Abstract

Significant improvements in the outcome of non–small cell lung carcinoma (NSCLC) have been reported in patients treated with the epidermal growth factor receptor (EGFR) inhibitor, erlotinib. To discover biomarkers for the enrichment of patients who might benefit from treatment, a pharmacogenomic approach was used to identify gene signatures that may predict erlotinib activity using \textit{in vitro} model systems. Erlotinib sensitivity in a panel of 42 NSCLC cell lines was determined by EGFR-mediated proliferative potential, \textit{EGFR} mutations, and/or \textit{EGFR} gene amplification, thus supporting an underlying biological mechanism of receptor activation. A strong multigene signature indicative of an epithelial to mesenchymal transition (EMT) was identified as a determinant of insensitivity to erlotinib through both supervised and unsupervised gene expression approaches. This observation was further supported by expression analysis of classic EMT marker proteins, including E-cadherin and vimentin. To investigate the clinical relevance of these findings, we examined expression of the epithelial marker E-cadherin by immunohistochemistry on primary tumor samples from subjects enrolled in a randomized NSCLC clinical trial in which erlotinib in combination with chemotherapy previously failed to show clinical activity. The majority (75%) of the 87 subjects tested showed strong E-cadherin staining and exhibited a significantly longer time to progression (hazard ratio, 0.37; log rank $P = 0.0028$) and a nonsignificant trend toward longer survival with erlotinib plus chemotherapy treatment versus chemotherapy alone. These data support a potential role for EMT as a determinant of EGFR activity in NSCLC tumor cells and E-cadherin expression as a novel biomarker predicting clinical activity of the EGFR inhibitor erlotinib in NSCLC patients.

Activation of the epidermal growth factor receptor (EGFR) via mutation, amplification, and/or overexpression of EGFR ligands has been implicated in a wide variety of epithelial cancers (1, 2). In non–small cell lung carcinoma (NSCLC), EGFR is expressed in the majority of tumor cells and selective blockade of the EGFR via small molecule tyrosine kinase inhibitors has shown clear activity in preclinical NSCLC models (3). Erlotinib and gefitinib, small molecule inhibitors of EGFR, have been extensively studied in NSCLC patients, either alone or in combination with chemotherapy. Only treatment with erlotinib (Tarceva, a quinazoline derivative EGFR tyrosine kinase inhibitor) in second/third line as a monotherapy has been shown to improve the survival of patients with advanced NSCLC (4). Although there have been multiple attempts to prove the clinical benefit of EGFR small molecule tyrosine kinase inhibitors in the first-line setting in combination with chemotherapy, none of these have been successful (5). One potential explanation could be that no patient selection strategies have been used in these trials to identify tumors with an activated EGFR pathway, which may have a better chance of responding to treatment. This may be particularly relevant when three drug combinations are being evaluated because the incremental benefit is likely to be small and, therefore, difficult to show without very large clinical trials. Thus, the discovery of molecular biomarkers that identify the subsets of NSCLC patients who are most likely to benefit from therapy targeted to EGFR has become an important area of investigation.

The status of the \textit{EGFR} gene and/or protein in comparison with clinical activity of EGFR tyrosine kinase inhibitors has been a focus of recent research. Several publications have reported \textit{EGFR} mutations as predictors of response (tumor shrinkage) after treatment with gefitinib or erlotinib (6–8). The prevalence of these mutations in Caucasians, however, is low (<15% in Caucasians; ~30–40% in Asians) and is unlikely to explain the majority of the survival benefit patients receive from...
treatment with erlotinib (4, 9). In a recent randomized, placebo-controlled phase III study evaluating erlotinib in NSCLC patients following failure of first-line or second-line chemotherapy, tumor-specific increase in copy number of the EGFR gene was shown to correlate with increased survival in NSCLC patients comparing erlotinib treatment with placebo (10). In the same study, a weaker correlation of EGFR protein expression with survival benefit was determined by immunohistochemical staining. Taken together, it is reasonable to assume that there are at least three mechanisms for EGFR activation supported by correlative clinical data: mutations in the EGFR kinase domain, increase in copy number of the EGFR gene, and overexpression of the EGFR protein. In addition, other EGFR activating mechanisms, such as overexpression of EGFR ligands (11) or heterodimerization with overexpressed coreceptors, cannot be excluded (12, 13), but have yet to be correlated with clinical benefit from EGFR inhibition.

Here, we identify a biological signature that would allow for the detection of erlotinib-sensitive NSCLC tumor cells with an activated EGFR pathway, independent from the mechanism leading to activation. We find that a gene signature indicative of an epithelial versus mesenchymal phenotype is not only predictive of erlotinib-mediated growth inhibition in NSCLC tumor cell lines but may also serve as a potential biomarker in predicting erlotinib clinical activity in NSCLC patients.

Materials and Methods

Cell culture experiments. Cell lines were obtained from either the American Type Culture Collection (Manassas, VA) or the National Cancer Institute Division of Cancer Treatment and Diagnosis tumor repository (Frederick, MD). All cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO) and 2 mmol/L L-glutamine. For erlotinib ICS determination, cells were plated in triplicate at 5 × 10^4 per well in 96-well plates in RPMI containing 0.5% fetal bovine serum (assay medium) overnight before stimulation with assay medium containing 3 nmol/L transforming growth factor-α (TGF-α). Erlotinib (Genentech, Inc., South San Francisco, CA) was added at multiple concentrations (10 μmol/L maximal) and 72 hours later, cell viability was measured using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI). The concentration of erlotinib resulting in the 50% inhibition of cell viability was calculated from a four-variable curve analysis and was determined from a minimum of three experiments. For assessing ligand-driven growth potential, cells were cultured for 72 hours in assay medium containing various concentrations of TGF-α (0.1-30 nmol/L) before determining cell viability as above. Stimulus indices were determined by dividing the average cell viability units of TGF-α-stimulated cultures by the average cell viability units of unstimulated cultures and are reported as the maximal stimulation index, independent of the concentration of TGF-α. For caspase activity determination, cells (1.2 × 10^5) were stimulated in assay medium containing 3 nmol/L TGF-α in the presence of a dose response of the EGFR inhibitors erlotinib or C225 (Calbiochem, La Jolla, CA). Caspase-3/7 activity was measured using the Apo-ONE Homogeneous Caspase-3/7 Assay (Promega) at either 24 hours (erlotinib) or 48 hours (C225) following treatment. Results are presented as a percentage of caspase activity relative to control (no drug treatment) cultures.

EGFR and KRAS gene status. For assessing sequence variations, PCR amplifications of genomic DNA isolated from NSCLC cell lines were carried out using nested primers to amplify EGFR exons 18 to 21 and KRAS exon 2, before the direct sequencing of products, as previously described (8). Genomic amplifications of the EGFR gene were determined by fluorescence in situ hybridization. NSCLC cell line tissue microarrays were constructed from formalin-fixed, paraffin-embedded cell pellets as previously described (14). Fluorescence in situ hybridization analysis was carried out on 3 μm sections using the LSI EGFR Spectrum Orange/CEP 7 Spectrum Green probe set (Vysis, Downers Grove, IL), as instructed in the protocol of the manufacturer. Fluorescence in situ hybridization results were manually scored in a blinded fashion and amplification was defined as an EGFR/CEP7 ratio >2.0, as described (15).

Quantitative expression analyses of HER receptors/ligands. HER family member receptor and ligand transcript expression levels were assessed by quantitative reverse transcription-PCR using standard Taqman techniques. Transcript levels were normalized to the housekeeping gene β-glucuronidase and results are expressed as normalized expression values (2^-ΔCt). The primer/probe sets for β-glucuronidase are forward, 5'-TGGTTGGAGACGCTCATTGGA-3'; reverse, 5'-GCACT-CTCCTCGCTGACTGT-3'; and probe, 5'(VIC)-TTTCCGATTCTC- GACT-(MGBNFQ)-3'. Primer/probe sets for the HER receptor family members and EGFR ligands were purchased from Applied Biosystems (Foster City, CA). The determination of EGFR cell surface expression levels was carried out by flow cytometry. Cells (1 × 10^6) were directly stained with the EGFR-FITC conjugated antibody (P54416F, Biodign, Saco, ME) or a murine isotype-FITC conjugated control (USB), washed, resuspended in 1 mL buffer containing 1 μg/mL propidium iodide, and live cells were subsequently analyzed for mean shift in fluorescence on a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ). Heat maps to visualize quantitative mRNA expression and mean shift in fluorescence values were generated using the software Cluster (16). Student’s t test was used to calculate statistically significant differences in gene/protein expression between erlotinib “sensitive” and “insensitive” groups.

Microarray studies. Basal gene expression analysis of NSCLC cell lines was carried out using RNA extracted from subconfluent cell cultures on the Affymetrix (Santa Clara, CA) microarray platform (HGU133Plus_2.0 chips). Preparation of complementary RNA, array hybridizations, and subsequent data analysis were carried out using the protocol of the manufacturer as described (17).

For unsupervised hierarchical clustering analysis, gene expression values were subjected to a variation filter to exclude genes with minimal variation across the samples being analyzed. This was done by excluding the maximum and minimum value for each probe set and then testing for a fold change (max/min > 10) and absolute variation (max-min > 500) over samples, and excluding genes not obeying both conditions. In addition, a single probe was selected to represent a gene, although probe sets with no Unigene identifier were assumed to represent different genes. Data preprocessing involved log transforming and median centering gene expression values and average linkage clustering was carried out using Cluster and TreeView software (16).

For the supervised identification of marker genes exhibiting differential expression between sensitive and insensitive cell lines, cell lines were classified as sensitive or insensitive based on a 0.85 SD window around the median erlotinib IC_{50} cell lines exhibiting IC_{50} < 2.0 μmol/L were classified as sensitive and IC_{50} >8.0 μmol/L as insensitive. Cell lines with intermediate erlotinib sensitivities (2.8 μmol/L IC_{50}) were excluded from the analysis. Gene expression values were subjected to a variation filter to exclude probe sets with minimal variation across the samples, and a signal to noise metric was subsequently used to identify marker probe sets using GenePattern software (http://www.broad.mit.edu/cancer/software/genepattern/).

To examine the expression of marker genes in primary tumors, gene expression profiles for 80 NSCLC tumors exhibiting ≥50% tumor content were extracted from the commercially available database, BioExpress (GeneLogic, Gaithersburg MD). A single probe set was selected for each gene, although probe sets with no Entrez gene identifier assigned were assumed to represent different genes. Only probe sets represented in both primary tumor and cell line data sets were considered. These included probe sets for 35 genes represented in the erlotinib-sensitive class and for 34 genes represented in the

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erlotinib-insensitive class. The average Pearson correlation coefficient was calculated for (a) all pairs of sensitive genes, (b) all pairs of insensitive genes, and (c) all pairs of genes in which one was from the sensitive set and one was from the insensitive set. None of the equivalent average correlations for 100,000 random sets of 35 and 44 genes met or exceeded those for the real sets.

**Western blot analysis.** NSCLC cell lines were lysed for 1 hour in Cell Extraction Buffer (BioSource, Camarillo, CA) supplemented with protease and phosphatase inhibitors and precleared by centrifugation before protein quantification using the BCA Protein Assay kit (Pierce, Rockford, IL). Proteins (20 μg) resolved by SDS-PAGE were electrophoretically transferred to polyvinylidene difluoride membrane and Western blots were carried out using standard techniques. The following primary antibodies were used in this study, E-cadherin (clone 67A4, Santa Cruz Biotechnology, Santa Cruz, CA), γ-catenin (clone H-1, Santa Cruz Biotechnology), N-cadherin (H-63, Santa Cruz Biotechnology), vimentin (clone RV202, Becton Dickinson), glyceraldehyde-3-phosphate dehydrogenase (clone 9484, Abcam, Inc., Cambridge, MA).

**Immunohistochemistry.** Immunohistochemistry for E-cadherin was done on freshly cut 5-μm sections of tumor samples assembled on a tissue microarray. Following deparaffinization, antigen retrieval was done by pretreating with Target Retrieval Solution at 110°C for 20 minutes (DakoCytomation, Carpinteria CA). The pretreated sections were then incubated with primary mouse monoclonal IgG2 antibody against E-cadherin (clone 36, PharMingen) at a concentration of 1 μg/mL for 60 minutes at ambient temperature. Primary antibody bound to the sections was detected using biotinylated horse anti-mouse IgG and visualized using the avidin-biotin peroxidase complex technique (Vectorstain ABC Elite, Vector Laboratories) and dianibenzidine as chromagen. The intensity of E-cadherin membranous/cytoplasmic expression was scored as follows: 0, absent; 1, weak; 2, moderate; 3, strong. Positive staining was prospectively defined as intensity scores 2 and 3, and negative staining as intensity scores 0 and 1.

**Patient samples.** TRIBUTE, a phase III randomized, placebo-controlled trial conducted in the United States sponsored by Genentech enrolled 1,079 chemotherapy-naïve patients with locally advanced or metastatic (stage IIIB or IV) NSCLC to compare the survival of patients who received erlotinib administered concurrently with a regimen of carboplatin and paclitaxel (n = 539) to patients who received carboplatin and paclitaxel alone (n = 540). The primary efficacy end point was duration of survival and the secondary efficacy end points were time to progression, objective response rate as defined by RECIST, duration of response, and time to symptomatic progression. The erlotinib-containing arm did not show any advantage for survival, objective response rate, time to progression, and duration of response over carboplatin and paclitaxel alone in the overall study population (18).

The TRIBUTE clinical protocol was approved by Independent Review Boards for each participating site and by the U.S Food and Drug Administration. All patients gave written informed consent for participation in the clinical study. Patients were given the option of providing an additional written informed consent to allow release of their archival tumor samples for research purposes. Only a subset of samples from patients who provided the second, fully optional written informed consent for tumor tissue research was included in this study. A tissue microarray was constructed, which contained triplicate 0.6-mm-diameter tumor tissue cores taken from donor specimens embedded in paraffin blocks. One hundred twenty-seven patients provided blocks containing tumor in sufficient quantity for microarray construction. Some cases suffered tissue loss or lack of tumor cell representation in the tissue microarray sections, such that E-cadherin immunohistochemistry scores were generated for 87 patients.

**Statistical analyses.** Demographic variables, best tumor response rate, time to progression, and overall survival were summarized by E-cadherin membranous/cytoplasmic (M/C) staining status and treatment received. Comparisons of best tumor response rate between the two treatment groups were made using the Fisher’s exact test. The Kaplan-Meier estimated curves for time to progression and overall survival were presented by E-cadherin M/C staining status. Median time to progression and overall survival were estimated using the Kaplan-Meier approach. The log-rank test was used to compare time to progression and overall survival between two treatment groups. Hazard ratios were estimated using Cox regression.

**Results**

**Characterizing epidermal growth factor receptor-mediated growth potential and erlotinib sensitivity in non–small cell lung carcinoma cell lines.** The growth inhibitory effect of erlotinib was investigated in a panel of 42 NSCLC-derived cell lines under low serum tissue culture conditions with added EGFR ligand (TGF-α, 3 nmol/L) to specifically activate EGFR. The degree of sensitivity to erlotinib was determined from a dose-response curve and the IC50 was assessed from multiple experiments (Fig. 1A, left axis). In addition, ligand-driven growth potential of each cell line was assessed at multiple concentrations of TGF-α, and the maximum degree of TGF-α-induced growth (indpendent of ligand concentration) was determined (Fig. 1A, right axis). NSCLC cell lines exhibited nearly a 2-log range in erlotinib IC50 (0.3 to ≥ 20 μmol/L), with the cell lines most responsive to the mitogenic effects of EGFR ligand also representing a majority of the erlotinib-sensitive lines. A few exceptions were noted, including the positive-control epidermal cell line, A431, which exhibits a submicromolar IC50, as reported previously (19). For subsequent analyses, cell lines were classified as sensitive or insensitive based on a 0.85 SD window around the median IC50, resulting in cell lines exhibiting IC50 < 2.0 μmol/L defined as sensitive and IC50 ≥ 8.0 μmol/L as insensitive. Cell lines exhibiting 2 to 8 μmol/L IC50 values were consequently defined to exhibit intermediate sensitivity.

To address the induction of apoptosis as a potential mechanism for erlotinib activity in the NSCLC cell line panel, caspase-3/7 activity was measured in response to erlotinib or the anti-EGFR antibody, C225, as a specificity control (Fig. 1B, summarized in Fig. 1A). Although erlotinib did not induce caspase activity in all erlotinib-sensitive lines (e.g., H1358), we did detect the dose-dependent induction of caspase activity in several sensitive NSCLC cell lines. The induction of apoptosis by erlotinib was likely specific to EGFR inhibition because cell lines exhibiting erlotinib-induced caspase activity always showed a similar effect with C225, albeit often at a somewhat lower level. In addition, one cell line (H1975) was identified that exhibited a dose-dependent induction of apoptosis with C225, but no apoptotic activity with erlotinib at any dose examined. We did not detect caspase induction in any of the tested cell lines that were insensitive to erlotinib. Thus, a subset of sensitive NSCLC cell lines can undergo apoptosis upon treatment with erlotinib.

**Characterizing determinants of epidermal growth factor receptor antagonist activity in non–small cell lung carcinoma cell lines.** Previously reported determinants of EGFR antagonist activity have included EGFR activating mutations, EGFR gene amplification, EGFR overexpression, KRAS status, and histopathologic subtypes (20). To determine whether such markers were associated with erlotinib sensitivity, we examined EGFR and KRAS status within the NSCLC cell line panel (Fig. 1A, bottom). EGFR activating mutations affecting the tyrosine kinase domain were identified in two NSCLC cell lines as reported previously, including an active site deletion in the
sensitive cell line H1650 (del.E746-A750) and a double mutation in the erlotinib-insensitive cell line H1975 (L858R, T790M; ref. 21). A novel genomic amplification in the EGFR gene was identified in H1838 cells by fluorescence in situ hybridization and the previously reported amplification in the control A431 cell line was confirmed (22). Both EGFR amplified cell lines were sensitive to the growth inhibitory effects of erlotinib; however, they exhibited minimal mitogenic response to exogenous ligand. KRAS mutations were identified in 31% of the NSCLC cell lines, which, interestingly, clustered primarily in cell lines of intermediate sensitivity (5-8 μmol/L IC50) to erlotinib. Two cell lines (Hop18 and H2122) with KRAS mutation showed exquisite sensitivity to erlotinib (IC50 < 1 μmol/L), with Hop18 also showing induction of apoptosis after erlotinib or C225 treatment. In addition, no specific histopathologic subtype of NSCLC cell line showed a striking correlation with sensitivity to erlotinib.

Expression levels of all HER family receptors and several relevant EGFR ligands were examined by quantitative reverse transcription-PCR and EGFR surface receptor status was established by fluorescence-activated cell sorting analysis (Fig. 1C). EGFR expression as assessed by quantitative reverse transcription-PCR and by cell surface protein expression showed higher levels in some erlotinib-sensitive lines, but showed no statistically significant correlation with sensitivity to erlotinib (P = 0.06; t test sensitive versus insensitive). Upon further analysis of HER2 and HER3, we found that HER3 transcript levels were elevated in erlotinib-sensitive cell lines relative to insensitive lines (P = 0.06; Fig. 2A). In contrast, HER2 transcript levels were elevated in erlotinib-insensitive cell lines (P = 0.0002), and amphiregulin (P = 0.0002), and amphiregulin (P = 0.01). Thus, sensitive cell lines exhibit either ligand-driven growth potential, elevated basal expression of select EGFR ligand transcripts, and/or EGFR mutations/amplifications.

Identification of gene signatures correlating with erlotinib sensitivity. Basal mRNA gene expression profiles were generated from the entire panel of 42 NSCLC cell lines using Affymetrix oligo microarrays. Using the 4,562 most variably expressed genes, unsupervised hierarchical clustering classified all NSCLC cell lines into two subclasses, with a majority of the erlotinib-sensitive (<2 μmol/L IC50) cell lines clustering within one of the two main branches (Fig. 2A). Only 2 of 16 sensitive cell lines (Sk-Mes-1 and H838) clustered with the insensitive/intermediate sensitive cell lines and, conversely, 2 of 14 insensitive lines (H1975 and H1781) and 2 of 11 intermediate sensitive lines (H647 and H441) clustered on the same branch as the erlotinib-sensitive lines. Similar results were obtained if cell lines were clustered using all genes on the Affymetrix oligo microarray (data not shown). To identify more accurately genes differentially expressed between the NSCLC cell lines classified as erlotinib sensitive or insensitive, a supervised gene identification analysis was carried out using the 19,592 probe sets overexpressed in the sensitive and insensitive cell lines, respectively (Fig. 2B; Table 1). Many of the 50 probe sets exhibiting elevated expression within erlotinib-sensitive cell lines detected epithelial-related genes, such as E-cadherin, plakophilin 3, and stratifin1. Conversely, primarily mesenchymal and/or morphogenesis-related genes were detected within the 50-probe erlotinib insensitive marker set. Upon inspection, a gene ontology analysis of these 50 marker probe sets revealed that 24% (12 of 50) represented morphogenesis-related biological processes. Noteworthy genes within this data set included the mesenchymal markers vimentin, TGFβ (ZEB-1), and epi morphin, which have been shown to play important roles in epithelial-mesenchymal interactions (23–25).

To confirm that the coregulated expression of the above-described marker genes is also reflected in the biology of tumors and is not specific to cell lines, the correlations between the transcriptional expression levels of these genes using Affymetrix oligo microarray data for 80 primary NSCLC tumor samples were analyzed (data not shown). There was a significant positive correlation regarding expression levels of both genes being up-regulated within the erlotinib-sensitive set and between genes showing higher expression within the erlotinib insensitive set (P < 1 e-5 in each case). There was also a significant negative correlation between pairs of genes in which one was from the sensitive set and one was from the insensitive set (P < 1 e-3). This suggests that the sets of genes associated with erlotinib sensitivity and insensitivity are coordinately and oppositely regulated in NSCLC tumors.

A two-dimensional, unsupervised hierarchical clustering approach was used to classify all NSCLC lines using several well-established epithelial to mesenchymal transition (EMT) marker genes; five of which were not in our top 100 gene list (23, 26) to determine if a potential EMT gene signature could differentially classify NSCLC cell lines based on erlotinib sensitivity. Using the complete panel of cell lines, these EMT marker genes clustered in two primary branches; with the epithelial genes E-cadherin, γ-catenin, and z-catenin forming one cluster and the mesenchymal genes, N-cadherin, vimentin, TGFα, and TGFβ forming the second cluster (Fig. 2C). With a few exceptions, the majority of sensitive and insensitive cell lines correctly fell within epithelial or mesenchymal branches, respectively. Taken together, these data support an epithelial to mesenchymal gene signature defining two distinct subsets of NSCLC cell lines and this gene signature associated with the sensitivity/insensitivity of the cell lines to treatment with erlotinib.

Expression of EMT protein markers in non–small cell lung carcinoma cell lines and primary tumor specimens. To evaluate further the potential role of EMT markers in defining erlotinib sensitivity, the protein expression levels of a few epithelial or mesenchymal-related proteins in the NSCLC cell lines were assessed (Fig. 3A). As was observed in the mRNA expression analyses, expression of the epithelial proteins E-cadherin and γ-catenin was high within the erlotinib-sensitive cell lines by Western blot analysis. Conversely, expression of the mesenchymal marker vimentin (and to some degree, N-cadherin) was detected primarily in the insensitive cell lines. An unsupervised hierarchical analysis of the semiquantitatively derived protein expression values, further shows that the majority of erlotinib-sensitive cell lines are represented within the epithelial subgroup (Fig. 3B).

To evaluate the potential for an epithelial versus mesenchymal phenotype to be predictive of erlotinib activity in patients, immunohistochemistry for the epithelial marker, E-cadherin, was done on formalin-fixed, paraffin-embedded
In tissues of the EMT marker proteins, E-cadherin was chosen based on the fact that it exhibited the best correlation with sensitivity in cell lines ($P < 0.0002$; Table 1) and robust immunohistochemical assays have been described (27, 28). E-cadherin expression was initially evaluated on a tissue microarray representing the panel of NSCLC cell lines and exhibited strong membranous and cytoplasmic staining in subsets of NSCLC cell lines, with expression levels by immunohistochemistry correlating well with expression as determined by Western blotting (Fig. 3A and C). Nuclear staining of E-cadherin, which was not correlated with membranous/cytoplasmic staining, was observed in several cell lines. Because some of the cell lines with nuclear staining had no detectable expression by mRNA or Western analysis, the nuclear staining is likely to be artifactual and was not assessed in subsequent analyses. Applying similar immunohistochemical staining criteria, robust membranous/cytoplasmic expression of E-cadherin was observed in epithelial tumor...
cells in primary NSCLC tissue specimens (Fig. 3C) and was, therefore, used to evaluate E-cadherin expression in clinical specimens. Tumors that exhibited E-cadherin immunoreactivity generally showed fairly consistent staining in >75% of the tumor cells, so only the intensity of membranous/cytoplasmic staining was used to score E-cadherin expression in further analyses. Again, nuclear staining was observed in some cases that did not correlate with membranous/cytoplasmic staining. Although membranous/cytoplasmic staining was limited to epithelial tumor cells, nuclear staining was also seen in stromal cells, consistent with artifactual staining.

**Relationship between E-cadherin staining and outcome in TRIBUTE.** To evaluate the potential relationship between E-cadherin expression and clinical activity of erlotinib, E-cadherin expression was evaluated in biopsies from chemotherapy-naïve patients with locally advanced or metastatic NSCLC from a randomized, placebo-controlled trial (TRIBUTE; ref. 18). Patients received either erlotinib administered concurrently with chemotherapy or chemotherapy alone. Baseline demographics and clinicopathologic characteristics are summarized in Table 2. For this study, we were able to analyze E-cadherin expression by immunohistochemistry on tumors from patients for whom adequate biopsy material was available for the construction of a tissue microarray. Immunohistochemical data for E-cadherin expression was obtained from 87 of 1,079 (8%) patients who participated in TRIBUTE. The clinical variables examined were balanced between those patients for whom E-cadherin expression could be determined versus all patients, with the exception of smoking history. There was a lower percentage of never smokers in patients with known E-cadherin expression (4 of 87 = 4.6% versus 116 of 1,078 = 10.8%). Within the E-cadherin status-known subgroup, patient characteristics were generally balanced between the two treatment arms.

Best tumor response rate in all patients as defined by RECIST criteria was 22% for the erlotinib- and chemotherapy-treated group and 19% for the chemotherapy alone group. Within the E-cadherin-positive staining intensity subgroup, the response rates were 18% and 14%, respectively, and for the E-cadherin-negative staining subgroup, response rates were 25% and 43%, respectively. None of the differences in response rate reached statistical significance.

Data from various sources point to the importance of stable disease as an important and meaningful outcome of treatment with erlotinib (29). Therefore, time to progression and overall survival are likely to be a more relevant outcome measures in the context of this study. For all patients treated with chemotherapy and erlotinib versus those treated with chemotherapy alone, time to progression was 23.7 versus 22.3 weeks [hazard ratio (HR), 0.90; log-rank \( P = 0.17 \)]. However, within the E-cadherin-positive staining subgroup, time to progression for those receiving erlotinib and chemotherapy versus chemotherapy alone was 34 versus 19.3 weeks (HR, 0.37; log-rank \( P = 0.003 \); Fig. 4A). Conversely for the E-cadherin-negative staining group, time to progression was 19.1 weeks for erlotinib and chemotherapy and 30 weeks for chemotherapy alone (HR, 1.63), although this difference was not statistically significant (log rank \( P = 0.40 \); Fig. 4B). An analysis with adjustment for baseline characteristics was not done due to small sample size.

Overall survival was not significantly different between the erlotinib and chemotherapy versus chemotherapy alone–treated groups in all patients (HR, 1.0). Within the positive staining subgroup, treatment with erlotinib was associated with a longer median survival time (15.1 versus 13.6 months; HR, 0.82), although this was not statistically significant (log rank \( P = 0.56 \); Fig. 4C). Within the negative staining group, treatment with chemotherapy alone was associated with a longer median survival time (11.3 versus 9.7 months; HR, 1.82), although not significantly (\( P = 0.28 \); Fig. 4D). In addition, E-cadherin-positive staining was associated with a longer median survival time irrespective of treatment (13.6 months versus 10.6 months), indicating that it may be a prognostic marker, as previously reported (27, 28), although this difference was not significant (\( P = 0.49 \)).
A pharmacogenomic approach using in vitro model systems was taken to identify predictive markers of erlotinib activity. Identification of clinical markers with significant predictive value requires the model system to be representative of the target pathway activity and the identified markers to be translatable to the clinical setting. We evaluated erlotinib activity under a culture condition that would allow us to assess both ligand-dependent and ligand-independent mechanisms of EGFR activity in a panel of NSCLC cell lines. The results strongly suggest that an underlying EGFR-driven biology is predictive of erlotinib activity as shown by the finding that erlotinib sensitivity could be predicted by either ligand-driven growth potential, ligand expression, and/or EGFR-activating mutations/amplifications. Specifically, NSCLC cell lines exhibiting the greatest sensitivity to erlotinib either harbored genetically activated EGFR, specifically a kinase domain deletion.
induce apoptosis in H1975 cells, although a dose-dependent
This is further shown by our finding that erlotinib fails to
confer resistance to EGFR small molecule antagonists (21, 30).
main, one of which (T970M) has been previously reported to
growth potential, minimal basal expression of EGFR ligands,
indicate a potential autocrine dependence for these cell lines. In
mechanism. Furthermore, nearly all of the erlotinib-sensitive
mitogenic activity, suggesting a ligand-independent growth
resulting in overexpression of mRNA and protein, exhibited no
by ligand. Two of the highly sensitive cell lines, H1838 and
-(H1650) and a genomic amplification (H1838), or exhibited
-byactivating mutation (Hop18). These
-H1975 harbors two mutations in the EGFR kinase do-
contrast, insensitive lines exhibited minimal ligand-dependent
growth potential, minimal basal expression of EGFR ligands,
and no EGFR-activating genetic alterations. The one excep-
tion, H1975, harbors two mutations in the EGFR kinase do-
main of which (T970M) has been previously reported to
counter resistance to EGFR small molecule antagonists (21, 30).
This is further shown by our finding that erlotinib fails to
induce apoptosis in H1975 cells, although a dose-dependent
induction of apoptosis was observed with an anti-EGFR anti-
body antagonist (C225; Fig. 1B). Taken together, the data sup-
ports both ligand-dependent and ligand-independent growth
mechanisms underlying erlotinib sensitivity in this cell line panel.
Mechanisms leading to erlotinib-dependent growth inhibi-
tion remain to be elucidated; however, EGFR mutations and
amplifications have been reported to sensitize cell lines to
antagonist-induced apoptosis (19, 31, 32). We found that in
addition to EGFR mutant (H1650) or amplified (A431 and
A431 (positive control) with genomic amplification of EGFR
-activating mutation (Hop18). These

### Table 1. Top 100 classifier probe sets predicting erlotinib sensitivity in NSCLC cell lines

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<th>S/N</th>
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<th>P</th>
<th>UniGene ID</th>
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(Continued on the following page)
findings would suggest that tumors bearing wild-type, non-amplified EGFR could also be equally responsive to erlotinib inhibition. This is in contrast to previous reports in which similar inductions of apoptosis were not observed in EGFR wild-type cells (19, 31). One explanation for this discrepancy could be that we have evaluated an apoptotic mechanism of action in the context of receptor activation with ligand, as opposed to high serum conditions only. Interestingly, we find that several erlotinib-sensitive cell lines, including the EGFR mutant H1650 line, are insensitive (IC50 > 10 μmol/L) to erlotinib-mediated growth inhibition when tested under high serum conditions without exogenous ligand (data not shown). Thus, it is possible that ligand activation of the EGFR is required to sensitize subsets of NSCLC cell lines to erlotinib-induced apoptosis.

Further characterization of these cell lines revealed additional associations with previously reported determinants of EGFR antagonist activity. Specifically, we detected a significant correlation of erlotinib sensitivity with HER3 expression levels. A similar correlation of HER3 expression levels and gefitinib activity was previously reported in smaller panels of NSCLC cell lines (13). However, we do not find a significant association with the level of HER2 overexpression in erlotinib-sensitive cell lines, contrary to previous reports (12, 33). Such a discrepancy could be due to many factors, including differences in tissue types (33), assessment of endogenous versus ectopically expressed HER2 (12), and/or assessment of mRNA compared with protein levels. Similarly, we did not detect a statistically significant correlation of EGFR expression levels with erlotinib sensitivity, although expression was elevated in some of the sensitive cell lines. Finally, KRAS mutations have been reported to associate with a lack of clinical activity to EGFR antagonists (8, 34). In our study, a majority of the KRAS mutant cell lines exhibit intermediate or low erlotinib sensitivity (n = 9); however, four KRAS mutant lines were sensitive to erlotinib. Two of these cell lines exhibited IC50 values <1 μmol/L and

### Table 1. Top 100 classifier probe sets predicting erlotinib sensitivity in NSCLC cell lines (Cont’d)

<table>
<thead>
<tr>
<th>Class: sensitive</th>
<th>S/N score</th>
<th>P</th>
<th>UniGene ID</th>
<th>Common gene name</th>
<th>Class: insensitive</th>
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one of these, Hop18, underwent apoptosis after erlotinib and C225 treatment. Thus, our findings do not support a major role of KRAS mutations in conferring resistance to inhibition of EGFR in NSCLC tumor cells.

A predominant subclassification of NSCLC cell lines can be based on epithelial or mesenchymal characteristics. During tumor progression, the morphologic transformation of a tumor from an epithelial to mesenchymal phenotype has been
associated with increased tumor aggressiveness and metastasis (23, 26, 35, 36). In particular, the loss of E-cadherin expression represents a hallmark of EMT, as the subsequent disassembly of adherens junctions upon E-cadherin loss results in the acquisition of a more motile and invasive tumor. Clinically, this has been significantly correlated with poor prognosis in NSCLC patients (27, 28, 37). The loss of various epithelial markers, highlighted by E-cadherin, also serves as a strong determinant of erlotinib insensitivity in the NSCLC cell lines tested in our study. Conversely, genes characteristic of a mesenchymal phenotype (i.e., vimentin), and genes specifically associated with EMT (i.e., TGF-β, TCF8, and epimorphin), are overexpressed in cell lines that are insensitive to erlotinib-mediated growth inhibition. For example, the E-cadherin transcriptional repressor TCF8 (ZEB1)

Table 2. Relationship between E-cadherin immunoreactivity and clinicopathologic variables/clinical response

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<td>E + chemo (n = 28)</td>
<td>Chemo (n = 14)</td>
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<td>63 ± 14</td>
<td>70 ± 9</td>
</tr>
<tr>
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<td>13 (35)</td>
<td>13 (46)</td>
<td>4 (29)</td>
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<tr>
<td>Ethnicity (%) White</td>
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</tr>
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</tr>
<tr>
<td>Others</td>
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<tr>
<td>Smoking history (%)</td>
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</tr>
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<td>ECOG performance status (%)</td>
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<tr>
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<td>8 (29)</td>
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<td>Cancer stage (%)</td>
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<tr>
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<tr>
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<td>25 (89)</td>
<td>13 (93)</td>
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<td>Histology (%)</td>
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<td>Squamous cell carcinoma</td>
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<td>Range</td>
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Best response, time to progression, and overall survival

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<th>E-cadherin positive</th>
<th>E-cadherin negative</th>
<th>All patients</th>
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<td>Chemo (n = 37)</td>
<td>E + chemo (n = 28)</td>
<td>Chemo (n = 14)</td>
</tr>
<tr>
<td>Response rate</td>
<td>5 (13.5)</td>
<td>5 (17.9)</td>
<td>6 (42.9)</td>
</tr>
<tr>
<td>95% CI for response rate</td>
<td>(2.5-24.5)</td>
<td>(3.7-32.0)</td>
<td>(16.9-68.8)</td>
</tr>
<tr>
<td>P (Fisher’s exact)</td>
<td>0.7341</td>
<td>0.6494</td>
<td>0.4059</td>
</tr>
<tr>
<td>Time to progression</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median time (wk)</td>
<td>19.3</td>
<td>34.0</td>
<td>30.0</td>
</tr>
<tr>
<td>HR</td>
<td>0.37</td>
<td>1.63</td>
<td>0.90</td>
</tr>
<tr>
<td>95% CI for HR</td>
<td>(0.19-0.73)</td>
<td>(0.50-5.33)</td>
<td>(0.78-1.05)</td>
</tr>
<tr>
<td>P (log rank)</td>
<td>0.0028</td>
<td>0.3976</td>
<td>0.1697</td>
</tr>
<tr>
<td>Overall survival</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median time (mo)</td>
<td>13.6</td>
<td>15.1</td>
<td>11.3</td>
</tr>
<tr>
<td>HR</td>
<td>0.82</td>
<td>1.82</td>
<td>1.00</td>
</tr>
<tr>
<td>95% CI for HR</td>
<td>(0.43-1.58)</td>
<td>(0.60, 5.49)</td>
<td>(0.86, 1.16)</td>
</tr>
<tr>
<td>P (log rank)</td>
<td>0.5604</td>
<td>0.2823</td>
<td>0.9517</td>
</tr>
</tbody>
</table>

Abbreviations: E, erlotinib; chemo, chemotherapy; ECOG, Eastern Cooperative Oncology Group.

*One patient did not have any baseline information collected. This patient was included in the overall survival analysis only.
is up-regulated in erlotinib-insensitive cell lines and, as shown
previously, can maintain E-cadherin repression in lung cancer
cell lines (38). Most importantly, these genes capable of sub-
classifying erlotinib-sensitive cell lines were also coregulated in
primary NSCLC tumors, suggesting that this EMT-associated
signature identified in vitro may also play a role in defining
EGFR antagonist activity in primary neoplasms. It remains to be
determined how the EGFR pathway and EGFR-dependent
growth is mechanistically linked to EMT. Previous reports have
implicated a “cross-talk” between the EGFR and E-cadherin,
specifically, suggesting that a critical link exists (39–41). Also,
whether a general HER family pathway activity is associated
with an epithelial signature is unclear. It is noteworthy that one of
the erlotinib-insensitive cell lines (H1781) exhibiting an epithelial
signature harbored a previously reported activating mutation
in HER2 (42). However, regardless of the mechanistic basis, our
data suggests that the underlying epithelial or mesenchymal
phenotypic characteristics of a tumor may serve as a determi-
nant of erlotinib activity.

The hypothesis that E-cadherin expression could serve as a
predictor of erlotinib-mediated clinical activity was evaluated by
performing E-cadherin immunohistochemistry on treatment-
naive biopsies from patients enrolled in TRIBUTE, a random-
ized, placebo-controlled clinical trial. In this trial, patients with
locally advanced or metastatic NSCLC received either erlotinib
administered concurrently with chemotherapy or chemo-
therapy alone but showed no clinical benefit to erlotinib in
combination with chemotherapy (18). However, we observed a
trend toward better outcome in all measures of clinical benefit
(response, time to progression, and overall survival) upon
comparison of the E-cadherin-positive group treated with
erlotinib + chemotherapy versus chemotherapy alone. Al-
though the difference for response and overall survival between
both arms was not significant, the HR and median time
difference for time to progression reached statistical significance
($P = 0.003$). The nonsignificant trend for improved overall
survival in the E-cadherin-positive groupings is not necessarily
at odds. This scenario is commonly seen in oncology trials and
may be due to the effect of subsequent therapy at the time of
progression, particularly because erlotinib treatment would
have been stopped at this time. Interestingly, analysis of the
small group of E-cadherin-negative patients in our study
revealed a worsened response rate, time to progression, and
overall survival within the patient group treated with erlotinib.
Although these findings were not significant and therefore have
to be treated with caution, this could potentially indicate that
a patient with a mesenchymal subtype tumor may fare better
with chemotherapy treatment alone.

Previous molecular subgroup analyses from TRIBUTE revealed
that patients with EGFR mutations have a higher response rate,
but no significant differences in time to progression and overall
survival comparing treatment with chemotherapy alone to

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![Fig. 4. Kaplan-Meier estimated time to progression (A and B) and estimated overall survival (C and D) for NSCLC patients treated with erlotinib + chemotherapy versus chemotherapy alone by E-cadherin positive (A and C) or negative (B and D) expression.](https://www.aacrjournals.org/doi/fig/10.1158/1078-0432.CCR-05-0363)
treatment with added erlotinib (8). In addition, a KRAS-mutated subgroup of patients seemed to derive a negative effect similar to that observed in our E-cadherin-negative group (8). Due to small sample sizes, we were not able to assess a possible correlation between E-cadherin expression and EGFR or KRAS status. Finally, it is important to bear in mind that the retrospective subgroup analyses described here should be viewed cautiously. Given the small numbers of patients within each subgroup, imbalances in patient characteristics that affect outcomes cannot be excluded. Furthermore, statistical adjustments for such imbalances are methodologically difficult due to the small numbers.

In conclusion, an EMT expression signature correlates with sensitivity of NSCLC cell lines to erlotinib, suggesting that patients with epithelial-type NSCLC tumors could have a better outcome after treatment with an erlotinib-containing regimen. Consistent with the preclinical hypothesis, this was supported by retrospective subgroup analyses in TRIBUTE. Patients with positive expression of the epithelial marker E-cadherin showed a significantly better time to progression when comparing erlotinib plus chemotherapy treatment to chemotherapy alone. As this clinical study was limited to the retrospective evaluation of a relatively small subset of patients, further evaluation in future studies is warranted.

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References

Epithelial versus Mesenchymal Phenotype Determines *In vitro* Sensitivity and Predicts Clinical Activity of Erlotinib in Lung Cancer Patients


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