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Amphiregulin triggered epidermal growth factor receptor activation confers \textit{in vivo} crizotinib-resistance of EML4-ALK lung cancer and circumvention by epidermal growth factor receptor inhibitors

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Key words
Amphiregulin, crizotinib-resistance, EML4-ALK, epidermal growth factor receptor, lung cancer

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Chinoderm microtubule-associated protein-like 4 (EML4)-anaplastic lymphoma kinase (ALK) positive lung cancer accounts for 3–5% of lung adenocarcinoma; it is more prevalent in younger people and non- or light-smokers.1–3 Crizotinib, a first-generation ALK-tyrosine kinase inhibitor (TKI), is known to be effective against EML4-ALK-positive non-small cell lung cancers. Nonetheless, the tumors subsequently become resistant to crizotinib and recur in almost every case. The mechanism of the acquired resistance needs to be deciphered. In this study, we established crizotinib-resistant cells (A925LPE3-CR) via long-term administration of crizotinib to a mouse model of pleural carcinomatous effusions; this model involved implantation of the A925LPE3 cell line, which harbors the EML4-ALK gene rearrangement. The resistant cells did not have the secondary ALK mutations frequently occurring in crizotinib-resistant cells, and these cells were cross-resistant to alectinib and ceritinib as well. In cell clone #2, which is one of the clones of A925LPE3-CR, crizotinib sensitivity was restored via the inhibition of epidermal growth factor receptor (EGFR) by means of an EGFR tyrosine-kinase inhibitor (erlotinib) or an anti-EGFR antibody (cetuximab) \textit{in vitro} and in the murine xenograft model. Cell clone #2 did not have an EGFR mutation, but the expression of amphiregulin (AREG), one of EGFR ligands, was significantly increased. A knockdown of AREG with small interfering RNAs restored the sensitivity to crizotinib. These data suggest that overexpression of EGFR ligands such as AREG can cause resistance to crizotinib, and that inhibition of EGFR signaling may be a promising strategy to overcome crizotinib resistance in EML4-ALK lung cancer.
Materials and Methods

Cell cultures and reagents. A human lung adenocarcinoma cell line, A925L, and its highly tumorigenic variant, A925LPE3, with an EML4-ALK fusion protein (variant 5a, E2:A20) were used in this study. All cells were maintained in RPMI-1640 medium supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (10 µg/mL) in a humidified CO2 incubator at 37°C. All cells were passaged for less than 3 months before renewal from frozen early-passage stocks. Cells were regularly screened for mycoplasma using a MycoAlert Mycoplasma Detection Kit (Lonza, Rockland, ME, USA). Erlotinib, alectinib and ceritinib were obtained from Selleck Chemicals (Houston, TX, USA), crizotinib was obtained from Active Biochem (Hong Kong, China), and cetuximab was obtained from Merck Serono (Darmstadt, Germany), recombinant AREG was obtained from R&D Systems.

Antibodies and western blot analysis. Protein aliquots of 25 µg each were separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad, Hercules, CA, USA) and transferred to polyvinylidene difluoride membranes (Bio-Rad). Membranes were washed three times and then incubated with Blocking One solution (Nacalai Tesque, Inc., Kyoto, Japan) for 1 h at room temperature. The membranes were incubated overnight at 4°C with primary antibodies against anti-ALK (C26G7), anti-phospho-ALK (Tyr1604), anti-phospho-EGFR (Tyr1068), anti-phospho-ALK (Ser473), cleaved PARP (Asp214), anti-AKT, anti-phospho-AKT (Ser473), anti-EGFR (1L9), anti-phospho-EGFR (Tyr1068), and antibodies against anti-ALK (C26G7), anti-phospho-ALK (Tyr1604), anti-phospho-EGFR (Tyr1068), anti-AKT, anti-phospho-AKT (Ser473), cleaved PARP (Asp214), and anti-β-actin (13E5) antibodies (1:1000 dilution each; Cell Signaling Technology, Danvers, MA, USA) and anti-human EGFR (1 µg/mL), anti-human/mouse/rat extracellular signal-regulated kinase (Erk1)/Erk2 (0.2 µg/mL), or anti-phospho-Erk1/Erk2 (T202/Y204) (0.1 µg/mL) antibodies (R&D Systems). The membranes were washed three times and then incubated for 1 h at room temperature with species-specific horseradish peroxidase-conjugated secondary antibodies. Immunoreactive bands were visualized with SuperSignal West Dura Extended Duration Substrate, an enhanced chemiluminescent substrate (Pierce Biotechnology, Rockford, IL, USA). Each experiment was performed independently at least three times.

Cell viability assay. Cell viability was measured using the MTT dye reduction method. Tumor cells (2–3 x 10^6 cells/100 µL/well) in RPMI 1640 medium with 10% FBS were plated onto 96-well plates and cultured with the indicated compound for 72 h. Afterwards, 50 µg of the MTT solution (2 mg/mL, 21; Sigma, St. Louis, MO, USA) was added to each well. Plates were incubated for 2 h, the medium was removed, and the dark blue crystals in each well were dissolved in 100 µL of DMSO. Absorbance was measured with a microplate reader at a test wavelength of 550 nm and a reference wavelength of 630 nm. Percent growth was determined relative to untreated controls. Experiments were repeated at least three times with triplicate samples.

Short interfering RNA knockdown. Duplexed Stealth RNAi (Invitrogen) against EGFR and AREG, Stealth RNAi-negative control low GC Duplex #3 (Invitrogen), and ALK (Dharmacon, Lafayette, CO, USA) were used for RNA interference (RNAi) assays (DCCS-S1). Briefly, aliquots of 1–2 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 h. The cells were transfected with siRNA (250 pmol) or scrambled RNA using Lipofectamine 2000 (5 µL) in accordance with the manufacturer’s instructions (Invitrogen).

Cytokine production. Cells (2 x 10^5) were cultured in RPMI-1640 medium with 10% FBS for 24 h, washed with PBS, and incubated for 48 h in 2 mL of the same medium. The culture medium was harvested and centrifuged, and the supernatant was stored at −70°C until analysis. Levels of AREG, β-cellulin, transforming growth factor-β (TGF-β), HB-EGF, and EGF were determined with Quantikine enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems) for those cytokines in accordance with the manufacturer’s protocols. All culture supernatants were tested twice. Color intensity was measured at 450 nm using a spectrophotometric plate reader. Concentrations of growth factors were determined in comparison to standard curves.

Tumor cell inoculation in mice with SCID. Five-week-old male mice with severe combined immunodeficiency (SCID) were obtained from Clea Japan (Tokyo, Japan). All animal experiments complied with the Guidelines of the Institute for Laboratory Animals, Advanced Science Research Center, Kanazawa University. For the pleural carcinomatosis model, an incision was made in the skin and subcutaneous tissue on the right side of the animal, the chest and abdomen was exposed. A 27-gauge needle was then used to inject tumor cells (1 x 10^6/0.1 mL) through the parietal pleura into the right thoracic cavity. The incision was subsequently sutured closed. Tumor luminescence and mouse body weight were measured twice a week. For the subcutaneous tumor model, cultured tumor cells (A925LPE3 and #2; 1.5 x 10^6 cells/0.1 mL) were subcutaneously implanted into the flanks of each mouse. The size of the subcutaneous tumors and the body weight of the mice were measured twice a week, using calipers, and tumor volume was calculated in mm³ (width² x length/2). At 10 days after inoculation, mice were anesthetized with the vehicle, crizotinib, or erlotinib orally, cetuximab intraperitoneally, or a combination for 15 days. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Education, Culture, Sports, Science, and Technology, Japan. The protocol was approved by the Ethics Committee on the Use of Laboratory Animals and the Advanced Science Research Center, Kanazawa University, Kanazawa, Japan (approval no. AP-153499). Surgery was performed once animals were anesthetized with sodium pentobarbital, and efforts were made to minimize animal suffering. According to institutional guidelines, mice were sacrificed using an overdose of sodium pentobarbital, when their tumor volume reached 1000 mm³.

Luciferase expression and radiographic analyses with an IVIS imaging system. After inoculation, the quantity of tumors was tracked in live mice by repeated noninvasive optical imaging of tumor-specific luciferase activity using the IVIS Lumina XR Imaging System (PerkinElmer, Alameda, CA, USA). Mice were anesthetized with 2% isoflurane and intraperitoneally injected with the luciferase substrate luciferin (150 mg/kg). Twenty minutes later, the mice were photographed under bright-field illumination and the images were overlaid with luminescence data gathered over the maximum exposure period (5–30 s). The intensity of the bioluminescence signal was analyzed with Living Image 4.0 software (PerkinElmer) by serially quantifying the peak photon flux in the selected region of interest (ROI) within the tumor. The intensity of the bioluminescence signal was corrected for the total area of the ROI.
and elapsed time during which bioluminescence signals were read by the CCD camera, and this value was expressed as photons/s/cm²/sr.

**Histological analyses of tumors.** Formalin fixed, paraffin embedded tissue sections (4 μm thick) were deparaffinized. Proliferating cells were detected by incubating tissue sections with Ki-67 antibody (Clone MIB-1; DAKO Corp, Glostrup, Denmark). Antigen was retrieved by microwaving tissue sections in 10 mM citrate buffer (pH 6.0). After incubation with secondary antibody and treatment with the Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, USA), peroxidase activity was visualized using the DAB reaction. The sections were counterstained with hematoxylin.

**Quantification of immunohistochemistry results.** The five areas containing the highest numbers of stained cells within each section were selected for histologic quantitation by light or fluorescent microscopy at 400-fold magnification.

**Statistical analysis.** Differences between groups were analyzed with one-way analysis of variance (ANOVA). All statistical analyses were performed using GraphPad StatMate 4 (GraphPad Software, Inc., San Diego, CA, USA). \( P < 0.05 \) was considered significant.

**Results**

**Crizotinib-resistant EML4-ALK-positive lung cancer cells were established in a model of carcinomatous pleurisy.** A925LPE3 cells were implanted in the thoracic cavity of mice with SCID in order to establish a model of carcinomatous pleurisy and obtain cells that were resistant to crizotinib. A925LPE3 and A925L had similar sensitivity to crizotinib (Fig. S1). Crizotinib was administered daily (50 mg/kg) starting on day 12 after the implantation of cancer cells. Tumor luminescence gradually increased starting from day 40 after implantation. These results suggested that the cells in the thoracic cavity were crizotinib-resistant. On day 70 after the implantation of cancer cells, the mice were sacrificed. The cells were collected from the pleural effusions and cultured in vitro to establish the A925LPE-CR (APE-CR) cell line. They were then cloned by limiting dilution to establish the #2 cell line (Fig. 1a). APE-CR and #2 cells had different morphologies than that of A925LPE3 cells (Fig. S2a).

APE-CR cells and #2 cells, clones of APE-CR cells were resistant to the ALK inhibitor crizotinib, as well as cross-resistant to alectinib and ceritinib (Fig. 1b). Secondary ALK mutations were not detected in APE-CR cells or #2 cells (data not shown). The ALK expression was knocked down with specific siRNA in order to examine whether resistance was dependent on the ALK. The viability of A925LPE3 cells (the parent cell line) was inhibited by si-ALK, but the viability of APE-CR cells and #2 cells was not inhibited (Fig. 1c), indicating that the latter two cell lines have the resistance mechanism independent of ALK. While epithelial to mesenchymal transition (EMT) is thought to be an important mechanism of resistance against various types of kinase inhibitors, \( ^{21,22} \) #2 cells did not show a typical mesenchymal phenotype (Fig. S2b). Thus,
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Fig 2. The effect of combination therapy ALK-TKI and EGFR-TKI or anti-EGFR antibody. (a) A925LPE3 and #2 cells (2 × 10^3 per well) were incubated with various concentrations of crizotinib with or without erlotinib (1 μM) or cetuximab (50 μg/mL) for 72 h. Cell viability was determined by MTT assay. The data shown represent the means ± SD of three independent experiments. (b) #2 cells (2 × 10^3 per well) were incubated with various concentrations of alectinib or ceritinib with or without erlotinib (1 μM) or cetuximab (50 μg/mL) for 72 h. Cell viability was determined by MTT assay. The data shown represent the means ± SD of three independent experiments. (c) A925LPE3 and #2 cells were treated with crizotinib (1 μM) and/or erlotinib (1 μM) or cetuximab (50 μg/mL) for 1 h. Cell lysates were evaluated for protein expression by western blot. Three independent experiments were performed, and a representative result is shown. (d) A925LPE3 and #2 cells were treated with siRNAs specific for EGFR (si-EGFR, respectively), or SCR. Left: #2 cells were treated with or without crizotinib (1 μM) for 1 h, following transfection with siRNA. Cell lysates were evaluated for protein expression by western blot. Three independent experiments were performed, and a representative result is shown. Right: #2 cells were treated with crizotinib (1 μM) following transfection with si-RNA. Cell viability was determined by MTT assay 72 h later. Data represents the mean ± SD. *P < 0.05 by Student’s t-test, the group treated with si-SCR and crizotinib versus the group treated with si-EGFR and crizotinib.
involve EGFR ligands. Accordingly, the levels of five EGFR ligands (AREG, β-cellulin, TGF-α, HB-EGF, and EGF) were measured in the cell culture supernatant by ELISA (Fig. 3a).

The #2 cells expressed higher levels of AREG than the A925LPE3 cells. However, #2 cells produced lower levels of other EGFR ligands than A925LPE3 cells. These findings suggest that AREG had the greatest impact on EGFR bypass signaling in #2 cells. Thus, AREG was knocked down with siRNAs specific for AREG (si-AREG1-3) to determine whether crizotinib sensitivity could be restored. When siRNAs specific for AREG were used along with crizotinib, crizotinib sensitivity was restored. The extent to which crizotinib sensitivity was restored coincided with the extent to which the expression of AREG was inhibited (Fig. 3b,c).

Conversely, we treated A925LPE3 cells with recombinant AREG, to examine whether AREG induces resistance to crizotinib. Exogenous recombinant AREG induced the resistance of A925LPE3 cells to crizotinib (Fig. S5). These results indicate that EGFR activation, caused predominantly by AREG, induced crizotinib resistance in #2 cells.

**Use of crizotinib and an EGFR inhibitor inhibited the growth of #2 tumor cells in vivo.** We sought to determine the effect of EGFR inhibitors when combined with crizotinib against tumors produced with #2 cells in vivo. A925LPE3 or #2 cells were subcutaneously implanted in SCID mice in order to produce tumors. A925LPE3 tumors shrank markedly when treated with crizotinib (Figs 4a and S6b). Western blotting indicated that the phosphorylation of AKT and ERK was inhibited in A925LPE3 tumors by treatment with crizotinib (Fig. 4c). In parallel experiments with #2 cells, the mice were divided into six groups and treated as follows: vehicle (control), erlotinib alone, cetuximab alone, crizotinib alone, crizotinib + erlotinib, and crizotinib + cetuximab. Compared to the control group, mice receiving crizotinib alone had slower tumor enlargement, but the tumor did not regress, indicating that #2 cells were resistant to crizotinib in vivo. Tumor enlargement was not inhibited in mice receiving erlotinib or cetuximab alone, but tumor shrinkage or arrested tumor enlargement was noted in mice receiving crizotinib combined with erlotinib or cetuximab (Figs 4b and S6b). Western blotting of the treated subcutaneous tumors indicated that phosphorylation of AKT and ERK was markedly inhibited in mice receiving crizotinib combined with erlotinib or cetuximab (Fig. 4d). Immunohistochemistry revealed that the number of Ki-67-positive proliferating tumor

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**Fig 3.** Increase of AREG caused the resistance for crizotinib. (a) Five EGFR ligands (AREG, β-cellulin, HB-EGF, EGF, and TGF-α) production by #2 cells. The cells were incubated in medium for 48 h and culture supernatants were harvested. The level of ligands in the supernatants was determined by ELISA, *P < 0.05 by Student t-test, #2 versus A925LPE3. (b) #2 cells were treated with or without crizotinib (1 μM) for 72 h following transfection with siRNAs for EGFR or AREG (si-EGFR and si-AREG, respectively), or SCR. Cell viability was determined by MTT assay. The data shown represent the means ± SD of three independent experiments. *P < 0.05 by Student’s t-test, versus the group treated with SCR. (c) AREG production by #2 cells were decreased after transfection with si-AREG. The cells were incubated in medium for 48 h and culture supernatants were harvested. The level of ligand in the supernatants was determined by ELISA. Data represents the mean ± SD. *P < 0.05 by Student’s t-test, versus the group treated with si-SCR.
EGFR activation. In these two reports, the involvement of amphiregulin expression and crizotinib resistance via EGFR knockdown was clearly demonstrated. The present study circumvention of crizotinib resistance by EGFR inhibitors or AREG on crizotinib resistance was not directly verified, but reports three novel findings. First, using EGFR, TGF-β, and crizotinib resistance to ALK-negative NSCLC cells (A925LPE3) expressing the unique EML4-ALK variant (E2:A20). To date, crizotinib resistance associated with AREG overexpression has been reported in NSCLC cells expressing other EML4-ALK variants (H3122 cells with E13:A20 and DFCI076 cells with E6:A20). Third, we demonstrated that AREG-triggered crizotinib resistance could be induced in a pleural effusion mouse model by daily oral treatment with crizotinib. To date, the association between AREG overexpression and crizotinib resistance has been demonstrated in: (i) cells that acquired crizotinib resistance through in vitro culture with increasing concentrations of crizotinib; and (ii) cells derived from clinical specimens of a patient who acquired crizotinib resistance. Our findings indicate that the pleural carcinomatosis mouse model with human EML4-ALK NSCLC cells would be an effective tool for identifying clinically relevant resistance mechanisms.

Activation of bypass signaling is a well-established resistance mechanism for targeted therapy. EML4-ALK-positive lung cancer is reported to develop resistance to crizotinib through bypass signaling via pathways such as c-KIT, EGFR, and IGFR-1. Resistance to alectinib is reported to develop as a result of mechanisms such as EGF ligands, MET gene amplification, overexpression of IGFR-1 and the activation of MET via HGF. Thus, the activation of EGFR may be a common route by which numerous types of cancer acquire the resistance to targeted therapy. Sustained
EGFR activity can be caused by ligand stimulation as well as EGFR mutations. In the present study, while the crizotinib-resistant #2 cells did not have any common EGFR mutations (exon 19 deletion or L858R) or the T790M mutation, they did produce a high level of EGFR ligand (AREG), activate EGFR, and thereby cause crizotinib resistance. Since #2 cells with high AREG expression showed cross-resistance to alectinib and ceritinib, AREG must be considered along with other EGFR ligands as common factors for resistance to ALK inhibitors.

Epidermal growth factor receptor ligands are not only produced by cancer cells, but also by stromal cells such as fibroblasts, macrophages, and vascular endothelial cells. We previously reported that exposing EML4-ALK-positive lung cancer cells to EGFR ligands (EGF, HB-EGF, and TGF-α) in a paracrine manner triggers ALK-TKI resistance. In contrast, crizotinib-resistant cell lines established in an in vivo model produced AREG and developed resistance in an autocrine manner (Fig. 3a). While mechanism by which AREG expression was upregulated in these cells is unknown at present, previous studies reported that the expression of the AREG was induced by activating the AMPK/PKA pathway via prostaglandin E₂, cigarette smoke, hypoxia, or by stimulation with inflammatory cytokines such as IL-1 and TNF-α. Further studies are warranted to clarify underlying mechanisms.

The pro-AREG protein that is expressed on the cell membrane after transcription and translation is cleaved after stimulation with inflammatory cytokines such as IL-1β, IL-8, and TNF-α that protein is secreted as AREG. Secreted AREG activates signaling via EGFR, which in turn produces autostimulatory feedback that activates the transcription of AREG. Thus, inflammatory cytokines play an important role in the expression of AREG. We established a crizotinib-resistant cell line from pleural effusion of mice with carcinomatous pleurisy where various cytokine may exist. Inflammatory cytokines in pleural effusion potentially led to an increased AREG level in cancer cells. Unexpectedly, expression of other EGFR ligands was not increased, or in fact decreased, in #2 cells in comparison to the A925L parental cell line. Tani et al. indicated that TGF-α levels increased in an alectinib-resistant cell line that was established in vitro. This disparity is assumed to be due to the cell line and drugs used, as well as differences in the conditions under which resistance was induced (in vitro versus in vivo).

Interestingly, the level of phosphorylated ALK in #2 cells was also lower than that in the parental cells. Therefore, it is possible that #2 cells were less dependent on ALK signaling for survival than the parental cells, though #2 cells still showed a low degree of sensitivity to crizotinib. In this unique situation, the survival signals from phosphorylated EGFR may confer crizotinib resistance in #2 cells, even though the level of phosphorylated EGFR in #2 cells was lower than that in the parental A925LPE3 cells. To clarify the underlying mechanisms by which low levels of phosphorylated EGFR were sufficient to induce crizotinib resistance in #2 cells, it is necessary to perform additional studies in the future.

Future topics include how to detect resistance as a result of EGFR bypass signaling in patients. Kim et al. reported that patients with crizotinib-resistant ALK-rearranged lung cancer have elevated levels of AREG in their pleural effusions. Therefore, in addition to the identification of gene mutations (ALK, EGFR, KRAS, etc.) that confer drug resistance in cancer cells in fluids, measurement of various ligands (including AREG) in the fluids may be crucial in cases where resistance developed as a result of cancer in the fluids (e.g. pleural effusion, ascites, and pericardial effusion).

In conclusion, we produced SCID mouse model of carcinomatous pleurisy with EML4-ALK lung cancer cells and induced acquired resistance to crizotinib by continuous oral treatment with crizotinib. We further established crizotinib-resistant cells and found that amphiregulin (AREG), an EGFR ligand, is largely responsible for the activation of EGFR in an autocrine manner and in turn leads to resistance to crizotinib. Moreover, we demonstrated that crizotinib resistance could be overcome by inhibiting EGFR bypass signaling in vivo. Therefore, inhibition of EGFR signaling may be a promising strategy to overcome crizotinib resistance in EML4-ALK lung cancer.

Disclosure Statement
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References
Amphiregulin confers crizotinib-resistance

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Crizotinib is equally effective against A925L and A925LPE3 cells.

Fig. S2. Morphological changes in each cell line.

Fig. S3. Crizotinib with either erlotinib or cetuximab is not sufficiently effective for APE-CR.

Fig. S4. The combined use of crizotinib and erlotinib or cetuximab led the higher expression of cleaved PARP than crizotinib alone.

Fig. S5. High concentration of AREG induced resistance of A925LPE3 cells to crizotinib.

Fig. S6. Body weight of mice and representative images of tumor-bearing mice.

DOC. S1. The siRNA target sequences.