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# Epithelial to Mesenchymal Transition in an *Epidermal Growth Factor Receptor*-Mutant Lung Cancer Cell Line with Acquired Resistance to Erlotinib

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**Introduction:** Mesenchymal status is related to "inherent resistance" to gefitinib or erlotinib in non-small cell lung cancer without *epidermal growth factor receptor* (*EGFR*) mutations. In addition, a recent report showed that the epithelial to mesenchymal transition (EMT) plays a role in acquired resistance to gefitinib in A549 cells, which harbor a *KRAS* mutation. However, recent clinical studies revealed that gefitinib or erlotinib are highly effective in the treatment of non-small cell lung cancer with *EGFR* mutations.

**Methods:** We developed resistant cells (HCC4006ER) from erlotinib-sensitive HCC4006 cells harboring an *EGFR* deletion mutation by chronic exposure to increasing concentrations of erlotinib. Acquired resistance mechanisms of HCC4006ER cells were analyzed.

**Results:** Neither known resistance mechanisms nor novel molecules that may confer erlotinib resistance were identified using candidate or comprehensive analyses. In addition, HCC4006ER cells lost dependency for EGFR. However, we found that HCC4006ER cells acquired a mesenchymal phenotype and exhibited down-regulation of E-cadherin expression ($2.7 \times 10^{-3}$ times compared with parental cells). We also found that the histone deacetylase inhibitor, MS-275, restored E-cadherin expression and moderate sensitivity to erlotinib in HCC4006ER cells, on the other hand, transforming growth factor beta, an inducer of EMT, led to moderate erlotinib resistance in HCC4006 parental cells.

**Conclusions:** This is the first report of a relationship between EMT and erlotinib acquired resistance in an erlotinib sensitive *EGFR*-mutant lung cancer cell line. Our results indicate that it would be important to consider the influence of EMT in the development of treatments against acquired resistance to gefitinib or erlotinib.

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Somatic mutations in the *epidermal growth factor receptor* (*EGFR*) gene are associated with significant clinical responses to orally available EGFR tyrosine kinase inhibitors (TKIs) in patients with non-small cell lung cancer (NSCLC). Although approximately 70 to 80% of the patients harboring *EGFR* mutations respond to these drugs,[1–4] acquired resistance develops in almost all patients, which limits the improvement of the outcomes of patients. *EGFR* secondary mutations that cause drug-binding deficiency or activation of alternative survival pathways have been reported as part of the molecular mechanisms underlying these acquired resistances.[5–10]

Mesenchymal status is related with "inherent resistance" to gefitinib or erlotinib in in vitro models,[11–14] in xenograft models,[11,14] and in clinical cases[13] of NSCLC. These reports provide a reason why some NSCLC cells without *EGFR* mutations have moderate sensitivity to gefitinib or erlotinib, whereas *EGFR*-mutant NSCLC cells virtually exhibit epithelial phenotypes.[15]

In this study, we developed resistant cells from the erlotinib-sensitive HCC4006 cell line, which harbors an *EGFR* mutation. Analyses of acquired resistance mechanisms led to the identification of epithelial to mesenchymal transition (EMT) features in cells exhibiting acquired resistance. This is the first report indicating the involvement of EMT in acquired resistance to EGFR-TKIs in erlotinib sensitive *EGFR*-mutant lung cancers.

## MATERIALS AND METHODS

### Cell Lines and Reagents

The *EGFR* mutant human lung adenocarcinoma cell line HCC4006 (del L747_E749, A750P) was the kind gift of Dr. Adi F. Gazdar. HCC4006 cells were cultured in RPMI1640 medium supplemented with 5% fetal bovine serum (FBS) and 1× antibiotic–antimycotic solution (Invitrogen, Carlsbad, CA) at 37°C in a humidified incubator with

5% $CO_2$. The identity of the HCC4006 cells was confirmed at the beginning of this study by the identification of the rare type of *EGFR* deletion mutation in this cell line.

Erlotinib was kindly provided by Hoffmann-La Roche, Inc. (Nutley, NJ). The selective MET inhibitor PHA-665,752 and the selective transforming growth factor beta (TGFbeta) receptor I inhibitor SD208 were purchased from Tocris Bioscience (Ellisville, MO) and Sigma-Aldrich Co. (St. Louis, MO), respectively. The allosteric MEK inhibitor PD0325901 and AKT 1/2 kinase inhibitor were purchased from Wako (Osaka, Japan). The histone deacetylase (HDAC) inhibitor MS-275 was purchased from Selleck Chemicals (Houston, TX). Human TGFbeta 1 was purchased from R&D Systems (Minneapolis, MN).

## Generation of In Vitro Erlotinib-Resistant HCC4006 Cells

Erlotinib-resistant HCC4006 (HCC4006ER) cells were developed by chronic, repeated exposure to increasing concentrations of erlotinib, from 20 nM to 2 $\mu$M, as described previously.[7] The concentration of erlotinib was increased stepwise when the cells resumed proliferation, similar to the pattern in untreated parental cells. Two clones (HCC4006ER4 and ER5) were isolated by limiting dilution.

## Cell Proliferation Assay

Cell proliferation was measured using TetraColor ONE (Seikagaku-kogyo, Tokyo, Japan), according to the manufacturer's instructions. Briefly, tumor cells ($3 \times 10^3$) were plated into each well of 96-well flat-bottomed plates and were grown in RPMI1640 containing 5% FBS. Twenty-four hours later, dimethyl sulfoxide (DMSO), erlotinib, PHA-665,752, PD0325901, AKT 1/2 kinase inhibitor, SD208, or a combination of these drugs was added to the indicated drug concentration, and cells were incubated for an additional 72 hours. MS-275 was added at the initial cell plating. A colorimetric assay was performed after addition of 10 $\mu$l TetraColor ONE to each well, and the plates were incubated at 37°C for 1 hour. Absorbance at 450 nm was read using a multiplate reader. Percent growth was determined relative to DMSO-treated controls.

## Preparation of DNA and RNA

Genomic DNA was extracted using a FastPure DNA Kit (Takara Bio, Otsu, Japan), according to the manufacturer's protocol. Total RNA was prepared using a mirVana miRNA Isolation Kit (Qiagen, Valencia, CA), according to the manufacturer's protocol. Random-primed, first-strand complementary DNA was synthesized from total RNA using Superscript II (Invitrogen), according to the manufacturer's instructions.

## Mutation Analysis

Mutation analysis of exons 18 to 21 of the *EGFR* gene and exons 1 to 2 of the *KRAS* gene was performed by direct sequencing after one-step reverse transcription polymerase chain reaction (RT-PCR) from total RNA using the Qiagen OneStep Reverse Transcription PCR Kit (Qiagen), as reported previously.[16]

## Gene Copy Number Analysis

The number of copies of the *MET* gene relative to a *LINE-1* repetitive element was measured using quantitative real-time PCR using the SYBR Green Method (Power SYBR Green PCR Master Mix; Qiagen) on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) as described previously.[7,17] Normal genomic DNA was used as a standard sample.

## Phospho-Receptor Tyrosine Kinase Array Analysis

A Human Phospho-RTK Array Kit (R&D Systems) was used to measure the relative level of tyrosine phosphorylation of 42 distinct receptor tyrosine kinases (RTKs). HCC4006 and HCC4006ER5 cells were cultured in RPMI1640 containing 5% FBS until subconfluency. The media was changed to 5% FBS containing DMSO or 2 $\mu$M erlotinib, respectively, for 24 hours, and the cells were lysed with NP-40 lysis buffer, according to the manufacturer's protocol. The arrays were blocked with blocking buffer and incubated with 450 $\mu$g of cell lysate overnight at 4°C. The arrays were washed, incubated with a horseradish-peroxidase-conjugated phosphotyrosine detection antibody, treated with ECL solution (GE Healthcare, Buckinghamshire, UK), and exposed to film.

## Phospho-Kinase Array Analysis

A Human Phospho-kinase Array Kit (R&D Systems) was used to measure the relative level of phosphorylation of 46 distinct intracellular kinases. HCC4006 and HCC4006ER5 cells were cultured in RPMI1640 containing 5% FBS until subconfluency. The media was changed to 5% FBS containing 2 $\mu$M erlotinib for 8 hours, and the cells were lysed using the lysis buffer provided. The arrays were blocked with blocking buffer and incubated with 450 $\mu$g of cell lysate overnight at 4°C. The arrays were washed and incubated with a biotinylated antibody for 2 hours. The arrays were washed again, incubated with a streptavidin–horseradish-peroxidase-conjugated detection antibody, treated with ECL solution, and exposed to film.

## Antibodies and Western Blot Analysis

Antiphospho-EGFR, anti-EGFR, antiphospho-insulin-like growth factor I receptor (IGF-IR), anti-IGF-IR, antiphospho-Akt, anti-Akt, antiphospho-extracellular signal-regulated kinase (ERK), anti-ERK, anti-E-cadherin, antivimentin, anti-SMAD2/3, antiphospho-SMAD2, and antiphospho-SMAD3 antibodies were purchased from Cell Signaling Technology (Beverly, MA). The antibeta actin antibody was purchased from Sigma (St Louis, MO). Anti-SMAD4, anti-TGFbeta-receptor I, and anti-TGFbeta-receptor II antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

The preparation of total cell lysates and immunoblotting was performed as described previously. Briefly, cells were cultured until subconfluency, and media was changed to 5% FBS containing DMSO or the indicated concentration of the various drugs. After 8 hours, cells were rinsed with phosphate-buffered saline, lysed in sodium dodecyl sulfate sample buffer, and homogenized. Total cell lysate (30 $\mu$g) was subjected to sodium dodecyl sulfate polyacrylamide gel

electrophoresis and transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore, Bedford, MA). After blocking with 5% nonfat dry milk, membranes were incubated with primary antibodies, washed with phosphate-buffered saline, reacted with secondary antibodies, treated with ECL solution, and exposed to film.

## EGFR siRNA Transfection

HCC4006 and HCC4006ER5 cells were reverse transfected using scrambled siRNA or one of two kinds of specific, validated siRNAs for EGFR (Applied Biosystems) using the Lipofectamine RNAiMAX transfection reagent (Invitrogen), according to the manufacturer's instructions.

## Microarray Analysis

Agilent human whole-genome microarray analyses were performed to assess differences in gene expression between HCC4006 and HCC4006ER5 cells. Each of the cell lines was cultured in RPMI1640 containing 5% FBS until subconfluency. The media was changed to 5% FBS containing 2 $\mu$M erlotinib for 8 hours, and total RNA was isolated. RNA quality was confirmed using the Agilent 2000 Bioanalyzer, and 200 ng of each total RNA was used for probe generation and hybridization. HCC4006ER5 cells (labeled with cyanine-5) were characterized by comparison with HCC4006 (labeled with cyanine-3) cells on a single slide. The microarray slide was read using an Agilent Scanner, and the Agilent Feature Extraction software was used to calculate gene expression values. We performed a gene-set enrichment analysis (GSEA) to identify gene-signature-based differences.[18]

## Quantitative Real-Time RT-PCR

Quantitative real-time RT-PCR was performed on first-strand complementary DNA using TaqMan probes and the TaqMan Universal PCR Master Mix (Applied Biosystems). TaqMan probes for *EGFR, human EGF receptor 2 (HER2), HER3, HER4,* and *phosphatase and tensin homolog (PTEN)* were purchased from Applied Biosystems, and the amplification was performed on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems), according to the manufacturer's instructions. Quantification was performed in triplicate, and the level of expression of 18S rRNA was used as an internal control.

# RESULTS

## In Vitro Erlotinib-Resistant HCC4006 Cells Did Not Harbor Known TKI-Resistance Mechanisms

First, we generated two HCC4006 cell clones that were resistant to erlotinib (designated as HCC4006ER4 and ER5) by growing cells in increasing concentrations of erlotinib (to a final concentration of 2 $\mu$M) for up to 4 months in vitro, as

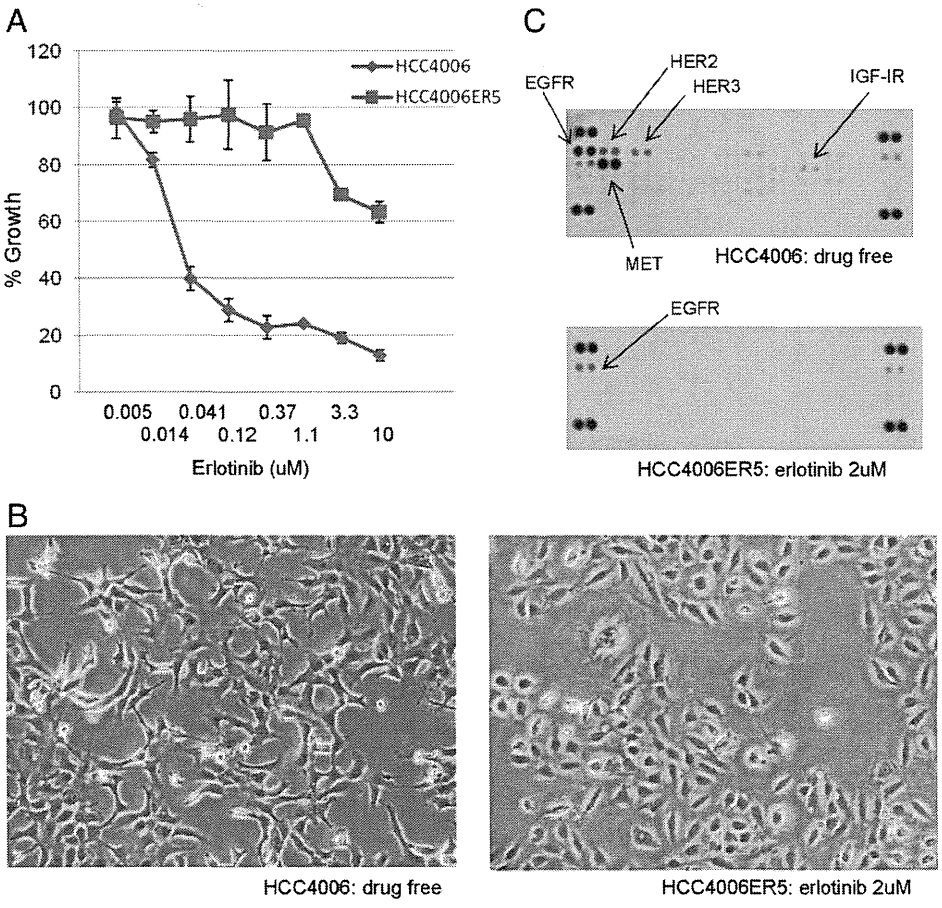


**FIGURE 1.** Establishment of HCC4006ER cells by chronic, repeated exposure to increasing concentrations of erlotinib. *A,* HCC4006ER cells were resistant to erlotinib. HCC4006 or HCC4006ER5 cells were incubated for 24 hours and an additional 72 hours with the indicated concentrations of erlotinib, and cell growth was assessed. *B,* Analysis of activated receptor tyrosine kinases (RTKs) using a Human Phospho-RTK Array Kit. Whole-cell extracts from HCC4006 and HCC4006ER5 cells exposed for 24 hours to the indicated drugs were incubated with the arrays, and phosphorylation status was determined. Each RTK was spotted in duplicate, and the pairs of dots in each corner are positive controls. *C,* Morphological differences observed between HCC4006 and HCC4006ER5 cells.

described previously.[7,19] HCC4006ER cells were more than 500 times more resistant to erlotinib (and to gefitinib, data not shown) compared with the parental HCC4006 cells (Figure 1*A*). We found remarkable differences between HCC4006ER cells and parental HCC4006 cells regarding their appearance. The morphological changes observed in the resistant cells included loss of intercellular connection and loss of polarity (Figure 1*B*, left and right panels).

First, we extracted RNA and DNA from HCC4006ER4 and ER5 cells and performed analyses of mutation, amplification, or gene expression for the various candidate genes. Mutation analyses revealed that neither secondary mutations in exons 18 to 21 of the *EGFR* gene (including T790M) nor mutations in exons 1 to 2 of the *KRAS* gene were detected in the resistant cells, although the resistant cells preserved *EGFR* deletion mutation in exon 19. The *MET* gene copy number in the resistant cells, as assessed using quantitative real-time PCR, was identical to that observed in the parental cells and to that of normal DNA. The expression of the *PTEN* gene in the resistant cells, as assessed using quantitative real-time RT-PCR, was also identical to that found in the parental cells. In addition, the MET inhibitor PHA-665,752 did not restore erlotinib sensitivity in resistant cells.

Therefore, we analyzed the activation of RTKs comprehensively using a phospho-RTK array. Although HCC4006 cells exhibited activation of HER family members and MET in the absence of erlotinib (Figure 1*C*, top), phospho-RTK array analysis of HCC4006ER5 cells showed a remarkable decrease in the phosphorylation of EGFR, without activation of any other RTKs (Figure 1*C*, bottom).

In addition, we analyzed the expression of EGFR and IGF-IR, the activation of which reportedly causes acquired resistance to gefitinib in A431 cells,[8] using immunoblot analysis (Figure 2*A*). The basal EGFR activity in HCC4006ER5 cells was lower, compared with that observed in parental cells. In addition, erlotinib inhibited the phosphorylation of EGFR effectively in both cell lines. In contrast, the level of phosphorylation of IGF-IR was slightly increased in HCC4006 parental cells in the presence of 1 μM erlotinib; however, phospho-IGF-IR was not detected in HCC4006ER5 cells, regardless of the concentration of erlotinib.

## HCC4006ER5 Cells Lost Dependency for EGFR

Next, we analyzed whether HCC4006ER5 cells retained dependency for EGFR using two kinds of validated siRNAs (Figure 2*C*). Although siRNA-mediated knockdown

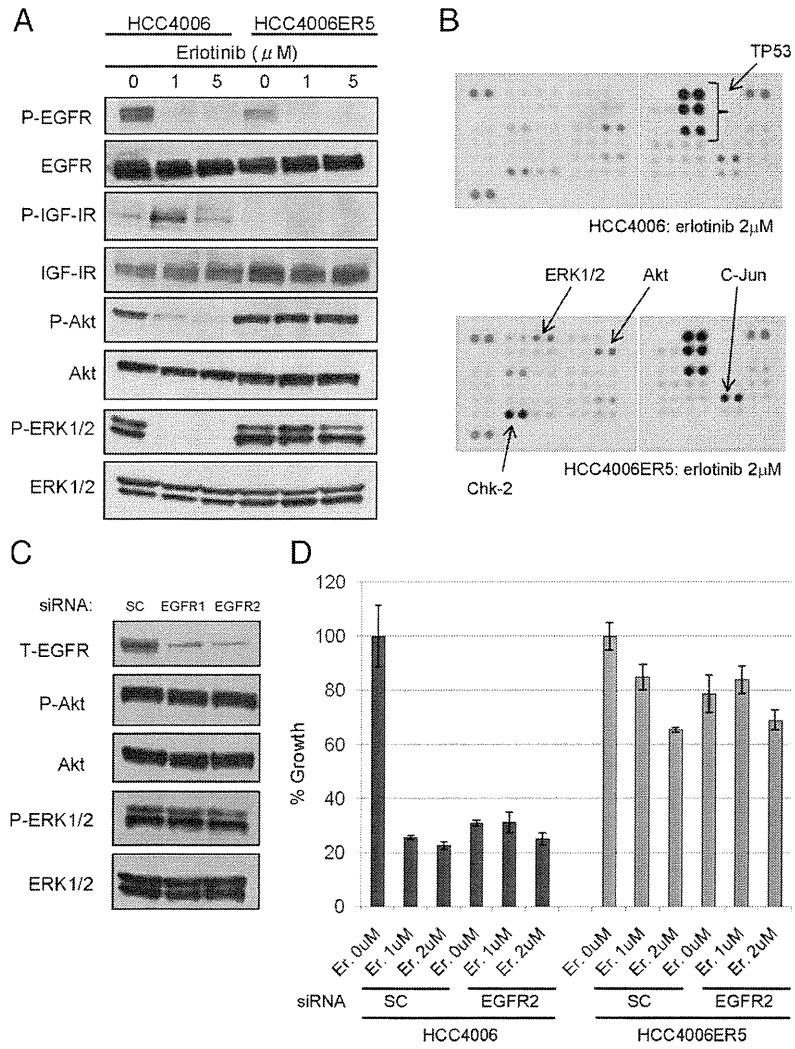


**FIGURE 2.** Analyses of intracellular signaling pathways and epidermal growth factor receptor (EGFR) dependency in HCC4006 and HCC4006ER5 cells. *A*, Cells were incubated for 8 hours with the indicated concentrations of erlotinib, and changes in EGFR- or insulin-like growth factor I receptor (IGF-IR)-related signals were analyzed using Western blotting. *B*, Analysis of activated intracellular kinases using the Human Phospho-kinase Array Kit. Whole-cell extracts from HCC4006 and HCC4006ER5 cells exposed to 2 μM erlotinib for 8 hours were incubated with the arrays, and phosphorylation status was determined. Each kinase was spotted in duplicate and the pairs of dots in each corner (with the exception of the right-lower corner) are positive controls. *C*, Confirmation of EGFR knockdown using two different siRNAs. HCC4006ER5 cells were reverse-transfected with the indicated siRNA, incubated for 72 hours, and EGFR-related signals were analyzed using Western blotting. *D*, HCC4006ER cells were EGFR independent. HCC4006 or HCC4006ER5 cells were reverse-transfected with control siRNA or EGFR2 siRNA, incubated for 24 hours and an additional 48 hours with the indicated concentrations of erlotinib (Er.), and cell growth was assessed.

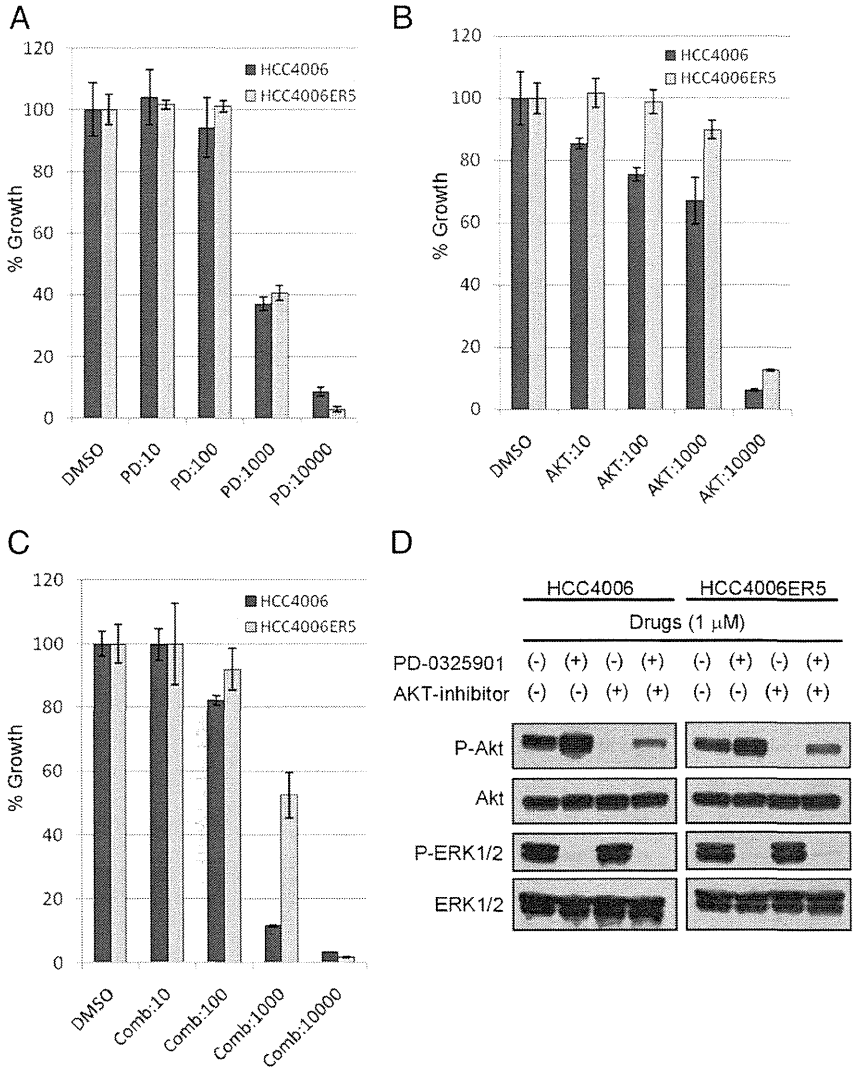


**FIGURE 3.** Growth inhibitory effects of the MEK inhibitor and/or the Akt inhibitor. *A–C*, HCC4006 or HCC4006ER5 cells were incubated for 24 hours and an additional 72 hours with indicated concentrations (nM) of the MEK inhibitor PD0325901 (PD; *A*), with the Akt inhibitor AKT 1/2 Kinase Inhibitor (AKT; *B*), or with the combination (Comb) of both drugs (*C*), and cell growth was assessed. *D*, Cells were incubated for 8 hours with 2 μM of the indicated drug(s), and activations of Akt or ERK were analyzed using Western blotting.

of EGFR suppressed the survival of the parental cells effectively to a level that was similar to that obtained after erlotinib addition, knockdown of EGFR did not affect cell viability in HCC4006ER5 cells irrespective of the presence of erlotinib (Figure 2*D*).

## HCC4006ER5 Cells Maintained the Activity of ERK and Akt in the Presence of Erlotinib

Next, we analyzed the activation of RTK downstream molecules using immunoblot analysis (Figure 2*A*) and identified that the activity of ERK and Akt was maintained in HCC4006ER5 cells, but not in parental cells, in the presence of erlotinib. This was consistent with the result that siRNA-mediated EGFR knockdown did not affect the phosphorylation of ERK and Akt in HCC4006ER5 cells (Figure 2*C*). In addition, we analyzed the differences in intracellular kinase activation comprehensively using a phospho-kinase array; however, we just confirmed the phosphorylation of ERK and Akt in HCC4006ER5 cells (Figure 2*B*).

Therefore, we examined whether the ERK inhibitor (PD0325901), the Akt inhibitor (AKT 1/2 Kinase Inhibitor), or the combination of both drugs can suppress the growth of HCC4006ER5 cells. HCC4006 parental cells and HCC4006ER5 cells both showed moderate sensitivity to PD0325901 (Figure 3*A*); however, HCC4006ER5 cells were more resistant to Akt inhibition compared with parental cells (Figure 3*B*). Combination of 1 μM of each drugs effectively inhibited the growth of HCC4006 parental cells but not of the resistant cells (Figure 3*C*), although both drugs worked well in both cell lines (Figure 3*D*).

## Gene-Expression Profiling for the Identification of Molecules and Pathways Involved in Acquired Resistance to Erlotinib in HCC4006ER5 Cells

We performed a DNA microarray analysis to identify genes that are overexpressed or suppressed in HCC4006ER5 cells compared with parental cells. The evaluation of the expression levels of RTKs led to the identification of a decrease in the expression of HER family members in HCC4006ER5 cells (Figure 4*A*), which was confirmed using

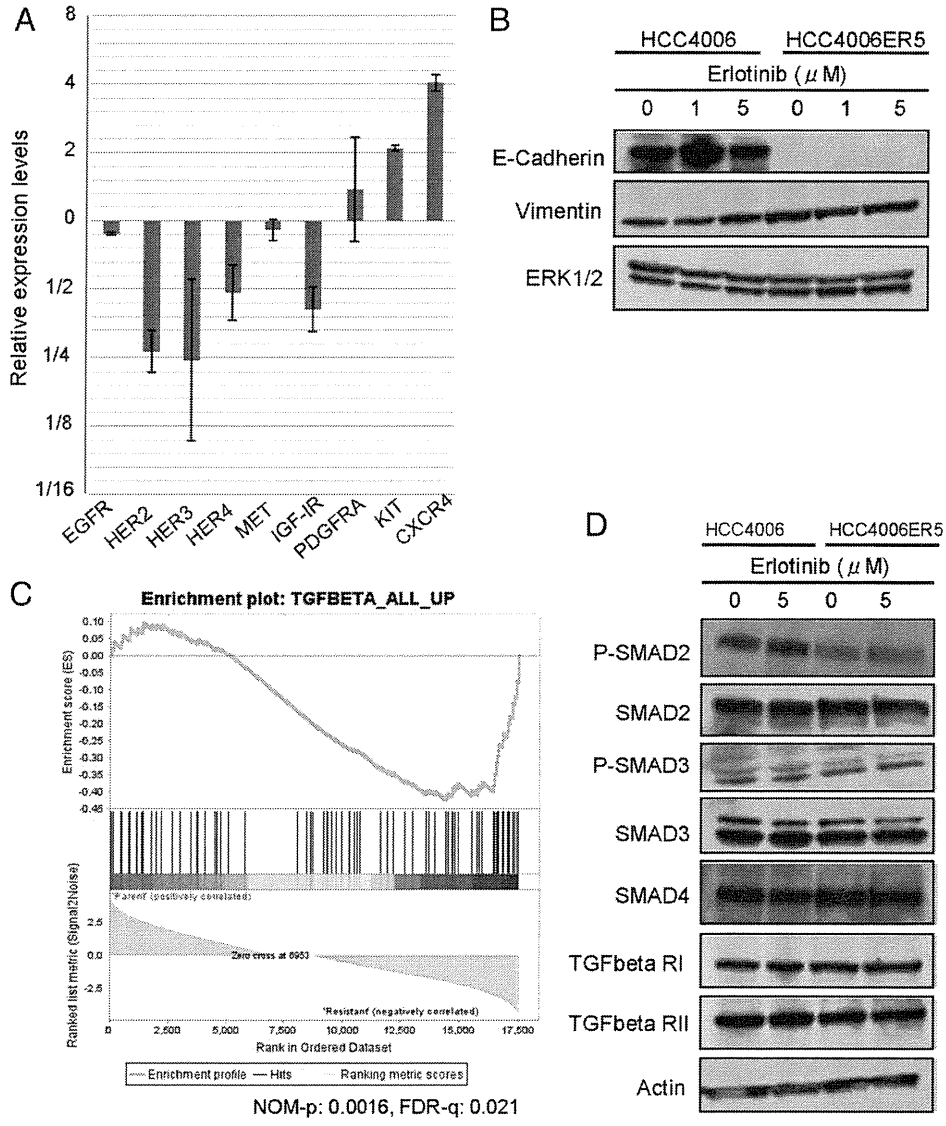


**FIGURE 4.** Epithelial to mesenchymal transition (EMT) and elevation of transforming growth factor beta (TGFbeta)-related signaling in HCC4006ER5 cells. *A*, Relative gene expression levels of several receptor tyrosine kinases (RTKs) determined using a gene expression array. HER family members were completely down-regulated in HCC4006ER5 cells. *B*, Loss of E-cadherin expression and up-regulation of vimentin in HCC4006ER5 cells, as assessed using Western blotting. *C*, Elevation of TGFbeta-related signaling in HCC4006ER5 cells, as assessed using gene-set enrichment analysis (GSEA). *D*, Downstream signaling of TGFbeta in HCC4006 and HCC4006ER5 cells was identified using Western blotting.

quantitative real-time RT-PCR. In contrast, we observed an increase in the expression of several RTKs in HCC4006ER5 cells (e.g., *fibroblast growth factor receptor 1, EPH receptor A2, platelet-derived growth factor receptor, alpha polypeptide [PDGFRA], PDGFRB, KIT, and chemokine [C-X-C motif] receptor 4 [CXCR4]*); however, most of these molecules were included in the phospho-RTK array analysis described earlier. We performed siRNA-mediated knockdown of CXCR4, which was not included in the phospho-RTK array; however, the suppression of CXCR4 did not restore erlotinib sensitivity in HCC4006ER5 cells (data not shown). We also found 16 times increase in the expression of *ATP-binding cassette, subfamily B (MDR/TAP), member 1 (ABCB1)* in HCC4006ER5 cells. However, simple involvement of multidrug resistance pumps for acquired resistance mechanism in HCC4006ER cells would not be possible, because HCC4006ER cells lost EGFR dependency (Figures 2*C, D*) and erlotinib effectively inhibited the phosphorylation of EGFR in HCC4006ER cells (Figure 2*A*).

The most notable gene expression feature observed in HCC4006ER5 cells was the down-regulation ($2.7 \times 10^{-3}$ times) of *E-cadherin*, which is a marker of the epithelial phenotype. Conversely, markers of the mesenchymal phenotype were up-regulated in HCC4006ER5 cells: *vimentin*, 2.2 times; *fibronectin*, 3.0 times; and *zinc finger E-box binding homeobox 1(ZEB1)*, 4.4 times. Loss of E-cadherin expression was confirmed using immunoblotting analysis (Figure 4*B*). These expression features and morphological changes, the loss of intercellular connection, and the loss of polarity were consistent with the presence of EMT in HCC4006ER5 cells.

Microarray data were ranked according to the ratio of the levels of expression detected in HCC4006ER5 cells to that observed in HCC4006 cells. Subsequently, we performed GSEA, which is a gene-expression profiling analytical method that was developed recently.[18] The results showed that gene sets involved in the TGFbeta signaling pathway were up-regulated in HCC4006ER5 cells (Figure 4*C*). This was consistent with the

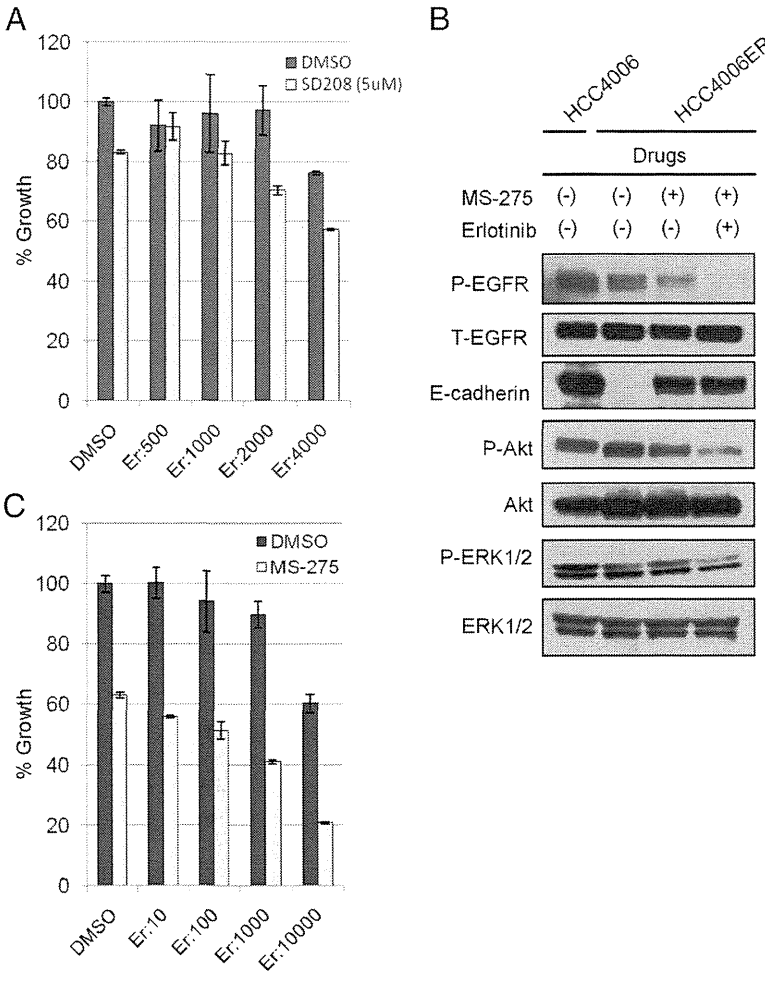


**FIGURE 5.** The histone deacetylase (HDAC) inhibitor but not the transforming growth factor beta (TGFbeta) inhibitor restored moderate sensitivity to erlotinib in HCC4006ER5 cells. *A*, SD208 did not restore remarkable erlotinib (Er.) sensitivity in HCC4006ER5 cells. HCC4006ER5 cells were incubated for 24 hours and an additional 72 hours with the indicated concentrations (nM) of erlotinib with/without 5 μM SD208, and cell growth was determined. *B*, Expression of E-cadherin and downstream signaling of EGFR in HCC4006 cells and in HCC4006ER5 cells treated with/without MS-275 and erlotinib were assessed using Western blotting. *C*, MS-275 restored moderate erlotinib sensitivity in HCC4006ER5 cells. HCC4006ER5 cells were incubated with/without 1 μM MS-275 for 24 hours and an additional 72 hours with indicated concentration of erlotinib (nM) with/without MS-275, and cell growth was determined.

EMT feature observed in HCC4006ER5 cells, as TGFbeta is a ligand that induces EMT.[20] Next, we analyzed the TGFbeta receptors I and II, as well as downstream molecules, using immunoblot analysis. However, the only obvious difference detected was the decrease in the phosphorylation of SMAD2 in HCC4006ER5 cells (Figure 4*D*). In addition, SD208, which is a selective TGFbeta receptor I kinase inhibitor, did not restore remarkable erlotinib sensitivity in HCC4006ER5 cells (Figure 5*A*).

## The HDAC Inhibitor Restored E-Cadherin Expression and Moderate Erlotinib Sensitivity in HCC4006ER5 Cells

Therefore, we analyzed whether the restoration of E-cadherin sensitize HCC4006ER5 cells to erlotinib. Referring to the previous report, we treated HCC4006ER5 cells with the HDAC inhibitor, MS-275, and identified that E-cadherin was restored after 72 hours treatment of 1 μM MS-275 (Figure 5*B*). Interestingly, MS-275 treatment induced moderate suppression of Akt and ERK activity in HCC4006ER5 cells in response to erlotinib (Figure 5*B*). In addition, we identified that the combination of 1 μM MS-275 and erlotinib moderately inhibited the growth of HCC4006ER5 cells (Figure 5*C*).

## Addition of TGFbeta Mimicked EMT and Acquired Resistance in HCC4006 Cells

To examine the involvement of EMT in acquired resistance in HCC4006 cells, we cultured these cells in the presence of 2 ng/ml of TGFbeta, a ligand that induces EMT, for 2 weeks. As shown in Figure 6*A*, HCC4006/TGFbeta cells acquired morphological changes that were similar to those of HCC4006ER5 cells. Analyses of response to erlotinib revealed that HCC4006/TGFbeta cells were moderately resistant to erlotinib compared with parental cells and that resistance was restored by the addition of 5 μM SD208 (Figure 6*B*). Immunoblot analyses showed an increase in the phosphorylation of SMAD2 and down-regulation of E-cadherin in HCC4006/TGFbeta cells, with maintenance of the level of phosphorylation of ERK and Akt in the presence of erlotinib (Figure 6*C*). The removal of TGF-beta for 2 weeks canceled the morphological changes and resistance to erlotinib observed in HCC4006/TGFbeta cells.

## DISCUSSION

EMT is a process in which epithelial cells that are organized, polarized, and tightly connected transdifferentiate into disorganized mesenchymal cells, which is accompanied

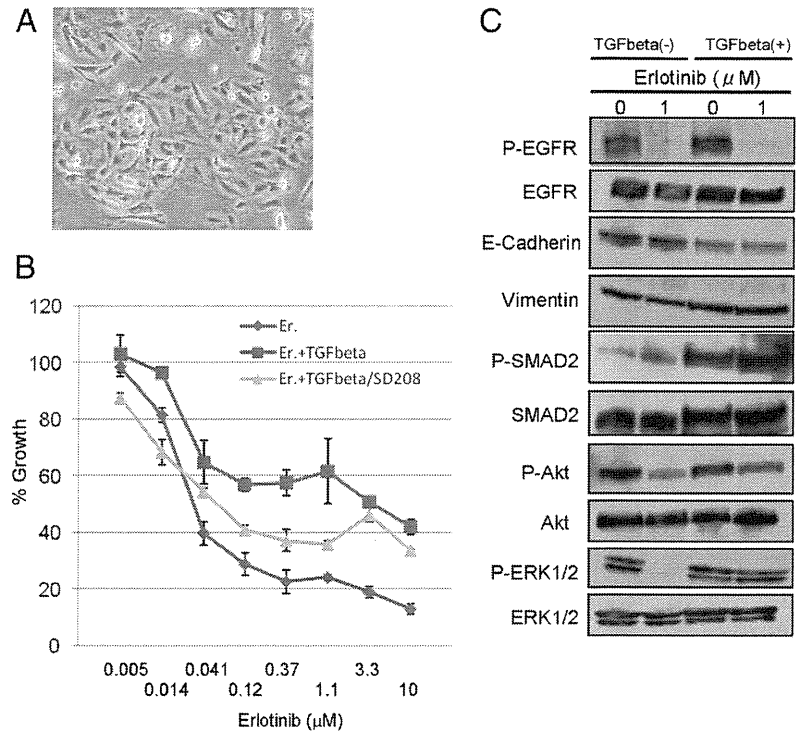


**FIGURE 6.** Transforming growth factor beta (TGFbeta) treatment reduced erlotinib sensitivity in HCC4006 cells. *A,* Morphological changes observed in HCC4006 cells after treatment with 2 ng/ml TGFbeta for 2 weeks. *B,* TGFbeta induced erlotinib resistance and SD208 restored sensitivity in HCC4006 cells. HCC4006 cells or HCC4006 cells treated with TGFbeta for 2 weeks were incubated for 24 hours and an additional 72 hours with the indicated concentrations of erlotinib (Er.) with/without 5 μM SD208, and cell growth was determined. *C,* Downstream signaling of TGFbeta and EGFR in HCC4006 cells and in HCC4006 cells treated with TGFbeta for 2 weeks, as assessed using Western blotting.

by changes in the expression of molecular marker proteins (e.g., down-regulation of E-cadherin and up-regulation of vimentin, fibronectin, and N-cadherin).[21,22] The relationship between EGFR-TKI sensitivity and changes from mesenchymal to epithelial status, or vice versa, has been reported in NSCLC without *EGFR* mutations.[12,23,24] Witta et al.[12] reported that transfection of E-cadherin into H157 cells (a *KRAS* mutant cells with a mesenchymal phenotype), or pretreatment with MS-275, which induces the expression of E-cadherin, increased their sensitivity to gefitinib. In contrast, Thomson et al.[23] reported that TGFbeta-treated H358 cells (a *KRAS* mutant cells with an epithelial phenotype) acquired a mesenchymal phenotype and lost their moderate erlotinib sensitivity. In addition, Rho et al.[24] generated a cell line that was more resistant to gefitinib from A549 cells (*KRAS* mutant), which are moderately resistant to gefitinib, and showed that EMT occurred in A549 gefitinib-resistant cells. Although these reports dealt with NSCLC without *EGFR* mutations, in this study we showed that an *EGFR*-mutant NSCLC cell line with acquired resistance to erlotinib also exhibited an EMT phenotype. Although our findings are the same with above previous studies, this study has much significance because EGFR-TKIs are very effective in patients with lung cancer with *EGFR* mutations but not in patients with wild-type *EGFR* (including those with *KRAS* mutations).

In this study, we found significantly increased expression of gene set that is related to the TGFbeta signaling pathway, as assessed using GSEA. Although very recent study by Yao et al.[25] has shown that erlotinib hyper-resistant cells established from H1650 cells (*EGFR* mutant, but erlotinib resistant due to *PTEN* deletion) displayed mesenchymal-like features and harbored increased TGFbeta-dependent IL-6 secretion, we failed to identify further evidence that showed the involvement of TGFbeta in acquired resistance to erlotinib. In addition, the expression level of IL-6 in HCC4006ER5 cells in this study was identical to that observed in parental cells, contrasting with the study by Yao et al. Because TGFbeta is a inducer of EMT, we suggested that unidentified cause(s), other than TGFbeta, increased expression of genes similar to those induced by TGFbeta addition and eventually conferred EMT-like phenotype on HCC4006ER cells.

HCC4006ER5 cells were also resistant to EGFR knockdown by siRNA transfection. This was in contrast with what was observed in parental HCC4006 cells, which indicates that HCC4006ER5 cells lost "EGFR addiction." This suggests the involvement of the activation of other oncoprotein(s) or other oncogenic pathway(s). First, we ruled out the involvement of MET[7] or IGF-IR,[8] which cause EGFR-TKI resistance in NSCLC. Down-regulation of *PTEN* also reportedly cause erlotinib primary resistance[26] or acquired resistance to cetuximab[27] or gefitinib,[28] respectively, in *EGFR*-mutant lung cancer cell lines. Nevertheless, the level of expression of *PTEN* in HCC4006ER cells was identical to that observed in parental cells. The involvement of autocrined hepatocyte growth factor was also ruled out, as the MET inhibitor did not restore erlotinib sensitivity in HCC4006ER cells. In addition, we did not identify any other "targetable oncoprotein" candidates in HCC4006ER5 cells (other than EGFR) using phospho-protein (RTK and intracellular kinase) array analyses or a gene expression assay. These results suggest that mesenchymal status, and not a specific oncogenic activated protein, confers resistance to erlotinib in HCC4006 cells.

Although the "primary change" observed in HCC4006ER cells was not clear, we found that the HDAC inhibitor, MS-275, restored E-cadherin expression and moderate erlo-

tinib sensitivity in HCC4006ER5 cells. We used MS-275 because this drug was used in the similar experiments[12] and was reported to reverse EMT in vivo.[29] Although the addition of MS-275 might confer combined effects rather than simple restoration of E-cadherin, our results would have clinical significance because HDAC inhibitors, including MS-275, are now under clinical development. Combination therapy for an HDAC inhibitor and erlotinib may be effective against tumors with acquired resistance to gefitinib or erlotinib by EMT.

The involvement of EMT in acquired resistance to gefitinib or erlotinib in clinically treated patients is unclear. However, it is also true that many of the resistance mechanisms identified using in vitro analyses have been found in clinically TKI-refractory samples. Moreover, a recent report that analyzed the expression profiles of epithelial and mesenchymal protein markers suggests the involvement of EMT in acquired resistance to gefitinib in *EGFR*-mutant lung cancer patients; although the interpretations included in the report had some weaknesses as discussed by the authors.[30]

In conclusion, our results suggest a role for EMT in acquired resistance to EGFR-TKIs in NSCLCs with *EGFR* mutations. The results of phase III studies reported recently[31,32] showed that many patients with NSCLC with *EGFR* mutations should be treated with EGFR-TKIs in the early phase of treatment. It may be important to consider the influence of EMT in the development of treatments for EGFR-TKI acquired resistance.

## ACKNOWLEDGMENTS

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