

Acquired Resistance to Alectinib in ALK-Rearranged Lung Cancer due to ABCC11/MRP8 Overexpression in a Clinically Paired Resistance Model



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ABSTRACT

Alectinib is used as a first-line treatment for anaplastic lymphoma kinase (ALK)-rearranged non-small cell lung cancer (NSCLC). Whereas other ALK inhibitors have been reported to be involved in resistance to ATP-binding cassette (ABC) transporters, no data are available regarding the association between resistance to alectinib and ABC-transporters. To investigate whether ABC-transporters contribute to alectinib resistance, ABC-transporter expression in alectinib-resistant cell lines derived from a patient with ALK-rearranged NSCLC and from H2228 lung cancer cells was evaluated and compared with that in each parent cell type. ATP-binding cassette subfamily C member 11 (ABCC11) expression was significantly increased in alectinib-resistant cell lines compared with that in alectinib-sensitive lines. ABCC11 inhibition increased sensitivity

to alectinib *in vitro*. ABCC11-overexpressing cells were established by transfection of an ABCC11 expression vector into H2228 cells, while control cells were established by transfecting H2228 cells with an empty vector. ABCC11-overexpressing cells exhibited decreased sensitivity to alectinib compared with that of control cells *in vitro*. Moreover, the tumor growth rate following alectinib treatment was higher in ABCC11-overexpressing cells than that in control cells *in vivo*. In addition, the intracellular alectinib concentration following exposure to 100 nmol/L alectinib was significantly lower in the ABCC11-overexpressing cell line compared with that in control cells. This is the first pre-clinical evidence showing that ABCC11 expression may be involved in acquired resistance to alectinib.

Introduction

In 2007, a novel driver oncogene, *EML4-ALK* fusion gene, in which the echinoderm microtubule-associated protein-like 4 (*EML4*) gene is fused to the anaplastic lymphoma kinase (*ALK*) gene, was identified in patients with non-small cell lung cancer (NSCLC; refs. 1, 2). Notably, the second-generation ALK inhibitor, alectinib, showed superior efficacy in primary treatment of ALK-positive NSCLC compared with that of crizotinib, a first-generation ALK inhibitor (3, 4). However, although alectinib is used as the first-line treatment for ALK-positive NSCLC, approximately 30% of patients treated with alectinib become refractory within a year of treatment owing to acquired resistance (4).

Several mechanisms of resistance to molecular-targeted therapeutic agents have been reported including: mutations of the drug target; activation of bypass signaling pathways; dysregulation of apoptosis; tumor microenvironment including the extracellular matrix, immune and inflammatory cells, and blood vessels; and increased protein

expression of drug efflux pumps such as ATP-binding cassette (ABC) transporters (5). Previous studies showed that the presence of secondary mutations such as I1171T/N/S, V1180L, L1196M, and G1202R in the kinase domain of ALK conferred resistance to alectinib (6, 7). Another major resistance mechanism involves bypass salvage signaling that maintains downstream signaling pathways to activate survival, antiapoptotic, and proliferation signals. In particular, activation of EGFR has reported to maintain survival signaling that induces alectinib resistance, and we have reported that coactivation of c-Src and MET functions as salvage signaling under alectinib exposure (8, 9).

ABC-transporters transport various molecules and are involved in multidrug resistance to anticancer agents. In particular, nine ABC-transporters, that is, multidrug resistance protein 1/ABCB1 (ABCB1), multidrug resistance-associated protein 1 (MRP1)/ABCC1 (ABCC1), MRP2/ABCC2 (ABCC2), MRP3/ABCC3 (ABCC3), MRP4/ABCC4 (ABCC4), MRP5/ABCC5 (ABCC5), MRP7/ABCC10 (ABCC10), MRP8/ABCC11 (ABCC11), and breast cancer resistance protein/ABCG2 (ABCG2) have been reported to be associated with anticancer drug resistance (10, 11). Moreover, several tyrosine kinase inhibitors (TKI; e.g., imatinib, nilotinib, dasatinib, ponatinib, gefitinib, erlotinib, lapatinib, vandetanib, sunitinib, and sorafenib) were recently reported as substrates for ABC-transporters including ABCB1, ABCC1, ABCG2, and ABCC10 (12). Notably, overexpression of ABCB1 was shown to be involved in the resistance to crizotinib and ceritinib, which constitute other ALK-TKIs, but not to alectinib (13). In addition, it was demonstrated that alectinib is not a substrate for ABCB1 and ABCG2 (14, 15). Thus, unlike other TKIs, the association between alectinib resistance and ABC-transporter expression is not fully understood. Therefore, the aim of this study was to determine the ABC-transporters that are associated with alectinib resistance in lung cancer.

We previously reported the establishment of a clinical paired resistant model (CPRM) comprised of patient-derived ALK-

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rearranged NSCLC cell lines from a treatment-naïve and subsequently, alectinib-refractory patient (9). In this study, we evaluated the expression of the nine chemoresistance-associated ABC-transporters in these cell lines, identifying that ABCC11 expression was significantly increased in alectinib-resistant cells compared with that in alectinib-sensitive cells. Therefore, in this study we investigated whether increased ABCC11 expression may constitute a mechanism underlying the acquired resistance to alectinib.

Materials and Methods

Clinical information and procedures for obtaining informed consent

The study protocol was prepared in accordance with the Declaration of Helsinki and approved by the Kyoto University Graduate School and Faculty of Medicine Ethics Committee (Kyoto, Japan; certification number: R0996). The patient provided written informed consent to participate in this study.

Establishment of patient-derived lung cancer cells

KTOR1 and KTOR1-RE (EML4-ALK variant 1 E13; A20) cells were established from a 29-year-old female patient with ALK-rearranged NSCLC, who regularly visited Kyoto University Hospital (Kyoto, Japan). The method used to establish the patient-derived cells was described previously (9). Briefly, 200 mL of pleural effusion was obtained from the patient with ALK-rearranged lung cancer. Tumor cells were separated by centrifugation at 800 rpm for 5 minutes, and then cultured and maintained in alectinib-free RPMI1640 Medium (Nacalai Tesque) supplemented with 8% heat-inactivated FBS (Sigma-Aldrich) and 1% penicillin/streptomycin (Gibco) at 37.0°C in 5% CO₂. Information about the patient was obtained from electrical medical records at Kyoto University Hospital (Kyoto, Japan).

Cells and reagents

NCI-H2228 (EML4-ALK variant 3a/b E6; A20) lung cancer cells were purchased in 2016 from ATCC. The alectinib-resistant H2228-AR1S cells were established by exposing NCI-H2228 cells to 300 nmol/L alectinib for 3 months *in vitro*. Similarly, KTOR1-AR was established from KTOR1 via exposure to 30 nmol/L alectinib for 6 months *in vitro*. All experiments were performed with cells that were within 20 passages. In 2019, all cells were confirmed to be negative for *Mycoplasma* using the MycoAlert Mycoplasma Detection Kit (Lonza). Alectinib was kindly provided by Chugai Pharmaceutical Co., Ltd. Crizotinib and ceritinib were purchased from LC Laboratories, and lorlatinib and brigatinib were purchased from ChemScene. Alectinib, crizotinib, ceritinib, and lorlatinib were dissolved in DMSO (Nacalai Tesque) at a concentration of 5 mmol/L and brigatinib was dissolved in DMSO at a concentration of 0.5 mmol/L. DMSO was also used as a vehicle control.

Cell viability and drug susceptibility assay

Cells were cultured overnight in 96-well plates at a density of 5,000 cells/well and treated with increasing concentrations of the reagents or vehicle control medium for 72–168 hours. Subsequently, cells were incubated with CellTiter-Glo 2.0 Luminescent Cell Viability Assay (Promega) for 20 minutes and luminescence was measured using an ARVO X3 Plate Reader (PerkinElmer). The IC₅₀ value was calculated using a nonlinear regression model with a sigmoidal dose response using GraphPad Prism 8.0 (GraphPad software). Three independent experiments were conducted.

Immunoblotting

SDS-PAGE and immunoblotting were performed as described previously (16). Briefly, antibodies against ABCC11 and GAPDH were purchased from Invitrogen. pALK (pY 1604) and secondary antibodies were purchased from Cell Signaling Technology. β-actin was purchased from Sigma-Aldrich (details are provided in Supplementary Table S1). Antibodies against ABCC11 (1:2,000), GAPDH (1:10,000), pALK (1:1,000), and β-actin (1:10,000), and secondary (1:2,000) antibodies were dissolved in 2.5% BSA/TBS with Tween 20. BSA was purchased from Nacalai Tesque. Quantification of protein bands was performed using ImageJ software (<https://imagej.nih.gov/ij/>).

Quantitative reverse transcription-PCR

Total RNA was extracted from cultured cells using the PureLink RNA Mini Kit (Thermo Fisher Scientific). Gene expression was evaluated by quantitative reverse transcription-PCR (qRT-PCR) using 100 ng of total RNA, the TaqMan RNA-to-Ct 1-Step Kit (Applied Biosystems), and a primer pair. A list of the primers purchased from Thermo Fisher Scientific is shown in Supplementary Table S2. Reactions were quantified using the 7300 Real-Time PCR System (Applied Biosystems).

Transfection with siRNA

ABCC11 siRNA oligonucleotides were purchased from Thermo Fisher Scientific (Stealth RNAi siRNA; Supplementary Table S3). A total of 3.0×10^5 cells were transfected with siRNA oligonucleotides at a final RNA concentration of 21 nmol/L using Lipofectamine RNAi-MAX Transfection Reagent (Invitrogen). The reverse transfection method was performed according to the manufacturer's protocol. RNA and total protein were extracted at 30 and 54 hours, respectively, following transfection. In the cell viability assay, transfected cells (5,000 cells/well) were cultured in a 96-well plate for 24 hours and treated with increasing concentrations of alectinib for 168 hours. Cell viability was assessed as described above.

Establishment of ABCC11-overexpressing H2228 cells

The ABCC11 expression vector (ABCC11/pcDNA3.1/V5-His) and empty vector (pcDNA3.1/V5-His C) were purchased from Invitrogen. The vector map of ABCC11/pcDNA3.1/V5-His is shown in Supplementary Fig. S1; the ABCC11 sequence inserted into the vector is shown in Supplementary Data S2. H2228 cells were transfected with the ABCC11 expression vector or empty vector using NEPA21 electroporator (Nepa Gene) in serum-free Opti-MEM (Thermo Fisher Scientific) to generate stably expressing cells. At 24 hours after transfection, cells were selected and cloned by limiting dilution in a medium containing geneticin selective antibiotic (Wako) for 2–3 weeks. Proteins were extracted from each clone and ABCC11 expression was analyzed by immunoblotting. Once increased expression of ABCC11 was confirmed, two cell lines derived from the transfected cells were selected as ABCC11-overexpressing cells (ABCC11a and ABCC11b). In the same manner, cells transfected with the empty vector were cloned and one cell line that exhibited similar ABCC11 expression as the parent cells was selected as the control cell line (mock).

Intracellular concentration measurement

Cells were cultured overnight in a low attachment 24-well plate (Greiner Bio-One) at a density of 2.5×10^5 cells/well and treated with 100 nmol/L alectinib for 2 and 4 hours. After washing the cells twice with cold PBS, cells were lysed with 50 μL of 0.1 mol/L sodium hydroxide solution (Nacalai Tesque) and homogenized. After

incubating the cells at 37°C for 1 hour, cells were neutralized with 50 μ L of 0.1 mol/L hydrochloric acid solution (Nacalai Tesque). Alectinib concentration was measured using a Shimadzu Prominence HPLC System (MD) and Qtrap 5500 Quadrupole Mass Spectrometer (AB Sciex). Mass spectrometry analysis was performed using electrospray ionization in positive ion mode. The multiple reaction monitoring mode m/z transition values were set at 483.22–396.10 for alectinib and 491.30–396.10 for d-alectinib as the internal standard.

Xenograft models

Six-week-old female BALB/c-nu mice (CAnN.Cg-Foxn1nu/CrlCrJ) were purchased from Charles River Laboratories. Xenograft models were generated by suspending $1-3 \times 10^6$ cells in Matrigel (Corning), which was injected subcutaneously into the backs of the mice. Mice with a tumor volume of 80–240 mm³ were randomly assigned to either alectinib or vehicle groups (day 0). Mice were treated with alectinib (8 mg/kg/day) or vehicle via oral gavage. Tumor volumes were evaluated using digital caliper measurements and calculated using the formula (length $\times$ width $\times$ width) $\times$ 0.52, where length and width represented the larger and smaller diameters, respectively. The mice were euthanized on day 11. For immunoblotting, mice with a tumor volume of 500–1,500 mm³ were treated with alectinib (8 mg/kg/day) and were humanely sacrificed 24 hours after alectinib treatment. Tumor tissues were collected, and immunoblotting was performed as

mentioned above. All animal experiments and protocols were approved by the Animal Research Committee of Kyoto University (Kyoto, Japan; ID: MedKyo 18298) and implemented according to the ARRIVE guidelines.

Statistical analysis

Continuous variable data are expressed as the means $\pm$ SEM. The significance of differences was assessed using the Student *t* test. Sidak multiple comparison test and Holm–Sidak multiple comparison test were used to compare the mean of more than three groups. $P < 0.05$ was defined as significant. All statistical analysis was performed using JMP Pro version 12.0 (SAS Institute) and visualized by GraphPad Prism 8.

Results

Establishment of alectinib-resistant cells

We established KTOR1 cells using cells derived from an alectinib-naïve patient with ALK-rearranged NSCLC, and KTOR1-RE cells were established from the same subsequently alectinib-refractory patient (ref. 9; Fig. 1A). The H2228-AR1S and KTOR1-AR cells were established from the H2228 and KTOR1 cells, respectively, through exposure to alectinib to investigate the determinants of acquired resistance to alectinib in lung cancer. KTOR1-RE, KTOR1-AR, and H2228-AR1S cells were more resistant to alectinib compared with the parental cells

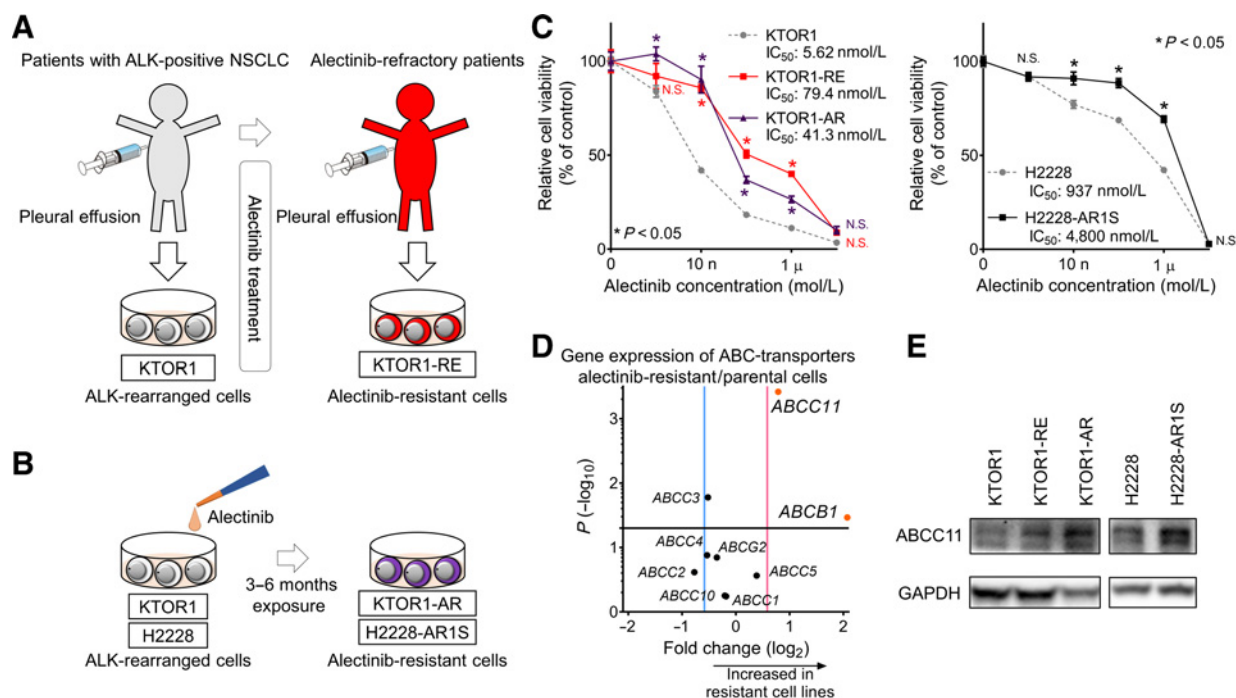


Figure 1.

ABCC11 as a potential candidate transporter associated with alectinib resistance. **A**, Schematic representation of establishment of patient-derived alectinib-resistant tumor cells (KTOR1-RE). Pleural effusion from a patient with ALK-rearranged NSCLC was obtained at disease progression and collected cancer cells were cultured *in vitro*. **B**, Schematic representation of conventional alectinib-resistant cells (H2228-AR1S and KTOR1-AR). H2228-AR1S cells were established by exposing parental H2228 cells to 300 nmol/L alectinib for 3 months. The KTOR1-AR cells were established by exposure of KTOR1 cells to 30 nmol/L alectinib for 6 months. **C**, Cell viability assay of KTOR1, KTOR1-RE, and KTOR1-AR (left) or H2228 and H2228-AR1S (right) cells treated with alectinib for 72 hours. P values were calculated using two-way ANOVA, Sidak multiple comparisons test ($n = 5$). **D**, Integrated results of the qRT-PCR analysis in the five cell types. Volcano plot representing gene expression of the ABC-transporters. For each gene, the fold change of alectinib-resistant cells (KTOR1-RE, KTOR1-AR, and H2228-AR1S) compared with that of parental cells (KTOR1 and H2228; horizontal line) and significance between alectinib-resistant ($n = 3$) and parental cells ($n = 2$) calculated using the *t* test [$-\log(P$ value); vertical line] was plotted. Expression of *ABCB1* and *ABCC11* was significantly increased in resistant cells (plotted in red). **E**, *ABCC11* protein expression of the five cell types was confirmed by immunoblotting. N.S., not significant.

Table 1. IC₅₀ values for alectinib treatment of H2228, H2228-AR1S, KTOR1, KTOR1-RE, and KTOR1-AR cells.

Cells	H2228		H2228-AR1S		KTOR1		KTOR1-RE			KTOR1-AR		
	Alectinib	IC ₅₀ (95% CI)	IC ₅₀	RR (95% CI)	IC ₅₀	(95% CI)	IC ₅₀	(95% CI)	RR	IC ₅₀	(95% CI)	RR
(nmol/L)	937	(577–1,450)	4,800	5.12 (3,340–7,900)	5.62	(4.27–7.36)	79.4	(35.0–185)	14.1	41.3	(30.1–56.3)	7.35

Abbreviations: 95% CI, 95% confidence interval; RR, relative resistance [(IC₅₀ in resistant subline)/(IC₅₀ in parental cells)].

(Fig. 1B). No secondary mutations (e.g., I1171N, V1180L, L1196M, or G1202R) were detected in the ALK tyrosine kinase–coding regions of KTOR1-RE, KTOR1-AR, or H2228-AR1S cells (ref. 9; Supplementary Fig. S2) as determined by ALK exon sequencing using ALK-sequencing primers (Supplementary Table S4). The IC₅₀ values for alectinib treatment of KTOR1-RE, KTOR1-AR, and H2228-AR1S revealed that the cells were approximately 14.1-, 7.35-, and 5.12-fold more resistant relative to the parental cells, respectively (Fig. 1C; Table 1). We have previously reported that the IC₅₀ of H2228 is approximately 100 nmol/L (9); notably, this was determined under low attachment conditions, under which H2228 cells tend to exhibit greater sensitivity to alectinib (Supplementary Fig. S3). In this study, we continued the experiments under attachment conditions to facilitate siRNA-knockdown experiments.

Expression levels of ABC-transporters in alectinib-resistant cells

To identify transporters that conferred resistance to alectinib, ABC-transporters exhibiting increased expression in alectinib-resistant cells were explored. In particular, gene expression of the nine ABC-transporters previously reported to be related to anticancer drug resistance was screened in the five cell types, and the expression ratio (resistant cells/parental cells) of the transporters was calculated (10–12). For each ABC-transporter, the fold change in gene expression (horizontal line) and significance between parental and alectinib-resistant cells calculated using the *t* test [–log(*P* value); vertical line] was plotted (Fig. 1D; Supplementary Fig. S4). Among the transporters, the expression of *ABCB1* and *ABCC11* genes was significantly increased in the resistant cells (fold change > 1.5; *P* < 0.05). We focused on *ABCC11* as a potential transporter candidate for alectinib resistance, as previous studies suggested that *ABCB1* was not involved in the extracellular efflux of alectinib (14, 15).

ABCC11 inhibition sensitizes H2228-AR1S and KTOR1-RE cells to alectinib

Cell viability in the presence of alectinib during siRNA inhibition of the *ABCC11* gene was measured to evaluate the functional role of *ABCC11* overexpression in alectinib resistance. H2228-AR1S cells transfected with siRNA targeting the *ABCC11* gene exhibited reduced cell viability of alectinib compared with that of cells transfected with negative control siRNA (Fig. 2C; Supplementary Table S5). KTOR1-RE cells transfected with siRNA targeting the *ABCC11* gene also demonstrated reduced cell viability following alectinib exposure (Fig. 2F; Supplementary Table S6).

ABCC11 overexpression confers alectinib resistance

To investigate whether *ABCC11* overexpression induced alectinib resistance, the *ABCC11* expression vector (*ABCC11*/pcDNA3.1/V5-His) was transfected into H2228 cells to establish two *ABCC11*-overexpressing cell lines, *ABCC11a* and *ABCC11b*. H2228 cells were also transfected with an empty vector (mock). Expression levels of the

ABCC11 gene and protein were increased in both *ABCC11a* and *ABCC11b* cells compared with those in mock cells (Fig. 3A and B). The negative impact of alectinib on cell viability was decreased in *ABCC11a* and *ABCC11b* cells compared with that on mock cells (Fig. 3C; Table 2). The loss of cell viability from crizotinib, ceritinib, lorlatinib, and brigatinib exposure was also significantly lower in *ABCC11a* and *ABCC11b* cells compared with that in mock cells (Fig. 3D).

ABCC11 overexpression decreases the intracellular alectinib concentration

To obtain evidence supporting that *ABCC11* effluxes alectinib, we measured the intracellular accumulation of alectinib in *ABCC11a* or mock cells using LC/MS. Following treatment with 100 nmol/L alectinib for 2 and 4 hours, the intracellular concentration of alectinib was significantly lower in *ABCC11a* cells compared with that in mock cells (Fig. 3E; Supplementary Fig. S5). To evaluate the effect of alectinib treatment on *ABCC11*-overexpressing cells, ALK phosphorylation was evaluated by immunoblotting in cells treated with increasing concentrations of alectinib. Treatment of cells with 10 and 30 nmol/L alectinib suppressed ALK phosphorylation in mock but not *ABCC11a* cells (Fig. 3F).

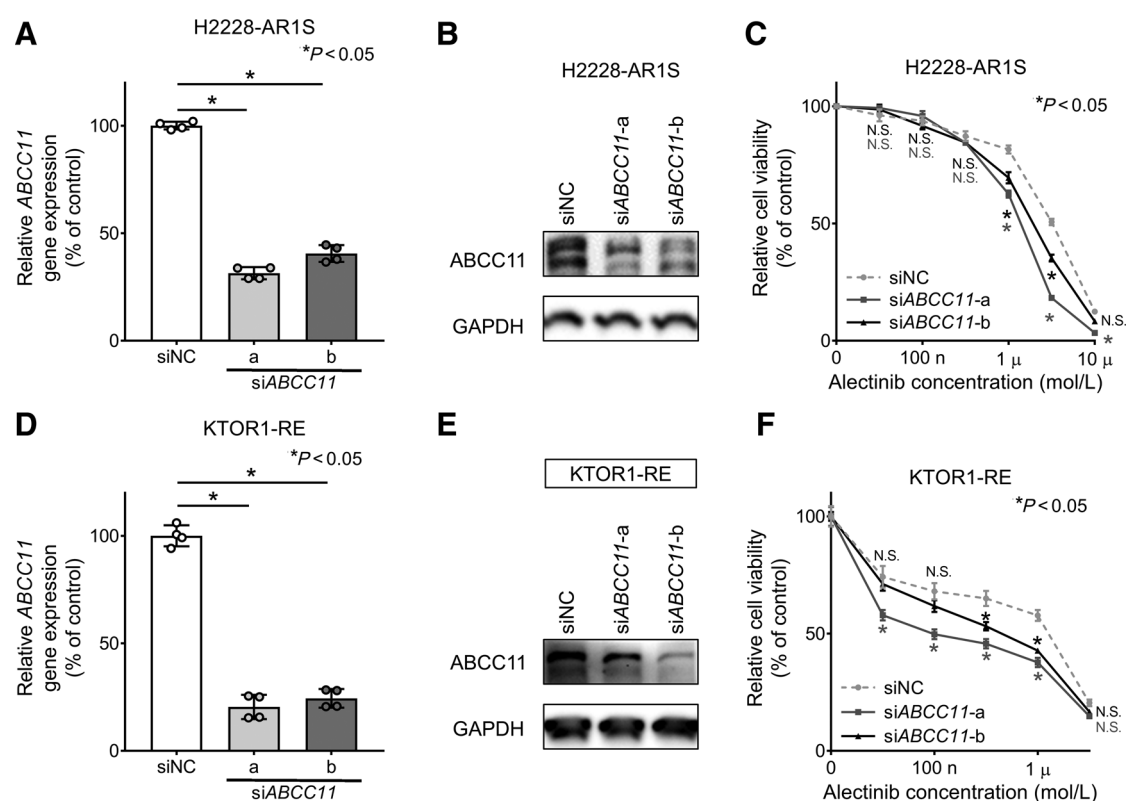
In vivo effect of ABCC11 overexpression on alectinib response

To determine whether *ABCC11* overexpression might alter sensitivity to alectinib *in vivo*, xenograft models using H2228 cells transfected with the *ABCC11* expression vector and cells transfected with empty vector were established and randomized into two groups: alectinib treatment and vehicle. The dosage of alectinib was selected as 8 mg/kg (10 days) in accordance with the human dose (600 mg/day/body; ref. 17). The tumor growth rate following alectinib treatment was higher with H2228 cells transfected with *ABCC11* expression vector compared with that from cells containing empty vector (–34% for *ABCC11a*, –24% for *ABCC11b*, and –77% for mock; Fig. 4A; Supplementary Fig. S6) and did not affect body weight (Fig. 4B). ALK phosphorylation after alectinib treatment in tumors established from *ABCC11a* and *ABCC11b* cells was not suppressed, but those in mock cells was suppressed (Fig. 4C). These results suggested that *ABCC11* overexpression could reduce the tumor response to alectinib treatment *in vivo*.

Discussion

The results of this study showed that *ABCC11* expression was increased in alectinib-resistant cells compared with that in the parental cells. We also revealed that altered *ABCC11* expression affected the cell viability following alectinib exposure. This is the first report to demonstrate that *ABCC11* overexpression may represent a mechanism involved in acquired resistance to alectinib.

ABC-transporters transport various molecules from the interior to the exterior of the cell. *ABCC11* was identified as an ABC-transporter in 2001 (18–20) and is expressed in various tissues

**Figure 2.**

Improved alectinib sensitivity in H2228-ARIS cells transfected with *ABCC11* siRNA. Gene expression of *ABCC11* following siRNA knockdown of *ABCC11* using two different *ABCC11*-directed RNAi sequences (*ABCC11-a* and *ABCC11-b*) in H2228-ARIS (A) and KTOR1-RE cells (D). *P* values were calculated using one-way ANOVA and the Dunnett test ($n = 4$). *ABCC11* protein expression detected using immunoblotting in H2228-ARIS (B) and KTOR1-RE cells (E) transfected with *ABCC11* siRNA was decreased compared with that in cells transfected with negative control siRNA. Cell viability assay of H2228-ARIS (C) and KTOR1-RE cells (F) transfected with *ABCC11* siRNA and negative control siRNA then treated with alectinib for 168 hours following 24-hour transfection. Statistical significance was calculated using two-way ANOVA followed by Sidak multiple comparisons test ($n = 5$). N.S., not significant.

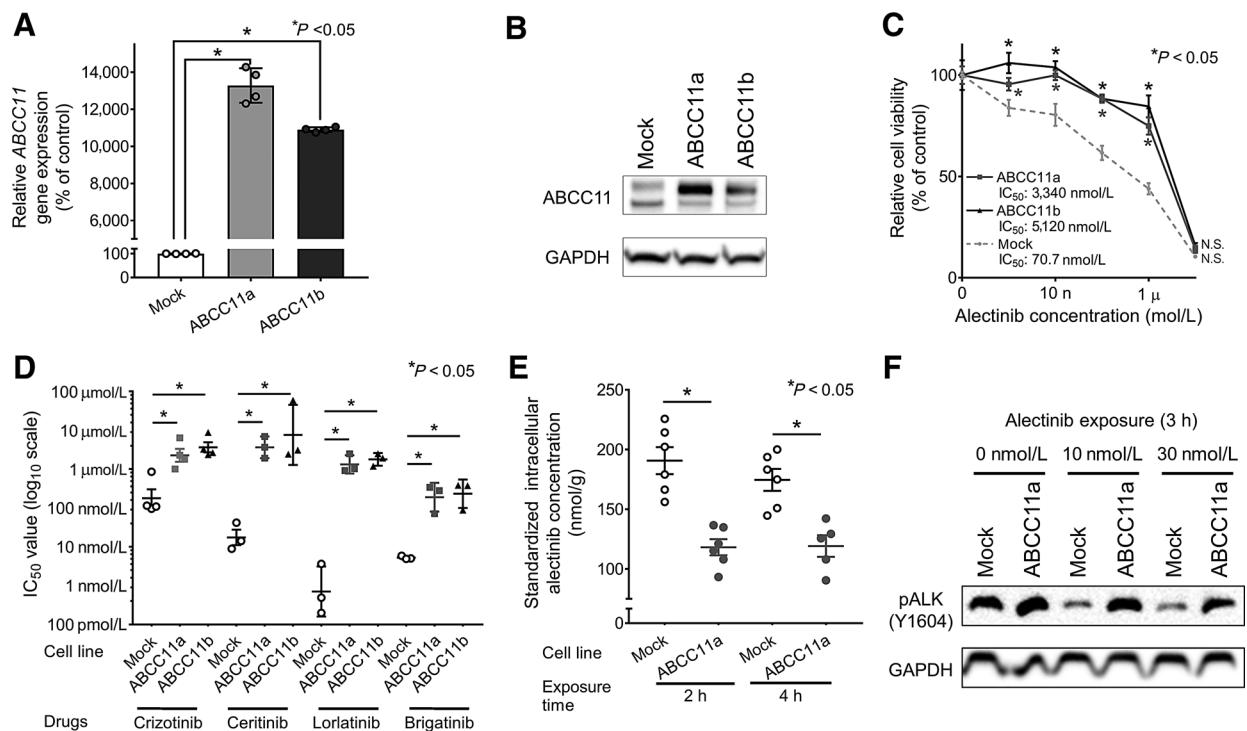
including the brain, lung, liver, kidney, and apocrine glands (21–24). *ABCC11* is also involved in the intracellular to extracellular efflux of dehydroepiandrosterone 3-sulfate, methotrexate, and tenofovir (25, 26), and is a substrate for cytotoxic anticancer agents including 5-fluorouracil and pemetrexed (19, 20, 27). However, to the best of our knowledge, molecular-targeted therapeutics have not previously been described as potential substrates for *ABCC11*. In this study, we showed that H2228 cells transfected with an *ABCC11* expression vector exhibited a lower intracellular alectinib concentration compared with that of cells transfected with empty vector, and exposure to alectinib suppressed ALK phosphorylation in cells transfected with empty vector but not in cells transfected with the *ABCC11* expression vector. These data suggested that *ABCC11* may be involved in intracellular to extracellular alectinib efflux (Fig. 4D).

In this study, *ABCC11* expression was increased in alectinib-resistant cells relative to the level in their parental cells. The cell viability of H2228-ARIS cells transfected with siRNA targeting *ABCC11* treated with alectinib was significantly decreased compared with that of cells transfected with negative control siRNA, whereas sensitivity to alectinib in H2228 cells transfected with the *ABCC11* expression vector was lower compared with that of cells transfected with empty vector. In addition, *ABCC11* expression was significantly lower in cells derived from the alectinib-naïve patient than in those

from the same alectinib-refractory patient. These results suggested that increased expression of *ABCC11* may be involved in acquired resistance to alectinib.

However, the clinical relevance of *ABCC11* in alectinib resistance remains unclear. *ABCC11* expression in other patients treated with alectinib could not be evaluated. Although several antibodies were evaluated to assess *ABCC11* expression in tumors using immunostaining, no useful antibodies could be obtained. We evaluated *ABCC11* expression using a CPRM that compares patient-derived resistant cells with patient-derived treatment-sensitive cells to provide information regarding acquired resistance. This study revealed that *ABCC11* expression was increased in KTOR1-RE compared with KTOR1 cells. Although the relative intracellular alectinib concentration in these cells was not evaluated, ALK phosphorylation was upregulated in KTOR1-RE cells compared with that in KTOR1 cells following treatment with 20 nmol/L alectinib (Supplementary Fig. S7). This result suggested that the intracellular alectinib concentration of KTOR1-RE cells might be lower than that of KTOR1 cells following alectinib exposure. Moreover, inhibition of *ABCC11* in KTOR1-RE cells increased sensitivity to alectinib (Fig. 2F). These results indicated that increased *ABCC11* expression may contribute to efflux the alectinib from the interior to the exterior of the cells in the evaluated patient. Further studies are required to evaluate whether high expression of *ABCC11* may be associated with acquired resistance to alectinib

ABCC11/MRP8 may Confer Alectinib Resistance

**Figure 3.**

ABCC11 overexpression leads to decreased alectinib intracellular concentration and low sensitivity to alectinib. *ABCC11* expression vectors were introduced into H2228 cells to establish *ABCC11*-overexpressing cell lines (*ABCC11a* and *ABCC11b*). Empty vector was transfected into H2228 cells to establish control cells (mock). **A**, Gene expression levels of *ABCC11* in *ABCC11a*, *ABCC11b*, and mock cell lines. *P* values were calculated using one-way ANOVA followed by Dunnett multiple comparison test ($n = 4$). **B**, Protein expression of *ABCC11* and GAPDH in mock, *ABCC11a*, and *ABCC11b* cell lines determined by immunoblotting. **C**, Cell viability assay of *ABCC11a*, *ABCC11b*, and mock cell lines treated with alectinib for 72 hours. *P* values were calculated using two-way ANOVA and *post hoc* Sidak multiple comparisons test ($n = 5$). **D**, IC_{50} value of mock, *ABCC11a*, and *ABCC11b* cell lines in the presence of crizotinib, ceritinib, lorlatinib, and brigatinib. *P* values were calculated using two-way ANOVA and *post hoc* Sidak multiple comparisons test. **E**, Intracellular alectinib concentrations of mock and *ABCC11a* cells exposed to 100 nmol/L alectinib. Raw data for intracellular concentrations prior to standardization using intracellular protein are shown in Supplementary Fig. S4. *P* values were calculated using two-way ANOVA and *post hoc* Sidak multiple comparisons test. **F**, ALK phosphorylation in mock and *ABCC11a* cell lines exposed to alectinib for 3 hours as determined using immunoblotting. N.S., not significant.

in other patients. We aim to continue to accumulate evidence to support our model using CPRM.

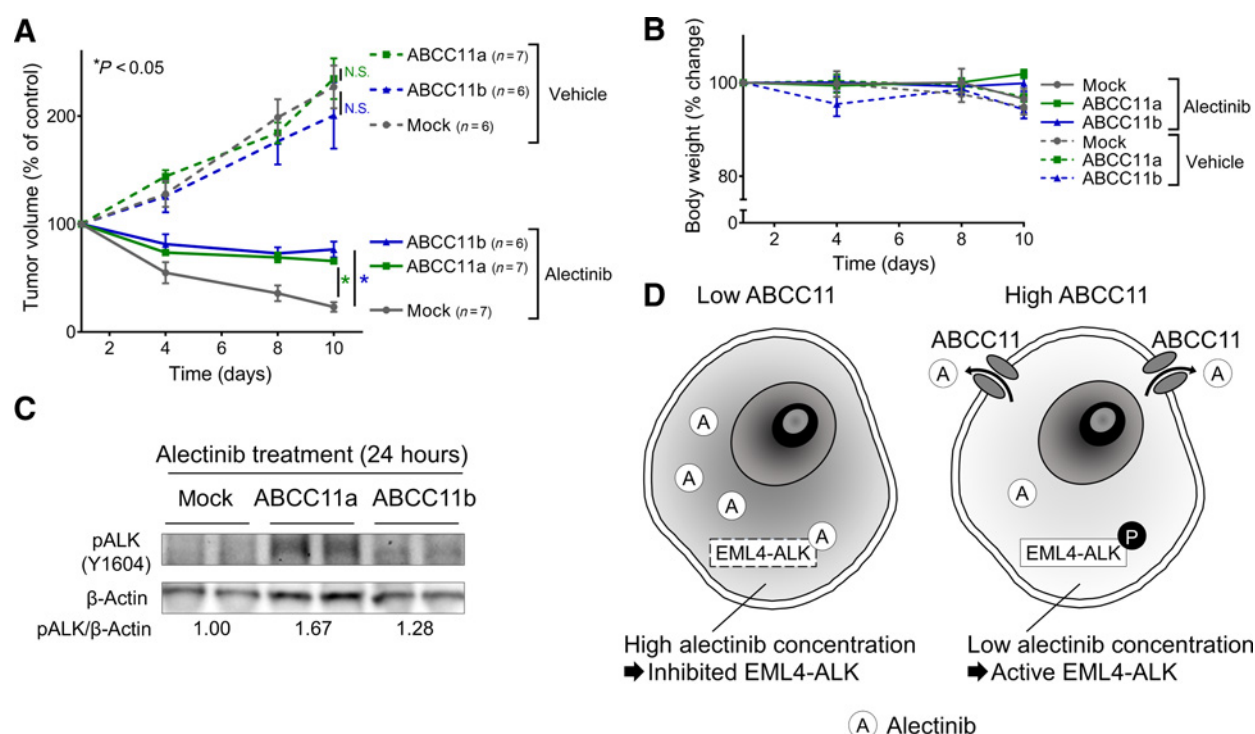
ABC-transporters are known to cause multidrug resistance (12, 19, 20). In this study, the sensitivity of crizotinib, ceritinib, lorlatinib, and brigatinib was lower in H2228-AR1S cells compared with that in H2228 cells (Supplementary Fig. S8). These results revealed that H2228-AR1S cells exhibit cross-resistance to other ALK inhibitors. In addition, as with alectinib, the cell viability impairment upon exposure to ALK inhibitors was decreased in H2228 cells transfected with the *ABCC11* expression vector compared with that of cells transfected with empty vector (Fig. 3D). Although we were unable to measure the intracellular concentration of ALK inhibitors other than alectinib in cells transfected with the *ABCC11* expression vector, these findings suggest that *ABCC11* expression may be involved in the acquired resistance to multiple ALK inhibitors.

As we were unable to clarify the mechanism underlying *ABCC11* overexpression in resistant cells, it is unclear whether exposure to alectinib selects cells with high expression of *ABCC11* prior to treatment or induces increased expression of *ABCC11* in these cells. Previous studies showed that cancer stem cells (CSC), which are innately resistant to many standard therapies, are associated with overexpression of *ABCB1* and *ABCG2* (5, 10, 28, 29). In particular, CD44 constitutes a CSC marker that exhibits strong negative correlations with patient survival and has been associated with the expression of ABC-transporters, most notably *ABCG2* (5, 28). *ABCB1* overexpression was also reported to be associated with short survival in stage 1 lung adenocarcinoma and rendered CSC-like properties (30, 31). These reports indicated that cancer cells may highly express ABC-transporters prior to treatment and that clonal selection of cells with high expression of ABC-transporters

Table 2. IC_{50} values for alectinib treatment of mock, *ABCC11a*, and *ABCC11b* cells.

Cells	Mock		ABCC11a		ABCC11b	
	IC_{50}	(95% CI)	IC_{50}	RR (95% CI)	IC_{50}	RR
Alectinib						
(nmol/L)	70.7	(25.9–172)	3,340	47.2 (2,260–5,430)	5,120	72.4
					(3,200–14,400)	

Abbreviations: 95% CI, 95% confidence interval; RR, relative resistance [$(IC_{50}$ in resistant subline)/(IC_{50} in parental cells)].

**Figure 4.**

ABCC11 overexpression limits the effect of alectinib. Xenograft models of mock, ABCC11a, and ABCC11b cell lines were established and randomized into two groups: alectinib treatment and vehicle. The alectinib dose was 8 mg/kg/day. **A**, Tumor volume following alectinib or vehicle treatment. In the alectinib group, tumor growth rate was significantly higher in the ABCC11a and ABCC11b groups compared with that in the mock group. P values were calculated using two-way ANOVA and Holm-Sidak multiple comparisons test. **B**, Body weights of the mice. **C**, ALK phosphorylation in tumors established from ABCC11a, ABCC11b, and mock cell lines treated with alectinib for 24 hours as determined using immunoblotting. Quantification of protein bands was performed using ImageJ software. Quantification values for each band were first normalized to corresponding values of β -actin. Then, the ratio between normalized values for ABCC11-overexpressing cells and mock cells was calculated. **D**, Schematic of ABCC11 role in alectinib resistance. *ABCC11* overexpression reduces alectinib intracellular concentrations and attenuates the effects of alectinib. N.S., not significant.

might be associated with acquired resistance to anticancer agents. Although an association between ABCC11 and CSC-like properties has not been reported, clonal selection of ALK fusion gene-positive lung cancer cells with ABCC11 overexpression may represent a mechanism of acquired resistance to alectinib.

Overall, our findings provide new insight into the mechanisms underlying alectinib resistance in ALK-rearranged NSCLC. We found that ABCC11 expression was significantly increased in cells derived from an alectinib-refractory patient using CPRM. We propose that alectinib may constitute a substrate for ABCC11 and that overexpression of ABCC11 may represent an important determinant of acquired resistance to alectinib, although other resistance mechanisms, including activation of salvage signaling pathways, may also be involved. Further studies are required to elucidate the mechanisms of alectinib resistance using CPRM to overcome the problem of acquired resistance in lung cancer.

Disclosure of Potential Conflicts of Interest

T.Y. Funazo reports receiving a commercial research grant from Japan Society for the Promotion of Science. T. Oguri has received speakers bureau honoraria from Chugai Pharma. Y. Yasuda has provided expert testimony for Japan Society for the Promotion of Science. Y.H. Kim has received speakers bureau honoraria from Chugai, Pfizer, and Novartis. T. Hirai reports receiving a commercial research grant from Chugai Pharmaceutical Co., Ltd. No potential conflicts of interest were disclosed by the other authors.

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Development of methodology: T. Tsuji, H. Ozasa

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