Proteasome inhibition overcomes ALK-TKI resistance in ALK-rearranged/TP53 mutant NSCLC via Noxa expression

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Abstract

Purpose:
In ALK-rearranged non-small cell lung cancer (NSCLC), impacts of concomitant genetic alterations on targeted therapies with ALK-tyrosine kinase inhibitors (ALK-TKIs) have not yet well understood. Here, we investigated genetic alterations related to ALK-TKI resistance using clinico-genomic data and explored effective therapies to overcome the resistance in preclinical models through the identification of underlying molecular mechanisms.

Experimental Design:
We used integrated clinical and next-generation sequencing data generated in a nationwide lung cancer genome screening project (LC-SCRUM-Japan). ALK-rearranged NSCLC cell lines expressing wild-type or mutant TP53 were used to evaluate cellular apoptosis induced by ALK-TKIs.

Results:
In 90 ALK-rearranged NSCLC patients who were treated with a selective ALK-TKI, alectinib, TP53 co-mutated patients showed significantly worse progression-free survival (PFS) than TP53 wild-type patients [median PFS, 11.7 months (95% CI, 6.3-not reached [NR]) vs. NR (23.6-NR); p=0.0008; hazard ratio, 0.33 (95% CI, 0.17-0.65)]. ALK-rearranged NSCLC cell lines which lost p53 function were resistant to alectinib-induced apoptosis, but a proteasome inhibitor, ixazomib markedly induced apoptosis in the alectinib-treated cells by increasing the expression of a pro-apoptotic protein, Noxa which bound to an anti-apoptotic protein, Mcl-1. In subcutaneous tumor models, combination of ixazomib and alectinib prominently induced tumor regression and apoptosis even though the tumors were generated from ALK-rearranged NSCLC cells with non-functional p53.

Conclusions:
These clinical and preclinical results indicate concomitant TP53 mutations reduce the efficacy of alectinib for ALK-rearranged NSCLC and the combined use of a proteasome inhibitor with alectinib is a promising therapy for ALK-rearranged/TP53-mutated NSCLC.
Translational Relevance

There is uncertainty as to whether concomitant TP53 mutations predict poor outcomes of alectinib, established here as the preferred ALK tyrosine kinase inhibitor (ALK-TKI), in TKI-naïve ALK-rearranged NSCLC patients. Additionally, little attention has been given to the rationale that p53 function has an influence on the effect of ALK-TKIs. We demonstrated that PFS was much shorter in the TP53 mutant group than those in the wild-type group and an enhancement of Noxa, a pro-apoptotic protein, by proteasome inhibition, overcame the tolerance to alectinib, which the lack of p53 function conferred. Moreover, enhanced Noxa bound to Mcl-1, one of the anti-apoptotic Bcl-2 family members, which was an important target for inducing apoptosis. The present study proposes a promising strategy targeting Mcl-1 through the use of proteasome inhibitors in ALK-rearranged NSCLC patients with p53 dysfunction.
Introduction

Anaplastic lymphoma kinase (ALK) gene rearrangement is detected in 3–5% of patients with non-small cell lung cancer (NSCLC) (1). ALK tyrosine kinase inhibitors (TKI) bring a significant clinical benefit to patients diagnosed with ALK-rearranged NSCLC. Alectinib, a second-generation selective ALK-TKI, significantly prolonged progression-free survival (PFS) and caused moderate adverse events compared with crizotinib, a first-generation ALK-TKI. Thus, alectinib is established as a standard first-line therapy in ALK-rearranged NSCLC patients (2). However, ALK secondary mutations (e.g. G1202R, I1171T/N/S), which decrease the TKI-binding affinity of ALK, were associated with >50% of mechanisms of acquired resistance to second-generation ALK inhibitors including alectinib (3).

There is a lack of research concerning the tolerance factor, unlike an acquired resistance to ALK-TKIs. Recently, however, some studies have claimed that TP53 mutations, whose frequency is about 25%, are relevant to poor prognosis in patients treated with ALK-TKIs, the majority of which were crizotinib (4-6). TP53 is a tumor suppressor gene and functional p53 proteins induce cell cycle arrest, DNA repair, senescence or apoptosis in response to cellular stress including chemotherapy (7).

However, there have been few reports about a correlation between treatment effect of alectinib and TP53 mutations in TKI-naïve patients in spite of a high incidence of TKI-induced secondary ALK mutations, which confer resistance to ALK-TKIs. Albeit TP53 mutations before treatment may be associated with tolerance to ALK-TKIs, whether this phenomenon can be reproduced by means of experimental validation has been little investigated.

Drug development for the targeting of tumor expressing mutant TP53 is still challenging, nonetheless, a few therapeutic strategies are being explored. Small molecules such PRIMA-1 analogs have been shown to bind to both mutant p53 and unfolded wild type p53 which behaved like mutant p53 (8). Promoting the proper conformation of the mutant and wild type proteins resulted in restoring the wild type p53 activity. Moreover, HDAC6 inhibition dissociated the mutant p53 from the Hsp70 and Hsp90 chaperone complex, which facilitated mutant p53 degradation by ubiquitin ligase, MDM2 and CHIP (9). Apart from these approaches of directly targeting either wild type or mutant TP53, an activation of the p53-independent apoptotic pathway was found to induce apoptosis in TP53 mutant tumors (10).

In the present study, we determined the impact of TP53 mutations on resistance to alectinib in patients with TKI-naïve ALK-rearranged NSCLC. Furthermore, we investigated the possibility of overcoming the resistance by upregulating p53-independent apoptotic protein in ALK-rearranged NSCLC cells lacking functional p53 in vitro and in vivo.
Materials and Methods

Nationwide genome screening project (LC-SCRUM-Japan)

In 2013, a nationwide lung cancer genome project (LC-SCRUM-Japan) were established to identify lung cancer patients with targetable gene alterations through large-scale molecular screening; a total of 263 institutions across Japan participated and more than 10,000 patients is currently enrolled in this project. Eligible tumor samples for molecular analyses were a pair of fresh/frozen tumor samples and 5 sections of a formalin-fixed/paraffin-embedded (FFPE) tumor specimen obtained from lung cancer patients. Malignant pleural effusion was also eligible. DNA and RNA were extracted from the samples and molecular analysis was performed in a Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory, SRL Inc. (Tokyo, Japan). Between February 2013 and March 2015, ALK, ROS1 and RET fusions were analyzed by RT-PCR and positivity was confirmed by fluorescence in-situ hybridization (FISH). Since April 2015, a targeted next-generation sequencing (NGS) system (Oncomine Comprehensive Assay ver. 1 or 3) was also used. Clinical data of enrolled patients were collected using an electronic data capture (EDC) system of LC-SCRUM-Japan. Baseline clinical characteristics were collected at the time of patient enrollment and subsequent clinical data from follow-ups including initiation dates for anti-cancer therapy, therapeutic regimens, tumor responses, and disease progression and prognosis dates were also collected and updated every year.

The protocol was approved by the institutional review board at each institution. Written informed consent was obtained from all patients. This study has been registered with the University Medical Hospital Information Network (UMIN) Clinical Trial Registry, number UMIN000010234.

Cell lines and reagents

EML4-ALK fusion-positive NSCLC cell lines, CCL-185IG and H2228 were purchased from the ATCC (Manassas, VA), and A925L was established from a surgical specimen of a Japanese male patient (11) kindly provided by Fumihiro Tanaka and Hidetaka Uramoto of the Second Department of Surgery, University of Occupational and Environmental Health, Japan. All three cell lines were subcultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and antibiotics, and passaged within 3 months of thawing the frozen stock. Mycoplasma infection in the cells was regularly checked using a MycoAlert Mycoplasma Detection Kit (Lonza, Rockland, ME). Alectinib was kindly provided by Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan) (12). Crizotinib, ixazomib, bortezomib, and S63845 were obtained from Selleck Chemicals (Houston, TX). All drugs were dissolved in DMSO and preserved at -30°C.

Next-generation sequencing

Tumor DNA and RNA obtained from CCL-185IG, H2228 and A925L cells were analyzed with Oncomine Comprehensive assay v3 (OCAv3) for detection of mutations, copy number variants and...
gene fusions in 161 genes (Thermo Fisher Scientific). To prepare DNA libraries, each tumor DNA (20 ng) was subjected to multiplex polymerase chain reaction (PCR) amplification using an Ion AmpliSeq Library Kit Plus and DNA OCAv3 (Thermo Fisher Scientific) and the PCR products were linked to Ion Xpress Barcode Adapters (Thermo Fisher Scientific). The libraries were pooled and sequenced with an Ion S5 XL System, Ion 540 Kit-Chef, and Ion 540 Chip (Thermo Fisher Scientific) after purification using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA). The DNA sequencing data obtained was accessed through the Torrent Suite ver.5.6 program (Thermo Fisher Scientific) and the reads were aligned with the hg19 human reference genome using the Torrent Suite program. Mutations and focal chromosomal copy number alteration in cancer cells were called using the Ion Reporter™ Software ver.5.6. The raw variant calls were filtered with a variant quality score of <100 and checked using the integrative genomics viewer (IGV; Broad Institute, Cambridge, MA). Germline mutations were excluded using the Human Genetic Variation Database (http://www.genome.med.kyoto-u.ac.jp/SnpDB) and Exome Aggregation Consortium database (http://exac.broadinstitute.org/). To prepare RNA libraries, each tumor RNA (20 ng) was subjected to reverse transcription using the SuperScript IV VILO Master Mix (Thermo Fisher Scientific). The libraries were generated using an Ion AmpliSeq Library Kit Plus and RNA OCAv3 (Thermo Fisher Scientific) and the PCR products were linked to Ion Xpress Barcode Adapters (Thermo Fisher Scientific). The libraries were pooled and then sequenced with an Ion S5 XL System, Ion 540 Kit-Chef, and Ion 540 Chip (Thermo Fisher Scientific) after purification using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA). The RNA sequencing data obtained were accessed through the Torrent Suite ver.5.6 program (Thermo Fisher Scientific) and the reads were aligned with the hg19 human reference genome using the Torrent Suite program. The fusions were analyzed using the Ion Reporter™ Software ver.5.6.

**Western blotting**

The proteins harvested were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred onto polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA), which were immersed in StartingBlock T20 (TBS) Blocking Buffer (Thermo Fisher Scientific, Waltham, MA) for 1 h at about 20°C, followed by incubation for more than 8 h at 4°C with antibodies against phospho-ALK (#3341, Tyr1604), ALK (#3633), cleaved PARP (#5625), cleaved caspase-3 (#9664), Noxa (#14766), Mcl-1 (#94296), Bak (#6947), p53 (#9282), p21 (#2947) and β-actin (#8457), which were purchased from Cell Signaling Technology (Danvers, MA). After being washed thrice in tris buffered saline with polyoxyethylene sorbitan monolaurate (TBST), the membranes were incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies. The proteins labeled with secondary antibodies were visualized using SuperSignal West Dura Extended Duration Substrate Enhanced Chemiluminescent.
Substrate (Thermo Fisher Scientific). Each experiment was carried out independently at least thrice.

**Cell viability assay**

Cell viability was measured using the MTT assay. The cells (2 × 10^5) cultured in the medium supplemented with 10% FBS were incubated in each well of the 96 well plate for 24 h. The drugs were then added to each well, and incubation was continued for another 72 h. Cell growth was measured using MTT solution at a stock concentration of 2 mg/mL (Sigma, St. Louis, MO).

**Small interfering RNA**

The cells (2×10^5) cultured in the medium containing 10% FBS (antibiotic free) for 24 h were treated with Silencer Select siRNA against TP53, Noxa and Mcl-1, and Silencer Select siRNA Negative Control Lo GC (Invitrogen, Carlsbad, CA) using Lipofectamine RNAiMAX (Invitrogen) for 48 h.

**Plasmid construction**

Lentivirus vectors expressing wild-type human TP53 and mutant TP53-R273H were purchased from Addgene (Watertown, MA). PcDNA3.1 plasmids expressing an empty vector and human Noxa were purchased from Thermo Fisher Scientific and Addgene. Lipofectamine LTX and PLUS Reagent (Invitrogen) were used to transfect cells with pcDNA3.1 plasmids following the manufacturer's instructions. Cells infected with the lentivirus vectors were continuously selected using blasticidin (Wako). Lysates obtained from the selected cells were analyzed using western blot.

**Small hairpin RNA**

Lentivirus vectors expressing shRNA against TP53 and negative control were purchased from Addgene. The cells (2×10^5) cultured in the medium containing 10% FBS (antibiotic free) for 24 h were infected with the lentivirus vectors, and continuously selected using puromycin (Sigma).

**Cell apoptosis assay**

Cellular apoptosis induced by the drugs was determined through the use of Fluoroskan Ascent™ FL (Thermo Fisher Scientific) using the Live or Dead cell viability Assay Kit (AAT Bioquest Inc, Sunnyvale, CA), which detected and quantified living cells with Cellbrite™ Orange at 630 nm and apoptotic cells with Blue™ DCS1 at 420 nm using the microplate reader.

**Immunocytochemistry**

The cells (5×10^4 in 1mL RPMI 1640 plus 10% FBS) were plated in each well of the 2-well chamber slides and incubated for 24 h and then the drugs were added to each well. For cell fixation and permeabilization, the cells in each well were immersed in 1mL of cold 100% methanol (Wako, Osaka,
Japan) for 10 minutes, and then with 1mL of Triton™ X-100 (Sigma) for 10 minutes. After blocking in TBST, the cells were incubated for more than 8 h at 4°C with antibodies against Noxa (Cell Signaling Technology, MA). After being washed thrice in phosphate buffered saline (PBS), the cells shielded from light were incubated for 1 h at room temperature with Anti-rabbit IgG Fab2 Alexa Flour as secondary antibodies (Abcam, Cambridge, United Kingdom). After being washed thrice in PBS, the cells on each slide were mounted using the VECTASHIELD Mounting Medium Hard Set with DAPI (Vector Laboratories, Burlingame, CA). The fluorescence of the cells labeled with secondary antibodies were visualized using BZ-X800 (Keyence, Osaka, Japan). Each experiment was carried out independently at least thrice.

**Ubiquitination Detection**

The cells (1×10^6 in 10 mL RPMI 1640 plus 10% FBS) treated with or without the drug were lysated using BlastR™ Rapid Lysate Prep (Cytoskeleton Inc, Denver, CO). Ubiquitinated proteins of the lysate were captured to ubiquitination affinity beads. The target proteins for ubiquitination were extracted from the beads, which were analyzed using western blotting.

**Co-immunoprecipitation**

Co-IP experiments were performed in A925L and H2228 cells using Noxa, Mcl-1, Bak (Cell Signaling Technology, MA) antibodies according to the Pierce Co-IP Kit protocol (Thermo Fisher Scientific). The cell cultures were lysed and placed in spin columns bound to agarose resin. The cell extracts were collected as prey proteins after centrifugation and placed in spin columns bound to the corresponding tubes bound to the covalent antibodies. They were incubated overnight with gentle rotation at 4°C. After being washed using the wash buffer, the protein complexes were eluted from the columns followed by western blotting.

**Xenograft studies**

Six-week-old male SHO-Prkdc<sup>scid</sup>H<sup>hr</sup> mice (Charles River, Yokohama, Japan) were injected with cultured tumor cells subcutaneously into both flanks (5 × 10<sup>6</sup> cells/0.1 mL/mouse). After the tumor volumes reached 500 to 600 mm<sup>3</sup>, the mice were randomized and treated by oral gavage with alectinib and/or ixazomib. Each tumor was monitored using an electronic caliper. Tumor volume was measured in two dimensions, and calculated using the following formula: tumor volume (mm<sup>3</sup>) = 1/2 × length (mm) × width (mm)<sup>2</sup>. After the mice were treated for 28 d, 2 tumors each in the control and treatment groups were excised, lysed, and subjected to western blot analysis. All animal experiments complied with the Guidelines for the Institute for Experimental Animals, Kanazawa University Advanced Science Research Center (approval No. AP-153499).
**Immunohistochemistry**

The tissues were fixed in 10% formalin overnight and embedded in paraffin. The paraffin embedded sections (4 µm-thick) were deparaffinized. Subsequently, heat-induced epitope retrieval was performed by boiling the tissues at 95°C in high pH buffer for 20 min. The tissues were incubated overnight at 4°C with the following primary antibodies: a mouse monoclonal anti-Noxa antibody (#ab13654, 1:500, abcam), a rabbit polyclonal anti-cleaved caspase-3 antibody (#9661, 1:100, Cell Signaling), and then with immune-enzyme polymer labeled with HRP (Nichirei Bioscience, Tokyo, Japan) at room temperature for 30 min. After incubation with DAB+ Chromogen for 5 min at room temperature, the tissues were observed under the microscope and photographed. All the sections were stained with hematoxylin and eosin (H&E) for routine histologic examinations.

**Statistical analysis**

Clinical and genetic data for ALK fusion-positive patients were statistically analyzed using EZR—a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). More precisely, it is a modified version of R commander designed to add statistical functions frequently used in biostatistics. Progression-free survival (PFS) was defined as the duration from start of TKI treatment to disease progression or death from any cause. The Kaplan–Meier methods were used to calculate PFS and logrank tests were applied to compare differences between the groups. Cox regression model was used to estimate the hazard ratio (HR) and 95% confidence interval (CI) of PFS. Experimental data were analyzed using one-way ANOVA. All statistical analyses were conducted using Graph-Pad Prism Ver. 6.05 (GraphPad Software Inc, San Diego, CA). The threshold for significance was P < 0.05.
Results

TP53 mutations are the most frequent co-alterations in ALK fusion-positive patients

From February 2013 to June 2019, a total of 7,844 lung cancer patients including 7,011 with non-small cell lung cancer (NSCLC) were enrolled in the LC-SCRUM-Japan (Supplementary Fig. S1). The submitted samples were available for molecular analysis in 6,444 NSCLC patients (91.9% success rate). ALK fusions were detected in 163 of the 6,444 NSCLC patients (2.5%), including 142 with NGS data. Of these, we evaluated 124 positive patients who received ALK-TKIs—either alectinib (n=90), crizotinib (n=25), or others (n=9) as the first TKI treatment. The molecular profile, baseline clinical characteristics, and PFS of each patient are shown in Fig. 1A. Variants of ALK fusion detected were EML4 exon 13-ALK exon 20 (variant 1, n=53, 42.7%), EML4 exon 6a-ALK exon 20 (variant 3, n=36, 29.0%), EML4 exon 20-ALK exon 20 (variant 2, n=25, 20.2%), other variants of EML4-ALK (n=8, 6.5%), HIP1-ALK (n=1, 0.8%), and STRN-ALK (n=1, 0.8%). At least one concomitant gene alteration with ALK fusions were detected in 77 of the 124 patients (62.1%); the most common one being TP53 mutation (n=31, 25.0%), including 20 missense, 8 frame-shift, and 3 nonsense mutations. Of the 20 missense mutations, 16 were annotated as oncogenic/likely oncogenic and/or pathogenic/likely pathogenic ones by referencing the public databases OncoKB (https://www.oncokb.org/) and ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), respectively (Supplementary Table S1). The frequencies of other co-altered genes were less than 10%: ATM (9.7%), RB1 (5.6%), TERT (5.6%), TSC2 (4.8%), TSC1 (4.0%), and others. TP53 mutations were significantly more frequent in smokers than in non-smokers (19/54 [61.3%] vs. 12/70 [38.7%]; Fisher’s exact test, \( p=0.0354 \)); however, there was no significant correlation between TP53 mutation status and other baseline characteristics such as age, sex, ECOG performance status, tumor stage, and existence of brain metastasis (Supplementary Table S2).

Concomitant TP53 mutations are associated with worse PFS in patients who received alectinib

We evaluated the association of ALK-TKIs efficacy with clinical or genetic factors. The PFS in ALK fusion-positive patients with alectinib as the first TKI treatment was significantly worse in those with concomitant TP53 mutations than without [median PFS, 11.7 months (95% CI, 6.3-not reached) vs. not reached (23.6-not reached); logrank, \( p=0.0008 \); HR, 0.33 (95% CI, 0.17-0.65)] (Fig. 1B). This significant correlation was also observed in all patients who received either of the ALK-TKIs—alectinib, crizotinib or others, as the first TKI treatment [median PFS, 9.2 months (95% CI, 7.4-13.1) vs. 27.9 months (19.9-43.4); logrank, \( p=0.0017 \); HR, 0.42 (95% CI, 0.24-0.73)] (Supplementary Fig. S2). Of the 31 concomitant TP53 mutations, oncogenic and/or pathogenic missense mutations, frameshift mutations, and nonsense mutations were considered loss-of-function mutations. A significantly worse PFS was also observed in patients with these loss-of-function mutations than in those without (Supplementary Fig. S3A-B). Meanwhile, ATM mutations, which
were the second most frequent concomitant mutations and were mutually exclusive with TP53 mutations in this study, did not affect the PFS of patients treated with alectinib or either ALK-TKIs (Supplementary Table S3 and Supplementary Fig. S4A-B). The comparative analysis of subgroups according to ALK fusion variants showed a non-significant trend towards better PFS in patients with EML4-ALK variant 1 than in those with other variants [median PFS, not reached (95% CI, 19.9-not reached) vs. 23.6 months (13.1-not reached); logrank, \( p = 0.34 \); HR, 0.72 (95% CI, 0.37-1.41)] (Supplementary Fig. S5A-B). In the EML4-ALK variant 1 subgroup, TP53 co-mutated patients also showed significantly worse PFS than in patients with wild type TP53 [median PFS, 7.4 months (95% CI, 1.1-not reached) vs. not reached (25.9-not reached); logrank, \( p = 0.0001 \)] (data not shown). On the other hand, no association was found between the clinical characteristics and PFS in patients with alectinib treatment. Although concomitant TP53 mutations were more frequent in smokers than non-smokers, there was no correlation between PFS and smoking status [smokers (n=41) vs. non-smokers (n=49); median PFS, 30.8 months (95% CI, 13.8-not reached) vs. not reached (4.2-not reached); logrank, \( p = 0.791 \); HR, 1.09 (95% CI, 0.57-2.10)].

**Loss of normal p53 function results in ALK-rearranged NSCLC resistance to ALK-TKIs**

Firstly, TP53 mutation statuses of three EML4-ALK fusion-positive NSCLC cell lines were identified using next-generation sequencing (Table 1). While CCL-185IG cells had wild type TP53, H2228 and A925L cells had nonsense and frameshift mutations, respectively, in TP53. To confirm whether these mutation statuses were relevant to p53 expression, we conducted western blotting on the ALK-rearranged NSCLC cells. The expressions of p53 and p21, whose transcription is regulated by functional p53, were much higher in CCL-185IG cells compared with H2228 and A925L cells (Fig. 2A). These results suggest that nonsense and frameshift TP53 mutations lead to loss of p53 function in H2228 and A925L cells, respectively. Next, we assessed the effect of ALK-TKIs on these cell lines with different TP53 genetic variations. CCL-185IG cells were more susceptible to either alectinib or crizotinib than H2228 and A925L cells with TP53 mutations (Fig. 2B). Although alectinib noticeably induced pro-apoptotic proteins, cleaved PARP and cleaved caspase-3 in CCL-185IG cells (Fig. 2C), the knock down of p53 attenuated expression of the pro-apoptotic proteins in alectinib-treated CCL-185IG cells (Fig. 2D). A925L cells, which had frameshift mutation and lost protein expression in p53, became susceptible to alectinib in the consequence of introducing wild type TP53. However, introducing TP53 with R273H mutation, which led to reduction in the DNA binding affinity (13), did not restore apoptosis induction by alectinib (Fig. 2D). To evaluate the impact of loss of p53 function on survival, we established p53 knock-downed CCL-185IG cells by introducing TP53-shRNA. Most p53 knock-downed CCL-185IG cells survived in the presence of alectinib or crizotinib for a long period (Fig. 2E). These findings suggest that loss of p53 function mediates resistance to ALK-TKIs in ALK-rearranged NSCLC.
Proteasome inhibitors enhance Noxa expression and the susceptibility to an ALK-TKI in ALK/TP53 co-mutated NSCLC

Several studies have validated that Noxa, one of the pro-apoptotic BH3-only subgroup of Bcl-2 family proteins, can induce apoptosis independently of p53 (10, 14). Additionally, the triggering of Noxa expression using proteasome inhibitors has been demonstrated in melanoma and myeloma cell lines (15). Through the boosting of Noxa expression in ALK-rearranged NSCLC with co-occurring TP53 mutations, we validated the effect of proteasome inhibition on Noxa induction in A925L and H2228 cells. A proteasome inhibitor, ixazomib, elevated the expression of Noxa, and that of cleaved PARP and cleaved caspase-3 markedly by combination with alectinib in these cells (Fig. 3A). Similarly, bortezomib, a proteasome inhibitor, enhanced the expression of Noxa and pro-apoptotic proteins by the combined use of alectinib (Supplementary Fig. S6A). Moreover, the combination of ixazomib and alectinib suppressed cell proliferation following long-term treatment (Supplementary Fig. S6B). The combination therapy decreased the proportion of calcine-AM-positive living cells, and markedly increased the proportion of DCS1-positive apoptotic cells (Fig. 3B-C). The knockdown of Noxa protein by Noxa-specific siRNA attenuated the induction of apoptosis by the combined use of alectinib and ixazomib (Supplementary Fig. S6C). In contrast, Noxa overexpression reinforced apoptosis induction in the presence of alectinib (Supplementary Fig. S6D). These results indicate that proteasome inhibitors induce Noxa protein expression so prominently that ALK-rearranged NSCLC cells with mutant TP53 become susceptible to an ALK-TKI. Next, we detected the ubiquitination of Noxa protein increasing as a result of proteasome inhibitor treatment (Fig. 3D and Supplementary Fig. S6E), suggesting that proteasome inhibition results in amplifying the undegraded Noxa protein sufficient enough to induce apoptosis. Meanwhile, to elucidate how increased levels of Noxa protein, which directly binds to the other apoptosis regulatory proteins including anti-apoptotic proteins and pro-apoptotic proteins, played a part in apoptosis induction, we investigated the interaction of Noxa and the other proteins. Consequently, at elevated levels, Noxa protein did not bind to a pro-apoptotic protein, Bak, but bound to Mcl-1, which is a member of the anti-apoptotic Bcl-2 family protein (Fig. 3E and Supplementary Fig. S6F).

Mcl-1 inhibition facilitates the potent antitumor effect of an ALK-TKI on ALK/TP53 co-mutated NSCLC

We suspected that Mcl-1 inhibition played a core role in the apoptosis induced by an ALK-TKI in p53-unfunctional NSCLC with ALK rearrangement. Suppression of Mcl-1 expression heightened the susceptibility to alectinib treatment in A925L and H2228 cells (Fig. 4A). Simultaneously, a knockdown of Mcl-1 noticeably induced the expression of cleaved PARP and cleaved caspase-3 in these cells which underwent alectinib treatment (Fig. 4B), indicating that Mcl-1 inhibition was
sufficient to activate apoptosis induced by an ALK-TKI. A small molecule, S63845, shows a high affinity for the BH3-binding groove of Mcl-1 and directly binds to Mcl-1 (16). The Mcl-1 inhibitor did not appreciably reduce proliferation of A925L and H2228 cells (Supplementary Fig. S7A) and thus we selected S63845 as a means of inhibiting Mcl-1 function. A combination therapy of alectinib and S63845 for these cells resulted in the eliciting of PARP and caspase-3 cleavage (Fig. 4C). In addition to western blotting, fluorescent staining demonstrated that the proportion of living cells diminished and that of apoptotic cells increased, following the combination therapy (Fig. 4D-E and Supplementary Fig. S7B-C). These results suggested that Mcl-1 inhibition potentiate an ALK-TKI effect on the ALK/TP53 co-mutated NSCLC.

Combination of ixazomib and alectinib subdue tumors with declining p53 function in ALK-rearranged NSCLC cells in vivo

To examine the efficacy and feasibility of combination therapy with alectinib and ixazomib in ALK/TP53 co-mutated NSCLC cells in vivo, we treated immunodeficient mice bearing A925L tumor xenografts with alectinib or ixazomib monotherapy, or the combination therapy of both. Although monotherapy with alectinib modestly decreased the size of the tumors for 18 d, the tumors regrew. In contrast, combined alectinib and ixazomib therapy caused remarkable tumor shrinkage, and 3 of the 8 tumors completely regressed (Fig. 5A and Supplementary Fig. S8A). Ixazomib did not cause weight loss throughout the treatment period regardless of the presence or absence of alectinib (Supplementary Fig. S8B). Furthermore, western blot analysis revealed that ixazomib markedly upregulated NOXA protein expression and apoptotic proteins, cleaved PARP and cleaved caspase-3 by the combined treatment with alectinib (Fig. 5B). Likewise, immunochemistry showed increased NOXA staining in tumors treated with ixazomib in combination with alectinib or without alectinib. Notably, decreasing cell population with necrosis and increasing expression of cleaved caspase-3 were observed in tumors treated with the concurrent therapy of alectinib and ixazomib (Fig. 5C).
Discussion

In this study, we demonstrated that concomitant TP53 mutations were highly correlated with unfavorable efficacy of alectinib in patients with TKI-naïve ALK-rearranged NSCLC extracted from our nationwide clinico-genomic database, and the combined use of a proteasome inhibitor with alectinib restored the unfavorable efficacy in our preclinical models.

To our knowledge, this is the first report showing negative impacts of TP53 mutations on the efficacy of alectinib for TKI-naïve ALK-rearranged NSCLC. This result is supported by several previous reports showing the association of TP53 mutations with efficacy of other ALK-TKIs, such as crizotinib (4-6). Kron et al. showed that the median PFSs of crizotinib-treated ALK-rearranged NSCLC patients with and without TP53 mutations were 5.0 and 14.0 months, respectively, with a HR of 2.89 (95% CI, 1.57-5.33) (6). These median PFSs were shorter than those in our alectinib-treated cohort, respectively, but the HR was similar to our results. In addition, we demonstrated the unfavorable efficacy of alectinib in TP53 co-mutated patients, irrespective of ALK fusion variants. In several previous reports, patients with EML4-ALK variant 1 showed longer PFS than those with other ALK fusion variants in the treatment with crizotinib or alectinib (17, 18). Although a similar tendency was observed in our alectinib-treated cohort, there was a more significant difference in alectinib efficacy between presence and absence of TP53 mutations, suggesting that TP53 mutation status had a stronger impact on alectinib efficacy than ALK fusion variants. In our cohort of ALK-rearranged NSCLC, TP53 mutations were the most frequent concomitant gene alterations, with a frequency of 25.0%, in consistent with a previous report (6). Therefore, more effective therapies to improve TP53 mutation-derived unfavorable efficacy are needed for a considerable fraction of ALK-rearranged NSCLC patients.

The proteasome degrades most of the damaged or misfolded proteins which are tagged with ubiquitin and controls the intracellular homeostasis in normal cells (19). On account of the aberrant proteins, cancer cells generally express more proteasome than normal cells. As a result of targeting the proteasome to treat tumors, endoplasmic reticulum stress by accumulation of unfolded proteins and inhibition of the pro-survival NFκB pathway by accumulation of IκBα (the inhibitor of NFκB) induces cytotoxicity in cancer cells (20, 21). Proteasome inhibitors have been tested for their clinical efficacy for NSCLC, which remains limited (22, 23). A third-generation proteasome inhibitor, ixazomib, which can improve drug compliance owing to oral administration, is approved for multiple myeloma (24, 25)(24). Our study demonstrated that ixazomib monotherapy was not sufficient to induce tumor regression in vivo, whereas it caused tumor bulk to shrink and had low toxicity under the presence of alectinib. Given that the combination of a proteasome inhibitor and the other targeted therapy for solid tumor remains unestablished, this result opens up new possibilities for developing a combination therapy in NSCLC. Some studies have suggested that alterations in TP53 should be independently associated with poor clinical outcomes of EGFR-TKI treatment in EGFR-mutant NSCLC patients.
Developing treatments against EGFR-mutant NSCLC concurrent TP53 mutations is as important as those against ALK-rearranged NSCLC because mutant TP53 account for about 50–60% in EGFR-mutant NSCLC. Thus, we need to examine the potential for the treatment strategy in our study to overcome tolerance in EGFR-mutant NSCLC with mutant TP53.

Whether differences among types of TP53 mutations alter the effect of ALK-TKIs remains an issue. In lung adenocarcinoma, missense mutations were the most common (50.0%), and frameshift (20.6%) and nonsense (17.6%) mutations were less frequent (29). There was a similar trend for our result, but more sample size is needed for understanding the consequence of each TP53 mutation relevant to the response to ALK-TKIs. The present study focused on TP53 mutations as a tolerance factor, meanwhile there is a possibility that TP53 mutations confer an acquired resistance to ALK-TKIs, judging from short PFS of ALK-TKIs in the patients whose TP53 were wild-type at baseline, but TP53 mutations were detected at progression (4). Therefore, it is worth considering that the combination of a proteasome inhibitor and an ALK-TKI overcomes the acquired resistance due to de novo TP53 mutations in ALK-rearranged NSCLC.

Induction of Noxa can occur in a p53-independent manner by proteasome inhibition, but Noxa is also involved in p53-dependent apoptosis via p53-mediated transcriptional induction (30). Most of the multiple myeloma patients not responding to bortezomib treatment harbored chromosomal loss or intragenic mutations of TP53, which did not have enough ability to upregulate high expression of Noxa (31). In ALK-rearranged NSCLC with TP53 mutations, proteasome inhibitors were able to enhance Noxa expression by a p53-independent manner. This may be because the extent of p53-mediated transcription of Noxa varies among types of tumors. BH3-only proteins of the Bcl-2 family which Noxa belongs to, did not activate Bak directly (32). Additionally, bortezomib did not elevate Bak expression in mesothelioma cells, coinciding with our results (33). These reports support our concept that accumulated Noxa indirectly accelerates alectinib-induced apoptosis through interaction with Mcl-1. In other words, targeting Mcl-1 directly is more reasonable to induce apoptosis than upregulating Noxa. S63845 monotherapy, an Mcl-1 inhibitor, was tolerated in mice at a dose which showed antitumor effects in lymphoma cells (16). Our study also provided the in vitro data regarding low cytotoxicity of S63845. Thus, further studies should be required to confirm tumor penetration and drug tolerance when S63845 is used in combination with an ALK-TKI in ALK-rearranged NSCLC.

In conclusion, this is the first study to underscore the tolerance to an ALK-TKI, alectinib, caused by concomitant TP53 mutations for ALK-rearranged NSCLC, both in clinical and preclinical data, and to identify a novel combination targeted therapy of alectinib and a proteasome inhibitor to restore the reduced efficacy. Even though a proteasome inhibitor monotherapy does not directly induce apoptosis in ALK-rearranged/TP53-mutated NSCLC cells, accumulated Noxa protein by proteasome inhibition contributes to notable apoptosis in combination with alectinib. Based on these data, this combination
therapy is needed to be validated in molecularly stratified clinical trials. Furthermore, targeting Mcl-1 by Noxa plays a crucial role in apoptosis induction and tumor shrinkage accordingly (Supplementary Fig. S9). The results of our study suggest that developing a Mcl-1 inhibitor combined with an ALK-TKI assist in establishing new strategy for ALK-rearranged/TP53-mutated NSCLC.

Table and figure legends

Table 1. TP53 mutant status and EML4-ALK variant in three ALK-rearranged NSCLC cells.

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Figure 1. Mutational landscape and progression-free survival (PFS) in 124 ALK fusion-positive lung cancer patients.
A, Overview of genetic alterations, baseline characteristics and PFS in ALK fusion-positive lung cancer patients who received an ALK-TKI as the first TKI treatment. B, Kaplan-Meier curves for PFS according to TP53 mutation status in patients who received alectinib as the first TKI treatment.

Figure 2. Loss of p53 function attenuates ALK-TKIs treatment sensitivity in ALK-rearranged NSCLC cells
A, Protein expression using western blotting in CCL-185IG cells (left), H2228 cells (middle), and A925L cells (right). B, Cell viability determined after 72 h with MTT assay in CCL-185IG cells, H2228 cells, and A925L cells which were treated with alectinib or crizotinib. The upper panel represents dose-response curves, and the lower represents the IC50 in each of the cell lines. Data shown are representative of at least three independent experiments. The data shown are the mean ± SEM of triplicate cultures. C, Western blotting of CCL-185IG cells, H2228 cells, and A925L cells which were treated with alectinib (3 µmol/L) for 48 h. D, Western blotting of CCL-185IG cells with/without TP53 knockdown (left) and A925L cells with wild-type or mutant R273H TP53 (right) which were treated with alectinib (3 µmol/L) for 48 h. E, Western blotting of CCL-185IG cells treated with shRNA targeting TP53 (left). The cells were treated with DMSO, alectinib (3 µmol/L) and crizotinib (3 µmol/L) for 9 days which were replenished every 72 h (right). The plates were stained with crystal violet and visually examined. A plate representative of three independent experiments is shown.

Figure 3. Proteasome inhibition overcomes apoptosis resistance to an ALK-TKI via Noxa protein expression in ALK/TP53 co-mutated NSCLC cells
A, Protein expression using western blotting in A925L cells and H2228 cells treated with alectinib (3...
µmol/L) and/or ixazomib (5 nmol/L) for 72 h. B, Quantification of the number of calcein-AM+ or DCS1+ cells determined by fluorometer. Calcein-AM+ cells in control and DCS1+ cells in combination were defined as 100% relative fluorescence units (RFU). The results are mean ± SEM of three independent experiments. *, P < 0.05 C, A925L cells and H2228 cells exposed to DMSO, alectinib (3 µmol/L), ixazomib (5 nmol/L), or concurrent treatment for 72 h. The cells were stained for calcein-AM and DCS1. Scale bars, 100 µm. D, Ubiquitinated Noxa protein in A925L cells and H2228 cells which were treated with ixazomib (5 nmol/L) for 48 h. N, C and I respectively correspond to non-specific, control and ixazomib. E, Protein expression in A925L cells and H2228 cells detected using western blot with immunoprecipitation of indicated proteins. The cell lines were treated with ixazomib (5 nmol/L) for 6 and 24 h.

The data shown are representative of at least three experiments with similar results.

Figure 4. Mcl-1 inhibition restores an ALK-TKI treatment sensitivity in ALK/TP53 co-mutated NSCLC cells

A, cell viability determined after 48 h with MTT assay in A925L cells and H2228 cells transfected by siRNA control (scramble) or Mcl-1 siRNA (si Mcl-1) which were treated with alectinib (3 µmol/L). Data shown are representative of at least three independent experiments. The data shown are the mean ± SEM of triplicate cultures. *, P < 0.05. B, protein expression in A925L cells and H2228 cells with/without Mcl-1 knockdown which were treated with alectinib (3 µmol/L) for 72 h. C, protein expression using western blotting in A925L cells and H2228 cells treated with alectinib (3 µmol/L) and/or S63845 (1 µmol/L) for 72 h. D, quantification of the number of calcein-AM+ or DCS1+ A925L cells determined by fluorometer. The results are the mean ± SEM of three independent experiments. *, P < 0.05. E, A925L cells stained for calcein-AM and DCS1 which were exposed to DMSO, alectinib (3 µmol/L), S63845 (1 µmol/L), or concurrent treatment for 72 h. Scale bars, 100 µm.

The data shown are representative of at least three experiments with similar results.

Figure 5. Combined use of alectinib and ixazomib successively regresses ALK/TP53 co-mutated NSCLC tumors

A, Tumor volume of A925L xenografts receiving the indicated treatments (n=8 for each group). Nude mice bearing the xenografts were treated with 25 mg/kg of alectinib daily and/or 5 mg/kg of ixazomib twice a week. All values are expressed as the mean ± SE and assessed using one-way ANOVA. *, P < 0.05 combination versus alectinib. B, protein expression of A925L tumor xenografts determined using western blotting. The each tumor was resected from mice treated with the indicated treatments for 28 days. C, immunochemical staining with antibodies specific for Noxa and cleaved caspase-3 in xenografts. Scale bars, 100 µm.
Acknowledgments

We would like to thank Yuri Murata for her great help with LC-SCRUM-Japan genome screening.
References


### Number at risk

| TP53 mut (-) | 65 | 44 | 28 | 14 | 4 | 1 | 0 |
| TP53 mut (+) | 25 | 10 | 4  | 1  | 1 | 0 | 0 |

### Kaplan-Meier Analysis

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Logrank, \( p = 0.0008 \)
Hazard ratio, 0.33 (95% C.I., 0.17-0.65)
**Figure 2**

**A**

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**B**

![Graphs showing the IC50 values for Alectinib and Crizotinib in CCL-185IG, H2228, and A925L cells.](image)

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Figure 3

A

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B

![Graph showing RFU for A925L and H2228](image)

C

![Images showing cell viability for A925L and H2228](image)

D

![Western blot images for A925L and H2228](image)

E

![Western blot images for Noxa IP](image)
**Figure 5**

A. Tumor volume (mm³) over days after treatment for Control, Alectinib, Ixazomib, and Combination.

B. Western blot analysis of tumor samples for Control, Alectinib, Ixazomib, and Combination groups. The proteins analyzed include p-ALK, ALK, Noxa, C-PARP, C-caspase-3, and β-actin.

C. Histological images stained with H&E and immunohistochemistry for Noxa and Cleaved-caspase-3 expression in Control, Alectinib, Ixazomib, and Combination groups.
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Abbreviations: E: EML4, A: ALK, ins: insertion, fs: frameshift
Clinical Cancer Research

Proteasome inhibition overcomes ALK-TKI resistance in ALK-rearranged/TP53 mutant NSCLC via Noxa expression

Azusa Tanimoto, Shingo Matsumoto, Shinji Takeuchi, et al.

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