Epithelial to mesenchymal transition predicts gefitinib resistance in cell lines of head and neck squamous cell carcinoma and non–small cell lung carcinoma

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Abstract
The modest response of patients with head and neck squamous cell carcinoma (HNSCC) and non–small cell lung carcinoma (NSCLC) to epithelial growth factor receptor tyrosine kinase inhibitors such as gefitinib and erlotinib indicates the need for the development of biomarkers to predict response. We determined gefitinib sensitivity in a panel of HNSCC cell lines by a 5-day 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and confirmed these responses with analysis of downstream signaling by immunoblotting and cell cycle arrest. Basal gene expression profiles were then determined by microarray analysis and correlated with gefitinib response. These data were combined with previously reported NSCLC microarray results to generate a broader predictive index. Common markers of resistance between the two tumor types included genes associated with the epithelial to mesenchymal transition. We confirmed that increased protein expression of vimentin combined with the loss of E-cadherin, claudin 4, and claudin 7 by immunoblotting was associated with gefitinib resistance in both HNSCC and NSCLC cell lines. In addition, the loss of the Ca2+-independent cell-cell adhesion molecules EpCAM and TROP2 in resistant lines was confirmed by immunofluorescence. Tumor xenografts derived from the gefitinib-sensitive UM-SCC-2 were growth-delayed by gefitinib, whereas the gefitinib-resistant 1483 xenografts were unaffected. These data support a role for epithelial to mesenchymal transition in establishing gefitinib resistance for both HNSCC and NSCLC, and indicate that clinical trials should address whether these biomarkers will be useful for patient selection. [Mol Cancer Ther 2007;6(6):1683–91]

Introduction
Head and neck cancers are the sixth most common neoplasms in the developed world, resulting in 13,000 deaths in the United States alone (1, 2). Despite improvements in treatment with the use of concurrent chemoradiotherapy in the management of advanced head and neck squamous cell carcinoma (HNSCC), local/regional control remains a problem and the 5-year survival rate of HNSCC patients remains at <50% (3).

In the last decade, the development of new therapeutic strategies targeting specific tumor-associated proteins has included monoclonal antibodies directed against growth factor receptors and small molecules that inhibit tyrosine kinases. Among these targeted molecules is the epithelial growth factor receptor (EGFR), which is overexpressed in many cancers including HNSCC, gliomas, and lung, ovarian, and pancreatic cancers (4). Only a minority of patients with HNSCC and non–small cell lung carcinomas (NSCLC) respond to EGFR-directed inhibition (5–9), indicating the need for predictive markers to select patients most likely to respond to small molecule EGFR-tyrosine kinase inhibitors (TKI) or monoclonal antibodies against EGFR. Although HNSCC and NSCLC are obviously different diseases, they share common risk factors, and similar gefitinib response rates would suggest that these tumor types might have similar resistance mechanisms.

The biological markers associated with objective responses to EGFR-TKIs in patients with NSCLC include activating somatic mutations in the kinase domain of the EGFR and increased EGFR gene copy number (10–13). Increased EGFR gene copy number by fluorescence in situ hybridization seems to be a predictive marker for survival (14), but amplification of the EGFR gene is less frequent in HNSCCs (15), and thus, is unlikely to be predictive for this tumor type. Mutations in the tyrosine kinase domain may predict response in NSCLC (16), but such mutations are uncommon in patients with HNSCC (17, 18). However, the truncation mutation EGFR variant III (EGFRvIII) has been identified in >40% of HNSCC tumors, but does not correlate with resistance to EGFR-TKIs (19). EGFR protein expression was associated with favorable survival in the randomized trials comparing gefitinib to placebo or erlotinib to placebo (14), but does not predict response to EGFR-directed therapies (9, 20).
Materials and Methods

Cell Culture

HNSCC cell lines UM-SCC-2, HN4, UM-SCC-8, HN31, UM-SCC-10A, MDA-1586, and MDA-584 were provided by Dr. Scott Weed (West Virginia University, Morgantown, WV) and UM-SCC-22B and UM-SCC-1483 were from Dr. Jenny Grandis (University of Pittsburgh). HNSCC cells were cultured in either DMEM (UM-SCC-2, UM-SCC-8, UM-SCC-10A, UM-SCC-22B, 1483, HN4, HN31) or RPMI (MDA1586, 584) supplemented with 5% heat-inactivated fetal bovine serum. Drs. John Minna and Adi Gazdar (University of Texas Southwestern Medical School, Dallas, TX) provided H1648, H157, and H460. Calu3, H1703, A549, and H520 were obtained from American Type Culture Collection. H322 was provided by Dr. Al Monstafa (National Research Council, Canada, Biotechnology Research Institute, Montreal, Quebec, Canada) and H358 was from Dr. Isaiah Fidler (University of Texas M.D. Anderson Cancer Center, Houston, TX). NSCLC cells were grown in RPMI with 10% heat-inactivated fetal bovine serum.

Determination of Gefitinib Response and the Effect of Gefitinib on EGFR Signaling Pathways and Cell Cycle Progression

Gefitinib (ZD1839, Iressa) was kindly provided by AstraZeneca. The response of NSCLC cell lines to gefitinib is reported elsewhere (29). The sensitivity of nine HNSCC cell lines to gefitinib was determined by a 5-day 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. In brief, 4,000 cells/100 μL were seeded into 96-well plates and allowed to attach overnight. Gefitinib was added to final concentrations of 0.03 to 10 μmol/L, and the plates were incubated for 5 days with standard incubation conditions. After 5 days, 50 μL of 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (2 mg/mL) was added to each well and incubated for 4 h. The supernatants were aspirated and the reduced dye was solubilized in 100 μL of a solution containing 75% isopropanol, 2% HCl, and 23% H2O. Absorbance was read at 490 nm with an automated plate reader.

HNSCC cells were plated to 80% confluence and treated with 1 μmol/L of gefitinib for 24 h. Following cell lysis, 50 μg of protein were loaded onto 8% SDS-PAGE gels. After transfer to polyvinylidene difluoride membranes, proteins were blotted for phospho-EGFR (Tyr1068), total EGFR, phospho-ERK1/2 (Thr202/Tyr204) and total ERK1/2 using antibodies from Cell Signaling Technology. β-Actin (clone ACTN05, LabVision) was used as a loading control.

Cell cycle distribution was determined by fluorescence-activated cell sorting analysis as previously described (30). After 24 h incubation with 0, 1, or 10 μmol/L of gefitinib, cells were trypsinized and stained with saponin, propidium iodide, and RNase A, then cells were analyzed by fluorescence-activated cell sorting using a Coulter EPICS, and ModFit software (Verity Software House) to calculate cell cycle distributions.

Microarray Analysis

Cells were plated at 2 × 10^6 in 10-cm plates 24 h before harvest. At harvest, plates were rinsed twice with PBS, and RNA was prepared using a RNeasy mini kit (Qiagen). RNA stabilization, isolation, and microarray sample labeling were carried out using standard methods for reverse transcription and one round of in vitro transcription (31). HG-U133 set microarrays were hybridized with 10 μg of cRNA and processed according to the protocols of the manufacturer (Affymetrix). Hybridization signals and detection calls were generated in BioConductor (32), using the gcrma and affy packages. Microarray data was analyzed using BRB ArrayTools v3.2 developed by Dr. Richard Simon and Amy Peng Lam. Hierarchical clustering and multidimensional scaling were done using the 415 genes comprising the gefitinib sensitivity–associated profile developed in NSCLC cell lines. This set of 415 genes was uncovered by comparing the baseline gene expression of NSCLC cell lines that were either highly sensitive or highly resistant to gefitinib (33).

Immunoblotting

The following antibodies and concentrations were used: mouse monoclonal antibodies for EpCAM (2 μg/mL, AbCam, Inc.), vimentin (1 μg/mL, Lab Vision), actin (0.5 μg/mL, Lab Vision), E-cadherin (0.1 μg/mL, BD Transduction Laboratories), claudin 4 (1 μg/mL, Zymed), and rabbit polyclonal antibodies for claudin 7 (1:2,000, Zymed) were used according to the recommendations of the manufacturer.

Immunofluorescence Staining

For EpCAM expression by flow cytometry, 2 × 10^5 cells were incubated with the mouse monoclonal B29.1 (VU-ID9)
Effect of Gefitinib on Tumor Growth

Athymic 4- to 6-week-old female mice were purchased from the National Cancer Institute and maintained in accordance with the institutional guidelines of the University of Colorado Health Sciences animal care facility. UM-SCC-2 cells and 1483 cells (1 × 10⁷/100 μL in 1:1 Matrigel/unsupplemented DMEM) were injected s.c. into the flank area of the mice. When tumors had reached 100 mm³, mice were randomized into two treatment groups of 10 mice each. The first group received vehicle solution and the second group received 60 mg/kg gefitinib, both for 5 d/wk by i.p. injection. Tumor volumes were measured weekly using a caliper and the following formula: V = π(smaller diameter)²(larger diameter) / 6. Mice were euthanized and tumors were formalin-fixed and paraffin-embedded.

Immunohistochemical Analysis

Paraffin-embedded tissues were used for the identification of E-cadherin, claudin 4, claudin 7, and vimentin. Sections were deparaffinized in xylene, treated with a graded series of alcohols and rehydrated in PBS. Sections were treated with citrate buffer (E-cadherin, claudin 7, and vimentin) or EDTA (claudin 4), and heat for epitope retrieval. Monoclonal antibodies for claudin 4 and claudin 7 (Zymed Laboratories), vimentin (DakoCytomation), and E-cadherin (Transduction Laboratories) were used according to the recommendations of the manufacturer.

Results

Gefitinib-Mediated Growth Inhibition of Human HNSCC Cell Lines In vitro

A broad range of responses to gefitinib were found in our panel of HNSCC lines when growth inhibition was assessed by a 5-day 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The IC₅₀ concentrations varied from 0.1 to >10 μmol/L as shown in Table 1. EGFR surface expression assessed by flow cytometry (fluorescence-activated cell sorting) correlated with response to gefitinib (P = 0.013). In a screen for mutations commonly found in NSCLC, the HNSCC cell lines in this study were all wild-type EGFR. In addition, all of the NSCLC lines included in this study were wild-type EGFR. EGFR gene expression by microarray did not correlate with gefitinib response, but cell surface expression as measured by fluorescence-activated cell sorting indicated that the sensitive HNSCC lines expressed more EGFR.

Figure 1 shows the results of immunoblotting for total and phosphorylated EGFR and ERK1/2 after treatment with 1 μmol/L of gefitinib for 24 h. Drug treatment inhibited EGFR phosphorylation of all HNSCC cell lines with an IC₅₀ of >1 μmol/L. In gefitinib-resistant lines, there was no change in EGFR phosphorylation of UM-SCC10A, whereas UM-SCC-22B and 1483 phosphorylation decreased, but ERK1/2 phosphorylation was unaffected. UM-SCC-2, HN4, and UM-SCC-8 ERK1/2 phosphorylation was inhibited by gefitinib, whereas MDA-1586 and HN31 were unaffected.

The results of cell cycle progression following gefitinib treatment (1 or 10 μmol/L for 24 h) support the immunoblot results (Table 1). The four gefitinib-resistant lines showed little or no G1 cell cycle arrest with 1 μmol/L, and UM-SCC-22B and 584 showed only modest G1 arrest with 10 μmol/L. The three cell lines with the greatest inhibition of ERK1/2 phosphorylation (UM-SCC-2, HN4, and UM-SCC-8) also had the highest G1 increase with 1 μmol/L, and 10 μmol/L of gefitinib did not greatly enhance the effect.

Microarray Analysis of HNSCC

To determine the genes associated with sensitivity/resistance of the HNSCC cell line, we examined the gene expression profiles of untreated cell lines. This places the focus on the primary differences between the cell lines which predispose them to gefitinib sensitivity. We then examined these HNSCC cell line data for the expression of the genes associated with sensitivity/resistance in the NSCLC cell lines with wild-type EGFR. This gene profile was generated by analyzing the differential expression of gefitinib-resistant and gefitinib-sensitive NSCLC cell lines. Gene expression was directed compared using a two-sample t test, with a nominal P cutoff of 0.001. There were 415 probe sets that met this nominal criterion (33). Sensitivity was defined as gefitinib IC₅₀ values of ≤1 μmol/L, and resistance was defined as IC₅₀ values of ≥8 μmol/L for both HNSCC and NSCLC cell lines. Hierarchical clustering of cell lines based on the expression of the 415 genes in the gefitinib sensitivity–associated profile showed that gefitinib-sensitive HNSCC cell lines clustered with sensitive NSCLC cell lines (Fig. 2). However, within this group of gefitinib-sensitive lines, HNSCC expression clustered together in a group distinct from NSCLC. Gefitinib-resistant HNSCC and NSCLC also formed a cluster separate from the sensitive lines and showed a similar pattern of separate groups at higher correlation. The one exception to this observation was the resistant HNSCC cell line, UM-SCC-10A, which had a profile more similar to NSCLC than HNSCC.
Changes in Gene Expression Associated with EMT Correlate with Gefitinib Response for Both HNSCC and NSCLC

We selected a subset of genes shown to be associated with EMT and evaluated the changes in gene expression in both HNSCC and NSCLC cell lines. These genes included those associated with adherens junctions (E-cadherin, EpCAM/TACSTD1/TROP1, TROP2/TACSTD2, N-cadherin, β-catenin), tight junctions (claudin 4, claudin 7, occludin, ZO1, ZO2, and ZO3), cell adhesion (EVA1), EMT signaling pathways (Smads 2, 3, and 4, TGFβ, TGFR1, and TGFR2), mesenchymal markers (vimentin and fibronectin), and genes that regulate EMT gene expression (ZEB1, ZEB2, Snail, LEF1, and RAB25).

As shown in Fig. 2, genes associated with epithelia such as E-cadherin, were expressed in both HNSCC and NSCLC cell lines sensitive to gefitinib but lost in resistant lines. Expression of the tight junction proteins claudin 4, claudin 7, and occludin, as well as associated ZO2, were also expressed in sensitive lines and suppressed in resistant lines. Resistant cell lines had high expression of the mesenchymal marker vimentin and most showed overexpression of fibronectin. In addition, the expression of the E-cadherin repressor ZEB1 was elevated in all of the HNSCC-resistant lines as well as the NSCLC lines.

Microarray Signals Correlate with Protein Expression of Selected Markers in Both HNSCC and NSCLC

We confirmed that the microarray signals for epithelial markers E-cadherin, claudin 4, claudin 7, and the mesenchymal marker vimentin corresponded to protein expression by immunoblotting using well characterized antibodies to these proteins. We found that gefitinib-resistant HNSCC cell lines lack E-cadherin protein expression (Fig. 3B). The loss of the epithelial tight junction proteins claudin 4 and claudin 7 in resistant HNSCC and NSCLC lines predicted by microarray analysis was also confirmed by immunoblotting (Table 2; Fig. 3C). The vimentin microarray signals of three sensitive lines, UM-SCC-2, Calu3, and H358, were elevated compared with the other sensitive lines, but...
protein expression for these three lines was similar to other sensitive lines (Fig. 3C). Consistent with the transition to the mesenchymal phenotype, the resistant HNSCC and NSCLC lines expressed vimentin protein by Western blotting (Fig. 3C).

Cell surface expression of the adherin junction proteins TROP2 and EpCAM (TROP1) were determined by flow cytometry. EpCAM gene expression was very low in our resistant HNSCC lines, and these results were confirmed by cell surface expression (Table 2). EpCAM expression in NSCLC was somewhat less consistent with gefitinib response. Whereas the majority of resistant lung lines had little or no EpCAM expression, the resistant line H520 expressed EpCAM by microarray analysis. This result was corroborated by both flow cytometry and immunoblotting. The loss of TROP2 expression was more predictive of gefitinib resistance in NSCLC than HNSCC (Table 2). Two of four resistant HNSCC lines expressed low levels of TROP2, whereas all of the resistant NSCLC lines showed no expression.

**Effect of Gefitinib on the Growth of UM-SCC-2 and 1483 Xenografts**

We chose one gefitinib-sensitive cell line (UM-SCC-2) and one gefitinib-resistant line (1483) for evaluation in a mouse xenograft model to determine if the *in vitro* data predicted gefitinib response *in vivo*. Gefitinib treatment resulted in growth delay of UM-SCC-2 tumors compared with control, whereas 1483 growth was uninhibited by treatment (Fig. 4A).

Finally, at the termination of the xenograft study, we assessed the expression of the epithelial markers E-cadherin, claudin 4 and claudin 7, and the mesenchymal marker vimentin in paraffin-fixed tumor tissue. In concordance with *in vitro* results, the expression of E-cadherin was highest in UM-SCC-2 tissue, with very little staining in 1483 sections, and only UM-SCC-2 tumors expressed claudin 4 and claudin 7 (Fig. 4B). Vimentin was strongly expressed in the gefitinib-resistant 1483 tumor tissue, with little staining in the UM-SCC-2 tissue.

**Discussion**

The mechanisms of gefitinib resistance in patients with HNSCC are poorly understood and the modest patient response in clinical trials suggests the need for developing a profile to identify those patients with HNSCC most likely to respond to gefitinib. We sought to identify the molecular markers associated with gefitinib response and compare these markers to those previously reported in NSCLC, with the goal of understanding gefitinib resistance across a broader scope of different tumor origins. We evaluated a panel of HNSCC cell lines for response to gefitinib by an *in vitro* assay and these results were correlated with genes differentially expressed in resistant and sensitive cell lines. The results of this study suggest that like NSCLC, the EMT in HNSCC corresponds to gefitinib resistance.

EGFR cell surface expression was elevated in HNSCC lines sensitive to gefitinib and the correlation between ICs₀ and EGFR MFI was significant ($r^2 = 0.60; P = 0.013$), indicating that gefitinib sensitivity is, in part, linked to EGFR expression. However, EGFR expression alone is not entirely predictive of response in our panel of HNSCC cell lines, in which the MFI in our most sensitive cell line, UM-SCC-8, was identical to MDA 1586, which had a 10-fold higher ICs₀. In addition, three of the four resistant lines expressed EGFR, suggesting that other underlying mechanisms contribute to gefitinib resistance. In our panel of NSCLC lines, EGFR cell surface expression did not correlate with gefitinib sensitivity (29).

In an effort to identify common mechanisms of gefitinib sensitivity and resistance in HNSCC and NSCLC, we combined baseline gene expression profiles from a panel of nine HNSCC lines (five sensitive and four resistant) and nine NSCLC lines (four sensitive and five resistant). Gene expression was more differential between response groups than between tumor origin, suggesting at least some common response markers. Within response group clusters, however, HNSCC was clearly delineated from NSCLC, indicating that each tumor type also had tumor-specific expression profiles.

From these data, we focused on several genes that were predictive in both tumor types. Of these common markers, all were associated either directly or indirectly with EMT. All of our resistant HNSCC cell lines have lost the ability to express E-cadherin, which is a hallmark of EMT. This is in agreement with our previously published data (27) and the study by Yauch et al. (25) in NSCLC cell lines in which E-cadherin was absent in resistant lines and overexpressed in sensitive lines. E-Cadherin is involved in cell-cell adhesion and the loss of expression has been observed during tumor progression (34). In one study, low E-cadherin expression in primary HNSCC tumors was correlated with the presence of nodal metastases (35),
although another investigation with paired HNSCC primary tumors and corresponding lymph node metastases failed to find reduced E-cadherin in metastatic tissue (36). In this same report, expression of the epithelial cell adhesion molecule EpCAM (TROP1/TACSTD1) was significantly lower in metastases compared with paired primary tumors. EpCAM is a Ca²⁺-independent molecule with two epithelial growth factor–like repeats strictly expressed in epithelia (37) and immunohistochemical analysis has shown no expression in mesenchymal, muscular, or neuroendocrine tissues (38, 39). EpCAM is not expressed in normal squamous epithelia, and active proliferation of epithelial tissue is associated with increased EpCAM expression (40). The loss of EpCAM expression in our resistant HNSCC and NSCLC cell lines is consistent with a mesenchymal phenotype. We also

Figure 3. Gene and protein expression of EMT-associated molecules correlate with gefitinib response. A, heat map of genes associated with EMT shows differential expression according to gefitinib response. Down-regulated (blue) and up-regulated genes (red). B, loss of E-cadherin expression is a signature of gefitinib resistance. E-Cadherin immunoblots of HNSCC cell lines characterized for gefitinib response showed a correlation between E-cadherin expression and gefitinib sensitivity. C, expression of epithelial- and mesenchymal-associated proteins differentiates between gefitinib-sensitive and gefitinib-resistant HNSCC and NSCLC cell lines. Western blot analysis of claudin 4 (epithelial), claudin 7 (epithelial), and vimentin (mesenchymal) showed expression of the claudins in HNSCC and NSCLC sensitive to gefitinib, and expression of vimentin in resistant lines.
found a correlation with the loss of the EpCAM-related TROP2 expression and gefitinib resistance, and this association was strongest in NSCLC. Although less is known about TROP2, it is also mainly expressed in epithelial cells and is involved in Ca2+ signaling (41).

EMT is characterized by the loss of cell-cell adhesion and the gain in cell motility. These processes are accompanied by a complete loss in epithelial cell polarity. Tight junctions are complexes that determine epithelial cell polarity and disappear during EMT. Tight junction strands consist of two types of membrane proteins, occludins and the claudins, which modulate some functions or provide the backbone of tight junctions (42). The loss of claudin 4 and claudin 7 expression is associated with EMT (43), and may lead to a more aggressive phenotype (44). All of our sensitive HNSCC and NSCLC lines expressed claudin 4 and claudin 7, and the loss or reduced expression seems to be correlated with gefitinib resistance. Our results suggest that the complete loss of claudin 4 is consistent with our resistant HNSCC lines and may be a better predictor of response than claudin 7 expression, where we found that two of four resistant lines expressed this protein. Conversely, loss of claudin 7 was a more reliable predictor of response in our NSCLC cell line panel. Taken together, however, the loss of one or both claudins indicates that these resistant lines are of the mesenchymal phenotype.

In addition to the loss of tight junctions and E-cadherin proteins, EMT is characterized by the expression of the mesenchymal marker vimentin. The results of vimentin expression and the association with gefitinib resistance in our NSCLC cell lines are in agreement with previous reports of vimentin expression in EGFR-TKI–resistant NSCLC cell lines (25, 26). We also found that gefitinib-resistant HNSCC cell lines expressed vimentin and that expression was completely absent in sensitive lines, and our xenograft results also suggest that vimentin expression was correlated with gefitinib resistance.

Mechanisms resulting in the re-expression of epithelial markers such as E-cadherin may increase gefitinib sensitivity in resistant cell lines. The zinc finger transcriptional repressor ZEB1 inhibits E-cadherin expression by recruiting histone deacetylases. We previously showed an inverse relationship between E-cadherin and ZEB1 expression in gefitinib-resistant NSCLC cell lines (27). Furthermore, the histone deacetylase inhibitor trichostatin A induced the re-expression of E-cadherin in lung cancer cell lines (45). We showed that a 24-h pretreatment with the histone deacetylase inhibitor MS-275, which is currently in phase I clinical trials, increased E-cadherin expression and increased gefitinib sensitivity in resistant NSCLC cell lines (27). Clinical trials are planned to evaluate the sequential administration of histone deacetylase inhibitors and EGFR-TKIs in patients with NSCLC lung cancer. This approach warrants investigation in both in vitro and in vivo studies in HNSCC as the same inverse correlation between E-cadherin and ZEB1 was shown by microarray analysis.

In conclusion, these data suggest that like erlotinib, EMT predicts resistance to gefitinib in NSCLC. The correlative results in HNSCC indicate that the EMT phenomenon may be a broad resistance signature, and further studies with additional tumor types are required to address this issue. It is unclear whether EMT represents an inherently more aggressive and EGFR-TKI–resistant phenotype, or changes

### Table 2. EpCAM (TROP1) and TROP2 expression by microarray and flow cytometry in a panel of HNSCC and NSCLC cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Gefitinib sensitivity</th>
<th>EpCAM (TROP1)</th>
<th>TROP2</th>
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<tr>
<td></td>
<td>Positive (%)</td>
<td>MFI</td>
<td>Array</td>
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Mol Cancer Ther 2007;6(6). June 2007

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in EMT-associated protein expression are directly related to response. What is clear, however, is that EMT signature profiling may aid in the prediction of patients with HNSCC and NSCLC most likely to respond to gefitinib.

Acknowledgments
We thank P. Schedin and P. Bell for excellent advice on immunohistochemistry.

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