Drug resistance mechanisms in Japanese anaplastic lymphoma kinase-positive non–small cell lung cancer and the clinical responses based on the resistant mechanisms

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Funding information
Nippon Foundation, Grant/Award Number: N/A; Japan Agency for Medical Research and Development, Grant/Award Number: JP19ck0106472h0001 and JP19cm0106203h0004; Japan Society for the Promotion of Science, Grant/Award Number: JP15H02368, JP16H04715, JP17H06327 and JP19H03524

Abstract
The treatment for anaplastic lymphoma kinase (ALK)-positive lung cancer has been rapidly evolving since the introduction of several ALK tyrosine kinase inhibitors (ALK-TKI) in clinical practice. However, the acquired resistance to these drugs has become an important issue. In this study, we collected a total of 112 serial biopsy samples from 32 patients with ALK-positive lung cancer during multiple ALK-TKI treatments to reveal the resistance mechanisms to ALK-TKI. Among 32 patients, 24 patients received more than two ALK-TKI. Secondary mutations were observed in 8 of 12 specimens after crizotinib failure (G1202R, G1269A, I1171T, L1196M, C1156Y and F1245V). After alectinib failure, G1202R and I1171N mutations were detected in 7 of 15 specimens. G1202R, F1174V and G1202R, and P-gp overexpression were observed in 3 of 7 samples after ceritinib treatment. L1196M + G1202R, a compound mutation, was detected in 1 specimen after lorlatinib treatment. ALK-TKI treatment duration was longer in the on-target treatment group than that in the off-target group (13.0 vs 1.2 months). In conclusion, resistance to ALK-TKI based on secondary mutation in this study was similar to that in previous reports, except for crizotinib resistance. Understanding the appropriate treatment matching resistance mechanisms contributes to the efficacy of multiple ALK-TKI treatment strategies.

KEYWORDS
anaplastic lymphoma kinase, mutation, progression-free survival, resistance mechanism, tyrosine kinase inhibitor
1 | INTRODUCTION

Soda et al (2007) found anaplastic lymphoma kinase (ALK) rearrangements in the specimens of patients with non–small cell lung cancer (NSCLC).\(^1\) ALK-positive (ALK [+] NSCLC accounts for only 3%-4% of all NSCLC cases.\(^2\) Crizotinib is the first ALK tyrosine inhibitor (ALK-TKI) to undergo clinical development.\(^3\) In the PROFILE 1007 and 1014 trials, which are randomized phase III studies comparing crizotinib and the standard first-line and second-line cytotoxic chemotherapies for ALK (+) NSCLC, patients who received crizotinib had significantly better progression-free survival (PFS) than those who received chemotherapy; thus, crizotinib became a standard treatment for ALK (+) NSCLC.\(^4,5\) Several second-generation and third-generation ALK-TKI have also been developed, including alectinib, ceritinib, brigatinib and lorlatinib. At present, among these ALK-TKI, four have been approved and are available in clinical practice in Japan, and five ALK-TKI are available in the USA and the EU.

Alectinib is a second-generation ALK-TKI, which has shown anti-tumor activity against ALK (+) NSCLC in patients previously treated with crizotinib.\(^6\) In addition, J-ALEX and ALEX, which are phase III studies, have shown that alectinib significantly improved PFS compared with crizotinib and had a better toxicity profile.\(^7,8\) Therefore, alectinib is commonly used as a first-line treatment for ALK (+) NSCLC. Ceritinib is another second-generation ALK-TKI. ASCEND-5, which is a phase III study, has shown that ceritinib significantly improved PFS compared with chemotherapy in patients with ALK (+) NSCLC who previously received chemotherapy and crizotinib.\(^9\) In addition, as a first-line treatment for ALK (+) NSCLC, ceritinib significantly improved PFS compared with platinum doublet chemotherapy.\(^10\) In September 2018, the use of lorlatinib, a third-generation ALK-TKI, which had better potency to secondary resistance mutation than that of prior generation ALK-TKI in a preclinical model,\(^11\) was approved for the treatment of patients whose disease has progressed after crizotinib failure or those who had been treated with another ALK-TKI based on the promising results of a phase I/II trial investigating the efficacy of such drugs in patients with ALK (+) lung cancer.\(^12,13\)

Due to these developments, several ALK-TKI are used sequentially in clinical practice at present.\(^14\) However, disease progression due to acquired resistance inevitably occurs after several years, resulting in a major problem in clinical practice.

Recently, several mechanisms of acquired resistance to ALK-TKI have been reported. Secondary mutations in the tyrosine kinase domain of ALK are among the major resistance mechanisms. Moreover, bypass pathway activation, such as epidermal growth factor receptor (EGFR), Met, KRAS, ALK amplification, cKIT amplification with SCF upregulation, overexpression of P-glycoprotein (P-gp) and SCLC transformation, has been identified as another resistance mechanism.\(^15-23\)

In contrast with T790M mutation of EGFR, which accounts for 50% of all resistance mechanisms of first- and second-generation EGFR-TKI, different mutations occurred during ALK-TKI treatment. First-, second- and third-generation ALK-TKI have different potencies for inhibiting secondary mutations, and they can potentially treat resistance to some secondary mutations associated with the use of other ALK-TKI.\(^22\) Therefore, the investigation of resistance mechanisms is extremely important in choosing the appropriate ALK-TKI, and the sequential use of ALK-TKI based on resistance mechanism in patients with ALK (+) lung cancer may result in better treatment outcomes.

In this study, we analyzed resistance mechanisms through serial tumor biopsy in Japanese patients during sequential treatment with ALK-TKI and revealed the sequential transition of resistance mechanisms, particularly those associated with the use of first-line alectinib.

2 | MATERIALS AND METHODS

2.1 | Patients and tissue sampling

We collected 112 tumor biopsy specimens or cavity fluid containing cancer cells from 32 ALK (+) patients during treatment. Of the 32 patients, 25 received more than two ALK-TKI. Moreover, 23 patients received crizotinib, 24 alectinib and 11 ceritinib. A total of 8 tissue samples were obtained via transbronchial lung biopsy and 80 from pleural or pericardial effusion. A section of the sample was frozen at −80°C.

Meanwhile, to establish cell lines, a part of the sample was cultured in RPMI 1640/F-12 supplemented with 15% FBS (RP/F12), 10-mmol/L HEPES and Antibiotic-Antimycotic (Invitrogen) or ACL-4 medium (Invitrogen) supplemented with 1% FBS and Antibiotic-Antimycotic (Invitrogen) or STEMPRO hESC SFM medium with Antibiotic-Antimycotic (Invitrogen) and 10 μmol/L of Y27632 (LC Laboratories).

2.2 | Analysis of resistant mechanisms

Genomic DNA was isolated from cell pellets or fresh frozen specimens with the DNeasy Blood & Tissue Kit (QIAGEN) according to the manufacturer’s protocol. Total RNA was isolated from cell pellets or fresh frozen specimens with the RNase Mini Kit (QIAGEN) according to the manufacturer’s instructions.

Genomic or complementary DNA was extracted from the specimens, and the sequence in the ALK kinase domain was assessed using Sanger sequencing with the primers shown below. If resistant secondary mutations were not identified, the tumor samples were assessed for the presence of ALK independent resistance mechanisms, including EGFR, MET, KRAS or ALK mutation/amplification, cKIT amplification, overexpression of P-gp\(^23\) or SCLC transformation, and cell lines, which are established from biopsy specimens through targeted next-generation sequencing (with Agilent HaloPlex custom panel and Illumina MiSeq as described in our previous paper\(^24\)), using the Proteome Profiler Human Phospho-RTK Array Kit (R&D
Systems), immunoblot, immune-histochemistry evaluation and histological diagnosis.

2.2.1 Primers for EML4-ALK PCR amplification

Forward: CACCATGGACGTTTCCGCGCA
Reverse: TCAAGGCCCCAGGGCTGTCATGC
Kinase domain forward: AGCCCGTACGAGCTGAGC
Kinase domain reverse: CCATATTCTATGGCGACTGC.

2.2.2 Sequencing primers

1F: TCCAGAAAAGCAAAGATGCTACTCC
1R: GTCAACATCGGAAGGAATGACATGG
2F: TGGGTTTTCCAAAACACGATGC
2R: AGGTGTCTGAGCTTGTACTCAGG
3F: TTGCTGTGGCGATAGAATATGG
3R: GGTGACAAACTCCAGAACTCC
4F: ACCGCTTTGCCGATAGAATATGG.

2.3 Statistical analysis

Between-group comparisons were performed using the $\chi^2$ test. Time-to-event endpoints (time-to-treatment failure [TTF] and overall survival) were estimated using the Kaplan-Meier method and the Graph Pad Prism 7 software, and significant differences were identified using the log-rank test. Other data, including clinical background information, were statistically analyzed using the JMP software version 14.2 (SAS Institute). A $P$ value <0.05 was considered statistically significant and a $P$ value <0.10 moderately significant.

The study protocol was approved by the institutional review board of the Japanese Foundation for Cancer Research (JFCR), and written informed consent was obtained from all patients. The clinical information of each patient obtained from the medical records was reviewed.

3 RESULTS

3.1 Baseline characteristics of the patients and treatment

Thirty-two patients with ALK (+) lung cancer who received at least one ALK-TKI at the Cancer Institute Hospital of JFCR from May 2011 to September 2018 were included. The characteristics of the patients are shown in Table 1 and are similar to those reported previously. The median age at diagnosis of lung cancer was 47 years, and a female predominance was observed. In terms of histological type, the patients presented with adenocarcinoma. Of 32 patients, 8 received one ALK-TKI, 13 received two ALK-TKI and 11 received three ALK-TKI. Twenty-three patients received crizotinib, 24 alectinib, 11 ceritinib and 3 lorlatinib.

To evaluate resistance mechanisms, 12, 15, 7 and 3 tissue samples were obtained from patients receiving crizotinib, alectinib, ceritinib and lorlatinib, respectively. The median attempt for re–biopsy in 1 patient was 2 (range: 1-20) times.

3.2 Analysis of resistance mechanism to anaplastic lymphoma kinase-tyrosine kinase inhibitors

Among the 112 tumor biopsy specimens used in 37 re–biopsy attempts, 18 specimens presented with secondary mutations in the tyrosine kinase domain of ALK, and four ALK independent resistance mechanisms (one EGFR activation, two P-gp overexpressed cases and one MET amplification) were identified in 4 specimens. In the remaining 15 samples, resistance mechanisms could not be identified (Table S1).

Resistance mechanisms to each ALK-TKI are presented in Figure 2 and Table S1. Mutations in the ALK kinase domain were considered the major drivers of resistance to ALK-TKI in our cohort: 8 (−66.7%) of 12, 7 (47%) of 15, 2 (−28.5%) of 7 and 1 (33%) of 3 specimens collected after crizotinib, alectinib, ceritinib and lorlatinib failure, respectively. The detailed resistance mechanisms were similar to those of previous reports. In crizotinib-resistant specimens, G1202R, G1269A, I1171T, L11196M, C1156Y and F1245V, as well as one EGFR activation working as
the bypass pathway, were the resistance mechanisms based on the cell line established using resistant specimens (Figure S1). The frequency of secondary mutations in crizotinib resistance patients seems to be higher than that reported in the USA.\textsuperscript{22} In alectinib-resistant specimens, G1202R and I1171N mutations were detected in the ALK, which accounted for approximately half of all alectinib-resistant cases. Meanwhile, the mechanisms in other specimens were not identified. In 2 of 7 ceritinib-resistant specimens, the following ALK secondary mutations were found: G1202R, F1174V and G1202R, and L1196M (with P-gp overexpression). However, resistance mechanisms to ceritinib in our cohort were more complicated than expected. F1174V harboring cells and G1202R mutated cells coexisted independently in 1 pleural fluid specimen. The overexpression of P-gp, a drug efflux transporter protein, was identified in 2 ceritinib refractory specimens but not in pre–treatment samples by immunohistochemistry and immunoblotting analysis as described in our previous paper.\textsuperscript{23} Of note, 1 of these specimens had P-gp overexpression concurrent with L1196M mutation after sequential treatment with crizotinib, alectinib and ceritinib. MET gene amplification, which is well-known as EGFR-TKI resistance, a cause of bypass pathway activation-mediated resistance to alectinib or ceritinib, was identified in 1 specimen. L1196M + G1202R, a compound mutation, was also a resistance mechanism to lorlatinib in patients with L1196M who previously experienced relapse while on crizotinib treatment.\textsuperscript{25}

### 3.3 | Efficacy of the sequential administration of anaplastic lymphoma kinase-tyrosine kinase inhibitors

In this study, to assess the advantage of sequential ALK-TKI therapy, the overall survival of patients with ALK (+) lung cancer was analyzed according to the number of ALK-TKI administered. Patients who received only one ALK-TKI had shorter survival than those who received two or more TKI (0.94 vs 4.1 vs 3.3 years; \(P < 0.0001\), Figure 3A). The inferiority of one ALK-TKI treatment might reflect the clinical situation in which patients received only crizotinib from September 2012 to October 2014. Contrary to our expectation, third ALK-TKI have less additional benefits based on the evaluation of the TTF of each line of ALK-TKI. Analysis of the median TTF of ALK-TKI in each order showed that third ALK-TKI had poor benefits compared with first and second ALK-TKI (3.0, 11.7, and 9.2 months, respectively; \(P = 0.0047\), Figure 3B).

To elucidate the reason why third ALK-TKI resulted in poor outcome, we retrospectively reviewed the relationship between resistance mechanisms and the administration of ALK-TKI from the viewpoint of whether ALK-TKI were administered as on-target or
off-target drugs. On-target indicates that the administered ALK-TKI is effective considering the molecular characteristic analysis results of the re–biopsy specimens: for example, alectinib to C1156Y or F1174C, ceritinib to I1171N, and lorlatinib to G1202R. Meanwhile, off-target indicates that the chosen ALK-TKI are not effective because they have limited activities or no activities at all based on pre-clinical data. The TTF of ALK-TKI for the on-target group was longer than that of the off-target group, as expected (Figure 3C, time on TKI: 13.0 vs 1.2 months, n = 4 and 7, respectively). Interestingly, the TTF of another group whose resistance mechanism was not identified via re–biopsy may be similar to that of the on-target group (median: 13.5 months, Figure 3C).

In our clinical experience, we encountered a patient who received alectinib after acquiring resistance to crizotinib via F1245V mutation (Figure 4A) and another patient with rapid disease progression who died while on alectinib treatment, which was administered after relapse with ceritinib due to MET gene amplification (Figure 4B). Of note, the cell line from the pleural fluid sample of this patient harbored MET gene amplification and showed partial sensitivity to crizotinib in vitro. Thus, the patient might have had a better
outcome after treatment with crizotinib instead of alectinib if c-Met amplification was identified within several days.

4 | DISCUSSION

Treatment for ALK (+) lung cancer has been changing since the introduction of ALK-TKI, such as crizotinib, alectinib, ceritinib and lorlatinib. A new class of ALK-TKI, which is used as front-line treatment, has improved PFS, and its sequential administration after relapse with prior ALK-TKI is beneficial. In parallel with the development of these ALK-TKI, several resistance mechanisms have been found. One of the major resistance mechanisms of ALK-TKI is secondary mutation in the kinase domain. Sensitivity of ALK-TKI is different in each secondary mutation, as previously reported in a preclinical model by Gainor et al. The approval of these ALK-TKI has changed clinical practice. That is, patients with ALK (+) lung cancer are generally treated with several ALK-TKI; this has raised some questions regarding how to accurately administer ALK-TKI in terms of resistance mechanisms.

In this study, several points focusing on resistance mechanisms associated with ALK-TKI treatment were presented via serial re-biopsies after treatment failure in clinical practice. First, the present study was the first to report on resistance mechanisms against alectinib in Japan with a measurable number of participants. Second, secondary mutation may be observed more frequently in patients with crizotinib resistance in our cohort compared to previous reports, which may be due to the relatively higher plasma concentrations of crizotinib among Japanese ALK (+) patients than among Caucasian patients, as indicated by Fujiwara et al. Third, additional mutation in the ALK kinase domain, which mediates acquired resistance to ALK-TKI, usually emerges during crizotinib, ceritinib and alectinib failure, whereas a compound mutation in the kinase domain was the only resistance mechanism identified in lorlatinib failure. The compound mutation leading to lorlatinib failure has been reported by Shaw AT et al, Yoda et al and Okada et al in a preclinical model and in some clinical cases. Although Okada and his colleagues have shown that the use of early-generation ALK-TKI and several BCR-ABL inhibitors might treat double mutation after lorlatinib failure, a strategy that can treat this type of resistance has not yet been established. Thus, a new strategy must be investigated as soon as possible because an increasing number of patients with ALK (+) lung cancer will receive lorlatinib in clinical settings.

In addition to the importance of overcoming these unresolved problems, our retrospective study of the relationship between the molecular characteristic analysis results and ALK-TKI indicated that the precise utilization of such medications would contribute to improving prognoses. Based on the study of Gainor et al, exploring resistance mechanisms using re-biopsy specimens might help to resolve this issue. In particular, if PFS of late-line ALK-TKI can be improved, the survival of patients with ALK (+) lung cancer will improve. A technique that can identify resistance mechanisms within a short period of time must be developed to prevent treatment with off-target drugs, which results in shorter PFS. The turnaround time for minutely examining resistance mechanisms using re-biopsy specimens will affect the possibility that treatment with the sequential administration of ALK-TKI would be more beneficial for patients. Cell-free plasma DNA analysis can make significant progress in this field.

The investigation of patients with unidentified mechanisms, as shown in Figure 3C, is another important issue because an optimal
treatment strategy for this group of patients is challenging to establish. Unfortunately, acquired resistance mediated by an unknown mechanism accounts for 30%–50% of all resistance cases. Using the Kaplan-Meier curve of the group with unidentified mechanisms, as shown in Figure 3C, some patients had favorable outcomes, whereas others experienced rapid disease progression. Those with favorable outcomes might have been coincidently treated with on-target drugs after progression due to conquerable resistance mechanisms or due to other factors, such as disease progression on prior ALK-TKI because of insufficient plasma concentration, which is associated with pharmacokinetics. Other possibilities should also be considered as some patients benefit from changing TKI due to an uncommon cause: for example, resistance by P-gp to ceritinib, which can be treated with alectinib or lorlatinib.

However, patients with poor outcomes in the group with unidentified mechanisms might be treated with ineffective off-target drugs. Further studies with detailed information for this group must be conducted in the future. A more accurate evaluation system of resistance mechanisms is required to identify patients who should receive on-target drugs or other optimal treatments. In addition to these efforts, we should continue to elucidate unknown alternative mechanisms that are now included in the off-target group. The identification of an alternative mechanism would lead to the identification of a new strategy that uses alternative drugs.

The present study has several limitations. First, this is a retrospective study that included a small number of patients at a single institution. Second, the analysis method for resistance mechanisms was not completely unified due to developments in detection and analysis methods during the course of this study. However, these points could be unavoidable for the following reasons. First, the number of patients with ALK (+) lung cancer is limited and, second, the amount and quality of re-biopsy samples varied significantly. Future studies should evaluate a larger number of samples using the unified NGS platform. An appropriate material should be used in re-biopsy to identify whether several re-biopsies at different sites at one relapse are necessary considering spatial heterogeneity and whether cell-free plasma DNA/RNA can be a surrogate marker of resistance mechanisms. Third, we did not evaluate the variant type of the ALK fusion gene. Recently, it was found that the resistance mechanism to ALK-TKI may depend on the variant type of ALK fusion.29 The G1202R, a highly refractory resistance mutation, is more likely to emerge from variant 3 than from variant 1.29 In future studies, whether the development of resistance mechanisms in view of temporary transition is observed according to variant type must be confirmed. Fourth, in relation to ALK-TKI resistance mediated by bypass pathway activation, such as EGFR activation or MET gene amplification, a strategy that can treat resistance has not been established in clinical practice. Thus, a therapeutic strategy that can overcome such resistance must be investigated.

In this study, the analysis of resistance mechanisms using re-biopsy specimens obtained from patients with ALK (+) lung cancer was performed, and results revealed that proper utilization of multiple ALK-TKI has become an important issue following the introduction of several ALK-TKI. In clinical practice, the precise sequential use of ALK-TKI (sometimes non–ALK-TKI) based on resistance mechanisms in patients with ALK (+) lung cancer has better treatment outcomes.

ACKNOWLEDGMENTS
We thank all laboratory members, especially Dr A. Takemoto and Ms T. Oh-hara at the Japanese Foundation for Cancer Research for fruitful discussions and technical advice regarding the in vitro experiments. This work was supported by grants from the Japan Society for the Promotion of Science KAKENHI (grant numbers JP16H04715 and JP19H03524, to RK, and JP15H02368 and JP17H06327, to NF) and grants from the Japan Agency for Medical Research and Development (grant numbers JP19cm0106203h0004 and JP19ck0106472h0001, to RK) and The Nippon Foundation (to NF).

CONFLICT OF INTEREST
R. Katayama reports research grants from Chugai, TAKEDA, TAIHO Pharmaceutical, TOPPAN Printing, FUJIFILM, and lecture’s fee from Pfizer, outside the submitted work. N. Yanagitani and F. Ohyanagi report personal fees from Chugai Pharmaceutical, outside the submitted work. M. Nishio reports grants and personal fees from Pfizer, Chugai Pharmaceutical, and Novartis, and grants from Astellas, outside the submitted work. N. Fujita reports grants from Api Co., Ltd. and TOPPAN Printing, and personal fees from Pfizer, outside the submitted work. Other authors have nothing to disclose related to this work.

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**SUPPORTING INFORMATION**

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**How to cite this article:** Yanagitani N, Uchibori K, Koike S, et al. Drug resistance mechanisms in Japanese anaplastic lymphoma kinase-positive non–small cell lung cancer and the clinical responses based on the resistant mechanisms. *Cancer Sci*. 2020;00:1–8. [https://doi.org/10.1111/cas.14314](https://doi.org/10.1111/cas.14314)