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Potential therapeutic strategy for EGFR mutant lung cancer with concomitant EML4-ALK rearrangement—combination of EGFR-TKIs and ALK inhibitors

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Abstract

Introduction: Although driver gene mutations have been believed to be mutually exclusive, some patients with non–small-cell lung cancer (NSCLC) and concomitant epidermal growth factor receptor (EGFR) mutations and echinoderm microtubule associated protein like 4-anaplastic lymphoma kinase (EML4-ALK) rearrangements have been reported. In this study, we reported a case of lung cancer patient harboring both EGFR mutation and the EML4-ALK rearrangement after acquiring resistance to the EGFR tyrosine kinase inhibitor treatment. EGFR mutant and ALK fusion proteins were detected in the same tumor cells through immunohistochemical analysis. Investigation of the molecular mechanisms of concomitant EGFR mutation and the EML4-ALK rearrangement in the same tumor cell can help discover an appropriate treatment for these patients.

Methods: PC-9 cells, expressing EGFR exon 19 deletion, were transfected with EML4-ALK variants 3a and 3b separately and selected, and the effect of EGFR and ALK inhibitors was evaluated in vitro and in vivo.

Results: PC-9/v3a-gef and PC-9/v3b-gef cells were resistant to gefitinib and ALK inhibitors alone, but ALK inhibitors enhanced gefitinib-induced cytotoxicity. In animal studies, gefitinib completely inhibited the tumor growth in PC-9/vector cells but not in PC-9/v3a-gef and PC-9/v3b-gef cells. A combination of ALK inhibitor and gefitinib was
found to be more potent than gefitinib alone in PC-9/v3a-gef and PC-9/v3b-gef cells.

Furthermore, combination treatment with osimertinib and ceritinib caused a decrease in liver tumor size of the patient with liver metastases.

**Conclusions:** Our data suggest that combination treatment with EGFR and ALK inhibitors can be a therapeutic strategy for treating NSCLC with concomitant *EGFR* mutation and *EML4-ALK* rearrangement.
Introduction

Oncogenic driver mutations result in abnormal activation of signaling pathways that contribute to cancer initiation \(^1\). Detection and inhibition of oncogenic driver mutations improve the efficacy of therapy for non–small-cell lung cancer (NSCLC)\(^2\). Several oncogenic driver mutations such as mutation of the epidermal growth factor receptor (EGFR) and fusion of the echinoderm microtubule associated protein like 4 (EML4)-anaplastic lymphoma kinase (ALK) genes have been identified in NSCLC \(^2,3\). Studies have reported that EGFR mutations and EML4-ALK fusions are mutually independent \(^4-6\). Moreover, the coexistence of the EGFR mutations and EML4-ALK fusions has been reported in lung cancers \(^7,8\). Furthermore, the coexistence of these two proteins was detected in the same tumor cell through immunohistochemical analysis \(^8\).

EGFR tyrosine kinase inhibitors (EGFR-TKIs) such as gefitinib, erlotinib, and afatinib are the standard therapies for advanced NSCLC patients with tumor cells carrying specific EGFR mutations \(^9\). All patients eventually develop acquired resistance to first- or second-generation EGFR-TKIs, and new-generation EGFR T790M inhibitors such as osimertinib overcome T790M-mediated resistance to first-line EGFR-TKI therapy \(^10\). Similarly, ALK inhibitors such as crizotinib and ceritinib are effective against NSCLC with the EML4-ALK fusion gene \(^11\).
Identification of the real driver gene is essential for physicians to determine appropriate treatments for patients. However, in patients with tumor cells carrying both EGFR mutation and EML4-ALK fusion, whether EGFR is the major driver in the cancer cells is uncertain. In addition, the expression of EML4-ALK rearrangement may be associated with EGFR-TKI resistance. Therefore, the efficacies of EGFR-TKIs and ALK inhibitors remain elusive in NSCLC harboring both EGFR mutation and EML4-ALK fusion.

In this study, we reported a case of a lung cancer patient with tumor carrying both mutant EGFR and EML4-ALK fusion genes. We explored the effect of EGFR-TKI and ALK inhibitors on lung cancer cells with concurrent EGFR mutation and EML4-ALK fusion both in vitro and in vivo. We further identified that a combination treatment with EGFR-TKIs and ALK inhibitors is a potential strategy for NSCLC with concomitant of EGFR mutations and EML4-ALK fusion.
Materials and methods

Lung cancer patient and gene sequencing: A patient was diagnosed with EGFR mutant advanced NSCLC and was treated at the National Taiwan University Hospital. The treatment course was recorded. A liver biopsy was performed upon progression to an EGFR T790M inhibitor. Targeted sequencing of a panel of 35 cancer-related genes and four lung cancer–related fusion genes, the ACT-Drug panel, was performed by ACT genomics (Taipei City, Taiwan). The patient’s medical record was reviewed. The use of patient data, including a waiver of informed consent from patients who were expired, was approved by the Research Ethics Committee of the National Taiwan University Hospital (NTUH REC 201612136RINC).

Immunohistochemistry study: Tissue sections (4 μm) were deparaffinized and rehydrated. Immunohistochemical analysis was performed using a Ventana BenchMark XT Autostainer (Ventana, Tucson, AZ, USA). The slides were allowed to react with an antibody specific for ALK (D5F3) CDx Assay (Ventana) and anti-EGFR (E746-A750del specific) antibody (clone 6B6, 1:150; Cell Signaling Technology). The results were evaluated by a pulmonary pathologist (Dr. Min-Shu Hsieh).

Cell lines and drugs: PC-9 is a human lung adenocarcinoma cell line with EGFR exon 19 deletion mutation. PC-9 cells were cultured in RPMI 1640 medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum. Gefitinib,
crizotinib, alectinib, MK2206, and selumetinib were purchased from Selleckchem (Houston, TX, USA).

Construction of EML4-ALK variant 3 and cell transfection: The coding sequence for EML4-ALK variant 3a or 3b was amplified from total cDNA of H2228 cells. The cDNA for EML4-ALK variant 3a or 3b was cloned into the pcDNA 3.3-TOPO vector (Invitrogen). The empty pcDNA 3.3-TOPO vector, pcDNA 3.3-TOPO-EML4-ALK variant 3a, and pcDNA 3.3-TOPO-EML4-ALK variant 3b were transfected into PC-9 cells by using the Xfect transfection reagent (Clontech Laboratories, Mountain View, CA, USA), and the cells were considered transiently expressing cells. To establish cells stably expressing transfected genes, the cells were further incubated in 500 mg/mL G418 (Geneticin, Gibco, Grand Island, NY, USA), and the selected cells were considered stably expressing cells. To obtain EML4-ALK variant 3a or 3b functional cells, PC-9 cells with a stable expression of EML4-ALK variant 3a or 3b were incubated with 1 μM gefitinib for 1 week, and the selected cells were expanded and used in further studies.

Cell viability assays. Cell viability upon drug treatment was determined using the sulforhodamine B assay as described previously. Percentages of cell viability were calculated by dividing the absorbance values of drug-treated cells by that of untreated cells.
Western blotting: Western blotting was performed as described previously\textsuperscript{13}. Primary antibodies used for immunoblot analyses were as follows: α-tubulin (Cell Signaling Technology, Beverly, MA, USA); ALK and phospho-ALK specific for Tyr1078, Tyr1278, Tyr1282/1283, Tyr1586, and Tyr1604 (Cell Signaling Technology), Akt, phospho-Akt (Ser473); extracellular-signal-regulated kinase (ERK); phospho-ERK (Cell Signaling Technology); EGFR; phospho-EGFR (Tyr1068); HER2; phospho-HER2 (Tyr1221/1222); HER3; phospho-HER3 (Tyr1289) (Cell Signaling Technology); poly (ADP-ribose) polymerase (PARP); Caspase-3; and Caspase-9 (Cell Signaling Technology).

Xenograft mouse model: 1 × 10\textsuperscript{6} cells were subcutaneously injected into the back of 6-week-old male Balb/c nude mice. The length and width of the tumors were measured using an electronic caliper, and the tumor volume was determined as (length × width\textsuperscript{2})/2. When tumors grew to 150 mm\textsuperscript{3}, mice were randomized into four groups and were administered vehicle, gefitinib (50 mg/kg once daily), alectinib (20 mg/kg once daily), or gefitinib plus alectinib. Gefitinib was suspended in 0.05% Tween 80 solution. Alectinib was suspended in a mixture of 30% PEG 400, 0.5% Tween 80, and 5% propylene glycol solution. These animals were maintained in individually ventilated cages according to the guidelines of “Guide for The Care and Use of Laboratory Animals.” The use of animals was approved by the Institutional
Animal Care and Use Committee of the National Taiwan University College of Medicine, Taipei, Taiwan.

**Statistical analyses:** Animal experimental data were analyzed using GraphPad Prism software. Statistical differences were determined using the Mann–Whitney nonparametric test. $P$ values of <0.05 were considered statistically significant.
Results

A lung cancer patient with tumor cells carrying both mutant *EGFR* gene and *EML4-ALK* fusion benefited from combination therapies

A 68-year-old woman with no smoking history had lung adenocarcinoma with brain, lung, and liver metastases; according to American Joint Committee on Cancer (AJCC) staging version 7, the tumor stage was cT1bN3M1b. *EGFR* exon 19 deletion (E746_A750 deletion) was identified in the tumor, and the immunohistochemical analysis of ALK protein was negative. She had received anti-cancer therapies, including afatinib and carboplatin plus pemetrexed. A biopsy performed upon progression to afatinib revealed *EGFR* exon 19 deletion plus T790M mutant adenocarcinoma. She was enrolled in a clinical trial of an EGFR T790M-specific inhibitor. Partial response was noted that persisted for 11 months until the development of progression to the liver. A genetic analysis of the tumor from the liver revealed the presence of four copies of the *EGFR* gene, *EGFR* exon 19 deletion plus T790M mutation, *TP53* mutation, and type-5 *EML4-ALK* fusion (Fig. 1A). Immunohistochemistry confirmed the coexpression of ALK and EGFR exon 19 deletion (E746_A750 deletion) proteins in tumor cells (Fig. 1B). Ceritinib, an ALK inhibitor, was administrated for 1 week, and a computed tomography (CT) scan revealed disease progression. She received an osimertinib plus ceritinib therapy. A CT
scan 6 weeks after the therapy suggested a modest decrease in the size of liver tumors (Fig. 1C). Unfortunately, a CT scan 12 weeks after osimertinib plus ceritinib suggested progression of a liver tumor which was different from the liver lesion we took biopsy. The patient was expired 6 months after start of osimertinib plus ceritinib.

**PC-9 cells that stably expressed the EML4-ALK fusion gene were resistant to gefitinib**

To explore whether cancer cells are simultaneously driven by both genes, we expressed the *EML4-ALK* fusion gene in PC-9 lung cancer cells carrying *EGFR* exon 19 deletion mutation. We first transiently expressed the *EML4-ALK* fusion variant 3a (PC-9/v3a-t) or variant 3b (PC-9/v3b-t) in PC-9 cells (Fig. 2A). No difference was observed in the cell viability of PC-9 cells and cells transiently expressing the EML4-ALK fusion variant 3a (PC-9/v3a-t) or variant 3b (PC-9/v3b-t) at various concentrations of the EGFR inhibitor gefitinib (Fig. 2B).

We then established PC-9 cells stably expressing *EML4-ALK* v3a and *EML4-ALK* v3b by incubating the cells with G418. After G418 selection, we obtained PC-9/v3a and PC-9/v3b cells that had low expression of EML4-ALK variant 3a and EML4-ALK variant 3b proteins, respectively (Fig. 2A and 3A). Localization of phospho-ALK was not observed in PC-9/v3a or PC-9/v3b cells (Fig. 2A and 3A), and
cell viability upon gefitinib treatment was similar to that of cells transfected with vector (PC-9/vector) (Fig. 3B). We incubated PC-9/v3a and PC-9/v3b cells with 1 µM of gefitinib and a minority of cells re-expanded after 2 weeks of the incubation. Notably, the gefitinib-treated cells, PC-9/v3a-gef and the PC-9/v3b-gef cells, were resistant to gefitinib (Fig. 3B).

We attempted to characterize the PC-9/v3a-gef and PC-9/v3b-gef cells. We observed ALK and phospho-ALK proteins in the PC-9/v3a-gef and PC-9/v3b-gef cells (Fig. 3A). No difference was observed in the expression of EGFR, HER2, and HER3 as well as their phosphorylated forms in PC-9/vector, PC-9/v3a-gef, and PC-9/v3b-gef cells (Fig. 3C). Stable expression of the EML4-ALK variant 3a or 3b did not influence the phosphorylation of downstream signals of EGFR, including Akt and ERK (Fig. 3D).

**A combination of gefitinib and alectinib inhibited growth and induced apoptosis of EML4-ALK expressing PC-9 cells**

Because PC-9/v3a-gef and PC-9/v3b-gef cells were resistant to gefitinib, we explored the role of the EML4-ALK fusion gene in these cells. No difference was observed in the cell viability of PC-9/vector, PC-9/v3a-gef, and PC-9/v3b-gef cells upon treatment with various concentrations of ALK inhibitors, alectinib, or crizotinib.
(Fig. 4A and Supplemental Fig. 1A). The sensitivity of PC-9/vector cells to gefitinib was not influenced by exposure to alectinib or crizotinib (Fig. 4B and Supplemental Fig. 1B). We observed a remarkable decrease in the cell viability of both PC-9/v3a-gef and PC-9/v3b-gef cells when the cells were cotreated with gefitinib and alectinib or crizotinib (Fig. 4C and D and Supplemental Fig. 1C and D). The findings suggested that the *EML4-ALK* fusion gene is responsible for the resistance to gefitinib in both PC-9/v3a-gef and PC-9/v3b-gef cells.

We then evaluated whether the change in cell viability was due to apoptosis induction. Exposure to gefitinib but not alectinib induced cleaved forms of PARP, caspase-9, and caspase-3 in the PC-9/vector cells (Fig. 4E). Gefitinib or alectinib alone induced modest amounts of cleaved forms of PARP, caspase-9, and caspase-3 in the PC-9/v3a-gef and PC9-v3b-gef cells. A combination of gefitinib and alectinib resulted in a remarkable elevation of cleaved forms of PARP, caspase-9, and caspase-3 in both cells (Fig. 4E). These findings indicated that PC-9/v3a-gef and PC-9/v3b-gef cells underwent apoptosis when they were exposed to a combination of EGFR inhibitor and ALK inhibitor.

A combination of gefitinib and alectinib inhibited the growth of *EML4-ALK* expressing PC-9 xenografts.
We evaluated the effect of EGFR-TKI and ALK inhibitors in vivo. The growth of PC-9/vector xenografts was attenuated by gefitinib or gefitinib plus alectinib treatment but not by alectinib alone (Fig. 5A). Further, the growth of PC-9/v3a-gef and PC-9/v3b-gef xenografts was inhibited by gefitinib plus alectinib treatment but not by gefitinib or alectinib alone (Fig. 5B and C).

**Complicated downstream signaling of EML4-ALK**

We explored the difference in signaling pathways between PC-9 cells with and without *EML4-ALK* translocation. Phosphorylation of EGFR but not ALK was inhibited by gefitinib in PC-9/v3a-gef and PC-9/v3b-gef cells (Fig. 6A). Akt and ERK were the two dominant downstream targets of EGFR and EML4-ALK. The EGF-induced ERK phosphorylation was inhibited by gefitinib alone in both PC-9/v3a-gef and PC-9/v3b-gef cells in a dose-dependent manner (Fig. 6B). However, the inhibitory effect of gefitinib on EGF-induced phosphorylation of Akt in PC-9/v3a-gef and PC-9/v3b-gef cells was weak compared with that in PC-9/vector cells (Fig. 6B and C). Alectinib treatment inhibited ALK phosphorylation in both PC-9/v3a-gef and PC-9/v3b-gef cells (Fig. 6C). However, phosphorylations of Akt and ERK were not influenced by alectinib alone. A combination of gefitinib and alectinib inhibited Akt phosphorylation to a greater extent in PC-9/v3a-gef and
PC-9/v3b-gef cells (Fig. 6C). STAT3 phosphorylation was not influenced by gefitinib, alectinib, or their combination in both PC-9/v3a-gef and PC-9/v3b-gef cells (Supplemental Fig. 2).

We next evaluated whether Akt or ERK inhibition influenced the gefitinib sensitivity in EML4-ALK stably expressing cells. No difference was observed in cell viability of PC-9/vector, PC-9/v3a-gef, and PC-9/v3b-gef cells in response to Akt inhibitor, MK2206 (Supplemental Fig. 3A). MK2206 or selumetinib (MEK1/2 inhibitor) modestly sensitized PC-9/v3a-gef and PC-9/v3b-gef cells to gefitinib, resulting in a decrease in cell viability (Supplemental Fig. 3B and C). The combination of MK2206 and selumetinib sensitized the PC-9/v3b-gef cells but not the PC-9/v3a-gef cells to gefitinib (Supplemental Fig. 3B and C). Our findings suggested that the EML4-ALK fusion gene might have multiple downstream signals. Blocking only one of these downstream pathways cannot increase the sensitivity of tumor cells to gefitinib in NSCLC with concomitant EGFR mutation and EML4-ALK translocation.
Discussion

We demonstrated that both the mutant EGFR and EML4-ALK fusion genes drove the cancer cells. We identified a patient with advanced EGFR mutant NSCLC, whose tumor on progression after EGFR inhibitor treatment carried a fusion of the EML4-ALK gene. The lung cancer patient with liver metastases exhibited a modest decrease in size of liver tumors after receiving osimertinib plus ceritinib. We further demonstrated, in vitro and in vivo, the efficacy of the combination therapy of an EGFR inhibitor and an ALK inhibitor in EGFR mutant lung cancer cells with the EML4-ALK fusion gene.

With few exceptions, oncogenic driver mutations in non-squamous cell NSCLC are generally mutually exclusive. Guibert et al. reported the detection of multiple genetic alterations involving driver mutations in 165 (0.93%) out of 17,826 untreated NSCLC samples. Concomitant EML4-ALK and EGFR mutation have been reported in lung cancers. In tumors harboring multiple potential driver mutations, little is known whether the concomitant mutations are essential or one mutation is dominant for cancer cell growth. In the patient-derived cancer cell line harboring both EGFR exon 19 deletion and PCBP2-BRAF fusion protein, Piotrowska et al. demonstrated that unlike osimertinib or BRAF inhibitors, the MEK1/2 inhibitor trametinib alone was able to abolish cell growth. Schmid et al. reported five patients with the EGFR
mutant de novo and ALK-positive NSCLCs. Partial responses were observed in four out of five patients who received the EGFR inhibitor or ALK inhibitor monotherapy, which was supportive of a single mutation that was dominant in tumors with de novo concomitant $EGFR$ mutation and $ALK$ fusion. In another study, Yang et al. described that the phosphorylation levels of EGFR and ALK in tumors harboring both $EGFR$ mutation and $EML4-ALK$ translocation responded to EGFR-TKI and crizotinib. In our patient, the in vitro findings revealed that either an EGFR inhibitor or ALK inhibitor alone was unable to inhibit cancer cell growth. In selected situations, cancer cells may be driven by multiple potential oncogenic drivers.

We explored the signaling pathways relating to the $EML4-ALK$-induced resistance to the EGFR inhibitors. The molecular mechanism of the EML4-ALK fusion protein is complicated, and it has been generally believed that the PI3K/Akt, JAK/STAT3, PLC$\gamma$, and MAPK cascades are the downstream signal pathways of ALK protein, and these pathways may mediate the malignant behavior of ALK-driven cancer cells (reviewed by Bayliss et al. 21). The downstream signal of EML4-ALK may be content dependent. Several EML4-ALK variant proteins exist, and different EML4-ALK variant proteins have different intra-cellular localizations and interact with different proteins. EML4-ALK variant 1, variant 2, and variant 5a localized diffusely in the cytoplasm, whereas variant 3 colocalized with microtubules in the
cytoplasm. We demonstrated that the phosphorylation of Akt and ERK was regulated by EGFR signaling but not by ALK signaling in PC-9/v3a-gef and PC-9/v3b-gef lung cancer cells (Fig. 6). Nevertheless, PC-9/v3a-gef and PC-9/v3b-gef cells were resistant to gefitinib. The combination of Akt and the MEK inhibitor did not sensitize the PC-9/v3a-gef and PC-9/v3b-gef cells to gefitinib (Supplemental Fig. 3B and C). Our findings suggested that multiple molecular cascades may drive the ALK signals in the PC-9/v3a-gef and PC-9/v3b-gef cells. Therefore, blocking a single pathway cannot increase the sensitivity of tumor cells to gefitinib in NSCLC with concomitant EGFR mutation and EML4-ALK translocation.

Mechanisms of acquired resistance to EGFR inhibitors have been widely studied. Although osimertinib is effective in the treatment of acquired resistance resulting from the acquired EGFR T790M mutation, which is responsible for approximately 50% of resistance to first- or second-generation EGFR inhibitors, few treatment strategies for treating acquired resistance to EGFR T790M mutation exist. Targeting the acquired amplification of MET gene has been widely studied clinically. In the phase III study, to evaluate the efficacy of the MET monoclonal antibody onartuzumab in patients with MET expressing advance NSCLC, the survival rate was shorter in EGFR mutant lung cancer patients who received onartuzumab plus erlotinib 25. In the phase I/II study on the MET inhibitor capmatinib (INC280) plus gefitinib in
EGFR mutant lung cancer patients, after the failure of EGFR inhibitors, a response rate of 47% and median progression-free survival of 5.5 months were observed among patients with tumor cells harboring more than six copies of the MET gene. Because amplification of the MET gene is observed only in approximately 5% of progressive tumors, an unmet need for exploring clinically relevant treatment strategies for the acquired resistance to first- or second-generation EGFR inhibitors still exists. In addition, mechanisms of resistance to osimertinib have been reported, and strategies to overcome the resistance are mostly under development. According to our knowledge, this is the first time the efficacy of the combination therapy of an EGFR inhibitor and ALK inhibitor in an NSCLC patient with tumor cells harboring both an EGFR mutation and EML4-ALK fusion gene has been reported. A comprehensive genomic study of tumors upon progression to EGFR inhibitors may guide the selection of treatment in advanced EGFR mutant lung cancer patients.

In our patient, we observed the existence of stable disease in response to osimertinib plus ceritinib. The EML4-ALK fusion in the patient was the EML4-ALK variant 5. It has been reported that the progression-free survival due to ALK inhibitors and chemotherapies were shorter in patients with EML4-ALK variant 3 lung cancers as well as their overall survival. In addition, Ba/F3 cells transformed by EML-ALK variant 3 or 5a proteins were less sensitive to ALK inhibition than cells
transformed by EML4-ALK variant 1 or 2 \textsuperscript{31}. The difference in the sensitivity may be due to a difference in protein (EML4-ALK) stability in the cells \textsuperscript{23}. The stable disease noted in our patient was compatible with clinical and in vitro findings reported previously. In addition, a CT scan taken 12 weeks after start of osimertinib plus ceritinib suggested progression of a liver tumor different from the one we took biopsy. Considering the potential intra-patient heterogeneity of tumors in heavily pretreated EGFR mutant lung cancer patient, we do not exclude the possibility that the progressive lesion may carry resistant mechanism other than the concomitant EML4-ALK rearrangement.

Notably, we observed that PC-9 cells that stably expressed EML4-ALK variant 3 were sensitive to gefitinib (PC-9/v3a and PC-9/v3b cells, Fig. 3B) unless they were incubated with gefitinib for 1 week and acquired resistance to gefitinib (PC-9/v3a-gef and PC-9/v3b-gef cells, Fig. 3B). The establishment of cancer cells resistant to a drug usually requires months (reviewed by McDermott et al. \textsuperscript{32}); however, the clinical relevance of the resistant cells in vitro is often questionable. The process of incubating EGFR mutant lung cancer cells in escalating concentrations of EGFR inhibitors in vitro to develop resistance against EGFR inhibitors has been found to occur over months \textsuperscript{13,33}. However, for the first time, we demonstrated that drug resistance can be
induced in cancer cells by incubation with the drug for a short period of time. Our model may aid future research on drug resistance.

To conclude, both mutant *EGFR* and the *EML4-ALK* fusion gene drive mutations in advanced EGFR mutant lung cancer patients upon progression to EGFR inhibitor resistance. Targeting mutant EGFR and ALK is a novel treatment strategy for treating NSCLC harboring *EGFR* mutation and *EML4-ALK* translocation.
References


Figure legends

**Figure 1.** Emergence of the *EML4-ALK* fusion gene in an EGFR mutant advanced NSCLC patient. (A) Deep sequencing of the progressive tumor suggests deletion of EGFR exon 19 (E746_A750del) and fusion of EML4 (exon 2) and ALK (exon 20). (B) Immunohistochemical analysis confirmed the expression of mutated EGFR (E746_A750del) and ALK proteins in the progressive tumor. (Original magnification: 400×) (C) Computed tomography images of liver lesions upon progression to an EGFR T790M inhibitor. Images were obtained 6 weeks prior to progression, start of osimertinib plus ceritinib treatment, and 6 weeks after osimertinib plus ceritinib treatment.

**Figure 2.** Transient ectopic expression of EML4-ALK variants 3a and 3b in PC-9 cells. (A) Expression of ALK and phospho-ALK in PC-9 cells after 24 and 96 hours of infection with pcDNA 3.3-TOPO vector (PC-9/vector), pcDNA 3.3-TOPO-EML4-ALK variant 3a (PC-9/v3a-t), and pcDNA 3.3-TOPO-EML4-ALK variant 3b (PC-9/v3b-t). Cells stably expressing EML4-ALK variants 3a and 3b were established by incubating the cells with G418 and were named as PC-9/v3a and PC-9/v3b, respectively. (B) Cell viability of PC-9, PC-9/vector, PC-9/v3a-t, and PC-9/v3b-t cells was tested after treatment with increasing gefitinib concentrations. Values are presented as means ± SD for three independent experiments.
Figure 3. Characterization of PC-9 cells stably expressing EML4-ALK variants 3a and 3b. (A) Expression of ALK and phospho-ALK in PC-9/vector, PC-9/v3a, PC-9/v3b, PC-9/v3a-gef, and PC-9/v3b-gef cells. The PC-9/v3a-gef and PC-9/v3a-gef cells were derived from the PC-9/v3a and PC-9/v3b cells through incubation with gefitinib for 2 weeks. (B) Cell viability of the PC-9/vector, PC-9/v3a, PC-9/v3b, PC-9/v3a-gef, and PC-9/v3b cells was tested at various concentrations of gefitinib. Values are presented as means ± SD for three independent experiments. (C) Expression of EGFR, HER2, and HER3 and their phosphorylated forms in the cells. (D) Expression of Akt and ERK and their phosphorylated forms in the cells.

Figure 4. Effect of the ALK inhibitor alectinib on sensitization of PC-9/v3a-gef and PC-9/v3b-gef cells to gefitinib. (A) Cell viability of PC-9/vector, PC-9/v3a-gef, and PC-9/v3b-gef cells on treatment with various concentrations of alectinib. Effect of the combination treatment of alectinib and gefitinib on the viability of (B) PC-9/vector cells, (C) PC-9/v3a-gef cells, and (D) PC-9/v3b-gef cells. Values are presented as means ± SD for three independent experiments. (E) Expression of PARP, caspase-9, and caspase-3 and their cleaved forms in PC-9/vector, PC-9/v3a-gef, and PC-9/v3b-gef cells upon gefitinib and/or alectinib treatment.

Figure 5. Effect of gefitinib and/or alectinib on xenograft models. (A) PC-9/vector xenografts, (B) PC-9/v3a-gef xenografts, and (C) PC-9/v3b-gef xenografts. T0: tumor
volume prior to drug treatment, Tn: tumor on day nth of drug treatment. Values are presented as means ± SEM. (*, $P < 0.05$; **, $P < 0.005$; $n = 4$).

Figure 6. Characterization of PC-9/v3a-gef and PC-9/v3b-gef cells. Effect of various concentrations of gefitinib on (A) expression of ALK and EGFR and their phosphorylated forms and (B) expression of Akt and ERK and their phosphorylated forms. (C) Expression of ALK, EGFR, Akt, and ERK and their phosphorylated forms upon gefitinib and/or alectinib treatment. All the cells were incubated in serum-free media for 3 hour, treated with gefitinib and/or alectinib at various concentrations for 1 hour, and then stimulated with 20 ng/mL EGF for 10 min.
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