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Baseline Gene Expression Predicts Sensitivity to Gefitinib in Non–Small Cell Lung Cancer Cell Lines

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Abstract

Tyrosine kinase inhibitors (TKI) of the epidermal growth factor receptor (EGFR) produce objective responses in a minority of patients with advanced-stage non–small cell lung cancer (NSCLC), and about half of all treated patients progress within 6 weeks of instituting therapy. Because the target of these agents is known, it should be possible to develop biological predictors of response, but EGFR protein levels have not been proven useful as a predictor of TKI response in patients and the mechanism of primary resistance is unclear. We used microarray gene expression profiling to uncover a pattern of gene expression associated with sensitivity to EGFR-TKIs by comparing NSCLC cell lines that were either highly sensitive or highly resistant to gefitinib. This sensitivity-associated expression profile was used to predict gefitinib sensitivity in a panel of NSCLC cell lines with known gene expression profiles but unknown gefitinib sensitivity. Gefitinib sensitivity was then determined for members of this test panel, and the microarray-based sensitivity prediction was correct in eight of nine NSCLC cell lines. Gene and protein expression differences were confirmed with a combination of quantitative reverse transcription-PCR, flow cytometry, and immunohistochemistry. This gene expression pattern related to gefitinib sensitivity was independent from sensitivity associated with EGFR mutations. Several genes associated with sensitivity encode proteins involved in HER pathway signaling or pathways that interrelate to the HER signaling pathway. Some of these genes could be targets of pharmacologic interventions to overcome primary resistance. (Mol Cancer Res 2006;4(8):521–8)

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Introduction

Lung cancer is the leading cause of cancer death in both men and women in the United States and is the leading cause of cancer death throughout the world (1, 2). The epidermal growth factor receptor (EGFR) is overexpressed in the majority of non–small cell lung cancers (NSCLC) and is a major target for new therapies. In 2005, the EGFR tyrosine kinase inhibitors (TKI) gefitinib (ZD1839, Iressa) and erlotinib (OSI-774, Tarceva) received Food and Drug Administration approval as single-agent therapy for the treatment of NSCLC following chemotherapy failure. In clinical trials, both agents produced objective responses in a small fraction of patients (9-26%) with advanced-stage NSCLC (3-6). However, the majority of patients with objective response treated with either gefitinib or erlotinib had symptom benefit; overall, 40% to 43% of treated patients had major improvement in cancer-related symptoms.

In a randomized trial, erlotinib-treated patients showed a significantly improved survival advantage compared with placebo in chemorefractory advanced-stage NSCLC (hazard ratio = 0.73; $P = 0.01$; ref. 7). In a similar trial, gefitinib showed a small nonsignificant survival advantage (hazard ratio = 0.89; $P = 0.89$; ref. 8). This lack of survival advantage led to the withdrawal of Food and Drug Administration approval. Expression levels of EGFR did not predict tumor response and some responses were noted in patients whose tumors failed to express EGFR by immunohistochemical analysis (9, 10). Subsequent studies have shown that a subset of tumors responsive to gefitinib or erlotinib harbor activating somatic mutations in EGFR (11-13), and correlative results have been reported *in vitro* (14). However, robust and durable responses have been observed in tumors harboring wild-type EGFR (13, 15). The mechanisms of primary resistance to EGFR-TKIs are not well understood but have been associated with somatic KRAS mutations in the absence of EGFR mutation (16). A better understanding of the mechanisms of primary resistance is essential for developing means of circumventing this resistance. Acquired resistance to EGFR TKIs has been shown to occur through a secondary "escape" somatic mutation to EGFR that abrogates drug binding (17). This same mutation (T790M) was recently associated with inherited susceptibility to lung cancer (18).

Both clinical and biological features were proposed as methods of patient selection. Clinical features of female gender, Asian ethnicity, never-smoking status, and adenocarcinoma (with or without brochoalveolar features) histology were

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reported to be associated with higher response rates to EGFR-TKIs (4, 5). However, the survival benefit observed with erlotinib was observed in all patient subsets examined (7). Biological features associated with response to EGFR-TKIs included EGFR, pAKT, and pMAPK protein expression by immunohistochemistry, EGFR and ERBB2 gene copy number by fluorescence *in situ* hybridization, and mutations in EGFR and KRAS by sequencing (9, 10, 19, 20).

NSCLC cell lines have a broad range of sensitivity to EGFR-TKIs *in vitro*, mimicking the wide sensitivity observed in human subjects. In this report, we used a panel of NSCLC cell lines with known sensitivity to gefitinib to establish a gene set that predicts sensitivity/resistance to gefitinib. The gene expression set was verified by applying it to a test set of cell lines with unknown gefitinib sensitivity and by confirming the differential expression of select genes and proteins by reverse transcription-PCR, immunohistochemistry, and flow cytometry. The "EGFR-dependent signature" may provide insight into biological pathways of oncogenesis, biomarkers for identification of tumors likely to respond to EGFR-TKIs, and novel strategies to overcome primary resistance.

Each of the 11 lines chosen for the training set harbors wild-type EGFR. The effect of EGFR mutations on TKI sensitivity *in vitro* is strong (14); therefore, we reasoned that additional important markers of primary sensitivity and resistance may only become apparent in the absence of this background. The NSCLC cell lines chosen for this comparison (test set), however, included examples of each of the NSCLC histologic subtypes, the three most common EGFR mutation types, and KRAS mutation status.

Results and Discussion

Determination of Gene Expression Pattern in the Training Set

The first steps in the development of a gefitinib sensitivity-related gene expression signature were the selection of a training set of cell lines and the initial analysis of the associated gene expression data. Our training set consisted of 11 NSCLC cell lines representing the extremes of gefitinib sensitivity: 5 lines were designated sensitive with $IC_{50} \leq 0.5$ μmol/L and 6 lines were designated resistant with $IC_{50} \geq 4.5$ μmol/L. This training set included examples of each of the NSCLC histologic subtypes as well as lines harboring mutant KRAS (Table 1A). Our intent was to include as wide a variety of NSCLC cell lines as possible while still sampling the extremes of gefitinib sensitivity and to thereby increase the likelihood that the features of any cell line chosen for the test set would be represented in the training set.

Affymetrix (Foster City, CA) HG-U133A and HG-U133B oligonucleotide microarrays were developed for each of the 11 lines comprising the training set, and the data were determined to be of high quality based on standard variables (21). In selected instances, cell line microarrays were repeated from the

Table 1. Characteristics of the NSCLC Cell Lines Used in this Study with EGFR and KRAS Mutational Status, Gefitinib IC_{50}, and Gene Expression Classification of Gefitinib Sensitivity

Cell line	Histology	EGFR	KRAS	IC_{50} (μmol/L)	Gene prediction
(A) NSCLC lines comprising the training set					
H358	BAC	Wild-type	Mutant	0.18	Sensitive
H322C	BAC	Wild-type	Wild-type	0.25	Sensitive
Calu-3	Adenocarcinoma	Wild-type	ND	0.3	Sensitive
H1334	Large	Wild-type	Wild-type	0.3	Sensitive
H1648	Adenocarcinoma	Wild-type	Wild-type	0.38	Sensitive
H125	Adenosquamous	Wild-type	Wild-type	4.8	Resistant
H1703	Squamous	Wild-type	Wild-type	8.0	Resistant
A549	Adenocarcinoma	Wild-type	Wild-type	9.6	Resistant
H157	Squamous	Wild-type	Mutant	12.8	Resistant
H460	Large	Wild-type	Mutant	12.9	Resistant
H520	Squamous	Wild-type	ND	13.6	Resistant
(B) NSCLC lines comprising the test set					
HCC827	Adenocarcinoma	Mutant*	Wild-type	0.005	Sensitive
HCC78	Adenocarcinoma	Wild-type	Wild-type	0.4	Sensitive
H2126	Large	Wild-type	Wild-type	1.0	Sensitive
H1648	Adenocarcinoma	Wild-type	Wild-type	1.3	Sensitive
HCC193	Adenocarcinoma	Wild-type	Wild-type	1.5	Sensitive
HCC95	Adenocarcinoma	Wild-type	Wild-type	1.9	Sensitive
HCC44	Adenocarcinoma	Wild-type	Mutant	7.9	Resistant
H460	Large	Wild-type	Mutant	8.0	Resistant
H2009	Adenocarcinoma	Wild-type	Mutant	8.8	Sensitive
HCC15	Squamous	Wild-type	Wild-type	9.4	Resistant
H157	Squamous	Wild-type	Mutant	13.8	Resistant
H1299	Large	Wild-type	Wild-type	14.7	Resistant
(C) Mutant EGFR NSCLC lines					
H3255	Adenocarcinoma	Mutant †	ND	0.015	Sensitive
HCC4006	Adenocarcinoma	Mutant*	Wild-type	0.02	Sensitive
HCC2279	Adenocarcinoma	Mutant*	Wild-type	0.03	Resistant
H1650	Adenocarcinoma	Mutant*	Wild-type	1.0	Sensitive
H820	Adenocarcinoma	Mutant*	Wild-type	3.0	Sensitive
H1975	Adenocarcinoma	Mutant †, ‡	Wild-type	8.0	Sensitive

*Activating mutation (deletion) in exon 19 of EGFR.
† Activating mutation (L858R) in exon 21 of EGFR.
‡ Escape mutation (T790M) in exon 20 of EGFR.

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same parental line cultured separately at intervals of several months. The overall gene expression pattern was found not to vary significantly (data not shown), strongly suggesting that the observed gene expression pattern was intrinsic to each individual line and not heavily influenced by temporal fluctuations. Gene expression was directly compared using a two-sample *t* test, with a nominal *P* cutoff of 0.001. There were 415 probe sets that met this nominal criterion (Fig. 1A; Supplementary Tables S1 and S2). False discovery rate analysis (22) suggested with 90% confidence that ≤50 false discoveries are listed among the 415 probe sets, and permutation testing strongly suggests with >98% likelihood that our assignment of these NSCLC cell lines to two sensitivity classes is reflected in a significantly different pattern of gene expression. These broad changes in gene expression are further illustrated in Fig. 1B and C, an "overabundance plot" (23). This plot shows that a greater number of gene expression differences are observed between these groups than would be expected by chance regardless of the *P* cutoff chosen.

Gefitinib Sensitivity Test Set

To establish the utility of this baseline gene expression signature of gefitinib sensitivity, we employed an independent "test set" of NSCLC cell lines and attempted to predict gefitinib sensitivity from their corresponding baseline gene expression profiles. Data on 28 NSCLC cell lines of unknown gefitinib sensitivity were collected on Affymetrix HG-U133A microarrays by collaborators at the University of Texas Southwestern (Dallas, TX). HG-U133A data from the University of Colorado Health Sciences Center (Denver, CO) training set were used for classification of these 28 cell lines based on six different prediction algorithms employed in BRB-ArrayTools: compound covariate predictor, 1- and 3-nearest neighbors, nearest centroid, support vector machine, and linear discriminant analysis. The full results are presented in Supplementary Table S3. Twelve cell lines were selected for experimental evaluation of gefitinib sensitivity at University of Colorado Health Sciences Center: Table 1B reports these prediction results and experimental IC_{50}s. Lines were selected if they were consistently classified as sensitive or resistant by all of the six different prediction algorithms and were currently available. Three of the 12 test set cell lines were also present in our training set of 11 cell lines (Table 1A); although these lines are not independent of the training set, they provided an opportunity to cross-validate the array measurements.

Our performance in predicting gefitinib sensitivity in the test set based on the training set "gene expression signature" was extremely good: each of the three nonindependent lines was correctly identified, and eight of the nine independent cell lines were also assigned to the correct gefitinib sensitivity group. The six cell lines correctly predicted to be sensitive had experimental IC_{50}s ranging from 5 nmol/L to 1.9 μmol/L, whereas the five predicted to be resistant ranged from 7.9 to 14.7 μmol/L. The assignment of the adenocarcinoma line H2009 to the gefitinib-sensitive group was the sole incorrect assignment in the test set. KRAS mutations have been associated with primary gefitinib resistance and H2009 does have mutant KRAS; however, there are NSCLC lines in the training set that are gefitinib resistant and have wild-type KRAS. H2009 is wild-type for EGFR expression in exons 19 and 21. The status of exon 20 (T790M), the secondary escape mutation that abrogates gefitinib binding, is unknown. The gefitinib sensitivity of each of the three nonindependent lines, present in the training set as well as in the test set, was correctly predicted. The gene expression pattern from the University of Texas Southwestern microarray data and the IC_{50} determined for the isolate obtained from University of Texas Southwestern was similar to that obtained with the University of Colorado Health Sciences Center isolate. The differences in gefitinib sensitivity-associated gene expression pattern between sensitive and resistant lines are illustrated in Fig. 2A. The gefitinib-induced growth inhibition in both training set and test set of NSCLC lines is shown in Fig. 2B.

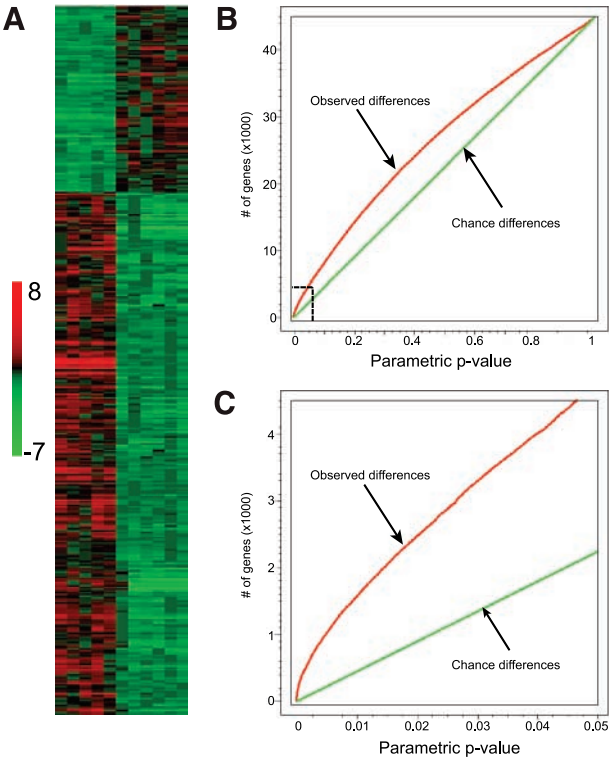

FIGURE 1. Differential expression between gefitinib-sensitive and gefitinib-resistant NSCLC cell lines. **A.** Heat map showing the relative expression levels of 415 probe sets differentially expressed between gefitinib-sensitive and gefitinib-resistant NSCLC cell lines. The five leftmost columns are each of the five sensitive lines, and the six rightmost columns are each of the six resistant lines. Red, relatively high expression; green, relatively low expression. **B** and **C.** Overabundance plot showing the number of genes with significant differences in expression between gefitinib-sensitive and gefitinib-resistant NSCLC cell lines plotted versus the indexed significance level for the statistical test (two-sample *t* test). **B.** Entire range of significance. **C.** Only the bottom left region indicated by the dotted lines in **B.** The number of observed differences (*green line*) exceeds the number of differences expected under the matching null hypothesis (*red line*) regardless of the significance level chosen for the test. This illustrates the robustness and the magnitude of the difference in gene expression between gefitinib-sensitive and gefitinib-resistant NSCLC cell lines.

EGFR Mutation Status and Gefitinib Sensitivity Prediction

Activating mutations in exons 19 and 21 that encode portions of the kinase domain of EGFR are strongly associated

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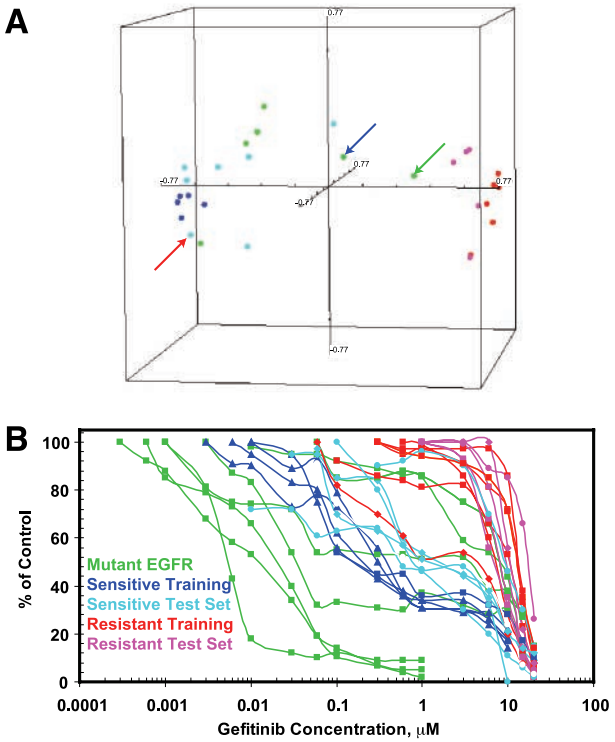

FIGURE 2. **A.** Multidimensional scaling plot of NSCLC cell lines based on the expression of genes in the gefitinib sensitivity signature. Each cell line is represented by a sphere, and the position of each sphere in space is determined by the three largest principal components of variance for that sample within the gefitinib sensitivity signature. The three largest principal components of variance are the *X*, *Y*, and *Z* axes of this multidimensional scaling plot. Gefitinib-resistant cell lines from the training set (*red*) and test set (*magenta*) are separate from sensitive cell lines from the training and test sets (*dark blue* and *light blue*, respectively). Cell lines bearing activating EGFR mutations (*green*) are more commonly associated to the gefitinib-sensitive group. The following cell lines H2009 (*red arrow*), H1975 (*blue arrow*), and HCC2279 (*green arrow*) exhibited gefitinib sensitivities inconsistent with this gene expression signature. **B.** Growth inhibition by gefitinib in NSCLC cell lines. Cells were treated with gefitinib at the indicated concentrations and growth inhibition was measured by MTT assays after 6 days of treatment. Percentage of cell growth is shown relative to untreated controls. Green lines, mutant EGFR NSCLC lines; dark blue lines, gefitinib-sensitive NSCLC lines from the training set; light blue lines, gefitinib-sensitive NSCLC lines from the test set; red lines, gefitinib-resistant NSCLC lines from the training set; magenta lines, gefitinib-resistant NSCLC lines from the test set.

with EGFR-TKI sensitivity in tumors and in cell lines (11-14). A secondary mutation in exon 20 has been shown to confer strong resistance (17). EGFR exons 19 and 21 were sequenced in our cell lines, and we found no mutations in the training set cell lines. One mutation (HCC827, exon 19 del) was found among the test set cell lines. Next, we collected six additional NSCLC cell lines known to harbor EGFR mutations. We determined the baseline gene expression pattern of the EGFR mutant lines and then predicted gefitinib sensitivity based on the gefitinib sensitivity-associated gene expression pattern. Gefitinib IC_{50}s were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays (Table 1C; Fig. 2B). In five mutant cell lines, the gefitinib IC_{50} was ≤1 μmol/L, and four of these had gene expression patterns predictive of gefitinib sensitivity. HCC2279 (IC_{50} = 0.03 μmol/L) had a gene expression pattern predictive of gefitinib resistance, suggesting that in this line that either the EGFR mutation overrides the gene expression pattern or that this line represents a subset of mutant EGFR lines in which there is a different gene expression pattern that predicts sensitivity. The line H1975 harbors the resistance-conferring exon 20 mutation in addition to the activating exon 21 mutation (17). Although the gene expression pattern for this line was consistent with the gefitinib-sensitive group, it was experimentally determined to be gefitinib resistant (IC_{50} = 8.0 μmol/L), suggesting that the desensitizing effect of the T790M escape mutation may be dominant.

Quantitative PCR Confirmation of Differential Gene Expression

Eight of the genes identified as differentially expressed between sensitive and resistant cell lines and listed in Supplementary Tables S1 and S2 were selected for confirmation by quantitative PCR (Table 2; details in Supplementary Table S4). In each case, the magnitude of differential expression determined by quantitative PCR was larger than that determined by microarray, but we found perfect agreement in the sign of differential expression between the two methods. These results are consistent with our prior experience and with published results (24), suggesting that microarray measurements of transcript fold changes consistently underestimate true fold differences.

Flow Cytometry Confirmation of Differential Protein Levels

Among the differentially expressed genes, we selected two that were known to encode cell surface proteins for confirmation by flow cytometry. Cell surface levels of TACSTD2 (TROP2) and ERBB3 were evaluated by flow cytometry for NSCLC cell lines from the training and test sets and are reported in Table 3 and Fig. 3. In most cases, the observation of these cell surface proteins was found to reflect the expression level of the corresponding gene. The cell line H125 is a notable exception, with very low microarray signal intensities for probe sets representing TACSTD2 and ERBB3, which is consistent with the assignment of this line to the gefitinib-resistant group, but flow cytometry analysis suggests that high levels of these proteins are present. The reason for this discrepancy is unknown. Three notable cases where cell lines exhibited gene expression patterns, which were discordant with the experimental gefitinib IC_{50} measurement, were H2009

Table 2. Quantitative Reverse Transcription-PCR Confirmation of Differential Expression of Selected Genes

Gene symbol	Probe set ID	Sensitive/resistant quantitative reverse transcription-PCR	Sensitive/resistant microarray
ALDH1A3	203180_at	13.3	8.8
ANXA9	211712_s_at	82.3	3.8
ARHGDIB	201288_at	357.8	16.3
BIK	205780_at	31.3	4.9
CDH1	201131_s_at	25.0	8.8
ERBB3	202454_s_at	16,691.0	13.3
PTK6	206482_at	194.0	2.5
RAB25	218186_at	329.8	53.4

NOTE: Gefitinib-sensitive and gefitinib-resistant cell lines from the training set were evaluated and the data are presented as a ratio.

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Table 3. Immunofluorescence Flow Cytometry Determination of Cell Surface Expression of TACSTD2 and ERBB3

Cell line	IC_{50} (μmol/L)	TACSTD2			ERBB3		
		% Positive	Median fluorescence intensity	Array	% Positive	Median fluorescence intensity	Array
Training set							
H358	0.18	96	21	8,895	99	6.4	554
H322C	0.25	94	34	10,421	96	5.2	685
Calu-3	0.3	99	35	7,141	86	4.4	1,444
H1648	0.38	98	25	14,348	69	1.8	561
H125	4.8	100	67	4	94	7.5	9
H1703	8.0	0	0	4	0	0	6
A549	9.6	0	0	4	42	3.9	51
H157	12.8	0	0	4	0	0	5
H460	12.9	0	0	4	0	0	5
H520	13.6	0	0	4	0	0	5
Test set							
HCC78	0.4	100	29	19,788	99	9.5	4,027
HCC193	1.5	100	59	13,425	98	4.9	200
HCC95	1.9	83	3.9	4,461	74	2.2	1,060
HCC44	7.9	89	3.5	316	0	0	9
H2009	8.9	99	29	11,980	86	6	1,246
HCC15	9.4	27	1.5	940	79	1.7	141
H1299	14.7	0	0	4	36	1.9	9
EGFR mutant							
HCC827	0.005	94	24	3,769	65	2.7	384
H3255	0.015	100	28	11,334	90	6.4	1,931
HCC4006	0.02	94	20	7,647	85	1.8	538
H2279	0.03	0	0	703	0	0	5
H1650	1.0	95	23	13,196	84	3.6	98
H820	3.0	99	30	7,338	95	3.9	504
H1975	8.0	46	38	2,329	39	3.8	197

(IC_{50} = 8.8 μmol/L) and H1975 (IC_{50} = 8.0 μmol/L), both predicted to be sensitive, and HCC2279 (IC_{50} = 30 nmol/L), predicted to be resistant. Here, the observation of cell surface expression of ERBB3 and TACSTD2 followed the pattern predicted by the microarrays: H2009 and H1975 both exhibit high levels of these proteins, whereas H2279 shows minimal ERBB3 and TACSTD2 by flow cytometry. This suggests that the incorrect prediction based on gene expression is not due to a disconnect between gene expression and protein levels but rather, as is possibly the case with the double (activating and escape) EGFR mutation bearing line H1975, due to a nongene expression-related dominant effect.

E-Cadherin Protein Levels

We next chose to examine the expression of E-cadherin by immunohistochemistry in a subset of the test and training set cell lines. Figure 4 shows this analysis in the eight cell lines chosen for confirmation by immunohistochemistry. High levels of E-cadherin are detected in gefitinib-sensitive cell lines (H358, H1648, H322C, and HCC78). No staining for E-cadherin is detected in most gefitinib-resistant cell lines (H460, H1703, and HCC15). The gefitinib-resistant cell line H520 shows no staining for E-cadherin in ~98% of cells but membrane-localized staining in ~2% of cells. These results are consistent with the gene expression levels detected in each of these lines. We and others have reported this association between E-cadherin levels and gefitinib sensitivity (25-27).

EGFR Family Gene Expression

The expression of genes encoding the EGFR family members (EGFR, ERBB2, and ERBB4) and the EGFR ligands (transforming growth factor-α, amphiregulin, ephiregulin, or epidermal growth factor) did not meet the significance criteria for inclusion in the gefitinib sensitivity-associated signature. We did detect a marked difference in the expression of ERBB3, which was found to be more highly expressed in gefitinib-sensitive cell lines. Although ERBB3 has little or no intrinsic tyrosine kinase activity, it has been shown to couple ligand binding to phosphatidylinositol 3-kinase pathway activation via the transautophosporylation of ERBB3 in heterodimers with

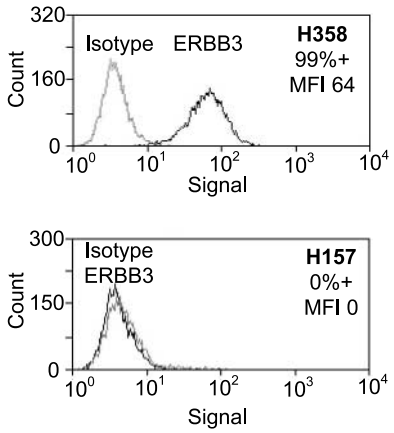

FIGURE 3. Expression of ERBB3 in two NSCLC cell lines as assessed by flow cytometry. Top, gefitinib-sensitive line H358 from the training set; bottom, gefitinib-resistant line H157 from the training set. The left peak (*gray*) represents the background staining of the isotype-matched control antibody and the right peak represents the cell surface staining with the anti-ERBB3 antibody. The H358 cell line was positive for ERBB3 cell surface expression and the H157 cell line was negative. MFI, median fluorescence intensity.

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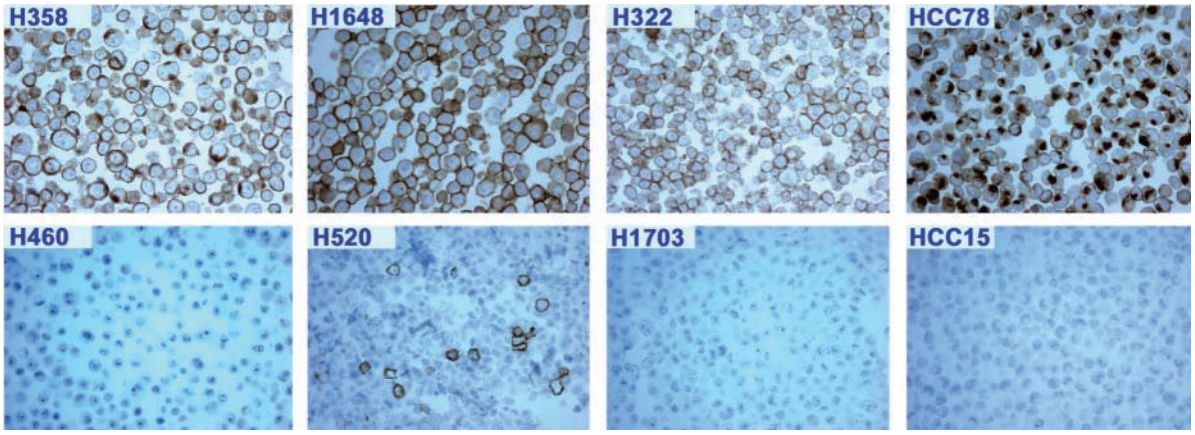

FIGURE 4. Expression of E-cadherin in eight NSCLC cell lines as assessed by immunohistochemistry. High levels of E-cadherin are detected in gefitinib-sensitive cell lines (*top row*: H358, H1648, H322C, and HCC78). No staining for E-cadherin is detected in most gefitinib-resistant cell lines (*bottom row*: H460, H1703, and HCC15). The gefitinib-resistant cell line H520 shows E-cadherin staining in a small proportion of cells.

other EGFR family members (28), and the expression of ERBB3 has been correlated previously with gefitinib sensitivity in NSCLC cell lines (29). However, enforced expression of ERBB3 did not convey sensitivity in gefitinib-insensitive NSCLC cell lines (29), and high polysomy or amplification of ERBB3 did not correlate with gefitinib sensitivity in a retrospective analysis (30).

Although the results of this study are correlative (i.e., the genes identified have not been shown to cause sensitivity or resistance), the pattern of expression of these genes is sufficient to predict the relative sensitivity of an independent set of cell lines, and the identity of the genes in this expression pattern may provide insight into the dependence of NSCLC cell lines on EGFR signaling and the various paths to transformation and carcinogenesis resulting in lung cancer. We have initiated the extension of this work into clinical samples, and the mechanistic relationship of E-cadherin expression to gefitinib sensitivity has been recently established (25) as well as the generalization of these results in other epithelial-derived cancer types. We anticipate that the combination of this gefitinib sensitivity-related signature with existing EGFR mutation and amplification tests will allow a much more accurate prediction of the clinical efficacy of gefitinib.

Materials and Methods

Cell Culture, Drugs, and MTT assay

Twenty-six different cell lines were studied (Table 1), representing squamous (H157, H520, H1703, and HCC15), adenocarcinoma (A549, Calu-3, H820, H1648, H1650, H1975, H2009, H3255, HCC44, HCC78, HCC95, HCC193, HCC827, HCC2279, and HCC4006), adenosquamous (H125), large cell (H460, H1299, H1334, and H2126), and bronchoalveolar (H322 and H358) histologies. The NSCLC cell lines H820, H1299, H1650, H2009, H2126, HCC15, HCC44, HCC78, HCC95, HCC193, HCC827, HCC2279, and HCC4006 were provided by Drs. John D. Minna and Adi Gazdar (University of Texas Southwestern Medical Center). The H322 line was provided by Dr. A. Moustafa (National Research Council Canada, Biotechnology Research Institute, Montreal, Quebec, Canada). The H358 line was provided by Dr. Isaiah J. Fidler (University of Texas Southwestern Medical Center). The H3255 line was provided by Dr. Bruce Johnson (Dana-Farber Cancer Institute, Boston, MA). All the other cell lines were obtained from American Type Culture Collection (Rockville, MD). All lines were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT) in 5% CO_2 with 100% humidity. Gefitinib was a gift from AstraZeneca (Alderley Park, United Kingdom). Stock solutions were prepared in DMSO and stored at −20°C. The drug was diluted in fresh medium before each experiment, and the final DMSO concentration was <0.1%. Growth inhibition was assessed using a modified MTT assay as described previously (31). Briefly, 2×10^3 NSCLC cells are plated in each well of 96-well flat-bottomed microtiter plates. Gefitinib was added the next day, and after 5-day incubation, a 2 mg/mL solution of MTT (50 μL) dissolved in RPMI 1640 was added to each well. The microtiter plates were incubated for 4 hours at 37°C. The visible absorbance of each well was measured using an automated plate reader (Molecular Devices, Sunnyvale, CA), and data were analyzed using SlideWrite to determine the drug IC_{50}.

Microarray Analysis of Gene Expression

Microarrays were run using RNA isolated from untreated (baseline) NSCLC cell lines growing in culture as described above. RNA stabilization, isolation, and microarray sample labeling were carried out using standard methods for reverse transcription and one round of *in vitro* transcription (32). HG-U133 set microarrays were hybridized with 10 μg cRNA and processed according to the manufacturer's protocol (Affymetrix). A MIAME checklist (33) containing extensive experimental details can be found in the Supplementary Tables. Hybridization signals and detection calls were generated in BioConductor using the "Robust Multi-Array Average" expression measure (34). Microarray data were analyzed using BRB-ArrayTools version 3.2 developed by Dr. Richard Simon and Amy Peng Lam. Class comparison using the univariate two-sample *t* test was done using all (44,792) noncontrol probe

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sets on the HG-U133 set. Multivariate permutation testing was conducted using the 462 available permutations of the class labels. Microarray data for the independent test set included only the HG-U133A chip; therefore, class prediction results were based on the 22,215 noncontrol probe sets included on that chip.

EGFR Sequencing

Exons 18, 19, and 21 of EGFR were sequenced using standard methods and modified primers.[5] The EGFR mutated NSCLC lines were sequenced for exons 19 to 21 at University of Texas Southwestern.

Real-time Reverse Transcription-PCR

Expression levels of genes of interest were assessed using real-time reverse transcription-PCR on an Opticon instrument (Bio-Rad, Hercules, CA) using SYBR Green detection chemistry (Molecular Probes, Eugene OR) according to the manufacturer's directions. Gene-specific primer sets are detailed in the Supplementary Tables.

Immunofluorescence Staining

For cell surface protein expression by flow cytometry, 2×10^5 cells were incubated with anti-ERBB3 (Neomakers, Inc., Fremont, CA), anti-TACSTD2 (R&D Systems, Minneapolis, MN), or an isotype-matched control. Counterstaining was conducted with goat anti-mouse IgG1-PE (for ERBB3) or goat anti-mouse IgG2A-FITC (for TACSTD2; both from Southern Biotechnology, Birmingham, AL). All staining was done on ice for 45 minutes followed by three washes with PBS and fixation with 1% formaldehyde. Cell fluorescence was measured by flow cytometry (EPICS-XL-MCL, Coulter, Hialeah, FL). The percentage of ERBB3 or TACSTD2 positively staining cells and their median fluorescence intensity were determined using the Coulter software.

Immunohistochemistry

The anti-E-cadherin antibody (mouse monoclonal; Transduction Laboratories, Lexington, KY) was applied at 1:100 dilution to sectioned paraffin-embedded cell lines. Antigen retrieval was done in citrate buffer using a Biocare Medical (Walnut Creek, CA) decloaking chamber. Peroxide blocking was preformed with 3% peroxide in absolute methanol. Blocking was done with Powerblock (Biogenics, San Ramon, CA) or avidin/biotin block. After incubation of primary antibodies at 37°C for 1 hour, the secondary antibody [DAKO (Carpinteria, CA) Biotinylated Multi-Link antimouse, immunoglobulin with 40% human serum] was applied for 30 minutes at room temperature. This was followed by application of streptavidin horseradish peroxidase enzyme complex and diaminobenzidine chromogen. The slides were then counterstained in hematoxylin and covered with a coverslip.

References

1. Jemal A, Murray T, Ward E, et al. Cancer statistics, 2005. CA Cancer J Clin 2005;55:10–30.

2. Parkin DM. Global cancer statistics in the year 2000. Lancet Oncol 2001;2:533–43.

3. Foon KA, Yang XD, Weiner LM, et al. Preclinical and clinical evaluations of ABX-EGF, a fully human anti-epidermal growth factor receptor antibody. Int J Radiat Oncol Biol Phys 2004;58:984–90.

4. Fukuoka M, Yano S, Giaccone G, et al. Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer (The IDEAL 1 Trial) [corrected]. J Clin Oncol 2003;21:2237–46.

5. Kris MG, Natale RB, Herbst RS, et al. Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non-small cell lung cancer: a randomized trial. JAMA 2003;290:2149–58.

6. Tiseo M, Loprevite M, Ardizzoni A. Epidermal growth factor receptor inhibitors: a new prospective in the treatment of lung cancer. Curr Med Chem Anti-Can Agents 2004;4:139–48.

7. Shepherd FA, Rodrigues PJ, Ciuleanu T, et al. Erlotinib in previously treated non-small-cell lung cancer. N Engl J Med 2005;353:123–32.

8. Thatcher N, Chang A, Parikh P, et al. Gefitinib plus best supportive care in previously treated patients with refractory advanced non-small-cell lung cancer: results from a randomised, placebo-controlled, multicentre study (Iressa Survival Evaluation in Lung Cancer). Lancet 2005;366:1527–37.

9. Cappuzzo F, Gregorc V, Rossi E, et al. Gefitinib in pretreated non-small-cell lung cancer (NSCLC): analysis of efficacy and correlation with HER2 and epidermal growth factor receptor expression in locally advanced or metastatic NSCLC. J Clin Oncol 2003;21:2658–63.

10. Hirsch FR, Varella-Garcia M, Bunn PA, Jr., et al. Epidermal growth factor receptor in non-small-cell lung carcinomas: correlation between gene copy number and protein expression and impact on prognosis. J Clin Oncol 2003;21:3798–807.

11. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 2004;304:1497–500.

12. Pao W, Miller V, Zakowski M, et al. EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. Proc Natl Acad Sci U S A 2004;101:13306–11.

13. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 2004;350:2129–39.

14. Sordella R, Bell DW, Haber DA, Settleman J. Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways. Science 2004;305:1163–7.

15. Taja-Chayeb L, Candelaria M, Brom R, Trejo-Becerril C, Meza F, Duenas-Gonzalez A. Response to gefitinib in bronchioloalveolar carcinoma in the absence of EGFR mutation. Lung Cancer 2005;50:259–63.

16. Miller VA, Kris MG, Shah N, et al. Bronchioloalveolar pathologic subtype and smoking history predict sensitivity to gefitinib in advanced non-small-cell lung cancer. J Clin Oncol 2004;22:1103–9.

17. Kobayashi S, Boggon TJ, Dayaram T, et al. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. N Engl J Med 2005;352:786–92.

18. Bell DW, Gore I, Okimoto RA, et al. Inherited susceptibility to lung cancer may be associated with the T790M drug resistance mutation in EGFR. Nat Genet 2005;37:1315–6.

19. Han SW, Hwang PG, Chung DH, et al. Epidermal growth factor receptor (EGFR) downstream molecules as response predictive markers for gefitinib (Iressa, ZD1839) in chemotherapy-resistant non-small cell lung cancer. Int J Cancer 2005;113:109–15.

20. Shigematsu H, Lin L, Takahashi T, et al. Clinical and biological features associated with epidermal growth factor receptor gene mutations in lung cancers. J Natl Cancer Inst 2005;97:339–46.

21. Tumor Analysis Best Practices Working Group. Expression profiling—best practices for data generation and interpretation in clinical trials. Nat Rev Genet 2004;5:229–37.

22. Korn EL, Troendle JF, McShane LML, Simon R. Controlling the number of false discoveries: application to high-dimensional genomic data. J Statist Plann Inference 2004;124:379–98.

23. Barash Y, Dehan E, Krupsky M, et al. Comparative analysis of algorithms for signal quantitation from oligonucleotide microarrays. Bioinformatics 2004;20:839–46.

24. Rajagopalan D. A comparison of statistical methods for analysis of high density oligonucleotide array data. Bioinformatics 2003;19:1469–76.

25. Witta SE, Gemmill RM, Hirsch FR, et al. Restoring E-cadherin expression increases sensitivity to epidermal growth factor receptor inhibitors in lung cancer cell lines. Cancer Res 2006;66:944–50.

[5] B.A. Helfrich, personal communication.

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26. Thomson S, Buck E, Petti F, et al. Epithelial to mesenchymal transition is a determinant of sensitivity of non-small-cell lung carcinoma cell lines and xenografts to epidermal growth factor receptor inhibition. Cancer Res 2005;65:9455–62.

27. Yauch RL, Januario T, Eberhard DA, et al. Epithelial versus mesenchymal phenotype determines *in vitro* sensitivity and predicts clinical activity of erlotinib in lung cancer patients. Clin Cancer Res 2005;11:8686–98.

28. Soltoff SP, Carraway KL III, Prigent SA, Gullick WG, Cantley LC. ErbB3 is involved in activation of phosphatidylinositol 3-kinase by epidermal growth factor. Mol Cell Biol 1994;14:3550–8.

29. Engelman JA, Janne PA, Mermel C, et al. ErbB-3 mediates phosphoinositide 3kinase activity in gefitinib-sensitive non-small cell lung cancer cell lines. Proc Natl Acad Sci U S A 2005;102:3788–93.

30. Cappuzzo F, Toschi L, Domenichini I, et al. HER3 genomic gain and sensitivity to gefitinib in advanced non-small-cell lung cancer patients. Br J Cancer 2005;93:1334–40.

31. Carmichael J, Mitchell JB, Degraff WG, et al. Chemosensitivity testing of human lung cancer cell lines using the MTT assay. Br J Cancer 1988;57:540–7.

32. Golpon HA, Coldren CD, Zamora MR, et al. Emphysema lung tissue gene expression profiling. Am J Respir Cell Mol Biol 2004;31:595–600.

33. Brazma A, Hingamp P, Quackenbush J, et al. Minimum Information About a Microarray Experiment (MIAME)—toward standards for microarray data. Nat Genet 2001;29:365–71.

34. Gentleman RC, Carey VJ, Bates DM, et al. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol 2004;5:R80.

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Baseline Gene Expression Predicts Sensitivity to Gefitinib in Non–Small Cell Lung Cancer Cell Lines

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