An Epithelial–Mesenchymal Transition Gene Signature Predicts Resistance to EGFR and PI3K Inhibitors and Identifies Axl as a Therapeutic Target for Overcoming EGFR Inhibitor Resistance


Abstract

Purpose: Epithelial–mesenchymal transition (EMT) has been associated with metastatic spread and EGF receptor (EGFR) inhibitor resistance. We developed and validated a robust 76-gene EMT signature using gene expression profiles from four platforms using non–small cell lung carcinoma (NSCLC) cell lines and patients treated in the Biomarker-Integrated Approaches of Targeted Therapy for Lung Cancer Elimination (BATTLE) study.

Experimental Design: We conducted an integrated gene expression, proteomic, and drug response analysis using cell lines and tumors from patients with NSCLC. A 76-gene EMT signature was developed and validated using gene expression profiles from four microarray platforms of NSCLC cell lines and patients treated in the BATTLE study, and potential therapeutic targets associated with EMT were identified.

Results: Compared with epithelial cells, mesenchymal cells showed significantly greater resistance to EGFR and PI3K/Akt pathway inhibitors, independent of EGFR mutation status, but more sensitivity to certain chemotherapies. Mesenchymal cells also expressed increased levels of the receptor tyrosine kinase Axl and showed a trend toward greater sensitivity to the Axl inhibitor SGI-7079, whereas the combination of SGI-7079 with erlotinib reversed erlotinib resistance in mesenchymal lines expressing Axl and in xenograft model of mesenchymal NSCLC. In patients with NSCLC, the EMT signature predicted 8-week disease control in patients receiving erlotinib but not other therapies.

Conclusion: We have developed a robust EMT signature that predicts resistance to EGFR and PI3K/Akt inhibitors, highlights different patterns of drug responsiveness for epithelial and mesenchymal cells, and identifies Axl as a potential therapeutic target for overcoming EGFR inhibitor resistance associated with the mesenchymal phenotype. Clin Cancer Res; 19(1); 279–90. ©2012 AACR.

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Affymetrix microarray results from the cell line training set were previously published and archived at the Gene Expression Omnibus repository (http://www.ncbi.nlm.nih.gov/geo/). GEO accession GSE4824.

Illumina v2 (GSE32989) and v3 (GSE32036) results have been deposited in the GEO repository and are available at the following links: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=lxatjamqwakaidw&acc=GSE32989.


BATTLE array results have been deposited in the GEO repository (GSE33072) and are available at the following links: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=xfwmxqsjyogaacajq&acc=GSE33072.

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doi: 10.1158/1078-0432.CCR-12-1558

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Identification of tumors that have not undergone EMT may help predict likelihood of benefit to EGFR-targeted therapy independent of EGFR status.

Introduction

Previous molecular profiling studies and recent mutation analyses have shown the molecular heterogeneity of non–small cell lung carcinoma (NSCLC). For EGFR receptor (EGFR) mutant and EMLA-ALK fusion subgroups, mutation status predicts response to targeted therapy with EGFR inhibitors or ALK inhibitors, respectively. Unfortunately only a minority of patients express these markers, with EGFR mutations detected in approximately 10% to 15% of lung adenocarcinomas (1–6) and EMLA-ALK fusions in approximately 4% (7). For the majority of patients with wild-type EGFR, a subgroup seems to benefit from EGFR inhibitor treatment, although there are currently no validated markers for identifying these patients (8–10). Thus, validated predictive markers are needed to accurately predict likelihood of benefit to EGFR-targeted therapy independent of EGFR mutation status, as well as for other targeted therapies.

Epithelial–mesenchymal transition (EMT) is a biologic program observed in several types of epithelial cancers including NSCLC. EMT is associated with loss of cell adhesion proteins, such as E-cadherin and increased invasion, migration, and cell proliferation (11–14). Preclinical and clinical data suggest that markers of EMT may be associated with limited responses to EGFR inhibitors, whereas reversion of an epithelial phenotype is associated with response even in patients without EGFR mutations (15–18). For example, high E-cadherin and low vimentin/fibronectin (i.e., an epithelial phenotype) have been associated with erlotinib sensitivity in cell lines and xenografts with wild-type EGFR (16). Clinically, E-cadherin protein expression has been associated with longer time to progression and a trend toward longer overall survival following combination erlotinib/chemotherapy, although EGFR mutation status of the patients was not known in this study (15). The ability to identify tumors that have not undergone EMT may help with the selection of patients most likely to benefit from EGFR inhibition, particularly in patients with wild-type EGFR. In addition, targeting EMT may reverse or prevent acquisition of therapeutic resistance to EGFR inhibitors, as illustrated by 1 study in which restoration of an epithelial phenotype in NSCLC cell lines restored sensitivity to the EGFR inhibitor gefitinib (19). Although a number of markers have been associated with EMT and EMT signatures have been described in other cancer types, there is no validated EMT signature in NSCLC. Furthermore, it is unknown to what extent EMT may predict response to other targeted drugs in NSCLC.

To better characterize EMT in NSCLC and its association with drug resistance, we conducted an integrated analysis of gene expression profiling from several microarray platforms together with high-throughput functional proteomic profiling. By cross-validating gene expression data from 2 independent microarray platforms in our training set of NSCLC cell lines, we were able to derive a robust EMT gene expression signature capable of classifying whether a NSCLC cell line had undergone EMT. We also conducted an integrated analysis of the EMT gene signature and high-throughput proteomic profiling of key oncogenic pathways to explore differences in signaling pathways between epithelial and mesenchymal lines. Finally, we tested the ability of the EMT signature to predict response to erlotinib and other drugs in preclinical models and patient tumor samples.

Materials and Methods

Cell lines

NSCLC cell lines were established by J.D. Minna and A. Gazdar at the National Cancer Institute and the University of Texas Southwestern Medical Center (Dallas, TX; refs. 20, 21) or obtained from the ATCC. Details of cell culture conditions are provided in the Supplementary Information.

Expression profiling of cell lines

Total RNA was isolated from cell lines and profiled as described in the Supplementary Information.

Selection of single best EMT marker probes

Because the NSCLC cell line panel was profiled on both Affymetrix and Illumina microarray platforms, we were able to select the single best Affymetrix probe sets for CDH1, VIM, CDH2, and FN1 on the basis of their strong correlation with other probes for the same gene within a microarray platform and/or across platforms (Supplementary Fig. S1). Details provided in the Supplementary Information.

Gene expression profiling of BATTLE tumors

Tumors collected during the Biomarker-Integrated Approaches of Targeted Therapy for Lung Cancer Elimination (BATTLE) clinical trial were subjected to microarray profiling, as detailed in the Supplementary Information.

Drug sensitivity of cell lines

For each drug, IC50 was measured by MTS assay 3 or more times in NSCLC cell lines derived from treatment-naive
patients and the average values were used for analysis as described previously and in the Supplementary Information (22).

Generation and characterization of AXL inhibitor SGI-7079

Details of SGI-7079 generation are provided in Supplementary Fig. S2 and Supplementary Information. To show inhibition of Axl activation by SGI-7079, HEK-293 cells were transiently transfected by electroporation with 1 mg FLAG-tagged plasmid containing the human Axl gene (OriGene Technologies) and allowed to incubate in standard media + 10% FBS for 24 hours. Cells were treated with SGI-7079 for 10 minutes at the indicated concentrations. Five minutes before lysis, the cells were stimulated with Gas6-containing WI38 conditioned media. Immunoprecipitation of FLAG-tagged Axl was conducted with anti-FLAG M2 agarose (Sigma-Aldrich). Immunoprecipitates were resolved by SDS-PAGE, and Western blotting was conducted with anti-PY20-HRP or anti-Axl (Santa Cruz Biotechnology Inc.).

Protein profiling by reverse-phase protein array and Western blot analysis

Reverse-phase protein array (RPPA) studies were conducted and analyzed as described previously and in the Supplementary Information (23).

Animal studies

Mice were obtained from Charles River Laboratories. All animal studies were conducted under an institutionally approved protocol and were compliant with NIH guidelines with respect to animal care and welfare assurance. A detailed description of these methods is included in Supplementary Information.

Results

A 76-gene EMT signature classifies NSCLC cell lines into distinct epithelial and mesenchymal groups

Using a training set of 54 NSCLC cell lines profiled on Affymetrix U133A, U133B, and Plus2.0 arrays, we selected genes for the EMT gene expression signature based on 2 criteria aimed at increasing the robustness and potential applicability of the signature across different platforms. First, we identified genes whose mRNA expression levels were either positively or negatively correlated with the single best probe for at least 1 of 4 putative EMT markers—E-cadherin (CDH1), vimentin (VIM), N-cadherin (CDH2), and/or fibronectin 1 (FN1; see Supplementary Information for details). These markers were selected on the basis of their previously established role as markers of EMT in lung cancer, as well as other epithelial tumors (15, 16, 24). From that set, we then selected only those genes whose mRNA expression followed a bimodal distribution pattern across the cell lines [bimodal index (BI) >1.5; ref. 25]. By limiting the EMT signature to genes expressed among the cell lines at either relatively high or low levels, but not in between, we expected to increase the likelihood that the signature could separate patient tumors into distinct epithelial and mesenchymal groups.

Using that approach, we identified 76 unique genes (the EMT signature) whose expression levels were (i) correlated with known EMT markers and (ii) bimodally distributed (Fig. 1 and Supplementary Table S1). Most genes in the signature (63/76) were highly correlated (positively or negatively) with CDH1 and/or VIM. In contrast, only 10 of 76 signature genes were highly correlated with FN1 and 3 of 76 with CDH2. CDH2 itself did not meet the criterion for bimodal distribution across the NSCLC cells and therefore was not included in the final 76-gene signature.

We then analyzed expression of the EMT signature in the training set using hierarchical clustering and principal component analysis. Clustering of the NSCLC lines based on their expression of signature genes separated them into distinct epithelial (n = 34/54 cell lines) and mesenchymal (n = 20/54) groups (Fig. 1A). Cell lines in the mesenchymal group expressed higher levels of genes activated by EMT transcription factors ZEB1/2 and/or SNAIL1/2, including matrix metalloprotease-2 (MMP2; refs. 26, 27), vimentin (28–30), and ZEB1 itself (a target of SNAIL; ref. 31). Consistent with these findings, mesenchymal cell lines also expressed significantly higher levels of TGFβ1, an inducer of SNAIL. AXL, a receptor tyrosine kinase (RTK) that is overexpressed in breast and pancreatic cancers that have undergone EMT (32–34), was also more highly expressed in mesenchymal NSCLC cells. In contrast, epithelial lines had higher expression of genes repressed by ZEB1 and SNAIL, such as CDH1 (28, 29, 31, 35), the vesicle protein RAB25 (36), MUC1 (31), and claudins 4 (CLDN4) and 7 (CLDN7; ref. 37). The EGFR family member ERBB3 and SPINT2, a regulator of hepatocyte growth factor (HGF), were also expressed higher levels in epithelial lines. Similar to the clustering results, first principal component analysis using the EMT signature also separated the cell lines into epithelial and mesenchymal groups (Fig. 1B).

As expected, all 9 EGFR-mutant cell lines in the training set were classified by the EMT signature as epithelial. Included were H1975 and H820, which carry the acquired-resistance mutation T790M (Fig. 1A). In contrast, KRAS mutations were more common in mesenchymal lines, comprising 60% of that group (n = 12/20), as compared with 18% of the epithelial cell lines [n = 6/34; P = 0.014 by Fisher exact test; 95% confidence interval (CI), 0.42–0.76; OR 0.19; Fig. 1A]. There was also a trend toward more frequent loss of STK11 (LKB1) in mesenchymal cell lines (56%) versus in epithelial cells (27.6%) and more SMARCA4 mutations/deletions in mesenchymal cell lines (46% vs. 15%), although these did not reach statistical significance possibly due to the small sample size (P = 0.11 for both; Supplementary Table S2). In contrast, CDKN2A and CDKN2B loss were more frequent in epithelial cell lines.

NSCLC cell lines in the training set included several histologies, although the predominant subtype was adenocarcinoma. Among 35 cell lines with adenocarcinoma
histology, 29 (83%) had epithelial signatures, and only 8 (23%) had mesenchymal signatures. That is, the adenocarcinomas more commonly expressed an epithelial signature ($P = 0.0016$ by $\chi^2$ test). The 4 cell lines with squamous histology were evenly distributed between epithelial and mesenchymal subsets, whereas lines with neuroendocrine, large cell, or large cell neuroendocrine all had mesenchymal signatures.

**Validation on alternate array platforms and in an independent testing set**

Because a major goal of this study was to develop a platform-independent signature, we next tested the performance of the EMT signature on a different microarray platform. Illumina WGv2 microarray data were available for 52 of 54 NSCLC cell lines used in the original training set. As with the Affymetrix platform, distinct differences were observed in the expression of Illumina probes corresponding to the 76 EMT signature genes, as reflected by hierarchical clustering and first principal component analysis (Fig. 1B). Strikingly, classification as epithelial or mesenchymal agreed across the 2 platforms for 51 of 52 cell lines tested, with only HCC1359 switching between groups (Fig. 1B).

The EMT signature was then tested in an independent set of 39 NSCLC cell lines profiled on a third microarray platform (Illumina WGv3), the EMT signature separated cell lines into distinct epithelial (green) and mesenchymal (red) groups by hierarchical clustering and principal component analysis.

**Figure 1.** The EMT gene expression signature separates NSCLC cell lines into distinct epithelial-like and mesenchymal-like groups independent of microarray platform. A, Affymetrix probes corresponding to the EMT signature genes were clustered by 2-way hierarchical clustering using Pearson correlation distance between genes (rows), Euclidean distance between cell lines (columns), and the Ward’s linkage rule. NSCLC cell lines separated into distinct epithelial (green bar) and mesenchymal (red bar) groups at the first major branching of the dendrogram. Mutation status for EGFR and KRAS are indicated by the color bars above the heatmap (dark blue, mutated; light blue, wild-type; and white, unknown). EGFR mutations were seen only in the epithelial group. KRAS mutations were more common in the mesenchymal group and expressed higher levels of FN1 and FN1-associated genes. B, cell line classifications were concordant across platforms, with the exception of H1395, which switched from epithelial to mesenchymal group when arrayed on the Illumina WG v2 platform. First principal component analysis shows good separation of the epithelial and mesenchymal groups on both Affymetrix and Illumina platforms. C, characteristic differences in morphology are seen between lines characterized as epithelial or mesenchymal by the EMT signature. D, in an independent set of 39 NSCLC cell lines profiled on a third platform (Illumina WGv3), the EMT signature separated cell lines into distinct epithelial (green) and mesenchymal (red) groups by hierarchical clustering and principal component analysis.

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into distinct epithelial and mesenchymal groups by hierarchical clustering and principal component analysis (Fig. 1D). Among these cell lines, only 1 contained a known EGFR mutation, and it was classified as epithelial (HCC4011).

**Integrated proteomic analysis**

Next, we conducted an integrated proteomic analysis to identify major differences in protein expression between cell lines classified by the signature as epithelial ($n = 29$) or mesenchymal ($n = 20$). More than 200 total and phosphorylated proteins were measured by RPPA, a highly quantitative assay that measures protein levels from cell lysates printed in a serial dilution series. Unsupervised hierarchical clustering of the cell lines based on their expression of all proteins showed separation of the epithelial and mesenchymal cell lines ($P = 0.001$ by $\chi^2$ test), reflecting major differences in protein expression and signaling between epithelial and mesenchymal cells (Fig. 2A). We then conducted a supervised analysis comparing the expression of each protein between epithelial and mesenchymal cell lines by $t$ test. Not surprisingly, E-cadherin was the most significantly different between the groups ($P < 0.0001$) with mean E-cadherin levels 7.42-fold higher in cell lines designated as epithelial as compared with mesenchymal (Fig. 2B and C). The EMT first principal component was also highly correlated with E-cadherin protein expression ($r = -0.90$; corresponding $P < 0.0001$; 95% CI, 0.83–0.94; Fig. 2B). Similarly, the EMT first principal component calculated from the Illumina Training (Affymetrix) ($r = -0.90$, $P = 2.2 \times 10^{-16}$) and Testing (Illumina WG v3) ($r = -0.67$, $P = 0.009$) datasets showed strong correlation with the EMT signature.

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**Figure 2.** Integrated analysis of protein expression and the EMT signature. A, RPPA analysis reveals that cell lines dichotomize into epithelial and mesenchymal groups based on their overall proteomic signature. B, E-cadherin protein levels quantified by RPPA were strongly correlated with the EMT signature first principal component in the training and testing cell line sets. C, hierarchical clustering of proteins strongly associated with an epithelial or mesenchymal signature showed higher expression of EGFR pathway proteins and Rab25 in epithelial lines. D, Axl expression was significantly higher in a subset of mesenchymal cell lines at the mRNA (using 2 different probes) and protein levels.
platform data also correlated strongly with E-cadherin ($r = 0.91; P < 0.0001$; CI, 0.84–0.95). In the independent testing set of 39 NSCLC cell lines, protein data were available for only 14 lines. However, despite the small numbers, EMT first principal component again correlated well with E-cadherin protein ($r = 0.68; P = 0.007$). In contrast, correlation of E-cadherin protein with any single CDH1 probe was highly variable ($r = 0.37–0.86$), supporting the rationale for using of a signature rather than any single gene to assess EMT from mRNA expression data (Supplementary Fig. S3).

Other proteins expressed at higher levels in epithelial cells lines included phosphorylated proteins in the EGFR pathway (e.g., pEGFR and pHER2 and downstream targets pSrc and pSTAT3, 5, and 6; $P < 0.006$) and Rab25 ($P < 0.001$; Fig. 2C). Rab25 is a trafficking protein involved with EGFR recycling. It was selected as one of the 76 EMT signature genes based on its strong correlation with CDH1 expression ($r = 0.8$) and high bimodal index (BI = 2.88, top 3% of signature genes). Rab25 protein was 1.5-fold higher in the epithelial group and correlated with E-cadherin protein at an $r$ value of 0.67. Although Rab25 has been described as a marker of EMT in breast cancer (33), this is the first time it has been associated with EMT in NSCLC.

Relatively few genes in the EMT signature were expressed at higher levels in the mesenchymal group, relative to the epithelial group. Among these, AXL mRNA expression correlated strongly with vimentin ($r = 0.60$) and N-cadherin ($r = 0.54$). Because Axl has previously been described as an EMT marker in breast and pancreatic cancer (32–34) and is a potential therapeutic target in NSCLC (38), we further investigated its expression at the protein level by RPPA. Consistent with the mRNA data, Axl protein expression was expressed at low levels in most epithelial lines but highly expressed in a subset of mesenchymal cell lines ($P = 0.001$ by $t$ test, 3.5-fold higher in mesenchymal; Fig. 2C and D).

**The EMT gene signature predicts resistance to EGFR and PI3K inhibitors in vitro**

Previously, E-cadherin expression has been associated with greater benefit from erlotinib in patients with NSCLC (15–18). Therefore, we tested the association between our
EMT Predicts EGFR and PI3K Inhibitor Resistance in NSCLC

Epithelial and mesenchymal classification and cell line sensitivity to erlotinib. Seventy-eight NSCLC cell lines derived from treatment-naïve patients were analyzed. Mesenchymal cell lines were highly resistant to erlotinib, with mean IC\textsubscript{50} \text{mol/L} that were 3.7-fold higher in the mesenchymal cell lines (n = 34) as compared with epithelial cell lines (n = 44; P = 0.002 by t test; Fig. 3 and Supplementary Figs. S4 and S5). Forty-five cell lines (29 epithelial and 16 mesenchymal) treated with gefitinib also showed greater sensitivity in the epithelial-like group (P = 0.0003 by t test; 5.5-fold difference in mean IC\textsubscript{50} values; Fig. 3 and Supplementary Figs. S4 and S5). Although cell lines with EGFR-activating mutations were among the most sensitive to erlotinib, in the subset of 40 cell lines (22 epithelial, 18 mesenchymal) with wild-type EGFR and wild-type KRAS, the correlation between EMT signature and erlotinib response was maintained with significantly greater resistance in mesenchymal-like cell lines (P = 0.023, 2-fold higher mean IC\textsubscript{50} values). Importantly, the EMT signature was a better predictor of erlotinib response than were mRNA probe sets for individual genes, such as CDH1 or VIM (Supplementary Fig. S6).

NSCLC cell lines with mesenchymal signatures were also more resistant to drugs targeting the PI3K/Akt pathway, such as the selective pan phosphoinositide 3-kinase (PI3K) inhibitor GDC0941 (Fig. 3B; P = 0.068, 1.9-fold higher IC\textsubscript{50} and 8-amino-adenosine, an adenosine analog that inhibits Akt/mTOR signaling (P = 0.003, 1.7-fold higher IC\textsubscript{50}; refs. 39, 40). A trend toward greater resistance was also seen in mesenchymal cell lines treated with the Akt-selective inhibitor MK2206 (Fig. 3B; P = 0.18, 1.5-fold difference IC\textsubscript{50}), although these did not reach statistical significance. In contrast to EGFR and PI3K inhibitors, mesenchymal cells were not more resistant to other targeted agents, such as sorafenib (Fig. 3B; P = 0.33) or to commonly used cytotoxic chemotherapies, including pemetrexed, docetaxel, paclitaxel, and platinum-doublets (P ≥ 0.2). Instead, a trend toward greater relative sensitivity was seen in mesenchymal cells as compared with epithelial for cisplatin (P = 0.11), gemcitabine (P = 0.06), and vinorelbine (P = 0.12; Fig. 3B). The observation of greater sensitivity in mesenchymal lines to some chemotherapies suggests that EMT is not a marker of pan-resistance, but may identify subgroups of cancers more or less likely to respond to inhibition by drugs with distinct pathway targeting or mechanisms of action.

Mesenchymal cells are sensitive to Axl inhibition

Because mesenchymal cell lines expressed higher levels of the RTK Axl, we next tested the activity of the Axl inhibitor SGI-7079 in mesenchymal versus epithelial NSCLC lines. As determined by Western blot analysis, SGI-7079 effectively inhibited Axl activation in the presence of exogenous Gas6 ligand (Fig. 3C). In keeping with their higher target expression, mesenchymal cell lines were 1.3-fold more sensitive overall to Axl inhibition, although this did not reach statistical significance (P value 0.17 by t test; Supplementary Fig. S5). Next, we tested whether Axl inhibition could reverse mesenchymal cell resistance to EGFR inhibition, as Axl inhibition has been shown to reverse the mesenchymal phenotype in other epithelial cancers (34). Mesenchymal cell lines expressing Axl were treated with SGI-7079 alone, erlotinib alone, or SGI-7079 and erlotinib in combination. In cell lines expressing high levels of Axl, erlotinib alone had little or no effect on cell growth. Conversely, these same cell lines were highly sensitive to SGI-7079 alone. However, when combined, the addition of Axl inhibition (SGI-7079) to EGFR inhibition (erlotinib) resulted in a striking synergistic effect as shown by the Chou-Talalay combination index (CI < 1.0 at IC\textsubscript{50} for combination; range, 0.46–0.72) in 4 of 6 cell lines (Table 1; ref. 41). In the 2 cell lines with highest Axl protein expression (Calu-1 and H2882), the combination was only synergistic at higher concentrations of SGI-7079, possibly reflecting a need for higher dosing in cells with higher expression levels of the target.

Axl blockade inhibits the growth of mesenchymal NSCLC tumors

We next tested the efficacy of SGI-7079 in a mouse xenograft model of NSCLC using the mesenchymal NSCLC cell line A549. Once the tumor volumes reached 100 mm\textsuperscript{3}, animals were randomized into treatment groups. SGI-7079 inhibited tumor growth in a dose-dependent manner, and at the maximum dose, inhibited tumor growth by 67%, compared with control (ΔT/ΔC 33%; Fig. 4A). The combined inhibition of Axl (SGI-7079) plus EGFR (erlotinib) was significantly more effective than either drug alone (P < 0.001 vs. erlotinib and P < 0.001 vs. SGI-7079 by t test). Notably, SGI-7079 + erlotinib (25/100 mg/kg) reduced the tumor growth by 82% (ΔT/ΔC 18% T/C; Fig. 4B and Supplementary Table S3).

| Table 1. Axl inhibition reverses EGFR resistance in mesenchymal cell lines |
|----------------------------------|-----------------|--------|--------|--------|--------|--------|--------|
| A549 | Calu-1 | H157 | H1299 | H460 | H2882 |        |
| Erlotinib IC\textsubscript{50}, μmol/L | 13.54 | >100 | 48.50 | >100 | >100 | >100 |        |
| SGI-7079 IC\textsubscript{50}, μmol/L | 0.92 | 2.44 | 0.74 | 1.74 | 2.01 | 4.29 |        |
| CI @ IC\textsubscript{50} | 0.46 | >1.00\textsuperscript{a} | 0.67 | 0.72 | 0.57 | >1.00\textsuperscript{a} |        |
| Combination: erlotinib + SGI-7079 IC\textsubscript{50}, μmol/L | 1.07 + 0.35 | 13.86 + 4.47 | 1.46 + 0.47 | 3.76 + 1.21 | 3.53 + 1.14 | 16.44 + 5.30 |        |

\textsuperscript{a} For Calu-1 and H2882, the combination was antagonistic at IC\textsubscript{50} but synergistic at higher concentration of SGI-7079.
EMT signature in patients with relapsed or metastatic NSCLC

Finally, we tested the EMT signature in previously treated NSCLC patients with advanced NSCLC enrolled in the BATTLE-1 trial (42). Consistent with what we observed in the cell lines—and despite all patients having advanced, metastatic disease—a majority of patients (approximately 2 of 3) showed epithelial signatures (Fig. 5). However, unlike the cell lines, clinical samples with EGFR and KRAS mutations were distributed more evenly between the 2 groups, possibly because of prior therapy (e.g., previous EGFR inhibitors in EGFR-mutant patients).

To assess the potential value of the EMT signature as a predictive marker of erlotinib response in patient tumors, we analyzed the association between EMT signature expression and clinical outcome. Analysis was limited to the EGFR wild-type/KRAS wild-type patients, as there are no validated markers of response to EGFR inhibitors in that group (whereas EGFR mutation is associated with response and KRAS mutation with resistance (3, 4, 43)). Although the numbers were small (n = 20), EGFR/KRAS wild-type patients with disease control at 8 weeks (the primary study endpoint) showed a more epithelial-like signature as compared with those without disease control, with the difference of borderline significance (P = 0.05, by t test). Six of 7 BATTLE patients with 8-week disease control had an epithelial EMT signature (defined as the first principal component of the EMT signature below the median), whereas only 1 of 5 patients with mesenchymal EMT signatures (principal components above the median) had disease control.

In contrast, among the full group of 101 of 139 clinically evaluable patients (all treatment arms), expression of EMT signature genes was not prognostic of 8-week disease control (P = 0.40) or progression-free survival (PFS) in the overall group (all treatment arms), nor was it associated with differences in disease control in other individual treatment arms (e.g., sorafenib-treated patients). These results suggest that the EMT signature may be a marker of erlotinib activity in EGFR wild-type/KRAS wild-type...
tumors, and not simply a prognostic marker of a less aggressive tumor phenotype.

Because of our interest in Axl targeting as a potential therapeutic approach in NSCLC, we also looked specifically at differences in Axl levels between BATTLE patients with mesenchymal and epithelial signatures. Axl mRNA levels were higher in mesenchymal patient tumors ($P < 0.01$ by $t$ test). Even more strikingly, the Axl ligand GAS6 was markedly increased in these tumors ($P < 0.0001$). Together, these data indicate that inhibiting GAS6 signaling through its receptor, Axl, might have therapeutic benefit within the subset of patients with NSCLC with mesenchymal tumors.

**Discussion**

In these studies, we developed and tested a robust EMT gene expression signature capable of assessing the degree to which NSCLC cells have undergone EMT status of NSCLC cells and tumors from patients. An integrated analysis of mRNA expression and proteomic data confirmed significant correlation of the EMT signature with E-cadherin protein levels. In addition, higher expression of activated EGFR pathway proteins were observed in epithelial cell lines, whereas higher protein expression of the RTK Axl (a signature gene associated with EMT in other epithelial cancers) was seen in mesenchymal lines. Finally, we have shown differences in drug response between epithelial and mesenchymal cancers. Cell lines and/or patients classified by the EMT signature as mesenchymal were more resistant to drugs inhibiting EGFR or the PI3K/Akt pathway but were more sensitive to certain chemotherapies and to the Axl inhibitor SGI-7079. Moreover, Axl inhibition reversed erlotinib resistance in a subset of mesenchymal cell lines, and in a mesenchymal xenograft model of NSCLC, combine blockade of Axl and EGFR was more effective at controlling tumor growth than inhibition of either single target.

A common limitation of gene expression signatures is their platform-dependence, resulting from the derivation of the signature on a specific microarray platform. One particular strength of the study presented here was the use of microarray data from 2 independent mRNA profiling platforms, Affymetrix and Illumina, for the initial development of the signature. In the training cell lines, this strategy allowed us to identify the most robust probe set for 4 EMT markers, which were then used to derive the 76-gene signature. The goal of selecting the best cross-platform probe sets was to increase the likelihood that the signature could be applied to samples profiled on different types of mRNA arrays and with emerging technologies such as RNA seq. The success of that approach was shown in the independent testing sets, which included cell lines profiled on Illumina v2 and v3 arrays and patient tumors profiled on Affymetrix Human ST 1.0 arrays.

We believe that the use of cross-validated, robust probe sets to derive the EMT signature also led to a signature enriched for genes with biologic relevance in EMT. Interestingly, the EMT first principal component correlated better with E-cadherin protein level than did even the best CDH1 RNA probe set. That observation supports our hypothesis that a signature incorporating several relevant markers is likely to be superior to any single marker for assessing complex biologic processes such as EMT. In addition, higher expression of 2 of the signature genes, Rab25 in epithelial lines and Axl in mesenchymal lines, was confirmed at the protein level. Those 2 genes are established EMT markers in other cancer types (32–34). However, to our knowledge, this is the first time they have been shown to be markers of EMT in NSCLC. This discovery has potential for therapeutic implications, particularly for mesenchymal NSCLC, given the rapid development of a number of Axl inhibitors that are currently in preclinical or clinical testing.

In addition, the similarities we observed between our EMT signature and EMT markers in other tumor types suggests that our EMT signature may also be applicable in other epithelial tumors, such as breast, colon, or head and neck.

Another important result of this study was that the EMT score predicted erlotinib sensitivity in both EGFR-mutant and EGFR wild-type NSCLC. Although the signature was derived in cell lines, it was validated in clinical samples in which it successfully identified EGFR wild-type patients who benefited from treatment with EGFR tyrosine kinase inhibitors (TKI). Currently, activating mutations of EGFR are the only validated biomarkers of response to EGFR TKIs in NSCLC. However, such mutations occur in only a minority of patients with NSCLC and cannot account for the subset of EGFR wild-type patients who have shown benefit from EGFR TKIs in several clinical trials (8–10). Therefore, our demonstration of greater clinical benefit from erlotinib in EGFR wild-type patients with tumors showing an epithelial phenotype from the BATTLE study suggests that EMT may be a clinically relevant predictive marker for patients lacking mutations known to be associated with drug sensitivity (EGFR mutation) or resistance (KRAS mutation), meriting further investigation. Consistent with these findings, we observed significantly greater EGFR pathway activation in epithelial cell lines (both EGFR mutant and wild-type) relative to mesenchymal lines in our protein analysis. Although the mechanism of activation in EGFR wild-type patients is not yet known, the greater frequency of EGFR pathway activation in epithelial-like NSCLC probably accounts for the trend toward greater sensitivity to erlotinib in the epithelial group.

Another major observation in this study was the significantly higher frequency of resistance to PI3K/AKT pathways inhibitors in mesenchymal cell lines. This was a class effect observed across 4 different drugs targeting this pathway. Coupled with the data from EGFR inhibitors, this suggests that mesenchymal cells may have decreased dependence on signaling from EGFR family of RTKs and downstream signaling pathways. Several PI3K/AKT inhibitors are in clinical development for NSCLC. Therefore, the identification of a negative predictive signature that identifies a group of patients unlikely to benefit from the drug has immediate clinical implications. To test this possibility, the EMT signature will be assessed in the ongoing BATTLE-2 study, which includes treatment arms with
erlotinib as well as 2 combinations with the AKT inhibitor MK2206. If the association between EMT and PI3K/AKT inhibitor resistance is confirmed in patients with NSCLC, it may also have relevance for other epithelial tumors such as breast cancer in which these drugs are also being investigated.

We also investigated whether EMT status predicted responsiveness to standard chemotherapy agents used for NSCLC and other targeted agents. There was no association between EMT status and drug response for sorafenib and most chemotherapeutics, indicating that the mesenchymal phenotype is not associated with pan-drug resistance. Gemcitabine and vinorelbine showed a mesenchymal-bias with more than 2-fold lower median IC_{50} in mesenchymal compared with epithelial cells. These findings indicate that certain chemotherapeutics or targeted agents may have greater activity in mesenchymal-type tumors, and provides a starting point for developing combination regimens tailored for mesenchymal-type tumors.

Finally, Axl was identified in this study as a novel marker of EMT in NSCLC and represents a potential new therapeutic target for NSCLC. In the NSCLC cell lines, Axl inhibition showed greater activity in the mesenchymal group. Moreover, inhibition of Axl—which has been shown in other cancers models to decrease invasion, migration, and other behaviors characteristic of cancers that have undergone EMT (34)—sensitized otherwise-resistant mesenchymal NSCLC lines to the EGFR inhibitor erlotinib in vitro and in vivo in a xenograft model of mesenchymal NSCLC. This suggests that in addition to single agent activity, Axl inhibition may have a role in reversing EGFR inhibitor resistance. Importantly, following the submission of this article, Zhang and colleagues (44) reported increased activation of Axl and evidence for EMT using in vitro and in vivo EGFR-mutant lung cancer models with acquired resistance to erlotinib. In these models, Axl inhibition restored sensitivity to erlotinib. Taken together, these results support further investigation of combined Axl and EGFR inhibition in mesenchymal tumors and in acquired EGFR inhibitor resistance, which is in some cases associated with EMT.

In conclusion, the present study establishes a robust, cross-platform EMT signature capable of classifying NSCLC cell lines and patient tumors as epithelial-like or mesenchymal-like. The ability to classify such tumors accurately, independent of microarray platform, will assist with future investigations into the growing field of EMT. Furthermore, the mesenchymal phenotype identified here seems to be a negative predictor of response to drugs that target EGFR or the PI3K/Akt pathway. Finally, this analysis identifies the tyrosine kinase Axl as a novel EMT marker and potential mesenchymal-associated target for therapy for NSCLC.

Disclosure of Potential Conflicts of Interest

D.J. Bears is employed by Tolero Pharmaceuticals as Founder Share Holder and has ownership interest (including patents) in Tolero Pharmaceuticals. S.L. Warner has ownership interest (including patents) in Tolero Pharmaceuticals. J.M. Foukis is employed by Astex Pharmaceuticals, Inc. as Sr. Manager, Discovery Biology and has ownership interest (including patents) in Astex Pharmaceuticals, Inc. G.R. Blumschein has a research grant from Bayer and is a consultant/advisory board member of Bayer. G.B. Mills has commercial research grants from AstraZeneca, Celgene, CeMines, Exelixis/Sanofi Aventis, GSK, LPATH, Roche, SDI, and Wyeth/Pfizer/Pfarma; has ownership interest (including patents) in Carina Pharmaceuticals, PTV Ventures, and Spindle Top Ventures; and is a consultant/advisory board member of Arcis Biotechnologies, Asuragen, Tau Therapeutics, Aushon, Catena Pharmaceuticals, Daiichi Pharmaceuticals, Targeted Molecular Diagnostics LLC, Foundation Medicine, Han Pharma, Korea Foundation, and Novartis. J.D. Minna has other commercial research support from Geron Pharmaceuticals and other (e.g., expert testimony) from Amgen. J.V. Heymach has honoraria from Speakers Bureau of Genentech. No potential conflicts of interest were disclosed by the other authors.

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Grant Support: L.A. Byers, J.D. Minna, J.V. Heymach

Acknowledgments

The authors thank Emily C. Brantley, Ph.D. for editorial assistance.

Grant Support

This work was supported by The University of Texas Southwestern Medical Center and The University of Texas MD Anderson Cancer Center Lung SPORE grant 5 P50 CA070907; DoD PROSPECT grant W81XWH-07-1-0306; DoD BATTLE grant W81XWH-06-1-0303 03; R01 CA166844-01; CCSG grant 5 P30 CA016672; Kleberg Center for Molecular Markers; Champions Fund for Bioinformatics in Personalized Cancer Therapy, 1 I324 CA143883; and the E.L. Wiegand Foundation. L.A. Byers is supported in part by the AACR-AstraZeneca-Peace Cancer Foundation Fellowship for Translational Lung Cancer Research and the Barbara Ratliff Advanced Fellowship Program.

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Received May 15, 2012; revised September 25, 2012; accepted October 9, 2012; published OnlineFirst October 22, 2012.

References

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