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

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Abstract

Treatment of second- and third-line patients with non–small-cell lung carcinoma (NSCLC) with the epidermal growth factor receptor (EGFR) kinase inhibitor erlotinib significantly increased survival relative to placebo. Whereas patient tumors with EGFR mutations have shown responses to EGFR inhibitors, an exclusive role for mutations in patient survival benefit from EGFR inhibition is unclear. Here we show that wild-type EGFR–containing human NSCLC lines grown both in culture and as xenografts show a range of sensitivities to EGFR inhibition dependent on the degree to which they have undergone an epithelial to mesenchymal transition (EMT). NSCLC lines which express the epithelial cell junction protein E-cadherin showed greater sensitivity to EGFR inhibition *in vitro* and in xenografts. In contrast, NSCLC lines having undergone EMT, expressing vimentin and/or fibronectin, were insensitive to the growth inhibitory effects of EGFR kinase inhibition *in vitro* and in xenografts. The differential sensitivity of NSCLC cells with epithelial or mesenchymal phenotypes to EGFR inhibition did not correlate with cell cycle status *in vitro* or with xenograft growth rates *in vivo*, or with total EGFR protein levels. Cells sensitive to EGFR inhibition, with an epithelial cell phenotype, did exhibit increased phosphorylation of EGFR and ErbB3 and a marked increase in total ErbB3. The loss of E-cadherin and deregulation of β-catenin associated with EMT have been shown to correlate with poor prognosis in multiple solid tumor types. These data suggest that EMT may be a general biological switch rendering non–small cell lung tumors sensitive or insensitive to EGFR inhibition. (Cancer Res 2005; 65(20): 9455-62)

Introduction

Epidermal growth factor receptor (EGFR) has been shown to be overexpressed in human cancers, including cancers of the lung [non–small-cell lung carcinoma (NSCLC)], central nervous system, head and neck, bladder, pancreas, and breast, and overexpression has been shown to correlate with poor survival (1). Inhibitors of EGFR function have shown clinical utility and the definition of key EGFR signaling pathways, which describe patient subsets most likely to benefit from therapy, has become an important area of investigation. Although paracrine and autocrine activation of EGFR plays a critical role in the maintenance of epithelial tissues, NSCLC lines show considerable variability in their cellular responses to EGFR inhibition and can be insensitive to pharmacologic withdrawal of EGFR signaling. The role of EGFR mutations (2, 3) as a principle mechanism in conferring sensitivity to EGFR inhibitors has been controversial (4). The variable sensitivities to EGFR inhibition of both cell lines and tumors containing wild-type (WT) EGFR have been shown to derive in part from EGFR-independent activation of the phosphatidylinositol 3′-kinase pathway, leading to a continued phosphorylation of the antiapoptotic serine-threonine kinase Akt (5). The molecular determinants of alternative routes of phosphatidylinositol 3′-kinase activation and consequent EGFR inhibitor insensitivity are poorly described, although the insulin-like growth factor I receptor, which strongly activates the phosphatidylinositol 3′-kinase pathway, has been implicated in cellular resistance to EGF inhibitors (6). Recent clinical data also suggest that *EGFR* gene amplification, as measured by fluorescence *in situ* hybridization, and EGFR protein, as measured by immunohistochemical methods, correlate with NSCLC patient benefit from EGFR inhibitors (7, 8). Here we report that human NSCLC cells containing WT EGFR show a variable sensitivity to erlotinib treatment *in vitro* and *in vivo*. Sensitivity to EGFR inhibition did not correlate with total EGFR levels, but did correlate with phospho-EGFR, phospho-ErbB3, and total ErbB3. The sensitivity of the cells and xenografts to erlotinib treatment can be predicted by whether the cells have undergone a process termed epithelial to mesenchymal transition (EMT). This transition is characterized by the combined loss of epithelial cell junction proteins such as E-cadherin and the gain of mesenchymal markers such as vimentin or fibronectin (9). It has become increasingly clear over recent years that EMT, already established as a critical developmental process, plays a major role in the progression of cancer (10, 11). The loss of E-cadherin has been associated with poor clinical outcome in NSCLC (12–16). The transition to a mesenchymal phenotype, mediated via transcriptional reprogramming by factors such as Twist (17, 18) and Snail (19, 20), increases the migratory potential of cancer cells and leads to metastasis. We discuss the implications of this transition with regards to erlotinib sensitivity and suggest that markers of these cellular changes may be used to identify patient groups who would maximally benefit from EGFR inhibition.

Materials and Methods

Cell culture, growth inhibition assay, and preparation of cell extracts. The human NSCLC lines (H292, H358, H322, H441, A549, Calu6, H460, H1703, SW1573, Calu3, Colo699, H2122, A427, H1437, H1299, Hop92, and H23) were cultured in the appropriate American Type Culture Collection recommended supplemented media. For growth inhibition assays, cells were plated and allowed to proliferate for 24 hours. Serial dilutions of erlotinib were added and the cells grown for a further 72 hours. Cell viability was assayed using Cell TiterGlow reagent (Promega Corp., Madison, WI). Cell

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extracts were prepared by detergent lysis in 50 mmol/L Tris-HCl (pH 8), 150 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, containing protease (Sigma, St. Louis, MO) and phosphatase inhibitor cocktails (Sigma). The soluble protein concentration was determined by micro-BSA (bovine serum albumin) assay (Pierce, Rockford, IL).

Protein identification and quantitation by liquid chromatography-tandem mass spectrometry peptide sequencing. Antiphosphotyrosine immunoaffinity resins were prepared by covalent coupling to solid support as previously described (21). Proteins isolated by antiphosphotyrosine affinity selection were digested with trypsin and peptides labeled with iTRAQ reagents essentially as described (22). Peptide masses, sequence information, and peptide quantitation were determined by electrospray liquid chromatography-tandem mass spectrometry (LC-MS/MS) and database searching as previously described (21).

Immunoblot analysis of non–small-cell lung carcinoma cell line extracts. Protein immunodetection was done by electrophoretic transfer of SDS-PAGE, separation of proteins on nitrocellulose, incubation with antibody, and chemiluminescent second-step detection (PicoWest; Pierce). The antibodies included E-cadherin, β-catenin, β-catenin, β-catenin, P-cadherin, N-cadherin, Zeb-1, ErbB3 and Brk (Santa Cruz Biotechnology, Santa Cruz, CA); vimentin and fibronectin (BD Biosciences, San Jose, CA); glyceraldehyde-3-phosphate dehydrogenase (GADPH) and cytokeratin 8/18 (AbCam, Cambridge, United Kingdom); and EGFR, pEGFR (pTyr1068), ErbB2, pErbB2 (pTyr1248), and pErbB3 (pTyr1289; Cell Signaling Technology, Beverly, MA).

Confocal microscopy. Cells grown on glass coverslips for 24 hours were washed and fixed with 3.7% formaldehyde in Dulbecco's PBS followed by permeabilization in 0.5% NP40. The cells were washed, blocked with 5% BSA in PBS, and incubated with primary antibody for 2 hours at room temperature and with diluted FITC-conjugated secondary antibody for 1 hour. Nuclei were stained with 4′,6-diamidino-2-phenylindole (300 nmol/L for 5 minutes). The images were captured using a spinning objective confocal microscope at ×60 magnification.

Fluorescence-activated cell sorting. Cells were seeded at 5×10^5/well and grown for 24 hours. The cells were trypsinized, harvested in cell culture media, washed once with PBS, and fixed by resuspension in ice-cold 70% ethanol for 24 hours. Cells were collected by centrifugation, resuspended in fluorescence-activated cell sorting (FACS) staining buffer (2% FCS, 0.01% sodium azide in PBS) containing 1 mg/mL RNase (Sigma), and incubated at 37°C for 60 minutes. Cells were stained with 5 μg/mL propidium iodide (Calbiochem, San Diego, CA) for 60 minutes at room temperature and analyzed using a Beckman Coulter EPICS XL MCL flow cytometer. The percentage of cells in each stage of the cell cycle was determined using the EXPO32 ADC analysis software.

***In vivo* xenograft modeling.** Female CD-1 *nu/nu* mice (Charles River Laboratories, Wilmington, MA) were implanted with harvested NSCLC tumor cells in a single s.c. site on the flank of the mice in the axillary region. Mice were injected with 5 to 10 million tumors cells per mouse in a 0.1 mL injection, depending on the model. The cells were counted with trypan blue and mice were not implanted if the viability was <90%. The mice were sorted by weight to randomize the defined tumor range. Tumors were allowed to grow to 200 ± 50 mm^3, at which time the animals were sorted into treatment groups of eight animals per group based on weight (± 1 g body weight), which randomized the tumor volume in the groups, and were tattooed on the tail for permanent identification. Tumor volumes and body weights were determined twice weekly. The tumor volume was determined by measuring in two directions with vernier calipers and calculated using the following formula: tumor volume = (length × width^2) / 2. The data were plotted as the percent change in mean values of tumor volume and body weight for each group. The tumor growth inhibition (%TGI) was determined as %TGI = 100(1 − Wt − Wc), where Wt is the median tumor volume of the treated group at time x and Wc is the median tumor volume of the control group at time x. Erlotinib was dosed in 6% Captisol (CyDex, Inc., Lenexa, KS) in Water for Injection solution and all control animals were dosed with an equal volume of the vehicle. Animals were dosed by oral gavage once a day for 14 days and tumor growth inhibition measured on day 15. Repeated measure ANOVAs were done for each experiment to test for differences between the percent tumor volume profiles of the two treatments (control and 100 mg/kg erlotinib). For immunoblot analysis, untreated tumors were grown for 3 days, excised, flash frozen in liquid N_2, pulverized, and proteins extracted as described above.

Results

Non–small-cell lung carcinoma cell lines containing wild-type epidermal growth factor receptor display a range of sensitivities to erlotinib *in vitro*. Analysis of erlotinib sensitivity in human NSCLC cell lines and mouse xenograft models, confirmed by DNA sequence analysis to express the WT EGFR polypeptide, indicated a wide range of sensitivity as measured by growth inhibition *in vitro* and *in vivo* (Table 1). The principle aim of this study was to determine the molecular determinants of erlotinib sensitivity in these NSCLC cell lines. We have thus broadly classified these cell lines into those that are insensitive (H1703, SW1573, H460, Calu6, H1437, H1299, Hop92, and H23), those that show an intermediate sensitivity (A549, Colo699, H2122, and A427), and those that are sensitive (H441, H358, H322, H292, and Calu3) to erlotinib-mediated growth inhibition both *in vitro* and in xenografts *in vivo*. Sensitivity was defined as >50% *in vitro* growth inhibition at an erlotinib concentration of <5 μmol/L, with statistically significant tumor growth inhibition *in vivo* if available (Table 1). In several model systems, differences in cellular sensitivity to EGFR inhibition can be correlated in part to a failure of the insensitive cell lines to show sufficient erlotinib-mediated inhibition of Akt/protein kinase B phosphorylation (5, 23). To expand on this, we examined total and phosphorylated levels of EGFR, ErbB2, and ErbB3 (Fig. 1). No correlation between total EGFR or ErbB2 and erlotinib sensitivity was observed. In contrast, ErbB3 expression was elevated in NSCLC cell lines sensitive to growth inhibition by erlotinib. Sensitive cell lines displayed markedly higher phosphorylation of ErbB3 and, to a lesser extent, EGFR, as compared with the insensitive lines. Phospho-ErbB2 was undetectable in all cases with the exception of H441. These data suggest activation of EGFR and ErbB3 pathways in erlotinib-sensitive cell lines and are in agreement with recent studies describing gefitinib sensitivity in NSCLC (24).

Changes in epithelial and mesenchymal cell markers correlate with sensitivity of non–small-cell lung carcinoma cell lines to erlotinib. To further understand the mechanisms by which cells escape growth inhibition by EGFR inhibitors, we examined protein expression differences between erlotinib-sensitive and -insensitive NSCLC lines. Initially, differences in protein tyrosine phosphorylation and complex formation between NSCLC lines sensitive or insensitive to erlotinib were measured. These experiments involved antiphosphotyrosine affinity selection of cell lysates, tryptic digestion, and protein identification based on LC-MS/MS fragment ion spectra (21). We observed a striking difference between the erlotinib-sensitive and -insensitive NSCLC lines with respect to the abnormal expression of vimentin and/or fibronectin (Fig. 2*A*). Typically, the expressions of vimentin, an intermediate filament protein, and fibronectin, a secreted extracellular matrix protein, are characteristic of mesenchymal cells and are absent or weakly expressed in epithelial cell lineages. Vimentin expression was primarily found in H1703 and Calu6, whereas fibronectin expression was observed in H460 cells, which correlated well with subsequent immunoblot results (see below). These three NSCLC lines were insensitive to growth inhibition by erlotinib *in vitro* and *in vivo* (Table 1). Little or no vimentin or fibronectin expression was found in the erlotinib-sensitive NSCLC lines H292 and H358 or in the intermediate line A549 by mass spectrometry analysis.

Table 1. Growth inhibition of human WT EGFR tumor cell lines and xenografts sensitive or insensitive to erlotinib

NSCLC cell line	EGFR genotype	Maximal inhibition (%)	EC_{50} (μmol/L)	TGI significance (rmANOVA)	Median %TGI, day 15	Classification
H292	WT	72	0.1	$P < 0.0001$	85	Sensitive
H322	WT	80	0.4	nd	nd	Sensitive
H358	WT	75	0.6	$P = 0.0073$	25	Sensitive
H441	WT	55	2	$P = 0.0002$	60	Sensitive
Calu3	nd	78	0.6	nd	67	Sensitive
A427	WT	62	2	nd	nd	Sensitive
A549	WT	44	5	$P = 0.5131$	49	Intermediate
Colo699	nd	45	3	nd	nd	Intermediate
H2122	WT	45	5	nd	44	Intermediate
H460	WT	29	5	$P = 0.9823$	6	Insensitive
Calu6	WT	36	>10	$P = 0.5884$	0	Insensitive
H1703	WT	30	7	nd	nd	Insensitive
SW1573	WT	25	9	nd	nd	Insensitive
H1437	nd	5	>10	nd	nd	Insensitive
H1299	WT	25	5	nd	nd	Insensitive
Hop92	WT	22	5	nd	nd	Insensitive
H23	WT	30	5	nd	nd	Insensitive

NOTE: Inhibition was assessed by two efficacy measurements, % maximal growth inhibition *in vitro* and statistically significant [repeated measures ANOVA (rmANOVA)] tumor growth inhibition (TGI) in xenografts treated with erlotinib (100 mg/kg, qd) or vehicle for 14 days, and one potency measurement, EC_{50}, the micromolar concentration required for half-maximal inhibition *in vitro*. Sensitivity was defined as >50% *in vitro* growth inhibition at an erlotinib concentration of <5 μmol/L, with statistically significant tumor growth inhibition if available.

Based on the expression of mesenchymal proteins in erlotinib-insensitive NSCLC lines, we analyzed an expanded panel of NSCLC cell lines for the presence or absence of markers characteristic of either epithelial or mesenchymal phenotypes (Fig. 2*B* and *C*; Table 2). E-cadherin was primarily observed in the sensitive cell lines (H441, H358, H322, H292, and Calu3) but was absent in the insensitive cell lines (H1703, SW1573, H460, Calu6, H1299, Hop92, and H23). The exception was the erlotinib-sensitive cell line A427 in which E-cadherin and P-cadherin expressions were absent but N-cadherin and β-catenin were coexpressed. In the erlotinib-insensitive cell lines (H1703, SW1573, H460, Calu6, H1299, Hop92, and H23), no E-cadherin was observed even on longer exposure of immunoblots. The intermediately sensitive cell line A549 showed low, but detectable, E-cadherin expression on longer immunoblot exposure. A similar loss of β-catenin was frequently observed in cells insensitive to erlotinib, with the exceptions of H460 and H1437. Therefore, the insensitive cell lines seem to have lost expression of epithelial cell junction proteins such as E-cadherin.

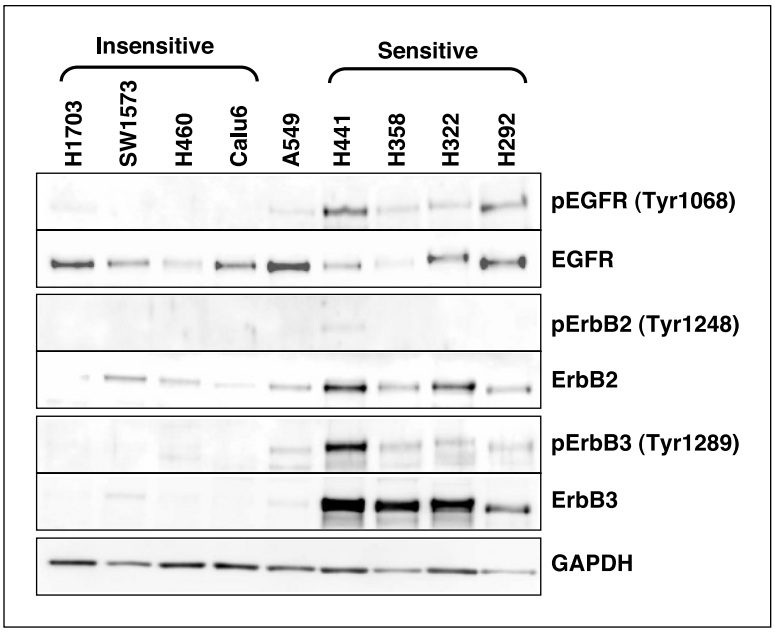

Figure 1. NSCLC cell lines sensitive, intermediately sensitive, or insensitive to growth inhibition mediated by EGFR kinase blockade with erlotinib (1 μmol/L) were evaluated for total and tyrosine phosphorylated forms of EGFR, ErbB2, and ErbB3 by immunoblot of detergent extracts. GAPDH served as a loading control. No correlation between total EGFR or ErbB2 and erlotinib sensitivity was observed. In contrast, ErbB3 expression was elevated in NSCLC cell lines sensitive to growth inhibition by erlotinib. Sensitive cell lines displayed markedly higher phosphorylation of ErbB3 and, to a lesser extent, EGFR, as compared with insensitive lines. Phospho-ErbB2 was undetectable in all cases with the exception of H441. These data suggest activation of EGFR and ErbB3 pathways in erlotinib-sensitive cell lines.

Next we asked whether these cell lines expressed the mesenchymal markers vimentin and/or fibronectin. The insensitive cell lines (H1703, SW1573, H460, Calu6, H1299, Hop92, and H23) clearly expressed vimentin and/or fibronectin (Fig. 2*B* and *C*; Table 2). H1437 cells were exceptional and retained E-cadherin and lost P-cadherin expression but had not undergone EMT, suggesting other pathways could override the epithelial phenotype to confer insensitivity to EGFR inhibition. Neither vimentin nor fibronectin was readily detectable in cell lines sensitive to erlotinib (H441, H358, H322, H292, and Calu3; Fig. 2*B* and *C*), with the exception of A427, which expressed coexpressed vimentin, N-cadherin, and β-catenin (Table 2). The intermediately sensitive cell line A549 again showed low, but detectable, levels of both vimentin and E-cadherin proteins. Interestingly, the majority of cell lines analyzed retained expression of the epithelial cell intermediate filament proteins cytokeratins 8 and/or 18 (data not shown). This suggests that although the insensitive cell lines have largely lost E-cadherin–dependent cell-cell junctions and began to express mesenchymal proteins, they maintained some epithelial cell proteins and proliferative potential.

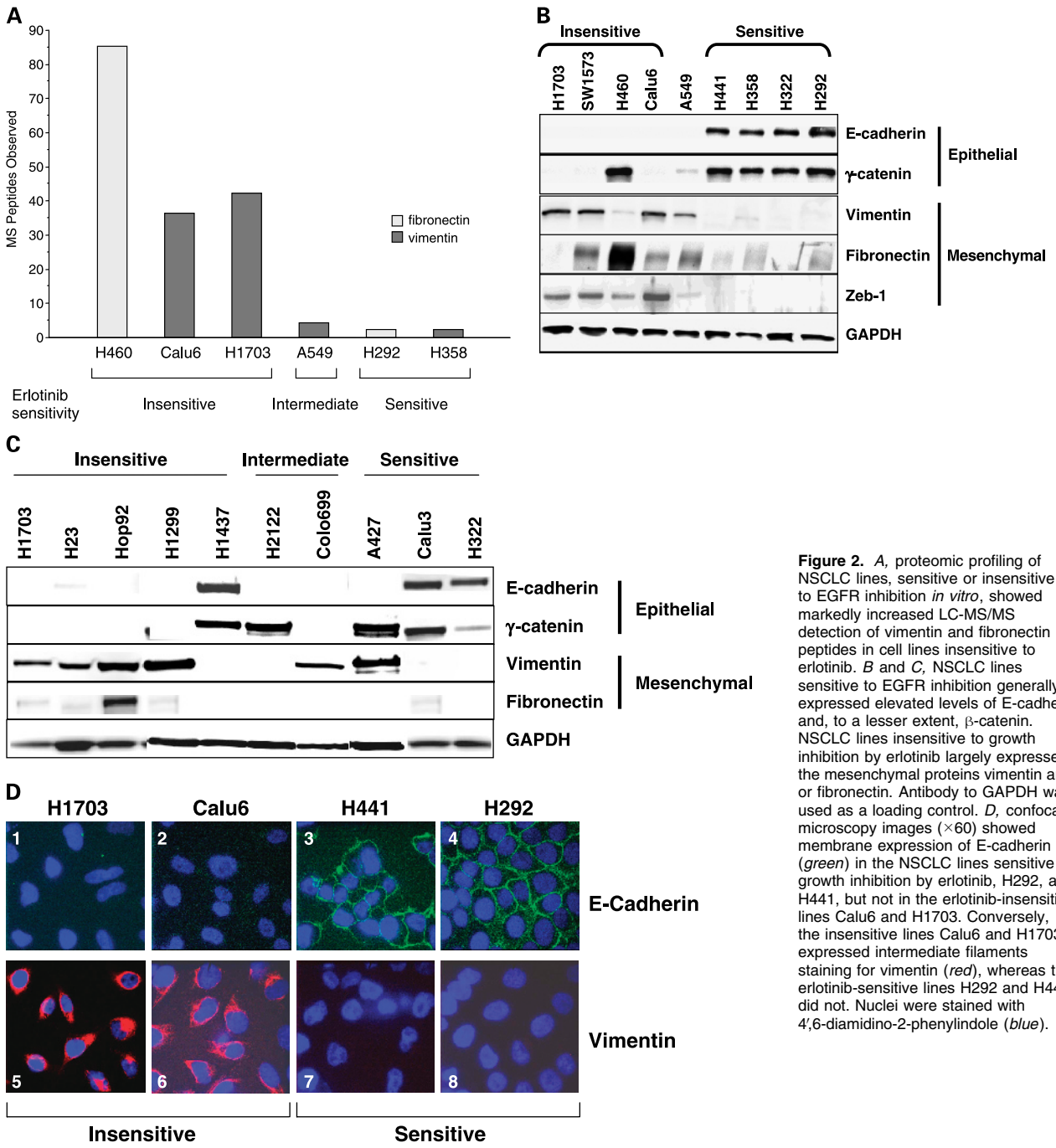

Figure 2. *A,* proteomic profiling of NSCLC lines, sensitive or insensitive to EGFR inhibition *in vitro*, showed markedly increased LC-MS/MS detection of vimentin and fibronectin peptides in cell lines insensitive to erlotinib. *B* and *C,* NSCLC lines sensitive to EGFR inhibition generally expressed elevated levels of E-cadherin and, to a lesser extent, β-catenin. NSCLC lines insensitive to growth inhibition by erlotinib largely expressed the mesenchymal proteins vimentin and/or fibronectin. Antibody to GAPDH was used as a loading control. *D,* confocal microscopy images (×60) showed membrane expression of E-cadherin (*green*) in the NSCLC lines sensitive to growth inhibition by erlotinib, H292, and H441, but not in the erlotinib-insensitive lines Calu6 and H1703. Conversely, the insensitive lines Calu6 and H1703 expressed intermediate filaments staining for vimentin (*red*), whereas the erlotinib-sensitive lines H292 and H441 did not. Nuclei were stained with 4′,6-diamidino-2-phenylindole (*blue*).

Table 2. Summary of EMT markers expressed in NSCLC cell lines *in vitro*

NSCLC cell line	Cell type	Erlotinib sensitivity	E-cadherin	P-cadherin	γ-catenin	N-cadherin	Vimentin	Fibronectin
H292	Mucoepedermoid	Sensitive	+	+	+	−	−	−
H322	Adeno	Sensitive	+	+	+	−	−	−
H358	Adeno	Sensitive	+	+	+	−	−	−
H441	Adeno	Sensitive	+	+	+	−	−	−
Calu3	Adeno	Sensitive	+	−	+	−	−	−
A427	Lung carcinoma	Sensitive	−	−	+	+	+	−
A549	Adeno	Intermediate	−	−	−	+	+	+
Colo699	Adeno	Intermediate	−	−	−	+	+	−
H2122	Adeno	Intermediate	−	−	+	−	−	−
H1703	Squamous	Insensitive	−	−	−	−	+	−
Sw1573	Squamous	Insensitive	−	−	−	−	+	+
H460	Large cell	Insensitive	−	−	+	−	+	−
Calu6	Lung cancer	Insensitive	−	−	−	−	+	+
H1437	Adeno	Insensitive	+	−	+	−	−	−
H1299	Large cell	Insensitive	−	−	−	+	+	−
Hop92	Large cell	Insensitive	−	−	−	nd	+	+
H23	Adeno	Insensitive	−	−	−	nd	+	−

NOTE: Expression of the individual proteins was determined by immunoblot analysis (Fig. 2*B* and *C* and data not shown). Those cell lines most sensitive to growth inhibition *in vitro* and in xenografts by erlotinib expressed the epithelial markers E-cadherin, P-cadherin, and β-catenin. Cell lines less sensitive to erlotinib have lost expression of E- and P-cadherin and gained expression of the mesenchymal proteins vimentin and fibronectin. The majority of cell lines also retained expression of cytokeratins 8 and/or 18, with the exception of H1703.

Transcription factors including Snail, Twist, and Zeb have been shown to promote cell transitions to a more mesenchymal phenotype. We examined the relative levels of these factors in NSCLC cells sensitive or insensitive to EGFR inhibition. Measurement of RNA abundance for Snail, Twist, Zeb-1/ΔEF-1, and Zeb-2/SIP in the panel of NSCLC lines by reverse transcription-PCR indicated Zeb-1 to be the most highly expressed (data not shown). The expression of the zinc finger and homeodomain transcriptional repressor Zeb-1 was observed only in the EGFR-insensitive lines with a mesenchymal phenotype (Fig. 2*B*), consistent with the role of Zeb-1 in promoting EMT in NSCLC.

The changes in cell lineage markers were further analyzed in two sensitive and two insensitive cell lines by confocal microscopy after immunostaining with antibodies toward E-cadherin and vimentin (Fig. 2*D*). No E-cadherin staining could be detected in either erlotinib-insensitive H1703 or Calu6 cells (Fig. 2*D*, *1* and *2*), whereas staining for intermediate filaments containing vimentin (Fig. 2*D*, *5* and *6*) was observed. The reverse was true for the sensitive cell lines H441 and H292, with clear E-cadherin staining at the plasma membrane of these cells (Fig. 2*D*, *3* and *4*) but no visible vimentin staining (Fig. 2*D*, *7* and *8*). In the erlotinib-insensitive cell lines, no redistribution of E-cadherin to the cytosol was observed by confocal microscopy, even after long exposure. Taken together, these data indicate that NSCLC cells that were insensitive to growth inhibition by erlotinib seemed to have undergone transition to a more mesenchymal cell type and expressed either vimentin or fibronectin. In contrast, cell lines that were sensitive to growth inhibition by erlotinib largely maintained an epithelial phenotype and expressed E-cadherin and ErbB3.

The transition from epithelial to mesenchymal phenotype has been generally associated with a decrease in cell proliferative potential. For example, when normal epithelial cells were exposed to transforming growth factor β1 *in vitro*, the resulting EMT was associated with a marked reduction of [^{3}H]thymidine incorporation (25). Similar finding were observed in transforming growth factor β3–induced EMT of NSCLC cells, in which a reduction of proliferation rate was correlated with a more mesenchymal phenotype.[3] To assess the possible effect of cell proliferation rates on sensitivity to erlotinib, cell cycle analysis of the NSCLC lines was done by FACS. No correlation between S-phase fraction and sensitivity to erlotinib or EMT status *in vitro* was observed (Table 3).

In summary, these data showed that the majority of erlotinib-sensitive lines were E-cadherin$^+$/vimentin$^-$ (five of six) and all expressed β-catenin. In contrast, erlotinib-insensitive cell lines seemed to have undergone EMT, as judged by the loss of E-cadherin and the expression of vimentin/fibronectin (seven of eight cell lines; Table 2).

Erlotinib sensitivity correlates with maintenance of epithelial markers during tumor growth *in vivo*. Tumor xenografts derived from NSCLC cell lines grown in mice displayed a similar degree of erlotinib sensitivity (Fig. 3*A*) to that observed for the respective cell lines *in vitro* (Table 1). Significant tumor growth inhibition was observed for H292, H441, and H358 xenografts in response to erlotinib treatment (Fig. 3*A*). We therefore examined whether the protein markers identified *in vitro* were also predictive of erlotinib sensitivity *in vivo*. Protein extracts were prepared from four independent tumor xenografts of H460, Calu6, A549, H441, and H292 cells. E-cadherin expression was not detectable in xenografts derived from the H460 and Calu6 cells insensitive to erlotinib (Fig. 3*B*). However, E-cadherin was expressed at low levels in xenografts derived from the A549 cells of intermediate sensitivity and was expressed at higher levels in H441 and H292 cell lines sensitive to erlotinib. Similar trends were observed on analysis of

[3] S. Thomson, F. Petti, and J.D. Haley, unpublished data.

Table 3. Cell cycle measurements of NSCLC cell lines sensitive or insensitive to EGFR inhibition

NSCLC cell line	G_0-G_1	G_2-M	S	Classification
H292	34.9 ± 2.4	27.8 ± 0.4	15.6 ± 0.1	Sensitive
H322	36.1 ± 1.4	31.8 ± 1.1	12.5 ± 1.8	Sensitive
H358	46.3 ± 1.9	20.2 ± 1.6	27.2 ± 2.1	Sensitive
H441	30.6 ± 1.9	31.1 ± 0.1	16.7 ± 0.2	Sensitive
A549	41.7 ±0.9	27.0 ± 0.7	22.3 ± 0.7	Intermediate
H460	51.3 ± 1.5	22.5 ± 1.4	14.5 ± 1.5	Intermediate
Calu6	24.6 ± 0.8	33.0 ± 1.7	19.1 ± 0.6	Insensitive
H1703	46.6 ± 1.9	19.5 ± 1.4	22.5 ± 1.2	Insensitive
SW1573	72.2 ± 1.1	08.8 ± 0.3	15.3 ± 0.3	Insensitive

NOTE: The percentages of cells of in G_0-G_1, G_2-M, and S phases of the cell cycle were measured by FACS. SEs from two independent biological experiments are indicated. No correlation between cell cycle status *in vitro* and sensitivity to EGFR inhibition or epithelial versus mesenchymal phenotype was observed.

β-catenin levels, with the exception of H460. Fibronectin and/or vimentin expression was only prominent in the erlotinib-insensitive xenograft extracts, suggesting minimal contribution from infiltrating mouse stromal tissue. These *in vivo* results further support the *in vitro* data and support the hypothesis that erlotinib sensitivity may be greatest in cells with an epithelial phenotype. Consistent with *in vitro* cell cycle analysis, no correlation between xenograft growth rates *in vivo* and sensitivity to erlotinib or EMT status was observed. The data suggest that cells that have undergone EMT become less dependent on EGFR signaling for cell proliferation and survival and are thus less responsive to erlotinib.

Discussion

The molecular determinants of patient sensitivity to EGFR inhibitors are not well defined and the role of mutations in conferring increased response and survival is still unclear. A correlation of total and phospho-ErbB3 family expression, and to a lesser extent phospho-EGFR, with erlotinib sensitivity was observed (Figure 1). These data suggest that NSCLC cells showing HER family signaling, either directly through EGFR-ErbB3 heterodimer formation or indirectly through Src family kinase activation of ErbB3, tend to be more sensitive to EGFR inhibition (23, 24). We observed preferential activation of EGFR and ErbB3 pathways in erlotinib-sensitive cell lines, in which the phosphorylation of ErbB3 was dependent on EGFR kinase activity (data not shown). These data suggest that cells which used EGFR to stimulate a phosphatidylinositol 3′-kinase signal via ErbB3 were those most sensitive to EGFR inhibition. Interestingly, erlotinib-sensitive cells also tended to express higher levels of the Src-like kinase Brk (data not shown), which has been shown to phosphorylate ErbB3 and promote Akt phosphorylation through phosphatidylinositol 3′-kinase (26). Whereas activations of EGFR and ErbB3 were shown to be associated with cellular sensitivity to EGFR inhibition, the nature of the insensitivity of NSCLC lines and xenografts was less clear.

EMT, defined by the combined loss of E-cadherin and gain of mesenchymal lineage marker expression (10), negatively affected cellular responses to EGFR inhibitors *in vitro* and in xenografts. Five of the six erlotinib-sensitive lines were E-cadherin$^+$/vimentin$^-$ and all expressed γ-catenin. Similarly, seven of the eight insensitive cell lines seem to have undergone EMT, as judged by the loss of E-cadherin and the expression of vimentin/fibronectin (Table 2). The loss of E-cadherin did not involve redistribution from membrane to cytosol and was a relatively complete loss. The expression of Zeb-1, a zinc finger and homeodomain transcription factor, was restricted to erlotinib-insensitive NSCLC lines that had undergone EMT (Fig. 2*B*). However, those cells continued to express cytokeratins 8/18 (data not shown), suggesting that a partial transition to a mesenchymal phenotype had occurred, in which cells express both epithelial (cytokeratin) and mesenchymal (vimentin) intermediate filament proteins and retain proliferative potential. H460 cells, although losing the expression of E-cadherin, maintained γ-catenin expression and showed elevated levels of fibronectin but only low levels of vimentin. Whether H460 cells represent a differing mesenchymal program remains to be determined. Initial data with the EGFR deletion mutant (Δ746-750) cell line, sensitive to erlotinib *in vivo*, showed an E-cadherin$^+$/vimentin$^-$ profile, consistent with the model proposed. Whether this holds true for other EGFR mutations in NSCLC (e.g., L858R) remains to be determined. The sensitivity of cell lines and xenografts to EGFR inhibitors seemed to be a continuum and not necessarily reflective of three distinct physiologic states. The binning of data into the three sensitivity classifications simply allows the observation that loss of E-cadherin and gain of vimentin/fibronectin expression correlated with sensitivity of NSCLC lines to erlotinib. These data suggest multiple stages or multiple mechanisms of EMT in NSCLC lines and xenografts that ultimately diminish cellular requirements for EGFR/ErbB3 signaling.

The elevated expression of transcription factor Zeb-1/ΔEF1 in cells showing loss of E-cadherin is consistent with transcriptional repression as a mechanism for down-regulation of E-cadherin and is supported by reverse transcription-PCR measurements showing loss of E-cadherin RNA in erlotinib-insensitive lines (data not shown). Zeb-1 has been shown to correlate with the loss of E-cadherin in NSCLC lines (27). The expression of Zeb-1 was recently shown to correlate with the degree of EMT in breast cancer cells (28), and E-cadherin has been shown to be highly regulated and repressed at the transcriptional level by factors including Snail, Slug, Twist, and ZEB (29, 30), likely requiring the recruitment of COOH-terminal binding protein as a corepressor (31, 32).

The loss of E-cadherin expression and the acquisition of a more mesenchymal phenotype have been shown to correlate with poor prognosis in multiple epithelial-derived solid tumors (33). One consequence of E-cadherin loss is the activation of components of the Wnt signaling pathway (11, 34). For example, cell-cell contacts mediated through the transmembrane protein E-cadherin can be linked to the actin cytoskeleton by recruitment of β-catenin. The disruption of cell-cell junctions mediated by E-cadherin loss releases β-catenin to translocate to the nucleus and transcribe genes required for cell cycle progression, such as myc and cyclin D. Interestingly, erlotinib-mediated down-regulation of cyclin D1 has been shown to correlate with erlotinib sensitivity *in vitro* and in human tumor samples (35). These data would be consistent with the presence of a functional E-cadherin/β-catenin signaling pathway.

The extent to which the mesenchymal proteins in NSCLC act as indicators versus functional participants in producing insensitivity to EGFR inhibitors is unclear at the present time. There is a

significant correlation between insensitivity to erlotinib and continued activation of Akt, and so these changes may initiate activation of the phosphatidylinositol 3′-kinase/Akt signaling pathway via non-EGFR-mediated routes. For example, in cells with a mesenchymal phenotype, the interaction of fibronectin and integrin (e.g., α5β1; ref. 36) has the potential to activate phosphatidylinositol 3′-kinase pathway signaling (5, 37, 38) in a manner independent of EGFR, potentially leading to erlotinib insensitivity. Fibronectin, increased by tobacco smoke injury to lung tissue, has been shown to increase expression of cyclooxygenase-2, leading to the production of prostaglandin E2 (39). The overexpression of cyclooxygenase-2 has been shown to exert antiapoptotic and proproliferative effects (40), in part through insulin-like growth factor I receptor modulation (41). Tumoral expression of fibronectin was shown to be elevated in NSCLC, and coexpression of integrin subunits α5 and β1 was associated with lymph node metastases (P = 0.04 and 0.005, respectively; ref. 42). Similarly, intermediate filaments containing keratins or vimentin, thought to be static structural elements of the cytoskeleton, have been shown to modulate and control cellular processes such as migration and likely proliferation (43, 44). In vimentin-null transgenic mice, cell migration and wound healing were impaired (45), suggesting a gain of migration function role in NSCLC cells having undergone EMT. It is likely that additional factors (e.g., phosphatase and tensin

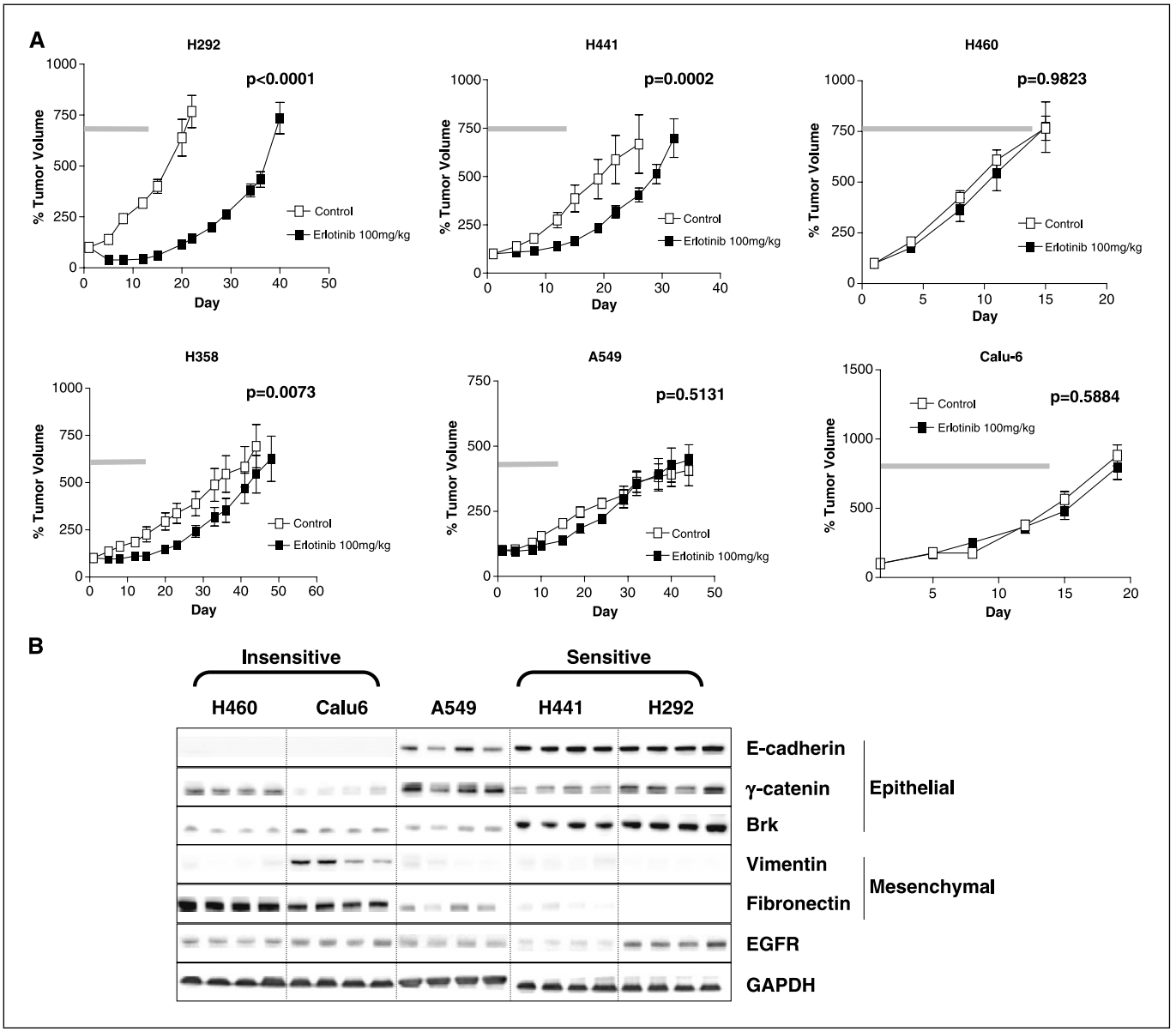

Figure 3. *A,* NSCLC lines were grown as s.c. xenografts in *nu/nu* mice and dosed for 14 days with erlotinib (100 mg/kg qd) or vehicle control. Statistical evaluation of percent tumor growth inhibition by repeated measures ANOVA was done. H292, H358, and H441 were found to be sensitive to EGFR inhibition *in vivo*. No correlation between tumor growth rate and sensitivity to EGFR inhibition was observed. *Solid bars,* erlotinib dosing period. *B,* tumor cell lysates were evaluated for the expression of epithelial and mesenchymal proteins. Tumors were grown to a volume of ~300 mm^3, excised, and flash frozen in liquid nitrogen. Tumor tissues were pulverized while frozen, subjected to detergent lysis and SDS-PAGE as described, and immunoblots probed with antibodies to E-cadherin, β-catenin, Brk, fibronectin, and vimentin. Similar with *in vitro* results, E-cadherin expression was restricted to erlotinib-sensitive lines and vimentin to insensitive lines. Antibody to GAPDH was used as a loading control.

homologue loss or phosphatidylinositol 3′-kinase mutations) may attenuate erlotinib sensitivity within E-cadherin$^+$/vimentin$^-$ tumor cells displaying an epithelial phenotype.

The suppression of E-cadherin expression has been reported to be a frequent event in multiple solid tumor types (33) including NSCLC (12–16). Whether mesenchymal-like cells are retained within tumors and whether they can be detected and measured distinctly from infiltrating stromal cells remain to be determined. Provided that robust, well-validated clinical assays for epithelial and mesenchymal markers can be established, these hypotheses can be tested in tumor samples from patients either sensitive or insensitive to EGFR inhibition with respect to both response rate and long-term survival. In conclusion, these findings suggest that EMT may play a general role in defining sensitivity to EGFR inhibitors, and provide a molecular signature (e.g., E-cadherin–positive and vimentin/fibronectin-negative) to define NSCLC tumors most likely to respond to treatment.

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