Paracrine receptor activation by microenvironment triggers bypass survival signals and ALK inhibitor-resistance in EML4-ALK lung cancer cells

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Statement of translational relevance:
Although crizotinib, a dual inhibitor of ALK and Met, shows dramatic effects against \textit{EML4-ALK} lung cancer cells, these cells can acquire resistance by several mechanisms, including ALK amplification and gatekeeper mutation. Selective ALK inhibitors may overcome crizotinib resistance due to these mechanisms, but these cells may become resistant to these inhibitors.

We demonstrate here that EGFR ligands produced by endothelial cells can cause \textit{EML4-ALK} lung cancer cells to become resistant to crizotinib and selective ALK inhibitors, by triggering bypass survival signals. By contrast, HGF produced by fibroblasts can induce resistance to selective ALK inhibitors, but not crizotinib. Since endothelial cells and fibroblasts are components of the microenvironment, our findings raise clinical questions about the class of ALK inhibitors more beneficial for \textit{EML4-ALK} lung cancer patients. Moreover, our results provide a rationale for targeting receptor ligands in the microenvironment for more successful treatment with ALK inhibitors.
Abstract

Purpose

Cancer cell microenvironments, including host cells, can critically affect cancer cell behaviors, including drug sensitivity. Although crizotinib, a dual tyrosine kinase inhibitor (TKI) of ALK and Met, shows dramatic effect against EML4-ALK lung cancer cells, these cells can acquire resistance to crizotinib by several mechanisms, including ALK amplification and gatekeeper mutation. We determined whether microenvironmental factors trigger ALK inhibitor resistance in EML4-ALK lung cancer cells.

Experimental Design

We tested the effects of ligands produced by endothelial cells and fibroblasts, and the cells themselves, on the susceptibility of EML4-ALK lung cancer cell lines to crizotinib and TAE684, a selective ALK inhibitor active against cells with ALK amplification and gatekeeper mutations, both in vitro and in vivo.

Results

EML4-ALK lung cancer cells were highly sensitive to ALK inhibitors. EGFR ligands, such as EGF, TGF-α, and HB-EGF, activated EGFR and triggered resistance to crizotinib and TAE684 by transducing bypass survival signaling.
through Erk1/2 and Akt. HGF activated Met/Gab1 and triggered resistance to TAE684, but not crizotinib, which inhibits Met. Endothelial cells and fibroblasts, which produce the EGFR ligands and HGF, respectively, decreased the sensitivity of EML4-ALK lung cancer cells to crizotinib and TAE684, respectively. EGFR-TKIs re-sensitized these cells to crizotinib and Met-TKI to TAE684 even in the presence of EGFR ligands and HGF, respectively.

**Conclusions**

Paracrine receptor activation by ligands from the microenvironment may trigger resistance to ALK inhibitors in EML4-ALK lung cancer cells, suggesting that receptor ligands from microenvironment may be additional targets during treatment with ALK inhibitors.

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Introduction

ALK fusion with EML4 in non-small cell lung cancer (NSCLC) was first detected in 2007(1), with 3% to 7% of unselected NSCLCs having this fusion gene (1-4). EML4-ALK lung cancer is more frequently observed in patients with adenocarcinoma than with other histologies, in young adults than in older patients, and in never smokers or light smokers (<15 pack-years) than in heavier smokers (2, 3). ALK kinase inhibitors show dramatic effects against lung cancers with EML4-ALK in vitro and in vivo (3, 4). In a phase 1-2 trial with crizotinib, a dual tyrosine kinase inhibitor (TKI) of ALK and Met, the overall response rate was 47 of 82 (57%) patients with EML4-ALK positive tumors (5). However, almost all patients who show a marked response to ALK-TKIs acquire resistance to these agents after varying periods of time (6, 7). Secondary mutations, including the gatekeeper L1196M mutation and others (F1174L, C1156Y, G1202R, S1206Y, 1151-T-ins, and G1269A), ALK amplification, KIT amplification, and autophosphorylation of EGFR, were shown to be responsible for acquired resistance to crizotinib in ALK translocated cancers (6-10).

Selective ALK inhibitors, including TAE684 and CH5424802, have been reported active against EML4-ALK lung cancer cells with ALK amplification and
secondary mutations. These cells, however, may develop resistance to this class of inhibitor, due to several mechanisms, including novel ALK mutations (L1152R, L1198P, and D1203N), coactivation of EGFR and ErbB2, and EGFR phosphorylation (3, 11, 12).

Most human cancers are composed of cancer cells that coexist with a variety of extracellular matrix components and cell types, including fibroblasts, endothelial cells, and immune cells, which collectively form the tumor microenvironment (13). This microenvironment can influence the growth, survival, invasiveness, metastatic ability, and drug sensitivity of cancer cells within these tumors (14). Paracrine signaling between cancer cells and host cells in the microenvironment, mediated by cytokines, chemokines, growth factors and other signaling molecules, play a critical role in tumor growth (15).

As receptors for these factors, the EGFR family of receptors and Met are of particular interest in lung cancer (16). The EGFR family consists of at least 4 receptor tyrosine kinases, including EGFR (ErbB1), Her2/neu (ErbB2), HER3 (ErbB3), and HER4 (ErbB4). To date, 7 ligands for EGFR have been identified: EGF, transforming growth factor α (TGF-α); heparin-binding EGF-like growth factor (HB-EGF); amphiregulin; betacellulin; epiregulin; and epigen (17).
contrast, Met is the only specific receptor for hepatocyte growth factor (HGF) and HGF binds only to Met (18). Many lung cancer cells express EGFR and Met, with these cells and others in their microenvironment expressing their ligands (19, 20), suggesting that these receptors and ligands modulate the sensitivity of cancer cells to molecular targeted drugs in their microenvironment.

We previously demonstrated that fibroblast-derived HGF induces EGFR-TKI resistance in EGFR mutant lung cancer cells by activating Met and downstream pathways (21, 22). However, the role of the microenvironment in the sensitivity of EML4-ALK lung cancer cells to ALK-TKIs has not been determined. We therefore examined whether factors in the microenvironment of EML4-ALK lung cancer cells trigger their resistance to crizotinib and TAE684, a selective ALK inhibitor, as well as clarifying their underlying mechanisms of action.

Materials and Methods

Cell culture

The H2228 human lung adenocarcinoma cell line, with the EML4-ALK fusion protein variant3 (E6;A20), the umbilical vein endothelial cell line HUVEC and the human bronchial epithelial cell line BEAS-2B, transformed with SV40 virus,
were purchased from the American Type Culture Collection (Manassas, VA, USA). The H3122 human lung adenocarcinoma cell line, with the EML4-ALK fusion protein variant1 (E13;A20), was kindly provided by Dr Jeffrey A. Engelman of the Massachusetts General Hospital Cancer Center (3). The MANA2 mouse lung adenocarcinoma cell line was established in Jichi Medical University from a tumor nodule developed in a transgenic mouse expressing \textit{EML4-ALK} variant 1 (E13;A20) (23). The MRC-5 and IMR-90 lung embryonic fibroblast cell lines were obtained from RIKEN Cell Bank. The human dermal microvessel endothelial cell line HMVEC was purchased from Kurabo (Osaka, Japan). The monocytic leukemia cell line U937 was purchased from Health Science Research Resources Bank. H2228 cells were cultured in RPMI 1640 medium, MANA2 cells were cultured in DMEM/F12+GlutaMAX-1, and MRC-5 (P 25-30) cells were cultured in DMEM medium, supplemented with 5% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (50 μg/mL), in a humidified CO\textsubscript{2} incubator at 37°C. HMVECs and HUVECs were maintained in HuMedia-MvG with growth supplements (Kurabo) and used for \textit{in vitro} assays at passages 2 to 5 and 2 to 4, respectively. BEAS-2B cells were maintained in LHC9/RPMI-1640 medium, as described (24) and used for \textit{in vitro} assays at
passages 42 to 46. Macrophage differentiation of U937 cells was induced by incubation in RPMI 1640 medium containing 10 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma Chemical Co.) (25) for 5 days, with floating cells removed by rinsing with phosphate-buffered saline (PBS), as described (26). Differentiated U937 cells (PMA-U937 cells) attached to the dishes were used for in vitro assays at passages 6 to 8. All cells were passaged for less than 3 months before renewal from frozen, early-passage stocks obtained from the indicated sources. Cells were regularly screened for mycoplasma using a MycoAlert Mycoplasma Detection Kit (Lonza, Rockland, ME).

**Reagents**

TAE684, crizotinib, BIBW2992, and WZ4002 were purchased from Seleck Chemicals (Houston, TX). Erlotinib hydrochloride was obtained from Chugai Pharmaceutical Co., Ltd (Tokyo, Japan). The anti-human EGFR antibody cetuximab was obtained from Merck Serono (Darmstadt, Germany). E7050 was synthesized by Eizai Co., Ltd., Ibaraki, Japan (27). Goat anti-human HGF antibody, control goat IgG, recombinant EGF, TGF-α, HB-EGF, IGF-1, and PDGF-AA were purchased from R&D Systems (Minneapolis, MN). Recombinant
HGF was prepared as described (28).

**Cell growth assay**

Cell proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) dye reduction method (17). Tumor cells at 80% confluence were harvested, seeded at $2 \times 10^3$ cells per well in 96-well plates, and incubated in appropriate medium for 24 hours. Several concentrations of TAE684, crizotinib, erlotinib, BIBW2992, WZ4002, E7050, cetuximab, anti-HGF antibody, and/or EGF, TGF-α, HB-EGF, IGF-1, PDGF-AA, and HGF were added to each well, and incubation was continued for a further 72 hours. To each well was added 50 μL MTT (2 mg/mL; Sigma, St. Louis, MO), followed by incubation for 2 hours at 37°C. The media were removed and the dark blue crystals in each well were dissolved in 100 μL of DMSO. Absorbance was measured with an MTP-120 microplate reader (Corona Electric, Ibaraki, Japan) at test and reference wavelengths of 550 nm and 630 nm, respectively. The percentage growth was calculated relative to untreated controls. Each assay was performed at least in triplicate, with results based on three independent experiments.
**Apoptosis assay**

H2228 and H3122 cells (3x10^3 cells) were seeded in 96-well, white walled plates and incubated overnight. The cells were treated with crizotinib (1μM) or vehicle (DMSO) for 48 h. Cellular apoptosis was determined by measuring caspase-3/7 activity using a luminometric Caspase-Glo 3/7 assay (Promega) according to the manufacturer’s protocol, with luminescence intensity measured using a Fluoroskan Ascent FL plate reader (Thermo Scientific). Cellular apoptosis was expressed relative to DMSO treated control cells.

**RNA interference**

Duplexed Stealth RNAi (Invitrogen) against EGFR, Met, ErbB3, Gab1, ALK, and Stealth RNAi Negative Control Low GC Duplex #3 (Invitrogen) were used for RNA interference (RNAi) assays. Briefly, aliquots of 1 x 10^5 cells in 2 mL of antibiotic-free medium were plated into each well of a 6-well plate and incubated at 37°C for 24 hours. The cells were transfected with siRNA (250 pmol) or scrambled RNA using Lipofectamine 2000 (5 μL) in accordance with the manufacturer’s instructions (Invitrogen). After 24 hours, the cells were washed twice with PBS, and incubated with or without crizotinib (100 nmol/L),
TAE684 (100 nmol/L), recombinant human EGF (100 ng/mL), TGF-α (100 ng/mL), HB-EGF (10 ng/mL), or HGF (50 ng/mL) for an additional 48 hours in antibiotic-containing medium. These tumor cells were then used for cell proliferation assays, with EGFR, Met, ErbB3, Gab1, and ALK knockdowns (#1, #2) confirmed by western blotting.

The siRNA target sequences were: EGFR, 5’-CGGAATAGGTATTGTTGAATTAAA-3’ and 5’-UUUAAUUCACCAAUACCUAUCCG-3’, Met, 5’-UCCAGAAGAUCGUUUCUAAUUA-3’ and 5’-UGAAUUAGGAAACUGAUCUUCUGGA-3’, ErbB3, 5’-UGGCAUGAAUGAAUUCUCUACUCUA-3’ and 5’-UGGCAUGAAUGAAUUCUCUACUCUA-3’, Gab1, 5’-UAGAGUAGCAGAGGAUGAAUCUGCC-3’ and 5’-GGCAGAUUCAUCCUCUGCUACUCUA-3’, ALK #1, 5’-UCAUUAUCCGGUAUACAGCCCAGG-3’ and 5’-CCGGGCGGUAUACCAGCCCAGG-3’, ALK #2, 5’-AAAGCUGCAGACCCAGCAUGACCGG-3’ and 5’-CCGGGCGGUAUACCAGCCCAGG-3’. Each assay was performed at
least in triplicate, with three independent experiments performed.

**Western blotting**

SDS polyacrylamide gels (Bio-Rad) were loaded with 40 μg total protein per lane; following electrophoresis, the proteins were transferred onto polyvinylidene difluoride membranes (Bio-Rad), which were incubated with Blocking One (Nacalai Tesque, Kyoto, Japan) for 1 hour at room temperature, followed by overnight incubation at 4°C with anti-ALK (C26G7), anti-phospho-ALK (Tyr1604), anti-phospho-EGFR (Tyr1068), anti-STAT-3(79D7), anti-phospho-STAT-3 (Y705), anti-Akt, anti-phospho-Akt (Ser473), anti-ErbB4 (111B2), anti-phospho-ErbB4 (Tyr1284), anti-Met (25H2), anti-phospho-Met (Y1234/Y1235) (3D7), anti-Gab1 (#3232), anti-phospho-Gab1 (Tyr627)(C32H2), anti-ErbB3 (1B2), anti-phospho-ErbB3 (Tyr1289) (21D3), or anti-β-actin (13E5) antibodies (1:1000 dilution each; Cell Signaling Technology, Danvers, MA), or with anti-human EGFR (1 μg/mL), anti-human/mouse/rat extracellular signal-regulated kinase (Erk)1/Erk2 (0.2μg/mL), or anti-phospho-Erk1/Erk2 (T202/Y204) (0.1 μg/mL) antibodies (R&D Systems, Minneapolis, MN). After washing three times, the membranes were incubated for 1 hour at room
temperature with secondary Ab (horseradish peroxidase-conjugated species-specific Ab). Immunoreactive bands were visualized with SuperSignal West Dura Extended Duration Substrate Enhanced Chemiluminescent Substrate (Pierce). Each experiment was performed at least three times independently.

**HGF, EGF, TGF-α, and HB-EGF production in cell culture supernatant**

Cells (2 x 10⁵) were cultured in 2 mL of RPMI 1640 or DMEM with 5% FBS for 24 hours. The cells were washed with PBS and incubated for 48 hours in RPMI 1640 or DMEM with 5% FBS. The culture medium was harvested and centrifuged, and the supernatant was stored at -70°C until analysis. HGF (Immunis HGF EIA: B-Bridge International, Mountain View, CA), EGF, TGF-α, and HB-EGF (Quantikine ELISA kits: R&D Systems) were assayed by ELISA, in accordance with the manufacturer’s procedures. All samples were run in triplicate. Color intensity was measured at 450 nm with a spectrophotometric plate reader. Growth factor concentrations were determined by comparison with standard curves. The detection limits for HGF, EGF, TGF-α, and HB-EGF were 0.1 ng/mL, 3.9 pg/mL, 15.6 pg/mL, and 31.2 pg/mL, respectively.
Co-culture of lung cancer cells with fibroblasts or endothelial cells

Cells were co-cultured in Transwell Collagen-Coated chambers separated by an 8-μm (BD Biosciences, Erembodegem, Belgium) or 3-μm (Corning Costar, Cambridge, MA) pore size filter. Tumor cells (8 × 10^3 cells/800 μL) with or without TAE684 (100 nmol/L) or crizotinib (100 nmol/L) in the lower chamber were co-cultured with MRC-5 (1 × 10^4 cells/300 μL) or HMVEC (1 × 10^4 cells/300 μL) cells, with or without 2 hours of pretreatment with anti-human HGF antibody (2 μg/mL), or cetuximab (2 μg/mL) in the upper chamber for 72 hours.

The upper chamber was then removed, 200 μL of MTT solution (2 mg/mL; Sigma) was added to each well and the cells were incubated for 2 hours at 37°C. The media were removed and the dark blue crystals in each well were dissolved in 400 μL of DMSO. Absorbance was measured with an MTP-120 microplate reader (Corona Electric) at test and reference wavelengths of 550 nm and 630 nm, respectively. The percentage growth was measured relative to untreated controls. All samples were assayed at least in triplicate, with each experiment performed three times independently.

Xenograft studies in SCID mice
Suspensions of H2228 cells (5 × 10^6), with or without MRC-5 cells (5 × 10^6), were injected subcutaneously into the backs of 5-week-old male severe combined immunodeficient (SCID) mice (Japan Clea). After 4 days (tumors diameter >4 mm), mice were randomly allocated into groups of six animals to receive TAE684 (1.25 mg/kg/day) or vehicle by oral gavage. Tumor size was measured with digital calipers, and tumor volume was calculated as 0.5 × length × (width)^2. All animal experiments complied with the Guidelines for the Institute for Experimental Animals, Kanazawa University Advanced Science Research Center (approval no. AP-081088).

**HGF production in tumor tissues**

Tumors obtained from SCID mice after 4 and 8 days were lysed in mammalian tissue lysis buffer containing a phosphatase and proteinase inhibitor cocktail (Sigma). HGF was quantitated by ELISA (Immunis HGF EIA; Institute of Immunology), with a detection limit of 0.1 ng/mL. All samples were assayed in triplicate.

**Statistical analysis**
Differences were analyzed by one-way ANOVA. All statistical analyses were performed using GraphPad Prism Ver. 4.01 (GraphPad Software, Inc., San Diego, CA). P < 0.05 was considered significant.

Results

*HGF and/or EGFR ligands reduced the sensitivity of EML4-ALK lung cancer cells to ALK inhibitor in vitro.*

We first examined the sensitivity of human H2228, human H3122, and mouse MANA2 lung cancer cell lines, all containing EML4-ALK translocations, to the ALK inhibitors crizotinib and TAE684, and to various EGFR-TKIs. Human H2228 cells with *EML4-ALK* variant 3 (E6;A20), and H3122 cells with *EML4-ALK* variant 1 (E13;A20), were insensitive to the EGFR-TKIs erlotinib (a reversible EGFR-TKI) and WZ4002 (selective for mutant EGFR), but sensitive to the ALK-TKIs crizotinib and TAE684 (Fig 1). MANA2 cells, established from lung tumors of an *EML4-ALK* variant 1 (E13;A20) transgenic mouse, were also sensitive to crizotinib and TAE684, although their viability was slightly inhibited by high concentrations (1 μM) of EGFR-TKIs.

Since several growth factors have been associated with poor patient prognosis
and/or drug resistance in lung cancer, we explored the effect of EGFR ligands (EGF, TGF-α, and HB-EGF), IGF-1, PDGF-AA, and HGF on the sensitivity of EML4-ALK lung cancer cells to ALK inhibitors. In the absence of ALK inhibitors, these growth factors slightly increased the viability of H2228, H3122, and MANA2 cells. In H2228 cells, all three EGFR ligands reduced sensitivity to crizotinib in a dose-dependent manner, but IGF-1, PDGF-AA, and HGF failed to do so (Fig 2, Supplementary Fig S1). Interestingly, HGF, as well as the EGFR ligands, reduced sensitivity to TAE684, but IGF-1 and PDGF-AA failed to do so. Similar results were observed in H3122 and MANA2 cells. To further confirm the effect of these growth factors on specific ALK inhibition, we knocked down ALK using two different specific siRNAs in H2228 cells. While H2228 cells were highly sensitive to ALK specific siRNAs, EGFR ligands and HGF restored cell viability inhibited by ALK knock down (Supplementary Fig S2). When we assessed the ability of crizotinib to induce apoptosis in H2228 and H3122 cells, we found that crizotinib induced apoptosis in H3122, but not H2228, cells (Supplementary Fig S3).

HGF and EGFR ligands trigger ALK inhibitor resistance via Met and EGFR,
respectively.

To assess the mechanism by which these growth factors reduced cell sensitivity to ALK inhibitors, we analyzed the phosphorylation status of ALK, receptors, and their downstream molecules in H2228, H3122, and MANA2 cells by western blotting. Crizotinib inhibited ALK phosphorylation, thereby suppressing the phosphorylation of Akt, Erk1/2 and STAT-3, as described (11) (Fig 3A, Supplementary Fig S4). The EGFR ligands, EGF, TGF-α, and HB-EGF, stimulated EGFR phosphorylation. Crizotinib inhibited ALK and STAT-3 phosphorylation even in the presence of EGFR ligands, but failed to inhibit phosphorylation of EGFR and downstream Akt, and Erk1/2. Phosphorylation of ErbB4, a potential receptor for HB-EGF, was not affected by crizotinib or EGFR ligands. To further confirm the involvement of EGFR in crizotinib resistance induced by EGFR ligands, we knocked down EGFR by specific siRNAs in H2228 and H3122 cells (Fig 3B). Although crizotinib markedly inhibited cell viability and all three EGFR ligands induced resistance in cells treated with scrambled siRNA, resistance to crizotinib was not induced by EGF, TGF-α, or HB-EGF in EGFR siRNA treated cells, indicating that EGFR ligand-triggered crizotinib resistance is mediated by EGFR.
In parallel experiments, TAE684 inhibited ALK phosphorylation, thereby suppressing the phosphorylation of Akt, Erk1/2 and STAT-3 (Fig 3C). HGF stimulated the phosphorylation of Met and its adaptor protein, Gab1, as described (29). TAE684 inhibited ALK and STAT-3 phosphorylation even in the presence of HGF, but failed to inhibit phosphorylation of Met and downstream Akt and Erk1/2. Phosphorylation of ErbB3, an adaptor of amplified but not HGF-stimulated Met (30), was not affected by TAE684 or HGF. To further confirm the involvement of Met and Gab1 in HGF-induced TAE684 resistance, we knocked down Met, ErbB3, or Gab1 by specific siRNAs in H2228 and H3122 cells (Fig 3D). TAE684 markedly inhibited the viability and HGF induced resistance in cells treated with scrambled siRNA. Importantly, treatment of cells with Met or Gab1, but not ErbB3, siRNA, induced TAE684 resistance, indicating the involvement of Met/Gab1 in HGF induced resistance to TAE684.

Cross talk of endothelial cells and fibroblasts reduces the sensitivity of EML4-ALK lung cancer cells to ALK inhibitors.

To determine which types of host cells could produce EGFR ligands and HGF, we investigated production of these growth factors by various types of host stromal cells, comparing lung epithelial cells and cancer cells. The endothelial
cell lines HMVEC produced discernible levels of EGFR ligands, including EGF, TGF-α, and HB-EGF, whereas fibroblasts produced a high level of HGF (Fig 4A). EML4-ALK lung cancer cells (H2228, H3122, and MANA2) and lung epithelial cells (BEAS-2B) produced low or no detectable levels of EGFR ligands or HGF. Interestingly, co-culture of H2228 or H3122 cells with fibroblasts (MRC-5) significantly reduced their sensitivity to TAE684, an effect abrogated by anti-HGF antibody (Fig 4B). Co-culture with endothelial cells (HMVEC) also reduced sensitivity to crizotinib, an effect inhibited by anti-EGFR antibody (Fig 4C).

These results suggest that host stromal cells, such as endothelial cells and fibroblasts, may regulate sensitivity to ALK inhibitors by secreting EGFR ligands and HGF, respectively.

HGF derived from fibroblasts induces TAE684 resistance of EML4-ALK lung cancer cells in vivo.

To investigate whether sensitivity to TAE684 could be affected by fibroblasts in vivo, we subcutaneously inoculated H2228 cells, with or without MRC-5 cells, into SCID mice. The tumors of mice injected with H2228 and MRC-5 cells grew slightly faster than those of mice injected with H2228 cells alone, but the
difference was not statistically significant by day 8 (Fig 5A). TAE684 treatment, beginning on day 4, caused marked regression of tumors in mice injected with H2228 cells alone, but not of tumors in mice injected with H2228 and MRC-5 cells, indicating that fibroblasts induced resistance to TAE684 in vivo (Fig 5A).

We confirmed that HGF was produced by MRC-5 cells in vivo. Although the tumors of mice injected with H2228 cells alone did not produce detectable levels of HGF, the tumors of mice injected with H2228 and MRC-5 cells produced high levels of HGF, started on day 4, but decreasing slightly on day 8 (Fig. 5B).

We further analyzed whether co-injection of MRC-5 cells restored the Akt pathway inhibited by TAE684 in the tumors. Western blotting showed that TAE684 treatment inhibited Akt phosphorylation, which was restored by co-injection of MRC-5 cells (Fig. 5C). These results suggest that fibroblasts produced HGF in the tumors and restored Akt phosphorylation as a survival signal, as well as inducing resistance to TAE684 in EML4-ALK lung cancer cells in vivo.

**Ligand triggered resistance to ALK inhibitors is abrogated by inhibitors of both HGF-Met and EGFR.**
To establish novel strategies to treat EGFR ligand- or HGF-triggered resistance to ALK inhibitors, we examined the effect of combinations of ALK inhibitors with EGFR inhibitors (anti-EGFR Abs, and reversible EGFR-TKIs) and HGF-Met inhibitors (anti-HGF Abs and Met-TKIs). Combined treatment with erlotinib, a reversible EGFR-TKI and cetuximab, an anti-EGFR Ab, successfully re-sensitized H2228 and H3122 cells to crizotinib even in the presence of the EGFR ligands, EGF (Fig 6A), TGF-α (Fig 6B), and HB-EGF (Fig 6C). Moreover, the combination of HGF with E7050 (Met-TKI) or anti-HGF Ab re-sensitized cells to TAE684 (Fig 6D).

Discussion

We have shown here that endothelial cells and fibroblasts, both components of the tumor microenvironment, secreted EGFR ligands and HGF, respectively, causing resistance to the ALK inhibitors crizotinib and/or TAE684 by activating bypass survival signals.

Of the EGFR ligands, EGF and TGF-α bind predominantly to EGFR, whereas HB-EGF binds to EGFR and ErbB4 (17). H2228 cells expressed both EGFR and ErbB4. Our results suggested that the bypass survival signal induced by
EGFR ligands is mediated mainly by EGFR, because EGFR ligands markedly activated the phosphorylation of EGFR, not ErbB4. Moreover, knock down of EGFR abrogated resistance caused by all EGFR ligands tested. EGFR ligand-triggered resistance was canceled by erlotinib or cetuximab, an anti-EGFR Ab, drugs approved for the treatment of patients with NSCLC and colorectal cancer. In addition, AP26113, an inhibitor of both ALK and EGFR, has been reported active against EML4-ALK lung cancer cells with amplified ALK and secondary mutations (7). Therefore, clinical trials are warranted to evaluate the efficacy and feasibility of combinations of an ALK inhibitor and these EGFR inhibitors to overcome ALK inhibitor resistance.

HGF, the sole ligand of Met (29), is important in EGFR-TKI resistance in EGFR mutant lung cancer. HGF derived from cancer cells or stromal fibroblasts activated Met phosphorylation and stimulated the downstream Akt and Erk1/2 pathways (21, 22, 30) utilizing Gab1, an adaptor protein for Met (31), triggering resistance to both reversible and irreversible EGFR-TKIs. In our Japanese cohort study of patients with EGFR mutant lung cancer, high HGF expression was detected in 61% of tumors with acquired resistance and in 29% of tumors with intrinsic resistance to EGFR-TKIs, suggesting the rationale of targeting
HGF to overcome EGFR-TKI resistance (32). We also found that HGF triggered TAE684 resistance by activating Met and stimulating downstream Akt and Erk1/2 pathways utilizing the adaptor protein Gab1. Since many anti-HGF Abs and Met-TKIs are being evaluated in clinical trials, HGF-triggered resistance to selective ALK inhibitors may be controlled by their combinations in the near future.

EGFR and Met have been shown to interact with each other and to mediate redundant signaling in lung cancer cells (33). In EGFR mutant lung cancer cells, Met amplification causes EGFR-TKI resistance by triggering bypass survival signals utilizing ErbB3, an adaptor protein (34). Met activation by HGF also triggers resistance to EGFR-TKIs that utilize Gab1 as an adaptor. In EML4-ALK lung cancer cells, both novel ALK second mutations and autocrine EGFR activation causes resistance to ALK inhibitors (11). We found that paracrine HGF and EGFR ligands could trigger ALK inhibitor resistance. Taken together, these findings suggest that signaling by EGFR and Met is crucial for the survival of lung cancer cells with EGFR mutations and EML4-ALK translocations under inhibition of these driver oncogenes.

We found that resistance to TAE684 was induced by both EGFR ligands and
HGF, whereas crizotinib resistance was induced by EGFR ligands alone, a finding that may be due to the dual activities of crizotinib on ALK and Met (5). Selective ALK inhibitors are expected to be effective against EML4-ALK lung cancer cells even, after acquiring ALK amplification and ALK second mutations and becoming refractory to crizotinib (7, 35). Our findings, however, suggest that HGF-triggered resistance may be directed against selective ALK inhibitors, not crizotinib. Future clinical trials with selective ALK inhibitors may reveal the class of ALK inhibitors that is more beneficial for EMK4-ALK lung cancer patients.

EML4-ALK and EGFR mutant lung cancers show dramatic responses to ALK inhibitors and EGFR-TKIs, respectively (5, 36, 37). Complete responses, however, are rarely achieved, despite these cells express the target (EML4-ALK or mutant EGFR) of the drug. Low expression of BIM, a pro-apoptotic molecule, may explain, at least in part, the variations in sensitivity of EGFR mutant lung cancer to EGFR-TKIs (38). This heterogeneous sensitivity may also be explained by HGF, because HGF is expressed more or less equally in EGFR mutant lung tumors sensitive to EGFR-TKIs (32). Therefore, EGFR ligands in EML4-ALK lung tumors may be involved in their heterogeneous response to
crizotinib. It is also curious whether ligand-triggered resistance is an independent mechanism or one that provided partial resistance when combined with another mechanism. Since crizotinib is expected to be approved in Japan to treat EML4-ALK lung cancer in 2012, we are planning a study to assess this possibility in clinical specimens.

In conclusion, we found that receptor ligands, such as EGFR ligands and HGF could cause resistance to the ALK inhibitors crizotinib and/or TAE684 by activating bypass survival signals. These ligands and growth factors may be produced by host stromal cells, which constitute the cancer microenvironment. Paracrine HGF from stromal fibroblasts may also trigger resistance to EGFR-TKIs in EGFR mutant lung cancer cells by activating bypass signals \((22)\). Collectively, these observations suggest that paracrine receptor activation by the microenvironment may be an important mechanism inducing resistance to molecular targeted drugs in oncogene-activated lung cancer cells. These findings suggest that targeting of receptor ligands may result in more successful therapy in lung cancer.
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Legends for Figure

Figure 1

EML4-ALK lung cancer cells are highly sensitive to the ALK inhibitors, crizotinib and TAE684.

The sensitivity of EML4-ALK lung cancer cells, human H2228, human H3122, and mouse MANA2, to the ALK inhibitors, crizotinib and TAE684, were determined by analyzing the effects of the EGFR-TKIs, erlotinib (reversible EGFR-TKI) and WZ4002 (mutant EGFR selective TKI). Tumor cell growth after 72 h was measured by the MTT assay. Each sample was assayed in triplicate, with each experiment repeated at least three times independently.

Figure 2

HGF and/or EGFR ligands (EGF, TGF-α, and HB-EGF) reduce the sensitivity of EML4-ALK lung cancer cells to ALK inhibitors in vitro.

H2228, H3122, and MANA2 cells were incubated with or without crizotinib (100
nmol/L), TAE684 (100 nmol/L) and/or EGF, TGF-α, IGF-I, or PDGF-AA (100 ng/mL); HB-EGF (10 ng/mL), or HGF (50 ng/mL), with cell growth determined after 72 hours. The percentage growth is shown relative to untreated controls. Each sample was assayed in triplicate, with each experiment repeated at least three times independently.

**Figure 3**

**HGF and EGFR ligands trigger ALK inhibitor resistance via Met/Gab1 and EGFR, respectively.**

(A) Crizotinib inhibited the phosphorylation of ALK and STAT-3 but did not that of EGFR, Akt and Erk1/2 in the presence of EGF, TGF-α, or HB-EGF. Tumor cells were treated with or without crizotinib (100 nmol/L) for 1 h and/or EGF (100 ng/mL), TGF-α (100 ng/mL), or HB-EGF (10 ng/mL) for 15 min. The cells were lysed and the indicated proteins were detected by immunoblotting. The results shown are representative of 3 independent experiments.

(B) Control or *EGFR*-specific siRNAs were introduced into H2228 and H3122 cells. After 24 hours, the cells were incubated with or without crizotinib (100 nmol/L), and/or EGF (100 ng/mL), TGF-α (100 ng/mL), or HB-EGF (10 ng/mL)
for 72 h and lung cancer cell growth was determined by MTT assays. *EGFR* knockdown was confirmed by immunoblotting. The percentage of growth is shown relative to untreated controls. Each sample was assayed in triplicate, with each experiment repeated at least three times independently.

(C) TAE684 inhibited the phosphorylation of ALK and STAT-3, but not of Met, Gab1, Akt and Erk1/2 in the presence of HGF. Tumor cells were treated with or without TAE684 (100 nmol/L) for 1 h and/or HGF (50 ng/mL) for 15 min. The cells were lysed and the indicated proteins were detected by immunoblotting. The results shown are representative of 3 independent experiments.

(D) Control or *Met*, *ErbB3*, or *Gab1*-specific siRNAs were introduced into H2228 and H3122 cells. After 24 hours, the cells were incubated with or without TAE684 (100 nmol/L) and/or HGF (50 ng/mL) for 72 h and lung cancer cell growth was determined by MTT assays. *Met*, *Gab1*, and *ErbB3* knockdowns were confirmed by immunoblotting. The percentage of growth is shown relative to untreated controls. Each sample was assayed in triplicate, with each experiment repeated at least three times independently.

**Figure 4**
Cross talk of endothelial cells and fibroblasts reduces sensitivity of EML4-ALK lung cancer cells to ALK inhibitors.

(A) Receptor ligand production was assayed in lung cancer (H2228, H3122, and MANA2,), human bronchial epithelial cell (BEAS-2B), fibroblasts (MRC-5 and IMR-90), endothelial cells (HUVEC and HMVEC), and the macrophage differentiated cell line (PMA-U937). The cells were incubated in medium for 48 h, culture supernatants were harvested, and EGF, TGF-α, HB-EGF, and HGF concentrations were determined by ELISA. All samples were assayed in triplicate.

(B) H2228 and H3122 cells were co-cultured with or without fibroblasts, MRC-5 cells and/or anti-HGF neutralizing antibody (2 μg/mL), in the presence or absence of TAE684 (100 nmol/L) for 72 h, with cell growth determined by MTT assays. *, P < 0.05 (one-way ANOVA). Each experiment included triplicate determinations, and each experiment was repeated at least three times independently.

(C) Endothelial cell-derived EGFR ligands induced crizotinib resistance in lung cancer cells with EML4-ALK fusion protein, an induction abrogated by blockade of EGFR. H2228 and H3122 cells were cocultured with or without endothelial
cells, HMVECs and/or anti-EGFR neutralizing antibody (2 μg/mL) in the presence or absence of crizotinib (100 nmol/L) for 72 h, with cell growth determined as in B. *, P < 0.05 (one-way ANOVA). Each experiment included triplicate determinations, with each experiment repeated at least three times independently.

Figure 5

HGF derived from fibroblasts induces TAE684 resistance of EML4-ALK lung cancer cells in vivo.

(A) Fibroblast-derived HGF induced TAE684 resistance in H2228 tumors in SCID mice. H2228 cells (5 × 10⁶), with or without MRC-5 cells (5 × 10⁶), were inoculated subcutaneously into SCID mice on day 0. Starting on day 4, mice received oral TAE684 (1.25 mg/kg/d) or vehicle alone, with tumor size measured on days 4 and 8. Tumor volumes were calculated as described in Materials and Methods. Data shown are the representative of 2 independent experiments. Error bars indicate standard errors of 6 mice. P < 0.05 was considered significant by one-way ANOVA. NS, not significant.

(B) HGF production by tumor tissues. Tumors were harvested on days 4 and 8
and lysed, and HGF in the lysates were assayed by ELISA. All samples were assayed in triplicate.

(C) Fibroblast-derived HGF induced TAE684 resistance via the Akt signal pathway in vivo. Tumors were harvested 2 hours after treatment on day 7 and lysed, and the lysates were analyzed by immunoblotting with the indicated antibodies, as described in Materials and Methods. The results shown are representative of 2 independent experiments.

Figure 6

Ligand triggered resistance to ALK inhibitors is abrogated by inhibitors of both HGF-Met and EGFR.

(A-C) The EGFR inhibitors erlotinib and cetuximab abrogated EGFR ligand-induced crizotinib resistance in EML4-ALK lung cancer cells. H2228 and H3122 cells were treated for 72 h with or without crizotinib (100 nmol/L) and/or EGF (100 ng/mL), TGF-α (100 ng/mL), or HB-EGF (10 ng/mL) in the presence or absence of erlotinib (1 μmol/L) or cetuximab (2 μg/mL). Cell growth was determined by MTT assays. *, P < 0.01 (one-way ANOVA). Each experiment included triplicate determinations, and each experiment was repeated at least
three times independently.

(D) Met-TKI E7050 or Anti-HGF antibody abrogated HGF-induced TAE684 resistance in EML4-ALK lung cancer cells. H2228 and H3122 cells were treated for 72 h with or without TAE684 (100 nmol/L) and/or HGF (50 ng/mL) in the presence or absence of E7050 (1 μmol/L) or anti-HGF neutralizing antibody (2 μg/mL). Cell growth was determined by MTT assays. *, P < 0.01 (one-way ANOVA). Each experiment included triplicate determinations, and each experiment was repeated at least three times independently.
Figure 1

% cell viability

Drug concentration (μM)

0 0.001 0.01 0.1

H3122

Erlotinib

Crizotinib

TAE684

WZ4002

H2228

% cell viability

Drug concentration (μM)

0 0.001 0.01 0.1

Crizotinib

Erlotinib

TAE684

WZ4002

MANA 2
Figure 3

A

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</tr>
<tr>
<td>EGF</td>
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</tr>
<tr>
<td>TGF-α</td>
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</tr>
<tr>
<td>HB-EGF</td>
<td>-</td>
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Crizotinib | - | + | - | + | - | + |

p-ALK | + | - | + | - | + | - |
ALK | + | - | + | - | + | - |
p-EGFR | + | - | + | - | + | - |
EGFR | + | - | + | - | + | - |
p-ErbB4 | + | - | + | - | + | - |
ErbB4 | + | - | + | - | + | - |
p-STAT-3 | + | - | + | - | + | - |
STAT-3 | + | - | + | - | + | - |
p-Akt | + | - | + | - | + | - |
Akt | + | - | + | - | + | - |
p-Erk1/2 | + | - | + | - | + | - |
Erk1/2 | + | - | + | - | + | - |
β-actin | + | - | + | - | + | - |

B

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p-ALK | + | - | + | - |
ALK | + | - | + | - |
p-Met | + | - | + | - |
Met | + | - | + | - |
p-ErbB3 | + | - | + | - |
ErbB3 | + | - | + | - |
p-Gab1 | + | - | + | - |
Gab1 | + | - | + | - |
p-STAT3 | + | - | + | - |
STAT3 | + | - | + | - |
p-Akt | + | - | + | - |
Akt | + | - | + | - |
p-Erk1/2 | + | - | + | - |
Erk1/2 | + | - | + | - |
β-actin | + | - | + | - |

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<td>siRNA Gab1</td>
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Crizotinib | - | + | - | + | - | + |
EGF | - | + | - | + | - | + |
TGF-α | - | + | - | + | - | + |
HB-EGF | - | + | - | + | - | + |

TAE684 | - | + | - | + |
HGF | - | + | - | + |

% cell viability

Crizotinib | - | + | - | + | - | + |
EGF | - | + | - | + | - | + |
TGF-α | - | + | - | + | - | + |
HB-EGF | - | + | - | + | - | + |

TAE684 | - | + | - | + |
HGF | - | + | - | + |
Figure 4

(A) Levels of EGF, TGF-α, HB-EGF, and HGF in various cell lines.

(B) Cell viability analysis of H2228 and H3122.

(C) Effect of Crizotinib and Anti-EGFR Ab on cell viability.

MRC-5, TAE684, Anti-HGF Ab, Crizotinib, and Anti-EGFR Ab are indicated.

* indicates significant difference.

EGF (pg/2×10^5 cells/48hr): H2228 <1.0, H3122 <10, MANA2 <10, BEAS-2B <1.0, MRC-5 <10, IMR-90 <10, HUVEC <1.0, HMVEC <1.0, PMA-U937 <10.

TGF-α (pg/2×10^5 cells/48hr): H2228 <10, H3122 <30, MANA2 <30, BEAS-2B <10, MRC-5 <10, IMR-90 <10, HUVEC <1.0, HMVEC <30, PMA-U937 <0.1.

HB-EGF (ng/2×10^5 cells/48hr): H2228 <10, H3122 <10, MANA2 <10, BEAS-2B <10, MRC-5 <10, IMR-90 <10, HUVEC <1.0, HMVEC <30, PMA-U937 <0.1.

HGF (ng/2×10^5 cells/48hr): H2228 <10, H3122 <10, MANA2 <10, BEAS-2B <10, MRC-5 <10, IMR-90 <10, HUVEC <1.0, HMVEC <30, PMA-U937 <0.1.
Figure 5

**A**

![Bar chart showing tumor volume comparison between H228/Control and H228/MRC-5/Control, as well as H228/TAE684 and H228/MRC-5/TAE684.](image)

- NS
- P < 0.001

**B**

![Graph showing HGF expression in H228 and H228+MRC-5 cell lines on Day 4 and Day 8.](image)

- H228
- H228+MRC-5
- Day 4
- Day 8

**C**

![Western blot analysis of TAE684, MRC-5, p-Akt, Akt, and β-actin expression levels.](image)

- TAE684
- MRC-5
- p-Akt
- Akt
- β-actin
Figure 6

A. H2228 and H3122 cell viability after treatment with Crizotinib and EGF.

B. H2228 and H3122 cell viability after treatment with Crizotinib and TGF-α.

C. H2228 and H3122 cell viability after treatment with Crizotinib and HB-EGF.

D. H2228 and H3122 cell viability after treatment with Crizotinib and E7050 anti-HGF Ab.
Paracrine receptor activation by microenvironment triggers bypass survival signals and ALK inhibitor-resistance in EML4-ALK lung cancer cells

Tadaaki Yamada, Shinji Takeuchi, Junya Nakade, et al.

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