Published OnlineFirst December 30, 2015; DOI: 10.1158/0008-5472.CAN-15-1010

Therapeutics, Targets, and Chemical Biology

Cancer Research

Non–Small Cell Lung Cancer Cells Acquire Resistance to the ALK Inhibitor Alectinib by Activating Alternative Receptor Tyrosine Kinases

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Abstract

Crizotinib is the standard of care for advanced non–small cell lung cancer (NSCLC) patients harboring the anaplastic lymphoma kinase (ALK) fusion gene, but resistance invariably develops. Unlike crizotinib, alectinib is a selective ALK tyrosine kinase inhibitor (TKI) with more potent antitumor effects and a favorable toxicity profile, even in crizotinib-resistant cases. However, acquired resistance to alectinib, as for other TKIs, remains a limitation of its efficacy. Therefore, we investigated the mechanisms by which human NSCLC cells acquire resistance to alectinib. We established two alectinib-resistant cell lines that did not harbor the secondary *ALK* mutations frequently occurring in crizotinib-resistant cells. One cell line lost the EML4-ALK fusion gene, but exhibited increased activation of insulin-like growth factor-1 receptor (IGF1R) and human epidermal growth factor receptor 3 (HER3), and overexpressed the HER3 ligand neuregulin 1. Accordingly, pharmacologic inhibition of IGF1R and HER3 signaling overcame resistance to alectinib in this cell line. The second alectinib-resistant cell line displayed stimulated HGF autocrine signaling that promoted MET activation and remained sensitive to crizotinib treatment. Taken together, our findings reveal two novel mechanisms underlying alectinib resistance that are caused by the activation of alternative tyrosine kinase receptors rather than by secondary *ALK* mutations. These studies may guide the development of comprehensive treatment strategies that take into consideration the various approaches ALK-positive lung tumors use to withstand therapeutic insult. *Cancer Res; 76(6); 1506–16. ©2015 AACR.*

Introduction

Targeted therapies for lung cancers harboring driver oncogenes are generally recognized because of the large influence they have on treatment outcomes. The Lung Cancer Mutation Consortium in the United States reported a median survival from the time of diagnosis of metastatic disease of 4.2 years in patients with lung adenocarcinomas harboring the echinoderm microtubule-associated protein-like 4 (EML4)-anaplastic lymphoma kinase (ALK) fusion gene when appropriate chemotherapies with molecular targeted agents were used (1). Soon after the discovery of *EML4-ALK* (2), Sakamoto and colleagues developed a selective tyrosine kinase inhibitor (TKI) for ALK called alectinib (3). The high selectivity of alectinib for ALK in preclinical data from Japanese phase-II trials indicated its high objective response rate (93.5%), long median progression-free survival (27.7 months), and favorable toxicity profile (4, 5). Based on these data, Japan was the first country to approve the use of alectinib. This agent is also effective in cases resistant to crizotinib, which is a first-generation ALK-TKI, including cases with metastasis to the central nervous system (6–9). Based on these positive results, alectinib received breakthrough therapy designation by the FDA. Two randomized clinical trials comparing alectinib with crizotinib in first- and second-line settings are currently underway.

Alectinib is expected to be a mainstay treatment for ALK-positive lung cancer in the future, but almost all patients eventually acquire resistance to alectinib as they would to crizotinib. The mechanisms of acquired resistance to crizotinib have been explored extensively and include secondary *ALK* mutations (10–15), *ALK* amplification (11), and bypass track activation via proteins, such as the human epidermal growth

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

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doi: 10.1158/0008-5472.CAN-15-1010

Published OnlineFirst December 30, 2015; DOI: 10.1158/0008-5472.CAN-15-1010

factor receptor (HER) family (13–15), v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (14), Kirsten rat sarcoma 2 viral oncogene homolog (15), and insulin-like growth factor-1 receptor (IGF1R; ref. 16). Crizotinib inhibits MET more strongly than ALK, so cells acquire resistance to the MET inhibitor as well as the ALK inhibitor presumably. Because alectinib is a selective TKI for ALK, unlike crizotinib, the mechanisms of resistance to the two drugs presumably differ. Therefore, we elucidated the mechanisms of alectinib resistance to discover strategies to overcome such resistance.

In this study, we used the H2228 and ABC-11 cell lines, which harbor *EML4-ALK*, to comprehensively investigate the mechanisms of acquired resistance to alectinib. Different from previously reported alectinib-resistant cell lines, neither of these resistant cell lines possess secondary *ALK* mutations (17). Discovering the mechanisms of resistance to alectinib with the goal of overcoming alectinib resistance will provide new insight into resistance to ALK-TKIs.

Materials and Methods

Cell lines and reagents

The PC-9 cells (epidermal growth factor receptor: EGFR Ex19 del E746_A750) were purchased from the European Collection of Cell Cultures in 2014. The H2228 (EML4-ALK variant 3a/b E6; A20) cell line was purchased from the American Type Culture Collection in 2008. They were authenticated using short tandem repeat analysis at each cell bank and resuscitated at least every 6 months from frozen master stocks. The ABC-11 (EML4-ALK variant 3b E6; A20) cell line was established at Okayama University from the pleural effusion of a patient with ALK inhibitor–naïve non–small cell lung cancer (NSCLC) harboring *EML4-ALK* (18) in 2011. Cells were cultured in RPMI 1640 medium (Sigma) supplemented with 10% heat-inactivated FBS at 37.0°C in 5% CO_2.

Alectinib was kindly provided by Chugai Pharmaceutical Co., Ltd. OSI-906 was kindly donated by Dr. Ebi (Kanazawa University, Ishikawa, Japan). Anti-human HGF-neutralizing antibody and human recombinant HGF were prepared as described previously (19). Human neuregulin 1 (NRG1) was purchased from Cell Signaling Technology, erlotinib from ChemieTek, and SGX325 and crizotinib from Selleck Chemicals.

Detection of EML4-ALK

IHC and FISH were performed on formalin-fixed, paraffin-embedded samples using an ALK antibody (5A4; Abcam) and a break-apart probe for the ALK gene (Vysis LSI ALK Break Apart Rearrangement Probe; Abbott Laboratories)at Hachioji Laboratories, SRL Inc, Tokyo, Japan. FISH-positive cases were defined as >15% split signals in cells. EML4-ALK mRNA expression was examined by RT-PCR. The primer sequences used have been described previously (20).

Drug sensitivity assays

Cells (2,000–3,000/well) were seeded in 96-well plates and cultured for 96 hours. Viable cells were measured using the modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) assay (21, 22).

Western blotting and receptor tyrosine kinase array

Western blotting was conducted as described previously (23). Antibodies information is provided in Supplementary Table S1. A phospho-receptor tyrosine kinase (RTK) Array kit was purchased from R&D Systems and used according to the manufacturer's recommendations. Bands and dots were detected using an LAS-4000 imager.

DNA and RNA extraction

DNA or RNA was extracted from cell line using a QIA-amp DNA Mini Kit (Qiagen) or an RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol, respectively.

Quantitative PCR and mRNA expression analysis

The MET copy-number gain was determined by qPCR analysis of DNA using TaqMan probes and primers. The HGF copy-number gain was determined by qPCR analysis of DNA using primers and the Power SYBR Green PCR Master Mix (Applied Biosystems). The probe and primer sequences are provided in Supplementary Table S2A. Gene dosage was calculated using a standard curve.

IGF1R, stem cell–related factors (ALDH1A1, SMO, and ABCG2), and HGF mRNA expression were analyzed by qRT-PCR of cDNA using primer and probe sets and the TaqMan Universal PCR Master Mix (Applied Biosystems), according to the manufacturer's protocol. The primer and probe set information is provided in Supplementary Table S2B.

PCR amplification was performed using the StepOne Plus Real-Time PCR instrument (Applied Biosystems), and gene dosage was calculated using a standard curve. The copy-number ratio of the target gene to GAPDH and their gene expression were calculated. Values for the resistant clones are expressed relative to the corresponding values for parental cells.

mRNA expression profile analysis

mRNA was profiled using Custom Microarray 4 × 44 K arrays (Agilent Technologies) according to the manufacturer's protocol. The original expression data have been submitted to Gene Expression Omnibus. The accession number is GSE73167.

Quantification of HGF by ELISA

Human HGF levels were determined using a Human Total HGF Assay kit (Immuno-Biological Laboratories Co.). Resistant clone data are expressed relative to the corresponding values for parental cells.

Conditioned medium

ABC-11/CHR3 cells (2×10^6/10 mL) were seeded and cultured in a flask in the absence of alectinib. The supernatant was harvested after 48 hours and centrifuged. Conditioned medium was prepared by mixing equal parts of the supernatant and fresh medium.

Xenograft models

Female BALB/c nu/nu mice were purchased at 7 weeks of age from Charles River Laboratories, Japan. Cells (2×10^6) were injected subcutaneously into the backs of the mice. Treatment was started when the tumor reached a certain volume (day 0). Mice were randomized to receive vehicle, alectinib (10 mg/kg/day), or crizotinib (50 mg/kg/day; refs. 3, 24, 25) for the same duration. Vehicle and drugs were administered orally once a day every third day by gavage. The tumor volume (width^2 × length/2) was determined periodically. Statistical data were analyzed on day 19.

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Statistical analysis

All data were expressed as the mean ± SE. All experiments were performed three times, and statistical analysis was determined by the two-tailed paired Student *t* test. *P* value of <0.05 was considered significant (*, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$, respectively).

Results

Establishing two alectinib-resistant cell lines using the dose-escalation method

We established the H2228/CHR and ABC-11/CHR alectinib-resistant cell lines from H2228 and ABC-11 cells, respectively, using a well-established dose-escalation method to explore the mechanisms underlying acquired resistance to alectinib comprehensively (Supplementary Fig. S1A; refs. 26, 27). Both cell lines became resistant within 5 months and then grew in the presence of 1 μmol/L alectinib.

As shown in Table 1, the H2228/CHR and ABC-11/CHR cells were 117- and 40-fold more resistant than was each parental cell line, respectively, in terms of relative resistance determined by the IC_{50} values. H2228/CHR cells also exhibited resistance to crizotinib, whereas ABC-11/CHR cells exhibited collateral sensitivity to crizotinib, which inhibited both ALK and MET.

No morphologic changes were detected compared with the parental cells (Supplementary Fig. S1B and S1C). Both resistant cell lines maintained downstream AKT and ERK phosphorylation even in the presence of 10 μmol/L alectinib. Surprisingly, the ALK protein was lost in H2228/CHR cells, but not in ABC-11/CHR cells (Fig. 1A). We confirmed that the resistant and parental cells had the same origin using PCR analysis of short tandem repeats (Supplementary Fig. S2A and S2B).

Loss of the EML4-ALK protein in H2228 cells following exposure to alectinib

As we were interested in H2228/CHR cells, which lack the ALK protein, we examined cells that were serially treated with 150 nmol/L or 600 nmol/L alectinib (designated H2228/150 nmol/L and H2228/600 nmol/L, respectively). ALK expression gradually decreased in H2228/150 nmol/L cells and was lost eventually (Fig. 1B). IHC revealed that pre-existing ALK-negative cells became dominant during chronic exposure to alectinib (Fig. 1C). In parallel with loss of the ALK protein, sensitivity to alectinib decreased (Fig. 1D).

To elucidate the resistance mechanism more precisely, we cloned three H2228/CHR sublines (designated H2228/CHR-1–3) from H2228/CHR cells by single-cell cloning. All clones exhibited the same characteristics as those of H2228/CHR, including resistance to alectinib and crizotinib and loss of ALK protein expression (Supplementary Fig. S3A and S3B). The statuses of the ALK protein and the EML4-ALK fusion gene in the H2228/CHR clones were confirmed by IHC, RT-PCR, and FISH (Fig. 1E–G).

We cultured H2228/CHR clones in the absence of alectinib for ≥1 year (designated H2228/CHR-r1–r3). The EML4-ALK fusion gene status was determined by multiplex RT-PCR and exon array analyses in the H2228/CHR-r cell lines (Mitsubishi Chemical Medience), and its loss was persistent (data not shown).

Survival of alectinib-resistant cells was maintained by activated IGF1R and HER3 signaling instead of EML4-ALK

As the *EML4-ALK* driver oncogene was lost in the H2228/CHR clones, we used a phospho-RTK array to identify tyrosine kinase receptors that contribute to survival. The results showed increased IGF1R and HER3 phosphorylation levels in H2228/CHR clones compared with parental H2228 cells (Fig. 2A). IGF1R upregulation was confirmed by qRT-PCR and Western blotting in H2228/CHR clones (Fig. 2B and C), but no changes in HER3 expression were observed (data not shown). Subsequently, we conducted mRNA array analysis to reveal high expression of several genes, which were more than 2-fold higher in H2228/CHR1 cells than parental H2228 cells. Remarkably, the NRG1, ligand of HER3 (28), was highly expressed in all resistant clones (Fig. 2D). We confirmed that external NRG1 enhanced HER3 phosphorylation and that downstream AKT and ERK phosphorylation were maintained regardless of treating parental cells with alectinib (Fig. 2E). In addition, both EGFR and HER3 phosphorylation were inhibited by the selective EGFR inhibitor erlotinib in H2228/CHR cells (Fig. 3A). These results suggest that HER3 may be activated through NRG1 and EGFR, leading to resistance to alectinib in H2228/CHR cells.

Taken together, these findings indicate that alectinib-resistant cells maintain survival by activating the IGF1R and NRG1/HER3 pathways rather than via EML4-ALK driver oncogene signaling.

Inhibition of IGF1R and HER3 overcomes alectinib resistance

As the survival of H2228/CHR clones that have lost *EML4-ALK* could depend on IGF1R and HER3 signaling, we examined whether inhibiting IGF1R or HER3 was effective for the H2228/CHR1-resistant clone. Although OSI-906 monotherapy (IGF1R inhibitor) or erlotinib (EGFR inhibitor) weakly suppressed survival signaling (AKT and ERK phosphorylation), the combination of OSI-906 and erlotinib clearly downregulated AKT survival signaling more remarkably than that of ERK1/2 (Fig. 3A). The combined therapy was significantly more effective for inhibiting growth of H2228/CHR1 cells than was either of the single treatments (Fig. 3B). We conclude that H2228/CHR cells became resistant to alectinib due to loss of the *EML4-ALK* driver oncogene and activation of alternative pathways, including IGF1R and NRG1/HER3 signaling.

The mRNA array results showed that several lung cancer stem cell–related markers (29) were highly expressed in the H2228/CHR clones. We confirmed upregulation of ALDH1A1, SMO, and ABCG2 in the H2228/CHR clones compared with that in parental

Table 1. Effects of ALK TKIs on parent and alectinib-resistant cells

	IC_{50} (μmol/L) values ± SE			IC_{50} (μmol/L) values ± SE		
Drugs	**H2228**	**H2228/CHR**	**RR**	**ABC-11**	**ABC-11/CHR**	**RR**
Alectinib	0.030 ± 0.014	3.5 ± 0.55[a]	117	0.13 ± 0.022	5.3 ± 0.64[a]	40
Crizotinib	0.17 ± 0.039	5.7 ± 0.98[b]	34	0.15 ± 0.020	0.098 ± 0.013[c]	0.65

NOTE: All experiments were performed three times, and statistical significance was determined by the two-tailed paired Student *t* test.
Abbreviation: RR, relative resistance.
[a]$P < 0.05$.
[b]$P < 0.01$.
[c]$P < 0.001$.

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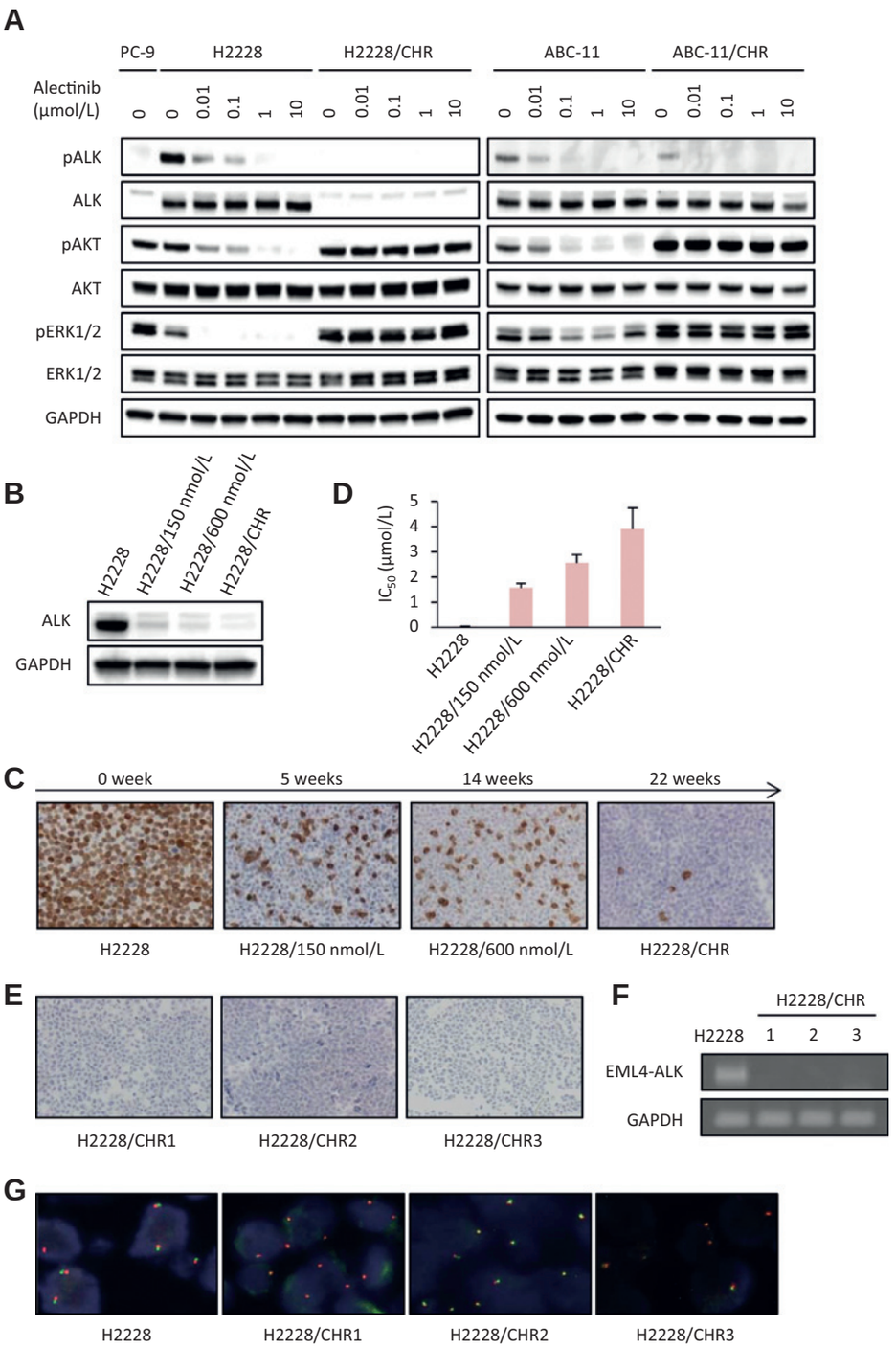

Figure 1.
Loss of *EML4-ALK* expression in H2228/CHR cells. A, effects of alectinib on the levels of phosphorylated ALK and downstream signaling in the alectinib-resistant cell lines. Each cell line was incubated with various concentrations of alectinib for 4 hours. Lysates were analyzed by Western blotting. B, Western blotting for ALK. C, IHC for ALK. Western blotting and IHC were conducted 0, 5, 14, and 22 weeks after beginning treatment on H2228, H2228/CHR (150 nmol/L), H2228/CHR (600 nmol/L), and H2228/CHR cells. D, sensitivity to alectinib. Cells were seeded and treated with various concentrations of alectinib. Viable cells were assessed by MTT assay. Antiproliferative effects are shown as IC_{50} values. E, IHC for ALK in the H2228/CHR clones. IHC of the parental cells is shown in B. F, detection of EML4-ALK mRNA. cDNA derived from H2228 and H2228/CHR clones was examined by PCR. G, FISH for the ALK fusion gene in the H2228/CHR clones (red, *ALK*3′; green, *ALK*5′).

cells by qRT-PCR (Fig. 3C). H2228/CHR cells may acquire a stem cell–like phenotype related to resistance as well.

Activation of MET in alectinib-resistant cells

Next, we investigated the mechanism underlying alectinib resistance in ABC-11/CHR cells. In contrast with H2228/CHR cells, ALK protein expression was maintained in ABC-11/CHR cells (Fig. 1A). We obtained three ABC-11/CHR clones (designated ABC-11/CHR-1–3) by single-cell cloning. All ABC-11/CHR clones exhibited the same characteristics as those of ABC-11/CHR (Supplementary Fig. S4A and S4B). Secondary *ALK* mutations, a frequent mechanism of acquired resistance to crizotinib, were not detected by direct sequencing of either clone (Supplementary Fig. S5). Phospho-RTK arrays revealed increased MET phosphorylation levels in the ABC-

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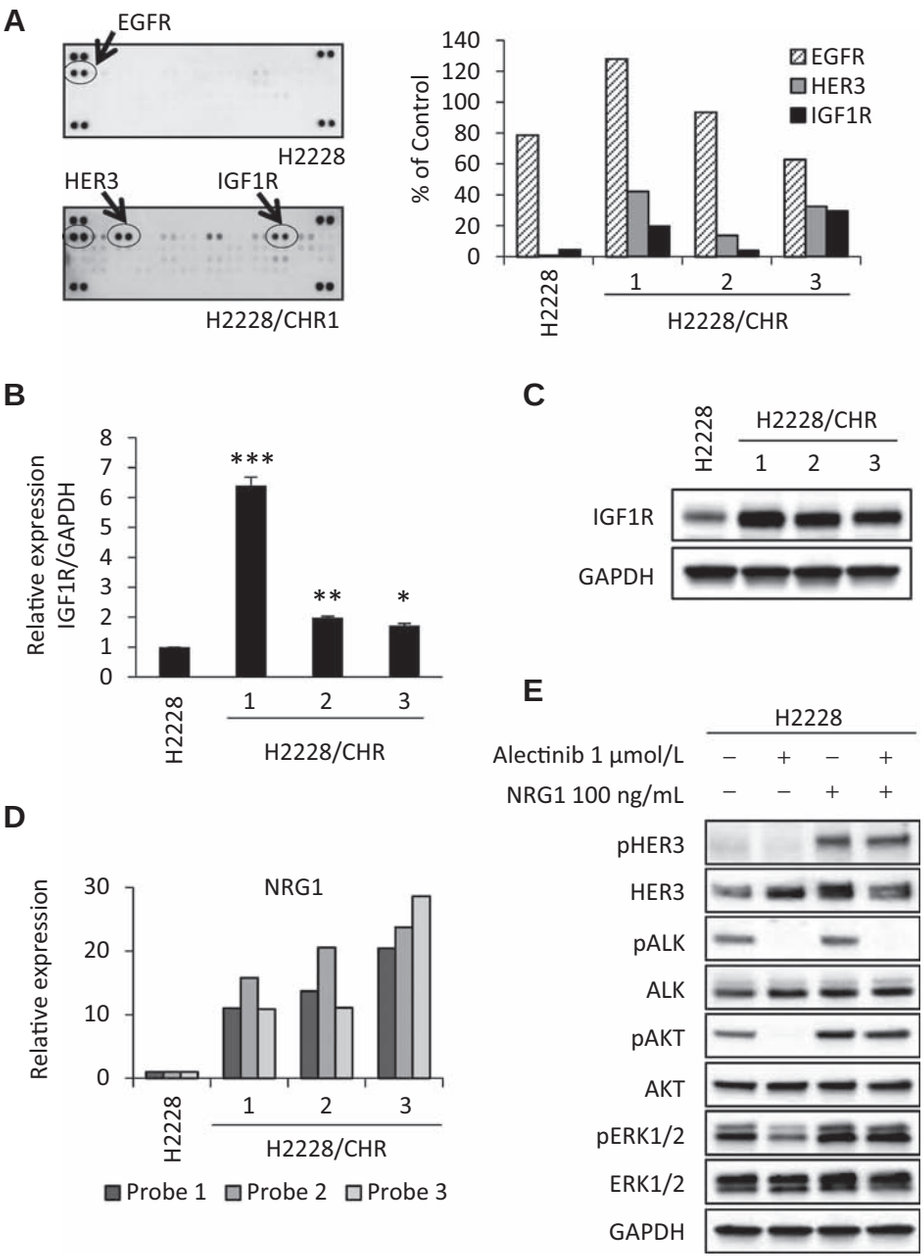

Figure 2.
IGF1R overexpression and HER3 activation with NRG1 overexpression in H2228/CHR cells. A, phospho-RTK arrays. Cells (H2228 and H2228/CHR1) were cultured, and lysates were hybridized to a phospho-RTK array. Hybridization signals located at the corners of the array served as controls. The expression levels in the bar graph were derived from the phospho-RTK arrays. B, IGF1R mRNA levels were determined by qRT-PCR. *, $P < 0.001$; **, $P < 0.01$; ***, $P < 0.05$. C, IGF1R protein expression. Cells were cultured, and lysates were analyzed by Western blotting. D, NRG1 expression levels were analyzed by mRNA array. The probe name corresponds to the code: Probe 1 (A_23_P315815), Probe 2 (A_33_P3284345), and Probe 3 (A_33_P3392192). E, effects of NRG1. Cells were seeded on 6-well plates and cultured. Thereafter, cells were incubated with alectinib at 0 or 1 μmol/L for 4 hours and with NRG1 at 0 or 100 ng/mL for 5 minutes, and lysates were analyzed by Western blotting.

11/CHR clones compared with the parental cells (Fig. 4A), which was confirmed by Western blotting (Fig. 4B). The ABC-11/CHR clones had a relative gene copy number (number of MET genes per GAPDH gene) less than 1.5-fold of that in parental cells (Fig. 4C).

Activated MET caused by HGF autocrine stimulation leads to alectinib resistance

To identify the main cause of MET activation, we focused on expression of HGF, which is a MET ligand. The HGF gene was significantly amplified in each of the ABC-11/CHR clones (Fig. 4D). mRNA expression increased markedly in the ABC-11/CHR clones (Fig. 4E). Consistent with this, HGF protein expression also increased in both cancer cell lines (Fig. 4F) and in the culture supernatants of ABC-11/CHR cells (Fig. 4G). Extrinsic HGF or conditioned medium led to resistance to alectinib in parental ABC-11 and H2228 cells (Supplementary Fig. S6A and S6B). In addition, extrinsic HGF or conditioned medium activated MET in ABC-11 cells, which was suppressed by the anti-HGF antibody (Fig. 4H and I). Combined alectinib and anti-HGF antibody treatment decreased the levels of phosphorylated MET and downstream signaling in ABC-11/CHR3 cells (Fig. 4J). The anti-HGF antibody inhibited growth dose-dependently, and combined therapy with alectinib was effective in ABC-11/CHR3 cells (Fig. 4K). Taken together, these results indicate that the cells acquired resistance to alectinib by activating MET resulting from HGF autocrine stimulation.

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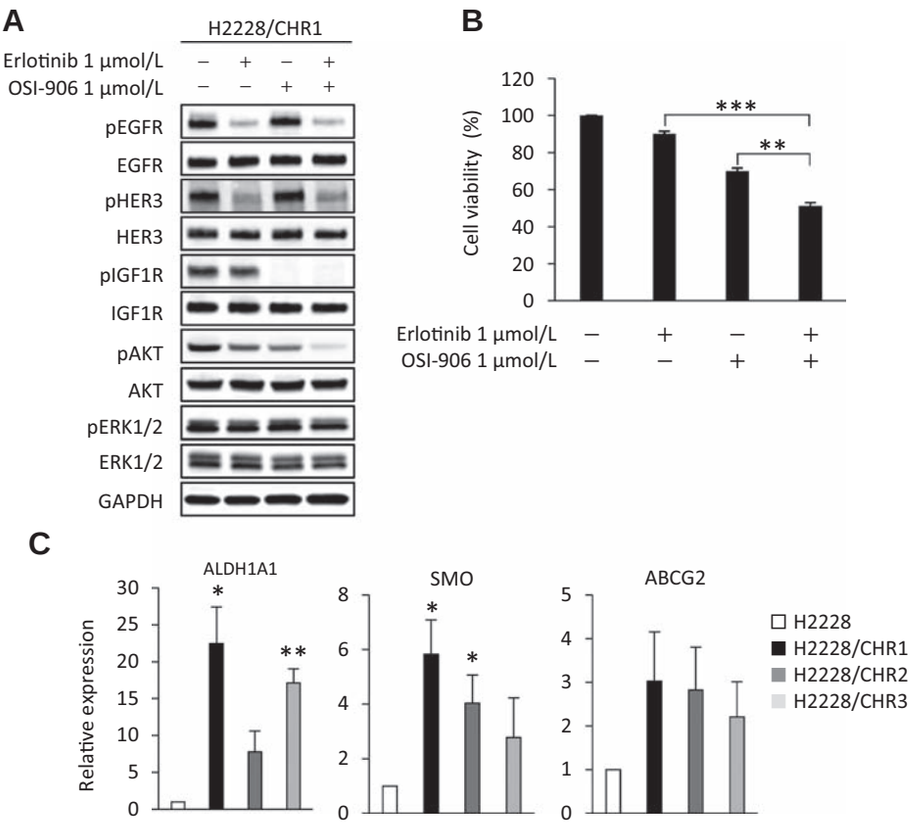

Figure 3.
IGF1R and HER3 as target proteins in H2228/CHR cells. A, effect of OSI-906 and erlotinib on survival signaling in resistant cells. H2228/CHR1 cells were left untreated or were incubated with 1 μmol/L OSI-906, 1 μmol/L erlotinib, or a combination of OSI-906 and erlotinib for 4 hours. Lysates were analyzed by Western blotting. B, sensitivity to erlotinib or OSI-906 in H2228/CHR1 cells. Cells were seeded and treated with 1 μmol/L erlotinib, 1 μmol/L OSI-906, or a combination of erlotinib and OSI-906 (1:1). Viable cells were assessed by MTT assay. C, relative gene expression levels of stem cell–related markers. ALDH1A1, SMO, and ABCG2 mRNA expression was confirmed by qRT-PCR. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

We continued culturing the ABC-11/CHR clones without alectinib for 3 months (designated ABC-11/CHR-r1–r3) to evaluate the reversibility of autocrine HGF secretion. All of the ABC-11/CHR-r clones continued to secrete HGF (Supplementary Fig. S7A) and remained resistant to alectinib but sensitive to crizotinib (Supplementary Fig. S7B).

Crizotinib overcomes resistance to alectinib

We examined whether inhibiting MET signaling was effective for overcoming alectinib resistance. The selective MET inhibitor SGX523 significantly inhibited growth of ABC-11/CHR3 cells (Fig. 5A). In addition, SGX523 combined with alectinib was more sensitive than SGX523 monotherapy (SGX523 vs. SGX523 + alectinib; $P = 0.01$). ABC-11/CHR3 cells exhibited high sensitivity to crizotinib, which inhibits both MET and ALK. MET and ALK phosphorylation and downstream AKT and ERK phosphorylation were suppressed markedly in the presence of 0.1 μmol/L crizotinib (Fig. 5B).

We also examined the effect of crizotinib in xenograft models. ABC-11 and ABC-11/CHR xenografts were treated with vehicle, alectinib, or crizotinib. ABC-11 and ABC-11/CHR xenograft tumors in vehicle-treated mice grew at the same rate. Alectinib led to almost complete inhibition of ABC-11 tumors and had a stronger effect than that of crizotinib (Fig. 5C, left top). In contrast, alectinib had little effect on ABC-11/CHR tumors (Fig. 5C, right top), which were extremely sensitive to crizotinib. No differences in body weight were observed among the vehicle-, alectinib-, and crizotinib-treated mice (Fig. 5C, bottom). Crizotinib almost completely blocked growth of ABC-11/CHR xenograft tumors for >30 days (Supplementary Fig. S8). These results suggest that first-generation crizotinib can overcome resistance to alectinib.

HGF was overexpressed in an alectinib-refractory patient

We obtained clinical samples from a 33-year-old female never-smoker with advanced NSCLC harboring the ALK fusion gene to determine if our findings are clinically relevant. Her tumor responded well to alectinib and subsequently acquired resistance. We performed IHC to detect HGF expression using the pre- and postalectinib biopsied samples. Consistent with our preclinical data, HGF was overexpressed in the postalectinib specimen but not in the prealectinib specimen (Fig. 5D). These results suggest that our findings are not limited to a preclinical setting.

Discussion

We established two alectinib-resistant cell lines to investigate the acquired resistance phenomenon in NSCLC harboring *EML4-ALK*. In contrast with previous studies, neither of the resistant cell lines had a secondary *ALK* mutation. "Mechanism 1" involves loss of *EML4-ALK* and activation of IGF1R and NRG1/HER3. "Mechanism 2" contains activation of MET as a result of autocrine stimulation by HGF (Fig. 6).

Alectinib-resistant H2228/CHR cells lost the *EML4-ALK* driver oncogene, and survival was completely independent of ALK signaling. Alternatively, IGF1R and NRG1/HER3 pathway were activated. Given that activated HER3 and overexpression of NRG1 have been described previously in the setting of crizotinib

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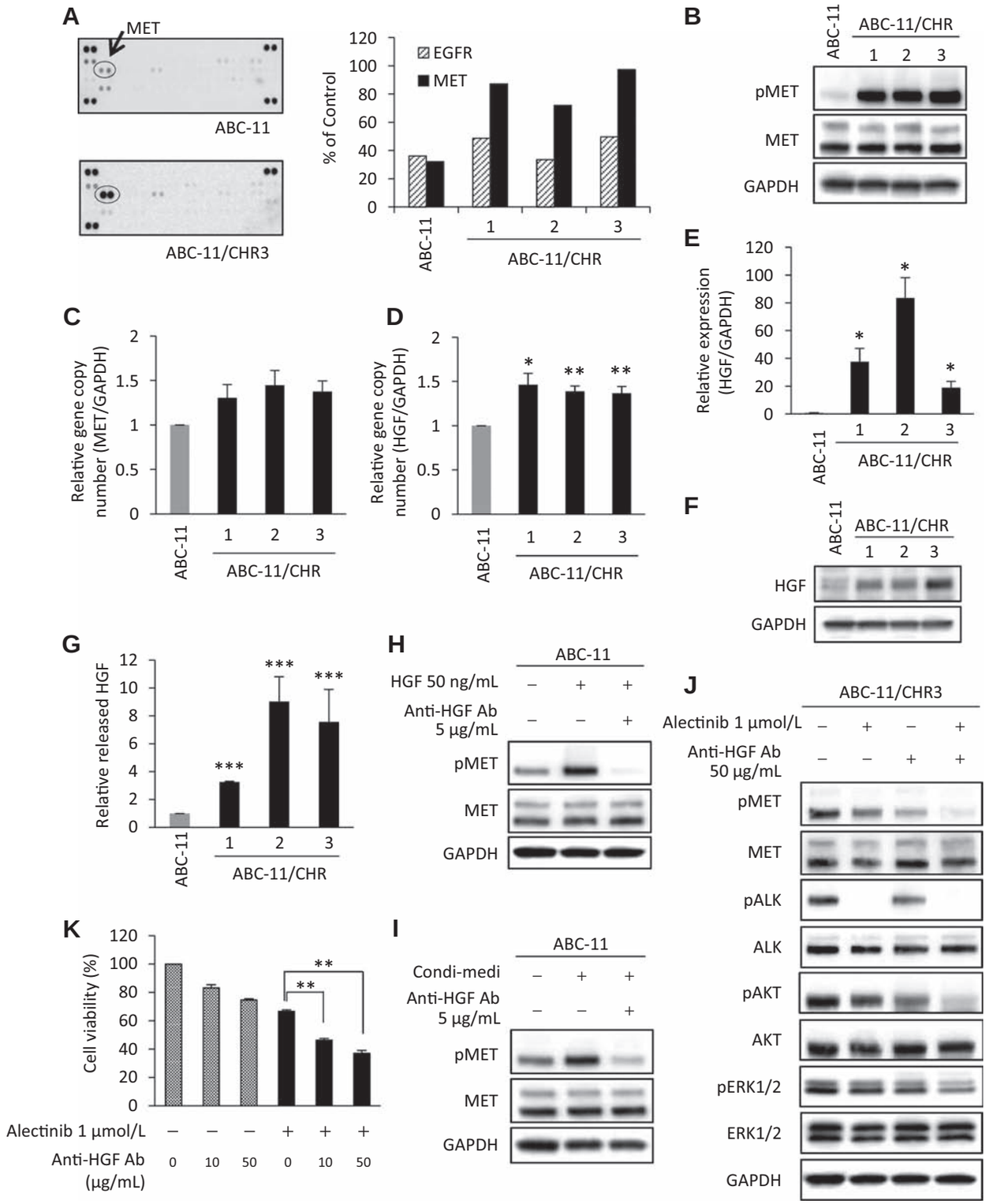

Figure 4.

HGF autocrine stimulation activates MET signaling in ABC-11/CHR cells. A, phospho-RTK arrays. B, MET protein expression and phosphorylation levels. Cells (ABC-11 and ABC-11/CHR clones) were cultured, and lysates were analyzed by Western blotting. C and D, MET and HGF gene-copy numbers. DNA derived from ABC-11 and ABC-11/CHR clones was extracted, and each gene-copy number relative to GAPDH was determined by qPCR. E, HGF mRNA expression levels. cDNA was extracted from the ABC-11 and ABC-11/CHR clones, and HGF expression levels relative to GAPDH were determined by qRT-PCR. F, HGF protein expression levels. Cells (ABC-11 and ABC-11/CHR clones) were cultured, and lysates were analyzed by Western blotting. (*Continued on the following page.*)

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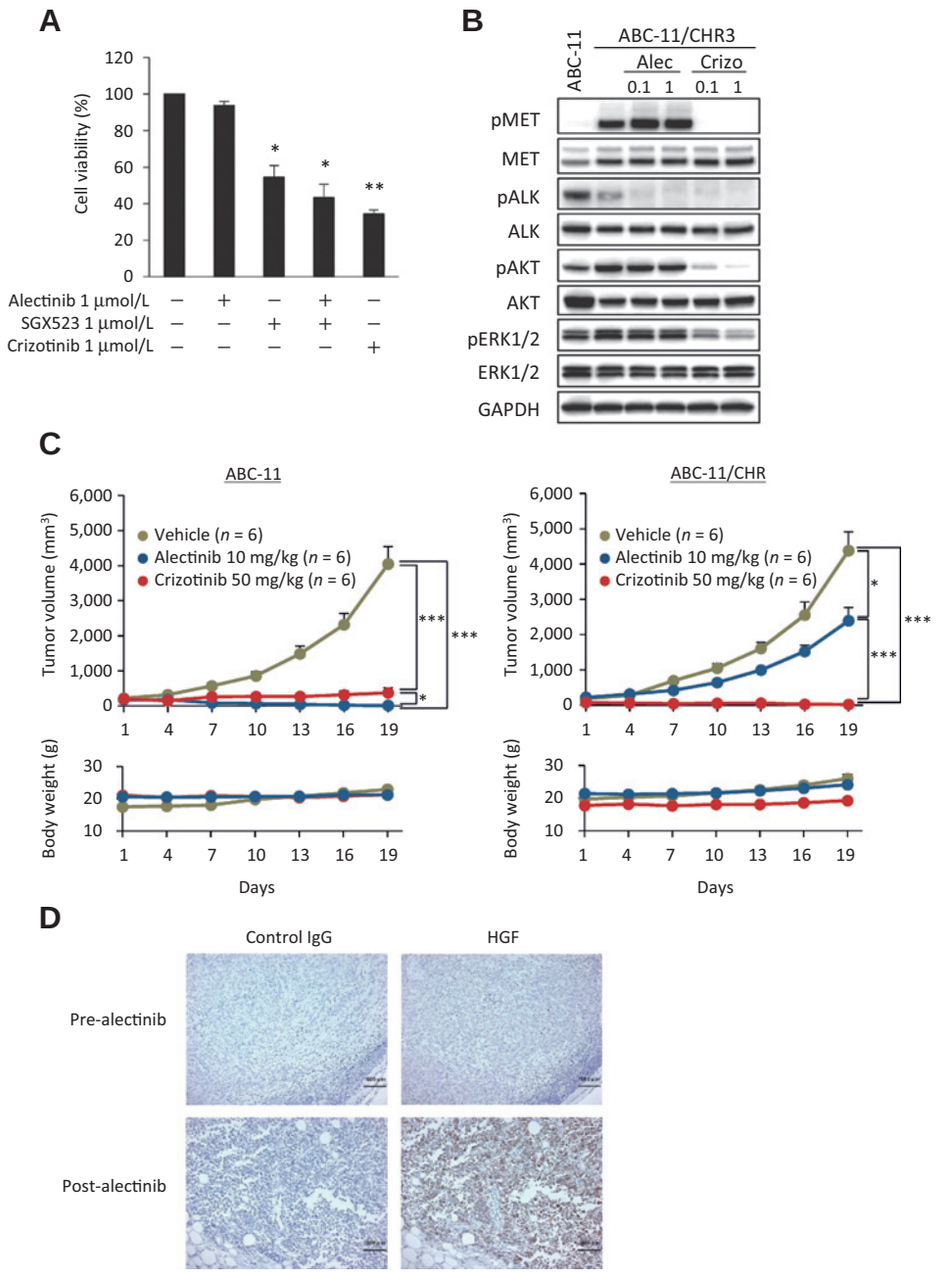

Figure 5.
Inhibition of MET significantly affected alectinib resistance. A, MET inhibitor sensitivity assay. ABC-11/CHR3 cells were seeded and treated with 1 μmol/L alectinib, SGX523 with or without alectinib, or crizotinib. Viable cells were assessed by MTT assay. *, $P < 0.001$; **, $P < 0.01$. B, effects of crizotinib on survival signaling in resistant cells. ABC-11/CHR3 cells were left untreated or were incubated with alectinib (0.1 or 1 μmol/L) or crizotinib (0.1 or 1 μmol/L) for 4 hours. Lysates were analyzed by Western blotting. C, effects of crizotinib on tumor growth and body weight in xenograft models. ABC-11 xenograft tumors and ABC-11/CHR xenograft tumors were treated with 10 mg/kg/day alectinib or 50 mg/kg/day crizotinib, respectively. The vehicle group and treated groups data were statistically analyzed on day 19. Tumor volume (top) and body weight (bottom) curves. *, $P < 0.001$; ***, $P < 0.05$. D, IHC was performed simultaneously using control rabbit IgG antibody or anti-HGF antibody. A postalectinib sample shows positive IHC staining for HGF. Scale bars, 100 μm.

resistance (14), it might be a common resistance mechanism of alectinib and crizotinib. Different from crizotinib resistance caused by NRG1, our alectinib-resistant model showed coincidental activation of IGF1R. Thus, the EGFR inhibitor when combined with the IGF1R inhibitor effectively overcame the resistance. The ALK protein was lost gradually with exposure to

(*Continued.*) G, HGF concentrations in the culture medium. Cells (ABC-11 and ABC-11/CHR clones; 2×10^5/well) were seeded in 6-well plates and incubated for 48 hours in 2-mL culture medium. Next, the culture supernatants were cleared by centrifugation. Viable supernatants were assessed by ELISA. H, influence of extrinsic HGF on parental cells. ABC-11 cells (2×10^5/well) were seeded on 6-well plates and cultured for 72 hours. Thereafter, the culture medium was replaced with fresh medium and incubated with 0 or 5 μg/mL anti-HGF antibody for 60 minutes and with 0 or 50 ng/mL HGF for 10 minutes. Lysates were analyzed by Western blotting. I, influence of conditioned medium on parental cells. ABC-11 cells (2×10^5/well) were seeded on 6-well plates and cultured for 24 hours. The culture medium was replaced with fresh medium or conditioned medium and cultured for 72 hours. Thereafter, the cells were incubated with 0 or 5 μg/mL anti-HGF antibody for 60 minutes and with HGF 0 ng/mL or 50 ng/mL for 10 minutes. Lysates were analyzed by Western blotting. J, effects of the anti-HGF antibody on survival signaling in resistant cells. ABC-11/CHR3 cells (2×10^5/well) were seeded in 6-well plates and cultured for 72 hours. They were then incubated with 0 or 50 μg/mL anti-HGF antibody for 60 minutes, and then 0 or 1 μg/mL alectinib was added for 4 hours. Lysates were analyzed by Western blotting. K, assay of sensitivity to the anti-HGF antibody. ABC-11/CHR3 cells were seeded and treated with anti-HGF antibody (0, 10, or 50 μg/mL) with or without 1 μmol/L alectinib. Viable cells were assessed by MTT assay. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Published OnlineFirst December 30, 2015; DOI: 10.1158/0008-5472.CAN-15-1010

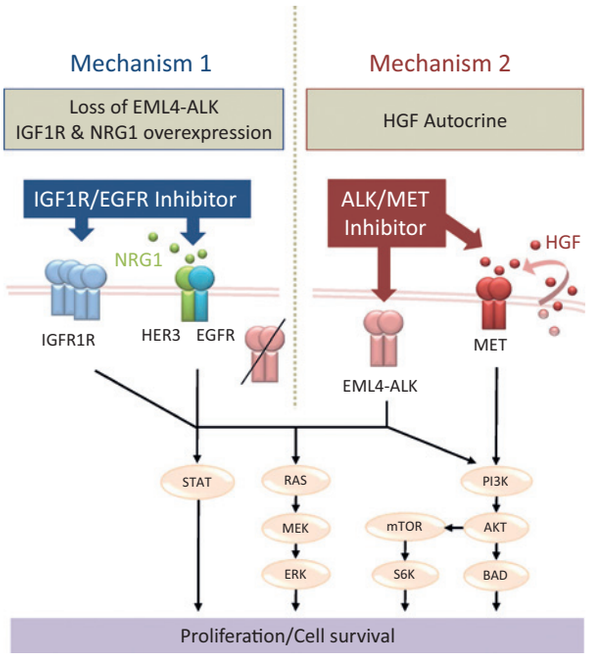

Figure 6.
Schematic mechanisms of alectinib resistance. Mechanisms 1 and 2 indicate H2228/CHR and ABC-11/CHR cells, respectively.

alectinib, and sensitivity to alectinib declined in parallel with loss of the ALK protein, suggesting that the IHC ALK-positive ratio might reflect efficacy to alectinib. More research using clinical samples is needed to confirm this hypothesis. Loss of the EML4-ALK fusion gene has also been reported in crizotinib-refractory patients (15, 30), so future research should not be limited to *in vitro* experiments. Gefitinib-resistant cells also showed loss of the *EGFR* driver oncogene and acquired stem cell–like properties (31). Consistent with this finding, H2228/CHR cells with loss of *EML4-ALK* demonstrated stem cell–like characteristics with high ALDH1A1, SMO, and ABCG2 expression. Alectinib-resistant cells might acquire a stem cell–like phenotype through loss of the driver oncogene.

The other resistant cell line, ABC-11/CHR, exhibited activated MET as a result of autocrine stimulation by HGF and sensitivity to the MET inhibitor. HGF causes acquired resistance to anticancer kinase inhibitors in oncogene-addicted cancer cells and has been presumed to be secreted into the tumor microenvironment by stromal cells, such as fibroblasts (19, 32–35). In contrast, we demonstrated that ABC-11 cells acquired the ability to secrete HGF from cancer cells themselves in an autocrine manner, but not stromal cells, resulting in resistance to alectinib. In addition, ABC-11/CHR cells exhibited significant collateral sensitivity to crizotinib. Based on these data, a phase II trial of crizotinib for alectinib-refractory patients according to HGF and MET status is ongoing (UMIN registration number 000015984). A recent case report described a patient who acquired resistance to alectinib thorough MET amplification and exhibited a dramatic response to crizotinib (36). Both this case and our model are similar, as acquired resistance is caused by activation of the MET pathway and sensitivity to crizotinib. Our model presumes that the MET pathway is activated by HGF autocrine without amplification of MET. We could not find such a case resistant to alectinib by checking MET amplification alone. Therefore, we should prepare to detect two different resistance mechanisms via the MET pathway.

Notably, H2228/CHR cells were not dependent on ALK signaling; thus, ALK-TKI was not effective at all. On the other hand, ABC-11/CHR cells were dependent on ALK signaling; thus, a dual MET and ALK inhibitor was more effective than was a MET inhibitor alone. Therefore, we need to assess the necessity of blocking ALK signaling in tumor specimens obtained by re-biopsy in patients refractory to alectinib.

Although an *ALK* secondary mutation is a common mechanism in patients with acquired resistance to crizotinib and accounts for approximately 30% of cases (37), such a mutation did not appear in our alectinib-resistant cell lines. Alectinib demonstrates selective and strong binding affinity [dissociation constant (Kd), 2.4 nmol/L; ref. 3] compared with that of the multitarget TKI crizotinib (Kd, 3.3 nmol/L; ref. 38). Nilotinib is a more potent and selective TKI for BCR-ABL than is imatinib in patients with chronic myeloid leukemia. A randomized clinical trial revealed that the incidence of secondary mutations caused by nilotinib treatment was approximately half than that by imatinib treatment (39). Therefore, we presume that bypass track activation occurs more frequently than do secondary mutations when a selective and strong TKI is used. Katayama and colleagues reported *ALK* secondary mutations as part of an alectinib-resistance mechanism: a V1180L ALK gatekeeper mutation in an *in vitro* experiment and an I1171T ALK mutation in a tumor from a patient who was treated with crizotinib followed by alectinib (17). More comprehensive research using clinical samples derived from alectinib-refractory patients is needed to confirm our speculation.

In summary, we elucidated novel mechanisms of resistance to alectinib using two types of ALK-positive cell lines. One cell line showed loss of *EML4-ALK*, and both cell lines showed activated alternative tyrosine kinase receptors that led to alectinib resistance. An IGF1R inhibitor combined with an EGFR inhibitor or crizotinib alone overcame the resistance. Our findings provide insight into next-generation therapeutic strategies for patients with ALK-positive lung cancer.

Disclosure of Potential Conflicts of Interest

N. Takigawa reports receiving commercial research grant from and has received honoraria from the Speakers Bureau of Chugai Pharma and Pfizer. K. Hotta has received honoraria from the Speakers Bureau of Chugai Pharma and Pfizer. K. Kiura reports receiving commercial research grant from Chugai Pharma and has received honoraria from the Speakers Bureau of Chugai Pharma, Novartis Pharma K.K., and Pfizer. No potential conflicts of interest were disclosed by the other authors.

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Other (provided grant): K. Kiura

Acknowledgments

The authors thank Hiromichi Ebi, MD and PhD (Kanazawa University), for the expert advice and for providing the IGF1R inhibitor OSI-906, Hiromi Nakashima for the excellent technical support, and our laboratory colleagues for the useful discussions.

Grant Support

This study was supported by the Ministry of Education, Culture, Sports, Science, and Technology, Japan [grants 24591182 (N. Takigawa) and 23390221, 15H04830 (K. Kiura)] and the Extramural Collaborative Research Grant of Cancer Research Institute, Kanazawa University.

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Received April 15, 2015; revised November 30, 2015; accepted December 13, 2015; published OnlineFirst December 30, 2015.

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Published OnlineFirst December 30, 2015; DOI: 10.1158/0008-5472.CAN-15-1010

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Published OnlineFirst December 30, 2015; DOI: 10.1158/0008-5472.CAN-15-1010

Cancer Research
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Non–Small Cell Lung Cancer Cells Acquire Resistance to the ALK Inhibitor Alectinib by Activating Alternative Receptor Tyrosine Kinases

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Cancer Res 2016;76:1506-1516. Published OnlineFirst December 30, 2015.