

Overcoming EGFR Bypass Signal-Induced Acquired Resistance to ALK Tyrosine Kinase Inhibitors in ALK-Translocated Lung Cancer

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Abstract

Activation of the EGFR pathway is one of the mechanisms inducing acquired resistance to anaplastic lymphoma kinase (ALK) tyrosine kinase inhibitors (TKI) such as crizotinib and alectinib. Ceritinib is a highly selective ALK inhibitor and shows promising efficacy in non-small cell lung cancers (NSCLC) harboring the ALK gene rearrangement. However, the precise mechanism underlying acquired resistance to ceritinib is not well-defined. This study set out to clarify the mechanism in ALK-translocated lung cancer and to find the preclinical rationale overcoming EGFR pathway-induced acquired resistance to ALK-TKIs. To this end, ceritinib-resistant cells (H3122-CER) were established from the H3122 NSCLC cell line harboring the

ALK gene rearrangement via long-term exposure to ceritinib. H3122-CER cells acquired resistance to ceritinib through EGFR bypass pathway activation. Furthermore, H3122 cells that became resistant to ceritinib or alectinib through EGFR pathway activation showed cross-resistance to other ALK-TKIs. Ceritinib and afatinib combination treatment partially restored the sensitivity to ceritinib.

Implications: This study proposes a preclinical rationale to use ALK-TKIs and afatinib combination therapy for ALK-translocated lung cancers that have acquired resistance to ALK-TKIs through EGFR pathway activation. *Mol Cancer Res*; 15(1): 106–14. ©2016 AACR.

Introduction

Lung cancer is the leading cause of cancer-related mortality worldwide (1). After the identification of the *EML4-ALK* gene rearrangement in 2007 (2), multiple ALK tyrosine kinase inhibitors (ALK-TKI) have been developed. These include crizotinib (3, 4), alectinib (5, 6), ceritinib (7, 8), brigatinib (9), and PF-06463922 (10). Crizotinib, a first-generation ALK-TKI, showed significant efficacy in ALK-translocated non-small cell lung cancer (NSCLC); the response rate was 57% (11) and 74% (12). Even in the presence of an initial favorable response, almost all lung cancers harboring the ALK gene rearrangement develop acquired resistance to crizotinib in 1 to 2 years. The

mechanisms underlying acquired resistance to crizotinib include mutations of the ALK gene such as L1196M, F1174L, C1156Y, G1202R, S1206Y, and G1269A or the activation of bypass pathways involving the EGFR or KIT (13–17). The *EML4-ALK* L1196M mutation is called a "gatekeeper" mutation because of the key location of the encoded amino acid substitutions at the entrance to a hydrophobic pocket at the posterior side of the ATP-binding cleft. This gatekeeper mutation induces a conformational change of the ATP-binding pocket of ALK, which consequently induces steric hindrance of crizotinib. Second-generation ALK-TKIs have been developed to overcome the acquired resistance to crizotinib through ALK gene mutations including L1196M. For example, alectinib and ceritinib showed promising efficacy in crizotinib-pretreated patients with NSCLC (18–20). Ceritinib is a second-generation ALK-TKI showing promising efficacy in patients with NSCLC harboring the ALK gene rearrangement. Indeed, recently, favorable results of ceritinib in patients with ALK-rearranged NSCLC were reported in a phase I and expansion phase study, which showed that the response rate was 58% among 114 patients who received at least 400 mg of ceritinib per day (20). In particular, ceritinib effectively inhibited ALK harboring the L1196M, G1269A, I1171T, and S1206Y mutations. In the aforementioned recent clinical trial, ceritinib showed promising efficacy in patients with crizotinib-pretreated NSCLC harboring ALK rearrangement. The response rate was 56% among 80 patients who had received crizotinib previously. In addition, ceritinib potently overcame crizotinib resistance in preclinical models (8). However, it is expected that lung cancer cells acquire resistance to ceritinib sooner or later. The mechanism

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underlying acquired resistance to ceritinib is not well studied yet. A recent study indicated a role of p-glycoprotein in mediating acquired resistance to ceritinib (21). We and others have repeatedly reported EGFR bypass pathway activation as one of the mechanisms underlying acquired resistance to ALK-TKIs in preclinical models (16, 22). However, currently, no clinically approved treatment exists for NSCLC harboring ALK gene rearrangement that shows EGFR bypass pathway activation.

In this study, we found EGFR pathway activation as a mechanism underlying acquired resistance to ceritinib. We confirmed the observation of EGFR pathway activation after ALK-TKI treatment in patient samples. Furthermore, we demonstrated that EGFR pathway-active H3122 cells showed cross-resistance to all clinically available ALK-TKIs. In addition, combination treatment consisting of the ceritinib and afatinib effectively inhibited the activation of both ALK and EGFR signals. We propose a preclinical rationale to use a combination therapy of afatinib and ALK-TKI for ALK-translocated lung cancers that acquired resistance to ALK-TKIs through EGFR pathway activation.

Materials and Methods

Cell lines

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). Cells were cultured in RPMI-1640 growth medium, supplemented with 10% FBS at 37°C in a humidified 5% CO₂ incubator. H3122-AR cells were established as previously described (22). Ba/F3 cells transduced with EGFR wild-type (Ba/F3 EGFR WT) were created as previously described (22). Ba/F3 EGFR WT cells were maintained in RPMI-1640 growth medium, supplemented with 10% FBS and EGF.

Reagents

Ceritinib was a gift from Novartis. Afatinib was purchased from LC Laboratories. Crizotinib, alectinib, and brigatinib were purchased from Selleck Chemicals. Total ALK antibody (#3791S), phospho-ALK (Y1282/1283) antibody (#9687S), total EGFR antibody (#4267S), 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 (22). Briefly, 3×10^3 cells per well were seeded in 96-well plates. Twenty-four hours after seeding the cells, various concentrations of each respective drug were added to each well. Control cells were treated with the same concentration of the vehicle (DMSO). The absorbance was measured 72 hours after treatment. All experiments were performed at least 3 times.

Immunoblotting analysis

Immunoblotting was performed as previously described (23). Briefly, cells were treated with ceritinib or afatinib at various concentrations. Cells were lysed in Cell Lysis Buffer

(Cell Signaling Technology). Equal amounts of protein per lane were loaded on SDS-PAGE. The separated proteins were transferred to polyvinylidene fluoride membranes. The membranes were incubated overnight with primary antibodies at 4°C and were subsequently incubated with secondary antibodies for 1 hour. For the detection of proteins, the membranes were incubated with LumiGLO reagent and peroxide (Cell Signaling Technology) under agitation followed by exposure to X-ray film.

Phospho-receptor tyrosine kinase array

The human phospho-RTK array kit was purchased from R&D Systems. Screening was performed according to the manufacturer's protocol. Signal intensity was calculated using the LumiVision Analyzer software (Aisin Seiki).

Apoptosis assay

The apoptosis assay was performed as previously described (22). Briefly, cells (1×10^5 cells/well) were seeded in 6-well plates. The cells were treated with ceritinib and afatinib, as single agents or combined. Cells were treated with ceritinib and afatinib at 1 μmol/L for 72 hours. Control cells were treated with the same concentration of the vehicle (DMSO). Apoptosis was assessed using the TACS Annexin V-FITC Apoptosis Detection Kit according to the manufacturer's protocol. The proportion of apoptotic cells was evaluated by flow cytometric analysis using the Gallios system (Beckman Coulter).

Quantitative RT-PCR

qRT-PCR experiments were performed as previously described (22). Total RNA was isolated from cells using an RNeasy Mini Kit (Qiagen). RNA was reverse transcribed using the High-Capacity RNA-to-cDNA Kit (Life Technologies) according to the manufacturer's protocol. Quantitative RT-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 primer sequences used in this study are available on request.

siRNAs targeting *EGF*, *TGFα*, and *HB-EGF*

Gene knockdown experiments using siRNA were performed as previously described (22). Cells were transfected with siRNAs that were specific for *EGF* (#s4520 and #s4521; Life Technologies), *TGFα* (#s14051 and #s14052; Life Technologies), *HB-EGF* (SASI_Hs01_00022632 and SASI_Hs01_00022633; Sigma), or *EGFR* (#s563 and #s564; Life Technologies), or with negative control siRNA. The Ambion Silencer Select Negative Control mix (Life Technologies) and siLentFect transfection reagent (Bio-Rad) were used according to the manufacturer's protocol. We confirmed knockdown of *EGF*, *TGFα*, and *HB-EGF* by qRT-PCR.

FISH

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

Human tissue sample study

The study was reviewed and approved by the Institutional Review Board of Keio University School of Medicine (Tokyo, Japan). Tumor samples were obtained with written informed consent. One patient with NSCLC harboring the *EML4-ALK* gene

rearrangement was included in this study. The phosphorylation levels of EGFR prior to administration of the ALK-TKI, crizotinib, and after disease progression were compared by immunohistochemistry (IHC).

Statistical analysis

Statistical analysis was performed using the GraphPad Prism software, version 4.0 (GraphPad Software). The Student *t* test was used for comparisons. All *P* values were 2-sided; values of *P* < 0.05 were regarded as statistically significant.

Results

Establishment of ceritinib-resistant H3122 cells

To clarify the mechanisms underlying acquired resistance to ceritinib in ALK-translocated lung cancer cells, we cultured H3122 cells, which harbor the *EML4-ALK* gene rearrangement, with ceritinib to establish ceritinib-resistant H3122 (H3122-CER) cells. The initial ceritinib concentration was 0.01 $\mu\text{mol/L}$, which was incrementally increased to 1 $\mu\text{mol/L}$. After an about 6-month exposure to ceritinib, H3122 cells acquired resistance to ceritinib (Fig. 1A). The calculated IC_{50} value of H3122 parental cells was 132 nmol/L whereas that of H3122-CER cells was 2574 nmol/L. Next, we performed FISH analysis of H3122 parental cells and H3122-CER cells because loss of *EML4-ALK* gene rearrangement has been shown as one of the mechanisms underlying acquired resistance to crizotinib (15).

We confirmed the existence of *EML4-ALK* gene rearrangement in both H3122 parental cells and H3122-CER cells (Fig. 1B). *EML4-ALK*-induced signaling is transduced to downstream signals including the PI3K/AKT and the ERK/MAPK pathways. Ceritinib effectively inhibited the phosphorylation of ALK, AKT, and ERK1/2 in H3122 parental cells, whereas the inhibition of AKT and ERK1/2 phosphorylation was attenuated in H3122-CER cells (Fig. 1C). In addition, the phosphorylation level of ALK was reduced in H3122-CER cells compared to that in H3122 parental cells, suggesting that H3122-CER cells are dependent on survival signals other than ALK. These data indicate that H3122-CER cells acquired resistance to ceritinib.

Increased phosphorylation of EGFR in H3122-CER cells

To clarify the mechanisms underlying acquired resistance to ceritinib, 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 to 28. Next, to identify the survival signal other than ALK in H3122-CER cells, we performed a human phospho-RTK array and found that ALK phosphorylation was attenuated in H3122-CER cells compared with that in H3122 parental cells (Fig. 2A and B). In contrast, EGFR phosphorylation was increased in H3122-CER cells (Fig. 2A and B). We confirmed the increased EGFR phosphorylation using immunoblotting, which further revealed that the expression of total EGFR was not affected

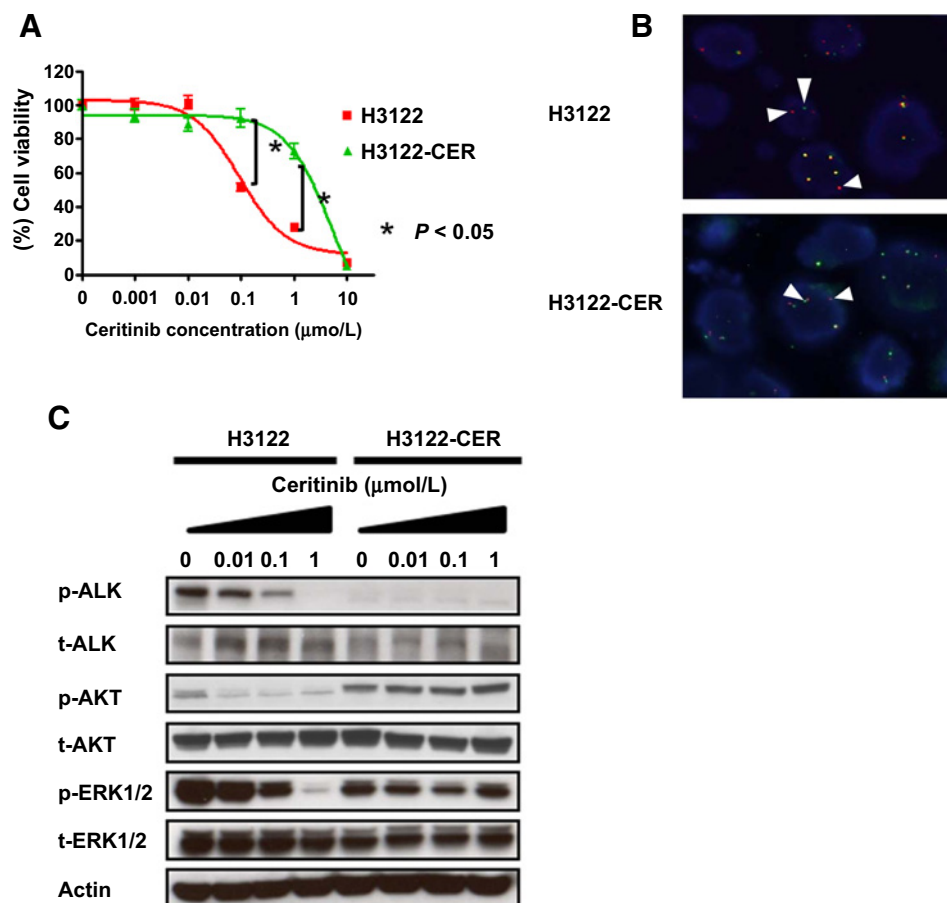


Figure 1.

Establishment of ceritinib-resistant H3122 cells. **A**, Results of MTS cell proliferation assays following cell treatment with the indicated ceritinib concentrations for 72 hours. Error bars indicate SD. **B**, Results of 2-color FISH analysis of H3122 and H3122-CER cells are shown. Arrowheads indicate isolated or split red signals. **C**, Immunoblotting analysis of H3122 and H3122-CER cells for phosphorylated (p-) and total (t-) ALK, AKT, and ERK1/2. Actin was used as a loading control. The cells were treated with ceritinib for 2 hours.

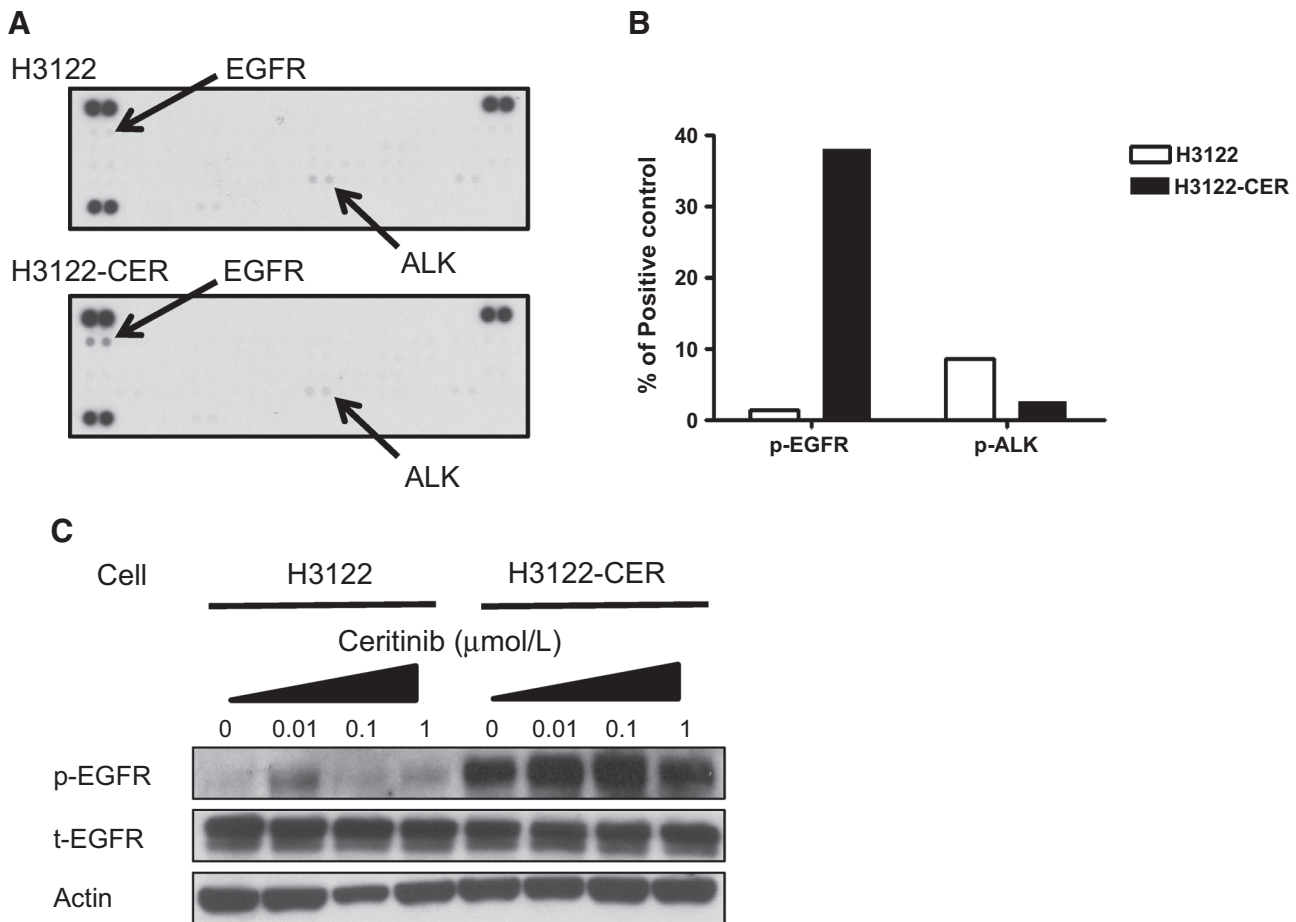


Figure 2. Increased phosphorylation of EGFR in H3122-CER cells. **A**, Results of human phospho-RTK array experiments in H3122 and H3122-CER cells. The spots for ALK and EGFR are indicated with arrows. **B**, Average proportional intensity of the spots for EGFR and ALK are shown. **C**, Immunoblotting analysis of H3122 and H3122-CER cells showing increased phosphorylation of EGFR (p-EGFR) in H3122-CER cells. Total EGFR (t-EGFR) is also shown. Actin was used as a loading control. The cells were treated with ceritinib for 2 hours.

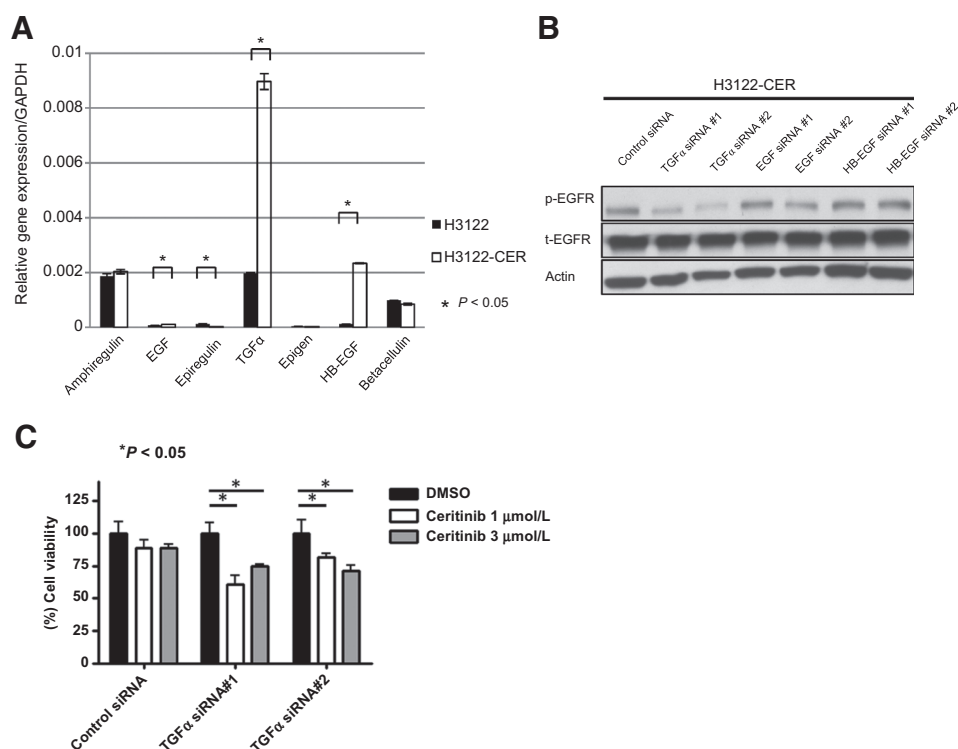
(Fig. 2C). We found that phosphorylation of EGFR in H3122-CER cells was not affected by ceritinib treatment. These results indicate that the EGFR pathway is activated in H3122-CER cells.

To clarify the mechanism underlying EGFR pathway activation, we first performed cDNA sequencing of the tyrosine kinase domain of the *EGFR* gene because a somatic mutation of the *EGFR* gene corresponding to the tyrosine kinase domain is a common mechanism of EGFR activation in NSCLC (24, 25). However, we found no mutation in the *EGFR* gene. Next, we examined the expression level of EGFR ligands by quantitative RT-PCR. We found that some ligands, including *TGF α* , were significantly increased in H3122-CER cells compared with those in H3122 parental cells (Fig. 3A). To assess whether this increased expression of EGFR ligands might contribute to EGFR pathway activation, we performed gene knockdown experiments using siRNA. We knocked down 3 EGFR ligands, that is, *TGF α* , *EGF*, and *HB-EGF*. We found that *TGF α* knockdown effectively inhibited the phosphorylation of EGFR (Fig. 3B). In addition, we found that *TGF α* knockdown partially restored the sensitivity of H3122-CER cells to ceritinib (Fig. 3C). These data indicate that increased expression of EGFR ligands

contribute to the activation of EGFR pathway in H3122-CER cells.

Increased phosphorylation of EGFR after ALK-TKI treatment in human tumors

We and others have previously reported EGFR pathway activation in acquired resistance to ALK-TKIs such as crizotinib and alectinib (16, 22). To determine the potential clinical relevance of our findings and to ascertain whether the activation of the EGFR pathway may also be found in human tumors, we sought to identify patients who had received ALK-TKI treatment and experienced disease progression. We identified one patient, a 51-year-old woman, who had NSCLC with the *EML4-ALK* gene rearrangement and had been treated with crizotinib. Her left neck lymph nodes had shrunk after crizotinib treatment, which was confirmed by computed tomography at 39 days after the initiation of crizotinib treatment; however, she experienced disease progression after 1 year of crizotinib treatment (Fig. 4A). Tumor samples were obtained and examined for phospho-EGFR using IHC. The surface of some cancer cells was stained in a pretreatment tumor sample (Fig. 4B). However, in a posttreatment tumor sample, the

**Figure 3.**

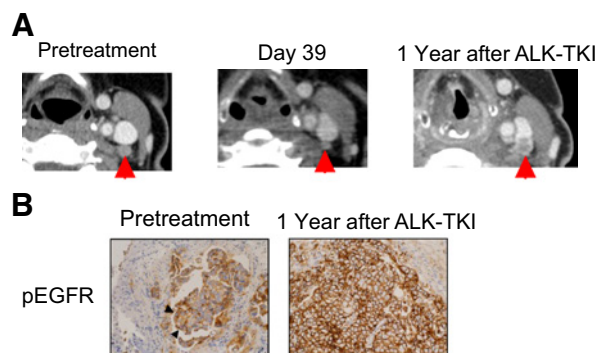
Contribution of EGFR ligands to the activation of the EGFR pathway in H3122-CER cells. **A**, Expression of the indicated genes is shown for H3122 and H3122-CER cells relative to the expression of *GAPDH*. Error bars indicate SD. **B**, Immunoblotting analysis of H3122-CER cells transfected with control siRNA, *TGFα*-targeted siRNAs (#1, #2), *EGF*-targeted siRNAs (#1, #2), or *HB-EGF*-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-CER cells treated with the indicated ceritinib concentrations are shown, following transfection with control siRNA or *TGFα*-targeted siRNAs (#1, #2). Error bars indicate SD.

surface of almost all cancer cells was strongly stained for phospho-EGFR (Fig. 4B). These data indicate that increased phosphorylation of EGFR after ALK-TKI treatment may also be found in human tumors.

H3122-AR cells and H3122-CER cells have cross-resistance to ALK-TKIs

We performed the MTS proliferation assay to examine whether EGFR pathway-activated, ALK-translocated lung cancer cells have cross-resistance to other ALK-TKIs. In addition to H3122-CER cells, we used H3122-AR cells, which are also H3122-derived cells. H3122-AR cells are alectinib-resistant H3122 cells, which acquire

resistance to alectinib through *TGFα*-induced EGFR pathway activation (22). We found that both H3122-AR cells and H3122-CER cells were resistant to all ALK-TKIs examined in this study, that is, crizotinib, alectinib, and ceritinib. The calculated IC_{50} values of crizotinib, alectinib, and ceritinib in H3122-AR cells were 1,927, $>1 \times 10^4$, and 1,611 nmol/L, respectively (Fig. 5A); in H3122-CER cells, these IC_{50} values were 3,979, $>1 \times 10^4$, and 4,632 nmol/L, respectively (Fig. 5B). Brigatinib is another second-generation ALK-TKI, which is reported to inhibit EGFR harboring gene-activating mutations in addition to ALK. The *in vitro* kinase assay data of brigatinib for more than 250 kinases, including ALK and EGFR, have been published previously (26). The IC_{50} values of brigatinib for ALK and EGFR were 0.62 and 129 nmol/L, respectively. We evaluated the efficacy of brigatinib in H3122 and H3122-CER cells. Brigatinib potently inhibited the proliferation of H3122 parental cells; however, its inhibition of H3122-CER cell proliferation was significantly less potent (Fig. 5C). The calculated IC_{50} value of brigatinib in H3122 and H3122-CER cells was 20 and 699 nmol/L, respectively. We performed immunoblotting to assess whether brigatinib specifically inhibited the phosphorylation of EGFR in H3122 and H3122-CER cells and found that brigatinib did not effectively inhibit EGFR phosphorylation at Tyr1086 (Fig. 5D). In addition, we performed the MTS assay using Ba/F3 cells transfected with wild-type EGFR. Brigatinib did not effectively inhibit the proliferation of these cells either (Supplementary Fig. S1). These data indicate that the effect of brigatinib is independent of EGFR pathway inhibition and that none of the tested ALK-TKIs can effectively inhibit ALK-translocated lung cancer cells that have acquired resistance through EGFR pathway activation. Moreover, they indicate that ALK-translocated lung cancer cells have cross-resistance to ALK-TKIs.

**Figure 4.**

Increased phosphorylation of EGFR in human tumors after ALK-TKI treatment. **A**, Representative computed tomography images of a patient who was treated with crizotinib (500 mg/d). **B**, Representative IHC images of pretreatment and posttreatment tumor staining for phosphorylated EGFR (p-EGFR). Arrowheads indicate cancer cells that are stained in the pretreatment sample.

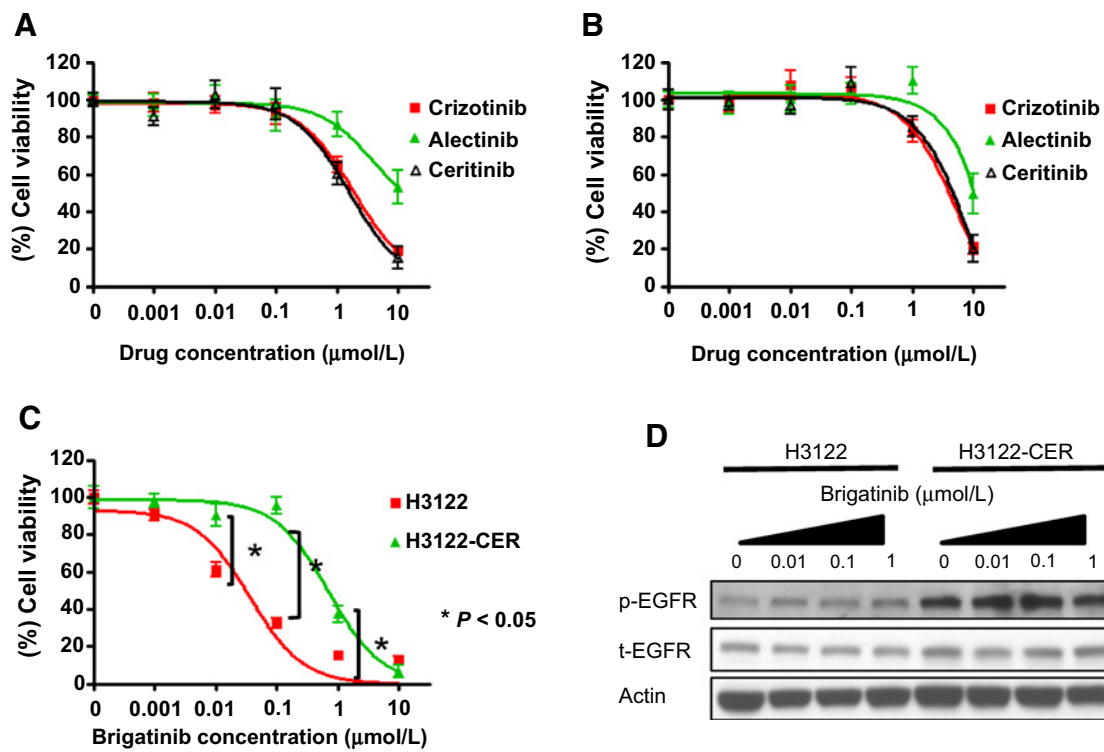


Figure 5.

H3122-AR cells and H3122-CER cells have cross-resistance to ALK TKIs. **A**, Results of the MTS cell proliferation assay. H3122-AR cells were treated with the indicated crizotinib, alectinib, and ceritinib concentrations for 72 hours. Error bars indicate SD. **B**, Results of the MTS cell proliferation assay. H3122-CER cells were treated with the indicated crizotinib, alectinib, and ceritinib concentrations for 72 hours. Error bars indicate SD. **C**, Results of the MTS cell proliferation assays following treatment with the indicated brigatinib concentrations for 72 hours. Error bars indicate SD. **D**, Immunoblotting analysis of H3122 and H3122-CER cells showing that brigatinib did not inhibit EGFR phosphorylation (p-EGFR). Total EGFR (t-EGFR) is also shown. Actin was used as a loading control. The cells were treated with brigatinib for 2 hours.

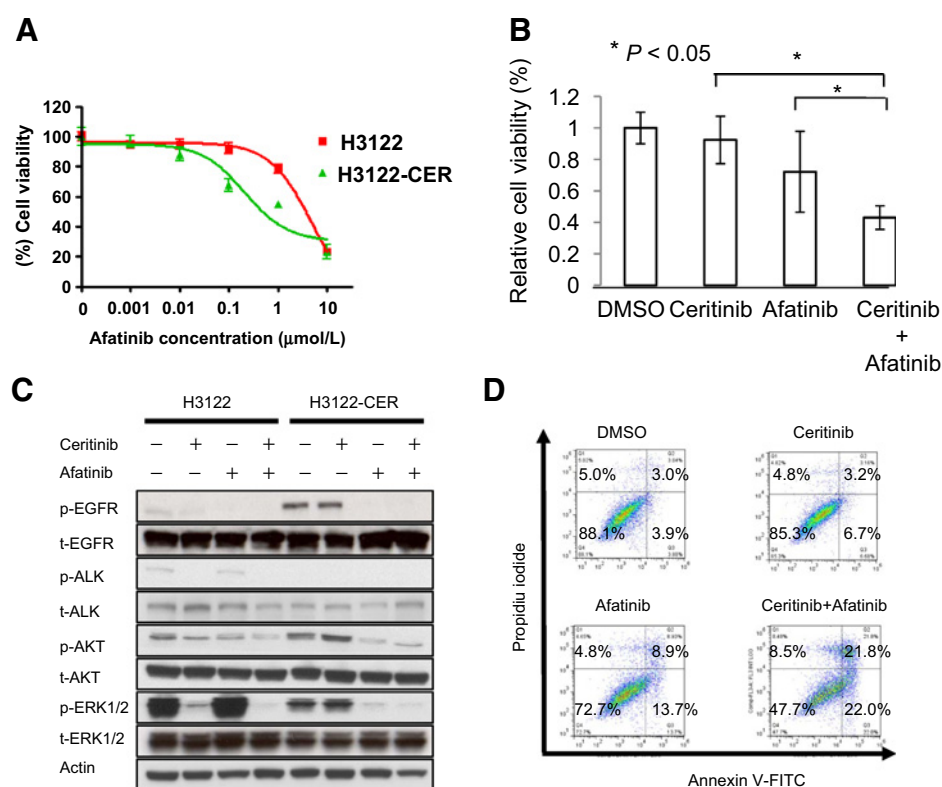
Overcoming EGFR pathway-induced acquired resistance by combination treatment with ceritinib and afatinib

We performed further *in vitro* experiments to obtain preclinical evidence that combination therapy targeting ALK and EGFR is a rationale for treatment of ALK-translocated lung cancer cells that acquired resistance to ceritinib through EGFR pathway activation. First, we treated H3122 parental cells and H3122-CER cells with afatinib, an EGFR-TKI (27), because of our observation of decreased ALK phosphorylation in H3122-CER cells. We found that the afatinib-induced inhibition of proliferation was more significant in H3122-CER cells compared with that in H3122 parental cells, indicating that H3122-CER cells were dependent mainly on EGFR (Fig. 6A). Next, we treated H3122 parental cells and H3122-CER cells with ceritinib or afatinib alone or in combination. We found that the ceritinib and afatinib combination treatment induced significant inhibition of proliferation compared with that obtained with single-drug treatment, indicating that H3122-CER cell proliferation was dependent on both ALK and EGFR (Fig. 6B). Using immunoblotting, we evaluated whether ceritinib and afatinib combination treatment effectively inhibited the activation of the ALK and EGFR pathway. Ceritinib effectively inhibited the phosphorylation of ALK, AKT, and ERK1/2 in H3122 parental cells. In contrast, ceritinib did not inhibit the phosphorylation of AKT and ERK1/2 in H3122-CER cells as mentioned above. However, ceritinib and afatinib combina-

tion treatment effectively inhibited the phosphorylation of AKT and ERK1/2 in H3122-CER cells after 2 hours of treatment (Fig. 6C). In addition, after 24 hours of treatment, ceritinib and afatinib combination treatment more effectively inhibited the phosphorylation of AKT in H3122-CER cells compared with afatinib alone (Supplementary Fig S2). Furthermore, apoptosis induction was significantly increased in H3122-CER cells treated with both ceritinib and afatinib compared with that in H3122-CER cells treated with either ceritinib or afatinib alone (Fig. 6D). These data indicate that ceritinib and afatinib combination treatment is effective against ceritinib-resistant, ALK-translocated lung cancer cells.

Discussion

Recent molecular characterizations of lung cancer have identified multiple "druggable" targets, such as *EGFR*, *ALK*, *ROS1*, or *RET* rearrangement (2, 25, 28, 29). To target these druggable targets, multiple molecular-targeted inhibitors have been developed. These inhibitors have dramatically improved the prognosis of patients with lung cancer and have changed the clinical treatment strategy for patients with lung cancer. However, still, most patients with lung cancer die. Although these molecular-targeted inhibitors are efficacious treatments for lung cancers harboring the aforementioned genetic alterations, cancer cells inevitably acquire resistance to the inhibitors. Indeed, overcoming acquired

**Figure 6.**

Overcoming EGFR pathway-induced acquired resistance using a ceritinib-afatinib combination treatment. **A**, Results of the MTS cell proliferation assay. H3122 and H3122-CER cells were treated with the indicated afatinib concentrations. Error bars indicate SD. **B**, Results of the MTS cell proliferation assay of H3122-CER cells treated with or without ceritinib (1 μmol/L) and afatinib (1 μmol/L) for 72 hours. Error bars indicate SD. **C**, Immunoblotting analysis of H3122 and H3122-CER cells. The cells were treated with or without ceritinib (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**, Proportion of Annexin V-positive and/or propidium iodide-positive cells was assessed by flow cytometry. H3122-CER cells were treated with or without ceritinib and afatinib for 72 hours.

resistance is important for improving the prognosis of patients with lung cancer.

EML4-ALK gene rearrangement was first identified in 2007 in about 7% of NSCLCs (2). Crizotinib, the first market-approved ALK-TKI, is effective against ALK-translocated lung cancers with a response rate of 57% (11) and 74% (12). However, in about 1 year, lung cancer cells acquire resistance to crizotinib. Alectinib and ceritinib are reported to be effective for crizotinib-treated and -resistant lung cancers. The response rate of alectinib and ceritinib for crizotinib-treated and -resistant lung cancers was 50% to 55% and 56%, respectively (19, 20, 30). Nevertheless, lung cancer cells acquire resistance to alectinib or ceritinib. Previously reported mechanisms underlying acquired resistance to alectinib included 2 novel *ALK* mutations, that is, V1180L and I1171T, as well as EGFR pathway activation (22, 31).

Ceritinib is an oral, ATP-competitive TKI showing a 20-fold more potent inhibition of ALK than is reported for crizotinib (7, 8). Ceritinib is not effective against G1202R and F1174C crizotinib-resistant mutations (8). However, the mechanisms underlying acquired resistance to ceritinib are not yet fully clarified.

Here, we demonstrated that EGFR pathway activation contributed to the acquired resistance to ceritinib. We showed that EGFR pathway activation is repeatedly found in cells exposed to ALK-TKIs *in vitro* and observed EGFR pathway activation in human lung cancer samples. In addition, by using H3122-AR cells and H3122-CER cells, we showed that ALK-translocated lung cancer cells that had acquired resistance to ALK-TKI through EGFR pathway activation have cross-resistance to all ALK-TKIs tested in this study. We believe this finding is clinically important, because generally, once lung cancer cells acquire resistance to one ALK-TKI, usually crizotinib, treatment is continued with

another ALK-TKI. However, given that the mechanism underlying resistance to ALK-TKI is through EGFR pathway activation as we demonstrate here, switching from one ALK-TKI to another one is not effective.

Brigatinib is a novel and potent ALK inhibitor. Previous work showed that brigatinib inhibited EGFR mutants in addition to ALK (9). Therefore, we expected that brigatinib might be effective against ALK-translocated lung cancer cells that had acquired resistance to ALK-TKIs through EGFR pathway activation. Indeed, in preclinical models, brigatinib inhibited wild-type EGFR at an IC_{50} value of 129 nmol/L (26). However, in the present study, brigatinib did not effectively inhibit the activation of EGFR. Considering the difference in the IC_{50} value of brigatinib for ALK (0.62 nmol/L) and EGFR (129 nmol/L), brigatinib may not be sufficiently potent to inhibit the activation of wild-type EGFR.

None of the ALK-TKIs tested in the present study showed effective inhibition of ALK-translocated lung cancer cells that had acquired resistance through EGFR pathway activation when used as mono treatment. Therefore, we propose a combination treatment using the ALK-TKI ceritinib and the EGFR-TKI afatinib because afatinib effectively restored the sensitivity of H3122-CER cells to ceritinib and significantly increased apoptosis. Thus, an afatinib-ALK-TKI combination treatment may be an effective therapy against ALK-translocated lung cancer cells that have acquired resistance to ALK-TKI through EGFR pathway activation.

Nevertheless, currently, it is not known whether this combination treatment is clinically safe and effective in patients with ALK-translocated lung cancer, especially because combination treatments occasionally cause a significant increase in treatment-related toxicity. A previously published safety profile of ceritinib indicated that the dose-limiting toxic morbidities included diarrhea, vomiting, nausea, dehydration, elevated alanine

aminotransferase levels, and hypophosphatemia (20). These toxicity-related morbidities were resolved after ceritinib discontinuation. The Lux-Lung 3 trial addressed the safety profile of afatinib, revealing that the most common afatinib-related grade 3 or 4 adverse events were rash or acne, diarrhea, and paronychia [37 (16%), 33 (14%), and 26 (11%) of 229 patients, respectively; ref. 32]. Thus, a dose-adjusted clinical trial is warranted to effectively evaluate the safety and efficacy of a ceritinib–afatinib combination treatment.

It is unclear how many ALK-translocated lung cancers acquire resistance through EGFR pathway activation. Given the considerable efficacy of ceritinib in patients with prior crizotinib treatment (the response rate of ceritinib was 56%), the majority of the mechanisms underlying acquired resistance to crizotinib appears to be ALK-induced resistance. To obtain an estimation of the proportion of EGFR-induced acquired resistance, it is necessary to compare the IHC data of phosphorylated EGFR between tumor samples obtained before and after ALK-TKI treatment.

In summary, here, we demonstrated EGFR pathway–induced resistance as mechanism underlying acquired resistance to ceritinib. We found that ALK-translocated lung cancer cells that had acquired resistance to ALK-TKI had cross-resistance to other ALK-TKIs. We propose a preclinical rationale for the use of a combination therapy consisting of the EGFR-TKI afatinib and the ALK-TKI ceritinib for the treatment of ALK-translocated lung cancers that have acquired resistance to ALK-TKIs through EGFR pathway activation.

Disclosure of Potential Conflicts of Interest

K. Soejima has provided expert testimony for Chugai, Ono, Taiho, Eli Lilly, AstraZeneca, Pfizer, and Shionogi. No potential conflicts of interest were disclosed by the other authors.

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