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Differential protein stability and clinical responses of *EML4-ALK* fusion variants to various ALK inhibitors in advanced *ALK*-rearranged non–small cell lung cancer

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ABSTRACT

Background

Anaplastic lymphoma kinase (ALK) inhibition using crizotinib has become the standard of care in advanced *ALK*-rearranged non–small cell lung cancer (NSCLC), but the treatment outcomes and duration of response vary widely. Echinoderm microtubule-associated protein-like 4 (*EML4*)-*ALK* is the most common translocation, and the fusion variants show different sensitivity to crizotinib *in vitro*. However, there are only limited data on the specific *EML4-ALK* variants and clinical responses of patients to various ALK inhibitors.

Patients and methods

By multiplex reverse-transcriptase PCR, which detects 12 variants of known *EML4-ALK* rearrangements, we retrospectively determined *ALK* fusion variants in 54 advanced *ALK* rearrangement-positive NSCLCs. We subdivided the patients into two groups (variants 1/2/others and variants 3a/b) by protein stability and evaluated correlations of the variant status with clinical responses to crizotinib, alectinib, or ceritinib. Moreover, we established the EML4-ALK variant-expressing system and analyzed patterns of sensitivity of the variants to ALK inhibitors.

Results

Of the 54 tumors analyzed, *EML4-ALK* variants 3a/b (44.4%) was the most common type, followed by variants 1 (33.3%) and 2 (11.1%). The 2-year progression-free survival (PFS) rate was 76.0% (95% confidence interval [CI] 56.8–100) in group *EML4-ALK* variants 1/2/others versus 26.4% (95% CI 10.5–66.6) in group variants 3a/b (P = 0.034) among crizotinib-treated patients. Meanwhile, the 2-year PFS rate was 69.0% (95% CI 49.9–95.4) in

group variants 1/2/others versus 32.7% (95% CI 15.6–68.4) in group variants 3a/b ($P = 0.108$) among all crizotinib-, alectinib-, and ceritinib-treated patients. Variant 3a- or 5a-harboring cells were resistant to ALK inhibitors with >10-fold higher half maximal inhibitory concentration *in vitro*.

Conclusion

Our findings show that group *EML4-ALK* variants 3a/b may be a major source of ALK inhibitor resistance in the clinic. The variant-specific genotype of the *EML4-ALK* fusion allows for more precise stratification of patients with advanced NSCLC.

Keywords: EML4-ALK, non–small cell lung cancer, crizotinib, ALK inhibitor, ALK translocation

Abbreviations

NSCLC, Non–Small Cell Lung Cancer;

ALK, Anaplastic lymphoma kinase;

EML4, Echinoderm microtubule-associated protein-like 4;

OS, overall survival;

PFS, progression free survival.

Key message: Multiple variants of the EML4-ALK fusion were identified in ALK-rearranged NSCLCs. We subdivided the patients into two groups (variants 1/2/others and variants 3a/b) by protein stability and found that patients with variants 3a/b may be a major contributor to ALK inhibitor resistance. Stratification of patients by the variant-specific genotype should help to predict responses to ALK inhibitors.

Introduction

Genetic alterations in the anaplastic lymphoma kinase (*ALK*) gene occur in 2% to 9% of non–small cell lung cancers (NSCLCs) [1-3]. Small-molecule tyrosine kinase inhibitors have been developed for *ALK*-rearranged NSCLC [1, 4]. Crizotinib is the first-generation ALK inhibitor and showed objective response rates of 61% to 74%, 2-year overall survival rate (OSR) of 54%, and a median progression-free survival (PFS) of 11 months, which is superior to PFS of standard first-line chemotherapy (7 months) [2, 5-7].

Resistance to crizotinib develops within 1 or 2 years after initial treatment via various mechanisms [8, 9] but crizotinib-resistant tumors still depend on ALK for growth and survival and are sensitive to second-generation ALK inhibitors such as ceritinib and alectinib [10, 11].

The predominant fusion partner in *ALK*-rearranged NSCLC is echinoderm microtubule-associated protein-like 4 (*EML4*) [12]. Multiple variants of the *EML4-ALK* fusion have been identified in NSCLC resulting from a translocation at different fusion points within the *EML4* gene, with variant 1 (V1, 33%), variant 2 (V2, 10%), and variants 3a/b (V3a/b, 29%) being the most frequent fusion mutants [13-15]. All variants have exons 20 through 29 of *ALK*; this region encodes the entire tyrosine kinase domain [14, 16]. EML4 has an N-terminal coiled-coil region, a basic region, a hydrophobic echinoderm microtubule-associated protein-like protein (HELP) motif, and WD (tryptophan-aspartic acid) repeats [17, 18]. The core HELP-WD region forms a novel tandem atypical β-propeller (TAPE) structure. The EML4 TAPE domain is truncated in many variants, which results in a partial structure that makes the EML4-ALK fusion proteins unstable. *EML4-ALK* variants 1, 2, 7, and others containing a partial TAPE domain are structurally unstable, whereas variants 3a/b and 5a/b lacking any core part of the TAPE structure are structurally stable *in vitro* [12, 19, 20]. *EML4-ALK* variants show differential sensitivity to crizotinib *in vitro* [21].

Here, we determined variant genotypes of *EML4-ALK* in patients with advanced *ALK*-rearranged NSCLC, and assessed correlations of the specific *EML4-ALK* variant status with clinical outcomes among the patients treated with various ALK inhibitors.

Patients and methods

Study design

We performed a retrospective analysis to assess the correlation between the treatment outcomes and *EML4-ALK* variants in patients with advanced *ALK*-positive NSCLC treated with ALK inhibitors using the Response Evaluation Criteria in Solid Tumor (RECIST) criteria version 1.1 [22]. None of the patients in this study had been previously treated with ALK-specific inhibitors.

We analyzed the sensitivity to ALK inhibitors in stably *EML4-ALK* V1-, V2-, V3a-, or V5a-expressing Ba/F3 cells and normal bronchial epithelial BEAS-2B cells transiently expressing one of the variants as well as two NSCLC cell lines (H2228 cells expressing variant 3b; H3122 cells expressing variant 1).

Patients

From June 2011 to August 2015, 1721 ALK-naive patients with advanced NSCLC at Asan Medical Center were tested with the Vysis FISH test to identify an *ALK* rearrangement [2]. The rearrangement was detected in 182 patients (10.6%). Of those, 113 patients were treated with the ALK inhibitors and had an Eastern Cooperative Oncology Group (ECOG) performance status [23] between 0 and 3. Among 81 enrolled patients who were tissue-available and approved by the institutional review board, 24 were excluded because of poor quality of genomic DNA or insufficient tissue samples, and three were lost to follow-up

(Figure 1). For the 54 enrolled patients, medical records were reviewed to extract clinicopathological data including sex, age, smoking status, diagnoses, therapeutic agents, and survival.

Assessment

Patients confirmed to have the *ALK* translocation were given one of the ALK inhibitors in clinical-practice or clinical-trial settings. They were assigned to receive oral crizotinib at a dose of 250 mg twice daily, alectinib 600 mg twice daily, or ceritinib 750 mg once a day administered every 4 weeks. The cycle was continued as long as the patients did not have the RECIST version 1.1-defined disease progression, unacceptable toxicity, death, or did not withdraw. Treatment responses were evaluated every two cycles using the RECIST criteria version 1.1. The safety or toxicity profile was evaluated every 2 weeks during the first one or two cycles and then every cycle using the Common Terminology Criteria for Adverse Events version 4.

Statistical analysis

Wilcoxon's rank-sum test and Fisher's exact test were used to assess the association between *EML4-ALK* variants and the clinicopathological characteristics.

Fisher's exact test was conducted for correlations between *EML4-ALK* variants and the objective response rate (ORR) or disease control rate (DCR). To estimate survival rates and compare the survival distribution, we used the Kaplan-Meier method and log-rank test, respectively. All statistical analyses were performed in the R software (version 3.1.3, the R Foundation for Statistical Computing, Vienna, Austria). Any P value <0.05 was assumed to indicate a statistically significant difference

Detection of the *ALK* gene rearrangement in daily practice

An *ALK* rearrangement was detected by FISH analysis using a break-apart probe specific for the *ALK* locus in Supplementary Methods, available at *Annals of Oncology* online.

Genotyping for *ALK*-positive patients

The *ALK*-rearranged patients treated with various ALK inhibitors were subtyped using Peptide nucleic acid (PNA)-mediated quantitative PCR (qPCR) assay (Supplementary Methods, available at *Annals of Oncology* online).

Responses of *EML4-ALK* variants-expressed cell lines to ALK inhibitors *in vitro*

To identify the correlation of the response and specific *EML4-ALK* variant expressed-cell lines treated with various ALK inhibitors, viability assay and ALK kinase assay were determined was determined using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI, USA) and the Universal Tyrosine Kinase Assay Kit (MK410; Takara Bio, Pittsburg, PA, USA), respectively (Supplementary Methods, available at *Annals of Oncology* online).

Results

Clinicopathological characteristics among the patients

A total of 54 patients with advanced *ALK*-positive NSCLC were analyzed. Baseline clinicopathological characteristics are showed in Supplementary Table S1, available at *Annals of Oncology* online. Among the 54 patients, crizotinib was a first-line treatment for 17 patients, second-line for 15, third-line for eight, and fourth or further-line therapy for seven patients. Alectinib or ceritinib were a first-line treatment for four patients, third-line for one, and

fourth-line for two patients.

Among the 54 patients with the *ALK* translocation, 51 had an *EML4-ALK* fusion. *EML4-ALK* variants 3a/b (24, 44.4%) was the most common group, followed by variants 1 (18, 33.3%) and 2 (6, 11.1%). Of the other six patients with rare *ALK* translocations, one had *EML4-ALK* variant 7, and two had novel variants EML4-ALK E14del2;del22A20 and E17;del70A20, which represent long *EML4-ALK* fusion transcripts similar to variant 7 (Table 1 & Figure 1). The remaining three patients had an *ALK* rearrangement with a fusion partner other than *EML4*. According to the expected stability of *EML4-ALK* variants [19, 21], all patients but three harboring other fusion partners of *ALK* were subdivided into two groups: variants 1/2/others and variants 3a/b. The baseline characteristics were well balanced between the two groups (Supplementary Table S2, available at *Annals of Oncology* online). Treatment-related adverse events and dose reduction are listed in Supplementary Tables S3–S5, available at *Annals of Oncology* online. Dose reduction was not related to disease progression (P = 0.767).

Treatment responses among patients with different *EML4-ALK* variants

Median overall survival (OS) and 2-year overall survival rate (OSR) of all the enrolled 54 patients were 36 months (95% CI 19 months to not available) and 57.8% (95% CI 42.1–79.3), respectively. Median PFS and 2-year progression-free survival rate (PFSR) were 19 months (95% CI 12 months to not available) and 45.1% (95% CI 27.8–73.1), respectively. Among the patients treated with crizotinib, 2-year PFSR was 76.0% (95% CI 56.8–100) in group *EML4-ALK* variants 1/2/others versus 26.4% (95% CI 10.5–66.6) in group variants 3a/b (P = 0.034; Figure 2A). When we included the patients treated with alectinib or ceritinib, 2-year PFSR was 69.0% (95% CI 49.9–95.4) for variants 1/2/others versus 32.7% (95% CI 15.6–68.4) for variants 3a/b (P = 0.108; Figure 2B). There was no significant difference in overall survival (OS) between the two groups (Supplementary Figure S1, available at *Annals of Oncology*

online). This is probably because the mortality rate was relatively low at the data cutoff (33.3%), and the proportion of patients treated with first line therapy was larger in the variant 3a/b group than in the variant 1/2/other group (54.2% vs. 29.6%, $P = 0.094$), which implies that the variant 1/2/others group included more heavily treated patients. Seven patients (four patients with variants 1/2/others and three patients with variants 3a/b) underwent re-biopsy among the 23 patients who developed progressive disease. There were no *ALK* mutations identified in those specimens.

In each assessment, an ORR and DCR of group variants 1/2/others were consistent with a strong response tendency as compared with group variants 3a/b (Supplementary Table S6, available at *Annals of Oncology* online). Overall, DCR was 100% in group variants 1/2/others and 87.5% in group variants 3a/b when we analyzed all patients treated with crizotinib, alectinib, or ceritinib; this result fell short of statistical significance ($P = 0.097$; Table 2).

Responses of cells with *EML4-ALK* variants to ALK inhibitors *in vitro*

To test whether the structural differences among EML4-ALK variants have effects on their kinase activities, we generated a system stably expressing EML4-ALK V1, V2, V3a, or V5a in an IL-3-dependent Ba/F3 cell line [24], which became IL-3-independent and ALK-dependent for growth. Western blotting analysis detected abundantly Tyr-1604-phosphorylated ALK in Ba/F3 cells expressing V3a or V5a (Figure 3A). A kinase assay confirmed greater activities in V3a- or V5a-expressing cells than in V1- or V2-expressing cells (Figure 3B). Viability assays were performed for ALK inhibitors—crizotinib, alectinib, and ceritinib—in the four cell lines, and IC_{50} values were determined (Figure 3C & 3D). All three ALK inhibitors significantly suppressed the growth of V1- or V2-expressing Ba/F3 cells. Ceritinib and alectinib inhibited the proliferation of these two cell lines with much lower IC_{50} values as compared with crizotinib. Nonetheless, V3a- or V5a-expressing cells showed

similar resistance to all ALK inhibitors, with IC_{50} > 500 nM.

Next, we analyzed the sensitivity to ALK inhibitors among NSCLC cell lines (H3122 cells with *EML4-ALK* V1 and H2228 cells with *EML4-ALK* V3b) and normal bronchial epithelial BEAS-2B cells transiently expressing an *EML4-ALK* variant. H3122 cells showed the highest sensitivity to ALK inhibitors among the cell lines examined; in contrast, H2228 and BEAS-2B cells expressing V3a or V5a exhibited only weak growth inhibition under the influence of ALK inhibitors (Figure 3E & 3F).

Discussion

Recently, Bayliss and colleagues resolved the unique β-propeller structure of EML proteins and explained how the different stability of EML4-ALK fusion proteins depend on the disruption of their β-propeller folding [19]. Although ALK inhibitors became a first-line treatment for advanced *ALK* rearrangement-positive NSCLC, a molecular companion test for *ALK* translocation does not discriminate between specific types of *EML4-ALK* fusion. Here, we explored the correlations between *EML4-ALK* variants and clinical responses to various ALK inhibitors in 54 patients with advanced *ALK* rearrangement-positive NSCLC.

We analyzed ALK fusion variants in 54 *ALK*-rearranged NSCLCs, and subdivided them into groups variants 1/2/others (27, 49.9%) and variants 3a/b (24, 44.4%) according to expected stability differences among EML4-ALK variant proteins. Given that group variants 1/2/others has the truncated incomplete tandem atypical propeller EML (TAPE) domain of EML4, whereas group variants 3a/b lacks any part of the TAPE domain [19], we hypothesized that group variants 1/2/others may have better treatment outcomes than the variants 3a/b group because of protein instability of variants 1/2/others. Our results revealed that PFS is significantly longer in group variants 1/2/others than in group variants 3a/b. The former group

also showed a tendency for greater ORR and DCR after treatment with crizotinib, alectinib, or ceritinib. These data suggest that *EML4-ALK* variants may be an important factor contributing to ALK inhibitor resistance in the large majority of tumors among patients with advanced *ALK*-rearranged NSCLC.

Our *in vitro* results on EML4-ALK variant-expressing Ba/F3 and BEAS-2B cells clearly showed that V3a- or V5a-harboring cells are resistant to crizotinib, ceritinib, and alectinib, and show >10-fold higher IC_{50} than do V1- or V2-expressing cells. These findings suggest that the "variants 3a/b" group of our patients will not benefit much from ALK signaling inhibition; this problem may be overcome by more potent ALK inhibitors or by combined treatment with other regimens.

Lei and colleagues reported no correlation between *EML4-ALK* variants and clinical responses to crizotinib, in contradiction to our data. They classified *EML4-ALK* variants into two groups of common variants (V1 and V3a/b, 65.6%) and uncommon variants (V2, V5, V7, and other partner-ALK fusions, 34.6%) [25]. We believe that the reason for the discrepancy is that they did not consider the stability of *EML4-ALK* variants. While we prepared this manuscript, Yoshida et al. reported that after treatment with crizotinib, PFS of the *EML4-ALK* V1 group (19 patients, 54%) is superior to that of the non-V1 group (V2, five patients, 14%; V3a/b, four patients, 12%; other variants, seven patients, 20%) [26]. The difference in PFSR after crizotinib treatment between the V1 and non-V1 groups in that study is much smaller than the difference between groups "variants 1/2/others and variants 3a/b" in our study (a stark difference in the graph, Figure 2). This is probably because classification of patients based on the V1 variant results in insufficient stratification of treatment responses. It should be emphasized that the V3a- and V5a- expressing cells did not respond well to treatment with advanced next-generation ALK inhibitors. In spite of a lack of clinical data, these *in vitro* data suggest that more potent and structurally distinct ALK inhibitors should be developed to

target the stable and treatment-resistant *EML4-ALK* variants.

In conclusion, our findings reveal that there is a subset of *ALK* rearrangement-positive NSCLCs responding differently to ALK inhibitors according to *EML4-ALK* variants. The ALK inhibitor-resistant patients harboring variants 3a/b represent 44% of our study population. Therefore, stratification of patients with advanced *ALK* rearrangement-positive NSCLC by the variant-specific genotype should help to predict clinical responses to ALK inhibitors.

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Disclosure

All the authors agree that there are no conflicts of interest. No competing financial interests exist.

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Figure Legends

Figure 1. The CONSORT flow chart.

Figure 2. Kaplan-Meier curves for the progression-free survival (PFS) in patients with *EML4-ALK* variants 1/2/others versus variants 3a/b. Two-year PFSR was 76.0% (95% CI 56.8–100) in group *EML4-ALK* variants 1/2/others versus 26.4% (95% CI 10.5–66.6) in group variants

3a/b among crizotinib-treated patients (N = 44) (A). Meanwhile, 2-year PFSR was 69.0% (95% CI 49.9–95.4) in group variants 1/2/others versus 32.7% (95% CI 15.6–68.4) in group variants 3a/b for all ALK inhibitors: crizotinib, alectinib, and ceritinib (N = 51) (B).

Figure 3. Sensitivity of EML4-ALK variant-expressing Ba/F3 cells to ALK inhibitors. Tyr-1604-phosphorylated ALK was detected by western blotting (A) and with an enzyme-linked immunosorbent assay (ELISA) kit (B) in Ba/F3 cells stably expressing EML4-ALK V1, V2, V3a or V5a. (C) EML4-ALK V1-, V2-, V3a-, and V5a-expressing Ba/F3 cells were treated with increasing doses of crizotinib, ceritinib, or alectinib for 72 h. Cell viability was determined using CellTiter-Glo assays. The IC_{50} values were calculated using the Prism 5.0 software. The mean of three experiments is shown in each column; the bars denote SD. (D) IC_{50} of ALK inhibitors in EML4-ALK variants-expressing Ba/F3 cells. (E) Sensitivity of NSCLC cells harboring an *ALK* translocation and EML-ALK variant-expressing bronchial epithelial cells, BEAS-2B, to ALK inhibitors. NSCLC cells (H3122 with *EML4-ALK* V1 and H2228 with *EML4-ALK* V3b) and BEAS-2B cells transiently expressing an EML4-ALK variant were incubated with increasing concentrations of ALK inhibitors for 72 h. The growth inhibitory effects of ALK inhibitors were determined using the CellTiter-Glo assay. The data points represent the means of three independent experiments; the bars denote SD. (F) IC_{50} of ALK inhibitors in H3122, H2228, and EML4-ALK variants-expressing BEAS-2B cells.

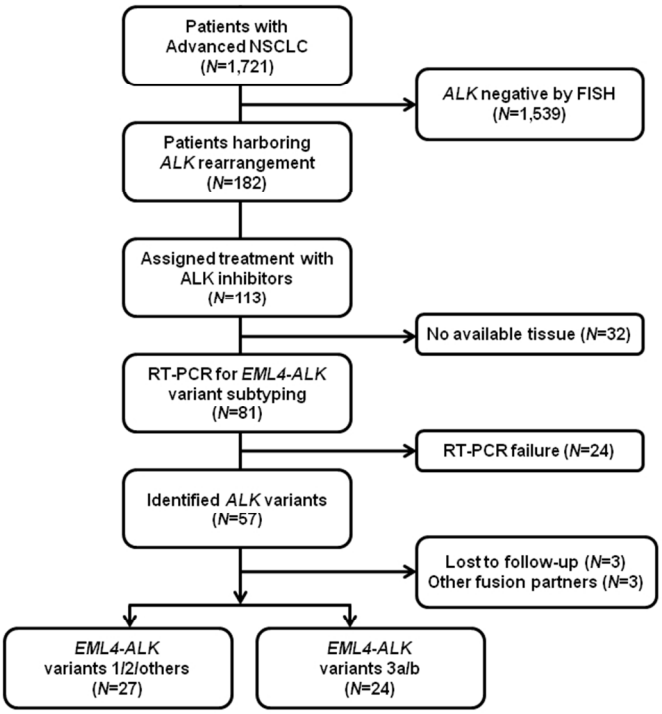

Figure 1

Figure 1

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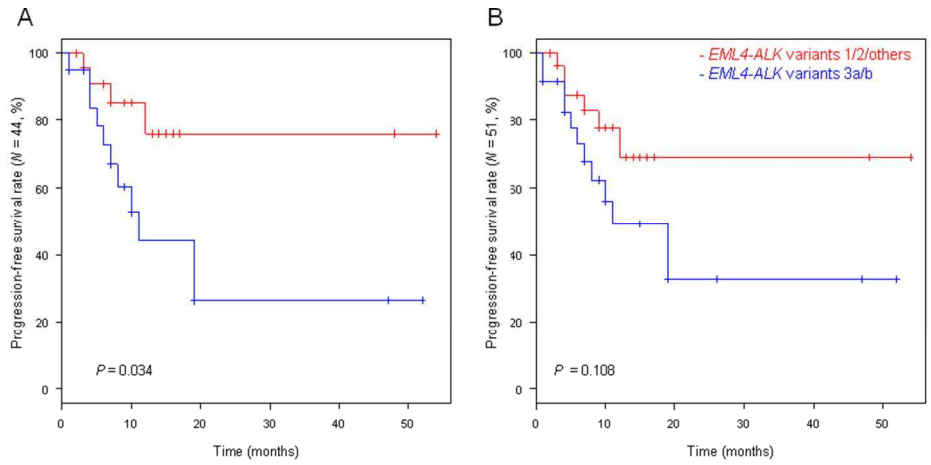

Figure 2

Figure 2

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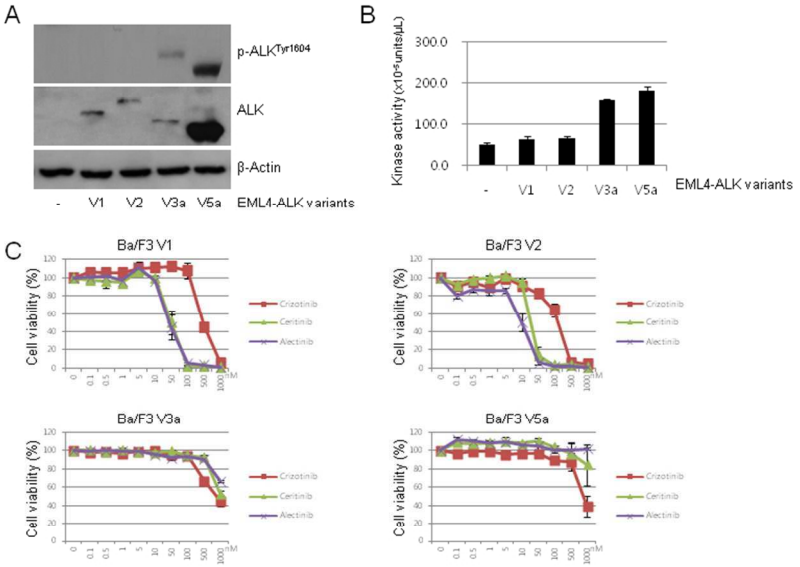


D

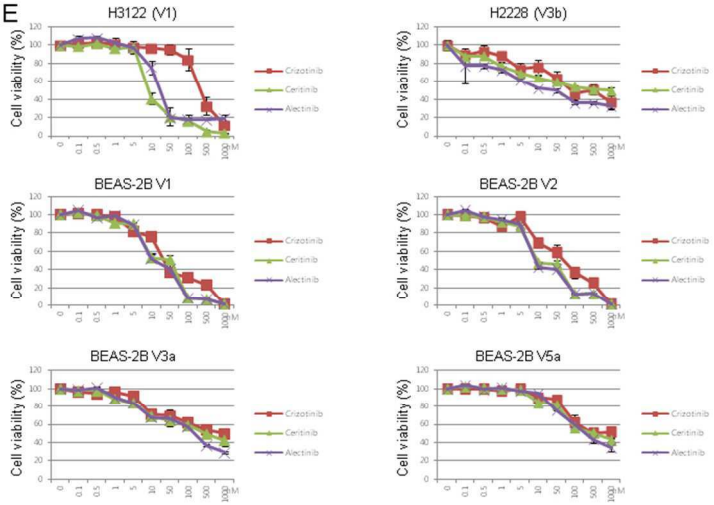
EML4-ALK variants	IC_{50} (nM)		
	Crizotinib	Ceritinib	Alectinib
Ba/F3 V1	471.2	48.16	42.55
Ba/F3 V2	268.8	28.34	14.99
Ba/F3 V3a	>500	>500	>500
Ba/F3 V5a	>500	>500	>500

Figure 3

Figure 3

254x190mm (96 x 96 DPI)

E



F

EML4-ALK variants	IC_{50} (nM)		
	Crizotinib	Ceritinib	Alectinib
H3122 (V1)	296.6	15.14	26.58
H2228 (V3b)	>100	>100	>100
BEAS-2B V1	24.5	22.82	17.12
BEAS-2B V2	57.62	16.48	12.94
BEAS-2B V3a	>500	>500	>500
BEAS-2B V5a	>500	>500	>500

Figure 3

Figure 3

254x190mm (96 x 96 DPI)

Table 1. Frequency of *ALK* translocations.

Groups	Variants	*N* (%)
Variants 1/2/others	*EML4-ALK* V1	18 (33.3)
	EML4-ALK V2	6 (11.1)
	EML4-ALK V7	1 (1.8)
	EML4-ALK unknown[a]	2 (3.7)
Variants 3a/b	*EML4-ALK* V3a/b	24 (44.4)
Other fusion partners		3 (5.6)
Total		54 (100)

[a]Unknown variants: E14del2;del22A20 and E17;del70A20.

Table 2. Comparison of clinical responses in ALK inhibitor-treated patients between groups *EML4-ALK* variants 1/2/others and variants 3a/b.

Response	Crizotinib [*N* (%)]			ALK inhibitors[a] [*N* (%)]		
	V1/2/others (*N* = 24)	V3a/b (*N* = 20)	*P* value	V1/2/others (*N* = 27)	V3a/b (*N* = 24)	*P* value
Objective response rate	20/24 (83.3)	15/20 (75.0)	0.709	23/27 (82.1)	17/24 (70.1)	0.310
Disease control rate	24/24 (100)	18/20 (90.0)	0.201	27/27 (100)	21/24 (87.5)	0.097

[a]ALK inhibitors include crizotinib, alectinib, and certinib.

Supplementary methods

Detection of the *ALK* gene rearrangement

An *ALK* rearrangement was detected by FISH analysis using a break-apart probe specific for the *ALK* locus (Vysis LSI ALK dual-color, break-apart rearrangement probe; Abbott Molecular, Abbott Park, IL, USA) in formalin-fixed, paraffin-embedded (FFPE) tumor tissue samples. FISH-positive samples were defined as those with more than 15% of tumor cells showing split signals or an isolated red signal (3′signal) as described previously [1, 2].

RNA extraction and cDNA synthesis

Total RNA was extracted from the FFPE cell blocks using the PureLink™ FFPE Total RNA Isolation Kit (Invitrogen, Carlsbad, CA, USA) with the following protocol modifications. The resulting RNA was eluted with 50 μL of elution buffer. The concentration and purity of the extracted RNA were determined by means of a Nanodrop (Thermo Fisher. USA). The extracted RNA was stored at −80°C until use. We used 250 ng of total RNA to generate cDNA using the Super Script VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA).

Peptide nucleic acid (PNA)-mediated quantitative PCR (qPCR) assay for *EML4-ALK* screening and genotyping

The *EML4-ALK* fusion RNA was detected using PANAqPCR™ *EML4-ALK* Fusion Gene Detection Screening and Genotyping Kit (Panagene, Daejeon, Korea), which tests for 28 types of known *ALK* rearrangements including E6;A19, E6;A20 (variant 3a), E6ins33;A20 (variant 3b, 3 subtypes), E6;ins18A20, E13;A20 (variant 1, 5 subtypes), E13;ins69A20 (variant 6, 2 subtypes), E20;A20 (variant 2, 2 subtypes), E20;ins18A20 (2 subtypes),

E14ins11;del49A20 (variant 4), E14;del12A20 (variant 7), E14;del36A20, E14ins2;ins56A20, E2;A20 (variant 5a), E2;ins117A20 (variant 5b), E17;ins30A20 (variant 8a), E17ins61;ins34A20 (variant 8b), E17ins65;A20, E17;ins68A20, and E17del58;ins39A20.

EML4-ALK translocation-positive samples were further genotyped to detect the presence of any of these 12 variants: E6;A20 (variant 3a), E6ins33;A20 (variant 3b), E13;A20 (variant 1), E13;ins69A20 (variant 6), E20;A20 (variant 2), E14ins2;ins56A20, E14ins11;del49A20 (variant 4), E14;del12A20 (variant 7), E2;A20 (variant 5a), E2;ins117A20 (variant 5b), E17;ins30A20 (variant 8a), and E17ins61;ins34A20 (variant 8b). The PCR was conducted under the following conditions: 2 min at 50°C; 15 min at 95°C; five cycles of 10 s at 95°C and 30 s at 58°C; and 45 cycles of 10 s at 95°C, 30 s at 58°C, and 15 s at 72°C. A positive result for *EML4-ALK* was defined as a threshold cycle (C_T) value <40, and the internal control was defined as a C_T value <36.

Cell culture and transfection

The *ALK* and *EML4*cDNAs were cloned by PCR from human cDNA (Clontech, Mountain View, CA, USA).*EML4-ALK* V1, V2, V3a, and V5a were constructed by ligating *EML4*cDNAs with *ALK* cDNA at the desired position by PCR as described previously [3]. Cell lines Ba/F3 (murine pro-B cells), BEAS-2B (human normal bronchial epithelial cells), H3122 (human NSCLC cells with *EML4-ALK* variant 1), and H2228 (human NSCLC cells with *EML4-ALK* variant 3b) were grown in RPMI 1640 (Invitrogen-GIBCO, Grand Island, NY, USA) with 10% of fetal bovine serum (FBS), 50 μg/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified 5% CO_2 incubator [4-6]. The medium for Ba/F3 cells was supplemented with IL-3 (0.5 ng/mL, Enzo, Farmingdale, NY, USA). To establish stable cell lines, Ba/F3 cells were transfected with *EML4-ALK* variant-encoding plasmids using the Neon^{TM} Transfection System (MPK5000; Invitrogen). After 48 hours, the medium for the

cells was replaced with an IL-3-free medium containing 200 μg/mL hygromycin B (10680-010, Invitrogen) to select EML4-ALK-expressing cells. BEAS-2B cells were transiently transfected with *EML4-ALK* variants using Lipofectamine 2000 (11668027, Invitrogen).

Compounds

ALK inhibitors including crizotinib (PF-02341066), ceritinib (LDK378), and alectinib (CH5424802) were purchased from Selleckchem (Houston, TX). Stock solutions were prepared in DMSO. The compounds were diluted with a fresh medium before each experiment, and the final concentration of DMSO was <0.1%.

Western blot analysis

Whole-cell lysates were prepared in RIPA lysis buffer [50 mMTris-HCl (pH 8.0), 150 mMNaCl, 0.5 mM EDTA, 1 mMdithiothreitol (DTT), 0.1% SDS, 1% NP-40] containing a protease inhibitor cocktail (BP-477; Boston BioProducts, Worcester, MA). Immunoblot analyses were carried out with anti-phospho-ALK (Tyr1604) (3341; Cell Signaling Technology, Beverly, MA), anti-ALK (104809; NOVO, OULU, Finland), and anti-β-actin (A5441; Sigma, St. Louis, MO) antibodies. The blots were visualized using the SuperSignal West Pico Chemiluminescent Substrate (34080; Pierce, Rockford, IL).

Viability assay

Cells were seeded in a complete growth medium in 96-well plates at 3×10^3 cells per well. After 24 hours, the cells were incubated with crizotinib, ceritinib, alectinib, or DMSO in the presence of 10% of FBS. After 72 hours of treatment, cell viability was determined using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI, USA). The half-maximal inhibitory concentration (IC_{50}) values were calculated from dose-response curves in

the Prism 5.0 software (GraphPad, San Diego, CA).

In vitro ALK kinase assay

Cells were lysed with RIPA lysis buffer. Three hundred nanograms of total cell extracts per reaction were analyzed using the Universal Tyrosine Kinase Assay Kit (MK410; Takara Bio, Pittsburg, PA, USA). Each experiment was repeated at least thrice.

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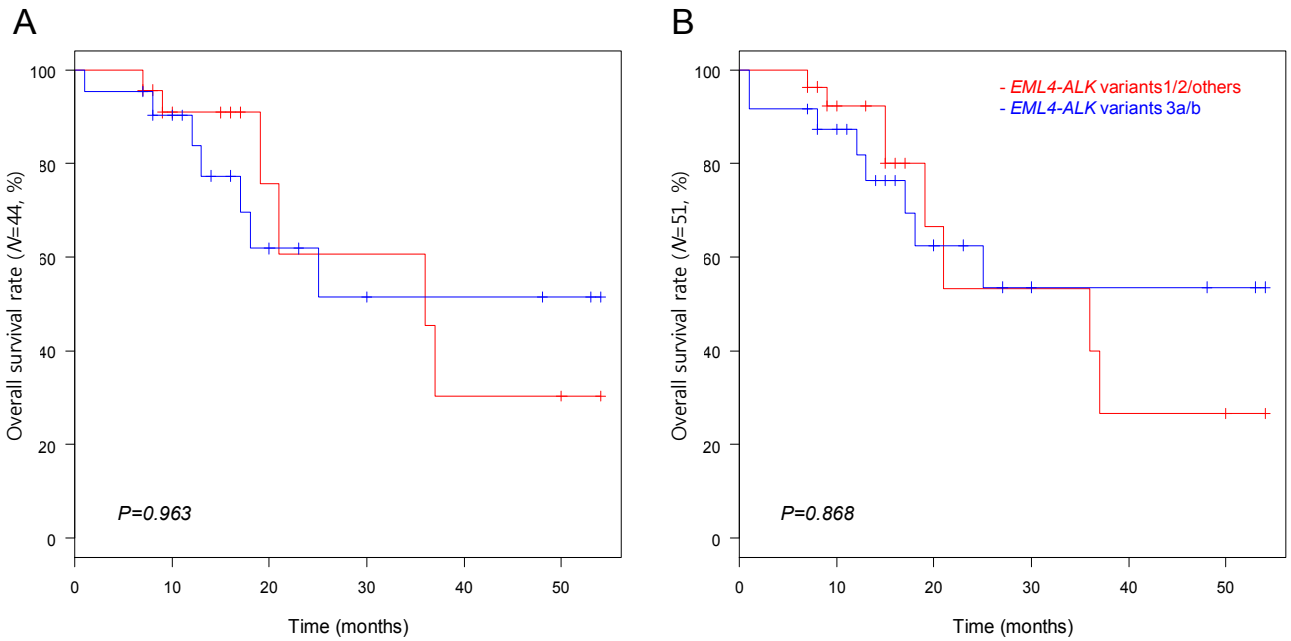

Figure S1

Figure S1. Kaplan-Meier curves for the overall survival (OS) in patients with *EML4-ALK* variants 1/2/others versus variants 3a/b among crizotinib-treated patients ($N = 44$) (A) and all ALK inhibitors-treated patients ($N = 51$) (B).

Table S1. Clinicopathologic characteristics of the analyzed patients (*N* = 54).

Parameters		*N* (%)
Sex	Male	26 (48.1)
	Female	28 (51.9)
Age in years, median (range)		54 (27 - 78)
Smoking history	Never	35 (64.8)
	≤10 pack-years	6 (11.1)
	>10 pack-years	13 (24.1)
Diagnosis	Adenocarcinoma	52 (96.3)
	Squamous cell carcinoma	1 (1.8)
	Large cell neuroendocrine carcinoma	1 (1.8)
Brain metastasis at the inclusion	Absent	36 (66.7)
	Present	18 (33.3)
Number of previous therapy	0	21 (38.9)
	1	15 (27.8)
	2	9 (16.7)
	≥3	9 (16.7)
ALK inhibitors	Crizotinib	44 (81.5)
	Crizotinib+onalespib (Hsp90 inhibitor)	3 (5.6)
	Alectinib	4 (7.4)
	Ceritinib	3 (5.6)
Follow up in months, median (range)		15 (1 - 53)

Table S2. Clinicopathologic characteristics of the ALK inhibitors[a]-treated patients.

Parameters - *N* (%)	V1/2/others (*N* = 27)	V3a/b (*N* = 24)	*P* value
Sex			1.000
Male	13 (48.1)	12 (50.0)	
Female	14 (51.9)	12 (50.0)	
Age in years, mean (range)	53 (27 - 68)	58 (37 - 77)	0.100
Smoking history			0.836
Never	17 (63.0)	16 (66.7)	
≤10 pack-years	4 (14.8)	2 (8.3)	
>10 pack-years	6 (22.2)	6 (25.0)	
Diagnosis			0.704
Adenocarcinoma	26 (96.3)	23 (95.8)	
Squamous cell carcinoma	1 (3.7)	0 (0)	
Large cell neuroendocrine carcinoma	0 (0)	1 (4.2)	
Number of previous therapy			0.201
0	8 (29.6)	13 (54.2)	
1	6 (22.2)	6 (25.0)	
2	6 (22.2)	3 (12.5)	
≥3	7 (25.9)	2 (8.3)	
Brain metastasis at the inclusion			0.372
Absent	16 (59.3)	18 (75.0)	
Present	11 (40.7)	6 (25.0)	
ALK inhibitors			0.648
Crizotinib	22 (81.5)	19 (79.2)	
Crizotinib+Onalespib	2 (7.4)	1 (4.2)	
Alectinib	1 (3.7)	3 (12.5)	
Ceritinib	2 (7.4)	1 (4.2)	
Follow up in months, median (range)	9.5 (1 - 49)	7 (1 - 53)	0.879

[a]ALK inhibitors include crizotinib, alectinib, and certinib.

Table S3. Adverse events after treatments with ALK inhibitors[a].

Adverse events - *N* (%)	Grade 1	Grade 2	Grade 3	Grade 4	No grade[b]	Total
AST/ALT elevation	19 (35.2)	8 (14.8)	4 (7.4)			31 (57.4)
Peripheral edema	2 (3.7)	1 (1.9)				3 (5.6)
Skin rash	1 (1.9)		3 (5.6)			4 (7.4)
Neutropenia		4 (7.4)	3 (5.6)	1 (1.9)		8 (14.8)
Anorexia		2 (3.7)	1 (1.9)			3 (5.6)
Thrombocytopenia		1 (1.9)		1 (1.9)		2 (3.7)
Nausea		1 (1.9)				1 (1.9)
Dizziness		1 (1.9)				1 (1.9)
Vomiting		1 (1.9)				1 (1.9)
Anemia			1 (1.9)			1 (1.9)
Creatinine elevation			1 (1.9)			1 (1.9)
Necrotizing fasciitis				1 (1.9)		1 (1.9)
Epigastric pain					2 (3.7)	2 (3.7)
Renal cysts					2 (3.7)	2 (3.7)

[a]ALK inhibitors include crizotinib, alectinib, and certinib.

[b]There were inadequate recorded toxicity grades.

Table S4. Dose reduction of ALK inhibitors.

Dose reduction - *N* (%)	V1/2/others (*N* = 27)	V3a/b (*N* = 24)	Others (*N* = 3)	Total (*N* = 54)
Crizotinib	5 (18.5)	4 (16.7)	2 (66.7)	11 (20.4)
Crizotinib+onalespib	1 (3.7)	1 (4.2)		2 (3.7)
Alectinib		1 (4.2)		1 (1.9)
Ceritinib	1 (3.7)	1 (4.2)		2 (3.7)
Total	7 (25.9)	7 (29.2)	2 (66.7)	16 (29.6)

Table S5. Progression according to dose reduction.

Dose reduction - *N* (%)	Progression	No progression	Total	*P* value
Absent	17 (73.9)	21 (67.7)	38 (70.4)	0.767
Present	6 (26.1)	10 (32.3)	16 (29.6)	
Total	23	31	54	

Table S6.1. Clinical responses in crizotinib-treated patients between groups *EML4-ALK* variants 1/2/others and variants 3a/b.

Assessment	Objective response rate, *N* (%)			Disease control rate, *N* (%)		
	V1/2/others (*N* = 24)	V3a/b (*N* = 20)	*P* value	V1/2/others (*N* = 24)	V3a/b (*N* = 20)	*P* value
1st	19/24 (79.2)	14/20 (70.0)	0.509	24/24 (100)	18/20 (90.0)	0.201
2nd	17/22 (77.3)	12/18 (66.7)	0.498	19/22 (86.4)	15/18 (83.3)	1.000
3rd	12/14 (85.7)	7/12 (58.3)	0.190	13/14 (92.9)	10/12 (83.3)	0.580
4th	8/10 (80)	6/7 (85.7)	1.000	10/10 (100)	7/7 (100)	1.000
5th	5/6 (83.3)	3/7 (42.9)	0.266	5/6 (83.3)	3/7 (42.9)	0.266

Table S6.2. Clinical responses in ALK inhibitor[a]-treated patients between groups *EML4-ALK* variants 1/2/others and variants 3a/b.

Assessment	Objective response rate, *N* (%)			Disease control rate, *N* (%)		
	V1/2/others (*N* = 27)	V3a/b (*N* = 24)	*P* value	V1/2/others (*N* = 27)	V3a/b (*N* = 24)	*P* value
1st	21/27 (77.8)	16/24 (66.7)	0.531	27/27 (100)	21/24 (87.5)	0.097
2nd	19/25 (76.0)	13/20 (65.0)	0.515	21/25 (84.0)	17/20 (85.0)	1.000
3rd	13/20 (65.0)	6/10 (60.0)	1.000	18/20 (90.0)	9/10 (90.0)	1.000
4th	9/12 (75.0)	8/9 (88.9)	0.603	12/12 (100)	9/9 (100)	1.000
5th	6/8 (75.0)	4/7 (57.1)	0.608	6/8 (75.0)	4/7 (57.1)	0.608

[a]ALK inhibitors include crizotinib, alectinib, and certinib.