

# Restoring E-Cadherin Expression Increases Sensitivity to Epidermal Growth Factor Receptor Inhibitors in Lung Cancer Cell Lines

Samir E. Witta,<sup>1</sup> Robert M. Gemmill,<sup>1</sup> Fred R. Hirsch,<sup>1,2</sup> Christopher D. Coldren,<sup>1</sup> Karla Hedman,<sup>1</sup> Larisa Ravdel,<sup>1</sup> Barbara Helfrich,<sup>1</sup> Rafal Dziadziuszko,<sup>1,4</sup> Daniel C. Chan,<sup>1</sup> Michio Sugita,<sup>2</sup> Zeng Chan,<sup>3</sup> Anna Baron,<sup>3</sup> Wilbur Franklin,<sup>2</sup> Harry A. Drabkin,<sup>1</sup> Luc Girard,<sup>5</sup> Adi F. Gazdar,<sup>5</sup> John D. Minna,<sup>5</sup> and Paul A. Bunn, Jr.<sup>1</sup>

Departments of <sup>1</sup>Medicine/Medical Oncology, <sup>2</sup>Pathology, and <sup>3</sup>Preventive Medicine and Biometrics, University of Colorado Health Sciences Center and University of Colorado Cancer Center, Aurora, Colorado; <sup>4</sup>Department of Oncology and Radiotherapy, Medical University of Gdansk, Gdansk, Poland; and <sup>5</sup>Hamon Center for Therapeutic Oncology Research, Dallas, Texas

## Abstract

The epidermal growth factor receptor (EGFR) is overexpressed in the majority of non-small cell lung cancers (NSCLC). EGFR tyrosine kinase inhibitors, such as gefitinib and erlotinib, produce 9% to 27% response rates in NSCLC patients. E-Cadherin, a calcium-dependent adhesion molecule, plays an important role in NSCLC prognosis and progression, and interacts with EGFR. The zinc finger transcriptional repressor, ZEB1, inhibits E-cadherin expression by recruiting histone deacetylases (HDAC). We identified a significant correlation between sensitivity to gefitinib and expression of E-cadherin, and ZEB1, suggesting their predictive value for responsiveness to EGFR-tyrosine kinase inhibitors. E-Cadherin transfection into a gefitinib-resistant line increased its sensitivity to gefitinib. Pretreating resistant cell lines with the HDAC inhibitor, MS-275, induced E-cadherin along with EGFR and led to a growth-inhibitory and apoptotic effect of gefitinib similar to that in gefitinib-sensitive NSCLC cell lines including those harboring *EGFR* mutations. Thus, combined HDAC inhibitor and gefitinib treatment represents a novel pharmacologic strategy for overcoming resistance to EGFR inhibitors in patients with lung cancer. (Cancer Res 2006; 66(2): 944-50)

## Introduction

Lung cancer is the leading cause of cancer death in men and women in the U.S. (1). Epidermal growth factor receptor (EGFR) is overexpressed in a large proportion of non-small cell lung cancers (NSCLC; ref. 2). The EGFR tyrosine kinase inhibitors (TKI) gefitinib (ZD1839, Iressa, AstraZeneca, Alderley Park, United Kingdom), and erlotinib (OSI 774, Tarceva, OSI/Genentech, South San Francisco, CA) were initially approved for use in the U.S. because they produce radiographic regression of 9% to 27% of tumors and erlotinib improves survival when compared with placebo in patients with chemotherapy-refractory advanced NSCLC (3–5). However, as many as 50% of patients have progressive disease within 8 months of therapy initiation and have no benefit. Recent studies indicate that the presence of activating mutations in the EGFR tyrosine kinase domain (6–9), increased *EGFR* copy number and/or expression of EGFR protein using immunohisto-

chemistry (9–11), correlate with response and survival after EGFR TKI therapy.

EGFR interacts with the cell adhesion molecule E-cadherin (E-cad, CDH1; refs. 12–14). E-Cadherin modulates EGFR activation and signaling through its downstream targets. Whereas E-cadherin can inhibit ligand activation of EGFR (13), it enhances AKT activation in neighboring cells (14). High levels of phosphorylated AKT may also predict for response to EGFR TKIs (15). In lung cancer cell lines, E-cadherin expression is regulated by  $\beta$ -catenin signaling and by zinc finger proteins including the Slug/Snail family, SIP1 and ZEB1 (TF-8, ZFH1A, AREB6, and  $\delta$ EF1; ref. 16). These transcription factors regulate the expression of genes via interaction with two 5'-CACCTG (E-box) promoter sequences (17). This regulation is facilitated by interaction with the transcriptional corepressor, CtBP, which recruits histone deacetylases (HDAC) leading to chromatin condensation and gene silencing (18). Inhibiting HDACs using trichostatin A in lung cancer cell lines led to reactivation of E-cadherin expression (16).

HDAC inhibitors are an emerging class of therapeutic agents that promote differentiation and apoptosis in hematologic and solid malignancies through chromatin remodeling and regulation of gene expression (19). MS-275 (Schering AG, Berlin, Germany), a benzamide HDAC inhibitor undergoing phase I investigation in hematologic and solid malignancies, leads to changes in histone acetylation that persist for several weeks following its administration (20).

In this study, we asked if E-cadherin expression was directly or indirectly related in response to EGFR TKIs. We found that E-cadherin and ZEB1 expression correlated with gefitinib sensitivity and restoring E-cadherin expression by transfection or HDAC inhibition resulted in an enhanced response to gefitinib. Clinical trials based on this observation are planned.

## Materials and Methods

**Cell culture, drugs and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.** Twenty-one NSCLC cell lines were used: squamous (H157, HCC95, HCC15, and H520), large cell (H460, H1299, H2126, and H1264, a derivative of H460), adeno (Calu3, A549, H1703, H2122, H1648, HCC78, HCC193, H2009, HCC44, and H3255), and bronchioalveolar (H358 and H322). The NSCLC cell lines HCC78, H2126, HCC95, H1299, HCC193, HCC44, HCC15, and H2009 were obtained from Drs. John Minna and Adi Gazdar (University of Texas Southwestern Medical School, Dallas, TX). The H3255 cell line was a gift from Dr. Bruce Johnson (Dana-Farber Cancer Center, Boston, MA). All the other cell lines were obtained from American Type Culture Collection (Rockville, MD). Gefitinib was a gift from AstraZeneca, MS-275 was a gift from Nihon Schering K.K. (Osaka, Japan). Epidermal growth factor (EGF) was purchased from R&D Systems, Inc.

**Requests for reprints:** Samir E. Witta, Department of Medicine/Medical Oncology, University of Colorado Health Sciences Center, Campus Box 8117, P.O. Box 6511, Aurora, CO 80045. Phone: 303-724-3876; Fax: 303-724-3889; E-mail: Samir.Witta@uchsc.edu.

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(Minneapolis, MN). Growth inhibition was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (21). The growth-inhibitory effect was based on cultures treated with gefitinib for 6 consecutive days (Table 1; Fig. 3). In Fig. 4, cell lines were treated with gefitinib for 5 days due to the increased sensitivity to the combination of MS275 and gefitinib.

**Cell lysis, Western blots, and immunohistochemistry.** Cells were disrupted in lysis buffer [10 mmol/L Tris-HCl (pH 7.5)/150 mmol/L NaCl/0.5% IGEPAL/0.5 mmol/L phenylmethylsulfonyl fluoride/10 µg/mL leupeptin/5 µg/mL pepstatin A/2.1 µg/mL aprotinin] on ice. After sonication, the Bradford assay was used for protein quantification. Protein lysates (30-50 µg) were separated by gel electrophoresis on 7.5% to 10% polyacrylamide and analyzed by Western blot using polyvinylidene difluoride membranes (Bio-Rad Laboratories, Inc., Richmond, CA). Anti-EGFR and the phospho-specific EGFR (pY1068) antibodies (Cell Signaling, Beverly, MA) were used at 1:1,000 dilutions. E-Cadherin and β-actin antibodies (BD Biosciences, San Jose, CA; Sigma-Aldrich, A5316, St. Louis, MO) were used at 1:3,000 and 1:5,000 dilutions, respectively. Detection was by horseradish peroxidase-conjugated secondary antibodies and chemiluminescence (Amersham Biosciences, Inc., Piscataway, NJ).

The anti-E-cadherin antibody (mouse monoclonal, clone 36; Transduction Laboratories, Lexington, KY) was applied at a 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 performed with 3% peroxide in absolute methanol. Blocking was done with Powerblock (Biogenics, San Ramon, CA) or avidin/biotin block. After incubation of primary antibodies for 1 hour at 37°C, the secondary antibody (Dako 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 and hematoxylin counterstained.

**RNA, primers, and quantitative real-time reverse transcription-PCR.** Total RNA was prepared from NSCLC cell lines using the RNeasy kit (Qiagen, Valencia, CA). Quantitative real-time reverse transcription-PCR assays were done from 0.3 µg total RNA using the SYBR Green RT-PCR Kit (Qiagen) using a GeneAmp 5700 Sequence Detector (Applied Biosystems, Foster City, CA). Amplification data were analyzed by using GENEAMP 5700 SDS software, converted into cycle numbers at a set cycle threshold (Ct values) and quantified in relation to a standard. Human adult lung RNA (Clontech Lab., Inc., Mountain View, CA) was used as a standard at 20, 100, and 500 ng in all the experiments. To normalize for the amount of input cDNA, the quantified relative amount of the generated product was divided by the amount generated for β-actin. All samples were done in triplicates. Primers were used as described, E-cadherin (16), ZEB1 (16), β-actin, forward 5'-ATC-CAC-GAA-ACT-ACC-TTC-AAC-TC-3', reverse 5'-GAG-GAG-CAA-TGA-TCT-TGA-TCT-TC-3'.

**Cell cycle analysis.** NSCLC cells were plated at a density of  $5 \times 10^5$  cells/well in six-well plates. Gefitinib was added to the medium after 24 hours, and the cells were incubated for another 48 hours. For the sequential treatment evaluation, MS-275 was added, followed by gefitinib 24 hours later and cells were incubated for an additional 48 hours. Cell death was evaluated using the Vybrant Apoptosis Assay Kit, propidium iodide (PI) or Annexin V-APC (V-13243 and A35110, respectively; Molecular Probes, Inc., Eugene, OR) and analyzed using flow cytometry according to the manufacturer's instructions.

**Microarray analysis of gene expression.** Following one round of *in vitro* transcription, 20 µg cRNA was hybridized with HG-U133 set microarrays and processed according to the manufacturer's protocol (Affymetrix, Foster City, CA). A MIAME checklist (22) containing extensive

**Table 1.** Relative expression of E-cadherin and ZEB-1 in NSCLC cell lines

Cell line	Gefitinib (IC <sub>50</sub> )	E-Cadherin		ZEB1	
		RT-PCR	Microarray	RT-PCR	Microarray
<b>Sensitive</b>					
H3225	0.015	13,926	7,018	9.4E-02	5.9
H358	0.18	10,453	4,701	5.02	7.4
H322	0.25	6,642	5,861	3.05	7.0
Calu3	0.3	4,359	5,532	6.98	7.8
H1648	0.38	6,642	2,825	1.29	7.6
HCC78	0.6	1,214	3,673	17	7.0
<b>Intermediate</b>					
H2126	1	709	3,946	9.4	7.2
HCC95	3	137	1,577	0.5	6.8
H441	4	925	926	13.6	8
HCC193	4	0.06	3,149	153	7.6
Colo699	4.2	9.8E-06	5	8.9	7.5
H2122	5.9	840	1,671	29	7.9
A549	8.2	132	1,508	388	30.3
HCC44	8.4	0.5	6	162	19.8
H1299	8.6	1.0E-04	5	130	46.1
H2009	9.8	558	6,959	2.6	6.5
<b>Resistant</b>					
HCC15	10	17	186	99	21.3
H1703	12	2.1E-02	5.4	123	35.6
H157	12.8	1.98E-04	5	356	40.5
H1264	12.9	3.4E-05	5	166	48.0
H460	12.9	1.4E-01	5	179	60.7
H520	13.6	58	84	337	10.1
<i>r</i>		-0.78	-0.76	0.76	0.75
<i>P</i>		<0.001	<0.001	<0.001	<0.001

experimental details can be found in the online data supplement. Hybridization signals and detection calls were generated in BioConductor, using the *gcrma* and *affy* packages.

**Statistical methods.** Normal distribution of the variables was assessed by Shapiro-Wilk test. Reported correlations were done using Spearman's correlation coefficient. The comparisons between gene expression in the groups of resistant and sensitive cell lines were done with Mann-Whitney *U* test. For variables with normal distribution, we used paired *t* test (comparisons within the same cell line under two different conditions), independent *t* test (comparison between two different cell lines) and one-way ANOVA (comparisons between multiple cell lines). All reported *P* values are two-sided.

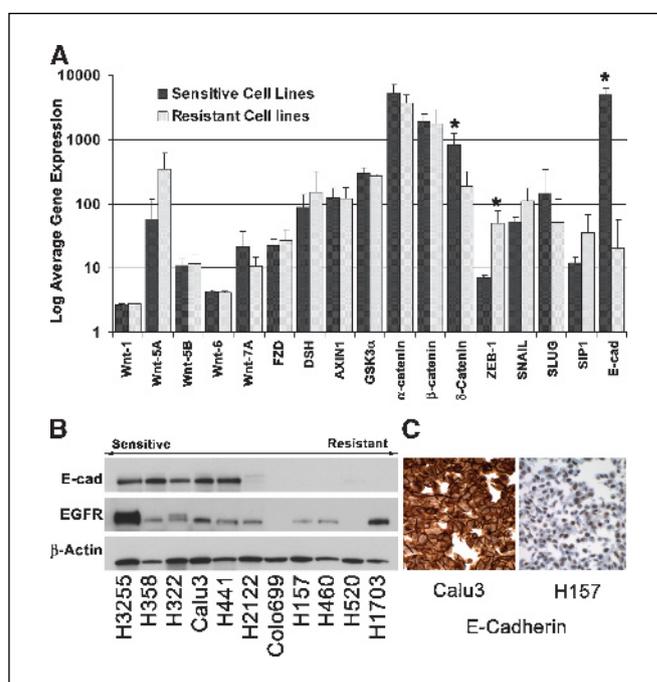
## Results

**Sensitivity of NSCLC cell lines to gefitinib and correlated molecules.** We analyzed a set of 22 NSCLC cell lines for their sensitivity to gefitinib using the MTT assay (Table 1). Six of these cell lines (H3255, H358, H322, Calu3, H1648, and HCC78) had an  $IC_{50}$  of  $<1 \mu\text{mol/L}$ , six (HCC15, H157, H460, H1703, H1264, and H520) had an  $IC_{50}$  of  $\geq 10 \mu\text{mol/L}$ , and the remaining 10 had an  $IC_{50}$  of 1 to  $10 \mu\text{mol/L}$ .

This diverse growth response to gefitinib was used to identify genes differentially expressed in sensitive versus resistant cell lines. RNA isolated from 22 cell lines was used to interrogate Affymetrix oligonucleotide microarrays. Expression of E-cadherin and related molecules in the Wnt pathway (Wnt1, Wnt5A, Wnt5B, Wnt6, Wnt7A, frizzled, axin1, disheveled, GSK3  $\alpha$ -catenin,  $\beta$ -catenin, and  $\delta$ -catenin) were compared in the six sensitive and six resistant lines (Fig. 1A). Expression of E-cadherin and  $\delta$ -catenin was significantly different between the sensitive and resistant cell lines ( $P = 0.004$  and  $P = 0.016$ , respectively). When expression of E-cadherin and  $\delta$ -catenin was evaluated in all the 22 cell lines, a significant correlation with gefitinib sensitivity was evident (E-cadherin,  $r = 0.76$ ;  $P < 0.001$ ;  $\delta$ -catenin,  $r = 0.69$ ;  $P < 0.001$ ). The highest E-cadherin RNA expression was detected in the most sensitive cell line, H3255 ( $IC_{50} = 0.015 \mu\text{mol/L}$ ) that harbors the EGFR mutation L858R (Table 1; refs. 6, 7). This correlation was confirmed in the 22 lines using real-time reverse transcription-PCR ( $r = 0.78$ ,  $P < 0.001$ ; Table 1). The data from the two methods of E-cadherin expression evaluation, real-time reverse transcription-PCR, and microarray analysis, correlated significantly ( $r = 0.85$ ,  $P < 0.0001$ ).

At the protein level, expression of E-cadherin was evaluated in 11 NSCLC cell lines by Western blot (Fig. 1B). E-Cadherin protein was present in the sensitive lines and undetectable in the resistant lines. We verified that these differences were readily detected by immunohistochemistry. E-Cadherin expression was detected in the sensitive cell line, Calu3, whereas expression was absent in the resistant cell line H157 (Fig. 1C).

E-cadherin expression is regulated by four zinc finger transcription factors: ZEB1, slug, snail and SIP1. Evaluation of the microarray data of the six sensitive cell lines and the six more resistant cell lines revealed that expression of ZEB1 significantly correlated with resistance to gefitinib ( $P = 0.004$ ; Fig. 1A) and this correlation was maintained when data on all 22 lines were included ( $r = 0.75$ ,  $P < 0.001$ ; Table 1). Expression levels of ZEB1 in all 22 cell lines were reevaluated using real-time reverse transcription-PCR and the correlation was maintained ( $r = -0.76$ ,  $P < 0.001$ ; Table 1). Significant negative correlations were evident between ZEB1 expression and either E-cadherin ( $r = -0.82$ ,  $P < 0.0001$ ) or  $\delta$ -catenin ( $r = -0.62$ ,  $P = 0.002$ ).

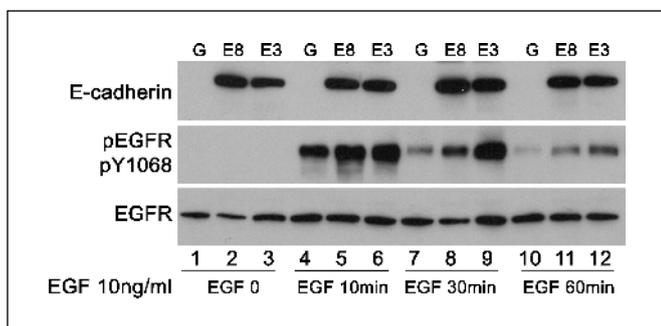


**Figure 1.** A, Affymetrix analysis of gene expression of members of the Wnt signaling pathway and zinc finger proteins in NSCLC cell lines. Log mean gene expression, SD, and two-tailed Wilcoxon test in gefitinib-sensitive (S) (H3255, H358, H322, Calu3, H1648, and HCC78) and gefitinib-resistant (R) (H157, H520, H460, H1703, H1264, and HCC15) cell lines. Gene [expression in gefitinib-sensitive cell lines (SD) and gefitinib-resistant (SD) cell lines; *P* value]: Wnt1 [2.7 (0.07), 2.8 (0.04);  $P = 0.87$ ]; Wnt5A [57.5 (59), 389 (253);  $P = 0.015$ ]; Wnt-5B [11 (2.7), 11 (3.5);  $P = 0.9$ ]; Wnt-6 [4.3 (0.23), 4.2 (0.3);  $P = 0.52$ ]; Wnt-7A [21.2 (16), 11 (3.2);  $P = 0.52$ ]; Frizzled3 (FZD) [22.4 (5.8), 27 (10);  $P = 0.42$ ]; disheveled (DSH) [88 (49), 130 (144);  $P = 1$ ]; Axin1 [122.4 (54), 138 (60);  $P = 0.5$ ]; GSK3 [303 (65), 270 (22);  $P = 0.42$ ];  $\alpha$ -catenin [5,303 (1,830), 3,806 (1,024);  $P = 0.2$ ];  $\beta$ -catenin [1,872 (619), 1,681 (943);  $P = 0.52$ ];  $\delta$ -catenin [837 (418), 245 (161);  $P = 0.016$ ]; ZEB1 [7.1 (0.6), 44 (25);  $P = 0.004$ ], Snail [52 (10), 108 (53);  $P = 0.05$ ]; Slug [149 (193), 45 (54);  $P = 0.337$ ]; SIP1 [12 (3), 31 (28);  $P = 0.15$ ]; E-cadherin [4,935 (1,394), 48 (68);  $P = 0.004$ ]. B, Western blot for E-cadherin and EGFR in 11 NSCLC cell lines. Blot was probed simultaneously with E-cadherin and  $\beta$ -actin antibodies and reprobed with EGFR antibody. C, immunohistochemistry of E-cadherin in Calu3 and H157 verifying high and low expression levels, respectively.

**E-cadherin modulates gefitinib-induced apoptosis and EGFR activation in NSCLC.** The correlation between E-cadherin and sensitivity to gefitinib led us to ask if E-cadherin could directly influence gefitinib responses. To address this question, an E-cadherin-negative, EGFR-positive gefitinib-resistant cell line, H157, was transfected with an E-cadherin expressing construct. Two stable G418-resistant clones were developed, H157-E-cad-3 (E3) and H157-E-cad-8 (E8), and E-cadherin expression was confirmed (Fig. 2, lanes 2-3).

The effect of E-cadherin transfection on EGFR activation was measured by pEGFR (PY1068) immunoblot. In the absence of EGF, transfection of E-cadherin did not lead to constitutive EGFR activation (phosphorylation; Fig. 2, lane 1-3). However, altered phosphorylation dynamics were detected on EGF treatment (Fig. 2, lanes 8, 9, 11, and 12). A sustained increase in pEGFR was detected in the E-cadherin-transfected cell lines compared with controls. This is in contrast with previous findings where E-cadherin inhibited activation of EGFR by EGF (13). There was no change in E-cadherin expression following treatment with  $10 \text{ ng/mL}$  EGF.

The effect of E-cadherin transfection on cell growth was evaluated with or without gefitinib treatment using the MTT assay. Reexpression of E-cadherin in the transfected cell lines, E3



**Figure 2.** Western blot for E-cadherin, pEGFR (pY1068) and EGFR with H157 cell line transfected with control (H157-GFP, G) or E-cadherin (H157-E-cad-8, E8 and H157-E-cad-3, E3) treated in the absence (0) or presence of 10 nmol/mL EGF and collected at 10, 30, and 60 minutes. The lower blot was stripped and reprobed with EGFR antibody.

and E8, resulted in a significant 30% ( $P = 0.002$ ) and 45% ( $P < 0.001$ ) decrease in growth compared with the parental cell line, respectively (Fig. 3A). This effect was further enhanced in the presence of gefitinib (Fig. 3B). E-Cadherin transfectants, E3 and E8, became more sensitive (gefitinib  $IC_{50}$  of 6 and 3  $\mu\text{mol/L}$ , respectively) to gefitinib compared with parental H157 cell line ( $IC_{50} = 12 \mu\text{mol/L}$ ; Fig. 3B).

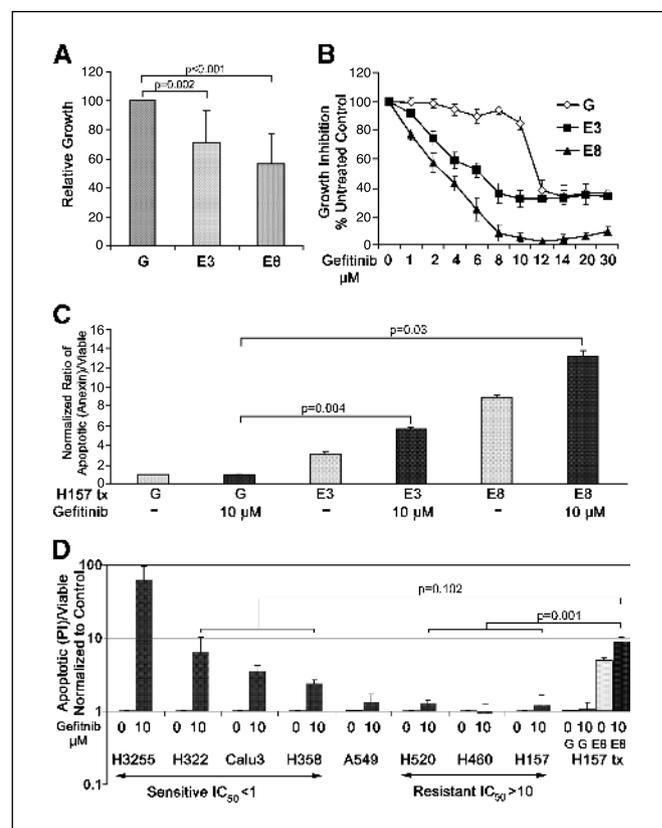
The effect of E-cadherin on cell death (apoptosis) in the presence and absence of gefitinib was evaluated using Annexin V and PI staining. Using Annexin V labeling, 3- and 9-fold increases in the ratio of apoptotic to viable cells was detected in E3 and E8, respectively, as compared with the control cell line H157-GFP ( $P = 0.083$  and  $P = 0.013$ , respectively; Fig. 3C). Significant 6- and 13-fold increases in the ratio of apoptotic to viable cells were detected in the E3 and E8 cell lines compared with control cells when treated with gefitinib ( $P = 0.004$  and  $P = 0.03$ , respectively; Fig. 3C). This indicated that expression of E-cadherin alone is able to drive some cell death and the apoptotic effect was further enhanced by gefitinib.

The apoptotic effect of gefitinib in the presence of E-cadherin was compared in the transfectant cell lines to that in cell lines sensitive to and resistant to gefitinib using PI staining (Fig. 3D). Treatment with 10  $\mu\text{mol/L}$  gefitinib induced a 9-fold increase in the ratio of apoptotic to viable cells in the E8 transfectant as compared with control cells. At the same gefitinib concentration, there was a 2.4- to 6.3- and 63-fold increase in cell death in the gefitinib-sensitive with wild-type EGFR cell lines (H322, H358, and Calu3) or the EGFR mutant cell line H3255, respectively (Fig. 3D). Only slight apoptotic or necrotic effects were detected in the more resistant cell lines. This indicated that transfection of E-cadherin significantly enhances the apoptotic effect of gefitinib on resistant cell lines as compared with the gefitinib-resistant lines ( $P = 0.001$ ). The degree of gefitinib sensitivity in the E-cadherin-transfected cell line was not significantly different from that in the wild-type sensitive cell lines ( $P = 0.102$ ) or in the EGFR mutant cell line, H3255 ( $P = 0.387$ ).

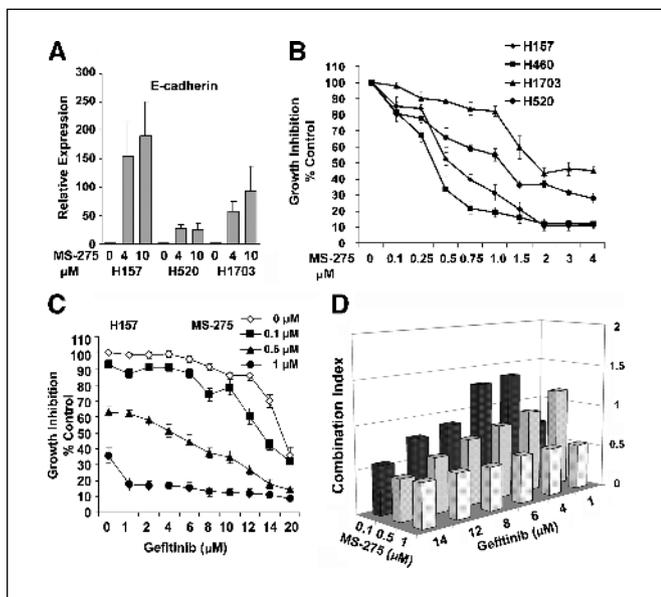
**HDAC inhibitors reverse resistance to gefitinib.** We tested whether the HDAC inhibitor MS-275 could increase expression of E-cadherin and EGFR, and thus increase response to gefitinib. E-Cadherin expression increased by 26- to 190-fold in the gefitinib-resistant NSCLC cell lines H157, H520, and H1703 following 24-hour exposure to MS-275 (Fig. 4A). In H520 cells that express neither E-cadherin nor EGFR, expression of EGFR increased by 27-fold after MS-275 exposure (data not shown).

The MS-275  $IC_{50}$  in the gefitinib-resistant lines H157, H460, H520, and H1703 was between 0.5 and 4  $\mu\text{mol/L}$  (Fig. 4B). The H157 cell line was treated separately with MS-275, gefitinib, or the sequential combination of MS-275 followed by gefitinib 24 hours later and evaluated for cell growth using MTT assay (Fig. 4C). An 81% inhibition in cell growth was detected with 1  $\mu\text{mol/L}$  of gefitinib when H157 was pretreated with 1  $\mu\text{mol/L}$  of MS-275 (from 99% to 18%; Fig. 1C). A synergistic effect was detected with the sequential use of MS-275 followed by gefitinib in this cell line. We calculated the combination index for each set of concentrations using the isobologram method (23). A combination index of  $<1$ , indicating synergy, was detected in the combination of gefitinib with MS-275 at most concentrations of each drug (Fig. 4D).

Next, we evaluated the effect of pretreatment with MS-275 on the apoptotic effect of gefitinib in the gefitinib-resistant NSCLC cell lines H157 and H520. A synergistic effect was detected with the sequential use of MS-275 followed by gefitinib in both of these cell lines. Increasing doses of MS-275 (0.5-4  $\mu\text{mol/L}$ ) resulted in a small increase in cell death in both H157 and H520 (4- to 6.7-fold and 2.4- to 4.8-fold increase, respectively; Fig. 5A and B). Similarly, treatment of these cells with increasing doses of gefitinib (10-14  $\mu\text{mol/L}$ ) resulted in almost no effect on apoptosis in these two cell lines (0.9- to 1.1-fold and 1.2- to 1.6-fold increase, respectively)



**Figure 3.** A, cell growth in H157 cell line transfected with control (H157-GFP, G) or E-cadherin (H157-E-cad-3, E3 and H157-E-cad-8, E8) measured by MTT assay. B, cell growth of E3, E8, and G in the absence (100%) or presence of gefitinib. C, cell death in E3, E8, and G cell lines in the absence (–) or presence of 10  $\mu\text{mol/L}$  gefitinib. Apoptosis was evaluated using Annexin V and the ratio of apoptotic to viable cells normalized to untreated H157-GFP (green fluorescent protein) is presented. D, relative apoptotic cells (PI) to live cells normalized to the untreated control in cell lines grown in the absence or presence of 10  $\mu\text{mol/L}$  gefitinib for 48 hours.



**Figure 4.** A, quantitative RT-PCR of E-cadherin in 300 ng of total RNA isolated from NSCLC treated with 4 or 10 μmol/L MS275 for 24 hours and compared with no treatment control (0). B, cell growth of H157, H1703, H520, and H460 cell lines in the absence (100%) or presence of MS-275. C, cell growth of H157 in the absence (100%) or presence of MS-275 followed by gefitinib. D, the combination index isobologram for the NSCLC line H157 with MS-275 followed by gefitinib. CI < 1 indicates synergism, CI = 1 indicates additive effects, and CI > 1 indicates antagonism.

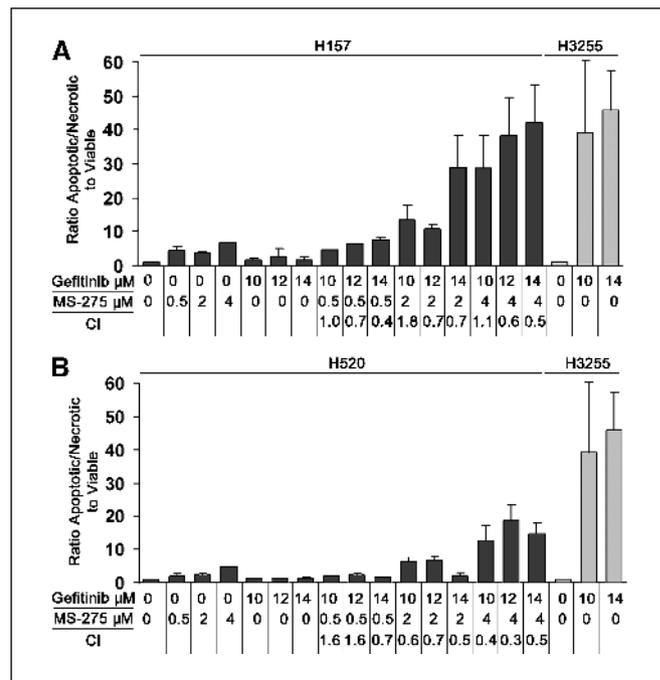
compared with the control (Fig. 5A and B). These doses of gefitinib were chosen because they fall within IC<sub>25-75</sub> for growth inhibition in the tested cell lines. When the two cell lines were treated with increasing doses of MS-275 for 24 hours prior to treatment with gefitinib, a significant and synergistic increase in cell death was detected. When the H157 cell line was treated with MS275 at 4 μmol/L followed by gefitinib at 10 to 14 μmol/L, a 36- to 50-fold increase in cell death was evident (Fig. 5A). In the H157 cell line, the apoptotic effect of MS-275 and gefitinib combination was similar to that of gefitinib in the mutant cell line H3255 (Fig. 5A). Similar treatments led to a 16- to 22-fold increase of cell death in the H520 cell line (Fig. 5B). Gefitinib and MS-275 produced additive to synergistic effects at nearly all of the concentrations tested.

**Discussion**

In this study, we show that NSCLC cell lines highly sensitive to EGFR TKIs express E-cadherin, whereas resistant lines do not. These data are consistent with the finding that E-cadherin expression is lost when a NSCLC cell line becomes erlotinib-resistant after exposing it to increasing levels of erlotinib (24). Our results suggest that E-cadherin expression would be a clinically relevant biomarker for a patient's selection of EGFR TKI therapy and responsiveness to EGFR TKIs. Cadherins are cell surface transmembrane molecules that play a major role in epithelial cell adhesion and connect the extracellular environment to the contractile cytoskeleton leading to the activation of certain nuclear responses (25, 26). Misregulated E-cadherin expression or function can alter the pattern of cell growth, differentiation, and cell invasiveness, influencing survival in patients with cancer (27). Epithelial to mesenchymal transition is a key step in the progression of tumors toward metastasis and invasion and may well play a part in determining sensitivity to EGFR TKIs (28-30).

Repression of E-cadherin by transcriptional regulation, e.g., ZEB1, might be involved in both epithelial to mesenchymal transition and resistance to EGFR TKIs. EGFR is localized in junctions and associates with the cadherin-catenin complex (31). EGF treatment of human tumor cells results in the disruption of cell-cell contacts and EGFR overexpression correlates with tumor progression, metastasis, and poor clinical prognosis (32, 33). Cell adhesion and growth factor receptor signaling are closely linked processes involved in tumor progression and invasiveness. The ability of cadherins to act as signal transducing molecules was shown in recent work indicating that assembly of E-cadherin-mediated adherens junctions is sufficient to trigger the activation of the phosphatidylinositol-3'-OH kinase/Akt cascade in epithelial cells (14). Furthermore, E-cadherin-mediated formation of calcium-dependent adherens junctions leads to ligand-independent activation of EGFRs and a rapid increase in the state of activation of mitogen-activated protein kinase (34), a process completely abolished by a EGFR TKI (34). These studies indicated that E-cadherin is involved with EGFR in activating downstream signaling pathways.

It is not fully understood why a subset of NSCLC EGFR-expressing tumors progress on treatment with EGFR TKI. EGFR TK mutations or the presence of EGFR-interacting molecules may predispose tumors for the action of EGFR TKI. A recent study showed that ErbB3 couples EGFR to the phosphoinositide-3-kinase/Akt pathway in gefitinib-sensitive NSCLC cell lines harboring wild-type and mutant *EGFRs* (35). In this study, we show that E-cadherin augments EGFR activation by EGF, which could serve as another way to regulate downstream events in the EGFR signaling pathway.



**Figure 5.** Cell death in H157 (A) and H520 (B) cell line grown in the absence or presence of 10, 12, or 14 μmol/L gefitinib or 0.5, 2, or 4 μmol/L MS-275 or with MS-275 for 24 hours followed by gefitinib for 48 hours. Apoptosis in H3255 cell line in response to 10 or 14 μmol/L gefitinib is displayed for comparison. Graphs show the ratio of apoptotic cells as measured by PI and YOPRO and divided by viable cells.

The value of identifying differentially expressed genes and proteins resides not only in developing biomarkers but also in strategies to overcome resistance to EGFR inhibitors. We evaluated the effect of E-cadherin on response to gefitinib in a cell line that expresses EGFR and is gefitinib-resistant (H157). Transfection of E-cadherin resulted in an increased activation of EGFR by EGF (Fig. 2). Recent studies indicated that the most EGFR TKI-sensitive cell lines have higher levels of EGFR activation (6), suggesting that expression of E-cadherin in cell lines increases dependence on EGFR for growth and survival.

Increasing E-cadherin expression both by transfection of E-cadherin and by exposure to MS-275 increased sensitivity to gefitinib to levels observed in sensitive NSCLC cell lines (Figs. 3-5). E-Cadherin transfection resulted in a 2- and 3-fold decrease in the  $IC_{50}$  of gefitinib and an apoptotic effect similar to that in wild-type gefitinib-sensitive cell lines (Fig. 3C and D). In lung cancer cells, we suggest that E-cadherin expression is not only a potential marker of response to EGFR inhibitors but also plays a role in the mechanism underlying response to these drugs.

Epigenetic changes leading to gene silencing are primary mechanisms modulating gene expression in cancerous cells (36). Histone acetylation and deacetylation is one mechanism that permits or halts gene expression, respectively (37). HDAC inhibitors act to restore gene expression leading to growth arrest, differentiation, and apoptosis of transformed cells (38). E-Cadherin expression is regulated by HDACs and its expression is restored with HDAC inhibitors (16). E-Cadherin can also be regulated by tumor acquired DNA promoter methylation, however, E-cadherin only undergoes methylation in ~18% of NSCLCs indicating that histone acetylation is probably the major mechanism of inactivation of E-cadherin expression in these tumors (39).

These findings have made it possible to test a clinically applicable treatment that would enhance E-cadherin expression and test the influence on response to gefitinib. We found a synergistic effect on growth inhibition and apoptosis from sequential treatment with MS-275 and gefitinib. In the presence of 1  $\mu\text{mol/L}$  MS-275, cell line growth was inhibited by 81% with 1  $\mu\text{mol/L}$  of gefitinib, a dose that does not lead to any growth inhibition in the absence of MS-275 (Fig. 4C). Those concentrations are clinically relevant as the maximum plasma concentrations ( $C_{\text{max}}$ ) achievable were 0.25 to 5  $\mu\text{mol/L}$  for gefitinib and

0.5 to 2  $\mu\text{mol/L}$  for MS-275 (20, 40). Pretreatment with MS275 also resulted in a 16- to 50-fold increase in apoptosis in the gefitinib-resistant cell lines tested. This increase in cell death was similar to what was detected in the mutant cell line H3255 treated with gefitinib alone (Fig. 5). The magnitude of increase in cell death exceeds what we detected with the ectopic expression of E-cadherin in H157 (Fig. 3D), indicating that other favorable genetic changes may also predispose these cell lines for the action of gefitinib or increase their apoptosis following treatment with MS275. This enhancement in the apoptotic effect of gefitinib was detected even in a cell line that lacks EGFR expression. However, this response could be explained by restoring EGFR expression by the pretreatment with MS275. HDACs affect many pathways (41-43), including those that favorably affect tumorigenesis such as the transforming growth factor- $\beta$  RI and RII and *p21<sup>WAF1/Cip1</sup>*. We confirmed the activation of these molecules by MS275 in gefitinib-resistant NSCLC cell lines (data not shown). This indicates that the synergistic effect of MS275 and gefitinib in the NSCLC cell lines could be, in part, a result of enhancing response to EGFR inhibitors by expressing molecules such as E-cadherin, and in part, affecting other pathways. This enhanced antitumor effect was previously reported when an HDAC inhibitor, NVP-LAQ842 was combined with a vascular endothelial growth factor receptor TKI, PTK787/ZK222584 (44).

In summary, we show that the cell adhesion molecule E-cadherin predicts and influences response to gefitinib in NSCLC cell lines and pretreatment of cells with the HDAC inhibitor MS-275 increases response to gefitinib. Drug concentrations that led to synergistic effects on cell growth are clinically achievable. Clinical trials are planned to evaluate the sequential combination of HDAC inhibitors and EGFR TKI in patients with advanced stage NSCLC.

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## Restoring E-Cadherin Expression Increases Sensitivity to Epidermal Growth Factor Receptor Inhibitors in Lung Cancer Cell Lines

Samir E. Witta, Robert M. Gemmill, Fred R. Hirsch, et al.

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