Delta-Crystallin Enhancer Binding Factor 1 Controls the Epithelial to Mesenchymal Transition Phenotype and Resistance to the Epidermal Growth Factor Receptor Inhibitor Erlotinib in Human Head and Neck Squamous Cell Carcinoma Lines

Yasmine Haddad, Woonyoung Choi, and David J. McConkey

Abstract

Purpose: Although the epidermal growth factor receptor (EGFR) is overexpressed in a majority of head and neck squamous cell carcinomas (HNSCC), only a minority of patients derive substantial clinical benefit from EGFR inhibitors. We initiated the present study to identify the mechanisms underlying erlotinib resistance in a panel of HNSCC cell lines.

Methods: We used [3H]thymidine incorporation to characterize the heterogeneity of responsiveness to erlotinib-mediated growth inhibition in a panel of 27 human HNSCC cells. We characterized the molecular mechanisms involved in resistance using a representative subset of six erlotinib-sensitive and erlotinib-resistant HNSCC lines.

Results: Erlotinib had heterogeneous effects on DNA synthesis in HNSCC cells that correlated closely with molecular markers of epithelial to mesenchymal transition (EMT). Specifically, the drug-sensitive lines expressed high levels of E-cadherin and showed limited invasion and migration capabilities. In contrast, the erlotinib-resistant HNSCC lines expressed high levels of the E-cadherin repressor delta-crystallin enhancer binding factor 1 (deltaEF1; Zeb-1) and other mesenchymal markers and low levels of E-cadherin, and they were highly invasive and migratory. Small interfering RNA - mediated knockdown of deltaEF1 in the erlotinib-resistant cell lines (1386LN and UMSCC1) resulted in up-regulation of E-cadherin and increased sensitivity to erlotinib in an E-cadherin - dependent manner.

Conclusions: DeltaEF1 controls the mesenchymal phenotype and drives erlotinib resistance in HNSCC cells. E-cadherin and deltaEF1 may prove to be useful markers in predicting EGFR inhibitor responsiveness.

Epidermal growth factor receptor (EGFR) overexpression occurs in the vast majority (up to 90%) of head and neck squamous cell carcinoma (HNSCC) cases and has been correlated with poor prognosis, resistance to both chemotheraphy and radiotherapy, increased risk of recurrence and metastasis, and reduced overall and disease-free survival (1, 2). Aberrant EGFR signaling has been implicated in many HNSCC malignant features, including uncontrolled proliferation and cell cycle progression, resistance to apoptosis, invasion, and metastasis (1). Erlotinib and gefitinib are two small-molecule tyrosine kinase inhibitors that have been developed to specifically inhibit EGFR in cancer (3). These drugs bind to and inhibit the EGFR tyrosine kinase thereby preventing initiation of downstream EGFR signal transduction pathways and causing p27-dependent cell cycle arrest (3, 4). The introduction of EGFR tyrosine kinase inhibitors in the clinic was greeted with tremendous enthusiasm, but they produced modest activity as single agents with response rates hovering between 5% and 15% (2).

One potential explanation for these disappointing results is that ineffective patient selection strategies were used to identify those HNSCC tumors that are actually dependent on EGFR signaling for their growth and/or survival. Studies in NSCLC have identified several features that seem to distinguish EGFR-dependent and EGFR-independent tumors that may help to prospectively identify the subset of patients who are most likely to benefit from EGFR-directed therapy (5). These include activating point mutations within the EGFR tyrosine kinase domain and EGFR gene amplification (present in drug-sensitive tumors) or the presence of mutant K-ras or loss of PTEN (associated with drug resistance; ref. 5). However, other studies have concluded that these features do not account for all EGFR sensitivity and resistance, nor does the level of EGFR expression, which served as a basis for patient selection in most of the clinical trials done with EGFR inhibitors to date (6). These observations have prompted new studies aimed at obtaining a better understanding of the biology of EGFR dependency in NSCLC and other solid tumors.
Translational Relevance

There is a growing appreciation for the importance of intertumoral heterogeneity in determining the outcome of epidermal growth factor receptor (EGFR) – targeted therapy in head and neck squamous cell carcinoma (HNSCC) and other solid malignancies. Our data show that the E-cadherin repressor delta-crystallin enhancer binding factor 1 (deltaEF1)/Zeb-1 mediates resistance to the EGFR inhibitor erlotinib and that small interfering RNA – mediated knockdown of deltaEF1 restores drug sensitivity. Therefore, measuring the expression of deltaEF1 and other markers associated with epithelial to mesenchymal transition may help to prospectively identify primary HNSCC tumors that are resistant to erlotinib and other EGFR inhibitors, and chemical or molecular inhibitors of deltaEF1 (including histone deacetylase inhibitors) could be used to reverse erlotinib resistance in patients.

Epithelial to mesenchymal transition (EMT) is a process that plays important roles in normal organ development and in cancer progression (7). EMT is characterized by the combined loss of E-cadherin and gain of mesenchymal markers such as fibronectin or vimentin and increased invasion and migration (7). Delta-crystallin enhancer binding factor 1 (deltaEF1), also known as TCF-8 or Zeb-1, is one of the transcription factors that have been implicated in EMT in tumor cells (7). It is characterized by the presence of two zinc-finger clusters located within its COOH and NH2 termini and a central homeobox domain (7). DeltaEF1 regulates E-cadherin transcription by simultaneous binding of the two zinc-finger domains to two high-affinity binding sites (known as E-boxes) located in the E-cadherin promoter region (7), and ectopic expression of deltaEF1 has been shown to be sufficient to down-regulate E-cadherin expression and induce EMT in normal mammary epithelial cells (8).

Here we report that resistance to the EGFR inhibitor erlotinib is linked to EMT in human HNSCC cells. We show that this EMT is driven by deltaEF1 and can be reversed by deltaEF1 knockdown. The results may have important implications for patient selection into EGFR inhibitor trials and identify deltaEF1 as a new therapeutic target in HNSCC.

Materials and Methods

Reagents, cell lines, and culture conditions. Erlotinib was obtained from the M. D. Anderson Pharmacy. Stock solutions were prepared in DMSO and stored in -20°C. Small interfering RNA (siRNA) targeting endogenous DeltaEF1 and an off-target control construct (targeting luciferase) were purchased from Dharmacon. Primary antibodies used for immunoblotting were from the following sources: Anti-EGFR and anti–p-EGFR(y1086) were obtained from Invitrogen; anti–Zeb-2/SIP1, anti-vimentin, anti-deltaEF1, and anti-fibronectin were obtained from Santa Cruz Biotechnology; anti-actin was from Sigma; anti-p27 was purchased from BD Biosciences Pharmingen; anti–E-cadherin was obtained from Zymed (Invitrogen); anti–lamin B was from Oncogene/Calbiochem; and anti–SNAIL was obtained from Abcam. Secondary antibodies were obtained from Amersham Biosciences. Dr. Gary Clayman (Department of Head and Neck Surgery, The University of Texas M. D. Anderson Cancer Center, Houston, TX) provided us with TU1138, MDA183, TU358B, UMSSC1, MDA1386LN, TU158LN, TU121LN, MDA686TL, MDA686LN, T404, TU167, T409, MDA886LN,TU182, MDA1483, TU159, and MDA1986LN. Dr. Reuben Lotan (Department of Head and Neck Medical Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, TX) provided us with 14A, 14B, 17A, 17B, 22A, 22B, and SQCCy1. Dr. Jeffrey Myers (Department of Head and Neck Surgery, The University of Texas M. D. Anderson Cancer Center, Houston, TX) provided us with the TU167LN and JMAR cell lines.

All were maintained in DMEM/F-12 Cellgro Mediatech cell culture medium containing 10% fetal bovine serum (Life Technologies), sodium pyruvate (BioWhittaker), l-glutamine (BioWhittaker), nontoxic amino acids (Life Technologies), vitamins (Life Technologies), and antibiotics (penicillin/streptomycin; BioWhittaker). Adherent monolayer cultures were incubated at 37°C in a mixture of 5% CO2 in air.

4[H]Thymidine incorporation assays. Cells were plated in 96-well plates at a density of 1 x 10^4 per well. Cells were exposed 24 h later to various concentrations of erlotinib (0.01-10 μmol/L) in serum-free DMEM. After 24 h, the medium was removed and replaced with fresh DMEM containing 10% fetal bovine serum and 10 μCi/mL 4[H]thymidine (Amersham Biosciences). The cells were pulsed with 4[H]thymidine for 1 h, lysed by the addition of 0.1 N KOH, and harvested onto fiberglass filters. The incorporated tritium was quantified in a scintillation counter.

Real-time quantitative PCR. For RNA extraction, cells were allowed to reach 70% confluence, medium was removed, and cells were immediately lysed in TRI Reagent (Sigma). Total RNA was prepared following the manufacturer’s instructions and further purified using Rneasy Mini Kit (Qiagen). For real-time PCR, the TaqMan system (Applied Biosystems) with Assays-on-Demand (Applied Biosystems) was used following the manufacturer’s instructions. A set of primers and TaqMan probes were DeltaEF1: Hs00232783-m1, E-cadherin: Hs00170423-m1, vimentin: Hs00185584-m1, fibronectin: Hs00415006-m1, and cyclophilin: Hs00170423-m1. The relative expressions of deltaEF1, E-cadherin, vimentin, and fibronectin were normalized to the amount of cyclophilin A in the same cDNA using the comparative CT method.

Immunoblot analyses. Cells were scraped from tissue culture plates with a rubber policeman into Triton lysis buffer [1% Triton X-100, 150 mmol/L NaCl, 25 mmol/L Tris (pH 7.5), 1 mmol/L glycerol phosphate, 1 mmol/L sodium orthovanadate, 1 mmol/L sodium fluoride, and one Complete Mini protease Inhibitor Cocktail tablet (Roche)] and were incubated for 1 h on ice. The lysates were then clarified by centrifugation before resolution by 10% SDS-PAGE. Polypeptides were transferred onto nitrocellulose membranes. Membranes were blocked for 1 h in 5% nonfat milk in a TBS containing 0.1% Tween 20 for 2 h at 4°C, incubated overnight with relevant antibodies, washed, and probed with species-specific secondary antibodies coupled to horseradish peroxidase (Amersham Biosciences), and then detected by enhanced chemiluminescence (Renais-
sance, New England Nuclear).

Cell cycle analysis. Cells were grown in six-well plates in the presence of 10% DMEM. After reaching 70% confluence, the cells were exposed to various concentrations of erlotinib for 24 h. Cells were harvested by trypsinization and pelleted by centrifugation. The pellets were then resuspended in PBS containing 50 μg/mL propidium iodide, 0.1% Triton X-100, and 0.1% sodium citrate. Samples were stored at 4°C for 1 h before analysis. Propidium iodide fluorescence was measured by fluorescence-activated cell sorting analysis (FACScan FL-3 channel; Becton Dickinson). Percentages of cell populations in different stages of the cell cycle were documented.

Wound healing assay and invasion assay. Cell motility and migration were determined by measuring the movement of cells to close an artificial wound created with a 10-μl pipette tip (time 0). The speed of wound closure was monitored by phase-contrast microscopy at 0-, 12-, and 24-h time points. For invasion assays, invasion chambers
containing Matrigel-coated polyethylene terephthalate membranes with 8-μm pores in a 24-well plate format were purchased from BD Biosciences. Cells (5 × 10⁴) were released from their tissue culture flask by EDTA (1 mmol/L), centrifuged, resuspended in DMEM, and placed in the upper compartment of the invasion chamber. Each cell line was plated in duplicate. NIH-3T3 conditioned medium was placed in the lower compartment as a chemoattractant. After incubation for 24 h at 37°C, the number of invading cells was counted in five separate fields per membrane.

**SiRNA-mediated knockdown of deltaEF1.** MDA1386LN and UMSCC1 cells were grown in six-well plates until they reached 60% confluence and then were transfected with siRNA targeting endogenous deltaEF1 or E-cadherin, both deltaEF1 and E-cadherin, or a non-targeting negative control using Oligofectamine (Invitrogen). All the siRNA constructs were obtained from Dharmacon Research, Inc. Sixty hours after transfection, total protein lysate was collected and analyzed by Western blotting, or total RNA was extracted and analyzed by real-time PCR. In a parallel experiments, 60 h posttransfection, siRNA-transfected cells were trypsinized and plated in 96-well plates for [³H]thymidine incorporation assays, subjected to EDTA and plated in invasion chambers for invasion assays, or allowed to reach 100% confluence for wound healing assays.

**Chromatin immunoprecipitation assay.** Cells were allowed to reach 70% to 80% confluence and then were processed for chromatin immunoprecipitation analyses using the CHIP-IT express kit (Active Motif) following the manufacturer’s protocol. PCR reactions were done with the primer for human E-cadherin promoter: forward 5’-AACCTCAAGCTAGGCGTCA-3’ and reverse 5’-GGGCTGGACTGCTGAACGTA-3’.

**Statistical analysis.** All assays were done at least three independent times. Numerical data are presented as mean ± SD. Comparisons between test and control groups were evaluated using Student’s t test. Statistical significance was set at P < 0.05.

## Results

**Heterogeneous effects of erlotinib on DNA synthesis in human HNSCC cells.** To determine their relative sensitivities to EGFR inhibition, we exposed a panel of 27 human HNSCC cells to increasing concentrations of erlotinib and measured DNA synthesis (as a marker of cell cycle progression) by [³H]thymidine incorporation. The lines displayed marked heterogeneity in responsiveness (Fig. 1), consistent with the data we and others have obtained in HNSCC and other solid tumors and visualized with an inverted microscope at ×10 magnification. The number of invading cells was counted in five separate fields per membrane.

### Table 1. Effects of erlotinib on cell cycle distribution in HNSCC cell lines

<table>
<thead>
<tr>
<th></th>
<th>Sensitive %G₁ ± SD</th>
<th>%S ± SD</th>
<th>Resistant %G₁ ± SD</th>
<th>%S ± SD</th>
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<tr>
<td>TU138</td>
<td></td>
<td></td>
<td>1386LN</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>54 ± 9.7</td>
<td>30 ± 6.8</td>
<td>Control</td>
<td>60 ± 4.2</td>
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<tr>
<td>Erl, 10 nmol/L</td>
<td>57 ± 8.3</td>
<td>27 ± 10.5</td>
<td>Erl, 10 nmol/L</td>
<td>64 ± 2.05</td>
</tr>
<tr>
<td>Erl, 100 nmol/L</td>
<td>66 ± 8.6</td>
<td>19 ± 5.9</td>
<td>Erl, 100 nmol/L</td>
<td>63 ± 7.3</td>
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<tr>
<td>Erl, 1 μmol/L</td>
<td>73 ± 11.5</td>
<td>14 ± 4.8</td>
<td>Erl, 1 μmol/L</td>
<td>61 ± 5.6</td>
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<tr>
<td>MDA 183</td>
<td></td>
<td></td>
<td>UMSCC1</td>
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<tr>
<td>Control</td>
<td>57 ± 5.8</td>
<td>27 ± 2.7</td>
<td>Control</td>
<td>58 ± 5.7</td>
</tr>
<tr>
<td>Erl, 10 nmol/L</td>
<td>64 ± 6.9</td>
<td>20 ± 4.1</td>
<td>Erl, 10 nmol/L</td>
<td>58 ± 2.2</td>
</tr>
<tr>
<td>Erl, 100 nmol/L</td>
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<td>Erl, 1 μmol/L</td>
<td>76 ± 3.1</td>
<td>8 ± 3.3</td>
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<tr>
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<td></td>
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<td>167LN</td>
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<tr>
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<td>24 ± 3.6</td>
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<td>Erl, 10 nmol/L</td>
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<td>Erl, 100 nmol/L</td>
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<td>Erl, 1 μmol/L</td>
<td>76 ± 3.1</td>
<td>8 ± 4.0</td>
<td>Erl, 1 μmol/L</td>
<td>72 ± 6.8</td>
</tr>
</tbody>
</table>

NOTE: Cells were allowed to reach 70% confluence and then were exposed to various concentrations of erlotinib (1 μmol/L, 10 nmol/L, and 100 nmol/L) for 24 h followed by propidium iodide staining for 1 h at 4°C. Propidium iodide fluorescence was measured using fluorescence-activated cell sorting analysis. Percentages of cell populations in different stages of the cell cycle were documented. Data are mean from three independent experiments (±SD).

Abbreviation: Erl, erlotinib.
(9–13). Based on their marked differences in erlotinib responsiveness, we selected three representative drug-sensitive cell lines (TU138, MDA183, and TU358B) and three representative drug-resistant cell lines (MDA1386LN, UMSCC1, and TU167LN) for further analysis.

To obtain an independent confirmation of the [3H]thymidine incorporation results, we used propidium iodide staining and fluorescence-activated cell sorting analyses to measure the percentages of cell populations in different stages of the cell cycle after exposure to increasing concentrations of erlotinib. Because cyclin-dependent kinase inhibitors have been implicated in growth arrest (4, 11), we also used immunoblotting to determine the effects of erlotinib on the expression of the cyclin-dependent kinase inhibitor p27 in the subset of six HNSCC cell lines. The three sensitive cell lines exhibited significantly induced increases in the percentages of cells that accumulated within the G1 phase of the cell cycle, with corresponding significant decreases in the percentages of cells found in S phase (Table 1). On the other hand, the three resistant cell lines showed minimal response with respect to cell cycle progression following exposure to increasing erlotinib concentrations (Table 1). Consistent with these observations, 1 μmol/L erlotinib increased expression of p27 in the sensitive cell lines after 24-h exposure, but erlotinib induced no change in p27 expression in the three resistant cell lines (Fig. 2A).

Baseline EGFR and p-EGFR expression does not correlate with response to erlotinib. To identify molecular markers predicting erlotinib sensitivity, we measured the expression of the erlotinib target protein EGFR as well as its active (phosphorylated) form (p-EGFR) in erlotinib-sensitive and erlotinib-resistant cell lines. Note that erlotinib inhibited baseline phosphorylation of EGFR in all of the erlotinib-sensitive cell lines but had no effect on the baseline phosphorylation of EGFR in the erlotinib-resistant cell lines.

Erlotinib-resistant cells display features of EMT. Recent studies have shown that EMT correlates with EGFR inhibitor resistance in diverse solid tumors including HNSCC (9–11, 13). Therefore, we used reverse transcription-PCR and immunoblotting to characterize the expression of E-cadherin, vimentin, fibronectin, and deltaEF1 in the panel of six HNSCC cell lines. Consistent with the previous studies (9), E-cadherin levels were high and the levels of vimentin, fibronectin, and deltaEF1 were low in the erlotinib-sensitive cells (Fig. 3). Conversely, E-cadherin levels were low whereas fibronectin and deltaEF1 levels were high in the erlotinib-resistant cells (Fig. 3).

Loss of intercellular adhesion and down-regulation of E-cadherin have been associated with enhanced motility and increased invasive and migratory potential (7). We therefore

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**Fig. 2.** Effects of erlotinib on (p27, EGFR, and p-EGFR) expression in HNSCC cell lines. A, Western blot analysis showed up-regulation of p27 in the three sensitive cell lines (TU138, MDA183, and TU358B) after 24-h exposure to 1 μmol/L erlotinib. The three resistant cell lines (MDA1386LN, UMSCC1, and TU167LN) exhibited no change in p27 expression levels after 24-h exposure to 1 μmol/L erlotinib. The expression of actin is shown as a loading control. B, status of baseline (EGFR and p-EGFR) in erlotinib-sensitive and erlotinib-resistant cell lines. Note that erlotinib induced baseline phosphorylation of EGFR in all of the erlotinib-sensitive cell lines but had no effect on the baseline phosphorylation of EGFR in the erlotinib-resistant cell lines.
performed wound healing assays to compare the migratory capabilities of the six HNSCC lines in the presence and absence of erlotinib. When cells reached 100% confluence, we created a scratch with a pipette tip and used light microscopy to monitor wound closure at 0-, 12-, and 24-hour time points. Resistant cell lines migrated faster to close the wound than did the sensitive cell lines (Fig. 4A). The most resistant cell line (MDA1386LN) exhibited the greatest migratory capability. It is important to note that MDA1386LN has the longest doubling time among the six HNSCC cell lines, indicating that MDA1386LN is able to close the wound quickly due to its advanced motile and migratory potential, and not due to its proliferation rate. Erlotinib had no effect on rate of wound closure in the “mesenchymal” cell lines (Fig. 4), indicating that cells that are resistant to the growth inhibitory effects of the drug are also resistant to its antimigratory effects. Conversely, the lines that were sensitive to the growth inhibitory effects of erlotinib also displayed delayed wound closure when they were exposed to the drug (Fig. 4), confirming that drug sensitivity was not limited to cell cycle inhibition. We then used Boyden chamber invasion assays to confirm and extend our findings with the wound healing assays and to determine the relative invasive capabilities of both sensitive and resistant cell lines. Consistent with their mesenchymal phenotypes, the resistant cell lines exhibited greater invasive potential compared with the sensitive cell lines. Furthermore, erlotinib did not affect the invasive capabilities of resistant cell lines, but it strongly inhibited invasion in the drug-sensitive lines (Fig. 4B). Together, these results strongly suggest that the antimetastatic effects of erlotinib are most prominent in HNSCC cells that are sensitive to erlotinib-induced growth arrest and display an “epithelial” molecular phenotype.

**DeltaEF1 induces EMT and drives resistance to erlotinib.** The correlation between deltaEF1 expression and erlotinib resistance suggested that a cause-effect relationship might exist between the two. To test this possibility, we knocked down deltaEF1 expression in two erlotinib-resistant lines (MDA1386LN and UMSCC1) and examined the effects on E-cadherin expression and erlotinib sensitivity. Immunoblotting and real-time PCR confirmed that silencing resulted in >50% knockdown of deltaEF1 and increased expression of E-cadherin (Fig. 5A) and increased cellular sensitivity to erlotinib as
measured by inhibition of \([^{3}H]\)thymidine incorporation (Fig. 5B). Combined knockdown of deltaEF1 plus E-cadherin reversed the erlotinib sensitization observed with knockdown of deltaEF1 alone (Fig. 5B), showing that erlotinib sensitization required E-cadherin reexpression. Furthermore, deltaEF1 knockdown dramatically decreased migration and invasion in 1386LN cell line (Fig. 5C and D). Together, these data establish that deltaEF1 is a critical component of the molecular

Fig. 4. Wound healing and invasion assays in resistant versus sensitive HNSCC cell lines. A, wound healing assays. Assays were done as described in Materials and Methods. Note that 24-h exposure to 1 μmol/L erlotinib had no influence on the migration rates of the resistant cell line but strongly decreased the migration rates of the sensitive cell line.

DeltaEF1 Controls EMT in HNSCC
mechanisms that drive EMT and EGFR inhibitor insensitivity in HNSCC cells.

**DeltaEF1 associates with the endogenous E-cadherin promoter.**
To determine whether deltaEF1 interacts directly with the endogenous E-cadherin promoter at the chromatin level, we performed chromatin immunoprecipitation experiments. A deltaEF1 antibody (directed against its NH₂ terminus) efficiently pulled down deltaEF1 together with chromatin fragments comprising the E-cadherin proximal regulatory promoter region in the resistant cell lines, whereas the antibody did not precipitate E-cadherin promoter fragments in the sensitive cell lines (Fig. 6). These results establish that deltaEF1 interacts

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**Fig. 4 Continued. B, invasion assays.** Modified Boyden chamber assays were done as described in Materials and Methods. Note that erlotinib had no effect on invasion in the resistant lines but caused significant reductions in invasion in the sensitive lines.
directly with the E-cadherin proximal promoter region in erlotinib-resistant cells. Together with the silencing data, they establish that deltaEF1 is responsible for suppressing E-cadherin expression and maintaining erlotinib resistance in the mesenchymal HNSCC cells.

Discussion

The results of clinical trials using EGFR inhibitors in diverse solid tumors indicate that tumors display remarkable heterogeneity in drug responsiveness that does not correlate well with target (EGFR) expression (2, 5, 6, 14). Here we used a panel of 27 human cell lines to obtain a sense of the heterogeneity in EGFR dependency that exists in HNSCC and to attempt to identify biological markers of drug sensitivity and resistance that might prove useful in distinguishing drug-sensitive from drug-resistant tumors. Our data show that cells that are sensitive to the EGFR inhibitor erlotinib express high levels of E-cadherin and low levels of deltaEF1 and display low invasive and migratory potential, whereas drug-resistant cells express high levels of deltaEF1 and low levels of E-cadherin and are highly invasive and migratory. Knockdown of deltaEF1 reversed the EMT phenotype associated with erlotinib resistance and restored erlotinib sensitivity in mesenchymal cell lines (MDA1386LN and UMSCC1), showing that deltaEF1 plays causative roles in both processes. The effects of deltaEF1 knockdown on erlotinib sensitivity required E-cadherin because combined knockdown of deltaEF1 plus E-cadherin reversed the erlotinib sensitization observed when deltaEF1 was silenced alone. Our data confirm and extend other recent findings in HNSCC (9) and other solid tumors (15), where EMT and, particularly, deltaEF1 have been shown to correlate with resistance to EGFR inhibitors. All of these studies strongly suggest that quantification of E-cadherin and deltaEF1 levels in...
pretreatment biopsies might allow for the prospective identification of solid tumors that are most likely to be sensitive to EGFR-directed therapy. This hypothesis must now be tested in well-designed clinical trials using pharmacodynamic markers that are capable of measuring drug-induced cell cycle arrest (p27 accumulation, decreases in Ki-67 or proliferating cell nuclear antigen, and possible intertumoral and intratumoral heterogeneity in the molecular EMT phenotype).

Other recent studies have shown that, like deltaEF1, E-cadherin also plays a causative role in determining EGFR inhibitor sensitivity (16). E-cadherin is known to form complexes with erbB family members that modulate downstream signal transduction (17–20), and it is also possible that the homotypic adhesion mediated by E-cadherin facilitates interactions between EGFR and surface-tethered ligands (heparin-binding epidermal growth factor and transforming growth factor α) expressed on neighboring cells. Preclinical studies have shown that knockdown of E-cadherin decreases EGFR inhibitor sensitivity (10); conversely, forced expression of E-cadherin in EGFR-independent cells can restore EGFR inhibitor sensitivity (16). These observations are somewhat inconsistent with previous work showing that the EGFR can drive EMT, invasion, migration, and metastasis (21, 22). It is possible that these observations can be reconciled if the EGFR can interact with other (mesenchymal) growth factor receptors to drive tumor progression in a manner that is insensitive to

Fig. 5. Continued. C, effects of deltaEF1 knockdown on cell migration. 1386LN cells were transfected with siRNAs targeting deltaEF1 or an off-target control for 48 h, and wound healing assays were done as described in Materials and Methods. Note that silencing decreased migration and that migration was further reduced by exposure to 1 μmol/L erlotinib. D, effects of knockdown on invasion. 1386LN cells were transfected with siRNAs targeting deltaEF1 or an off-target control and then analyzed in modified Boyden chambers as described in Materials and Methods. Note that similar effects of silencing and erlotinib were observed in the invasion and migration assays.
the currently available EGFR antagonists. This possibility is consistent with our observation that the constitutive EGFR phosphorylation that is observed in mesenchymal HNSCC cells is refractory to erlotinib (Fig. 2B). Another important question that remains unresolved is how deltaEF1 expression is regulated in HNSCC cells. One attractive possibility is that deltaEF1 expression is increased by cytokines and other signals, including the transforming growth factor-β, Shh, Notch, cyclooxygenase-2, and nuclear factor-κB pathways, which have been implicated in EMT from other models (7, 23). However, recent work has shown that members of the miR-200 family of microRNAs control deltaEF1 expression and the EMT phenotype in the NCI-60 and other model systems (24–27). Specifically, the transcripts encoding deltaEF1 (Zeb-1) and its homologue Zeb-2 (Sip-1) contain multiple binding sites for these microRNAs, and forced expression of members of the miR-200 family result in their direct binding to the deltaEF1 and Zeb-2 transcripts, decreasing the translation of their protein targets (24–27). Furthermore, profiling studies have shown that miR-200 family microRNAs are lost in cell lines that display high levels of deltaEF1 and Zeb-2, low levels of E-cadherin, and a mesenchymal phenotype (24). We have found that expression of miR-200c inversely correlates with deltaEF1 expression in bladder and pancreatic cancer cells, and reintroduction of the microRNA restores an epithelial phenotype and EGFR inhibitor sensitivity in mesenchymal bladder cancer cells. We measured miR-200c expression in the subset of six HNSCC lines characterized here and found that it is expressed at low levels in the most mesenchymal line (MDA1386LN) but not in UMSCC1 or TU167LN cells even though the latter are also erlotinib resistant, highly migratory, and express high levels of fibronectin (data not shown). We are currently expanding these studies to include our whole panel of HNSCC lines and additional members of the miR-200 family, but these preliminary results suggest that other mechanisms may be involved as well.

DeltaEF1 represses gene expression by serving as a scaffold for recruitment of histone deacetylases to chromatin (7). This observation provides an immediate means of translating our findings into clinical application. Histone deacetylase inhibitors are being developed aggressively for cancer therapy in large part because early studies showed that they could promote differentiation in erythroid leukemia cells (28), a process that is reminiscent of EMT reversal. Vorinostat (suberoylanilide hydroxamic acid) is the first of this class of agents to receive Food and Drug Administration approval for cancer treatment (for the therapy of cutaneous T-cell lymphoma; refs. 29–31). Although it displays little, if any, single agent activity in solid tumors, preclinical studies have shown that MS-275 is capable of reversing EMT and restoring sensitivity to EGFR inhibitors in NSCLC cells (16), and our own results in pancreatic cancer cells confirm these observations. Importantly, the EMT reversal induced by vorinostat in vitro or in vivo is relatively long lasting, suggesting that proper scheduling might maximize the beneficial effects of the drug in solid tumors while avoiding some of the toxic effects that might be caused by combination therapy. We are currently characterizing the effects of histone deacetylase inhibitors on sensitivity to erlotinib and other drugs in preclinical in vitro and in vivo models of HNSCC to determine whether they can be used to restore sensitivity to EGFR inhibitors and other agents.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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**Fig. 6.** DeltaEF1 interacts with the E-cadherin promoter in erlotinib-resistant cells. Chromatin immunoprecipitation assays were done with an anti-deltaEF1 antibody and primers designed to amplify the region containing the E-boxes present in the E-cadherin promoter. Note that deltaEF1 interacts with the E-cadherin promoter in all of the erlotinib-resistant lines tested but does not interact with the promoter in the erlotinib-sensitive cells. Also note that other candidate regulators of E-cadherin promoter activity and EMT (Snail and Zeb-2) were not detected on chromatin in these assays.


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