

# Activation of EGFR Bypass Signaling by TGF $\alpha$ Overexpression Induces Acquired Resistance to Alectinib in *ALK*-Translocated Lung Cancer Cells

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## Abstract

Alectinib is a highly selective ALK inhibitor and shows promising efficacy in non-small cell lung cancers (NSCLC) harboring the *EML4-ALK* gene rearrangement. The precise mechanism of acquired resistance to alectinib is not well defined. The purpose of this study was to clarify the mechanism of acquired resistance to alectinib in *ALK*-translocated lung cancer cells. We established alectinib-resistant cells (H3122-AR) from the H3122 NSCLC cell line, harboring the *EML4-ALK* gene rearrangement, by long-term exposure to alectinib. The mechanism of acquired resistance to alectinib in H3122-AR cells was evaluated by phospho-receptor tyrosine kinase (phospho-RTK) array screening and Western blotting. No mutation

of the ALK-TK domain was found. Phospho-RTK array analysis revealed that the phosphorylation level of EGFR was increased in H3122-AR cells compared with H3122. Expression of TGF $\alpha$ , one of the EGFR ligands, was significantly increased and knockdown of TGF $\alpha$  restored the sensitivity to alectinib in H3122-AR cells. We found combination therapy targeting ALK and EGFR with alectinib and afatinib showed efficacy both *in vitro* and in a mouse xenograft model. We propose a preclinical rationale to use the combination therapy with alectinib and afatinib in NSCLC that acquired resistance to alectinib by the activation of EGFR bypass signaling. *Mol Cancer Ther*; 15(1); 162–71. ©2015 AACR.

## Introduction

Lung cancer is the leading cause of cancer-related mortality worldwide (1). Recent molecular characterization of lung cancer enabled us to identify driver oncogenes, such as *EGFR*, *KRAS*, and the *EML4-ALK* fusion oncogene in non-small cell lung cancer (NSCLC; refs. 2–6). The *EML4-ALK* gene rearrangement was first identified in 2007 in 6.7% of NSCLCs (6). In addition to *EML4*, several other *ALK* fusion partners, such as *KIF5B*, *TFG*, and *KLC1* were identified (7–9). Fusions with the products of these partners induce oligomerization of the ALK, anaplastic lymphoma receptor tyrosine kinase, which leads to its constitutive activation (10). The activation of ALK subsequently induces the activation of downstream pathways, including the PI3K/protein kinase B (PI3K/AKT) and the extracellular signal-regulated kinase (ERK)/MAPK pathways. After the 2007 study, ALK tyrosine kinase was

regarded as an effective target in a subgroup of patients with NSCLC that harbor the *EML4-ALK* gene rearrangement. Crizotinib, a first generation ALK tyrosine kinase inhibitor (ALK-TKI) that targets ALK, MET, and ROS1 (11–14), showed great efficacy in *EML4-ALK* NSCLC (15); the response rate of patients was 57%.

However, almost all NSCLCs that harbored the *EML4-ALK* gene rearrangement acquired resistance to crizotinib within 1 to 2 years, similar to NSCLCs that harbor *EGFR* mutations, which acquired resistance to the EGFR-TKIs gefitinib and erlotinib (16, 17). The mechanisms of acquired resistance to EGFR-TKIs have been studied extensively. In general, they can be clearly classified into two types, one alteration of the *EGFR* gene itself, the *EGFR* T790M mutation (16, 17), and the other activation of a bypass signaling pathway, such as via MET, AXL, or FGFR1 (18–20). Similar mechanisms of acquired resistance were identified for crizotinib in several studies. These include secondary mutations of the *ALK* gene itself (such as L1196M, F1174L, C1156Y, G1202R, S1206Y, and G1269A), gene amplification, or activation of a bypass signal pathway, such as via KIT or EGFR (21–25). The *EML4-ALK* L1196M and *EGFR* T790M mutations are called "gatekeeper" mutations, because of the key location of the encoded amino acid substitutions at the entrance to a hydrophobic pocket at the back of the ATP binding cleft (26). These gatekeeper mutations induce a conformational change of the ATP binding pocket of ALK or EGFR, which induces steric hindrance of TKIs; refs. 21, 26).

To overcome acquired resistance to crizotinib, several second-generation ALK-TKIs have been developed (27–29). Of these, alectinib is a highly selective ALK inhibitor and has high activity against NSCLC with the *EML4-ALK* rearrangement. Alectinib also

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**Note:** Supplementary data for this article are available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

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has activity against the product of the *EML4-ALK* L1196M gatekeeper-mutant gene (30). In a recent report, alectinib showed promising efficacy in crizotinib-untreated and -treated patients with NSCLC (31, 32). The response rate was 94% in crizotinib-naïve patients. Strikingly, alectinib was also effective in crizotinib-treated patients, in whom the response rate was 55%.

Until now, the mechanism of acquired resistance to alectinib has not been well defined. In a recent report, two novel *ALK* mutations were identified, V1180L and I1171T, which conferred alectinib resistance (33). However, other mechanisms of acquired resistance to alectinib have not been clarified. In this study, we found that the activation of a TGF $\alpha$ -EGFR signaling pathway contributes to acquired resistance to alectinib. We propose the possibility that treatment that concomitantly targets the *ALK* and *EGFR* pathways may overcome resistance to alectinib in the subgroup of NSCLCs that harbor *ALK* translocations.

## Materials and Methods

### Cell line

The NCI-H3122 cell line, which harbors the *EML4-ALK* E13; A20 fusion, was a gift from S. Kobayashi (Beth Israel Deaconess Medical Center, Boston, MA). No authentication was done by the authors. Cells were cultured in RPMI1640 growth medium, supplemented with 10% FBS at 37°C in a humidified 5% CO<sub>2</sub> incubator.

### Reagents

Alectinib was a gift from Chugai Pharmaceutical Co. Ltd (Japan). Afatinib and erlotinib were purchased from LC Laboratories. Cetuximab was purchased from Keio University Hospital (Tokyo, Japan). Total *ALK* antibody (#3791S), phospho-*ALK* (Y1282/1283) antibody (#9687S), total *EGFR* antibody (#2232), total *AKT* antibody (#9272), phospho-*AKT* (S473; D9E) antibody (#4060), total p44/42 *MAPK* antibody (#9102S), and phospho-p44/42 *MAPK* (T202/204) antibody (#9101S) were purchased from Cell Signaling Technology. Phospho-*EGFR* (Y1068) antibody (44788G) was purchased from Invitrogen/Life Technologies. Actin antibody was purchased from Sigma-Aldrich.

### Cell proliferation assay

The MTS cell proliferation assay was performed as previously described (34). Briefly, 3 × 10<sup>3</sup> cells/well were seeded in 96-well plates. Twenty-four hours after seeding the cells, various concentrations of alectinib or afatinib were added to each well. Control cells were treated with the same concentration of the vehicle, dimethyl sulfoxide (DMSO). Seventy-two hours after treatment, absorbance was measured. All experiments were carried out at least three times.

### Western blotting analysis

Cells were treated with alectinib or afatinib at various concentrations. Alectinib was used at concentrations of 0.1 to 1  $\mu$ mol/L, and afatinib was used at a concentration of 1  $\mu$ mol/L. Cells were lysed in Cell Lysis Buffer (Cell Signaling Technology). Equal amounts of protein per lane were loaded on SDS-polyacrylamide gels. Separated proteins were transferred to polyvinylidene fluoride membranes. The membranes were incubated overnight with primary antibodies at 4°C and then incubated with secondary antibodies for 1 hour. For the detection of proteins, the membranes were incubated with agitation in LumiGLO reagent and

peroxide (Cell Signaling Technology), and then exposed to X-ray film.

### Phospho-receptor tyrosine kinase array

The human phospho-receptor tyrosine kinase (phospho-RTK) array kit was purchased from R&D Systems and screened according to the manufacturer's protocol, with 150  $\mu$ g of protein being used for each experiment. Signal intensity was calculated using the LumiVision Analyzer software (Aisin Seiki, Japan).

### Apoptosis assay

Cells (30,000/well) were seeded in 6-well plates. The cells were treated with alectinib and afatinib, as single agents or in combination. Cells were treated with alectinib and afatinib at 1  $\mu$ mol/L for 72 hours. Control cells were treated with the same concentration of the vehicle, DMSO. We analyzed the apoptotic status of cells using the TACS Annexin V-FITC Apoptosis Detection Kit (R&D Systems), according to the manufacturer's protocol. The proportion of apoptotic cells was evaluated by flow cytometric analysis, using the BD FACSCalibur system (Becton Dickinson).

### Quantitative RT-PCR

Total RNA was isolated from cells using an RNeasy Mini Kit (Qiagen). The RNA was subjected to reverse transcription using the High-Capacity RNA-to-cDNA Kit (Life Technologies), according to the manufacturer's protocol. Quantitative RT-PCR (qRT-PCR) was performed using the fluorescent SYBR Green dye methodology and an ABI Prism 7000 Sequence Detection System (Life Technologies). Human *GAPDH* was used for normalization of input cDNA. The sequences of primers used in this study are summarized in Supplementary Table S1.

### siRNAs targeting *EGF*, *TGF $\alpha$* , and *EGFR*

Cells were transfected with siRNAs that were specific for *EGF* (#s501229 and #s4519; Life Technologies), *TGF $\alpha$*  (#s14051 and #s14052; Life Technologies), or *EGFR* (#s563 and #s564; Life Technologies), or with negative control siRNA. The Ambion Silencer Select Negative Control mix (Life Technologies) was used according to the manufacturer's protocol. The siLentFect transfection reagent (Bio-Rad) was used according to the manufacturer's protocol. We confirmed knockdown of *EGF*, *TGF $\alpha$* , and *EGFR* by qRT-PCR.

### FISH

FISH analysis of the *EML4-ALK* gene rearrangement was performed by SRL.

### Mouse xenograft model

All animal experiments were approved by the Laboratory Animal Center, Keio University School of Medicine. Female BALB/*c-nu* mice were purchased from Charles River. Mice were anesthetized with ketamine. H3122-AR cells were suspended in Matrigel and injected subcutaneously. Tumor volume was monitored using a caliper. Once average tumor volume reached 200 mm<sup>3</sup>, mice were randomized, and received vehicle, alectinib (6 mg/kg) alone, afatinib (20 mg/kg) alone, or alectinib and afatinib in combination; mice were treated once a day by intragastric administration. Animals were humanely sacrificed and tumor tissues were harvested. For Western blotting, proteins were extracted from representative tumor tissues using Cell Lysis Buffer

(Cell Signaling Technology). Western blotting was performed as mentioned above.

### Statistical analysis

Statistical analysis was performed using the GraphPad Prism software, version 4.0 (GraphPad Software). Student *t* test was used for comparisons. All *P* values were two sided; values of *P* < 0.05 were regarded as statistically significant.

## Results

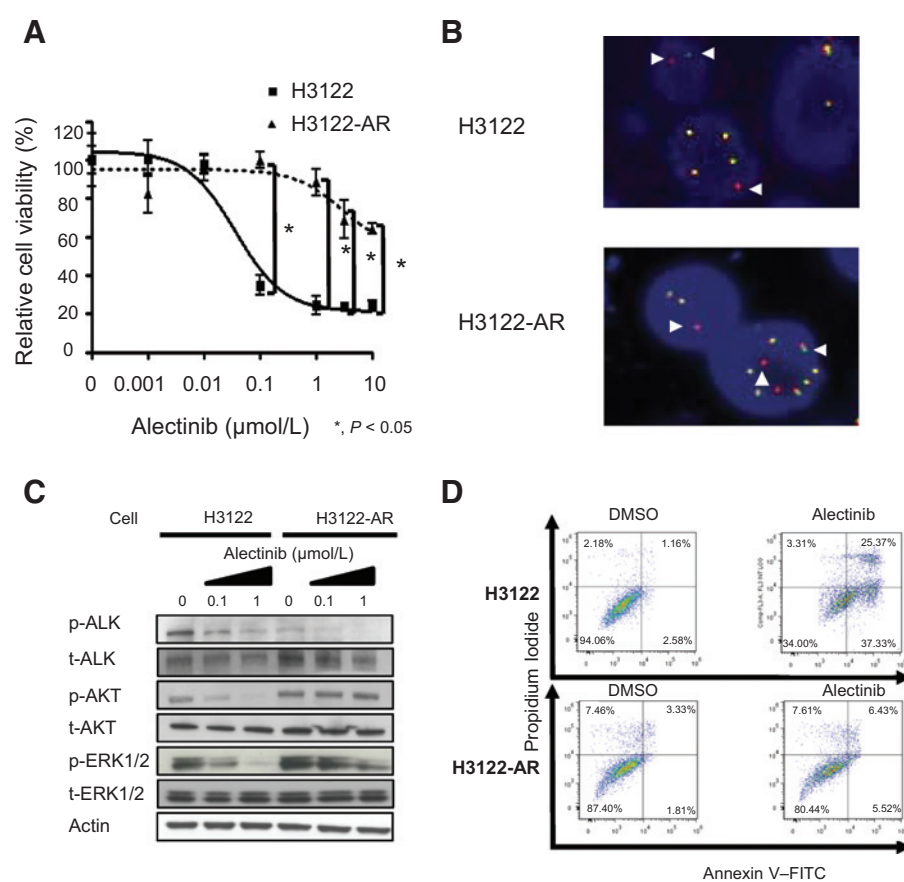
### Establishment of alectinib-resistant H3122 cells

H3122 cells, which harbor the *EML4-ALK* gene rearrangement, were cultured with alectinib to establish alectinib-resistant H3122 (H3122-AR) cells. The initial alectinib concentration was 0.01  $\mu\text{mol/L}$ , and this was incrementally increased to 1  $\mu\text{mol/L}$ . After 6 months of exposure to the drug, the H3122-AR cells were established. Their resistance to alectinib was confirmed by the MTS cell proliferation assay (Fig. 1A). The calculated  $\text{IC}_{50}$  values for alectinib were 38.2 nmol/L for alectinib-naïve H3122 parental cells and >3  $\mu\text{mol/L}$  for H3122-AR cells. To evaluate the reversibility of the resistance in H3122-AR cells, we removed alectinib for two weeks and performed MTS cell proliferation assay after the 2 weeks. We found that resistance was retained after removing alectinib (Supplementary Fig. S1). To confirm that H3122 cells and H3122-AR cells retained the *EML4-ALK* gene rearrangement, we performed FISH analysis, because loss of the *EML4-ALK* gene rearrangement was previously reported in crizotinib-resistant

tumors (23). We found that both H3122 and H3122-AR cells retained the *EML4-ALK* gene rearrangement (Fig. 1B). Next, we performed Western blotting for H3122 and H3122-AR cells to examine the effect of alectinib on downstream pathways, including the PI3K/AKT pathway and the MAPK pathway (Fig. 1C). The phosphorylation of ALK, AKT, and ERK1/2 was efficiently inhibited by alectinib in H3122 cells. The phosphorylation of ALK was also efficiently inhibited by alectinib; however, the phosphorylation of AKT and ERK1/2 was less efficiently inhibited by alectinib in H3122-AR cells compared with H3122 cells. To examine whether H3122-AR cells were resistant to apoptosis induced by alectinib, the level of apoptotic cells in H3122 and H3122-AR cultures following alectinib treatment was evaluated by flow cytometry; the proportion of propidium iodide-positive and annexin V-positive cells was evaluated (Fig. 1D). Alectinib efficiently induced apoptosis in H3122 cells. However, alectinib induced apoptosis less efficiently in H3122-AR cells. These results indicate that H3122-AR cells have acquired resistance to alectinib.

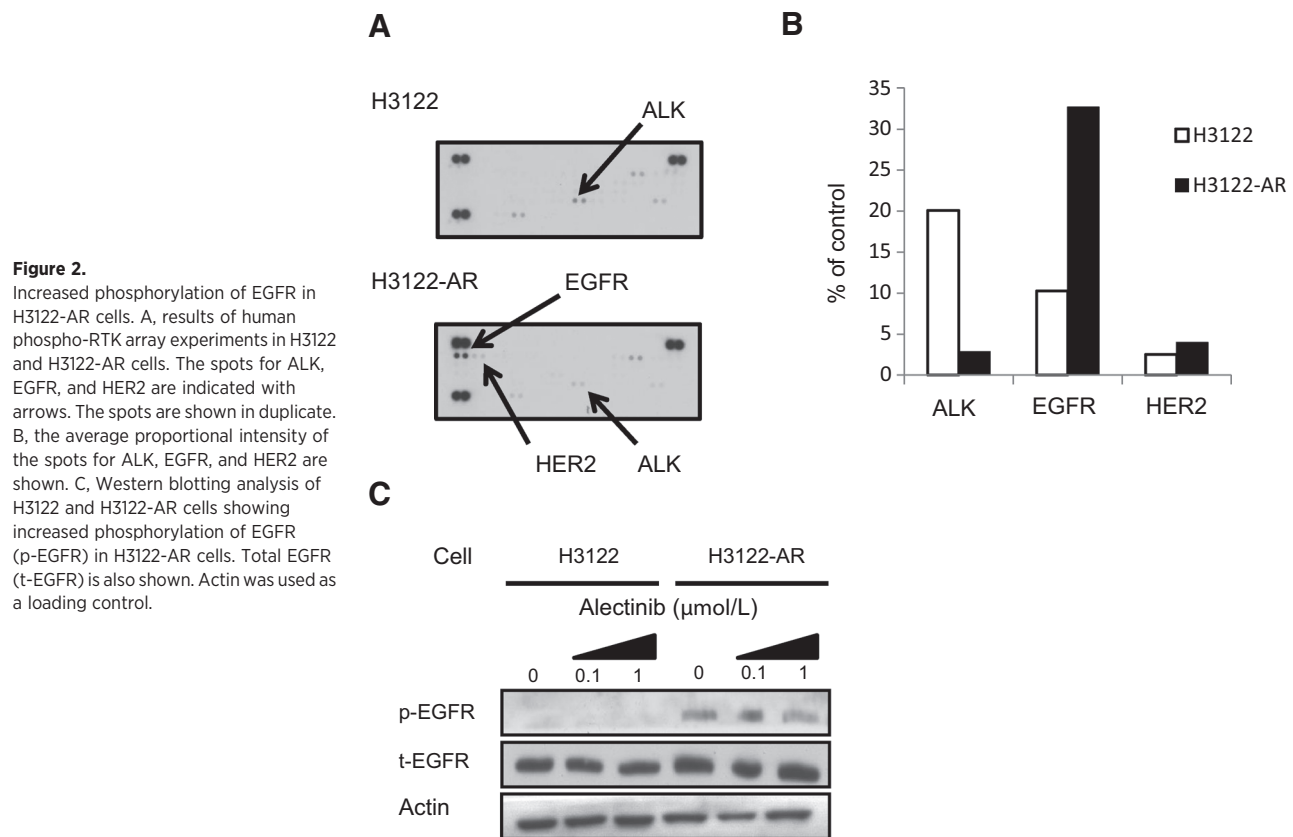
### Increased phosphorylation of EGFR in H3122-AR cells

Until now, the mechanisms of acquired resistance to ALK-TKIs (crizotinib or TAE684) were reported to be alteration of the *ALK* gene itself or activation of bypass signaling (21, 24, 35). To clarify the mechanism of acquired resistance to alectinib in H3122-AR cells, we first performed DNA sequencing of a cDNA representing the *ALK* tyrosine kinase domain. We did not find any novel mutations affecting the *ALK* tyrosine kinase domain, spanning exons 21–28. Next, to evaluate the activation of bypass pathways,



**Figure 1.**

Establishment of alectinib-resistant H3122 cells. A, the results of MTS cell proliferation assays are shown, following treatment of cells with the indicated alectinib concentrations for 72 hours. Error bars, SD. B, results of two-color FISH analysis of H3122 and H3122-AR cells are shown. Arrowheads, isolated or split red signals. C, Western blotting analysis of H3122 and H3122-AR cells, for phosphorylated (p-) and total (t-) ALK, AKT, and ERK1/2, is shown. Actin was used as a loading control. D, flow cytometric data are shown for H3122 and H3122-AR cells treated with dimethyl sulfoxide (DMSO) vehicle or alectinib for 72 hours. The numbers (%) indicate the proportion of annexin V-FITC- and/or propidium iodide-stained cells.



we examined the phosphorylation level of other receptor tyrosine kinases, using a human phospho-RTK array kit, in both H3122 and H3122-AR cells. Phospho-RTK array experiments revealed that the phosphorylation level of EGFR in H3122-AR cells was increased compared with H3122 cells, and the phosphorylation level of ALK in H3122-AR cells was reduced compared with H3122 cells (Fig. 2A and B). Furthermore, we found a slight increase in the phosphorylation of HER2. To confirm the increased phosphorylation of EGFR, we performed Western blotting of cells that had been treated with or without alectinib (Fig. 2C). As expected, the phosphorylation level of EGFR (Y1068) was increased in H3122-AR cells compared with H3122 parental cells, even though the expression level of total EGFR was not increased. We found that the phosphorylation level of EGFR in H3122-AR cells was not affected by alectinib treatment. These results indicate that the EGFR pathway is activated in H3122-AR cells.

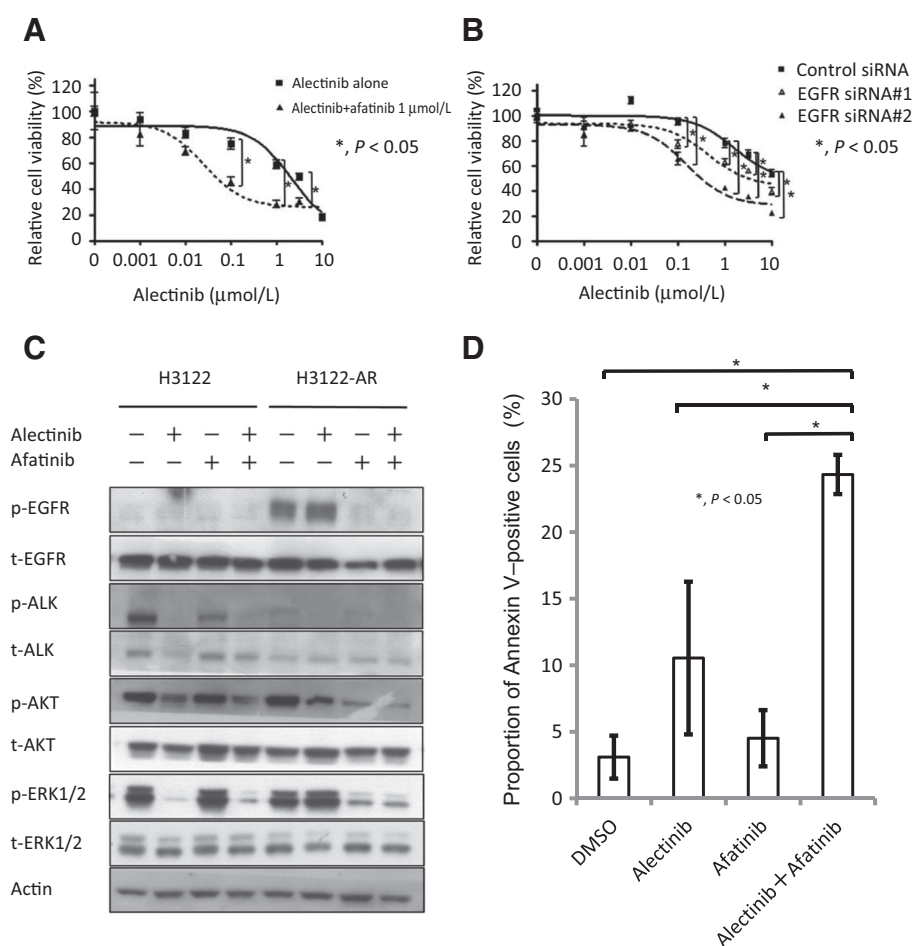
#### EGFR pathway activation contributes to acquired resistance to alectinib in H3122-AR cells

To investigate whether EGFR pathway activation has a functional role in the acquired resistance to alectinib in H3122-AR cells, we performed the MTS cell proliferation assay with afatinib, a second-generation EGFR-TKI (36), alone or afatinib and alectinib in combination for H3122-AR cells. Afatinib as a single agent could not inhibit the proliferation of H3122-AR cells (Supplementary Fig. S2A). Next, we used the MTS assay to examine whether combined treatment with alectinib and afatinib, could inhibit the proliferation of H3122-AR cells. We found that addition of afatinib partially restored the sensitivity of H3122-AR cells

to alectinib (Fig. 3A). In addition, we performed similar MTS assay with alectinib and erlotinib, another EGFR-TKIs. Again, we found that addition of erlotinib partially restored the sensitivity of H3122-AR cells to alectinib, although the effect was less significant when compared with afatinib (Supplementary Fig. S2B). Because afatinib is a panhuman epidermal growth factor receptor family inhibitor and TKIs induce non-specific, off-target effects, especially at high concentrations, we performed *EGFR* gene-specific knockdown by siRNA in H3122-AR cells (Fig. 3B). The knockdown of *EGFR* gene expression was confirmed by qRT-PCR. We found that *EGFR* gene knockdown restored the sensitivity of H3122-AR cells to alectinib. These data indicate that EGFR contributes to the acquired resistance to alectinib in H3122-AR cells. To examine whether EGFR pathway inhibition induces additional sensitivity to alectinib in H3122 parental cells, we performed MTS cell proliferation assay with afatinib or EGFR siRNA (Supplementary Fig. S3). We found no additional effect of afatinib or EGFR siRNA. These data indicate that EGFR pathway activation contributes to acquired resistance to alectinib in H3122-AR cells but not in H3122 parental cells.

To investigate whether alectinib/afatinib combination treatment effectively inhibited the activation of pathways downstream of ALK and EGFR, including the PI3K/AKT pathway, and MAPK pathway, in H3122-AR cells, we performed Western blotting for both H3122 and H3122-AR cells (Fig. 3C). We found that alectinib efficiently inhibited the phosphorylation of ALK, AKT, and ERK1/2 in H3122 parental cells; in contrast, afatinib did not alter the phosphorylation level of ALK, AKT, or ERK1/2 in these cells. In H3122-AR cells, the phosphorylation level of ALK was



**Figure 3.**

The contribution of EGFR pathway activation to acquired resistance to alectinib in H3122-AR cells. A, results of the MTS cell proliferation assay are shown. H3122-AR cells were treated with the indicated alectinib and afatinib concentrations. Error bars, SD. B, results of the MTS cell proliferation assay for H3122-AR cells treated with the indicated alectinib concentrations are shown, following transfection with control siRNA or *EGFR*-targeted siRNAs (#1, #2). Error bars, SD. C, Western blotting analysis of H3122 and H3122-AR cells is shown. The cells were treated with or without alectinib (1 μmol/L) and afatinib (1 μmol/L) for 2 hours. The phosphorylated (p-) and total (t-) forms of the indicated proteins were examined. Actin was used as a loading control. D, the proportion of Annexin V-positive cells examined by flow cytometry is shown. H3122-AR cells were treated with or without alectinib and afatinib for 72 hours. The cells were stained with annexin V-FITC and propidium iodide. Error bars, SD.

decreased compared with H3122 parental cells. We found that alectinib/afatinib combination treatment efficiently inhibited the phosphorylation of AKT and ERK1/2. Interestingly, the phosphorylation of AKT and ERK1/2 in H3122-AR cells was partially inhibited by afatinib alone. Although there are some discrepancies, between the aforementioned MTS assay data and Western blotting data for the analysis of proteins downstream of ALK and EGFR, the Western blotting data indicate that signals are mainly transduced to downstream signaling proteins through EGFR rather than ALK in H3122-AR cells.

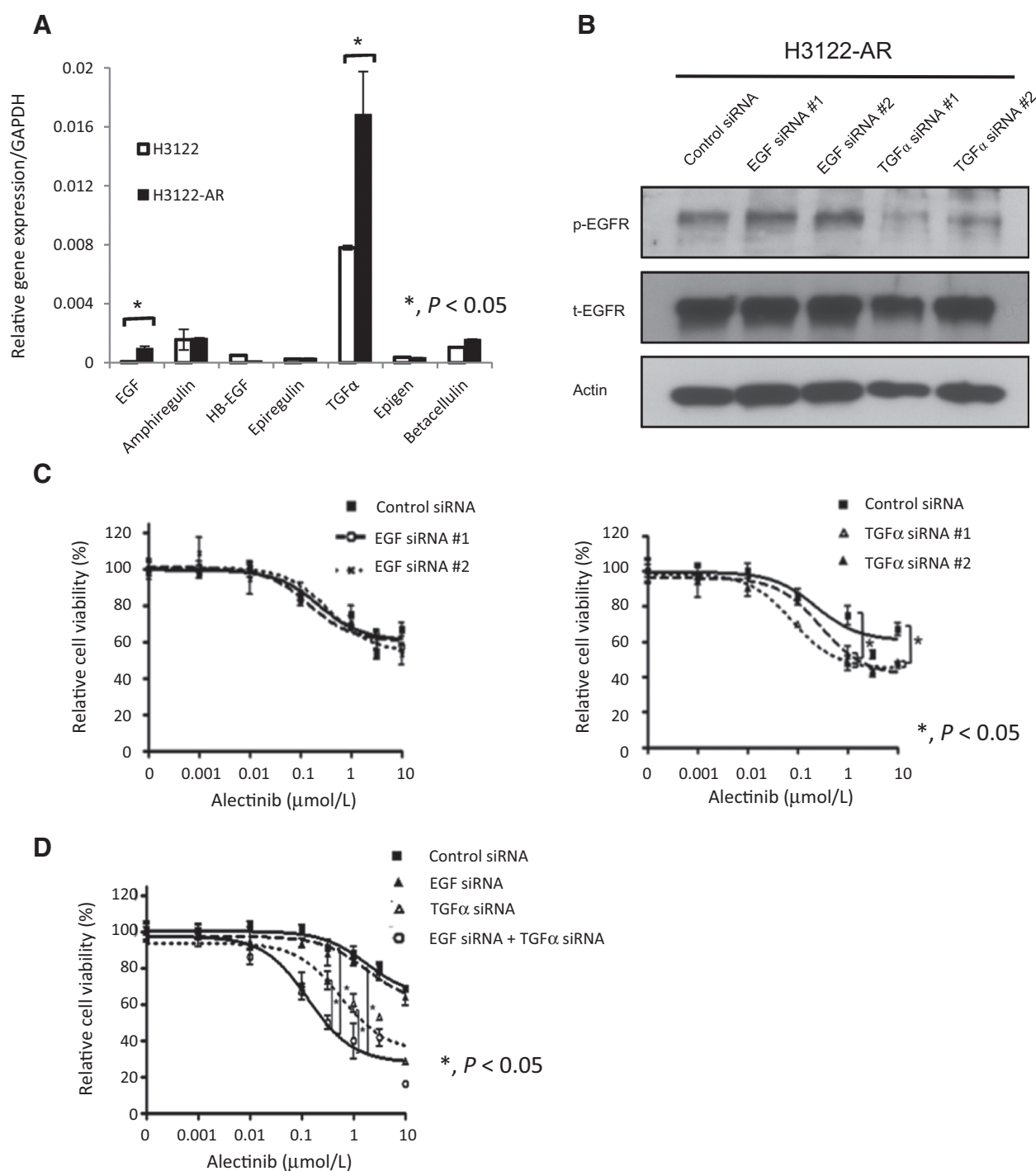
To investigate whether alectinib/afatinib combination treatment effectively induced apoptosis in H3122-AR cells, the proportion of apoptotic cells, annexin V-positive cells, in H3122-AR cultures following treatment with alectinib, afatinib, or alectinib/afatinib in combination was evaluated by flow cytometry. Compared with alectinib alone, alectinib/afatinib combination treatment increased the proportion of apoptotic cells from 10.54% to 24.33% ( $P < 0.05$ ; Fig. 3D). Again, we noticed some discrepancies between the results of the Western blotting (Fig. 3C) and the apoptosis assay (Fig. 3D). None of the signal pathways measured were inhibited more by the combination treatment relative to any single agent after 2 hours of treatment. We expected that the duration of the treatment could partially explain the discrepancy. We performed Western blotting at longer treatment duration, because the data of the MTS assay or the apoptosis assay was obtained after 72 hours of treatment. As expected, the inhibition

of downstream pathway was more efficient in combination treatment compared to alectinib or afatinib alone at 24 hours (Supplementary Fig. S4). These data indicate that the combination treatment induce more sustained inhibition of downstream pathways compared with alectinib or afatinib alone.

In summary, these data indicate that activation of the EGFR signaling pathway contributes to an acquired resistance to alectinib in H3122-AR cells, and that treatment with alectinib/afatinib in combination effectively eliminates H3122-AR cells *in vitro*.

#### Contribution of TGF $\alpha$ to the acquired resistance to alectinib in H3122-AR cells

Activation of EGFR occurs through ligand stimulation or mutation of *EGFR* affecting the tyrosine kinase domain (37, 38). To clarify the mechanism of EGFR pathway activation in H3122-AR cells, we performed DNA sequencing of a cDNA representing the tyrosine kinase domain of EGFR; however, no mutations were detected. To evaluate the possibility of ligand-induced activation of EGFR, we examined the expression level of EGFR ligands. Until now, seven EGFR ligands, namely EGF, amphiregulin, heparin-binding EGF-like growth factor (HB-EGF), epiregulin, TGF $\alpha$ , epigen, and betacellulin have been reported (39). We found that the expression of *EGF* and *TGF $\alpha$* , but not the other five ligands, were significantly increased in H3122-AR cells compared with H3122 parental cells (Fig. 4A). To clarify the time course of *EGF* and *TGF $\alpha$*  induction following alectinib treatment, we performed



**Figure 4.**

Contribution of TGF $\alpha$  to the acquired resistance to alectinib in H3122-AR cells. A, expression of the indicated genes is shown for H3122 and H3122-AR cells relative to the expression of *GAPDH*. Error bars, SD. B, Western blotting analysis is shown for H3122-AR cells transfected with control siRNA, *EGF*-targeted siRNAs (#1, #2), or *TGF $\alpha$* -targeted siRNAs (#1, #2). Phosphorylated (p-) and total (t-) EGFR are shown. Actin was used as a loading control. C, results of the MTS cell proliferation assay for H3122-AR cells treated with the indicated alectinib concentrations are shown, following transfection with control siRNA, *EGF*-targeted (#1, #2), or *TGF $\alpha$* -targeted siRNAs (#1, #2). Error bars, SD. D, results of the MTS cell proliferation assay for H3122-AR cells treated with the indicated alectinib concentrations are shown, following transfection with control siRNA, *EGF*-targeted alone, *TGF $\alpha$* -targeted alone, or in combination. Error bars, SD.

qRT-PCR using H3122 cells which were preserved at multiple alectinib concentrations during the process of alectinib exposure. We found the levels of *EGF* and *TGF $\alpha$*  expression started to

increase at around 0.1–0.3  $\mu\text{mol/L}$  of alectinib (Supplementary Fig. S5). To determine whether the inhibition of EGFR ligand binding restores the sensitivity to alectinib in H3122-AR cells, we

performed MTS assay with cetuximab, an EGFR antibody which block ligands binding. We found cetuximab restored the sensitivity to alectinib in H3122-AR cells (Supplementary Fig. S2C), indicating the possibility that EGFR ligands contribute to the acquired resistance to alectinib in H3122-AR cells.

To evaluate whether the increased expression of *EGF* or *TGF $\alpha$*  had a functional role in H3122-AR cells, we performed *EGF* or *TGF $\alpha$*  gene-specific knockdown by siRNA. The knockdown of *EGF* or *TGF $\alpha$*  gene expression was confirmed by qRT-PCR. We found that the phosphorylation level of EGFR was decreased in *TGF $\alpha$*  knockdown, but not *EGF* knockdown (Fig. 4B). To examine whether *EGF* or *TGF $\alpha$*  knockdown restore the sensitivity to alectinib, we performed MTS assay. We found that *TGF $\alpha$*  gene knockdown, but not *EGF*, partially restored the sensitivity of H3122-AR cells to alectinib (Fig. 4C). These data indicate increased expression of *TGF $\alpha$*  contributes to the acquired resistance to alectinib in H3122-AR cells. To determine whether the combined knockdown of *EGF* and *TGF $\alpha$*  can further restore the sensitivity of H3122-AR cells to alectinib, we performed MTS assay. Interestingly, the combined knockdown of *EGF* and *TGF $\alpha$*  further restored the sensitivity of H3122-AR cells to alectinib compared with either single knockdown (Fig. 4D). These data indicate that increased expression of *EGF* also contributes to the acquired resistance to alectinib in H3122-AR cells, even though the restoration of sensitivity was limited.

The fact that the combination knockdown restored the sensitivity to alectinib in H3122-AR cells promoted us to examine the heterogeneity of H3122-AR cells. To examine the heterogeneity of H3122-AR cells, we established single cell-derived clones from H3122-AR cells. We confirmed the increased expression of *EGF* and *TGF $\alpha$*  in almost all clones established. We performed MTS assay with *EGF* and/or *TGF $\alpha$*  knockdown for representative clones (Supplementary Fig. S6). We found that *TGF $\alpha$*  gene knockdown partially restored the sensitivity to alectinib in all clones examined. Interestingly, in one clone, H3122-AR3, the combined knockdown of *EGF* and *TGF $\alpha$*  further restored the sensitivity of H3122-AR cells to alectinib compared with either knockdown alone. These data indicate the possibility that *TGF $\alpha$*  contributes to the acquired resistance to alectinib in a major population of H3122-AR cells, and that *EGF* contributes to the acquired resistance to alectinib in a minor population of H3122-AR cells, representing the heterogeneity of H3122-AR cells.

All these data indicate the main mechanism of acquired resistance to alectinib in H3122-AR was exerted through the *TGF $\alpha$* -EGFR pathway.

#### Alectinib/afatinib combination treatment is effective against H3122-AR cells *in vivo*

To examine whether alectinib/afatinib combination treatment was effective against H3122-AR cells *in vivo*, we employed a mouse xenograft model. Mice with H3122-AR-induced tumors were treated with vehicle, alectinib, afatinib, or alectinib/afatinib in combination. During treatment, the body weight of the mice (Supplementary Fig. S7) and tumor size were monitored. Tumor formation was confirmed by macroscopic analysis (Fig. 5A) and pathologic evaluation of tumors revealed the histologic phenotype of poorly differentiated adenocarcinoma (Fig. 5B). We found that, after approximately 20 days, alectinib/afatinib combination treatment effectively and significantly inhibited tumor progression, compared with vehicle, alectinib, or afatinib alone (Fig. 5C).

In addition, to see whether alectinib/afatinib combination treatment effectively inhibits the downstream pathways of ALK and EGFR, we performed Western blotting using tumor samples. We found that alectinib/afatinib combination treatment efficiently inhibited the phosphorylation of AKT and ERK1/2. These data indicate that alectinib/afatinib combination treatment is effective against H3122-AR cells *in vivo*.

## Discussion

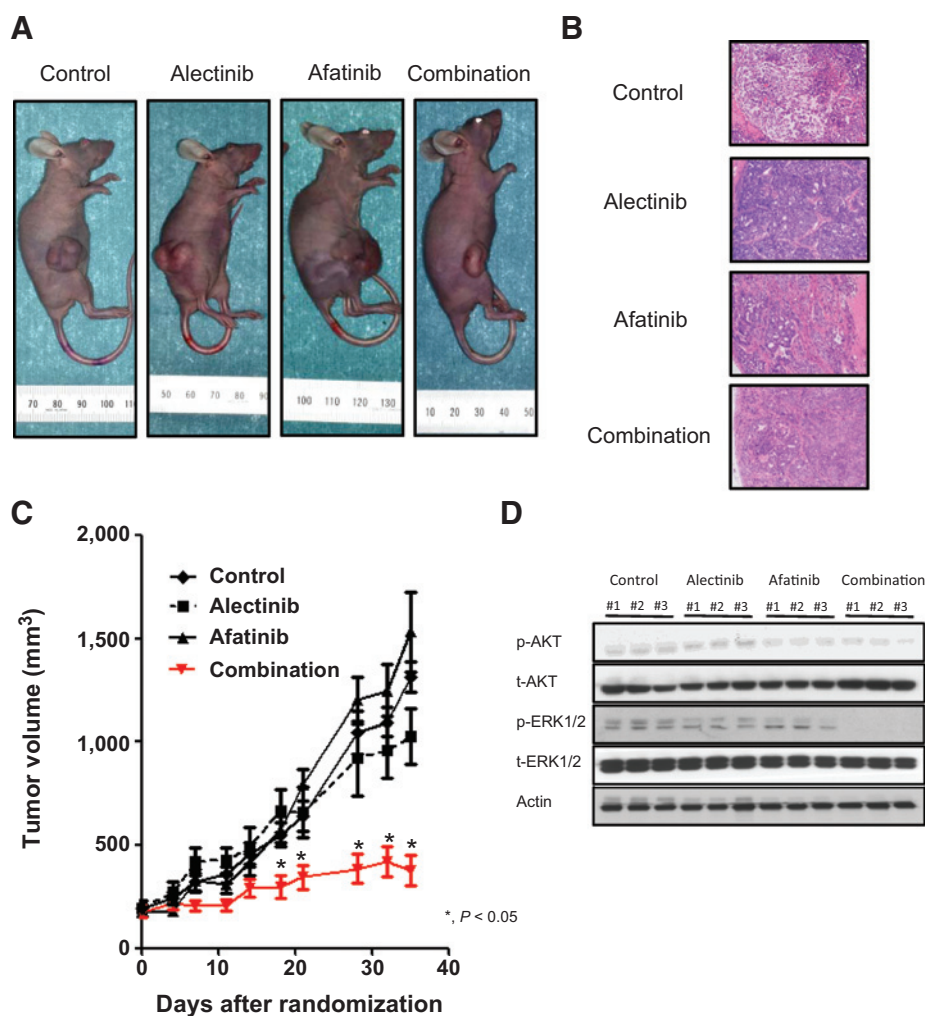
Recent advancements in the understanding of cancer biology have revealed tyrosine kinases' oncogenic function, and tyrosine kinase-specific targeted therapies have been developed. In NSCLC, several tyrosine kinases have been identified as oncogenic drivers; these include EGFR, ALK, FGFR1, ROS1, and RET (2, 4, 6, 40–42). To target these driver tyrosine kinases, many small-molecule TKIs have been developed. Of especial note, EGFR-TKIs, such as gefitinib and erlotinib, and ALK-TKIs, such as crizotinib, have made a significant contribution to the improved prognosis of patients with lung cancer (43–45). Although these EGFR-TKIs and ALK-TKIs are powerful treatments for *EGFR*-mutated or *ALK*-translocated NSCLCs, cancer cells inevitably acquire resistance to TKIs. To improve further the prognosis of patients with lung cancer, a thorough understanding of the mechanisms of acquired resistance is essential. The mechanisms of acquired resistance to EGFR-TKIs, such as gefitinib and erlotinib, have been well defined; they include gatekeeper mutation, *EGFR* T790M, of the *EGFR* gene itself or alternative bypass-pathway activation. In the case of EGFR-TKIs, MET, AXL, and FGFR1 signaling pathways contribute to the acquired resistance to EGFR-TKIs. In the case of ALK-TKIs, activation of the EGFR signaling pathway was reported as a mechanism of acquired resistance to crizotinib or TAE684 (24, 35), but not alectinib.

Alectinib is a second-generation and highly selective ALK-TKI. Alectinib showed an objective response rate of 94% in *ALK* translocation-positive/*ALK* inhibitor-naïve Japanese patients with NSCLC in a phase I/II clinical study, with an excellent safety profile (31). As a consequence, the Pharmaceutical and Medical Devices Agency approved alectinib for use from July 2014 in Japan. It was reported that alectinib also has activity against NSCLC that harbors the gatekeeper *ALK* L1196M mutation as well as *EML4-ALK* translocation (30). Considering its higher efficacy and safety compared with crizotinib, alectinib is expected to take the place of crizotinib as the front-line treatment for most patients with *ALK* translocation-positive NSCLC in the near future. Therefore, elucidation of the mechanisms of acquired resistance to alectinib is of great importance. Recently, a novel mechanism of acquired resistance to alectinib was reported (33). The authors used an *in vitro* model of chronic alectinib exposure in H3122 cells and found two novel *ALK* mutations. However, until now, EGFR pathway activation in acquired resistance to alectinib has not been identified.

In this study, we have identified a novel mechanism of acquired resistance to alectinib; we show that EGFR pathway activation by *TGF $\alpha$*  induces resistance to alectinib. Recently, it was reported that exogenous ligands stimulation, including HGF, EGF and *TGF $\alpha$* , induce resistance to alectinib in several *ALK*-translocated lung cancer cells (46). In this study, we found endogenous overexpression of *TGF $\alpha$*  induces resistance to alectinib. Alectinib/afatinib

**Figure 5.**

Alectinib/afatinib combination treatment is effective against H3122-AR cells *in vivo*. Tumor-bearing mice were randomized into vehicle, alectinib, afatinib, or alectinib/afatinib treatment groups. A, representative images of tumor-bearing mice. B, representative images of tumor samples stained with hematoxylin-eosin. C, tumor size was measured and tumor volume calculated. The values indicate average tumor volume in each group. \*,  $P < 0.05$  for the combination of alectinib/afatinib versus other treatments (vehicle, alectinib alone, and afatinib alone). D, Western blotting analysis of tumor samples is shown. The mice were treated with vehicle, alectinib, afatinib or alectinib/afatinib combination. The phosphorylated (p-) and total (t-) forms of the indicated proteins were examined. Actin was used as a loading control.



combination treatment was effective against H3122-AR cells both *in vitro* and *in vivo*. The phosphorylation of AKT and ERK1/2 was inhibited by treatment with afatinib alone (Fig. 3C). These data indicate the possibility that the signal to the PI3K/AKT and MAPK pathways was mainly transduced through EGFR rather than ALK in H3122-AR cells. We speculate that the function as driver oncogene has shifted from ALK to EGFR. However, there was discrepancy between data from western blotting of downstream proteins, and data from the MTS cell proliferation or apoptosis assays (Fig. 3). Afatinib alone did not inhibit cell proliferation, nor did it induce apoptosis; only alectinib/afatinib combination treatment induced apoptosis in H3122-AR cells. This discrepancy was partially explained by the results of the Western blotting after 24 hours of treatment (Supplementary Fig. S4). The activation of downstream signals was persistently inhibited only in the alectinib/afatinib combination treatment. These data indicate the possibility that EGFR and ALK cooperate to transduce signals to downstream proteins.

Furthermore, there are some limitations to the current study. First, it is unclear whether long-term alectinib treatment itself increased TGF $\alpha$  expression in H3122 cells, or whether alectinib selectively eliminated H3122 cells that expressed low levels of TGF $\alpha$  such that only cells expressing high levels of TGF $\alpha$

survived as H3122-AR cells. Considering tumor heterogeneity and the finding that increased expression of EGF and TGF $\alpha$  was not observed in early alectinib exposure phase (Supplementary Fig. S5), we speculate that the latter case is more likely, although difficult to prove. Second, we could not identify a patient who was treated with alectinib, and experienced relapse after alectinib treatment. It remains elusive whether acquired resistance to alectinib through EGFR activation occur in human ALK-translocated lung cancers. Third, there seems to be a discrepancy between the *in vitro* MTS assay data and the *in vivo* efficacy data, with the *in vivo* data appearing to be more impressive. This suggests that other mechanisms of acquired resistance could be involved. For further characterization of acquired resistance to alectinib, additional *in vitro*, *in vivo*, and human studies are necessary.

At present, we do not know what proportion of acquired resistance to alectinib can be explained by EGFR pathway activation; however, we expect that some patients with ALK-translocated lung cancer will benefit from the addition of EGFR-TKIs to their therapeutic regime after disease progression on alectinib.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.



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## References

- Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. *CA Cancer J Clin* 2013;63:11–30.
- Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, et al. EGFR mutations in lung cancer: Correlation with clinical response to gefitinib therapy. *Science* 2004;304:1497–500.
- Imielinski M, Berger AH, Hammerman PS, Hernandez B, Pugh TJ, Hodis E, et al. Mapping the hallmarks of lung adenocarcinoma with massively parallel sequencing. *Cell* 2012;150:1107–20.
- Pao W, Girard N. New driver mutations in non-small-cell lung cancer. *Lancet Oncol* 2011;12:175–80.
- Slebos RJC, Kibbelaar RE, Dalesio O, Kooistra A, Stam J, Meijer CJLM, et al. K-ras oncogene activation as a prognostic marker in adenocarcinoma of the lung. *New Engl J Med* 1990;323:561–5.
- Soda M, Choi YL, Enomoto M, Takada S, Yamashita Y, Ishikawa S, et al. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. *Nature* 2007;448:561–U3.
- Takeuchi K, Choi YL, Togashi Y, Soda M, Hatano S, Inamura K, et al. KIF5B-ALK, a novel fusion oncokine identified by an immunohistochemistry-based diagnostic system for ALK-positive lung cancer. *Clin Cancer Res* 2009;15:3143–9.
- Takeuchi K, Soda M, Togashi Y, Ota Y, Sekiguchi Y, Hatano S, et al. Identification of a novel fusion, SQSTM1-ALK, in ALK-positive large B-cell lymphoma. *Haematol-Hematol J* 2011;96:464–7.
- Togashi Y, Soda M, Sakata S, Sugawara E, Hatano S, Asaka R, et al. KLC1-ALK: a novel fusion in lung cancer identified using a formalin-fixed paraffin-embedded tissue only. *Plos One* 2012;7:e31323.
- Shaw AT, Hsu PP, Awad MM, Engelman JA. Tyrosine kinase gene rearrangements in epithelial malignancies. *Nat Rev Cancer* 2013;13:772–87.
- Camidge DR, Bang YJ, Kwak EL, Iafrate AJ, Varella-Garcia M, Fox SB, et al. Activity and safety of crizotinib in patients with ALK-positive non-small-cell lung cancer: updated results from a phase 1 study. *Lancet Oncol* 2012;13:1011–9.
- Ou SHI, Bazhenova L, Camidge DR, Solomon BJ, Herman J, Kain T, et al. Rapid and dramatic radiographic and clinical response to an ALK inhibitor (Crizotinib, PF02341066) in an ALK translocation-positive patient with non-small cell lung cancer. *J Thorac Oncol* 2010;5:2044–6.
- Bergthron K, Shaw AT, Ou SHI, Katayama R, Lovly CM, McDonald NT, et al. ROS1 rearrangements define a unique molecular class of lung cancers. *J Clin Oncol* 2012;30:863–70.
- Yasuda H, de Figueiredo-Pontes LL, Kobayashi S, Costa DB. Preclinical rationale for use of the clinically available multitargeted tyrosine kinase inhibitor crizotinib in ROS1-translocated lung cancer. *J Thorac Oncol* 2012;7:1086–90.
- Kwak EL, Bang YJ, Camidge DR, Shaw AT, Solomon B, Maki RG, et al. Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. *New Engl J Med* 2010;363:1693–703.
- Kobayashi S, Boggon TJ, Dayaram T, Janne PA, Kocher O, Meyerson M, et al. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. *New Engl J Med* 2005;352:786–92.
- Pao W, Miller VA, Politi KA, Riely GJ, Somwar R, Zakowski MF, et al. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. *PLoS Med* 2005;2:225–35.
- Engelman JA, Zejnullahu K, Mitsudomi T, Song YC, Hyland C, Park JO, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science* 2007;316:1039–43.
- Zhang ZF, Lee JC, Lin LP, Olivas V, Au V, LaFramboise T, et al. Activation of the AXL kinase causes resistance to EGFR-targeted therapy in lung cancer. *Nat Genet* 2012;44:852–+.
- Terai H, Soejima K, Yasuda H, Nakayama S, Hamamoto J, Arai D, et al. Activation of the FGF2-FGFR1 autocrine pathway: a novel mechanism of acquired resistance to gefitinib in NSCLC. *Mol Cancer Res* 2013;11:759–67.
- Choi YL, Soda M, Yamashita Y, Ueno T, Takashima J, Nakajima T, et al. EML4-ALK mutations in lung cancer that confer resistance to ALK inhibitors. *New Engl J Med* 2010;363:1734–9.
- Katayama R, Shaw AT, Khan TM, Mino-Kenudson M, Solomon BJ, Halmos B, et al. Mechanisms of acquired crizotinib resistance in ALK-rearranged lung cancers. *Sci Transl Med* 2012;4:120ra17.
- Doebele RC, Pilling AB, Aisner DL, Kutateladze TG, Le AT, Weickhardt AJ, et al. Mechanisms of resistance to crizotinib in patients with ALK gene rearranged non-small cell lung cancer. *Clin Cancer Res* 2012;18:1472–82.
- Sasaki T, Koivunen J, Ogino A, Yanagita M, Nikiforov S, Zheng W, et al. A novel ALK secondary mutation and EGFR signaling cause resistance to ALK kinase inhibitors. *Cancer Res* 2011;71:6051–60.
- Sasaki T, Janne PA. New strategies for treatment of ALK-rearranged non-small cell lung cancers. *Clin Cancer Res* 2011;17:7213–8.
- Yun CH, Mengwasser KE, Toms AV, Woo MS, Greulich H, Wong KK, et al. The T790M mutation in EGFR kinase causes drug resistance by increasing the affinity for ATP. *Proc Natl Acad Sci U S A* 2008;105:2070–5.
- Galkin AV, Melnick JS, Kim S, Hood TL, Li NX, Li LT, et al. Identification of NVP-TAE684, a potent, selective, and efficacious inhibitor of NPM-ALK. *Proc Natl Acad Sci U S A* 2007;104:270–5.
- Kinoshita K, Asoh K, Furuichi N, Ito T, Kawada H, Hara S, et al. Design and synthesis of a highly selective, orally active and potent anaplastic lymphoma kinase inhibitor (CH5424802). *Bioorgan Med Chem* 2012;20:1271–80.
- Marsilje TH, Pei W, Chen B, Lu WS, Uno T, Jin YH, et al. Synthesis, structure-activity relationships, and in vivo efficacy of the novel potent and selective anaplastic lymphoma kinase (ALK) inhibitor 5-chloro-N2-(2-isopropoxy-5-methyl-4-(piperidin-4-yl)phenyl)-N4-(2-(isopropylsulfonyl)phenyl)pyrimidine-2,4-diamine (LDK378) currently in phase 1 and phase 2 clinical trials. *J Med Chem* 2013;56:5675–90.
- Sakamoto H, Tsukaguchi T, Hiroshima S, Kodama T, Kobayashi T, Fukami TA, et al. CH5424802, a selective ALK inhibitor capable of blocking the resistant gatekeeper mutant. *Cancer Cell* 2011;19:679–90.
- Seto T, Kiura K, Nishio M, Nakagawa K, Maemondo M, Inoue A, et al. CH5424802 (RO5424802) for patients with ALK-rearranged advanced

- non-small-cell lung cancer (AF-001JP study): a single-arm, open-label, phase 1-2 study. *Lancet Oncol* 2013;14:590–8.
32. Gadgeel SM, Gandhi L, Riely GJ, Chiappori AA, West HL, Azada MC, et al. Safety and activity of alectinib against systemic disease and brain metastases in patients with crizotinib-resistant ALK-rearranged non-small-cell lung cancer (AF-002JG): results from the dose-finding portion of a phase 1/2 study. *Lancet Oncol* 2014;15:1119–28.
  33. Katayama R, Friboulet L, Koike S, Lockerman EL, Khan TM, Gainor JF, et al. Two novel ALK mutations mediate acquired resistance to the next-generation ALK inhibitor alectinib. *Clin Cancer Res* 2014;20:5686–96.
  34. Yasuda H, Park E, Yun CH, Sng NJ, Lucena-Araujo AR, Yeo WL, et al. Structural, biochemical, and clinical characterization of epidermal growth factor receptor (EGFR) exon 20 insertion mutations in lung cancer. *Sci Transl Med* 2013;5:216ra177.
  35. Tanizaki J, Okamoto I, Okabe T, Sakai K, Tanaka K, Hayashi H, et al. Activation of HER family signaling as a mechanism of acquired resistance to ALK inhibitors in EML4-ALK-positive non-small cell lung cancer. *Clin Cancer Res* 2012;18:6219–26.
  36. Yu HA, Riely GJ. Second-generation epidermal growth factor receptor tyrosine kinase inhibitors in lung cancers. *J Natl Compr Canc Netw* 2013;11:161–9.
  37. Yun CH, Boggon TJ, Li YQ, Woo MS, Greulich H, Meyerson M, et al. Structures of lung cancer-derived EGFR mutants and inhibitor complexes: Mechanism of activation and insights into differential inhibitor sensitivity. *Cancer Cell* 2007;11:217–27.
  38. Martini M, Vecchione L, Siena S, Tejpar S, Bardelli A. Targeted therapies: how personal should we go? *Nat Rev Clin Oncol* 2012;9:87–97.
  39. Roskoski R. The ErbB/HER family of protein-tyrosine kinases and cancer. *Pharmacol Res* 2014;79:34–74.
  40. Hammerman PS, Lawrence MS, Voet D, Jing R, Cibulskis K, Sivachenko A, et al. Comprehensive genomic characterization of squamous cell lung cancers. *Nature* 2012;489:519–25.
  41. Takeuchi K, Soda M, Togashi Y, Suzuki R, Sakata S, Hatano S, et al. RET, ROS1 and ALK fusions in lung cancer. *Nat Med* 2012;18:378–81.
  42. Lipson D, Capelletti M, Yelensky R, Otto G, Parker A, Jarosz M, et al. Identification of new ALK and RET gene fusions from colorectal and lung cancer biopsies. *Nat Med* 2012;18:382–4.
  43. Shepherd FA, Pereira JR, Ciuleanu T, Tan EH, Hirsh V, Thongprasert S, et al. Erlotinib in previously treated non-small-cell lung cancer. *New Engl J Med* 2005;353:123–32.
  44. Mok TS, Wu YL, Thongprasert S, Yang CH, Chu DT, Saijo N, et al. Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. *New Engl J Med* 2009;361:947–57.
  45. Shaw AT, Kim DW, Nakagawa K, Seto T, Crino L, Ahn MJ, et al. Crizotinib versus chemotherapy in advanced ALK-positive lung cancer. *New Engl J Med* 2013;368:2385–94.
  46. Tanimoto A, Yamada T, Nanjo S, Takeuchi S, Ebi H, Kita K, et al. Receptor ligand-triggered resistance to alectinib and its circumvention by Hsp90 inhibition in EML4-ALK lung cancer cells. *Oncotarget* 2014; 5:4920–8.