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Elucidation of Resistance Mechanisms to Second-Generation ALK Inhibitors Alectinib and Ceritinib in Non–Small Cell Lung Cancer Cells

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Abstract

Crizotinib is the first anaplastic lymphoma kinase (ALK) inhibitor to have been approved for the treatment of non–small cell lung cancer (NSCLC) harboring an ALK fusion gene, but it has been found that, in the clinic, patients develop resistance to it. Alectinib and ceritinib are second-generation ALK inhibitors which show remarkable clinical responses in both crizotinib-naive and crizotinib-resistant NSCLC patients harboring an ALK fusion gene. Despite their impressive activity, clinical resistance to alectinib and ceritinib has also emerged. In the current study, we elucidated the resistance mechanisms to these second-generation ALK inhibitors in the H3122 NSCLC cell line harboring the EML4-ALK variant 1 fusion *in vitro*. Prolonged treatment of the parental H3122 cells with alectinib and ceritinib led to two cell lines which are 10 times less sensitive to alectinib and ceritinib than the parental H3122 cell line. Although mutations of ALK in its kinase domain are a common resistance mechanism for crizotinib, we did not detect any ALK mutation in these resistant cell lines. Rather, overexpression of phospho-ALK and alternative receptor tyrosine kinases such as phospho-EGFR, phospho-HER3, and phospho-IGFR-1R was observed in both resistant cell lines. Additionally, NRG1, a ligand for HER3, is upregulated and responsible for resistance by activating the EGFR family pathways through the NRG1-HER3-EGFR axis. Combination treatment with EGFR inhibitors, in particular afatinib, was shown to be effective at overcoming resistance. Our study provides new mechanistic insights into adaptive resistance to second-generation ALK inhibitors and suggests a potential clinical strategy to combat resistance to these second-generation ALK inhibitors in NSCLC.

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Introduction

The unprecedented success enjoyed by epidermal growth factor receptor (EGFR) inhibitors in the treatment of non–small cell lung cancer (NSCLC) patients with an EGFR active mutation has made targeted therapy a promising new anticancer strategy for NSCLC [1]. Specific genetic alterations of anaplastic lymphoma kinase (ALK) [2]; hepatocyte growth factor receptor, also known as MET [3]; Kirsten rat sarcoma viral oncogene homolog (KRAS) [4]; B-Raf proto-oncogene [5]; Ros proto-oncogene1 (Ros1) [6]; and Ret proto-oncogene [7] were subsequently identified, and small-molecule inhibitors that target these specific tyrosine kinases have been pursued as novel anticancer treatments for NSCLC [8]. Crizotinib, a potent ALK inhibitor, has demonstrated impressive clinical activity in NSCLC patients whose tumors harbor the EML4-ALK fusion gene

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and has become the second class of successful targeted therapy for the treatment of NSCLC [2]. Unfortunately, most patients treated with crizotinib relapse after a remarkable initial response, with a median progression-free survival of 10.5 months in clinical trials [9]. Various mutations in the ALK kinase domain, alternative bypass pathway activation, and epithelial to mesenchymal transition have been identified as contributors to crizotinib resistance [9–12].

To overcome crizotinib resistance, second-generation ALK inhibitors have been developed. Ceritinib (LDK378) was the first second-generation ALK inhibitor approved by the US Food and Drug Administration (FDA) in 2014 and is now being used for the treatment of NSCLC patients who had progressed during crizotinib treatment or were intolerant of crizotinib [13,14]. Another second-generation ALK inhibitor, alectinib (CH5424802), has also shown impressive responses in clinical studies in crizotinib-naive or crizotinib-resistant ALK-positive NSCLC patients [15–17]. These two second-generation ALK inhibitors are not only more efficacious than crizotinib but also show activity toward the resistant ALK mutations that arise during crizotinib treatment.

Perhaps unsurprisingly, a number of recent studies have now shown that resistance also develops to alectinib and ceritinib in both preclinical settings and in ALK-positive patients. Novel ALK-resistant mutations (V1180L and G1123S) and refractory ALK-resistant mutations (G1202R and F1174C) have been reported to exhibit resistance to these second-generation ALK inhibitors [18–21]. The V1180L-resistant mutation to alectinib was developed in an *in vitro* study using an NSCLC cell line but is still sensitive to ceritinib. The ALK-G1123S mutation was identified in an NSCLC patient who progressed on ceritinib treatment, but this patient was still responsive to alectinib treatment, suggesting that the G1123S ALK mutation is sensitive to alectinib. However, the ALK-G1202R and F1174C mutations showed significant resistance to both alectinib and ceritinib. Moreover, in an ongoing clinical study with ceritinib, several patients (6 out of 10, 60%) developed ceritinib resistance, but their tumors did not harbor any detectable ALK-resistant mutations [13]. These clinical data suggest that, in addition to ALK mutations, other resistance mechanisms exist for these second-generation ALK inhibitors.

In this study, we have investigated the mechanisms of resistance to ceritinib and alectinib using the NCI-H3122 NSCLC cell line which harbors the EML4-ALK fusion gene variant 1. Our data showed that no ALK-resistant mutations were detected when the NCI-H3122 cells acquired resistance *in vitro* to these next-generation ALK inhibitors. Instead, the primary resistance mechanism to ceritinib and alectinib found in our study was the activation of alternative receptor tyrosine kinase (RTK) pathways, in particular the NRG1-HER3-EGFR axis. Accordingly, we explored strategies to overcome resistance to these second-generation ALK inhibitors and found that the combination of ALK inhibitors with afatinib, a small-molecule inhibitor targeting both wild-type and mutated EGFR, is effective in overcoming resistance to these second-generation ALK inhibitors.

Materials and Methods

Reagents

Alectinib (CH5424802), ceritinib (LDK378), crizotinib, erlotinib, AZD9291, AZD 8931, afatinib, AP26113, and PF06463922 were purchased from Selleckchem (Houston, TX). Epithelial growth factor (EGF), amphiregulin, neuregulin-1 (NRG1), and insulin growth factor (IGF) were purchased from R&D systems (Minneapolis, MN). All reagents were stored at −20°C.

Cell Culture and Cell Viability Assay

The NCI-H3122 cell line harboring the fusion gene EML4-ALK variant 1, and the Karpas 299 cell line and the SU-DHL-1 cell line harboring the fusion gene NPM-ALK were purchased from American Type Culture Collection (Manassas, VA) and cultured in RPMI1640 medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum (Gibco). Cells were maintained in a cell culture incubator at 37°C in a humidified atmosphere with 5% CO_2.

Cell viability was evaluated by a WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] assay (Dojindo Molecular Technologies, Inc., Rockville, MD). Cells were plated in 96-well plates and cultured overnight to allow cells to attach, and then the drug was added at indicated concentrations for 96 hours. Cell culture media containing the drug were washed, 10% WST-8 dye (100 μl) was added to each well and incubated for an additional hour, and the absorbance at 450 nm was measured in a microplate reader (Molecular Devices, Sunnyvale, CA). Cell growth inhibition was evaluated as the ratio of the absorbance of the drug-treated samples to that of the DMSO-treated control and analyzed by Prism 6 software. All experiments were carried out in triplicate.

Establishment of In Vitro ALK Resistance Models

Alectinib- and ceritinib-resistant models were established by exposing cells to a high drug concentration (1 μM) for 3 days. The drug-tolerant cells were allowed to expand and regain proliferation rates comparable to those of the parental cells. The surviving cells were then exposed to drugs again for 3 days. This process was repeated until the cells grew at a comparable rate in either the absence or the presence of 1 μM alectinib or ceritinib.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR), Sequencing, Quantitative Real-Time PCR (qRT-PCR)

Total RNA was isolated from 5×10^5 cells using the RNeasy Mini Kit (Qiagen, Valencia, CA), cDNA was generated *via* a SuperScript III one-step RT-PCR system (Invitrogen, Carlsbad, CA) according to manufacturer's instructions, and cDNA was then PCR amplified with specific primers that cover the whole ALK kinase domain coding region (primers: forward: GTACAAGCTGAGCAAGCTCCGCAC; reverse: AGGCACTTTCTCTTCCTCTTCCAC) [9]. The PCR products were purified using a PCR Purification Kit (Qiagen) prior to Sanger dideoxynucleotide sequencing (Sequencing Core at the University of Michigan). For qRT-PCR, Taqman probes ALK (Hs01058318_m1), EGFR (Hs01076078_m1), HER3 (Hs00176538_m1), IGF-1R (Hs00609566_m1), EGF (Hs01099999_m1), IGF (Hs01547656_m1), amphiregulin (Hs00950669_m1), NRG1 (Hs00247620_m1), and GAPDH (Hs02758991_g1) as an endogenous control were purchased from Life Technologies (Carlsbad, CA). qRT-PCR was performed according to manufacturer's instructions, and data were analyzed using the △△Ct method.

Western Blotting and Phospho-RTK Array

Total protein was isolated from cells as indicated in each experiment. The following primary antibodies were used and purchased from Cell Signaling Technology (Beverly, MA): rabbit anti-total EGFR, total

ALK, total HER3, total IGF-1R, total ERK1/2, total AKT, phospho-ALK (pTry1604), phospho-EGFR (pTyr1068), phospho-HER3 (pTyr1222), phospho-IGF-1R (pTry1135), phospho-ERK1/2 (pThr202/Tyr204), and phospho-AKT (pSer473). The primary antibodies were diluted 1:1000 in blocking buffer and incubated with the membranes overnight at 4°C. Membranes were washed with Tris-Buffered Saline and Tween 20 (TBST) buffer three times, and goat anti-rabbit IgG secondary antibody was applied for 1 hour at room temperature. HRP-conjugated anti-GAPDH antibody was purchased from Life Technologies and diluted 1:2000 for use.

To detect phosphorylation of human kinases, the Proteome Profiler Human Phospho-Kinase Array Kit from R&D Systems (Minneapolis, MN) was employed. For this assay, 300 μg of total proteins was isolated from cells, and the Phospho-Kinase Array Kit was used according to manufacturer's instructions to detect the phosphorylation of 43 human kinases.

Immunoprecipitation Assay

A total of 1000 μg of protein was extracted from cells; then 1 μg of anti-HER3 antibody (Cell Signaling Technology) was added to the protein lysate and incubated for 1 hour at 4°C with gentle mixing. For each sample, 20 μl of Protein A Agarose beads (Sigma-Aldrich, St. Louis, MO) was added to 1.5-ml tubes and washed with lysis buffer four times before being added to the protein lysate containing the anti-HER3 antibody and incubated overnight at 4°C. The beads containing the antibody-target protein complexes were washed five times and then pelleted by centrifugation, SDS loading buffer was added, and samples were boiled for 5 minutes to separate beads and proteins. Beads were pelleted by centrifugation, and supernatants were collected and submitted to Western blot analysis for detection of p-EGFR as described above. EGFR pulled-down products were also collected to detect p-HER3 presence, and EGFR antibody for immunoprecipitation (IP) study was also purchased from Cell Signaling Technology.

Transfection of siRNA

siRNAs [ON-TARGET plus Human ERBB3 (2065) siRNA–SMART pool, L-003127-00] against the HER3 target sequence purchased from GE Healthcare (Pittsburgh, PA) were used to knock down HER3. Cells were seeded in six-well plates, and siRNAs were transfected into cells with the help of Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol.

Software and Statistical Analysis

Dose-response curves were analyzed using Graphpad Prism 6 software. qRT-PCR data were analyzed using the ΔΔCt method, normalized to the proper housekeeping gene (GAPDH).

Results

Establishment of Two Adaptive Drug Resistance Cell Models to Alectinib and Ceritinib

Two adaptive drug resistance cell models, to alectinib and ceritinib, were generated by exposing H3122 cells harboring the EML4-ALK variant 1 to these drugs for an extended period of time. After 5 months of intermittent exposure to 1 μM alectinib, the cells became resistant and were named CHR H3122. After 7 months of intermittent exposure to 1 μM ceritinib (LDK378), the cells became resistant and were named LDKR H3122. Both resistant cells were then cultured in the presence of 1 μM of their respective ALK inhibitor to maintain their resistance. As shown in Figure 1, *A* and *B*, the resistant cells displayed approximately 10-fold lower sensitivities to ALK inhibitors when compared with the parental cells. Cross--resistance to these two ALK inhibitors was also observed (Figure 1, *A* and *B*). Additionally, both CHR H3122 and LDKR H3122 cells

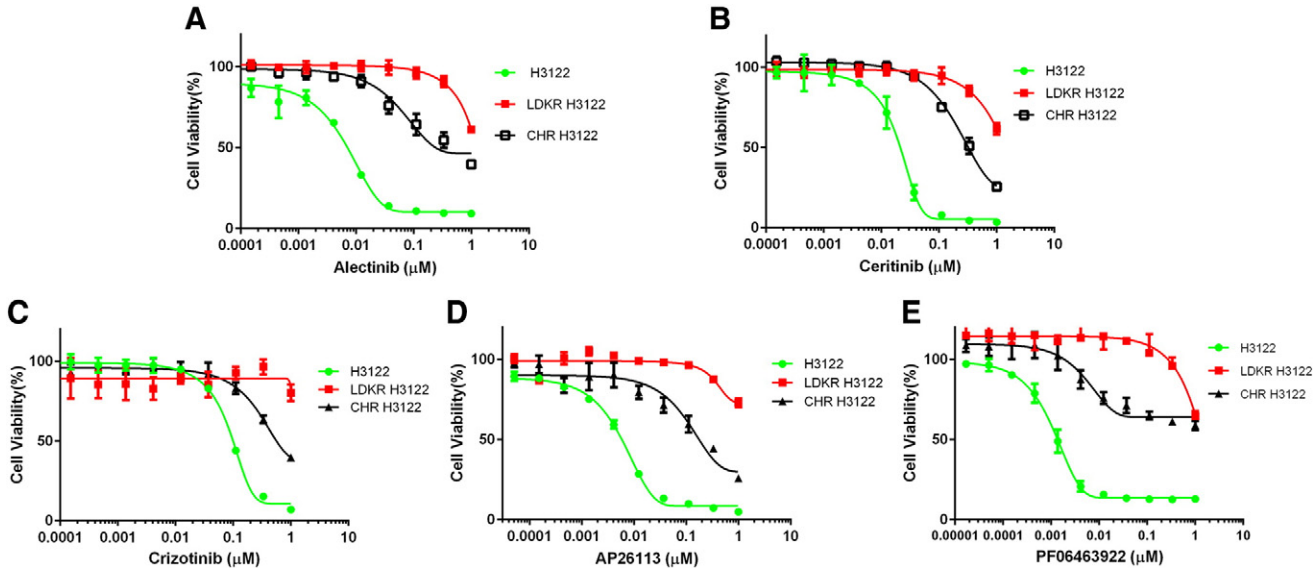

Figure 1. Development of two ALK models resistant to alectinib and ceritinib conferring cross-resistance to crizotinib and all second-generation ALK inhibitors. (A and B) Second-generation ALK inhibitors resistant cells CHR H3122 (developed by exposing to alectinib) and LDKR H3122 (developed by exposing to ceritinib) were treated with alectinib or ceritinib for 4 days; cell viabilities were determined by WST assay. Resistant cells showed 10-fold lower drug sensitivity and were resistant to both second-generation ALK inhibitors. (C–E) Both resistant cell lines also showed different levels of resistance to the first-generation ALK inhibitor crizotinib and additional two novel ALK inhibitors, AP26113 and PF06463922. (Data were representative of three independent experiments)

showed not only high resistance to crizotinib but also resistance to AP26113 and PF06463922, two other new-generation ALK inhibitors currently in clinical development [22,23] (Figure 1, *C–E*).

We next investigated if the resistance is permanent or reversible. When the CHR H3122 and LDKR H3122 resistant cell line pools were cultured in drug-free media, the resistant cells regained their sensitivities to both ALK inhibitors after 6 weeks. We also attempted to identify single-cell resistant clones by expanding single cells in drug-free media and found that all the expanded clones lost their resistance to ALK inhibitors during this process. These data clearly show that the resistance developed to alectinib and ceritinib is reversible.

To further investigate if the resistant cells existed *de novo* in the parental cell pools or if the resistance was gained during drug treatment, the parental H3122 cells were seeded in 96-well plates (one cell per well) (see Supplementary Information 1) to generate single-cell clones. After 3 weeks of culture, these single-cell clones were examined for their proliferative rate and their sensitivity to alectinib in a cell growth assay. Our data showed that, in the bulk of tumor cells, individual clones possessed different proliferative rates. Clones with higher proliferative rates were more sensitive to alectinib, and three relatively quiescent clones were less sensitive to alectinib. Interestingly, once these quiescent clones expanded, they displayed sensitivities to alectinib similar to those of the sensitive clones.

Based upon our data, we propose that the H3122 parental cell line has considerable heterogeneity, and a small population of cells (dormant cells) has a lower proliferative rate and exhibits considerable *de novo* resistance to ALK inhibitors. Furthermore, when the H3122 parental cell line was treated with new-generation ALK inhibitors, these small population drug-tolerant cells became dominant in the presence of an ALK inhibitor but lost their resistance once expanded in the absence of the drug.

Evaluation of ALK-Resistant Mutations and Phospho-ALK Levels in Resistant Cells

ALK mutations and overexpression have been identified as important resistance mechanisms for ALK inhibitors [9,10,18–21]. We next determined if ALK mutations were developed in the resistant cells obtained from our study.

We isolated the total RNAs from the CHR H3122 and LDKR CHR H3122 cell pools. Sanger dideoxynucleotide sequencing using ALK-specific primers which expand the whole ALK kinase domain (1006 bp) was performed but failed to identify any mutation in the ALK kinase domain in both the CHR H3122 and LDKR CHR H3122 cell pools. Additionally, we performed Sanger sequencing of dozens of single-cell clones derived from the resistant cell pools and again failed to detect any ALK mutation (data not shown).

We next determined the levels of ALK mRNA by qRT-PCR, and total ALK protein and phospho-ALK by Western blot. A two-fold increase in the levels of ALK mRNA was detected in both resistant cell lines (Figure 2*C*), but no changes in the total ALK protein levels were observed in these resistant cells or in the resistant cells treated with either alectinib or ceritinib (Figure 2*A and B*). Interestingly, although the levels of p-ALK (Tyr1604) were slightly reduced upon treatment with either alectinib or ceritinib in the parental cells, both resistant cell lines have upregulation of p-ALK upon treatment with these ALK inhibitors at all doses tested (Figure 2*A and B*). These results show that p-ALK levels had been increased in the resistant cells and these new generation ALK inhibitors are also unable to reduce the levels of pALK in the resistant cells.

Alternative Activation of Receptor Tyrosine Kinase Pathways (RTK) as a Major Mechanism Mediating Adaptive Drug Resistance to New Generation ALK Inhibitors

Activation of alternative RTK pathways is known to be an important drug resistance mechanism for different classes of RTK inhibitors. Previous reports have also identified activation of EGFR, KIT, and some other RTK pathways as important resistance mechanisms for crizotinib [9,11,24–26].

To investigate activation of RTK in both CHR H3122 cells and LDKR H3122 cells without bias, we employed an RTK array and found that both resistant cell lines displayed a significant increase of phospho-EGFR protein (Figure 3*A*). Increased levels of phospho-IGF-1R and phospho-HER3 were also observed in the LDKR H3122 cells (Figure 3*A*).

We investigated mRNA levels of these RTKs by qRT-PCR and found that levels of EGFR mRNA, HER3 mRNA, and IGF-1R mRNA were all increased in the resistant cells (Figure 3*B*). In

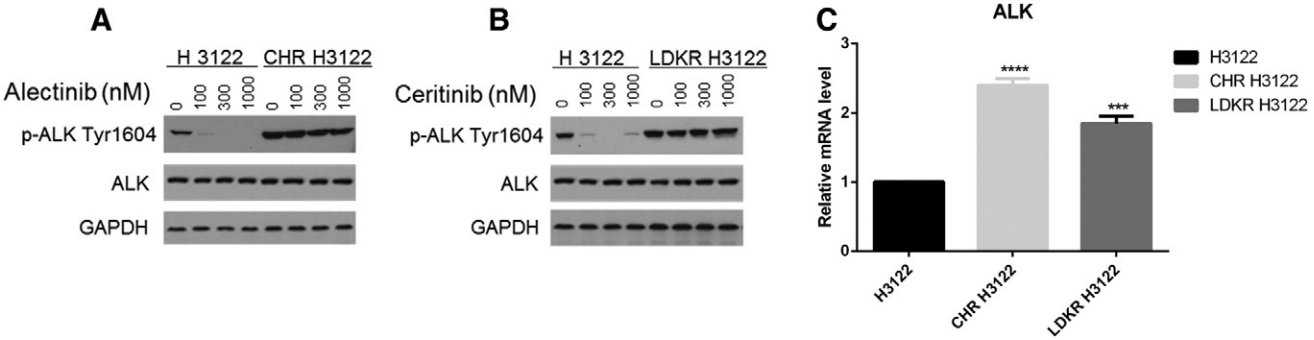

Figure 2. Upregulation of activated p-ALK contributes to drug resistance. (A and B) Parental and resistant cells were treated with the indicated concentrations of drugs for 1 hour and then collected for protein isolation. Western blot was performed using the indicated primary antibodies. (C) Total RNA was isolated from cells and qRT-PCR was performed with ALK-specific primers according to manufacturer's instructions. GAPDH primer was used for normalizing ALK mRNA expression levels ($P < .001$***, $P < .0001$****). (All experiments have been repeated three times)

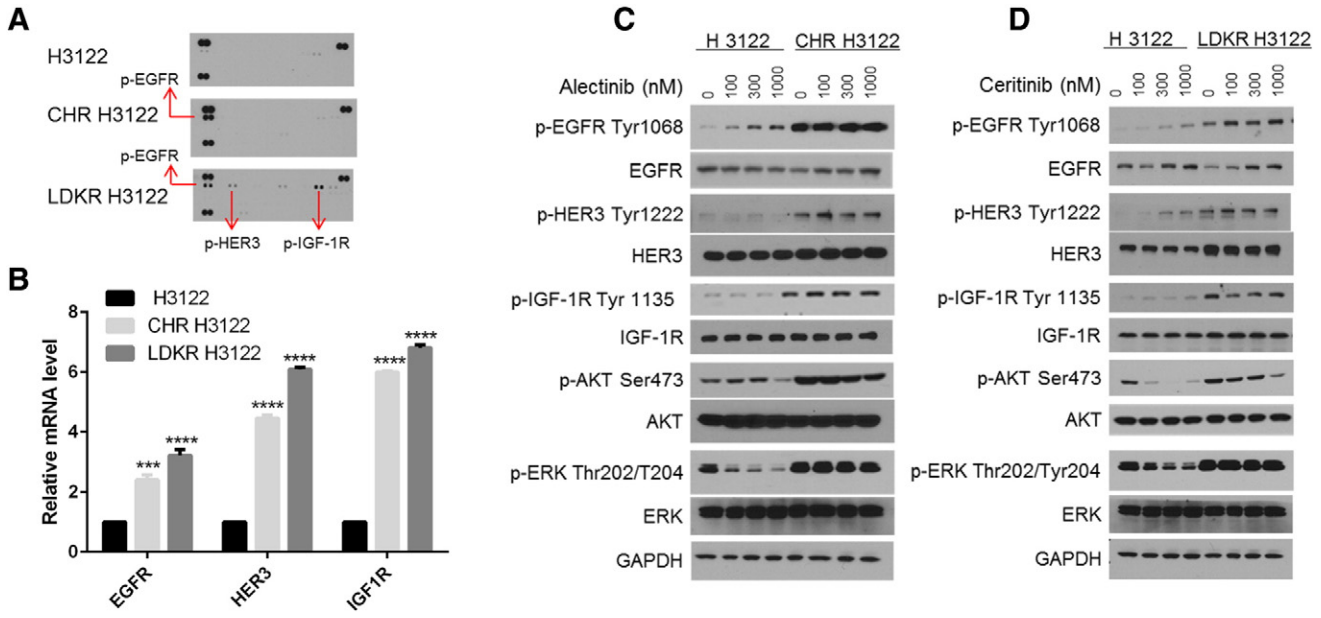

Figure 3. RTKs alternative pathway activation contributes to resistance to second-generation ALK inhibitors. (A) Three hundred micrograms of proteins was isolated from cells and then used in the RTK array according to manufacturer's instructions; compared with parental H3122 cells, CHR H3122 cells showed upregulation of phosphor-EGFR, and LDKR H3122 cells showed upregulation of phosphor-EGFR, phosphor-IGF-1R, and phosphor-HER3. (B) Total RNA was isolated from H3122, CHR H3122, and LDKR H3122 cells and analyzed by qRT-PCR, demonstrating overexpression of RTKs in the resistant cells ($P < .001$***, $P < .0001$****). (C and D) Cells were treated with indicated drugs for 1 hour; immunoblotting for RTKs in both resistant cell lines showed upregulation of phosphor-EGFR and phosphor-HER3 when compared with parental cells. (All experiments have been repeated three times)

particular, the mRNAs of HER3 and IGF-1R were increased by more than five-fold.

Western blotting analysis (Figure 3, *C* and *D*) showed that although no obvious increase in the total level of EGFR protein was detected in both resistant cell lines, the level of p-EGFR (Tyr1068) was significantly increased. Similarly, whereas the total HER3 and IGF-1R protein levels were not changed in both resistant cell lines, the levels of p-HER3 (Tyr 1222) and p-IGF-1R (Try 1135) were greatly increased. AKT and ERK kinases are downstream signaling molecules of ALK, EGFR, HER3, and IGF-1R kinases, and whereas

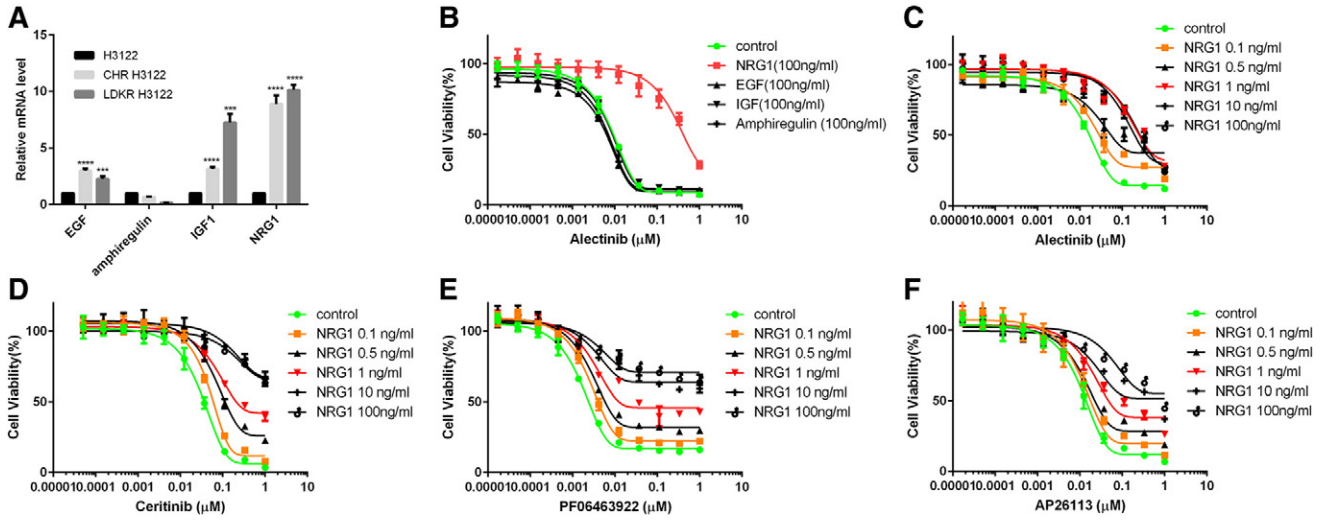

Figure 4. Role of RTK ligands in the development of acquired resistance. (A) Total mRNA was isolated from cells and qRT-PCR was performed using specific primers for EGF, IGF, and NRG1. GAPDH was used as internal reference ($P < .001$***, $P < .0001$****). (B) Parental H3122 cells were seeded in 96-well plates overnight to allow for attachment and were incubated with stepwise increased concentrations of ALK inhibitors in the presence of indicated EGFR ligands (EGF and amphiregulin), HER3 ligand (NRG1), and IGF1R ligand (IGF) at a concentration of 100 ng/ml for 4 days. WST was performed to evaluate cell viabilities. (C–F) Parental H3122 cells were incubated with different concentrations of NRG1 and stepwise increased concentrations of ALK inhibitors for 4 days. WST assay was then performed.

there is no increase in the total levels of AKT and ERK proteins in both resistant cell lines as compared with the parental H3122 cell line, the levels of p-AKT (Ser473) and p-ERK (Thr202/Try204) proteins were increased in the resistant cell lines. Notably, whereas both alectinib and ceritinib effectively reduce the levels of p-AKT (Ser473) and p-ERK (Thr202/Try204) proteins in a dose-dependent manner in the parental H3122 cell line, these ALK inhibitors are ineffective in reducing the levels of p-AKT (Ser473) and p-ERK (Thr202/Try204) proteins in both resistant cell lines. Interestingly, although the AXL pathway was reported to contribute to crizotinib resistance, we did not observe an increase of p-AXL expression in both resistant cell lines (data not shown).

Increased Expression of EGF-Like Ligand NRG1 as a Major Contributor to Resistance

We next asked how these RTKs are activated. Previous work revealed that the ligands of RTKs secreted in a tumor microenvironment were the main contributors of RTK activation [9,25,27]. Hence, we investigated the mRNA levels of EGFR ligands (EGF and amphiregulin), HER3 ligand (NRG1), and IGF-1R ligand (IGF) in cells using qRT-PCR. Our data showed (Figure 4*A*) that expression levels of EGF mRNA in both resistant cells were about two-fold higher than those in the parental H3122 cells; CHR H3122 and LDKR H3122 cells expressed two- and six-fold higher IGF mRNA, respectively, than the parental cell line; and both resistant cell lines expressed more than eight-fold higher NRG1 mRNA than the parental cell line.

To investigate if the increased ligands could induce drug resistance, we exogenously added 100 ng/ml [24–27] of one of these ligands (EGF, amphiregulin, NRG1, IGF) to the cell culture media of the parental H3122 cells. As shown in Figure 4*B*, parental cells became resistant to ALK inhibitors in the presence of NRG1 but not EGF, amphiregulin, or IGF. In fact, NRG1 at concentrations as low as 200 pg/ml was sufficient to induce the H3122 parental cell line to become resistant to alectinib and ceritinib (Figure 4,*C* and *D*). Additionally, NRG1 also induces the H3122 parental cells to become resistant to AP26113 and PF06463922, two new-generation ALK inhibitors in clinical development.

Hence, our data showed that increased expression of NRG1, but not EGF, amphiregulin, or IGF, is a major contributor to the adaptive resistance of the H3122 cells to new-generation ALK inhibitors.

The NRG1-HER3-EGFR Axis Responsible for Resistance to New Generation ALK Inhibitors

As an important ligand for HER3 receptor, NRG1 can activate the HER3-AKT pathway [28,29]. Indeed, in the parental H3122 cells, exogenous NRG1 induced an increase of p-HER3 and p-AKT

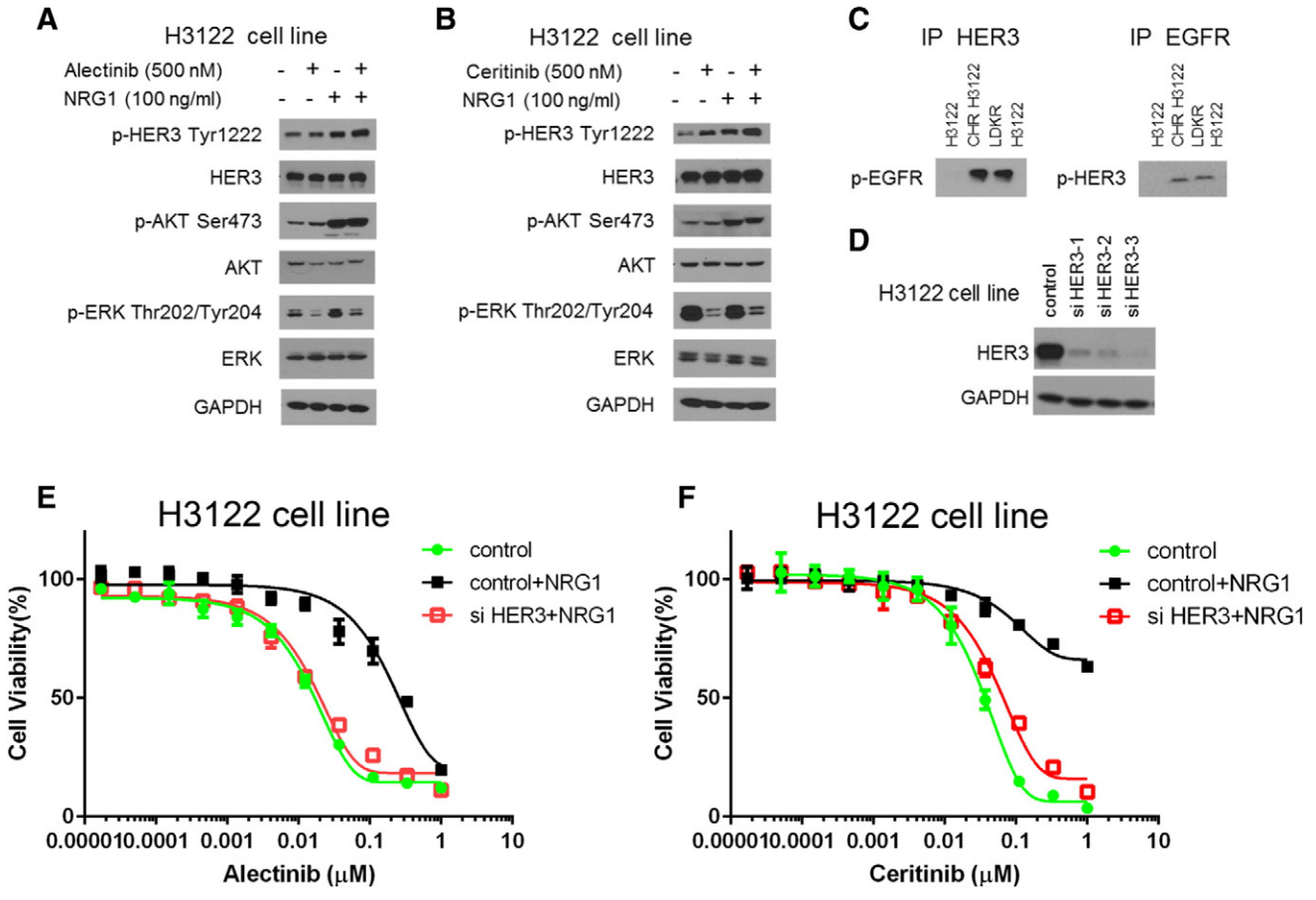

Figure 5. The NRG1-HER3-EGFR axis is responsible for activated EGFR family–induced drug resistance. (A and B) Indicated concentrations of NRG1 and alectinib/ceritinib were added to cell culture media. Cells were incubated for 1 hour and lysed, and proteins were analyzed by Western blot with the indicated primary antibodies. (C) One thousand micrograms of protein was isolated for immunoprecipitation study; p-EGFR/p-HER3 was used as primary antibody to detect its expression in the HER3/EGFR pulled-down products. (D) Efficient knockdown of HER3 by three different siRNA constructs in H3122 cells. (E and F) H3122 cells and HER3 knockdown H3122 cells were incubated with stepwise increased concentrations of ALK inhibitors in the presence of 100 ng/ml of NRG1 for 4 days. WST was performed to evaluate cell viabilities.

(Figure 5*A* and *B*). Interestingly, alectinib and ceritinib failed to inhibit p-HER3 and p-AKT and in fact increased p-HER3 in either the presence or absence of NRG1 (Figure 5*A* and *B*).

It has been reported that the NRG1-EGFR-HER3 autocrine signaling axis can mediate acquired lapatinib resistance in HER2-positive breast cancer models [30]. We therefore investigated if there is an interplay between activated EGFR and HER3 in the resistant cells. In both resistant cell lines but not in the parental cell line, we detected p-EGFR presence in the HER3 pulled-down complexes, and conversely, p-HER3 was also detected in EGFR pulled-down complexes. These data suggest the formation of HER3-EGFR heterodimer in both resistant cell lines.

To investigate if NRG1-induced resistance is HER3 dependent, we knocked down HER3 by siRNA in the parental cells and tested the sensitivity of alectinib and ceritinib in both parental and HER3 knocked-down cells (Figure 5*D*). Our data (Figure 5*E* and *F*) showed that effective knockdown of HER3 reverses the resistance of the H3122 cells induced by NRG1 to both ALK inhibitors. In addition, we employed Karpas 299 and SU-DHL-1 cell lines, two anaplastic large cell lymphoma cell lines which harbor ALK fusion gene (NPM-ALK) but express only a very low level of HER3, to test the sensitivities of these ALK inhibitors in these two cell lines in the presence of NRG1 (Supplementary Information 2) and found that NRG1 did not affect the sensitivities to ALK inhibitors in these two anaplastic large cell lymphoma cell lines. Taken together, our data support a key role of HER3 in NRG1-mediated resistance to alectinib and ceritinib, and the NRG1-HER3-EGFR axis is an important mechanism mediating activation of bypass RTK pathways and resistance to these ALK inhibitors.

Overcoming Resistance to ALK Inhibitors Using EGFR-Family Tyrosine Kinase Inhibitors (TKIs)

Our results highlight that the NRG1-HER3-EGFR axis is an important mechanism of resistance to these new-generation ALK inhibitors and suggest that an inhibitor targeting this axis could be effective in overcoming such resistance.

Because there is no specific NRG1 inhibitor available, we investigated if EGFR-family TKIs can overcome the resistance to ALK inhibitors. We evaluated several EGFR inhibitors, either already FDA approved or currently in clinical trials (Table 1), including erlotinib (a first-generation EGFR inhibitor), afatinib (a second-generation irreversible EGFR inhibitor that targets wild-type EGFR, the T790M EGFR mutant, and HER2), AZD9291 (a third-generation irreversible EGFR inhibitor selectively targeting the T790M EGFR mutant), and AZD8931 (a reversible, ATP competitive broad-spectrum inhibitor of EGFR, HER2, and HER3).

Whereas each of these EGFR inhibitors as a single agent had only a moderate effect at concentrations as high as 1 μM in the CHR H3122 resistant cell line, combination of these EGFR inhibitors at 500 nM with alectinib can partially overcome the resistance to alectinib (Figure 6*B*). Interestingly, whereas these EGFR inhibitors as a single agent had a greater effect at 1 μM in the LDKR H3122 cell line than in the CHR H3122 cell line, their combination with ceritinib only had an additive effect in overcoming resistance of the LDKR 3122 cells to ceritinib (Figure 6*C*).

In both the CHR H3122 and LDKR H3122 cells, afatinib at 500 nM is effective in overcoming resistance, and we next determined the minimal concentration needed for afatinib to overcome resistance of ALK inhibitors in these two resistant cell lines. Our data showed that afatinib at concentrations as low as 31.25 nM already achieved the maximum effect in inhibition of cell growth in its combination with alectinib or ceritinib in these two resistant cell lines (Figure 6, *B* and *C*). Additionally, concurrent treatment of alectinib or ceritinib with afatinib helped to eradicate the remaining cell population obtained from treatment with an ALK inhibitor alone in the H3122 parental cell line (Supplementary Information 3). Western blotting analysis showed that although combination of alectinib with either afatinib or afatinib can effectively block the EGFR Tyr 1068 phosphorylation, the former combination is more effective than the latter in inhibition of p-EGFR, providing a mechanistic explanation for the strong synergistic effect between alectinib and afatinib in CHR H3122 resistant cells (Figure 6, *D* and *E*).

Discussion

Crizotinib, the first ALK inhibitor approved as a targeted therapy for NSCLC [2], showed impressive clinical responses in patients harboring ALK gene fusion. However, ALK fusion–positive patients developed resistance to crizotinib fairly quickly, highlighting a clear need to develop new treatment strategies to combat crizotinib-resistance. Investigations into possible resistance mechanisms to crizotinib have identified mutations of ALK in its kinase domain as a major mechanism. Accordingly, second-generation ALK inhibitors alectinib and ceritinib were developed and were shown to be effective in overcoming most of the resistant ALK mutations developed during crizotinib treatment [13,15]. Despite their impressive clinical data, recent studies have also revealed that clinical resistance began to develop to these second-generation ALK inhibitors, and novel ALK mutations have been found in patients who developed resistance to these second-generation ALK inhibitors [18–21]. Interestingly, not all of the patients who developed resistance to these second-generation ALK inhibitors have a novel ALK mutation [13].

To investigate resistance mechanisms for these second-generation ALK inhibitors, we obtained two resistant cell lines using the H3122 NSCLC cell line harboring the EML4-ALK variant 1 fusion gene and alectinib and ceritinib. After a 5-month treatment of the H3122 NSCLC cells with a relatively high concentration (1 μM) of alectinib, we obtained a resistant cell line (the CHR H3122 cell line), and after a 7-month treatment of the H3122 NSCLC cells with 1 μM of ceritinib, we obtained a second resistant cell line (the LDKR H3122 cell line). Both of these two resistant cell lines are approximately 10

Table 1. EGFR Inhibitors Used in This Study.

EGFR Inhibitors	IC50 on Different EGFR Family Members				Reversibility	Clinical Development Stage
	EGFR (L858R)	EGFR (wt)	HER2	HER3		
AZD9291	11.4 nM	493 nM	NA	NA	No	Phase III
AZD8931	NA	4 nM	3 nM	4 nM	Yes	Phase I
Afatinib	0.4 nM	0.5 nM	14 nM	NA	No	Approved for NSCLC
Erlotinib	2 nM	29 nM	NA	NA	Yes	Approved for EGFR mutated NSCLC

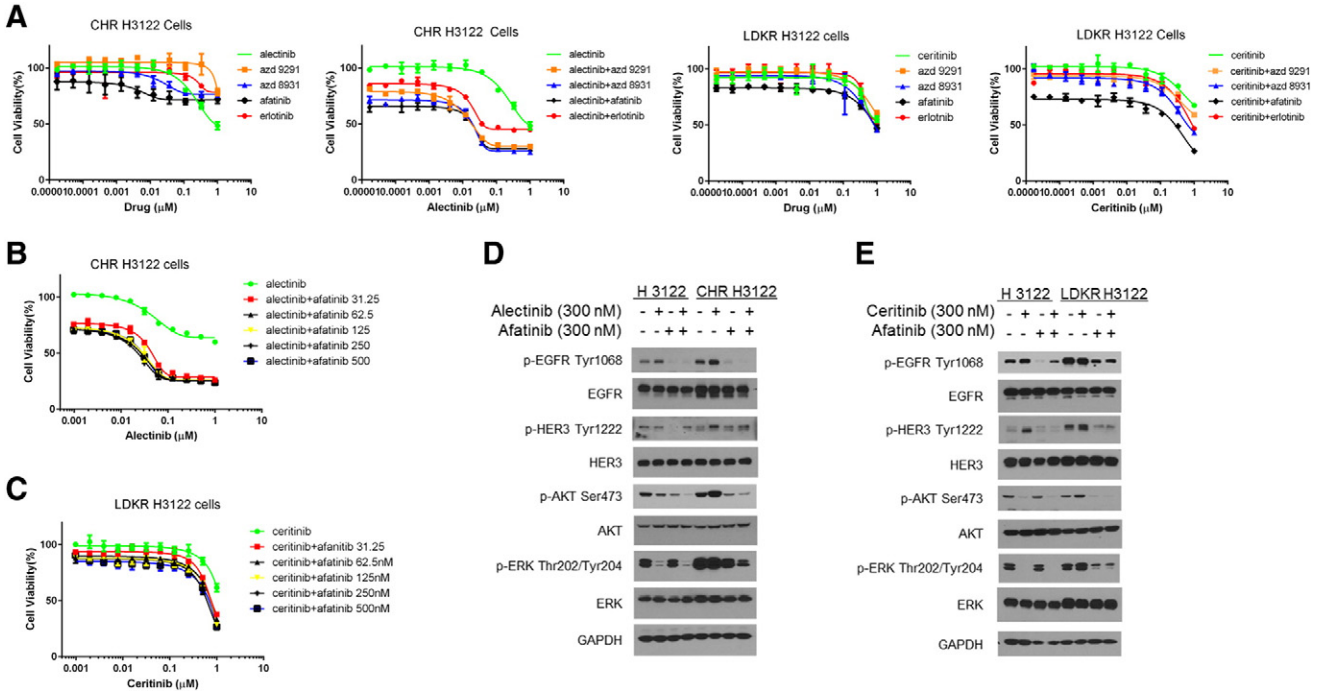

Figure 6. Combination treatment to overcome resistance. (A) Both resistant cell lines CHR H3122 and LDKR H3122 were seeded in 96-well plates and cultured with indicated single drugs or 500 nM combination drugs for 4 days; WST assay was then performed. (B and C) Different concentrations of afatinib (from 31.25 nM to 500 nM) were combined with ALK inhibitors and added to cell culture media for 4 days. Viability was assessed by WST assay. (D and E) Proteins were collected after treatment with the indicated drugs for 2 hours, and immunoblotting was performed. Combination with afatinib induced downregulation of p-EGFR and its downstream signals.

times less sensitive than the parental H3122 cell line to alectinib and ceritinib. To examine if these resistant cell lines also become insensitive to other new-generation ALK inhibitors in clinical development, we tested AP26113 and PF06463922. AP26113 [22] has been reported to be a dual inhibitor of ALK and EGFR mutants, and PF06463922 [23] has been reported to be effective against all known ALK-resistant mutations. Our data showed that both of these resistant cell lines become much less sensitive to AP26113 and PF06463922 than the parental H3122 cell line, suggesting that the resistance developed with alectinib and ceritinib in the H3122 cell line cannot be overcome by other ALK inhibitors. These data showed that resistance to second-generation ALK inhibitors can indeed be developed, although an extended treatment time may be necessary.

Investigations into the possible resistance mechanisms in these two resistant cell lines failed to identify any ALK mutation in its kinase domain. Instead, we found increased activation of p-ALK and some other RTKs such as p-EGFR, p-HER3, and p-IGF-1R in both resistant cell lines, and both alectinib and ceritinib became ineffective inhibitors of p-AKT and p-ERK in both resistant cell lines (Figures 2 and 3).

Activation of alternative RTKs (EGFR, KIT, and IGF-1R) has been reported to mediate crizotinib resistance in both clinical and preclinical studies [9,11,24,25], but whether or not these RTKs can mediate resistance to these second-generation ALK inhibitors was not known.

We next investigated how the bypass pathways were activated. The EGFR ligands (EGF, amphiregulin), IGF-1R ligand (IGF), and c-Met ligand (HGF) have been reported to be increased in cell culture media and to contribute to bypass pathways of RTK activation [9,25,27]. Higher concentrations of EGFR ligands were also reported in the supernatant fluid from crizotinib-resistant patients with malignant pleural effusion [10]. In the current study, we detected increased mRNA expression of NRG1, EGF, and IGF in both resistant cell lines (Figure 3).

Among all the upregulated ligands, we found that NRG1 expression was increased by more than eight-fold in both resistant cell lines. Furthermore, our data showed that exogenous NRG1, but not EGF and IGF, can cause the parental H3122 cells to become resistant to all second-generation ALK inhibitors (Figure 4), highlighting NRG1 upregulation as an important contributor to the development of resistance to these second-generation ALK inhibitors. Our finding is consistent with recent studies which showed that NRG1 is a resistance driver in ALK-dependent lung cancer cell systems [26,31].

NRG1 is a ligand for HER3, and the autocrine loop between HER3 and NRG1 has been reported in ovarian, head and neck, melanoma, and prostate cancers [28,29,32,33]. In this study, we show that NRG1 indeed induces upregulation of p-HER3, which abrogates the effective inhibition of downstream p-AKT achieved by ALK inhibitors in the parental H3122 cells (Figure 5). Furthermore, knockdown of HER3 in the parental H3122 cells blocked NRG1-induced drug resistance, and in ALK-positive but HER3-negative cell lines, and NRG1 failed to induce resistance. Collectively, these results demonstrate that the resistance induced by NRG1 to ALK inhibitors is HER3 dependent.

We investigated the interplay between HER3 and other EGFR family members. As a pseudokinase, HER3 can form heterodimers with other EGFR family members and lead to activation of other EGFR members [30,34–36]. Our RTK assay did not detect phosphorylated HER2 expression in the resistant cells (Figure 3*A*). We also investigated the p-HER2 expression using Western blot but failed to detect p-HER2 expression, strongly suggesting that HER2 is not a contributor to ALK resistance. Since both EGFR and HER3

were activated in the resistant cells (Figure 3), and the EGFR-HER3 heterodimer was previously shown to be involved in acquired lapatinib resistance in HER2-positive breast cancer models [30], we then asked if the EGFR-HER3 heterodimer contributes to the activation in the resistant cells. We detected p-EGFR in the HER3 protein complexes in both ALK-resistant cells but not in the parental H3122 cells (Figure 5), and found that treatment of the resistant cells with an EGFR inhibitor (afatinib) decreased p-EGFR, as well as p-HER3 (Figure 6, *D* and *E*). These data suggest that EGFR is important in maintaining HER3 activation in the resistant cells.

Based upon our data, we propose that the NRG1-HER3-EGFR axis plays a key role in mediating the resistance to second-generation ALK inhibitors. Interestingly, although AP26113 is a ALK/EGFR dual inhibitor, it showed limited activity in overcoming alectinib and ceritinib resistance (Figure 1*D*).

We next tested strategies to combat the resistance by targeting the NRG1-HER3-EGFR axis. To date, no specific NRG1 inhibitor has been reported. Mohamad Younes et al. recently reported that tumor cells expressing NTS (NRG1) were responsive to EGFR inhibitors and that EGFR TKIs could reduce EGFR family expression and their downstream molecules [37]. We therefore evaluated a number of FDA-approved or clinically developed small-molecule inhibitors of the EGFR family proteins in combination with ALK inhibitors. Our data showed that several EGFR inhibitors are very effective in overcoming alectinib-induced drug resistance and afatinib is the most effective in overcoming ceritinib-induced drug resistance (Figure 6). Combination of afatinib with either alectinib or ceritinib effectively eliminates the remaining small population of tumor cells when treated with an ALK inhibitor alone in the H3122 parental cell line. Since afatinib has been approved for clinical use in late-stage NSCLC patients a combination of afatinib and ALK inhibitors can be tested in the clinic in patients who develop resistance to ALK inhibitors but lack detectable ALK-resistant mutations.

Conclusion

In the present study, we investigated the mechanisms of acquired resistance of alectinib and ceritinib, two second-generation ALK inhibitors, in the H3122 NSCLC cell line harboring the EML4-ALK variant 1 fusion *in vitro*. Our data showed that alectinib and ceritinib can develop resistance in the H3122 cells, although prolonged drug treatment was needed. While mutations of ALK in its kinase domain are a common resistance mechanism for crizotinib, the first-generation ALK inhibitor, we did not detect any ALK mutation in these resistant cell lines. Instead, overexpression of phospho-ALK and alternative RTKs such as phospho-EGFR, phospho-HER3, and phospho-IGFR-1R was observed in resistant cell lines. Furthermore, we found that NRG1, a ligand for HER3, is upregulated and responsible for resistance by activating the EGFR family pathways through the NRG1-HER3-EGFR axis. Combination treatment of an ALK inhibitor with an EGFR inhibitor, in particular afatinib, a potent inhibitor of both wild-type and mutated EGFR, can effectively overcome resistance. Our study provides new mechanistic insights into adaptive resistance to these second-generation ALK inhibitors and suggests a potential clinical strategy to combat resistance ALK-positive lung cancer cells to these second-generation ALK inhibitors in NSCLC.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.neo.2016.02.001.

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