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Research Paper

P-glycoprotein Mediates Ceritinib Resistance in Anaplastic Lymphoma Kinase-rearranged Non-small Cell Lung Cancer

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ABSTRACT

The anaplastic lymphoma kinase (ALK) fusion oncogene is observed in 3%–5% of non-small cell lung cancer (NSCLC). Crizotinib and ceritinib, a next-generation ALK tyrosine kinase inhibitor (TKI) active against crizotinib-refractory patients, are clinically available for the treatment of ALK-rearranged NSCLC patients, and multiple next-generation ALK-TKIs are currently under clinical evaluation. These ALK-TKIs exhibit robust clinical activity in ALK-rearranged NSCLC patients; however, the emergence of ALK-TKI resistance restricts the therapeutic effect. To date, various secondary mutations or bypass pathway activation-mediated resistance have been identified, but large parts of the resistance mechanism are yet to be identified. Here, we report the discovery of p-glycoprotein (P-gp/ABCB1) overexpression as a ceritinib resistance mechanism in ALK-rearranged NSCLC patients. P-gp exported ceritinib and its overexpression conferred ceritinib and crizotinib resistance, but not to PF-06463922 or alectinib, which are next-generation ALK inhibitors. Knockdown of ABCB1 or P-gp inhibitors sensitizes the patient-derived cancer cells to ceritinib, in vitro and in vivo. P-gp overexpression was identified in three out of 11 cases with in ALK-rearranged crizotinib or ceritinib resistant NSCLC patients. Our study suggests that alectinib, PF-06463922, or P-gp inhibitor with ceritinib could overcome the ceritinib or crizotinib resistance mediated by P-gp overexpression.

1. Introduction

The *anaplastic lymphoma kinase* (*ALK*) fusion oncogene caused by chromosomal rearrangement has been observed in a variety of human malignancies, including ALK-rearranged non-small cell lung cancer (NSCLC), which was identified in 2007 (Soda et al., 2007). *ALK* gene rearrangement results in the constitutive expression and activation of an ALK fusion protein, which has been shown to strongly drive oncogenesis. To target ALK-rearranged NSCLC, the oral ALK and *v-ros* avian ur2 sarcoma virus oncogene homolog 1 (ROS1) inhibitor crizotinib have been used. Two randomized phase 3 studies of crizotinib showed significantly longer progression-free survival (PFS; 7.7 months vs 3.0 months in the second-line study and 10.9 months vs 7.0 months in the first-line study) and higher overall response rate [ORR; 65% (113/173) vs 20% (34/174) in the second-line study and 74% (128/172) vs 45% (77/171) in the first-line study] compared with those of chemotherapy (Shaw

Abbreviations: ALK, anaplastic lymphoma kinase; TKI, tyrosine kinase inhibitor; P-gp, P-glycoprotein; ROS1, *v-ros* avian ur2 sarcoma virus oncogene homolog 1; PFS, progression-free survival; ORR, overall response rate; EGFR, epidermal growth factor receptor; BBB, blood–brain barrier; MRP1, multidrug Resistance-associated Protein 1; BCRP, breast cancer resistance protein; ATP, adenosine triphosphate; ABC, adenosine triphosphate (ATP)-binding cassette; CAF, cyclophosphamide, doxorubicin, and fluorouracil; OS, overall survival; FISH, fluorescence in situ hybridization; IHC, immunohistochemical; IRB, institutional review board; TNM, tumor-node-metastasis; CT, computed tomography; K562/VCR, K562-derived vincristine-resistant; RPMI, Roswell Park Memorial Institute; FBS, fetal bovine serum; IC50, half-maximal inhibitory concentration; (sh)RNA, small hairpin; CSCs, cancer stem/initiating cells; LCNEC, large cell neuroendocrine carcinoma; BAC, bronchioloalveolar carcinoma; SP, side population.

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et al., 2013; Solomon et al., 2014). However, although crizotinib has shown significant treatment efficacy in ALK fusion-positive NSCLC patients, tumor relapse because of acquired resistance has been observed. Crizotinib resistance was shown to be caused by various types of secondary mutations in the ALK kinase domain, by *ALK* fusion gene amplification, or by activation of the epidermal growth factor receptor (EGFR) or KIT (*v-kit* hardy-zuckerman 4 feline sarcoma viral oncogene homolog)-mediated bypass pathways (Doebele et al., 2012; Katayama et al., 2012; Sasaki et al., 2011). Crizotinib has also been shown to be relatively ineffective for cancer that has metastasized to the brain because of poor blood–brain barrier (BBB) penetration by P-glycoprotein (P-gp) overexpression (Costa et al., 2011; Chuan Tang et al., 2014).

To overcome crizotinib resistance, various next-generation ALK inhibitors have been evaluated in clinical trials. Among these, two ALK-tyrosine kinase inhibitors (TKIs) alectinib and ceritinib, have revealed prominent responses in both ALK-TKI-naïve and crizotinib-treated patients (Sakamoto et al., 2011; Shaw et al., 2014; Gadgeel et al., 2014; Seto et al., 2013; Marsilje et al., 2013). Encouraged by these significant clinical responses (Shaw et al., 2014), ceritinib was approved for clinical use by the US Food and Drug Administration (FDA) in 2014 and European Medicines Agency (EMA) in 2015, and alectinib was approved by the Pharmaceuticals and Medical Devices Agency of Japan in 2014 and FDA in 2015 (Seto et al., 2013). However, it is expected that next-generation ALK inhibitor-resistant tumors will also eventually develop via multiple mechanisms. To date, a few ceritinib-resistant mutations in the ALK kinase domain have been identified in patients who experienced a relapse during ceritinib therapy (Friboulet et al., 2014).

In human cancer, ABCB1/P-gp, ABCC1/multidrug resistance-associated protein 1 (MRP1), and ABCG2/breast cancer resistance protein (BCRP) are well-known causes of multidrug resistance to multiple chemotherapeutic agents, such as taxane and vinca alkaloids (Gottesman et al., 2002). Therefore, much effort has been devoted for developing the ATP-binding cassette (ABC)-transporter inhibitors. Among the ABC-transporter inhibitors, dofequidar fumarate (MS209) was identified as an orally active, quinoline-derived inhibitor of ABCB1/P-gp. In preclinical studies, MS209 reversed multidrug resistance in ABCB1 — and ABCC1-expressing cancer cells *in vitro*. In addition, oral administration of MS209 enhanced the antitumor activity of adriamycin, vincristine, and docetaxel *in vivo* (Naito et al., 2002; Nakanishi et al., 1997; Sato et al., 1995). Thus, MS209 has been a promising multidrug resistance-reversing agent and has been evaluated in clinical trials. The results of phase III clinical evaluations of MS209 for breast cancer treatment showed a relative improvement and increased response rate in patients who received MS209 plus cyclophosphamide, doxorubicin, and fluorouracil (CAF); however, differences did not reach statistical significance. However, the subgroup analysis suggested that MS209 plus CAF therapy displayed significantly improved PFS and overall survival (OS) in therapy-naive patients (Saeki et al., 2007). To date, a large number of ABC-transporter inhibitors have been developed, but failed because of undesirable toxicities (Fletcher et al., 2010).

Here, we report on our experience with a metastatic lung adenocarcinoma patient positive for *ALK* rearrangement who initially responded to ceritinib treatment. Meanwhile, the disease ultimately relapsed and failed to respond to subsequent chemotherapy or crizotinib therapy. We identified a ceritinib resistance mechanism caused by P-gp overexpression in the tumor. In vitro studies using ceritinib-resistant patient-derived cells have revealed that ceritinib is a P-gp substrate, and P-gp overexpression confers ceritinib and crizotinib resistance but does not confer alectinib or PF-06463922 resistance. In cell-line and murine-xenograft experiments, patient-derived P-gp-overexpressing cells responded to ceritinib plus P-gp inhibitor combination treatment, or alectinib or PF-06463922 which is not a P-gp substrate. In addition, we identified P-gp overexpression in two out of 10 crizotinib- or ceritinib-resistant metastatic ALK rearranged NSCLC patients and in one out of 31 ALK-TKI naïve tumors. The ALK-TKI naïve patient with P-gp overexpression had large-cell neuroendocrine carcinoma. These results suggest that P-gp overexpression potentially affects the response to ALK-TKIs, signifying the importance of obtaining biopsy samples following precise characterization of both pre- and post- ALK-TKI-treated tumors in ALK-rearranged NSCLC patients to achieve maximum therapeutic effects.

2. Material and Methods

2.1. Patients

Biopsies of ALK-TKI-resistant tumors from 10 ALK-positive NSCLC patients with acquired resistance were obtained between January 2010 and December 2013. Standard histopathological analysis was performed to confirm the diagnosis of malignancy and the histological subtype. For all samples, total nucleic acid was isolated as described below. In cases with sufficient tissue samples, we also performed fluorescence in situ hybridization (FISH) and immunohistochemical (IHC) analyses as described below. Electronic medical records were retrospectively reviewed to obtain clinical information in accordance with an institutional review board (IRB)-approved protocol.

The patient submitted written informed consent for genetic and cell biological analyses, which were performed in accordance with protocols approved by the IRBs of the Japanese Foundation for Cancer Research or Massachusetts General Hospital Cancer Center (Boston, MA, USA).

2.2. Establishment of Patient-derived Cancer Cell Lines

Tumors from patients with ALK-positive NSCLC patients were biopsied. Standard histopathological analysis was performed to confirm a diagnosis of malignancy and the histological subtype. We also performed FISH and IHC analyses as described below. Cell lines were established from malignant fluid or biopsy specimens after a sufficient amount of tissue was obtained. JFCR018-1 cell line was established from malignant fluid from an ALK-TKI-naïve ALK-rearranged NSCLC patient. JFCR018-1 cells harbor EML4-ALK v1 without secondary mutation.

2.3. JFCR013 Patient Treatment History

In May 2012, a 47-year-old woman with no history of smoking was diagnosed with metastatic lung adenocarcinoma with an *ALK* translocation at a tumor-node-metastasis (TNM) clinical stage of T4N3M1a. After two cycles of chemotherapy (cisplatin and pemetrexed), disease progression was confirmed by computed tomography (CT) scan (Fig. S1C). The patient was subsequently enrolled in a phase-I clinical trial of ceritinib (LDK378) treatment, and 6 weeks after initiation of ceritinib treatment, a remarkable response was observed (PR, 38.9% tumor shrinkage, Fig. S1A). However, 6 months later, the disease had progressed on ceritinib therapy. To identify the mechanisms of ceritinib resistance, we obtained a biopsy specimen and pleural fluid with malignant cells from ceritinib-resistant tumors in the patient, with which we established the cell lines JFCR013-1 and JFCR013-2. The existence of ALK rearrangement was confirmed by FISH analysis. After ceritinib therapy, the patient received one cycle of docetaxel, to which she failed to respond, and we established the cancer cell line JFCR013-3 from malignant fluid in the right pleural cavity. The patient was then treated with crizotinib for 6 weeks. Because there was no measureable region, and the thoracentesis was performed during crizotinib treatment, the response to crizotinib was not evaluable (NE). During crizotinib treatment, we obtained two additional specimens of malignant fluid from the right pleural cavity and established the cell lines JFCR013-4 and JFCR013-5. The patient died, and an autopsy was performed. From all disease sites that were examined, tumor samples were stored and cell lines were established from primary and metastatic tumors, which included normal lung tissue (JFCR013-6-4) (Table S1 and Fig. S1D).

2.4. Genetic Studies and Other Assays

FISH analysis of *EGFR*, *cMET*, and *cKIT* and the break-apart ALK FISH assay were used to identify gene copy numbers and ALK gene rearrangements in malignant tissue specimens. Complementary DNA from malignant tissues and cells was used to amplify EML4-ALK and ABCB1 (P-gp). RT-PCR products were sequenced using the Sanger method. Genomic DNA extracted from primary cancer cells from a malignant fluid sample was used for deep sequencing of a panel of 48 cancer-related genes (MiSeq Cancer Panel; Illumina, Inc., San Diego, CA, USA). Cell culturing, sequencing, IHC, and FISH procedures and characterization of P-gp are described in detail in the following methods.

2.5. Deep Sequencing

The samples were processed on the MiSeq platform with a TruSeq Amplicon Cancer Panel (Illumina, Inc.), which is designed to detect well-known somatic mutations of 48 genes (Table S2). Somatic variants were called using the Somatic Variant Caller (Illumina, Inc.) and annotated using the ANNOVAR (Wang et al., 2010) software tool (http://www.openbioinformatics.org/annovar/) and in-house scripts.

2.6. Reagents

Alectinib, ceritinib (LDK378) and PF-06463922 were purchased from Active Biochem (Hong Kong, China). TAE684 was purchased from Chemietek (Indianapolis, IN, USA), crizotinib from Shanghai Biochempartner (Shanghai, China), and verapamil and MS209 from Sigma-Aldrich (St. Louis, MO, USA). ^{14}C-labeled ceritinib (Fig. S7A) was kindly provided by Novartis Pharma AG (Basel, Switzerland). ^{3}H-labeled paclitaxel was purchased from American Radiolabeled Chemicals Inc. Each compound was dissolved in dimethyl sulfoxide for *in vitro* experiments.

2.7. Isolation of Genomic DNA, Total RNA Preparation, and Sequencing of the ALK Fusion Gene

Genomic DNA was isolated from cell pellets or fresh frozen specimens using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Total RNA was isolated from cell pellets or fresh frozen specimens using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Each exon of the ALK kinase domain (exon 21–27), EML4-ALK, or ALK kinase domain was PCR-amplified from genomic DNA or cDNA synthesized from total RNA with oligo-dTs using the KOD Plus v2 kit (Toyobo Co., Ltd., Osaka, Japan) and bidirectionally sequenced by the Sanger method.

2.8. FISH Analysis

FISH analyses for *ALK, MET, KIT*, and *EGFR* were performed using formalin-fixed paraffin-embedded tissues with in-house probes made from BAC clones (the exact clone names are available upon request). Images were captured with an Olympus BX51 fluorescent microscope equipped with a charge-coupled device camera (DP71; Olympus, Tokyo, Japan).

2.9. Cell Culture Conditions

H3122, K562, and K562-derived vincristine-resistant (K562/VCR) cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS; R-10). The H3122 cell line was obtained from the MGH/CMT Lab. JFCR013 cell lines derived from a ceritinib-resistant patient and the JFCR018-1 cell line established from ALK-TKI-naïve patient were cultured in RPMI 1640/F-12 medium supplemented with 15% FBS and 20 mM Hepes buffer (pH 7.5).

2.10. Survival Assays

For 72-h drug treatments, 2000–3000 cells were plated in replicates of 3–6 into 96-well plates. Following drug treatment, cells were incubated with CellTiter-Glo assay reagent (Promega Corp., Madison, WI, USA) for 10 min and luminescence was measured using a Centro LB 960 microplate luminometer (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). The data were graphically displayed using GraphPad Prism graphing software (v5.0; GraphPad Software, Inc., San Diego, USA). Half-maximal inhibitory concentration (IC50) values were determined using a non-linear regression model with a sigmoidal dose–response curve (GraphPad).

2.11. Immunoblot Analysis

Lysates were prepared as previously described (Engelman et al., 2007b; Katayama et al., 2012). In brief, equal amounts of lysates were electrophoresed and immunoblotted with antibodies against phospho-ALK (Tyr1604), ALK (C26G7), phospho-STAT3 (Thr705), STAT3, phospho-p42/44 ERK/MAPK (Thr202/Tyr204), p42/44 ERK/MAPK, phospho-Akt (Ser473) (D9E), panAkt (C67E7), phospho-S6 ribosomal protein (Ser240/244, D68F8), S6 ribosomal protein (54D2), P-gp (Cell Signaling Technology, Inc., Beverly, MA, USA), GAPDH (6C5; EMD Millipore, Billerica, MA, USA), and β-actin (Sigma-Aldrich).

2.12. IHC Analysis

Formalin-fixed, paraffin-embedded tissue specimens were sliced by a thickness of 4 μm, and the sections were placed on silane-coated slides. For antigen retrieval, slides were heated for 45 min at 105 °C in an antigen retrieval solution at pH 9.0 (Nichirei Biosciences, Inc., Tokyo, Japan). Antigen–antibody complexes were visualized with Histofine Simple Stain MAX PO detection reagent (Nichirei Biosciences, Inc.). The ALK Detection Kit (Nichirei Biosciences, Inc.), which is based on the intercalated antibody-enhanced polymer (iAEP) method (Takeuchi et al., 2009), was used for anti-ALK IHC analysis. The staining procedure was performed using the Histostainer automated staining system (Nichirei Biosciences, Inc.). For IHC of P-gp, anti-ABCB1 (P-gp) monoclonal antibody (6C4.2, Chemicon International Inc.) was used. Positivity for P-gp was standardized using KB3-1 (negative control) and ABCB1 overexpressed KB3-1 (KB3-1/ABCB1, positive control).

2.13. Retroviral Infection

Retroviral expression vectors encoding shRNA against P-gp or control shRNA were purchased from Sigma-Aldrich and viruses were produced as previously described (Engelman et al., 2007a). After retroviral infection, cells were kept in puromycin (1.0 μg/mL) for 2 weeks.

2.14. Cellular Uptake of ^{14}C-ceritinib and ^{3}H-paclitaxel

Cellular uptake of ^{14}C-ceritinib and ^{3}H-paclitaxel was measured in a culture medium. Tumor cells (1×10^6) were incubated at 37 °C in the presence of [^{14}C]ceritinib (100 nM; specific activity, 7.4 MBq/mg) and verapamil or MS209. After incubation, the cells were washed with PBS and lysed with lysis buffer containing 1% Triton X-100. The cell lysates and liquid scintillation cocktail (ACS II) were mixed well, and the cellular uptake of ceritinib was measured using a liquid scintillation counter (Beckman Coulter, Inc., Pasadena, CA, USA).

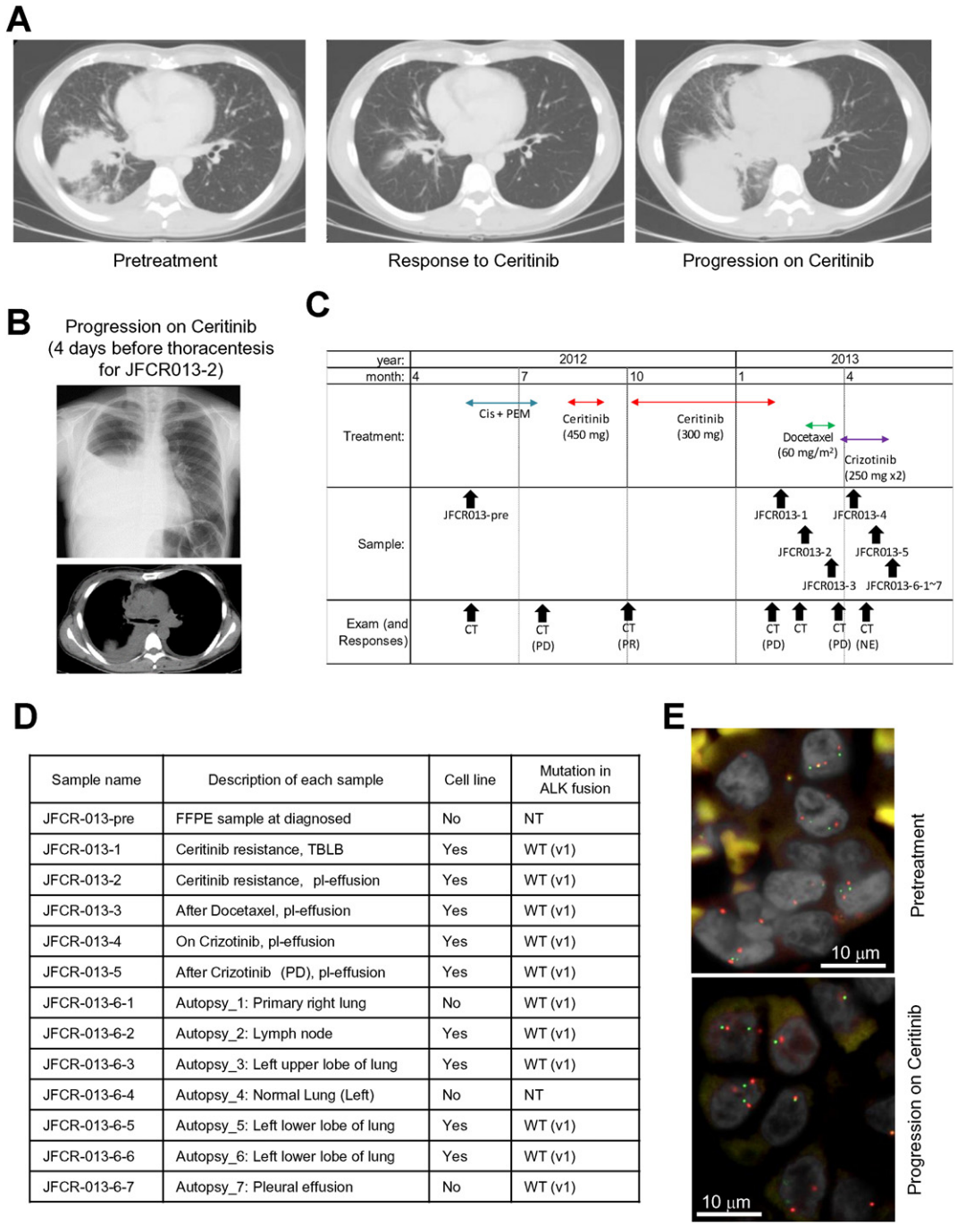
2.15. Mouse Experiment

JFCR013-2 cells (1×10^6) were subcutaneously injected into 5–6-week-old female BALB/c-nu/nu (nude) mice (Charles River Laboratories, Yokohama, Japan). The mice were checked twice weekly for palpable tumor formation and then euthanized. The tumors were

resected and diced and a piece was sequentially subcutaneously injected into another 5–6-week-old female BALB/c nude mouse. Tumor size was measured every other day using a caliper and tumor volumes were defined as (longest diameter) × (shortest diameter)^2/2. When the tumor size reached approximately 100 mm^3, the mice were sorted into five equal groups. Then, the nude mice were orally administered ceritinib (50 mg/kg) with or without MS209 (200 mg/kg) or alectinib (CH5424802). MS209 was administered 30 min prior to ceritinib treatment. Body weight and tumor size were measured every 3 days. All animal procedures were performed in accordance with protocols approved by the Japanese Foundation for Cancer Research Animal Care and Use Committee.

2.16. Statistical Analysis

All data are presented as means ± standard deviation. Statistical analysis was performed using the two-tailed Student's *t*-test or



Sample name	Description of each sample	Cell line	Mutation in ALK fusion
JFCR-013-pre	FFPE sample at diagnosed	No	NT
JFCR-013-1	Ceritinib resistance, TBLB	Yes	WT (v1)
JFCR-013-2	Ceritinib resistance, pl-effusion	Yes	WT (v1)
JFCR-013-3	After Docetaxel, pl-effusion	Yes	WT (v1)
JFCR-013-4	On Crizotinib, pl-effusion	Yes	WT (v1)
JFCR-013-5	After Crizotinib (PD), pl-effusion	Yes	WT (v1)
JFCR-013-6-1	Autopsy_1: Primary right lung	No	WT (v1)
JFCR-013-6-2	Autopsy_2: Lymph node	Yes	WT (v1)
JFCR-013-6-3	Autopsy_3: Left upper lobe of lung	Yes	WT (v1)
JFCR-013-6-4	Autopsy_4: Normal Lung (Left)	No	NT
JFCR-013-6-5	Autopsy_5: Left lower lobe of lung	Yes	WT (v1)
JFCR-013-6-6	Autopsy_6: Left lower lobe of lung	Yes	WT (v1)
JFCR-013-6-7	Autopsy_7: Pleural effusion	No	WT (v1)

Fig. 1. Response to ceritinib and obtained tumor samples from ALK-rearranged NSLCL patient. (A) An axial CT scan of the chest showing the patient's tumor burden prior to ceritinib treatment (left), after response to ceritinib treatment (center), and at the time of disease progression while on ceritinib (right). (B) X-ray and CT scan of the chest showing accumulation of pleural effusion at the time of disease progression on ceritinib. The picture was taken 4 days before thoracentesis from which the JFCR013-2 cell line was established. (C) Treatment history of JFCR013 and time line of the treatment, examination, and sample acquisition. (D) A summary of all obtained specimens and genetic alteration of ALK analysis by Sanger sequencing of the EML4-ALK and ALK kinase domain and deep sequencing of the ALK kinase domain (Illumina TruSeq amplicon panel). (E) Break-apart FISH analysis with a 5′-ALK probe (red) and a 3′-ALK probe (green) of a formalin-fixed paraffin-embedded specimen from the patient. ALK rearrangement was indicated by the presence of individually isolated red 3′-ALK green probes. Nuclei were stained with 4′,6-diamidino-2-phenylindole.

Mann–Whitney U test (for mice experiments). Significant probability (P)-values are indicated as $^{***}P < 0.001$, $^{**}P < 0.01$ and $^{*}P < 0.05$.

3. Results

3.1. Identification of Ceritinib Resistance Without a Secondary Resistance Mutation in ALK

A metastatic *ALK*-rearranged lung adenocarcinoma patient was enrolled in a phase-I clinical trial of ceritinib (LDK378) treatment. Evaluation performed 6 weeks after initiation of ceritinib treatment revealed a marked treatment response (PR, 38.9% tumor shrinkage). However, 6 months later, it was observed that the disease had progressed while the patient was still receiving ceritinib therapy (Fig. 1A). To identify the mechanisms of ceritinib resistance, we obtained a biopsy specimen and malignant pleural fluid from ceritinib-resistant tumors in the patient by which we established the cell lines JFCR013-1 and JFCR013-2 (Fig. 1B–D, Table S1). The *ALK* rearrangement was confirmed by FISH analysis (Fig. 1E), and the fusion of exon 13 of *EML4* to exon 20 of *ALK* was identified by RT-PCR from JFCR013-2 cells (Fig. 1D). Upon relapse, the patient received one cycle of docetaxel, but the tumor failed to respond, and we established the JFCR013-3 cancer cell line from malignant pleural fluid in the right lung (Fig. 1C). The patient was subsequently treated with crizotinib, during which we obtained two additional samples of malignant fluid from the right pleural cavity and established the cell lines JFCR013-4 and JFCR013-5 (Fig. 1C, D, and Table S1). The patient died, and an autopsy was performed. From all disease sites that were examined, tumor samples were stored and cell lines established from primary and metastatic tumors, which included the normal lung tissue (Fig. 1D, S1, and Table S1). None of the tumor sites carried a mutation in the kinase domain of the EML4-ALK fusion protein (Fig. 1D). To further identify potential resistance mechanisms, we performed amplicon sequencing analysis of a panel of 48 genes typically implicated in cancer (Table S2). The analysis confirmed the absence of

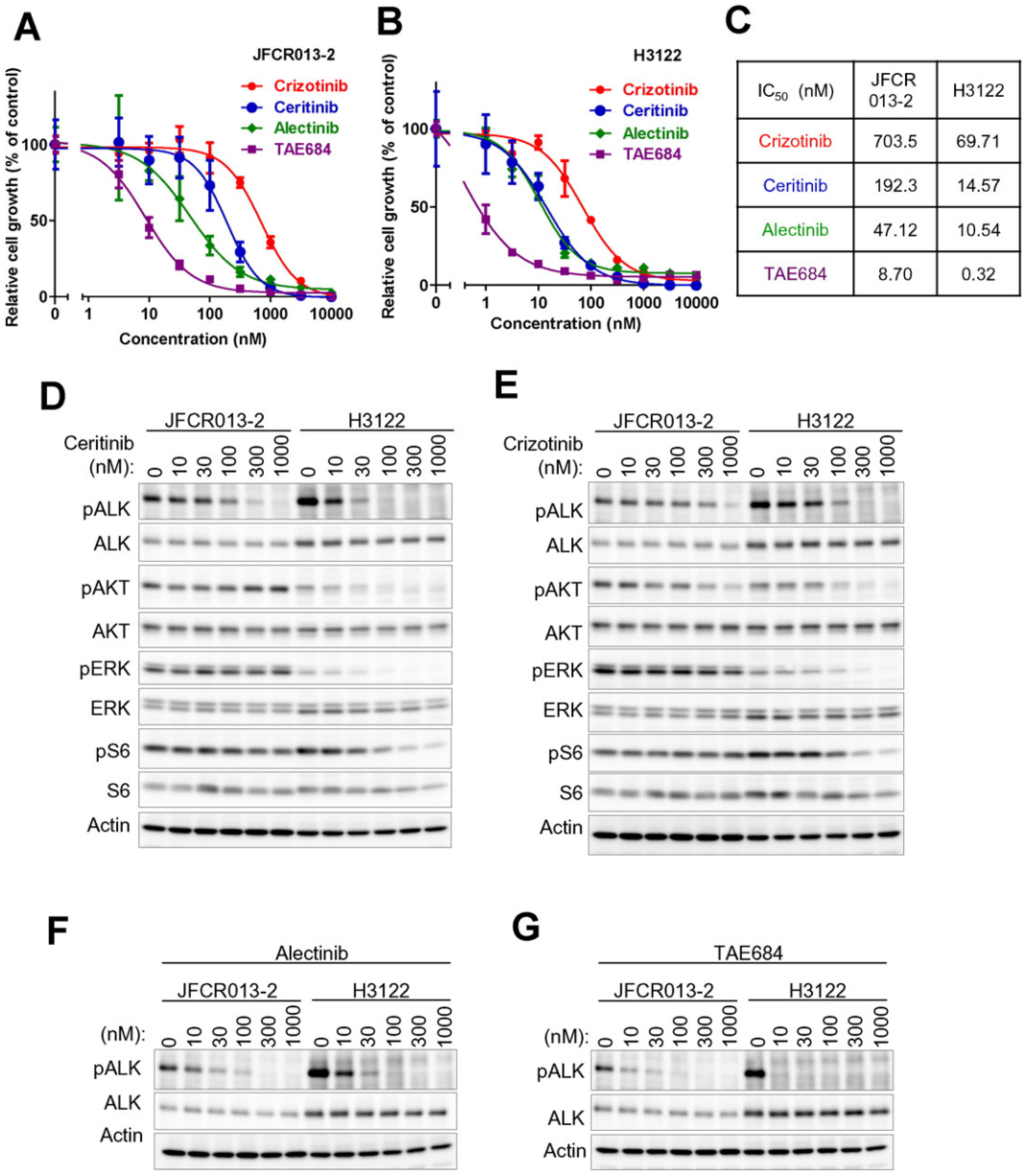


Fig. 2. Ceritinib-refractory patient-derived cells were resistant to ceritinib and crizotinib but not to alectinib. (A–C) JFCR013-2 or H3122 cells were treated with the indicated concentrations of crizotinib, ceritinib, alectinib, or TAE684 for 72 h. After incubation, cell viability was measured using the CellTiter-Glo assay. Calculated IC50 values are shown in the table (C). (D–G) JFCR-013-2 and H3122 cells were treated with the indicated concentration of ceritinib (D), crizotinib (E), alectinib (F), or TAE684 (G) for 3 h. Cell lysates were analyzed by immunoblotting with the indicated antibodies.

somatic mutation in other major oncogenes. FISH analysis of *EGFR*, *cMET*, and *cKIT* revealed no amplification in JFCR013-2 and JFCR013-5 specimens (data not shown).

3.2. P-gp Overexpression-mediated Ceritinib and Crizotinib Resistance

Next, we examined the sensitivity of various selective ALK inhibitors to JFCR013-2 cells derived from ceritinib-resistant tumor samples. JFCR013-2 cells showed marked crizotinib and ceritinib resistance, but were sensitive to alectinib and TAE684 (Fig. 2A–C). Furthermore, ceritinib as well as crizotinib effectively inhibited ALK phosphorylation and downstream phospho-AKT and phospho-ERK activation in H3122 cells, which are EML4-ALK harboring ALK-TKI sensitive cells. On the other hand, JFCR013-2 cells required higher concentration of crizotinib or ceritinib to suppress phospho-ALK than H3122 cells (Fig. 2D and E). In contrast, TAE684 and alectinib inhibited phospho-ALK in both H3122 and JFCR013-2 cells to a similar extent (Fig. 2F and G). Because the disease did not respond to docetaxel treatment after relapsed on ceritinib, we treated JFCR013-2 cells with docetaxel. As the result, compared with H3122 or K562 cells, JFCR013-2 cells were highly resistant to

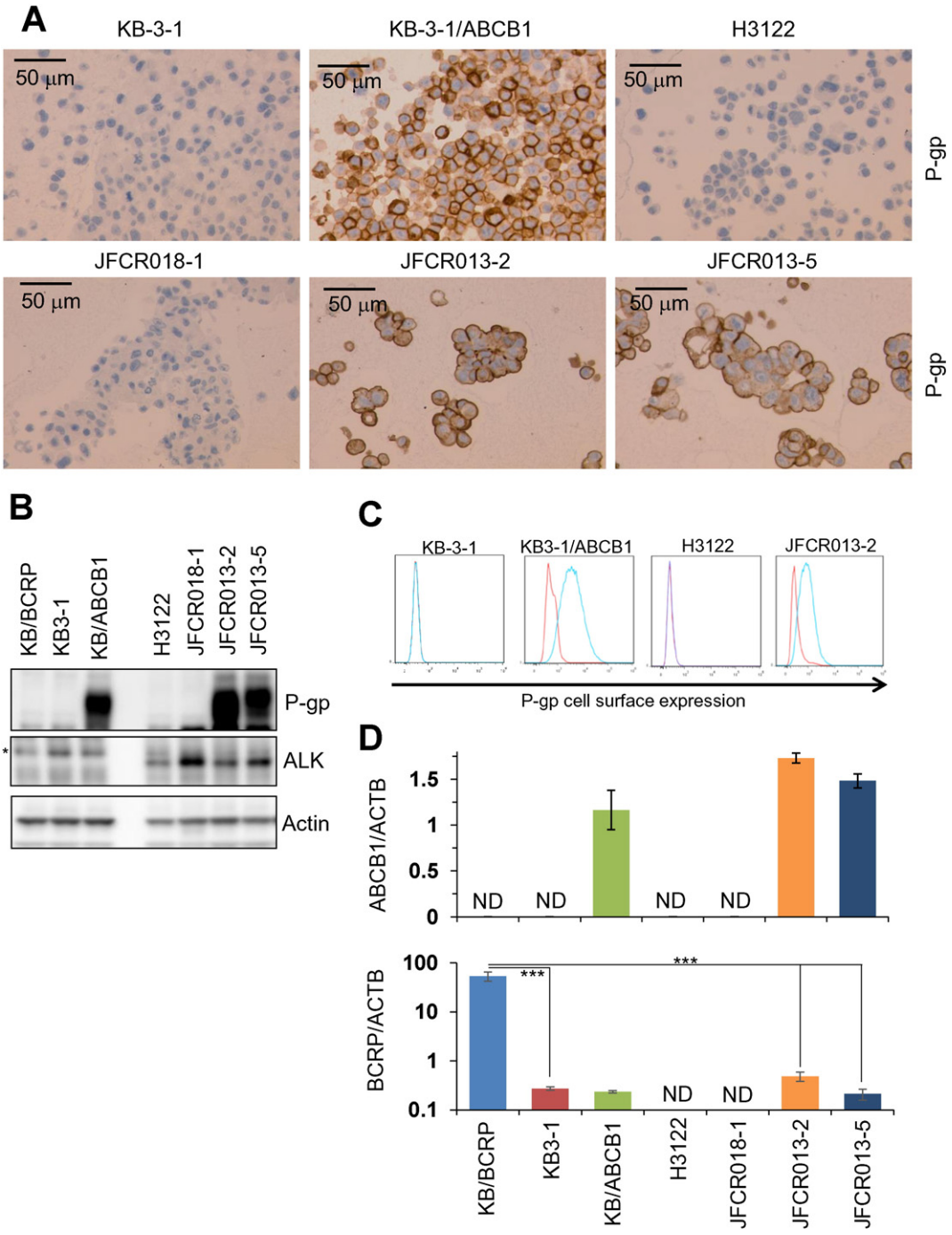


Fig. 3. P-gp was highly expressed in JFCR-013 cells. (A) P-gp expression in the indicated cell lines was analyzed by immunohistochemistry. (B) P-gp, ALK or β-actin protein expression analyzed by immunoblotting. (C) Cell surface P-gp expression analyzed by flow cytometry after incubation of each cell line with P-gp monoclonal antibody (blue) or control IgG (red). (D) *ABCB1* or *BCRP* (*ABCG2*) mRNA expression levels analyzed by quantitative RT-PCR. Relative mRNA expression was calculated using *β-actin* (*ACTB*) mRNA expression as reference. ND (not detected) means no PCR amplicon of ABCB1 or ABCG2 was detected.

docetaxel to a similar extent as K562/VCR cells (Fig. S2A) that had previously been established from a vincristine-resistant K562 cell line by treating increasing concentration of vincristine, and that had been shown to be resistant to docetaxel by overexpressing P-gp via *ABCB1* gene amplification (Fig. S2B, S2C) (Sugimoto et al., 1987; Naito et al., 2002). On the other hand, each of these cell lines showed similar sensitivity to cisplatin which is not a substrate of P-gp (Fig. S2D). ABC transporters such as P-gp (ABCB1) or BCRP (ABCG2), play an important role in chemotherapeutic drug resistance (Gottesman et al., 2002). In addition, it is also known that ABC transporters are highly expressed in the cancer stem/initiating cells (CSCs), and confer CSC resistance to multiple chemotherapeutic drugs (Katayama et al., 2009). Therefore, we hypothesized that ceritinib and crizotinib were actively exported from the JFCR013-2 cells by ABC transporter(s), resulting in ceritinib and crizotinib resistance. Thus, we examined expression levels of the ABC transporter P-gp (ABCB1). As we expected, P-gp expression was significantly upregulated in the established ceritinib-resistant cell lines JFCR013-2 and JFCR013-5 at a level similar to that in ABCB1-transfected KB3-1 cells, but it was not upregulated in parental KB-3-1 cells or JFCR018-1 cells established from another ALK-TKI treatment-naïve ALK-rearranged NSCLC patient (Fig. 3A and B). Using the anti-P-gp monoclonal antibody MRK-16, which recognizes the extracellular domain of P-gp (FitzGerald et al., 1987), we confirmed surface P-gp expression in JFCR013-2 cells (Fig. 3C). Furthermore, the mRNA levels of *ABCB1*, but not those of *ABCG2*, were upregulated in the ceritinib-resistant cells lines and tumors (Fig. 3D). Next, we examined P-gp expression in the stored clinical specimens. P-gp overexpression and upregulation of *ABCB1* mRNA were also observed in the ceritinib-resistant tumor (JFCR013-2 and JFCR013-6-1) but not in the pre-ceritinib-treated tumor biopsy specimen (JFCR013-pre; Fig. 1A, C and Table S1), and ALK expression was maintained throughout the treatment period (Fig. 4A). In contrast to the K562/VCR cells, JFCR013-2 cells showed no marked amplification of *ABCB1* (Fig. S2C), and no *ABCB1* mutation in the JFCR013-2 cell line was observed.

3.3. P-gp-knockdown or Overexpression Affects the Sensitivity to Ceritinib and Crizotinib

To examine whether P-gp overexpression contributes to ceritinib resistance, we established *ABCB1*-knockdown cells using shRNA. sh-ABCB1 (#86 and #87), but not control shRNA, decreased P-gp expression and sensitized the cells to ceritinib and crizotinib (Fig. 5A and B). Conversely, we established H3122 cells overexpressed P-gp (Fig. 5C). As shown in Fig. 5D, E, S3 and S4, P-gp overexpressing H3122 cells

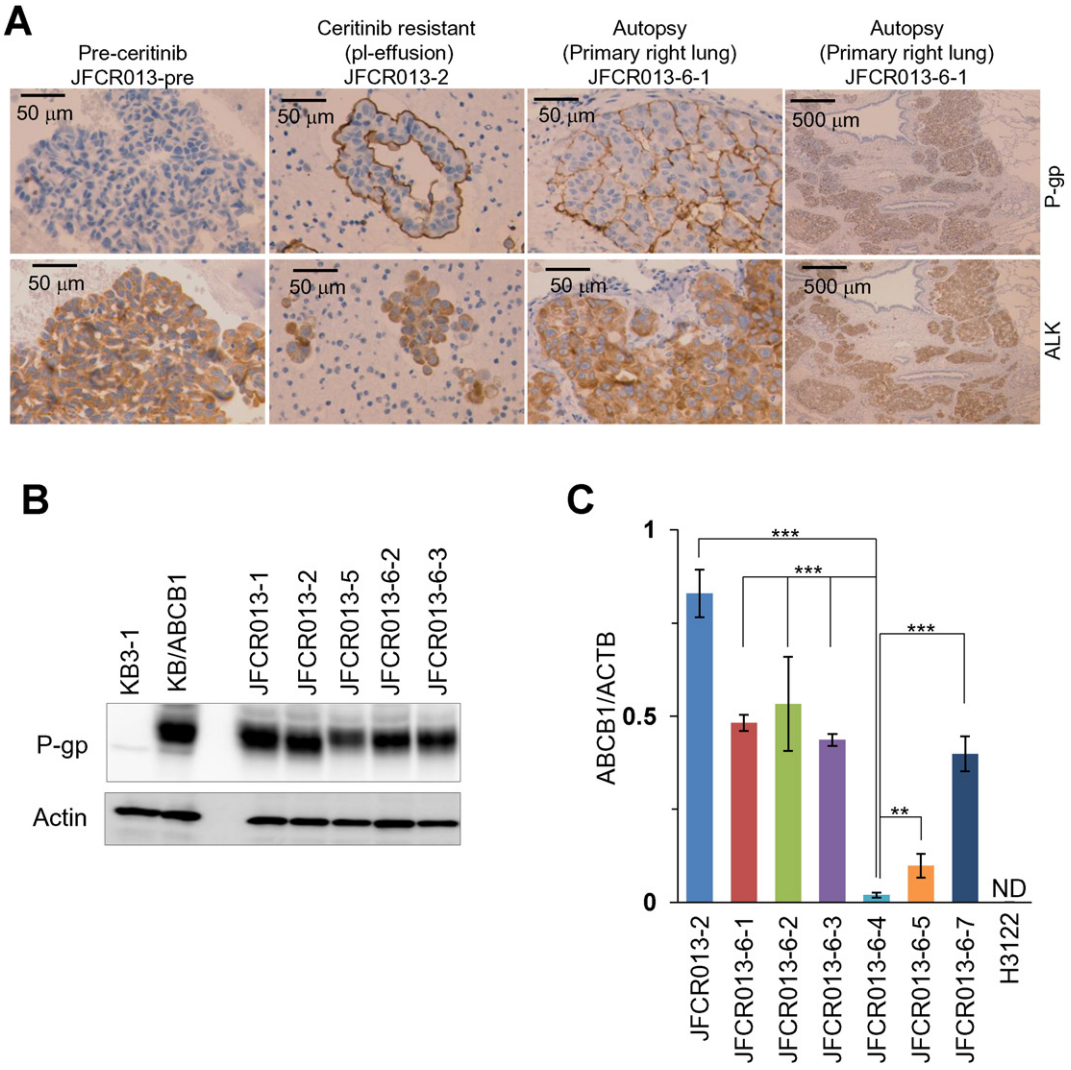
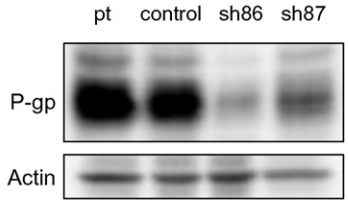


Fig. 4. P-gp overexpression was observed only in the tumor after ceritinib treatment. (A) IHC analysis of P-gp or ALK protein expression in JFCR-013-pre and ceritinib-resistant JFCR013-2 and JFCR013-6-1 (autopsy) specimens. (B) P-gp protein expression in the indicated cell lines and in autopsy specimens analyzed by immunoblotting. (C) ABCB1 mRNA expression level in autopsy specimens was analyzed by quantitative RT-PCR. In particular, JFCR013-6-4 reflects normal lung tissue. Relative mRNA expression was calculated using *β-actin* (*ACTB*) mRNA expression as a reference.

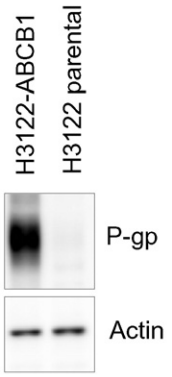
A



B

Cells	IC_{50} (nM) to ceritinib	IC_{50} (nM) to crizotinib
JFCR013-2	180.4	408.5
013-2_sh-control	141.3	458.5
013-2_sh-ABCB1 (#86)	60.49	155.5
013-2_sh-ABCB1 (#87)	97.06	251.7

C



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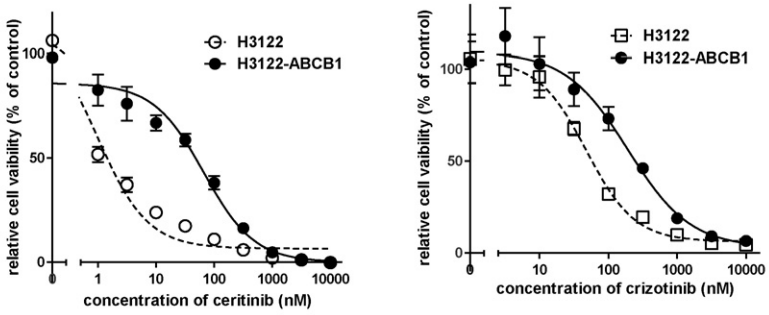


E

relative IC_{50}	Ceritinib	Crizotinib	Alectinib	PF-06463922
H3122	1	1	1	1
H3122-ABCB1	162	5.4	1.9	1.1

Fig. 5. P-gp overexpression confers ceritinib and crizotinib resistance. (A) P-gp protein expression in the indicated shRNA-induced cells analyzed by immunoblotting. (B) JFCR013-2- and shABCB1-induced JFCR013-2 cells treated with serially diluted concentrations of ceritinib or crizotinib for 72 h. After incubation, cell viability was measured by CellTiter-Glo Assay. Calculated IC_{50} values are shown in the table. (C) P-gp and β-actin protein expression in H3122 and ABCB1-overexpressing H3122 cells analyzed by immunoblotting. (D, E) H3122 or ABCB1-overexpressing H3122 cells treated with the indicated concentrations of ceritinib (left) or crizotinib (right) for 72 h. After incubation, cell viability was measured using the CellTiter-Glo assay. Calculated IC50 values are shown in (E).

showed significant ceritinib and crizotinib resistance, but did not show alectinib or PF-06463922 resistance, a potent next-generation ALK inhibitor under clinical evaluation, which is also showed active against multiple crizotinib resistant mutants (Johnson et al., 2014). Indeed, the patient-derived JFCR013-2 cells were highly sensitive to PF-06463922 (Fig. S5).

3.4. P-gp-mediated Ceritinib Pump-out Confers Resistance

Next, we examined if ceritinib was in fact exported by the ABC transporter P-gp. Ceritinib uptake was measured by incubating the cells with ^{14}C-labeled ceritinib (Fig. S6A). In ceritinib-resistant JFCR013-2 cells, the incorporation of ^{14}C-ceritinib became constant after 8 h, whereas treatment with the P-gp inhibitor MS209 (Sato et al., 1995) or verapamil (Tsuruo et al., 1981) constantly increased cellular uptake of ^{14}C-ceritinib. In contrast, sh-ABCB1 inhibited ceritinib pump-out, resulting in a continuous intercellular accumulation of ^{14}C-ceritinib (Fig. 6A). Similar results were observed in vincristine-resistant P-gp-overexpressing K562/VCR cells, but not in K562 parental cells (Fig. 6B). In addition, when JFCR013-2 cells were incubated with ^{3}H-paclitaxel, which is a substrate of P-gp, the incorporation of ^{3}H-paclitaxel was restored by MS209 or verapamil treatment (Fig. S6B). These results demonstrated that ceritinib is a P-gp substrate.

3.5. P-gp Inhibitors Resensitized Cells to Ceritinib, Crizotinib, or Docetaxel

To overcome ceritinib resistance caused by P-gp overexpression, we treated each cell line with ceritinib and the P-gp inhibitor MS209, verapamil, or the BCRP inhibitor fumitremorgin C (FTC). MS209 and verapamil, but not FTC, sensitized patient-derived JFCR013-2 cells as well as P-gp overexpressing H3122 clone to ceritinib, crizotinib and docetaxel (Figs. 7A, S7A and S7B). Consistently, ceritinib with MS209 or verapamil effectively suppressed ALK phosphorylation (Fig. 7B) and inhibited phospho-ALK reactivation when the cells were treated with 1 μM of ceritinib (Fig. 7C). In addition, MS209 treatment also re-sensitized the

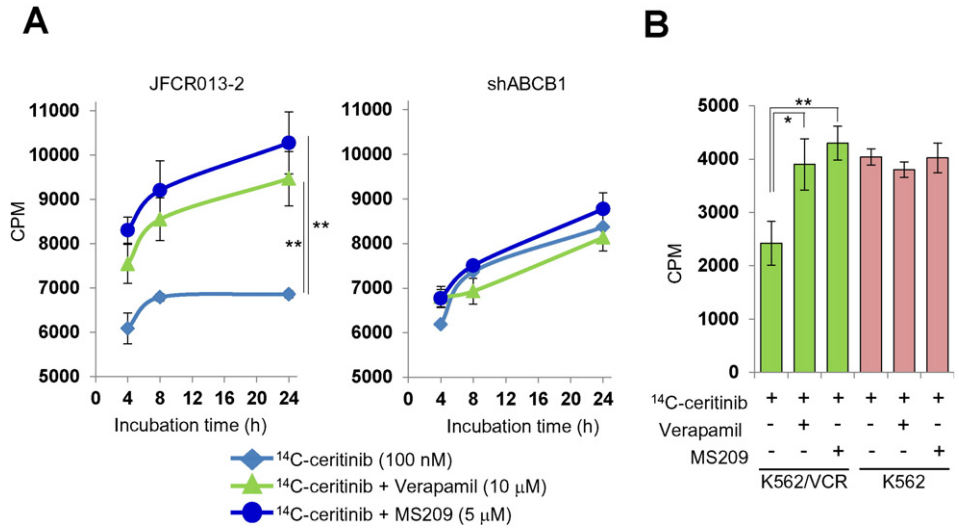


Fig. 6. Ceritinib is a P-gp substrate and P-gp inhibition reduces drug export. (A) JFCR013-2 cells and shABCB1-(#86) induced JFCR013-2 cells incubated with ^{14}C-labeled ceritinib (100 nM) plus verapamil (10 μM) or MS209 (5 μM) at 37 °C. At the indicated time points, incorporated ^{14}C-ceritinib was measured using a liquid scintillation counter. (B) K562 and P-gp-overexpressing K562/VCR cells incubated with ^{14}C-labeled ceritinib (100 nM) plus verapamil (10 μM) or MS209 (5 μM) for 8 h at 37 °C. After washing the cells, incorporated ^{14}C-ceritinib was measured using a liquid scintillation counter.

P-gp overexpressing H3122 cells to crizotinib and ceritinib, but not to alectinib or PF-06463922 (Figs. S3 and S4).

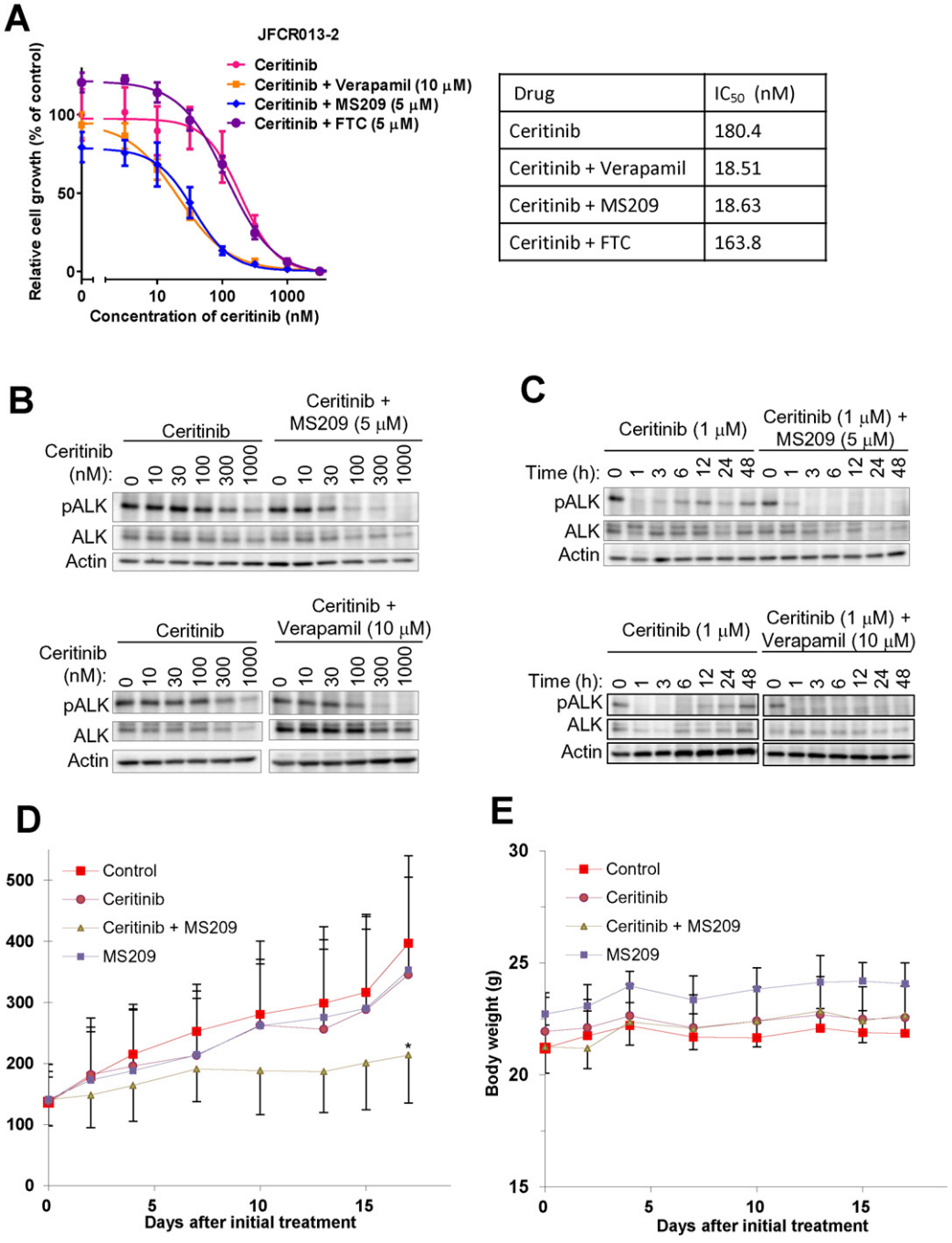
Because MS209 was subjected to clinical evaluation in an advanced breast cancer patient and found to be both safe and tolerable (Saeki et al., 2007), we evaluated the efficacy of ceritinib with MS209 in vivo. JFCR013-2 cells were subcutaneously injected into mice, which were subsequently treated with ceritinib plus MS209. The results showed that the co-administration of ceritinib with MS209, but not ceritinib monotherapy, inhibited tumor growth without affecting body weight (Figs. 7D, E, S8A, and S8B). Ceritinib and MS209 in combination inhibited phospho-ALK activation in the JFCR013-2 xenograft tumor (Fig. S8C). In addition, alectinib and PF-06463922 more effectively inhibited the growth of the JFCR013-2 xenograft tumor (Fig. S9).

3.6. P-gp Overexpression Mediated Drug Resistance in ALK-rearranged NSCLC

To further examine the significance of P-gp overexpression in ceritinib and/or crizotinib resistance in ALK-rearranged NSCLC, we examined P-gp expression by IHC analysis in 30 treatment-naïve surgical specimens of ALK-rearranged NSCLC but found no tumor overexpressing P-gp (Fig. S10). Next, we examined P-gp expression in tumors of progressed ALK-rearranged NSCLC treated with either of the ALK-TKIs and found two cases of P-gp overexpression in ALK-rearranged NSCLC (Fig. 8A). In one case, JFCR025 showed P-gp overexpression in both pre-ALK-TKI-treated and post-ALK-TKI-treated tumors obtained from an ALK-rearranged large cell neuroendocrine carcinoma patient (LCNEC; Fig. 8B and Supplementary Fig. S11). From the ceritinib-treated resistant tumor biopsy, we established the cell line JFCR025 and found that JFCR025 cells harbor the L1196M gatekeeper mutation with P-gp overexpression (Figs. 8A, C, and Supplementary Fig. S12A). The JFCR025 cells showed ceritinib resistance but were sensitive to ceritinib + MS209 (Fig. 8D and Supplementary Fig. S12B). In the second case, we identified P-gp overexpression in a metastatic ALK-rearranged lung adenocarcinoma patient who received crizotinib following ceritinib (MGH015). P-gp overexpression was observed in the solid components of the crizotinib-resistant tumor but not in the bronchiolo-alveolar carcinoma (BAC) component of crizotinib-resistant tumor. P-gp overexpression was also observed in bone-metastatic regions relapsing after 8 months of ceritinib treatment (Figs. 8E and S13). As described in our previous report (Katayama et al., 2012), crizotinib-resistant tumors in the solid component, but not in the BAC component, showed high Ki67 levels, *cKIT* gene amplification, and overexpression of its ligand, SCF. These results suggest that P-gp overexpression decreased cellular crizotinib or ceritinib levels and contributed to the resistance.

4. Discussion

We identified an acquired ceritinib-resistance mechanism caused by P-gp overexpression in an ALK-rearranged NSCLC patient who was receiving ceritinib therapy. We also demonstrated that ceritinib is a P-gp substrate and that P-gp inhibition reversed ceritinib and crizotinib resistance. While our manuscript was under review, it was reported that brain accumulation of ceritinib is restricted by P-gp and BCRP (Kort et al., 2015). For ALK-rearranged NSCLC patients, treatment with ALK-TKIs, such as crizotinib, ceritinib, or alectinib often leads to conspicuous tumor shrinkage; however, tumors inevitably relapse because of acquired resistance approximately within 1–2 years of treatment, similar to what is known for imatinib-treated CML with BCR-ABL- (Branford et al., 2002) or EGFR-TKI-treated EGFR-mutated NSCLC (Engelman and Janne, 2008; Kobayashi et al., 2005). Unlike lung cancers with EGFR mutations, various crizotinib resistance mutations have been identified in crizotinib-treated ALK-rearranged NSCLC. In addition, *ALK* fusion gene amplification has also been identified as a crizotinib resistance mechanism. Ceritinib is a potent oral ALK inhibitor, which has been shown to be effective for the treatment of naïve and crizotinib-treated ALK-rearranged NSCLC patients (Shaw et al., 2014). However, acquired ceritinib resistance also occurs, such as G1202R or F1174C/V mutations in the ALK kinase domain (Friboulet et al., 2014). Similarly, G1202R or I1171T/N/S mutations in ALK have been identified as an alectinib resistance mutation. In addition, *MET* gene amplification has also been identified in an alectinib-resistant patient in whom the tumor responded to crizotinib, which is a potent MET inhibitor. Apart from alterations in the *ALK* gene, bypass pathway activation mediated crizotinib resistance, such as EGFR activation, *KIT* amplification with SCF upregulation, or IGF1R activation has been identified. However, the mechanism underlying crizotinib, ceritinib and alectinib resistance remains largely unknown in many cases (Katayama et al., 2012; Shaw and Engelman, 2013; Gainor et al., 2013; Lovly et al., 2014). Recently, crizotinib was shown to be exported by P-gp, resulting in decreased penetration of the blood–brain barrier by P-gp (Chuan Tang et al., 2014). Because the use of crizotinib following ceritinib treatment showed minimal effect in our patient (JFCR013), we suspected P-gp upregulation as a



Drug	IC_{50} (nM)
Ceritinib	180.4
Ceritinib + Verapamil	18.51
Ceritinib + MS209	18.63
Ceritinib + FTC	163.8

Fig. 7. P-gp inhibitor treatment sensitized the cells to ceritinib in vitro and in vivo. (A) JFCR-013-2 cells treated with the indicated concentrations of ceritinib plus verapamil (10 μM), MS209 (5 μM), or FTC (5 μM) for 72 h. After incubation, cell viability was measured using the CellTiter-Glo assay. Calculated IC50 values are shown in the lower table. (B, C) JFCR-013-2 cells treated with the indicated concentrations of ceritinib with or without P-gp inhibitor MS209 (5 μM) or verapamil (10 μM) for 3 h (B) or for 1–48 h (C). Cell lysates were analyzed by immunoblotting with the indicated antibodies. (D, E) JFCR013-2 cells were subcutaneously injected into nude mice. After the tumor achieved a size of approximately 100 mm^3, the mice were randomized by tumor size and daily treatment with 50 mg/kg of ceritinib with or without 200 mg/kg of MS209 was started. Tumor volumes were measured as follows: 0.5 × length × width × width. Tumor sizes relative to day 0 are shown in (D), and changes in body weight are shown in (E).

mechanism of crizotinib resistance. Among the family of ATP-binding cassette proteins, P-gp is most extensively characterized. Its overexpression is shown to be sufficient to confer chemotherapeutic drug resistance to both cell cultures and animal models of human cancers. To date, attempts to improve anticancer therapy by the co-administration of various P-gp inhibitors, including MS209, with chemotherapeutic agents have been disappointing. In addition, the limited success of a combination therapy of a P-gp inhibitor with a chemotherapeutic agent was mainly because of undesirable toxicities. Although we did not observe any severe adverse effects in our mouse experiments, we must carefully consider the benefits and undesirable toxicities of molecular-targeted drugs in combination with the use of a P-gp inhibitor. Many studies have investigated P-gp-associated drug resistance, but very few have described clinical cases of P-gp-mediated drug resistance; in particular, molecular-targeted agent resistance in NSCLC. In the JFCR013 cells, P-gp overexpression was not caused by *ABCB1* gene amplification. mRNA expression in JFCR013 cell lines was substantially higher than that in H3122 or ALK-TKI-naïve patient-derived JFCR018-1 cells, in which P-gp expression was barely detectable. When we continuously treated the H3122 tumor-bearing mice with ceritinib

A

Patient ID	ALK mutation after (1st line ALK-TKI)	ALK mutation after (2nd line ALK-TKI)	ALK mutation after (3rd line ALK-TKI)	P-gp positivity in the specimen		
JFCR010	G1269A (Crizotinib)			Negative (-)		
JFCR011	L1196M (Crizotinib)			Negative (-)		
JFCR013	WT (Ceritinib)	WT (Crizotinib)		pre-Ceritinib	post-Ceritnib	
				Negative (-)	**Positive (++)**	
JFCR021	G1269A (right lung) (Crizotinib)	F1174V (left lung) and G1202R (right lung) (Ceritinib)		negative		
JFCR025*	NT (Alectinib)	NT (Crizotinib)	L1196M (Ceritinib)	pre-Alectinib	post-Ceritnib	
				Positive (+)	**Positive (++)**	
JFCR027	WT (Crizotinib)			negative		
JFCR041	NT (Alectinib)	G1202R (Ceritinib)		negative		
MGH015	WT (Crizotinib)	WT (Ceritinib)		pre-Crizotinib	post-Crizotinib	post-Ceritinib
				Negative (-)	**Positive (++)** in solid part	**Positive (++)** in bone meta
MGH034	WT (Crizotinib)	WT (Ceritinib)		Negative (-)		
MGH049	WT (Crizotinib)	WT (Ceritinib)		Negative (-)		
MGH051	WT (Crizotinib)	G1202R (Ceritinib)		Negative (-)		

*JFCR025 is LCNEC

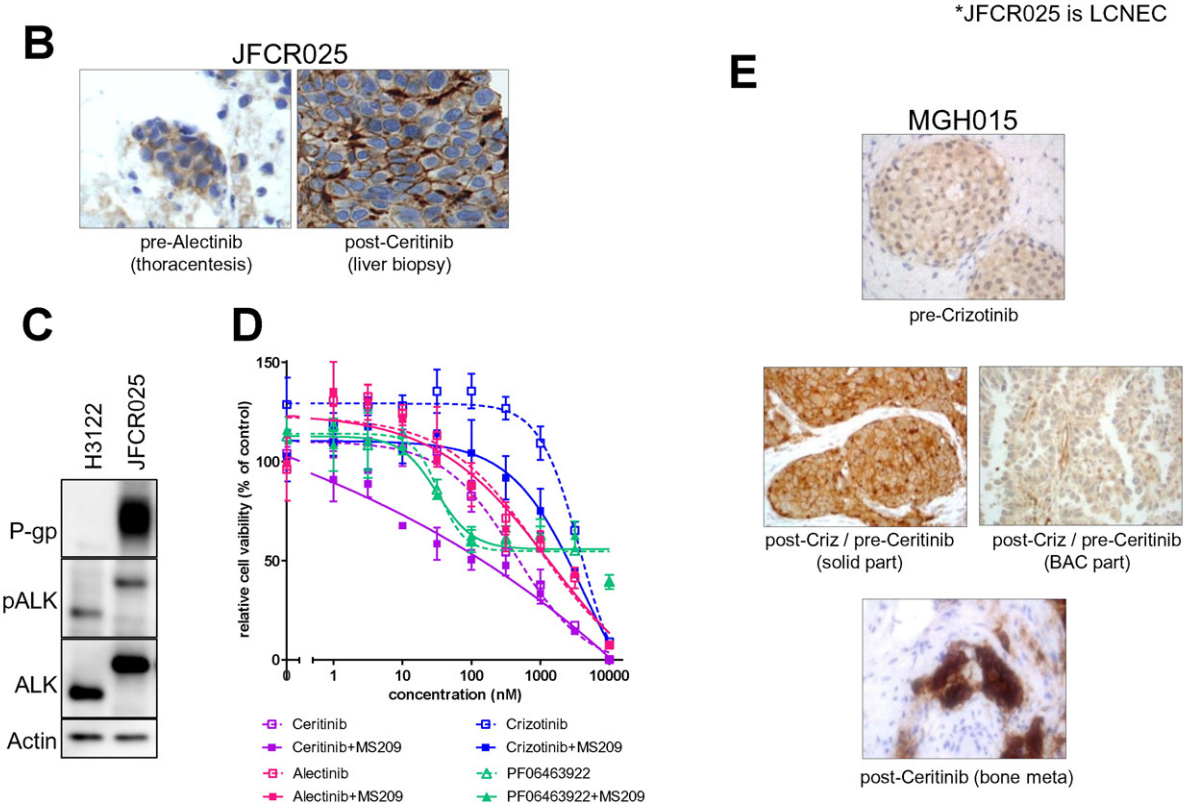


Fig. 8. P-gp overexpression in ALK-rearranged NSCLC clinical specimens. (A) ALK mutation status and P-gp expression status in an ALK-TKI-resistant tumor before and after ALK-TKI treatment. (B, E) IHC analysis of P-gp protein expression in pre-alectinib- and post-ceritinib-treated JFCR025 specimens (B) and pre-crizotinib-, post-crizotinib-/pre-ceritinib-, or post-ceritinib-treated MGH015 specimens (E). (C) H3122 and ceritinib-resistant patient-derived JFCR025 cells were analyzed by immunoblotting with the indicated antibodies. (D) JFCR025 cells were treated with the indicated concentrations of ceritinib, crizotinib, alectinib or PF-06463922 with or without MS209 (5 μM) for 72 h. After incubation, cell viability was measured using the CellTiter-Glo assay.

more than 2 months, we found that one tumor overexpressed *ABCB1* mRNA (230 fold higher than the untreated H3122 xenograft tumor) without *ABCB1* gene amplification (data not shown). Thus, ABCB1 overexpression may be epigenetically regulated. Hence, further studies are needed to clarify the mechanism of ABCB1 upregulation in ceritinib-resistant cell lines.

In addition, a single case of LCNEC harboring an EML4-ALK rearrangement that confers crizotinib resistance was recently reported (Omachi et al., 2014). However, in that study, the investigators did not explore the potential mechanisms of resistance. In the present study, we experienced a case of LCNEC overexpressing P-gp in both pre- and post-ALK-TKI-treated specimens, for which the tumor responded to alectinib. Indeed, we found that less than 1% of treatment-naïve lung cancers overexpressed P-gp by tissue microarray examination of 1477 lung cancer surgical specimens. Because we only detected few P-gp positive cases, we do not know whether P-gp expression links with cancer phenotypes. In the alectinib-, crizotinib-, and ceritinib-resistant tumor cells (JFCR-025), the L1196M gatekeeper mutation with P-gp overexpression was identified. In addition to our data in this study, alectinib and PF-06463922 were found not to be a P-gp substrate in a former study (Kodama et al., 2014; Johnson et al., 2014). In another case (MGH015) with P-gp overexpression, we previously identified *KIT* amplification with upregulation of the KIT ligand SCF in the solid component of a crizotinib-resistant tumor. In our previous study, we showed that SCF treatment conferred moderate crizotinib resistance in H3122 cells overexpressing KIT. However, in this study, we found that P-gp was also overexpressed in the solid part of the tumor. Furthermore, P-gp overexpression was observed in a ceritinib-resistant tumor metastasized to the bone. Because P-gp contributes to the pump-out of the ALK inhibitors crizotinib and ceritinib, resulting in decreased sensitivity to crizotinib or ceritinib, activation of the bypass pathway (e.g., KIT) with P-gp overexpression may confer complete ALK-TKI resistance. Therefore, further studies on the frequency and mechanisms of P-gp overexpression in resistant tumors are needed to determine therapeutic strategies to prevent or overcome drug resistance in ALK-positive NSCLC. In particular, we also found that the feedback activation of EGFR and MET was also identified by the RTK-array when the JFCR013-2 cells were completely inhibited ALK by ceritinib with MS209 or alectinib treatment. However, RTKs re-activation did not largely contribute to ALK-TKI resistance in JFCR013-2, because erlotinib (EGFR inhibitor) and PHA665752 (MET inhibitor) slightly sensitized the cells to ALK inhibition (alectinib or ceritinib plus P-gp inhibition). These results suggest that P-gp overexpression is the main cause of ceritinib resistance (and crizotinib) in JFCR013-2 cells. As described in the result section, gene amplification or mutation of *EGFR*, *MET* and *KIT* was not identified in JFCR013-2 cells.

In addition, ABC-transporters are important markers for the isolation and analysis of stem cells and cancer propagating (stem) cells (Goodell et al., 1996; Zhou et al., 2001; Chiba et al., 2006; Haraguchi et al., 2006; Hirschmann-Jax et al., 2004; Kondo et al., 2004). We previously reported that ABCG2 was highly expressed on the surface of cancer stem-like side population (SP) cells from various cancer cell lines. Treatment with MS209 (dofequidar), an inhibitor of both P-gp and ABCG2, effectively sensitize the cancer stem-like SP cells to chemotherapeutic agent such as mitoxantrone or CPT-11 (Katayama et al., 2009). In this study, we showed that P-gp overexpression plays an important role in acquired crizotinib and ceritinib resistance. In addition, our results imply that the existence of ABC transporters-overexpressing cells as a minor population in the TKI treatment-naïve cancer may be a seed of resistant tumor development, resulting in the prevention of complete cure of cancer by TKIs, such as ceritinib or crizotinib. Further studies are needed to clarify the importance of ABC transporters (P-gp) in acquired TKI resistance and innate TKI resistance.

At this moment, multiple ALK-TKIs are used in the clinic and others are under clinical evaluation. Each next-generation ALK-TKI has a different sensitivity to crizotinib-resistant mutations. From this study, we found that P-gp overexpression causes ceritinib and crizotinib resistance in patients; crizotinib and ceritinib, but not alectinib and PF-06463922, are substrates of P-gp. Thus, our results suggest that determination of P-gp expression by IHC in addition to testing the genetic alteration, and choosing ALK-TKIs based on the P-gp expression may be very helpful to maximize ALK-TKIs therapy.

Author Contributions

Experimental design: R.K. Cell line, in vitro, and in vivo studies: R.K., T.S., S.K., L.F. and S.S. Sequencing analysis of patient-derived samples: R.K. and S.K. Manuscript preparation: R.K., N.Y. and N.F. Identification and organization of patients and repeat biopsies: N.Y., A.H., S.S., Y.T., S.K., J.F.G., A.T.S., J.A.E., and M.N. Immunohistochemistry and pathology of all repeat biopsy samples: M.M.-K., A.J.I., M. N., H.N. Y.I. and K.T. Deep sequencing: A.D. and K.T. Overall experimental supervision: R.K., A.T.S., J.A.E., M.N., and N.F. Specimen collection and genetic/immunohistochemistry testing: A.J.I. and Y.I. Autopsy: N.Y., A.H., and H.N.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2015.12.009.

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