Loss of homotypic cell adhesion by epithelial-mesenchymal transition or mutation limits sensitivity to epidermal growth factor receptor inhibition

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Abstract

Overexpression and enhanced activation of the epidermal growth factor receptor (EGFR) is frequently observed in human carcinomas. Inhibitors of EGFR signaling have shown clinical utility; however, understanding response at the molecular level is important to define patient subsets most likely to benefit, as well as to support the rational design of drug combinations. Pancreatic and colorectal tumor cell lines insensitive to EGFR inhibition were those that had lost or mutated the epithelial junction constituents E-cadherin and γ-catenin, had lost homotypic adhesion, and often gained proteins associated with an epithelial to mesenchymal–like transition, such as vimentin, zeb1, or snail. In matched pairs of colorectal tumor cells, the epithelial lines showed an average 7-fold greater sensitivity than mesenchymal-like lines. In human pancreatic and colorectal tumor tissues, gain of mesenchymal characteristics and loss of epithelial characteristics correlated with advancing tumor stage. These data indicate an especially sensitive patient subset as well as a rationale for the combination of EGFR antagonists with agents that affect the epithelial to mesenchymal–like transition process as a mechanism to enhance sensitivity for more advanced mesenchymal-like tumors. [Mol Cancer Ther 2007;6(2):532–41]

Introduction

Signaling through the epidermal growth factor receptor (EGFR) is frequently deregulated in solid tumors, leading to abnormal activation of pro-proliferative and antiapoptotic pathways, notably the phosphatidylinositol 3-kinase/Akt, Ras/Raf/Mek/extracellular signal-regulated kinase, and signal transducers and activators of transcription 3/5 pathways (1–3). Inhibition of EGFR kinase activity has shown clinical utility in the treatment of non–small cell lung cancer (NSCLC; 4, 5) and pancreatic cancer (4, 6–9). Although these trials proved successful despite the enrollment of a broad base of patients with no prior selection for tumor type or biomarkers, the selection of patients who will most benefit from treatment with specific molecularly targeted therapies is an increasingly important goal. However, the molecular determinants governing tumor cell sensitivity to EGFR inhibitors have been multiple and complex, leading to uncertainty in how to identify patients who will best respond to EGFR inhibitors. Two types of markers potentially indicative of EGFR inhibitor treatment outcome have been considered—those pharmacodynamically associated with drug action and those predictive in pretreatment biopsy of drug efficacy. Markers reflective of drug action, including the attenuation of extracellular signal-regulated kinase (10), Akt (10–12), and/or signal transducers and activators of transcription 3/5 (13) phosphorylation or the activation of c-fos (14), have been documented. However, in the absence of suitable imaging techniques, markers of this type require biopsy during treatment and may not be suitable for a broad population of patients. Markers predictive of treatment outcome at first biopsy would circumvent the need for additional tumor tissue procurement. Candidate markers in this class have been described and include EGFR (as measured by immunohistochemistry and fluorescence *in situ* hybridization; refs. 15–17), phosphorylated Akt (18), EMP-1 (19), EGFR mutation (20–22), K-Ras mutation (18, 20, 23), ErbB3 (11, 24), and, more recently, markers reflective of an epithelial to mesenchymal (EMT)–like transition (25, 26).

Multiple factors have been proposed to predict clinical benefit from EGFR kinase inhibitor treatment. The identification of mutations in the EGFR kinase domain (21–23, 27) and the demonstration that mutant EGFRs (notably 746–750/3 deletion and L858R mutants) were more sensitive to erlotinib and gefitinib (23, 28) led to the hypothesis that these inhibitors would only be effective against tumors bearing heterozygous mutations in the *EGFR* gene. The *in vitro* demonstration of prolonged signaling from these mutated receptors and hypersensitivity of cell lines bearing these mutations suggested that these types of tumors would be more addicted to EGFR signaling and so be more susceptible to inhibition of the pathway. However, there remains some controversy as to the importance of EGFR mutations in the clinical setting. Initial

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retrospective analyses of gefitinib-treated NSCLC tumors for EGFR mutation suggested that those testing positive for mutation received the most benefit from gefitinib treatment as measured by both response rate and survival (10), providing support for the mutation hypothesis. In contrast, analysis of the only placebo-controlled EGFR inhibitor study thus far carried out (BR21) concluded that there was a survival benefit for both wild-type and mutant EGFR patients treated with the EGFR inhibitor erlotinib (5). Data from several phase III studies (4, 29) also suggest that patients with mutation in *EGFR* survive longer than those with wild-type EGFR irrespective of treatment. However, the concept that EGFR mutations may indicate a more positive prognosis contradicts the widely held belief that EGFR overexpression and activation are poor prognostic factors in solid tumors.

An additional factor suggested to predict the efficacy of EGFR inhibitors has been the mutational status of the small GTPase K-Ras in NSCLC. K-Ras is often mutationally activated in NSCLC and it has been suggested that subsequent activation of the Ras pathway would override any potential benefit of EGFR inhibition. Studies *in vitro* do not seem to support this hypothesis, as there is no correlation between K-Ras mutation status and erlotinib sensitivity in either NSCLC (26, 30) or pancreatic cancer cell lines (24). The data from the clinic seem less clear. Clinical studies of patients with K-Ras mutations from several trials (20, 27) suggest that these mutations correlate with reduced response or time to progression in patients receiving EGFR inhibitors. However, the potential cosegregation of K-Ras mutation and smoking history obscures interpretation.

The molecular mechanisms that promote cell proliferation and mediate cell survival differ among tumor cells, but are responsible for the varying degrees of sensitivity to EGFR inhibition. As tumor cells use these varying mechanisms for proliferation and survival, even among tumors of the same primary origin, identifying biomarkers for erlotinib sensitivity is critical for selecting patients who are likely to receive the most clinical benefit from this EGFR inhibitor. The serine threonine protein kinase Akt, activated by upstream phosphatidylinositol 3-kinase/3′-phosphoinositide-dependent kinase 1 and mammalian target of rapamycin/Rictor signaling (31), is an important component of cellular cell cycle progression and survival pathways. Tumor cells in which EGFR and Akt are sequentially activated seem to be the most sensitive to EGFR inhibition (11, 24). We have previously shown that for both pancreatic and colorectal tumor cell lines, signaling pathways important for erlotinib sensitivity are, in part, mediated by the ability of transphosphorylated ErbB3 to recruit the p85 subunit of phosphatidylinositol 3-kinase and transmit signals to Akt (11, 24). The measurement of the expression of EGFR as a predictive marker for total EGFR inhibitor efficacy has remained controversial. EGFR levels, as measured by immunohistochemistry or immunoblot, have not correlated well with sensitivity to EGFR inhibition, *in vitro* and clinically (32), although some correlation has been observed by measuring *EGFR* gene copy number using fluorescence *in situ* hybridization (15–17). Additional determinants of sensitivity to EGFR inhibitors need to be defined.

In NSCLC, an EMT has been shown to be associated with erlotinib sensitivity (25, 26), although the precise changes that alter the cellular requirements for EGFR signaling are obscure. It was observed that carcinoma cell lines expressing epithelial proteins, such as E-cadherin and α- and γ-catenins, were sensitive to growth inhibition by erlotinib, whereas those tumor cell lines that had undergone an EMT-like transition associated with the loss of epithelial marker expression (e.g., E-cadherin and P-cadherin) and the gain of mesenchymal cell protein expression (e.g., vimentin, fibronectin, and the transcription factor zeb1/TCF8/δEF1) were less sensitive to EGFR inhibition (25). The changes in cell markers observed were indicative of the cells having undergone an EMT-like transition. This transition is believed to render the cells more motile and invasive and is thought to be a critical step in the progression to metastasis (33). Similarly, a retrospective analysis of a phase III trial in NSCLC for erlotinib plus chemotherapy compared with chemotherapy alone showed that E-cadherin expression was a significant predictive marker for efficacy of EGFR inhibition by erlotinib, as measured by progression-free survival (26).

Here, we have extended these studies to investigate whether these observations apply to other solid tumor types, specifically pancreas and colorectal. We describe the expression of epithelial and mesenchymal markers in a panel of 12 pancreatic and 14 colorectal tumor cell lines. We find that those pancreatic cell lines that have higher expression of zeb1, snail, and twist and have undergone EMT are less sensitive to growth inhibition by erlotinib. These cell lines have lost the tight cell-to-cell junctions characteristic of epithelial cells and exhibit a spindle-like phenotype that is representative of mesenchymal cells. A similar relationship was found between sensitivity to EGFR inhibition and the formation of adhesion junctions in colorectal tumor cell lines. Epithelial colorectal tumor cell lines were more sensitive than those that had undergone EMT. We examined other colorectal tumor cell lines that have not undergone EMT but contain mutations in the genes encoding E-cadherin and β-catenin, which disrupt their ability to participate in forming desmosomes important for homotypic adhesion (34, 35). We considered if these specific mutations and the associated changes in cell morphology would similarly affect sensitivity to growth inhibition by erlotinib, and indeed these cell lines also show reduced sensitivity to erlotinib compared with lines showing an epithelial morphology. Collectively, these data indicate that colorectal tumor cell lines expressing E-cadherin and β-catenin and morphologically showing an epithelial phenotype are sensitive to erlotinib, whereas those that had lost an epithelial phenotype, either through mutation or by EMT, are less sensitive. The expression of E-cadherin and vimentin were found to correlate with tumor stage in both pancreatic cancer and colorectal cancer, supporting a role for an EMT-like transition in the

progression of these tumor types *in vivo*. Collectively, these data suggest the use of biomarkers reflective of the EMT state (e.g., E-cadherin and vimentin) as predictors of EGFR inhibitor treatment outcome in multiple tumor types.

Materials and Methods

Cell Lines and Growth Inhibition Assays

The pancreatic cancer cell lines HPAC, CFPAC, BxPCX3, Panc1, MiaPaca2, A1165, Hs766T, SW1990, Capan-1, Capan-2, and HPAF-II and colorectal cancer cell lines RKO, HCT-116, Colo205, HT-29, SW620, Colo201, WiDr, SW480, CBS, GEO, FET, DLD1, HCT-8, and HCT-15 were cultured in the appropriate American Type Culture Collection (Manassas, VA)–recommended supplemented medium. For growth inhibition assays, cells were plated and allowed to proliferate for 24 h. After 24 h, cells had reached ~15% confluency, at which time serial dilutions of erlotinib were added and the cells were grown for a further 72 h. Cell viability was assayed using the Cell TiterGlow reagent (Promega Corp., Madison, WI).

Preparation of Lysates and Western Blotting

Cell extracts were prepared by detergent lysis [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/L 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 0.8 μmol/L aprotinin, 20 μmol/L leupeptin, 40 μmol/L beestatin, 15 μmol/L pepstatin A, 14 μmol/L E-64 (1:100 dilution of protease inhibitor cocktail P8340); Sigma, St. Louis, MO] and sodium orthovanadate, sodium molybdate, sodium tartrate, and imidazole (1:100 dilution of phosphatase inhibitor cocktail P5726; Sigma). The soluble protein concentration was determined by micro–bovine serum albumin assay (Pierce, Rockford, IL). Protein immunodetection was done by electrophoretic transfer of SDS-PAGE separated proteins to nitrocellulose, incubation with antibody, and chemiluminescent second-step detection (PicoWest; Pierce). The antibodies included the following: E-cadherin, β-catenin, vimentin, γ-catenin, δ-catenin, and zeb1. The antibody for vimentin was obtained from BD (Franklin Lakes, NJ); all others were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Immunohistochemistry and Tissue Scoring

Tissue microarrays for pancreatic and colorectal tumors were obtained from U.S. Biomax (Rockville, MD) and Imgenex (San Diego, CA) and were stained with E-cadherin (H-108) and vimentin (clone V9; Santa Cruz Biotechnology; Dako, Carpinteria, CA) at a 1:100 dilution for 30 min. Antigen retrieval was carried out in a steamer using Dako Target Retrieval solution (pH 6 for E-cadherin and pH 9 for vimentin). A Vectastain Elite ABC rabbit kit (Vector Laboratories, Inc., Burlingame, CA) was used for the E-cadherin and a Vectastain Elite ABC mouse kit for the vimentin. Sections were incubated for 10 min with a diaminobenzidine tetrahydrochlorine substrate (DAB+, Dako) and counterstained with hematoxylin. Tissue sections were examined by light microscopy. Sections were subjectively scored by assigning an intensity score of 0 to 3 and a percentage of tumor cells selectively staining at the highest intensity stain. E-cadherin scoring was limited to membrane staining, and vimentin was scored based on cytoplasmic staining.

Reverse Transcription-PCR Conditions

Taqman probe and primer sets for zeb1, snail, and twist were obtained from Applied Biosystems (Foster City, CA). Quantitation of relative gene expression was conducted as described by the manufacturer using 30 ng of template. To determine relative expression across cell lines, amplification of the specific genes was normalized to amplification of the gene for glyceraldehyde-3-phosphate dehydrogenase.

Confocal and Light Microscopy

Cells were grown on glass coverslips, washed twice with PBS, and then fixed using paraformaldehyde. Cells were then probed with antibodies directed to either E-cadherin or vimentin and detected using a fluorescent-conjugated secondary antibody. Confocal images were taken by using a Perkin-Elmer (Norwalk, CT) Ultra View spinning disc scanner coupled to an Olympus 1 × 70 microscope.

Results

The sensitivity to growth inhibition by the EGFR kinase inhibitor erlotinib for a panel of 12 pancreatic cell lines is shown in Fig. 1A. Growth inhibition achieved by 10 μmol/L erlotinib was selected to compare cell lines as this criteria has been shown to closely mirror the percentage of tumor growth inhibition from *in vivo* xenograft studies, indicating a correlation with biological responses. Trough, peak, and average steady-state plasma concentrations in treated patients reach 9.7, 15, and 11 μmol/L, respectively. Therefore, responses at the 10 μmol/L concentration lie within the window of clinical relevance (25, 36, 37). Moreover, growth inhibition by erlotinib for sensitive cell lines is dose dependent (24). Cell lines in this group were derived from both primary (BxPC3, HPAC, Capan-2, Panc-1, SW1990, MiaPaca-2, and A1165) and metastatic (AsPC1, Hs766T, HPAFF-II, CFPAC, and Capan-1) sites. These cell lines show a continuum of sensitivities and were divided into three groups (sensitive, intermediate sensitivity, and insensitive) based on their average sensitivity as a group (intermediate sensitivity) and the surrounding 95% confidence intervals (sensitive and insensitive). The *in vivo* sensitivities of select cell lines within this panel, in addition to sensitivities for cell lines of other tumor types, closely match sensitivity measurements derived from *in vitro* experiments (24, 25). The mutation status for the genes encoding APC, p53, K-Ras, phosphatidylinositol 3-kinase, and PTEN is described in Table 1. We find that the mutation status for these genes is not a strong determinant of cellular sensitivity to EGFR kinase inhibition. We determined the expression levels for proteins associated with either an epithelial (E-cadherin, γ-catenin, β-catenin, and δ-catenin) or mesenchymal (vimentin and zeb1) phenotype by immunoblot (Fig. 1B). E-cadherin expression could be detected in all four of the most sensitive cell lines but not in any of the insensitive cell lines. E-cadherin was

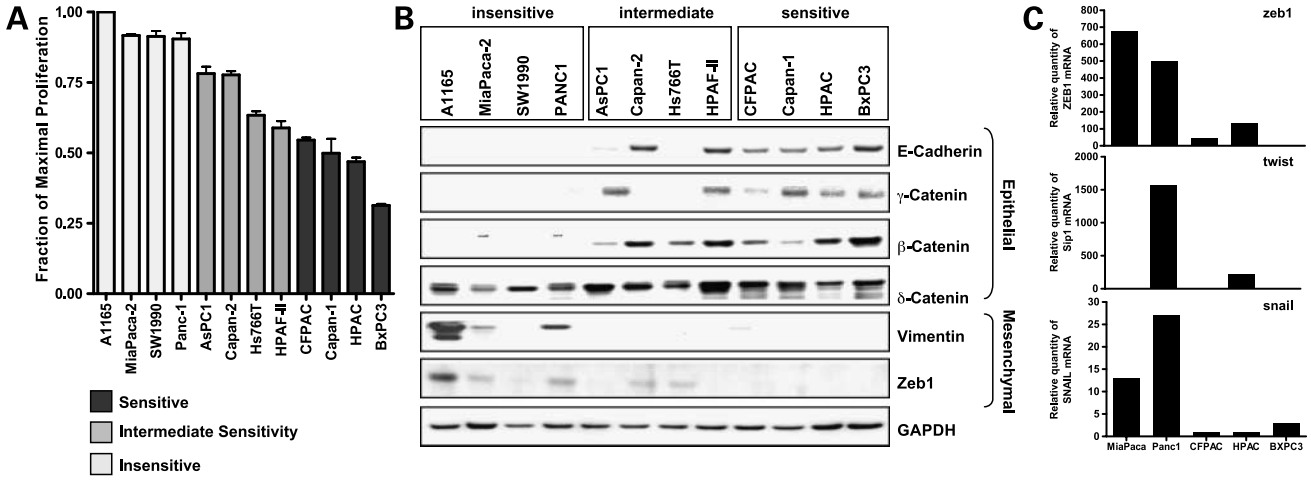

Figure 1. **A,** sensitivity of a panel of 12 pancreatic tumor cell lines to growth inhibition by erlotinib. Cell lines show a continuum of sensitivities to erlotinib. Cell lines were divided into three groups (sensitive, intermediate, and insensitive) based on their maximal percentage growth inhibition by 10 mmol/L erlotinib. Intermediate cell lines were defined as those having the mean inhibition ±5%. Sensitive and insensitive cell lines were defined as those that showed maximal inhibition outside of this 95% confidence interval. Results are typical of three independent experiments. **B,** Western blot for expression of epithelial (E-cadherin, γ-catenin, β-catenin, and δ-catenin) and mesenchymal (vimentin and zeb1) proteins in the panel of pancreatic tumor cell lines. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a protein loading control. **C,** RNA expression levels of three transcriptional repressors of E-cadherin (zeb1, twist, and snail) by quantitative PCR for a panel of five pancreatic tumor cell lines (MiaPaca-2, Panc1, CFPAC, HPAC, and BxPC3).

expressed in two of four cell lines of intermediate sensitivity. Consistent observations were found for γ-catenin and β-catenin, although we observed expression of δ-catenin in both sensitive and insensitive cell lines. The microfilament vimentin, a mesenchymal marker, was expressed in three of four insensitive cell lines but not in any cell line that was characterized as sensitive or of intermediate sensitivity. Accordingly, the transcriptional repressor of E-cadherin, zeb1, was found in three of four of the insensitive cell lines but not in sensitive cell lines. A1165, the cell line that showed the lowest level of erlotinib sensitivity, expressed the highest protein levels for both vimentin and zeb1. Therefore, epithelial and mesenchymal markers distinguished the pancreatic cell lines tested as sensitive or insensitive to erlotinib.

Zeb1 is one of several transcription factors that down-regulate E-cadherin expression and mediate EMT (38–40). As robust commercial antibodies were not available for other members (notably snail and twist), we measured their RNA levels by quantitative PCR in a representative panel of three sensitive (HPAC, CFPAC, and BxPC3) and two insensitive (Panc1 and MiaPaca-2) cell lines. RNA levels for zeb1 correlated with protein levels, with the highest levels in Panc1 and MiaPaca-2 (Fig. 1C). Likewise, we found high levels of zeb1, snail, and twist RNA in these two insensitive cell lines, but not in the three sensitive cell lines. Although RNA levels for zeb1 were higher than those for snail or twist in Panc1 and MiaPaca-2, it seems this suite of transcriptional repressors might work in conjunction to establish and/or maintain a mesenchymal phenotype (33, 41).

We used immunofluorescence to visualize the localization of E-cadherin and vimentin in sensitive and insensitive cell lines. The insensitive cell line MiaPaca-2 shows vimentin expression at the cell front, and this is absent in the sensitive cell line BxPC3 (Fig. 2A). Conversely, we observed E-cadherin expression at the cell-to-cell junctions of BxPC3 but not in MiaPaca-2. A comparison of the morphology of representative sensitive and insensitive cell lines shows a cobblestone pattern with tight cell-to-cell junctions, characteristic of epithelial cells, for the EGFR inhibitor–sensitive cell lines, whereas the erlotinib-insensitive cell lines show a fibroblastic spindle-like morphology (Fig. 2B).

Select colorectal tumor cell lines have been reported to harbor mutations in the genes encoding E-cadherin and β-catenin (34, 35). Cell lines that contain E-cadherin and β-catenin mutations have been reported to be very migratory and highly metastatic. Therefore, these cell lines seemed to have gained a mesenchymal-like behavior through mutation of E-cadherin. The expression levels of epithelial and mesenchymal markers for a panel of colorectal cell lines were determined by immunoblot (Fig. 3A). Nine of 14 cell lines (HCT-15, HCT-8, DLD1, FET, GEO, CBS, SW480, WiDr, and HT-29) showed robust expression of wild-type E-cadherin and β-catenin. Three of 14 cell lines (HCT-116, Colo205, and Colo201) showed expression of E-cadherin and β-catenin, but these cell lines harbor mutations in the genes encoding these junctional proteins that inhibit their function. Two of 14 cell lines (RKO and SW620) showed either no E-cadherin expression or only very low expression for both E-cadherin and β-catenin. SW620 expressed vimentin, indicating that this cell line has undergone an EMT-like transition. The relative expression of the EMT-associated transcription factors zeb1 and snail was determined by quantitative PCR for a select panel of cell lines

Table 1. The mutation status for the genes encoding APC, p53, K-Ras, phosphatidylinositol 3-kinase, and PTEN for a panel of pancreatic and colorectal tumor cell lines

Cell line	Tumor type	APC	p53	K-Ras	PI3K	PTEN
HCT-116	CRC	WT	WT	Mutant	Mutant	WT
RKO	CRC	WT	WT	WT (Raf mutant)	Mutant	ND
WiDr=HT29	CRC	WT	Mutant	WT	WT	WT
HT-29=WiDr	CRC	WT	Mutant	WT	WT	WT
FET	CRC	ND	ND	ND	WT	ND
CBS	CRC	ND	ND	ND	WT	ND
GEO	CRC	ND	ND	ND	WT	ND
HCT-15	CRC	WT	Mutant	Mutant	Mutant	WT
DLD1	CRC	Mutant	WT	Mutant	Mutant	WT
HCT8	CRC	ND	ND	Mutant	Mutant	WT
Colo201	CRC	WT	Mutant	WT (Raf mutant)	WT	WT
Colo205	CRC	WT	Mutant	WT	WT	WT
SW480	CRC	Mutant	Mutant	Mutant	WT	WT
SW620	CRC	WT	Mutant	Mutant	WT	WT
BxPC3	Pancreatic	WT	Mutant	WT	WT	WT
HPAC	Pancreatic	ND	ND	Mutant	ND	ND
CFPAC	Pancreatic	WT	Mutant	Mutant	WT	WT
MiaPaca2	Pancreatic	WT	Mutant	Mutant	ND	ND
Panc1	Pancreatic	ND	ND	Mutant	ND	ND
Capan1	Pancreatic	ND	ND	Mutant	ND	ND
Capan2	Pancreatic	WT	WT	Mutant	WT	WT
HPAF-II	Pancreatic	WT	Mutant	Mutant	WT	WT
AsPC1	Pancreatic	WT	Mutant	Mutant	WT	WT
A1165	Pancreatic	ND	ND	ND	ND	ND
SW1990	Pancreatic	WT	WT	Mutant	WT	WT

NOTE: Wellcome Trust Sanger Institute Cancer Genome Project Web site (http://www.sanger.ac.uk/genetics/CGP).
Abbreviations: CRC, colorectal cancer; WT, wild-type; PI3K, phosphatidylinositol 3-kinase; ND, not determined.

(Fig. 3B). Two cell lines that express wild-type E-cadherin (DLD1 and GEO) showed the lowest expression of zeb1 and snail. The highest level of zeb1 was observed for RKO cells, a cell line that lacks E-cadherin expression although it has not acquired expression of vimentin or other filamentous proteins associated with a mesenchymal state, including fibronectin (data not shown). The expression levels for zeb1 and snail were ~2-fold higher for the cell line SW620 derived from a colorectal cancer metastasis compared with the matched cell line SW480, derived from the primary tumor of the same patient. Elevated expression of zeb1 or snail was not observed in Colo205, indicating that the E-cadherin and β-catenin mutations in Colo205 seem to have not triggered more global cellular changes associated with EMT. The comparison of the morphologies for cell lines with epithelial and mesenchymal-like phenotypes is shown in Fig. 3C. Two cell lines (HCT-15 and SW480) that express wild-type E-cadherin and β-catenin show tight cell-to-cell junctions. This is in contrast to the lack of cobblestone morphology for the mesenchymal-like

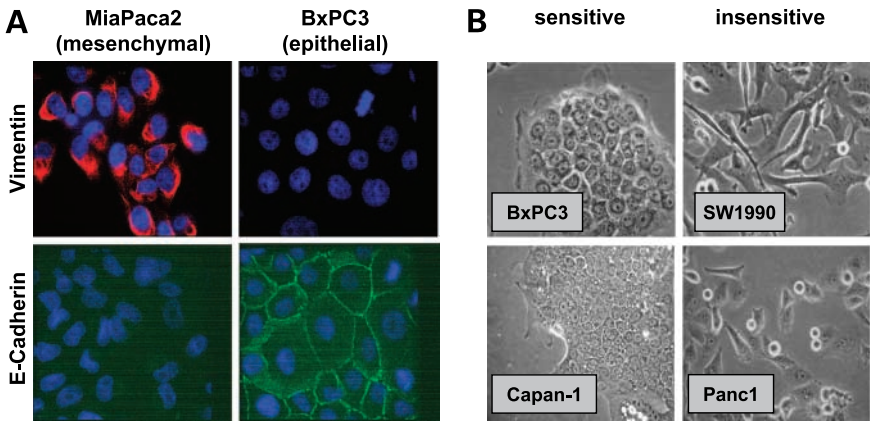

Figure 2. **A,** localization of vimentin (*red*) and E-cadherin (*green*) expression in MiaPaca-2 (EGFR inhibitor insensitive; mesenchymal phenotype) or BxPC3 cells (EGFR inhibitor sensitive; epithelial phenotype) as determined by immunofluorescence. **B,** comparison of the cell morphology for two erlotinib-sensitive (BxPC3 and Capan-1) and two erlotinib-insensitive (SW1990 and Panc1) cell lines.

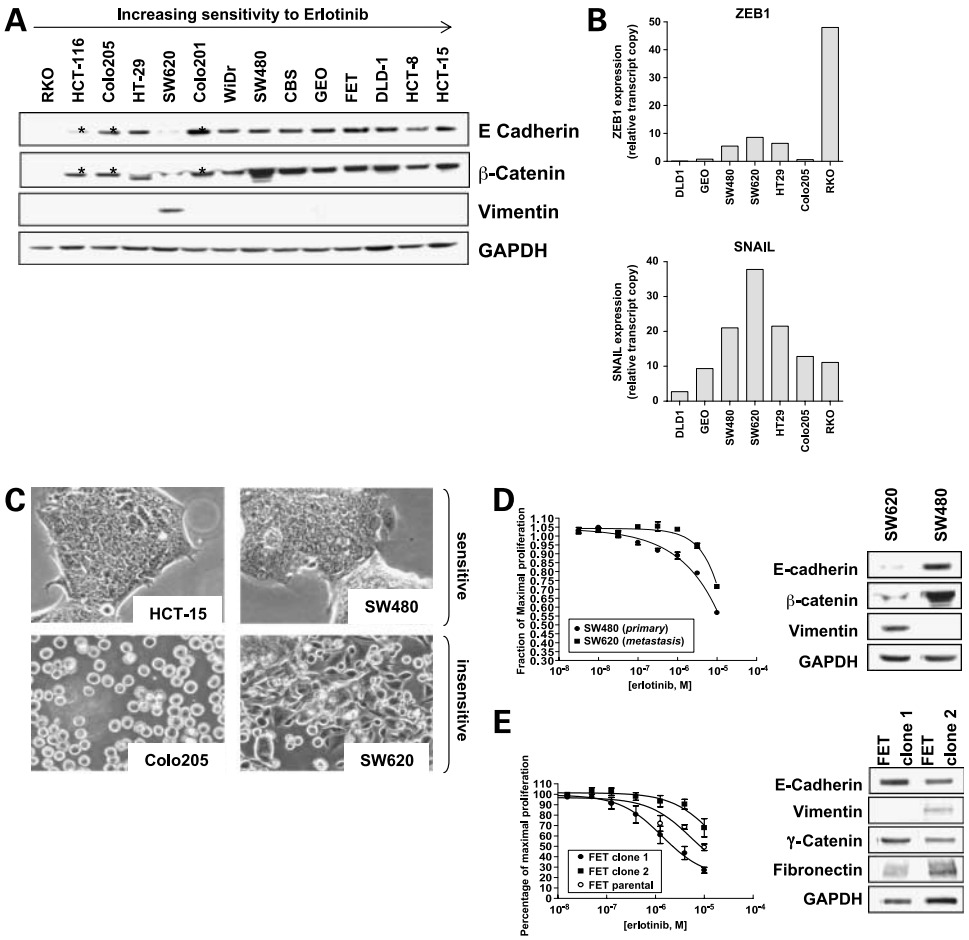

Figure 3. **A,** Western blot for the expression of epithelial (E-cadherin and β-catenin) and mesenchymal (vimentin) marker proteins in a panel of 14 colorectal tumor cell lines. *, bands that correspond to mutated versions of either E-cadherin or β-catenin. **B,** relative levels of mRNA for zeb1 (*top*) or snail (*bottom*) in the cell line panel DLD1, GEO, SW480, SW620, HT29, Colo205, and RKO as determined by quantitative PCR. **C,** comparison of the morphologies of two erlotinib-sensitive (HCT-15 and SW480) cell lines with two erlotinib-insensitive (Colo205 and SW620) cell lines. Both HCT-15 and SW480 express wild-type E-cadherin. SW620 is a mesenchymal cell line that expresses vimentin, and Colo205 expresses mutated versions of both E-cadherin and β-catenin. **D,** effect of varying concentrations of erlotinib on the growth of SW620 and SW480 cells (*left*). Western blot for the expression of E-cadherin, β-catenin, and vimentin in SW620 and SW480 (*right*). **E,** effect of varying concentrations of erlotinib on the growth of FET parental cells and two clones (clones 1 and 2) derived from the parental population (*left*). Western blot for the expression of E-cadherin, vimentin, γ-catenin, and fibronectin in FET clones 1 and 2 (*right*). Results for proliferation are representative of three or more independent experiments.

lines Colo205 and SW620. Colo205, harboring mutations in the genes encoding E-cadherin and β-catenin (Colo205), grew in suspension. SW620 has undergone EMT, expresses vimentin, and has a fibroblastic morphology. We asked how either mesenchymal status or E-cadherin mutation would affect cellular sensitivity to EGFR inhibition for this cell line panel. We determined the sensitivity to growth inhibition by erlotinib. These cell lines showed a spectrum of sensitivities to erlotinib (EC_{50} values from 0.5 to >10 μmol/L, maximal inhibition from 8% to 67%; Table 2). All of the most erlotinib-sensitive cell lines express wild-type E-cadherin and β-catenin, whereas none of the cell lines that are either mesenchymal (RKO and SW620) or harboring mutations in E-cadherin (Colo205, Colo201, and HCT-116) show sensitivity to growth inhibition >35%; the EC_{50} values for all were more than 10 μmol/L. Therefore, for colorectal tumor cell lines, either mesenchymal status or specific mutations in the genes encoding E-cadherin and β-catenin predicted a limited response to EGFR inhibition. A direct comparison of the SW620 and SW480 cell lines (derived from the same patient; SW480 from the primary tumor site and SW620 from the liver metastasis) illustrates that the primary tumor line SW480 has epithelial characteristics, whereas the metastatic tumor line SW620 derived from the same patient as SW480 has a more mesenchymal phenotype (Fig. 3C). SW480 expresses the epithelial proteins E-cadherin and β-catenin and lacks expression of the mesenchymal protein vimentin, whereas the converse is true for the metastatic variant SW620 (Fig. 3D, *right*). This is accompanied by a decrease in the potency for growth inhibition by erlotinib of ~3-fold (Fig. 3D, *left*).

For several of the erlotinib-sensitive cell lines in our panel, including FET and GEO, erlotinib was fairly potent (EC_{50} of ≤1 μmol/L); however, it maximally inhibited cell growth by only ~50% (data not shown). We considered whether a mixed population of FET cells was responsible for this lack of complete efficacy. We established single cell clones from the FET parental line. Comparison of cell clones showed a difference in the expression levels of epithelial and mesenchymal proteins. As shown in Fig. 3E (*right*), FET clone 2 expressed vimentin and fibronectin, with attenuated expression of E-cadherin and γ-catenin. These changes in markers were accompanied by a decrease in growth inhibition by erlotinib. The FET cell clone with a more epithelial phenotype (clone 1) is 12-fold more sensitive to EGFR inhibition than the more mesenchymal clone 2 (Fig. 3E; *left*). Moreover, the maximal efficacy of erlotinib is ~70% for clone 1, suggesting that a less

Table 2. Sensitivity of a panel of colorectal tumor cell lines to growth inhibition by erlotinib

Cell line	E-cadherin status	Morphology	EC_{50} (μmol/L)	% Inhibition
RKO	Wild-type	Fibroblastic	>10	10
HCT-116	Mutation	Fibroblastic	>10	8
SW620	Wild-type	Fibroblastic	>10	10
Colo205	Mutation	Suspension	>10	15
HT-29	Wild-type	Mixed	>10	25
Colo201	Mutation	Suspension	>10	36
WiDr	Primary	Mixed	>10	37
SW480	Wild-type	Epithelial	2.2	44
CBS	Not reported	Epithelial	0.5	50
GEO	Not reported	Epithelial	0.5	50
FET	Not reported	Epithelial	1.0	51
DLD-1	Wild-type	Epithelial	3.0	61
HCT-8	Wild-type	Epithelial	1.6	64
HCT-15	Wild-type	Epithelial	3.3	67

NOTE: A description of the morphology for the cell lines is also indicated.

sensitive and more mesenchymal subpopulation of cells within the FET parental line contributes to a decrease in maximal growth inhibition by erlotinib. Collectively, these data indicate that functional homotypic adhesion mediated through E-cadherin is a useful predictor of erlotinib sensitivity for colorectal tumors. Finally, for a panel of 34 cell lines derived from NSCLC, colorectal, or pancreatic tumors, we observed a strong correlation between functional E-cadherin expression and growth inhibition by EGFR kinase blockade (Fig. 4). These data indicate the utility of E-cadherin as a robust biomarker to predict sensitivity to EGFR inhibition across multiple tumor types.

Decreased E-cadherin expression has been proposed as a marker for more advanced, aggressive tumors. We sought to determine how E-cadherin and vimentin expression would correlate with disease stage in pancreatic and colorectal tumor tissues. We measured the expression of E-cadherin and vimentin for pancreatic and colorectal tissue microarrays containing tumors of varying stages. Overall score for E-cadherin and vimentin expression was calculated by multiplying the staining intensity score (on a scale of 0–3) by the fraction of tumor cells that showed expression. For pancreatic tumors, we observed a continuous decrease in the extent of E-cadherin expression with increased disease stage, consistent with previous reports (Fig. 5A, *top*; refs. 42, 43). Conversely, we observed expression of vimentin only in stage II and stage III tumors, with the highest level of vimentin expression in stage III tumors (Fig. 5A, *bottom*). A comparison of E-cadherin and vimentin expression in representative stage I and stage III tumors is shown in Fig. 5B. The stage I tumor shows a high degree of organized ductal morphology, and this is accompanied by a high level of E-cadherin expression, while lacking vimentin expression. The stage III tumor shows a lack of differentiation that is accompanied by an absence of E-cadherin expression and robust expression of vimentin. These data indicate that cells in many of the more advanced tumors had either undergone an EMT-like transition and/or recruited infiltrating stroma. Collectively, these data indicate that EGFR inhibitors would likely show enhanced efficacy for earlier-stage pancreatic tumors, as these have not yet undergone EMT. For colorectal tumors, we observed a similar correlation between EMT status and disease stage. The expression level of E-cadherin was lower in stage II to IV tumors than in normal control tissues, and the expression of vimentin was observed only in stage III to IV tumors (Fig. 5C). Therefore, we suggest that EGFR inhibitors may also show enhanced efficacy toward early-stage colorectal tumors.

Discussion

Biomarkers associated with cellular EMT status have been reported to be predictors of EGFR inhibitor sensitivity in NSCLC cell lines, xenografts, and patient samples (25, 26, 44). Erlotinib has been recently approved for the treatment

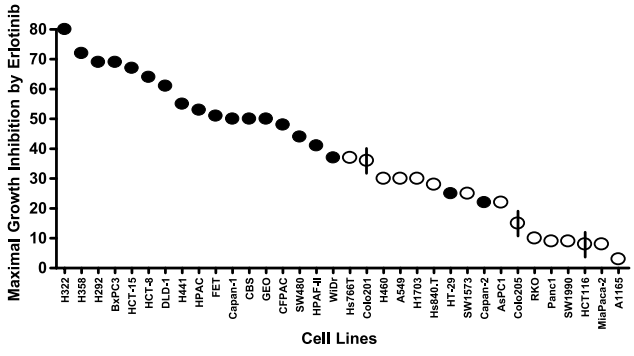

Figure 4. Correlation of E-cadherin expression with erlotinib sensitivity in three tumor types (NSCLC, pancreatic, and colorectal). Sensitivity is expressed as maximal growth inhibition by 10 μmol/L erlotinib. ●, high E-cadherin expression; ○, low E-cadherin expression; ϕ, E-cadherin is expressed but mutated and nonfunctional.

of pancreatic cancer patients and we sought to determine the utility of E-cadherin as a biomarker for erlotinib sensitivity in this setting. Herein, we show that E-cadherin also has the potential to select for pancreatic cancer patients that might maximally benefit from treatment with EGFR inhibitors. In a panel of 12 pancreatic tumor cell lines, the four that were most sensitive to growth inhibition by erlotinib all expressed epithelial markers, including E-cadherin. This is in contrast to the lack of E-cadherin expression in the four least sensitive cell lines in this panel. Insensitive cell lines expressed vimentin and zeb1, indicating that they had undergone EMT. Several studies have shown that inhibition of Akt activity by EGFR inhibitors occurs only in cell lines that are sensitive to growth inhibition by EGFR inhibitors (11, 24). We have previously reported that EGFR is coexpressed with ErbB3 in erlotinib-sensitive pancreatic and colorectal tumor cell lines, and EGFR transactivation of ErbB3 was important for activation of Akt (24). Transcriptional repressors of E-cadherin, such as snail, also repress the gene encoding ErbB3, suggesting that there is a link between EMT and loss of signaling proteins that mediate Akt activation by EGFR. From analysis of patient tumors, we observed decreased E-cadherin expression and increased vimentin expression with advancing disease stage. Erlotinib is currently indicated for advanced stage, nonresectable, and metastatic pancreatic tumors. Here, erlotinib has been shown to increase overall 1 year survival from 19% to 24%. Our findings would suggest that there would be a greater sensitivity to erlotinib in an early-stage disease setting, perhaps in the neoadjuvant or adjuvant setting after resection in which peripheral tumor cells have not yet developed a mesenchymal phenotype. Preliminary data from pancreatic tumors obtained by Whipple resection suggest that these tumors are especially sensitive to growth inhibition by erlotinib (data not shown). In this regard, high E-cadherin status has been shown to be a positive prognostic factor for Whipple resectable patients (45).

We further showed that functional homotypic adhesion is a predictor of erlotinib sensitivity for colorectal tumor cell lines. In colorectal tumors, the genes encoding E-cadherin and β-catenin are frequently mutated (34, 35). This occurs most often in tumors that are replication error positive. Those tumor cell lines that have acquired these somatic mutations in E-cadherin are reported to be very invasive and highly aggressive. Furthermore, they exhibit a phenotype that is very characteristic of mesenchymal cells and in contrast to the tightly adherent clusters of their E-cadherin–positive counterparts. We find that colorectal tumor cell lines that have gained a mesenchymal phenotype, either through EMT (SW620 and RKO) or through mutation (Colo201, Colo205, and HCT-116), are relatively insensitive to EGFR inhibition. For Colo205 cells, mutations in the genes encoding E-cadherin and β-catenin were not accompanied by the expression of transcription factors (zeb1 or snail) associated with EMT. In this instance, the data suggest that mutations in E-cadherin and β-catenin specifically, and not other global changes associated with EMT, were sufficient to render Colo205 cells insensitive to EGFR inhibition, supporting the notion that functional E-cadherin and β-catenin themselves might directly participate in regulating dependency for EGFR signaling. In a direct comparison of 34 cell lines derived from three tumor types (NSCLC, pancreatic, and colorectal), there is a strong correlation between functional E-cadherin expression and sensitivity to erlotinib.

Recent data suggest that E-cadherin might not merely be a bystander cellular biomarker but rather an active participant in mediating EGFR activity that is important for cellular growth in erlotinib-sensitive cell lines. E-cadherin has been reported to bind to EGFR and modulate receptor activation (46–49). Although there are reports for

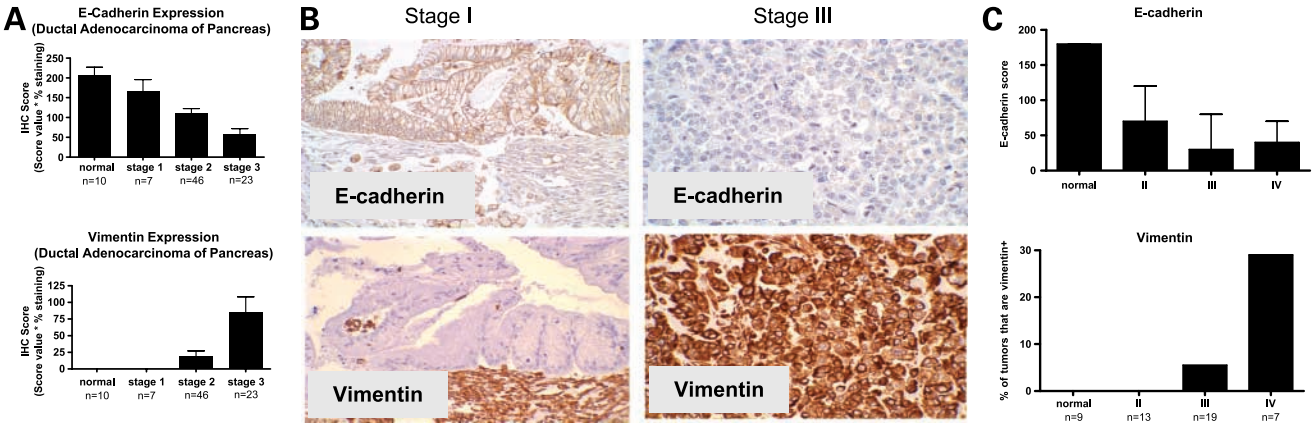

Figure 5. Expression of E-cadherin and vimentin in pancreatic tumors of various stages as determined by immunohistochemistry. **A,** the immunohistochemical expression score for E-cadherin (*top*) and vimentin (*bottom*) was calculated by multiplying the staining intensity (on a scale of 0–3) by the percentage of cell that stained positive. **B,** representative tissue sections from stage I (×20 magnification) or stage III (×40 magnification) pancreatic tumors, which show the expression pattern for E-cadherin and vimentin. **C,** expression of E-cadherin and vimentin in colorectal tumors of various stages as determined by immunohistochemistry. The immunohistochemical expression score for E-cadherin (**A**) and vimentin (**B**) was calculated by multiplying the staining intensity (on a scale of 0–3) by the percentage of cells that stained positive.

E-cadherin abrogating ligand-induced receptor activation, E-cadherin has also been shown to activate the EGFR in a ligand-independent manner (49, 50). E-cadherin expression can modulate the mobility of EGFR by promoting the localization of EGFR to sites of cell-to-cell contact. By increasing effective concentration of EGFR in this cellular microenvironment, E-cadherin promotes homodimeric and heterodimeric receptor activation in a ligand-independent manner. This ligand-independent EGFR activation has been implicated in the downstream activation of both Akt and extracellular signal-regulated kinase (51). Elucidating the exact mechanism for E-cadherin–mediated cellular sensitivity to EGFR inhibition is the subject of further investigation.

In summary, the results herein show the potential for both the broad applicability of E-cadherin and vimentin as biomarkers to stratify patients across several tumor types and provide a rationale for combining EGFR inhibitors with agents that target the mesenchymal process as a mechanism to restore heightened dependence on EGFR signaling. Trials to test these hypotheses are both planned and in progress. Several agents that have shown effectiveness in reversing or preventing the mesenchymal phenotype are currently being tested in combination with EGFR inhibitors in the clinic, including histone deacetylase and cyclooxygenase II inhibitors (44, 52, 53). The data presented here provide a mechanistic insight underlying these combinations.

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