR wild-type cell lines, whereas EGF and TGF-α are rare. Concentrations of amphiregulin above 20 pmol/L have been associated with cell proliferation (42, 43). B: detection of amphiregulin mRNA by quantitative PCR. The high amphiregulin-producing cell lines (blue) express significantly (P < 0.05, paired t test) higher quantities of amphiregulin mRNA than cell lines, which produce low amounts of amphiregulin (red). Green, EGFR mutant cell lines. Y-axis, fold changes compared with HCC4006.](www.aacrjournals.org)
phase of the cell cycle consistent with the lack of growth inhibition in this group of cell lines (Fig. 1A). In all of the cell lines in which there was an increase in the G1-S phase following cetuximab or gefitinib treatment, there were corresponding decreases in the S and G2-M phases (data not shown).

Gefitinib and cetuximab preferentially inhibit ERK1/2 signaling in amphiregulin-producing cell lines. We next examined EGFR signaling in the two groups of cell lines at baseline and following treatment with cetuximab or gefitinib. The high amphiregulin-producing cell lines expressed phospho-EGFR more frequently and to a greater degree than the low amphiregulin producing cells (Fig. 3). In addition, Western blotting demonstrated that the amphiregulin producing cells appeared to express greater amounts of total EGFR. However, there were no differences in EGFR copy number between these two groups of cell lines (mean EGFR copy number high amphiregulin vs. low amphiregulin producing cell lines 2.30 vs. 2.05; \( P = 0.44 \)). Five of the six high amphiregulin producing cell lines also expressed phospho-ERBB3 compared to only 1/6 of the low amphiregulin producing cell lines (HCC95). HCC95 was one of the two cell lines that also produced heregulin (Supplementary Fig. S1). Expression of phospho-ERBB3 has been previously been shown to be associated with efficacy of gefitinib in vitro and all EGFR mutant sensitive NSCLC cell lines express phosphorylated ERBB3 (29). Furthermore, gefitinib treatment of EGFR mutant NSCLC cell lines leads to down regulation of ERBB3 and AKT phosphorylation (29, 46).

We next examined the effects of gefitinib and cetuximab on EGFR signaling in 2 high amphiregulin-producing cell lines (HN11 and H1648) and in 1 low amphiregulin-producing cell line (HN28). In both HN11 and H1648, gefitinib treatment led only to a minimal inhibition of ERBB3 and Akt phosphorylation (Fig. 4). In contrast, gefitinib led to a more significant decrease in ERK1/2 phosphorylation in these cell lines and an increase in p27 expression, which is a known mediator of G1-S arrest. The effects on p27 were more pronounced following 24 h of drug exposure consistent with the results observed by fluorescence-activated cell sorting analyses (Fig. 2B). Similarly, cetuximab treatment did not result in inhibition of ERBB3 or Akt phosphorylation but lead to a down-regulation of ERK1/2 phosphorylation and an increase in p27 expression. Neither gefitinib nor cetuximab had a significant effect on AKT or ERBB3 phosphorylation (29). However, gefitinib treatment of EGFR mutant NSCLC cell lines leads to downregulation of ERBB3 and AKT phosphorylation (29, 46).

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We further evaluated the role of amphiregulin on the growth of HNSCC and NSCLC cell lines by using an anti-amphiregulin neutralizing antibody (Fig. 5A). We evaluated 2 concentrations (1 and 5 \( \mu \)g/mL) of the amphiregulin antibody and compared it to an isotype control IgG using 4 cell lines (3 high amphiregulin-producing cell lines (HN11, HN12, and H358) and 1 low amphiregulin-producing cell line (HN28)). Both concentrations of the anti-amphiregulin neutralizing antibody but not the control antibody significantly (\( P < 0.05 \), paired \( t \) test, for all 3 cell lines) inhibited the growth of all 3
amphiregulin-producing cell lines (Fig. 5A), whereas both antibodies did not alter the growth of HN28 cells. There were no differences in growth between the two concentrations of the anti-amphiregulin antibody (Fig. 5A). Treatment of HN11 cells with the anti-amphiregulin antibody also resulted in a decrease in ERK1/2 phosphorylation as well as a decrease in SHC phosphorylation (Fig. 5B). SHC is a known mediator of EGFR-dependent ERK1/2 signaling (47). We also inhibited amphiregulin production in HN11 and HN28 cells using an amphiregulin-specific siRNA, which was associated with a significant \( p < 0.01 \) paired \( t \) test growth inhibition in HN11 but not HN28 cells (Fig. 5C). We further examined the effects of CI-1040, a specific MEK inhibitor, in 3 high (HN11, H1648, and H1661) and 3 low (HN28, H2126, and H661) amphiregulin-producing cell lines to determine if it could alone recapitulate the effects of gefitinib or cetuximab. If growth inhibition by cetuximab and gefitinib or by inhibition of amphiregulin were mediated by down-regulation of multiple signaling pathways (in addition to ERK1/2 signaling), then inhibition of ERK1/2 alone may not be sufficient to cause growth arrest. However, all 3 amphiregulin-producing cell lines were growth inhibited by CI-1040 (IC\(_{50}\) values, 30-64 nmol/L; Supplementary Table S1), whereas the low amphiregulin-producing cells were relative more resistant with a \( >10 \)-fold IC\(_{50}\) values (1-8 \( \mu \)mol/L; Supplementary Table S1). Treatment with CI-1040 also led to a dose-dependant inhibition of ERK1/2 phosphorylation in HN11 cells, an increase in p27 expression and to G\(_{1}\)-S cell cycle arrest (Fig. 5D; data not shown). Together, these findings suggest that, in the high amphiregulin-producing cell lines, gefitinib, cetuximab, or inhibition of amphiregulin itself results in growth inhibition predominantly through down-regulation of ERK1/2 signaling. To further determine a role for amphiregulin in the proliferation of NSCLC and HNSCC cell lines, we evaluated the effects of exogenous amphiregulin (10 ng/mL) on the growth of high (HN11 and H358) and low (HN28 and H661) amphiregulin-producing cells (Fig. 5E). Exogenous amphiregulin lead to a significant \( P < 0.05 \) paired \( t \) test proliferation of only HN11 and H358 cells (Fig. 5E), which was associated with activation of ERK1/2 signaling in HN11 but not HN28 cells (Fig. 5F). These findings further support the role of amphiregulin in the growth of NSCLC and HNSCC cells that have autocrine production of amphiregulin.

**Amphiregulin expression can be detected by immunohistochemistry and is associated with stable disease in EGFR wild-type NSCLC patients treated with erlotinib or gefitinib.** Our studies in EGFR wild-type NSCLC and HNSCC suggest that in a subset of these cell lines EGFR is activated by an autocrine production of amphiregulin, which promotes cell growth. Furthermore, direct inhibition of amphiregulin by an antibody or a siRNA, or its binding to EGFR (by cetuximab) or inhibition EGFR kinase activity (by gefitinib), leads to growth inhibition through cell cycle arrest. These in vitro findings raise the possibility that the presence of amphiregulin in tumor specimens could be used as a potential biomarker to help choose NSCLC patients likely to develop stable disease from EGFR-targeted therapies. To evaluate this possibility, we evaluated the expression of amphiregulin using immunohistochemistry. We first evaluated amphiregulin expression using immunohistochemistry in HN11 (high amphiregulin-expressing) and HN28 (low
Fig. 5. Amphiregulin modulates growth of NSCLC and HNSCC cell lines. **A**, amphiregulin neutralizing antibody inhibits cell proliferation of amphiregulin-producing cell lines. High (HN11, HN12, and H358) or low (H28) amphiregulin-producing cells were either untreated, treated with the amphiregulin neutralizing antibody at 1 or 5 μg/mL, or treated with a control IgG for 6 days. Mean ± SD. *, *P* < 0.05, compared with control or IgG treated.

**B**, Western analysis of HN11 cells treated with cetuximab, gefitinib, or the amphiregulin-neutralizing antibody. The cells were treated for 6 h. Cells were lysed and the indicated proteins were detected by immunoblotting.

**C**, amphiregulin siRNA inhibits growth of HN11 cells. Control or amphiregulin siRNAs were transfected into HN11 cells (see Materials and Methods). Cell growth was assayed 6 days following (left) transfection. The amphiregulin siRNA led to a significant decrease in amphiregulin expression (right) as measured by quantitative PCR (see Materials and Methods). ***, *P* < 0.01, compared with control. Y-axis, fold changes compared with HCC4006.

**D**, Western analysis of HN11 cells treated with CI-1040. The cells were treated with CI-1040 at indicated concentrations for 6 h. Cells were lysed and the indicated proteins were detected by immunoblotting.

**E**, amphiregulin stimulates the growth of HN11 and H358 cells. Control or amphiregulin-treated (10 ng/mL) cells were counted in triplicate 6 days following treatment. *, *P* < 0.05.

**F**, Western analysis of HN11 and HN28 cells treated with amphiregulin. The cells were treated with amphiregulin (10 ng/mL) for 3 h. Cells were lysed and the indicated proteins were detected by immunoblotting.
amphiregulin-expressing) cell lines (Supplementary Fig. S2). Both immunohistochemistry and flow cytometry detected significant cytoplasmic and cell surface expression of amphiregulin in HN11 but not HN28 cell lines. We thus evaluated the expression of amphiregulin in formalin-fixed, paraffin-embedded tumor specimens from NSCLC patients using the conditions optimized with the HN11 and HN28 cell pellets (Fig. 6A). We observed different quantities of amphiregulin expression and graded these from 0 to 3+. No 4+ staining specimens were identified. We used a previously established scoring system an multiplied the intensity of staining with the degree of tumor cell staining (39). We evaluated amphiregulin expression by immunohistochemistry in tumor specimens from 24 NSCLC patients, all of whom were EGFR wild-type, had been treated with single-agent erlotinib (n = 18) or gefitinib (n = 6), and had achieved either stable disease (n = 10) or

Fig. 6. A, expression of amphiregulin by immunohistochemistry in NSCLC tumor samples. The expression intensity of amphiregulin ranges from 0 to 3+. No specimens that stained 4+ were identified (data not shown). B, summary of amphiregulin scores in NSCLC patient specimens. The amphiregulin scores (see Materials and Methods) are shown for patients with stable disease (red; left) or progressive disease (blue; right) following erlotinib or gefitinib treatment. A score of 100 (black line) separates the two categories (P = 0.0005, Fisher’s exact test).
activating mutations are only found in the minority (progressive disease (<100; 13 of 14; \(P = 0.028\), Wilcoxon signed-rank test). We also used a cutoff value to 100, which could separate patients who developed stable disease (mean, 145; range, 20-270) than in patients with progressive disease (mean, 40.7; range, 0-140; \(P = 0.0005\), Fisher’s exact test). There was no difference in the duration of stable disease in patients with and without \(KRAS\) mutations (data not shown).

### Discussion

EGFR-targeted therapies are effective treatments in a variety of malignancies including NSCLC, HNSCC, and colorectal cancers (11, 12, 48). However, not all patients derive benefit from these agents and the degree of benefit varies among different patient subsets. Several studies have focused on identifying molecular markers associated with benefit from EGFR-targeted therapies with the ultimate goal that such markers could be used to prospectively select appropriate patient subsets for therapy. In patients with NSCLC, \(EGFR\) activating mutations are associated retrospectively and prospectively with a 60% to 80% likelihood of dramatic tumor regressions and prolonged times to disease progression following treatment with EGFR TKIs gefitinib or erlotinib (19–22). However, in Caucasian NSCLC patients, \(EGFR\)-activating mutations are only found in the minority (\(\sim 10-15\%\)) of all NSCLC patients (6). A significant portion of NSCLC patients develop stable disease following treatment with \(EGFR\) inhibitors (11). This is an equally important clinical benefit from EGFR-targeted therapy especially for a disease where the median survival is 8 to 10 months for advanced NSCLC patients (49). Furthermore, recent studies of imatinib and sunitinib treatment in gastrointestinal stromal tumors suggested that survival of patients that developed stable disease was similar to those that developed tumor shrinkage (50, 51). However, no biomarkers to help identify patients likely to develop stable disease following EGFR TKI therapy have been developed.

In the current study, we evaluated EGFR ligands amphiregulin, EGF, and TGF-\(\alpha\), in \(EGFR\) wild-type NSCLC and HNSCC and their relationship with the efficacy of the EGFR-directed antibody cetuximab and the EGFR TKI gefitinib. Our studies suggest that the growth of lung cancer and head and neck cancer cell lines that produce amphiregulin can be inhibited by either an \(EGFR\) kinase inhibitor or an \(EGFR\)-directed antibody. Amphiregulin stimulates the growth of these gefitinib/cetuximab-sensitive cell lines and inhibition of amphiregulin alone (either by a neutralizing antibody or by siRNA) is sufficient to inhibit cell growth. Furthermore, unlike in \(EGFR\) mutant NSCLC cell lines in which gefitinib leads to apoptosis, both gefitinib and cetuximab treatment lead to cell cycle arrest (23, 26). Thus, cell cycle arrest could be considered an \textit{in vitro} equivalent of stable disease following drug treatment. In fact, our limited series of gefitinib- or erlotinib-treated \(EGFR\) wild-type NSCLC patients suggest that patients whose tumors express significant amounts of amphiregulin by immunohistochemistry are more likely to have stable disease rather than disease progression (Table 1). There were no differences in \(EGFR\) expression by immunohistochemistry in tumors from patients that developed stable disease compared with disease progression following gefitinib treatment (data not shown). Our clinical findings on amphiregulin expression are hypothesis-generating and need to be further validated in prospective clinical studies and furthermore in tumor specimens from NSCLC patients that have not been treated with an \(EGFR\) inhibitor to determine whether amphiregulin expression is also a prognostic factor.

Amphiregulin mRNA expression as assessed by microarray analyses has been associated with disease control rate (combination of response and stable disease) and longer progression-free survival in colorectal cancer patients treated with single-agent cetuximab (27). Our current study shows that amphiregulin expression assessed by mRNA, protein assay, and immunohistochemistry maybe an appropriate biomarker for identifying patients who will benefit from treated with cetuximab in both NSCLC and HNSCC and that it is also a biomarker of stable disease for \(EGFR\) kinase inhibitors in NSCLC. Thus, unlike \(EGFR\) mutations, which are almost uniformly restricted to NSCLC, amphiregulin expression may serve as a biomarker across different cancers (lung, head and neck, and colorectal cancers) in which \(EGFR\)-targeted therapies are widely in clinical use. Prior studies have also evaluated serum levels of amphiregulin in NSCLC patients treated with gefitinib (52). In these studies, high levels of serum amphiregulin were associated with a lack of benefit from gefitinib treatment (52). It is possible that there are differences in the local tumor concentrations of amphiregulin that are not reflected in the systemic circulation, which accounts for these different observations. The correlation between tumor

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. patients ((N = 24))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
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</tr>
<tr>
<td>Male</td>
<td>10</td>
</tr>
<tr>
<td>Female</td>
<td>14</td>
</tr>
<tr>
<td>Histology</td>
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</tr>
<tr>
<td>Adenocarcinoma</td>
<td>14</td>
</tr>
<tr>
<td>Adenocarcinoma with BAC</td>
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<tr>
<td>SCC</td>
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<tr>
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</tr>
<tr>
<td>Prior systemic chemotherapy</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>18</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>(EGFR) mutation</td>
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<td>Mutant</td>
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</tr>
<tr>
<td>Wild-type</td>
<td>24</td>
</tr>
<tr>
<td>(KRAS) mutation</td>
<td></td>
</tr>
<tr>
<td>Mutant</td>
<td>6</td>
</tr>
<tr>
<td>Wild-type</td>
<td>15</td>
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<td>3</td>
</tr>
<tr>
<td>(EGFR) TKI treatment</td>
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</tr>
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<td>Erlotinib</td>
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</tr>
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<td>Gefitinib</td>
<td>6</td>
</tr>
<tr>
<td>Clinical outcome</td>
<td></td>
</tr>
<tr>
<td>Stable disease</td>
<td>10</td>
</tr>
<tr>
<td>Disease progression</td>
<td>14</td>
</tr>
</tbody>
</table>
expression of amphiregulin and serum amphiregulin was only modest in the colorectal cancer study (27). Further studies on the role of amphiregulin in NSCLC should include both tumor- and serum-based studies to help determine which detection method is most predictive clinically. In addition, at least one preclinical study suggested that exogenous amphiregulin could in fact result in an increase in gefitinib IC50 in NSCLC cell lines (53). These differences may be a result of the use of different cell lines and/or to the role of amphiregulin in cancer cells, which have an amphiregulin autocrine loop, such as those in the current study, compared with cell lines where its production is induced by a growth factor receptor or where it is added exogenously (53).

KRAS mutations are associated with lack of response to EGFR-targeted therapies in both NSCLC treated with gefitinib or erlotinib or in colorectal cancer treated with cetuximab (27, 54). Consistent with these findings, none of the patients treated with erlotinib or gefitinib with a KRAS mutation had a radiographic response to treatment (Table 1). However, KRAS mutations were detected in 4 of 9 patients that had stable disease and tumors from these patients expressed amphiregulin. Although limited by the number of patients, the duration of stable disease did not significantly differ between KRAS wild-type and KRAS mutant patients (data not shown). Similarly, the H358 cell line contains a KRAS mutation (Fig. 3). Thus, it remains possible that although KRAS mutations are associated with a lack of radiographic responses with gefitinib or erlotinib treatment, some patients, especially those whose tumors concurrently express amphiregulin, may still benefit from gefitinib or erlotinib treatment by developing stable disease. This hypothesis needs to be further evaluated in prospective clinical trials.

In the EGFR wild-type cell lines that express abundant amphiregulin, gefitinib and cetuximab treatment both led to significant inhibition of ERK1/2 phosphorylation. In contrast, gefitinib only modestly inhibited ERBB3 and Akt phosphorylation, whereas cetuximab had no effect on either ERBB3 or Akt phosphorylation (Fig. 4). Furthermore, inhibition of ERK1/2 signaling alone by CI-1040 was sufficient to cause growth inhibition and G1/S arrest in the amphiregulin-producing cell lines. The findings are consistent with the lack of apoptosis following treatment with gefitinib and cetuximab but are different than what is observed in EGFR mutant NSCLC cell lines. In EGFR mutant NSCLC cell lines, gefitinib effectively inhibits both PI3K/Akt signaling (by inhibiting ERBB3 and Akt phosphorylation) and ERK1/2 signaling (29, 46). In the current study, we did not observe a down-regulation of ERBB3 phosphorylation in the EGFR wild-type cell lines. The mechanism(s) accounting for these observed differences in EGFR mutant and wild-type NSCLC cell lines remains to be determined but may provide important insights into EGFR signaling and are currently being evaluated. They also raise the possibility that a combination therapeutic strategy of a PI3K inhibitor with an EGFR-targeted agent in amphiregulin-expressing NSCLC or HNSCC may be an effective combination therapeutic strategy and should be evaluated preclinically and in clinical trials.

Disclosure of Potential Conflicts of Interest

P.A. Jänne is a consultant with AstraZeneca and Genentech and has received honoraria from Roche; D.M. Jackman and B.E. Johnson are consultants with Genentech. B.E. Johnson and P.A. Jänne are part of a patent application on EGFR mutations.

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Kimio Yonesaka, Kreshnik Zejnullahu, Neal Lindeman, et al.


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