

Efficacy of gilteritinib in comparison with alectinib for the treatment of *ALK*-rearranged non-small cell lung cancer

Chihiro Ando¹ | Eiki Ichihara²  | Tatsuya Nishi¹ | Ayako Morita¹ | Naofumi Hara¹ | Kenji Takada¹ | Takamasa Nakasuka¹ | Hiromi Watanabe¹ | Hirohisa Kano¹  | Kazuya Nishii¹ | Go Makimoto³  | Takumi Kondo⁴ | Kiichiro Ninomiya² | Masanori Fujii² | Toshio Kubo²  | Kadoaki Ohashi²  | Ken-ichi Matsuoka¹ | Katsuyuki Hotta⁵ | Masahiro Tabata³ | Yoshinobu Maeda¹ | Katsuyuki Kiura²

¹Department of Hematology, Oncology and Respiratory Medicine, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan

²Department of Allergy and Respiratory Medicine, Okayama University Hospital, Okayama, Japan

³Center for Clinical Oncology, Okayama University Hospital, Okayama, Japan

⁴Department of Hematology and Oncology, Okayama University Hospital, Okayama, Japan

⁵Center for Innovative Clinical Medicine, Okayama University Hospital, Okayama, Japan

Correspondence

Eiki Ichihara, Okayama University Hospital, Shikata-cho Kita-ku, Okayama, Japan.

Email: ichih-e@md.okayama-u.ac.jp

Funding information

Japan Lung Cancer Society

Abstract

Gilteritinib is a multitarget tyrosine kinase inhibitor (TKI), approved for the treatment of FLT3-mutant acute myeloid leukemia, with a broad range of activity against several tyrosine kinases including anaplastic lymphoma kinase (ALK). This study investigated the efficacy of gilteritinib against *ALK*-rearranged non-small cell lung cancers (NSCLC). To this end, we assessed the effects of gilteritinib on cell proliferation, apoptosis, and acquired resistance responses in several *ALK*-rearranged NSCLC cell lines and mouse xenograft tumor models and compared its efficacy to alectinib, a standard *ALK* inhibitor. Gilteritinib was significantly more potent than alectinib, as it inhibited cell proliferation at a lower dose, with complete attenuation of growth observed in several *ALK*-rearranged NSCLC cell lines and no development of drug tolerance. Immunoblotting showed that gilteritinib strongly suppressed phosphorylated *ALK* and its downstream effectors, as well as mesenchymal–epithelial transition factor (MET) signaling. By comparison, MET signaling was enhanced in alectinib-treated cells. Furthermore, gilteritinib was found to more effectively abolish growth of *ALK*-rearranged NSCLC xenograft tumors, many of which completely receded. Interleukin-15 (IL-15) mRNA levels were elevated in gilteritinib-treated cells, together with a concomitant increase in the infiltration of tumors by natural killer (NK) cells, as assessed by immunohistochemistry. This suggests that IL-15 production along with NK cell infiltration may constitute components of the gilteritinib-mediated antitumor responses in *ALK*-rearranged NSCLCs. In conclusion, gilteritinib demonstrated significantly improved antitumor efficacy compared with alectinib against *ALK*-rearranged

Abbreviations: AE, adverse event; *ALK*, anaplastic lymphoma kinase; AML, acute myeloid leukemia; EGFR, epidermal growth factor receptor; ERK1/2, extracellular signal-regulated kinases 1/2; FLT3, FMS-like tyrosine kinase 3; HGF, hepatocyte growth factor; IC₅₀, half-maximal inhibitory concentrations; IL15, interleukin-15; ITD, internal tandem duplication; KRAS, V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; MET, mesenchymal–epithelial transition factor; NK, natural killer; NSCLC, non-small cell lung cancer; PFS, progression-free survival; TKI, tyrosine kinase inhibitor.

This is an open access article under the terms of the [Creative Commons Attribution-NonCommercial-NoDerivs](https://creativecommons.org/licenses/by-nc-nd/4.0/) License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

© 2023 The Authors. *Cancer Science* published by John Wiley & Sons Australia, Ltd on behalf of Japanese Cancer Association.

NSCLC cells, which can warrant its candidacy for use in anticancer regimens, after further examination in clinical trial settings.

KEYWORDS

alectinib, ALK, gilteritinib, non-small cell lung cancer, TKI

1 | INTRODUCTION

The discovery of various gene alterations, such as those found in *EGFR*, *ALK*, and *ROS-1*, has markedly changed the treatment strategy for non-small cell lung cancer (NSCLC). Rearrangement of *ALK* occurs in 3%–5% of patients with NSCLC,¹ and in this regard tyrosine kinase inhibitors (TKIs) against anaplastic lymphoma kinase (ALK) have shown significant clinical benefits in the treatment of *ALK*-rearranged NSCLC.² Several *ALK*-TKIs are currently used for the targeted treatment of *ALK*-rearranged NSCLC.^{3–5} Among these, alectinib is a highly selective *ALK*-TKI that has been shown to achieve significantly better progression-free survival (PFS) rates for patients than crizotinib, according to a phase III ALEX study (HR: 0.43; 95% CI: 0.32–0.58; median PFS: 34.8 months vs. 10.9 months)⁶ while additionally exhibiting lower toxicity (serious adverse events [AEs]: 3.9% vs. 4.6%; grade ≥ 3 AEs: 44.7% vs. 51.0%). Based on these data, alectinib is currently the preferred first-line standard treatment for patients with advanced *ALK*-rearranged NSCLC.

Nevertheless, despite the efficacy of *ALK* inhibitors, including alectinib, patients invariably relapse because of tumor cells' acquired resistance to the treatment. The mechanisms of tolerance development can be divided into two types: *ALK*-independent and *ALK*-dependent. The *ALK*-independent resistance mechanisms include activated bypass pathways involving epidermal growth factor receptor (*EGFR*), cytoplasmic mesenchymal–epithelial transition factor (*MET*), *V-Ki-ras2* Kirsten rat sarcoma viral oncogene homolog (*KRAS*), *AXL*, and histological transformation.^{7–11} The *ALK*-dependent drug insensitivity is associated with secondary mutations in *ALK* and/or amplification of *ALK* fusion genes.^{12–14}

Gilteritinib is a TKI inhibitor developed primarily against *FMS*-like tyrosine kinase 3 (*FLT3*) and approved in the treatment of *FLT3*-mutant acute myeloid leukemia (AML).¹⁵ However, it is a multitarget compound with a broad range of activity against several tyrosine kinases, including *AXL* and *ALK*,¹⁶ and is reportedly effective against *ALK*-rearranged anaplastic large cell lymphoma.¹⁷ Furthermore, a recent study demonstrated the efficacy of gilteritinib in sufficiently treating *ALK*-rearranged NSCLC with acquired resistance to the third-generation *ALK*-TKI lorlatinib.¹⁸ Several double mutations have been identified, which confer tolerance to all approved *ALK*-TKIs, such as I1171N+F1174I and I1171N+L1198H. Gilteritinib has exhibited potent activity against *ALK*-TKI-resistant *EML4*-*ALK* I1171N/S compound mutants. In addition, gilteritinib also had an inhibitory effect on *ALK*-TKI-resistant single mutants, with the exception of G1202R, D1203N, and I1171N. However, the benefit of gilteritinib against treatment-naïve *ALK*-rearranged NSCLC has not yet been fully elucidated. Therefore, in the present study we aimed

to comparatively investigate the efficacies of gilteritinib and alectinib against treatment-naïve *ALK*-rearranged NSCLC.

2 | MATERIALS AND METHODS

2.1 | Cell lines and reagents

The H2228 (*EML4*-*ALK* fusion) and A549 cell lines were purchased from the American Type Culture Collection. The HCC827 (*EGFR* Ex19 del E746_A750), H3122 (*EML4*-*ALK* fusion), and HCC78 cells (*SLC34A2*-*ROS1* fusion) were kindly provided by Dr. William Pao (Vanderbilt University). The H3255 cells were kindly provided by Fujimoto and Kurie (MD Anderson Cancer Center). The ABC-11 (*EML4*-*ALK* fusion) and ABC-19 cells (*EML4*-*ALK* fusion) were established in our laboratory. Gilteritinib was provided by Astellas Pharma Inc. Alectinib, and tepotinib were purchased from Selleck Chemicals. Recombinant human hepatocyte growth factor (HGF) (carrier-free) was purchased from BioLegend. All compounds were dissolved in dimethyl sulfoxide (DMSO) for in vitro studies.

2.2 | Cell proliferation assay

Growth inhibition was measured using a modified MTT assay.¹⁹ Briefly, cells were seeded in 96-well plates at densities of 2000–3000/well and continuously treated with each drug for 96 h.

2.3 | Antibodies, immunoblotting, and receptor tyrosine kinase array

The following antibodies were obtained from Cell Signaling Technology: anti-phospho-*EGFR* (Tyr1068), anti-*EGFR*, anti-phospho-*ALK* (Tyr1282/1283), anti-*ALK*, anti-phospho-*ROS1* (Tyr2274), anti-*ROS1*, anti-phosphorylated extracellular signal-regulated kinases (ERK) 1/2 (Thr202/Tyr204), anti-ERK1/2, anti-phospho-*AKT* (Ser473), anti-*AKT*, anti-*GAPDH*, and horseradish peroxidase-conjugated anti-rabbit IgG. For immunoblotting, cells were harvested, washed in PBS, lysed in radioimmunoprecipitation assay buffer (1% Triton X-100, 0.1% SDS, 50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 10 mmol/L β -glycerol-phosphate, 10 mmol/L NaF, and 1 mmol/L sodium orthovanadate), and additionally supplemented with a protease inhibitor cocktail (Roche Applied Sciences).

Proteins were separated using SDS-PAGE and transferred onto membranes that were subsequently incubated with the

indicated primary and secondary antibodies. Chemiluminescence was detected using an enhanced chemiluminescence reagent (Cytiva). Phospho-receptor tyrosine kinase arrays were performed using a phospho-receptor tyrosine kinase array kit (R&D Systems) in accordance with the manufacturer's instructions. Bands and dots were detected using Amersham ImageQuant 800 (Cytiva).

2.4 | Xenograft mouse models

Female BALB/c nu/nu mice (6 weeks old) were purchased from Charles River Laboratories. All mice were provided with sterilized food and water and housed in a barrier facility under a 12-h light/dark cycle. Cancer cells ($3\text{--}5 \times 10^6$) were injected subcutaneously into the back of the mice in a bilateral manner. Vehicle and compound-containing agents were administered by gavage once per day for a total of five times per week. Tumor volume was measured twice per week as $\text{width}^2 \times \text{length} / 2$, for 4 weeks. Tumor volumes measured on day 28 were used for further statistical analysis.

2.5 | Quantitative PCR of interleukin-15 (IL-15) expression

Cells were treated with either DMSO, 100 nM alectinib, or 100 nM gilteritinib for 24 h, and RNA was extracted using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen). The primer sequences are provided in Table S1. The PCR was performed on a LightCycler Real-Time (RT) PCR System (Roche Applied Science), and the gene dosage was calculated using a standard curve. The copy number ratio of the target gene to GAPDH was then calculated and used for statistical comparisons.

2.6 | ELISA

Cells were seeded in 3.5-cm cell culture dishes at $12\text{--}14 \times 10^5$ cells/dish and treated with either DMSO, 100 nM alectinib, or 100 nM gilteritinib. Cell supernatants were collected following incubation for 48 h. The IL-15 levels were measured using a Human IL-15 Quantikine ELISA kit (R&D Systems) following the manufacturer's instructions.

3 | RESULTS

3.1 | Gilteritinib effectively suppresses proliferation of ALK-rearranged NSCLC cell lines in vitro

We first investigated the effects of gilteritinib on various oncogene-driven NSCLC cell lines, including ALK-rearranged cells. Cell proliferation assays were conducted in ALK-rearranged (H3122, ABC-11),

EGFR-mutant (HCC827, H3255), KRAS-mutant (A549), and ROS1-rearranged (HCC78) NSCLC cells (Figure 1A). The NSCLC cells with oncogenic alterations other than ALK rearrangement exhibited resistance or moderate sensitivity to gilteritinib. In EGFR-mutant HCC827 and H3255 cells, the half-maximal inhibitory concentrations (IC_{50}) of gilteritinib were 667 and 370 nmol/L, respectively. In KRAS-mutant A549 cells and ROS1-rearranged HCC78 cells, the IC_{50} were 394 and 46 nmol/L, respectively. In contrast to these oncogene-driven NSCLC cells, the ALK-rearranged H3122 and ABC-11 cell lines showed high sensitivity to gilteritinib, with IC_{50} values of 0.8 and 5.4 nmol/L, respectively, indicating that gilteritinib was highly effective against ALK-rearranged NSCLC cells.

3.2 | Improved efficiency of gilteritinib over alectinib against treatment-naïve ALK-rearranged NSCLC cells

Next, we focused on comparing the efficacy of gilteritinib to that of alectinib in the context of inhibiting growth of ALK-rearranged NSCLCs. Cell proliferation assays were performed to individually examine gilteritinib's and alectinib's ability to attenuate growth of ALK-rearranged NSCLC cell lines, including H3122, H2228, ABC-11, and ABC-19 cells (Figure 1B). Alectinib performed according to expectations, as it effectively halted the proliferation of H3122 (IC_{50} : 12 nmol/L), H2228 (IC_{50} : 235 nmol/L), ABC-11, (IC_{50} : 109 nmol/L), and ABC-19 (IC_{50} : 74 nmol/L) cell lines. However, gilteritinib consistently surpassed alectinib in potency, as it achieved similar levels of growth inhibition for all ALK-rearranged NSCLC cell lines (H3122, H2228 ABC-11, and ABC-19), at significantly lower dosages (IC_{50} : 0.8, 45, 5.4, and 7.8 nmol/L, respectively) compared to alectinib (Figure 1B).

Finally, we determined the efficacy of gilteritinib against cells that had developed resistance to alectinib (Figure S1). H2228/CHR and ABC-11/CHR are cell lines that became resistant to alectinib, established from H2228 and ABC-11 through long-term exposure to alectinib.²⁰ The IC_{50} of gilteritinib was 361 nmol/L in H2228/CHR and 660 nmol/L in ABC-11/CHR, respectively. Gilteritinib demonstrated only moderate activity against these resistant cell lines.

In addition, we investigated the apoptotic effects of gilteritinib in ALK-rearranged NSCLC cells (Figure 1C). In H3122 and ABC-19 cells, both cleaved PARP and high caspase-3 levels were detected at gilteritinib concentrations ≥ 100 nM, whereas these were not observed at alectinib concentrations $< 1 \mu\text{M}$. Similarly, cleaved PARP was detected at a lower dose of gilteritinib than that of alectinib in H2228 and ABC-11 cells. To determine the long-term efficacy of gilteritinib against ALK-rearranged NSCLC, H3122 cells were seeded in 96-well plates and cell viability following long-term exposure to each drug was examined (Figure 1D). After 2 weeks of continuous drug exposure, no resistance was observed in either alectinib- or gilteritinib-treated cells. However, after 4 weeks, approximately 36% of the alectinib-treated cells developed tolerance to the compound,

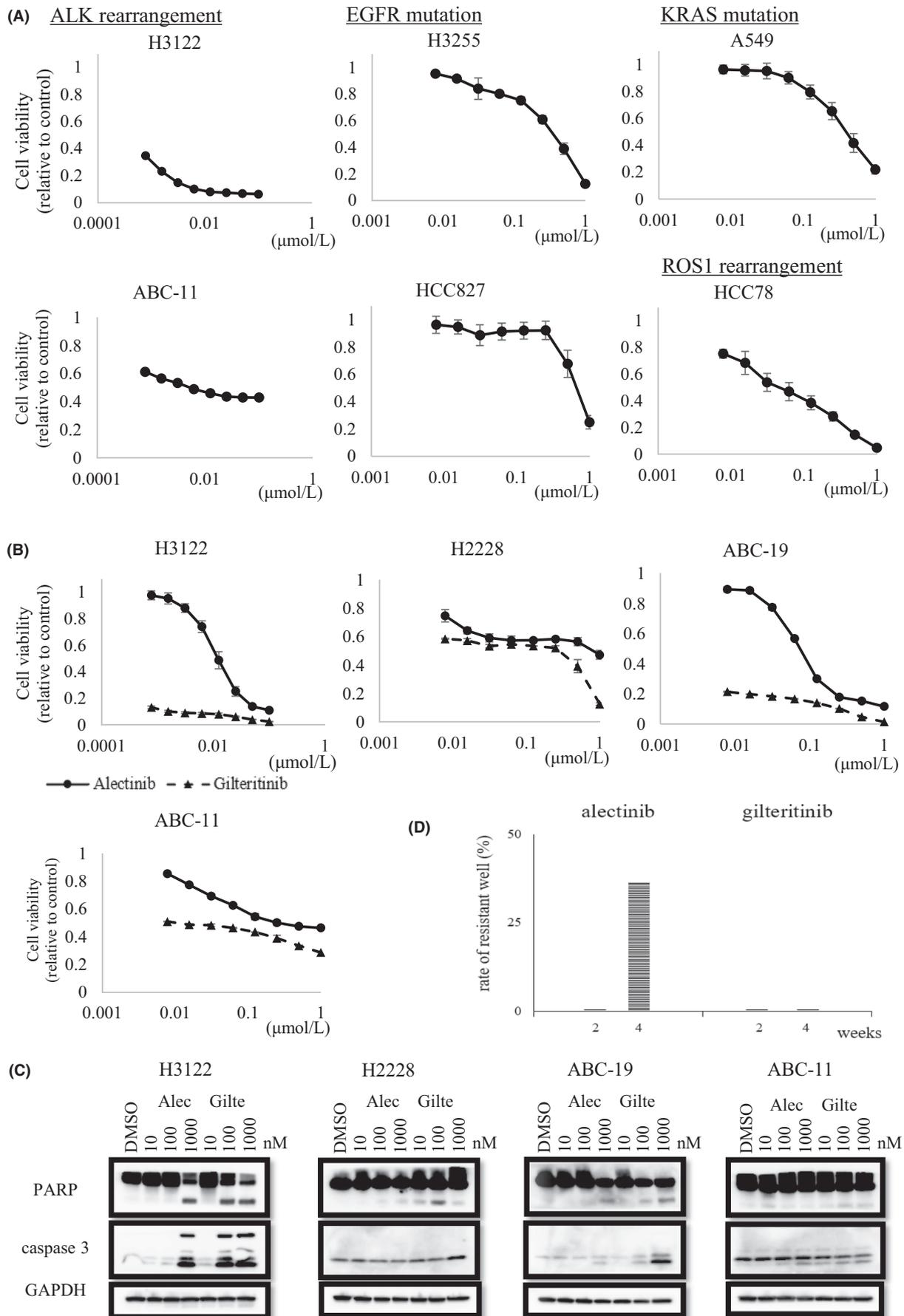


FIGURE 1 Efficacy of gilteritinib on multiple oncogene-driven NSCLC cell lines. (A) Cell proliferation assay using gilteritinib in cell lines with different gene mutations. Error bars indicate SD. (B) Cell proliferation assay with alectinib and gilteritinib in *ALK*-rearranged NSCLC cell lines. Error bars indicate SD. (C) Western blot analysis of PARP and cleaved caspase-3 levels in H3122, H2228, ABC-19, and ABC-11 cell lines. The control group for each cell line was treated with DMSO at the same concentration of alectinib or gilteritinib for 72 h. (D) H3122 cells were seeded in 96-well plates at 5000 cells/well and treated with 100 nM alectinib or 100 nM gilteritinib the following day (day 1). The culture medium was carefully changed twice a week using an eight-channel pipette to avoid disturbing the cells. Cell growth was evaluated using the MTT assay at weeks 2 and 4, and wells with absorbance values equal to or greater than two times that of day 1 were considered to contain cells resistant to the drug.

whereas all gilteritinib-treated cells remained responsive to the treatment (Figure 1D).

Collectively, these results indicate that gilteritinib was more effective than alectinib at inhibiting growth and inducing apoptosis in *ALK*-rearranged NSCLC cell lines at a considerably lower dosage, while also preventing the onset of acquired resistance, within a 1-month timeframe of continuous administration.

3.3 | Gilteritinib mediates antitumor responses through strong inhibition of *ALK* and *MET* signaling

To elucidate the mechanism underlying the favorable effects of gilteritinib over alectinib, we examined the cell signaling pathways affected, using immunoblotting (Figure 2A). As observed in ABC-11, ABC-19, H2228, and H3122 cell lines, gilteritinib inhibited *ALK* phosphorylation and its effector signaling proteins *AKT* and *ERK* at lower concentrations than alectinib. These results indicated that gilteritinib was more potent than alectinib in regard to *ALK* inhibition.

Considering that gilteritinib is a multitarget TKI, while alectinib is *ALK* specific, we assessed gilteritinib's efficacy on potential bypass pathways. We first focused on *AXL*, a characterized target of gilteritinib¹⁶ and monitored the effect of gilteritinib on the phosphorylation of this kinase in *ALK*-rearranged cell lines (Figure 2B). In H3122 cells, *AXL* phosphorylation was only moderately inhibited by gilteritinib and alectinib at a comparable level, while in H2228, ABC-11, and ABC-19 cell lines, *AXL* inhibition by gilteritinib yielded inconclusive results. These data suggested that the effect of gilteritinib was likely not mediated by *AXL* inhibition.

The effects of gilteritinib on multiple receptor tyrosine kinases were compared with those of alectinib using a phospho-receptor tyrosine kinase array in H3122 cells (Figure 2C). Interestingly, *MET* phosphorylation was abolished following treatment with gilteritinib but was slightly increased in alectinib-treated cells. Immunoblotting also corroborated that alectinib induced *MET* phosphorylation in *ALK*-rearranged cell lines, whereas gilteritinib did not (Figure 3A). These data implied that gilteritinib's effect on inhibiting cell proliferation was also mediated by *MET* attenuation.

To confirm our observations, we examined the combined effect of alectinib and the *MET* inhibitor tepotinib. Although the effect was modest, tepotinib enhanced the growth-inhibiting effect of alectinib in several *ALK*-rearranged cell lines (Figure 3B). In addition, exposure to HGF, a *MET* ligand, led to the development of resistance to alectinib but only partial resistance to gilteritinib (Figure 3C). These data

highlighted that the superior efficacy of gilteritinib over alectinib is at least partly due to the latter's inhibition of *MET* function.

3.4 | Gilteritinib irreversibly attenuates tumor growth in *ALK*-rearranged xenograft models

Next, we assessed the effect of gilteritinib compared with that of alectinib *in vivo* using murine xenograft models (Figure 4). Both alectinib and gilteritinib significantly suppressed tumor growth in the H3122 xenograft model, but gilteritinib showed distinctly higher efficacy. Gilteritinib administration resulted in a decrease in tumor size, whereas no similar effect was observed for alectinib (Figure 4A). No apparent weight loss was observed in either group. Similar to our findings in H3122 tumors, gilteritinib also showed significantly improved tumor growth attenuation compared with alectinib in ABC-11 (Figure 4B) and ABC-19 (Figure 4C) xenograft models, without any observable weight loss in mice.

Notably, gilteritinib completely inhibited all the examined ABC-11 tumors (10/10 tumors), and no tumor regrowth was detected 4 months after drug discontinuation. In contrast, alectinib only partially inhibited growth of all tumors tested across the different *ALK*-rearranged NSCLC xenograft models (Figure 4B). Gilteritinib caused the complete inhibition in six out of the ten investigated ABC-19 tumors (Figure 4C), and pathological complete inhibition was confirmed in them (Figure 4D). Together, these observations suggested that gilteritinib was more potent than alectinib and with a longer-lasting effect on tumor recession *in vivo*, as assessed in *ALK*-rearranged NSCLC xenograft models.

3.5 | Gilteritinib increases the infiltration of NK cells into the tumor

Gilteritinib reportedly promotes the anticancer effects of natural killer (NK) cells by inducing *IL-15* expression in *FLT3* internal tandem duplication (ITD)-mutant leukemia cells.²¹ Given that *IL-15* has been shown to be involved in the maturation and activation of T and NK cells,²² we hypothesized that the marked effect of gilteritinib on xenograft tumors might involve the activation of NK cells through induction of *IL-15* expression. We first investigated whether gilteritinib induced *IL-15* expression in *ALK*-rearranged NSCLC cells *in vitro*, using qRT-PCR and ELISA. Gilteritinib-treated cell lines showed higher *IL-15* expression than DMSO- or alectinib-treated cells

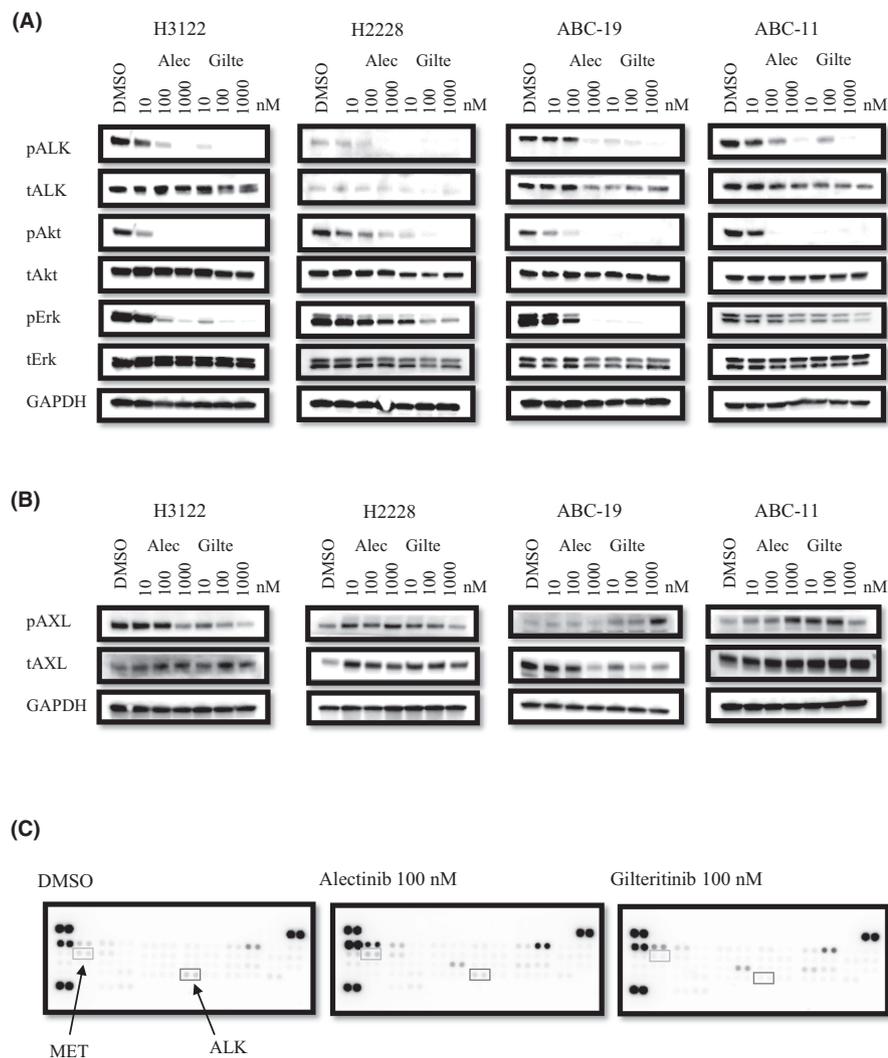


FIGURE 2 Efficacy of gilteritinib in comparison with alectinib in ALK-rearranged cell lines. (A) Suppression of phospho-ALK and effector proteins in untreated ALK-positive cell lines were evaluated by Western blotting. Cells were treated with the indicated concentrations of alectinib or gilteritinib for 24 h. All experiments were performed in triplicate. (B) Phospho-AXL expression in ALK-rearranged cell lines was evaluated using western blotting. Cells were treated with the indicated concentrations of alectinib or gilteritinib for 24 h. All experiments were performed in triplicate. (C) Relative levels of tyrosine phosphorylation of human receptor tyrosine kinases (RTKs) in H3122 cells were examined. Cells were treated with DMSO, 100 nM alectinib, or 100 nM gilteritinib for 24 h.

(Figure 5A; Figure S2). This supported our hypothesis that gilteritinib induced higher IL-15 expression in ALK-rearranged NSCLC cells.

Next, we assessed NK cell infiltration into tumor cells using immunostaining with an anti-NCR1 antibody. A small number of NK cells was observed in the vehicle group, mainly at the edge of the tumor (Figure 5B,C). For both, alectinib- and gilteritinib-treated tumors, although the number of peripheral NK cells did not significantly increase, the amount of infiltrating NK cells observed within the tumor was distinctly elevated. Interestingly, a considerably higher number of NK cells was detected inside the gilteritinib-treated tumors compared with the alectinib-treated group. Collectively, these results demonstrated that drug-induced production of IL-15 in the xenografted tumors facilitated the increased recruitment of NK cells on site and their infiltration within the tumor, with this effect being more pronounced in the gilteritinib- than in the alectinib-treated group.

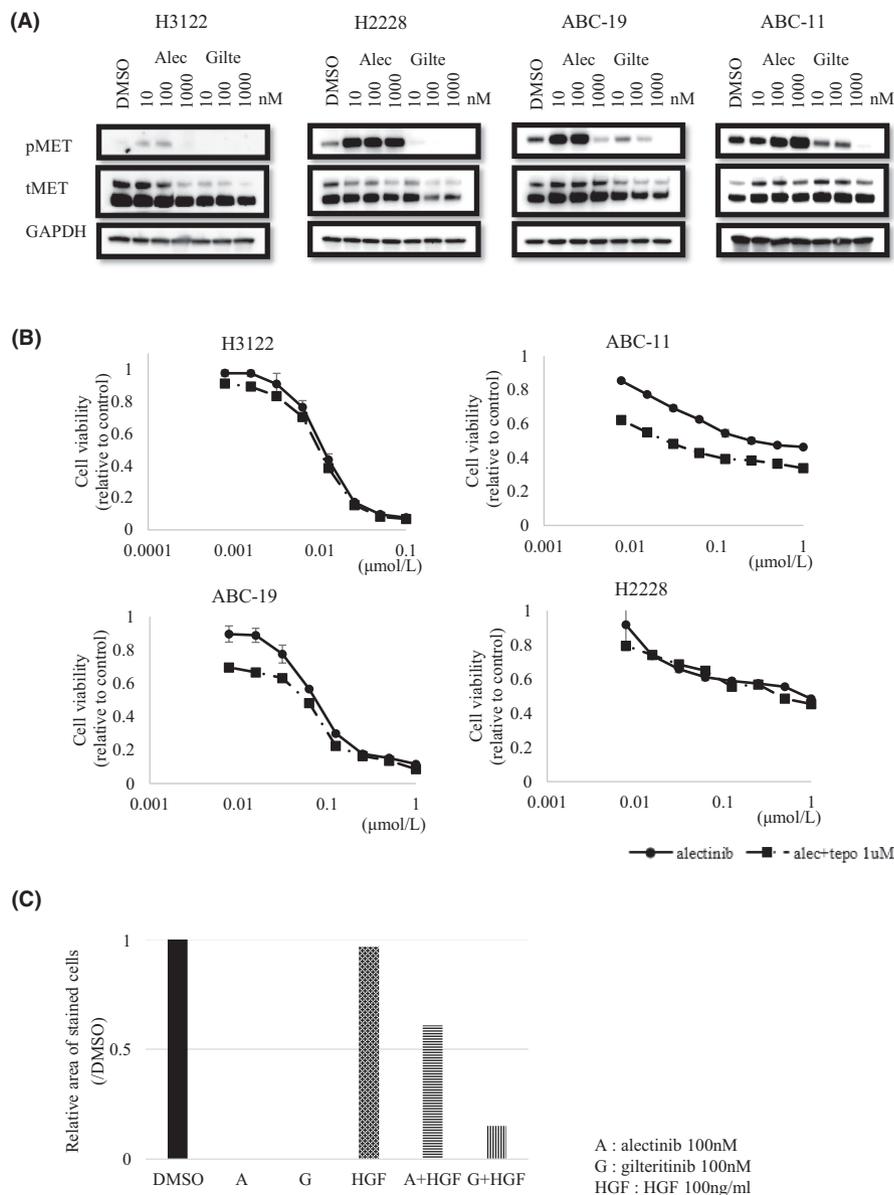
4 | DISCUSSION

The efficacy of gilteritinib in treatment-naïve ALK-rearranged NSCLC has not been established to date. The current study highlighted that

gilteritinib was effective against untreated ALK-rearranged NSCLC, with superior efficacy compared with the commonly used alectinib. Although ALK-TKIs, including alectinib, are highly effective for ALK-rearranged tumors, patients ultimately develop resistance to treatment,²³ and satisfactorily efficient alternative treatment modalities remain to be successfully implemented. In this regard, we showcased herein the antitumor effects of gilteritinib against ALK-rearranged NSCLC, which support its candidacy as a more efficient multitarget TKI that may be used in first-line therapy.

Unlike other more recently developed compounds, gilteritinib has already been used against *FLT3*-mutant AML, and its clinical feasibility in this context has been confirmed. In a phase 3 clinical trial for *FLT3*-mutant AML, AEs of grade 3 or higher, and serious AEs, occurred less frequently in the gilteritinib group than in the chemotherapy group. The most common AEs of grade 3 or higher in this trial included hematological toxicity, such as febrile neutropenia, anemia, and thrombocytopenia.²⁴ Therefore, extending its application to ALK-rearranged NSCLC as well could be a simpler task than for other compounds. This remains to be evaluated in follow-up clinical trials. Mizuta et al. recently reported that gilteritinib is effective against ALK-rearranged NSCLC cells which have acquired resistance

FIGURE 3 Effects of gilteritinib on MET signaling in ALK-rearranged cell lines. (A) Phospho-MET levels are evaluated by Western blotting. Cells were treated with the indicated concentrations of alectinib or gilteritinib for 24 h. All experiments were performed in triplicate. (B) Cell proliferation assay showing the combined effect of alectinib and MET inhibitors in ALK-rearranged NSCLC cell lines. Error bars indicate SD. (C) Evaluation of HGF-mediated resistance development in H3122 cells. Alectinib (100 nM), gilteritinib (100 nM), or HGF (100 ng/μL) was administered, and the acquired resistance of cells to treatment was evaluated using a crystal violet assay after 7 days. Data were quantified using the ImageJ software.



to various ALK-TKIs.¹⁸ In their study, gilteritinib successfully overcame the previously developed tolerance to the third-generation ALK-TKI lorlatinib, which was caused by secondary ALK mutations.¹⁸ These data highlighted the promising targeting of ALK by gilteritinib. Although the efficacy of gilteritinib in acquired resistance has been investigated, its potency against treatment-naive ALK-rearranged NSCLC, particularly in comparison with alectinib, has not been fully clarified.

The third-generation EGFR-TKI osimertinib was initially developed for EGFR-mutant NSCLC with acquired resistance to first- and second-generation EGFR-TKIs,²⁵ but it has been shown to be more effective when used upfront.²⁶ Cancer cells become progressively more heterogeneous, acquiring multiple resistance mechanisms during TKI therapy and as such, a potent initial therapy could help toward preventing tumor cell heterogeneity.²⁷ In this context, more potent TKIs may be used in first-line treatment regimens in order to achieve long-term tumor suppression, and gilteritinib

could be preferentially and more efficiently employed at this stage of cancer therapy.

The results of our study indicated that gilteritinib showed potential therapeutic effects against ALK-rearranged xenograft tumors. We then proceeded to further delineate the mechanisms underlying the effects of gilteritinib on ALK-rearranged NSCLC cells. Our findings showed that gilteritinib effectively inhibited phosphorylated ALK at a 10-times lower concentration than alectinib. Consistent with this observation, gilteritinib inhibited cell proliferation at a correspondingly 10-times lower IC₅₀ value than alectinib. This suggested that the gilteritinib-mediated strong ALK activity attenuation contributed to the observed potency of the compound in inhibiting growth rates of ALK-rearranged NSCLC cells.

In addition to ALK inhibition, we assessed the downregulatory effects on other substrate kinases of gilteritinib. Current evidence supports that gilteritinib also exhibits activity against AXL. In EGFR-mutant NSCLC, AXL signaling promotes adaptive responses

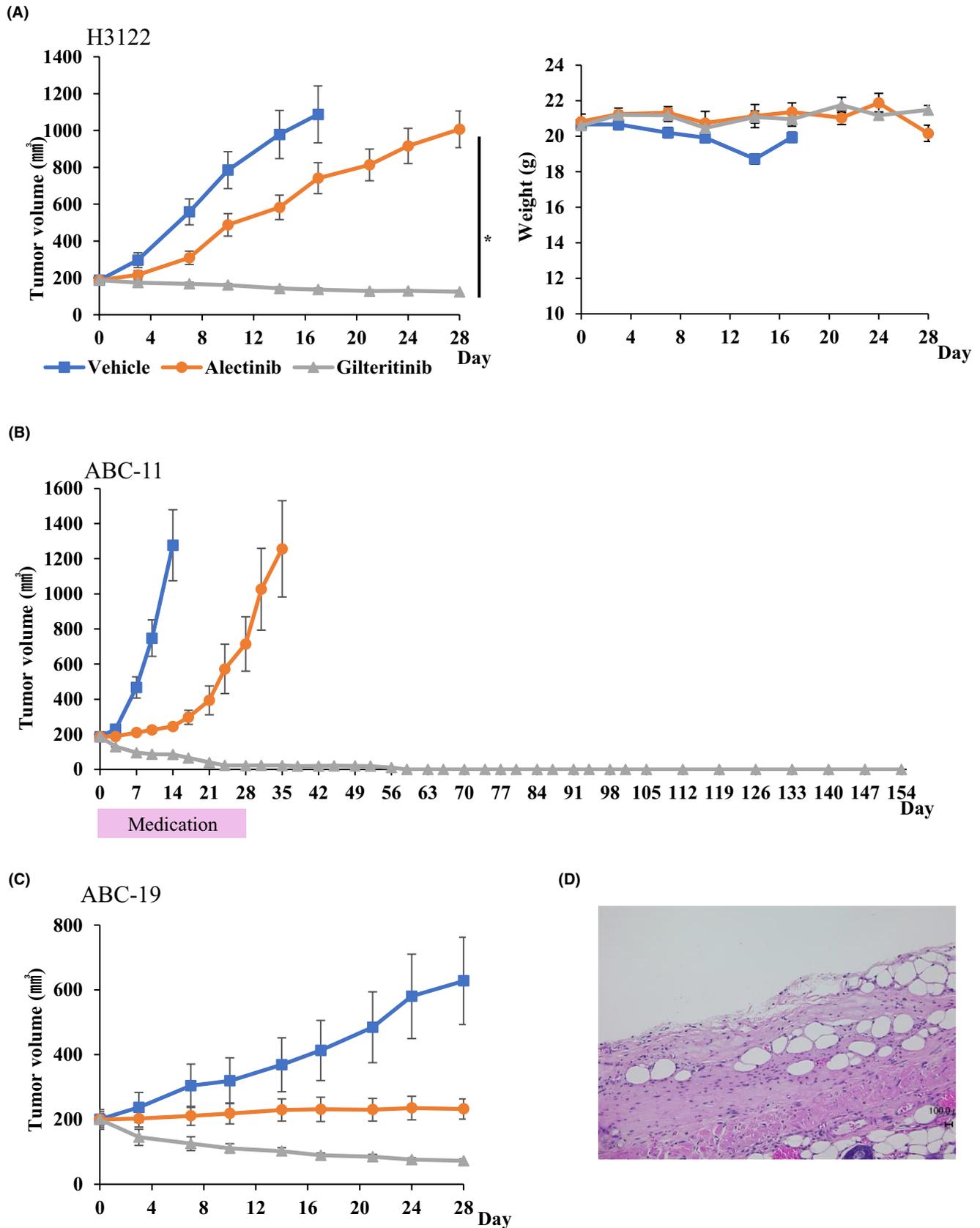
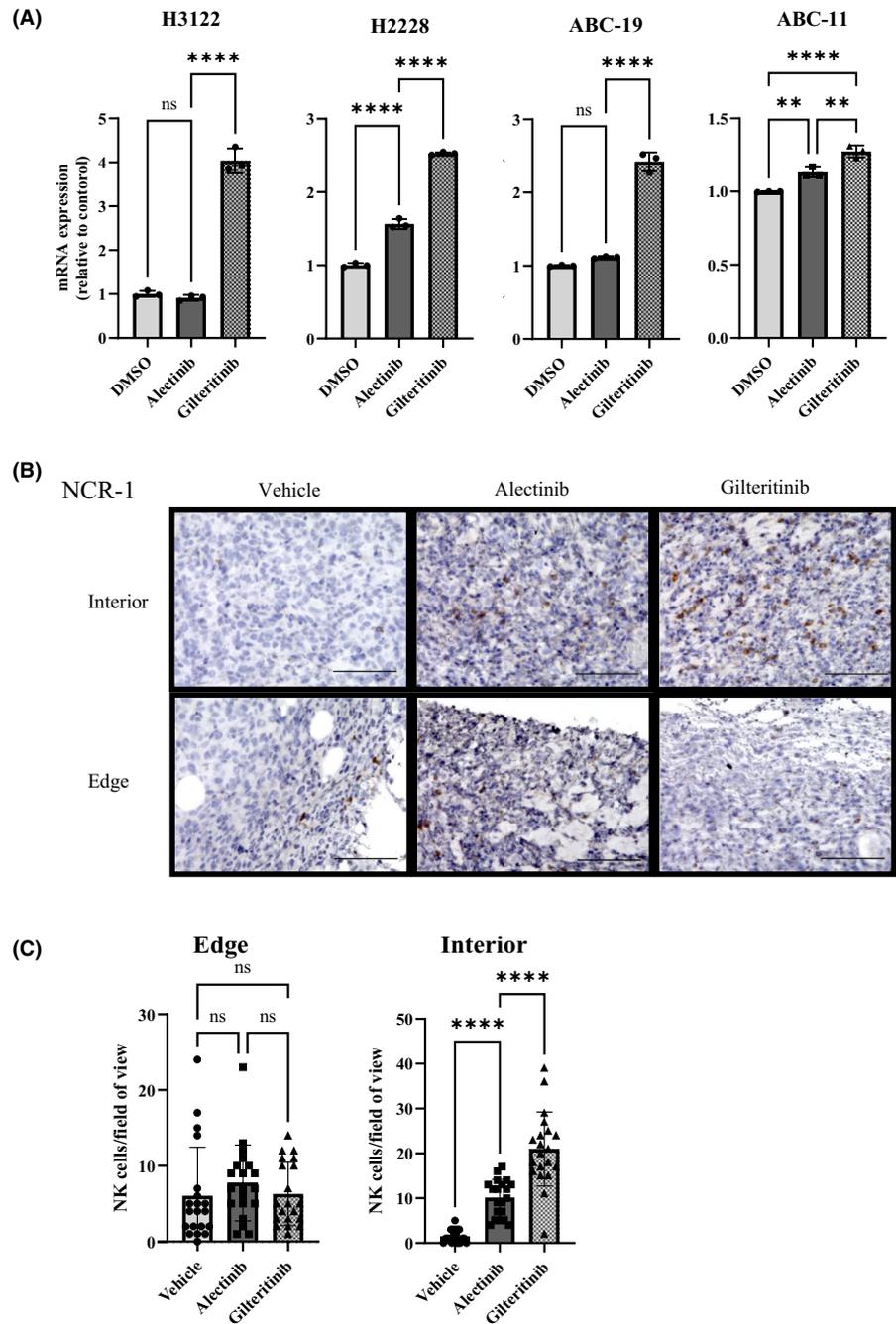


FIGURE 4 Effects of gilteritinib on ALK-rearranged xenograft tumors. (A–C) Mice injected with H3122, ABC-11, or ABC-19 cells for tumor growth. The TKI agents (alectinib or gilteritinib, 10 mg/kg/day) were orally administered 5 days per week for 28 days. No body weight loss was observed. Error bars represent standard errors; * $p < 0.01$ (Student's *t*-test). (B) The ABC-11 xenograft group was followed up with no medication after 28 days of drug administration. (D) In the ABC-19 xenograft model, which showed consistent tumor shrinkage 72 days post treatment, there were no identifiable tumor cells (HE staining), although there were slight subcutaneous traces. Scale bar, 100 μ m.

FIGURE 5 Interleukin (IL)-15 production and natural killer (NK) cell recruitment in gilteritinib-treated ALK-rearranged NSCLC cells. (A) qPCR quantification of IL-15 mRNA levels relative to GAPDH in ALK-rearranged NSCLC cell lines. The cells were treated with DMSO, 100nM alectinib, or 100nM gilteritinib for 24 h. All experiments were performed in triplicate. Error bars represent standard error. *p*-Values were calculated using one-way ANOVA with post-hoc Tukey's test for group comparisons. All experiments were performed in triplicate. ***p* < 0.005, *****p* < 0.0001. (B, C) NK cell infiltration in ABC-11 xenograft tumors (alectinib or gilteritinib 10mg/kg/day on day 4), assessed by immunohistochemical analysis. Scale bar, 100µm. NCR1-positive NK cells were visually quantified and evaluated separately at the edges and inside the tumor. The positive cells were quantified visually. Data are presented as means. Error bars represent standard error. *p*-Values were calculated using one-way ANOVA with post-hoc Tukey's test for group comparisons. *****p* < 0.0001; ns, not significant.



to EGFR-TKIs, while in contrast, AXL inhibition enhances the effects of EGFR-TKIs.²⁸ Based on this evidence, we posited that AXL signaling may attenuate the effects of ALK TKIs in ALK-rearranged NSCLC cells and that the overall efficacy of gilteritinib may be at least partially attributed to the compound's inhibition of AXL. However, in our experimental setting, gilteritinib had no observable effect on AXL in ALK-rearranged NSCLC, indicating that the drug's antitumor efficacy may be potentially mediated in an AXL-independent manner. However, gilteritinib inhibited MET, which was by contrast activated by alectinib. The attenuation of MET signaling by gilteritinib, although moderate, was identified to be one of the mechanisms underlying the compound's efficacy against ALK-rearranged NSCLC cells. Crizotinib exhibits strong activity

against both MET and ALK. However, in a clinical setting, crizotinib has been shown to be less effective than alectinib.²⁹ This may be due to crizotinib's lower activity against ALK compared with alectinib. Studies indicate that crizotinib inhibits wild-type ALK at a concentration in the double-digit nM range, while alectinib does so at a concentration in the single-digit nM range.²⁹

Gilteritinib has been reported to promote anticancer activity by inducing IL-15 expression in *FLT3-ITD*-mutant leukemia cells. Given that IL-15 has been shown to be involved in the maturation and activation of NK cells, we therefore hypothesized that the anti-tumor mechanisms induced by gilteritinib in xenograft tumors may involve activation of NK cells through induction of IL-15 expression. According to the results from our in vitro models, IL-15 levels

were consistently higher in all gilteritinib-treated cell lines relative to DMSO- or alectinib-treated cells. Our *in vivo* observations aligned with our *in vitro* findings, as we detected significantly higher numbers of infiltrating NK cells inside gilteritinib-treated tumors compared with the alectinib-treated counterparts. These findings indicated that in addition to its direct inhibitory effect on cancer cell growth, gilteritinib treatment also led to the increased production of IL-15, which corresponded to the higher infiltration of NK cells within the tumor. The direct and indirect effects of gilteritinib may at least partially account for the compound's high anticancer potency in our xenograft tumor models. The innate immune-mediated antitumor effect of NK cells has recently attracted considerable attention,³⁰ and gilteritinib may have similar mechanisms of action as indicated by our study, particularly in ALK-rearranged lung cancer.

In conclusion, here we provided further supporting evidence for gilteritinib as a candidate first-line anticancer drug against untreated ALK-rearranged NSCLC. Gilteritinib exhibited more potent antitumor effects than alectinib both *in vitro* and *in vivo* through enhancing the suppression of ALK and MET signaling, as well as promoting IL-15-mediated NK cell activation. Importantly, gilteritinib-treated tumors did not acquire resistance to the compound, and inhibition of tumor growth was extensively sustained following termination of treatment within the timeframe of our study. Our findings provide insight in gilteritinib's antitumor mechanisms and could be used as reference for future clinical trials examining the efficacy of the compound in first-line treatment approaches against ALK-rearranged NSCLC.

AUTHOR CONTRIBUTIONS

Chihiro Ando: Conceptualization, methodology, formal analysis, investigation, writing—original draft. Eiki Ichihara: conceptualization, methodology, formal analysis, writing—original draft, project administration, funding acquisition, supervision. Tatsuya Nishi: investigation, writing—review and editing. Naofumi Hara: investigation, writing—review and editing. Ayako Morita: investigation, writing—review and editing. Naofumi Hara: investigation, writing—review and editing. Kenji Takada: investigation, writing—review and editing. Takamasa Nakasuka: investigation, writing—review and editing. Hiromi Watanabe: investigation, writing—review and editing. Hirohisa Kano: investigation, writing—review and editing. Kazuya Nishii: investigation, writing—review and editing. Go Makimoto: methodology, writing—review and editing. Takumi Kondo: methodology, writing—review and editing. Kiichiro Ninomiya: methodology, writing—review and editing. Masanori Fujii: writing—review and editing. Toshio Kubo: writing—review and editing. Kadoaki Ohashi: writing—review and editing. Ken-ichi Matsuoka: writing—review and editing. Katsuyuki Hotta: writing—review and editing. Masahiro Tabata: writing—review and editing. Yoshinobu Maeda: writing—review and editing, supervision. Katsuyuki Kiura: writing—review & editing, project administration.

ACKNOWLEDGMENTS

The authors would like to thank H. Nakashima and K. Maeda for their technical support. Gilteritinib was provided by Astellas Pharma Inc.

FUNDING INFORMATION

This work was supported by grants from the Japan Lung Cancer Society.

CONFLICT OF INTEREST STATEMENT

E. Ichihara reports grants from Nippon Kayaku Co., Ltd., MSD K.K., Janssen Pharmaceutical K.K., Pfizer Japan Inc., Bristol-Myers Squibb K.K., Ono Pharmaceutical Co., Ltd., and Takeda Pharmaceutical Company Limited and personal fees from Chugai Pharmaceutical Co., Ltd., Eli Lilly Japan K.K., AstraZeneca K.K., Takeda Pharmaceutical Company Limited, Ono Pharmaceutical Co., Ltd., Pfizer Japan Inc., Bristol-Myers Squibb K.K., Boehringer Ingelheim Co., Ltd., Janssen Pharmaceutical K.K., and Novartis Pharma K.K. outside the submitted work. G. Makimoto reports personal fees from Chugai Pharmaceutical Co., Ltd. outside the submitted work. T. Kondo reports personal fees from Kyowa Kirin Co. and Asahi Kasei Pharma Corporation outside the submitted work. K. Ninomiya reports personal fees from AstraZeneca K.K., Nippon Boehringer Ingelheim Co., Ltd., Eli Lilly Japan K.K., MSD K.K., Ono Pharmaceutical Co., Ltd., Nippon Kayaku Co., Ltd., TAIHO Pharmaceutical Co., Ltd., Kyowa Kirin Co., Ltd., and Chugai Pharmaceutical Co., Ltd. outside the submitted work. M. Fujii reports personal fees from AstraZeneca K.K., Takeda Pharmaceutical Company Limited, and Teijin Pharma Limited. outside the submitted work. K. Ohashi reports grants from Nippon Boehringer Ingelheim Co., Ltd., Chugai Pharmaceutical Co., Ltd., Eli Lilly Japan K.K., Daiichi Sankyo Company, Limited, and Amgen K.K. and personal fees from Eli Lilly Japan K.K., Novartis Pharma K.K., Chugai Pharmaceutical Co., Ltd., AstraZeneca K.K., and Kyowa Kirin Co., Ltd. and received research reagents from Genentech, Inc. and Novartis Pharma K.K. outside the submitted work. K. Matsuoka reports grant from Ono Pharmaceutical Co., Ltd. and personal fees from Astellas Pharma Inc., Novartis Pharma K.K., Janssen Pharmaceutical K.K., CSL Behring K.K., Nippon Shinyaku Co., Ltd., Chugai Pharmaceutical Co., Ltd., Kyowa Kirin Co., Ltd., Daiichi Sankyo Company, Amgen K.K., AbbVie GK, Mallinckrodt Pharma K.K., and Sanofi K.K. outside the submitted work. K. Hotta reports grants from MSD K.K., AstraZeneca K.K., Chugai Pharmaceutical Co., Ltd., Eli Lilly Japan K.K., Bristol-Myers Squibb K.K., and AbbVie GK and personal fees from Pfizer Japan Inc., AstraZeneca K.K., Chugai Pharmaceutical Co., Ltd., Eli Lilly Japan K.K., Takeda Pharmaceutical Company Limited, MSD K.K., Bristol-Myers Squibb K.K., Ono Pharmaceutical Co., Ltd., Nippon Boehringer Ingelheim Co., Ltd., and Nippon Kayaku Co., Ltd. outside the submitted work. Y. Maeda reports grants from Asahi Kasei Pharma Corporation, Eisai Co., Ltd., Chugai Pharmaceutical Co., Ltd., Otsuka Pharmaceutical Co., Ltd., Kyowa Kirin Co., Ltd., KYORIN Pharmaceutical Co., Ltd., Takeda Pharmaceutical Company Limited, TAIHO Pharmaceutical Co., Ltd., Japan Blood Products Organization, and Mallinckrodt Pharma K.K. and personal fees from AstraZeneca K.K., Astellas Pharma Inc., Amgen K.K., AbbVie GK, Eisai Co., Ltd., Otsuka Pharmaceutical Co., Ltd., Kyowa Kirin Co., Ltd., Ono Pharmaceutical Co., Ltd., Sanofi K.K., Celgene K.K., Bristol-Myers Squibb K.K., CSL Behring K.K., Daiichi Sankyo Company, Limited, Takeda Pharmaceutical Company Limited, TERUMO

CORPORATION, Chugai Pharmaceutical Co., Ltd., Nippon Shinyaku Co., Ltd., Pfizer Japan Inc., Meiji Seika Pharma Co., Ltd., Novartis Pharma K.K., Janssen Pharmaceutical K.K., Yakult Honsha Company, Limited, KYORIN Pharmaceutical Co., Ltd., Sumitomo Pharma Co., Ltd., Mundipharma K.K., Human Life CORD Japan Inc., Bayer Yakuhin, Ltd., and Medical Review Co., Ltd. outside the submitted work. K. Kiura reports grants from Nippon Boehringer Ingelheim Co., Ltd., Ono Pharmaceutical Co., Ltd., Chugai Pharmaceutical Co., Ltd., TAIHO Pharmaceutical Co., Ltd., Nippon Kayaku Co., Ltd., Teijin Pharma Limited, Shionogi Pharma Co., Ltd., Novartis Pharma K.K., Takeda Pharmaceutical Company Limited, KYORIN Pharmaceutical Co., Ltd., Merck Biopharma Co., Ltd., MSD K.K., and Pfizer Japan Inc. and consulting fees from Daiichi Sankyo Company, Limited, Nippon Kayaku Co., Ltd., and Nipro Pharma Corporation and personal fees from Nippon Boehringer Ingelheim Co., Ltd., Daiichi Sankyo Company, Limited, Eli Lilly Japan K.K., Bristol-Myers Squibb K.K., Pfizer Japan Inc., Merck Biopharma Co., Ltd., Takeda Pharmaceutical Company Limited, TAIHO Pharmaceutical Co., Ltd., Nippon Kayaku Co., Ltd., Nipro Pharma Corporation, Chugai Pharmaceutical Co., Ltd., AstraZeneca K.K., Novartis Pharma K.K., and Ono Pharmaceutical Co., Ltd. outside the submitted work. No disclosures were reported by the other authors.

ETHICS STATEMENT

Approval of the research protocol by an Institutional Reviewer Board: N/A.

Informed Consent: N/A.

Registry and the Registration No. of the study/trial: N/A.

Animal Studies: All animal experiments were performed under the auspices of the Institutional Animal Care and Research Advisory Committee of the Department of Animal Resources, Okayama University Advanced Science Research Center (Okayama, Japan).

ORCID

Eiki Ichihara  <https://orcid.org/0000-0002-2966-106X>

Hirohisa Kano  <https://orcid.org/0000-0003-4327-7547>

Go Makimoto  <https://orcid.org/0000-0002-0638-8435>

Toshio Kubo  <https://orcid.org/0000-0002-2545-3052>

Kadoaki Ohashi  <https://orcid.org/0000-0002-5180-3933>

REFERENCES

- Soda M, Choi YL, Enomoto M, et al. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. *Nature*. 2007;448(7153):561-566. doi:10.1038/nature05945
- Solomon BJ, Mok T, Kim DW, et al. First-line crizotinib versus chemotherapy in ALK-positive lung cancer. *N Engl J Med*. 2014;371(23):2167-2177. doi:10.1056/NEJMoa1408440
- Hida T, Nokihara H, Kondo M, et al. Alectinib versus crizotinib in patients with ALK-positive non-small-cell lung cancer (J-ALEX): an open-label, randomised phase 3 trial. *Lancet*. 2017;390(10089):29-39. doi:10.1016/S0140-6736(17)30565-2
- Shaw AT, Bauer TM, de Marinis F, et al. First-line Lorlatinib or Crizotinib in advanced ALK-positive lung cancer. *N Engl J Med*. 2020;383(21):2018-2029. doi:10.1056/NEJMoa2027187
- Camidge DR, Kim HR, Ahn MJ, et al. Brigatinib versus Crizotinib in ALK-positive non-Small-cell lung cancer. *N Engl J Med*. 2018;379(21):2027-2039. doi:10.1056/NEJMoa1810171
- Camidge DR, Dziadziuszko R, Peters S, et al. Updated efficacy and safety data and impact of the EML4-ALK fusion variant on the efficacy of Alectinib in untreated ALK-positive advanced non-Small cell lung cancer in the global phase III ALEX study. *J Thorac Oncol*. 2019;14(7):1233-1243. doi:10.1016/j.jtho.2019.03.007
- Yamada T, Takeuchi S, Nakade J, et al. Paracrine receptor activation by microenvironment triggers bypass survival signals and ALK inhibitor resistance in EML4-ALK lung cancer cells. *Clin Cancer Res*. 2012;18(13):3592-3602. doi:10.1158/1078-0432.CCR-11-2972
- Doebele RC, Pilling AB, Aisner DL, et al. Mechanisms of resistance to crizotinib in patients with ALK gene rearranged non-small cell lung cancer. *Clin Cancer Res*. 2012;18(5):1472-1482. doi:10.1158/1078-0432.CCR-11-2906
- Katayama R, Shaw AT, Khan TM, et al. Mechanisms of acquired crizotinib resistance in ALK-rearranged lung cancers. *Sci Transl Med*. 2012;4(120):120ra17. doi:10.1126/scitranslmed.3003316
- Fujita S, Masago K, Katakami N, Yatabe Y. Transformation to SCLC after treatment with the ALK inhibitor Alectinib. *J Thorac Oncol*. 2016;11(6):e67-e72. doi:10.1016/j.jtho.2015.12.105
- Hrustanovic G, Olivass V, Pazarentzos E, et al. RAS-MAPK dependence underlies a rational polytherapy strategy in EML4-ALK-positive lung cancer. *Nat Med*. 2015;21(9):1038-1047. doi:10.1038/nm.3930
- Lin JJ, Riely GJ, Shaw AT. Targeting ALK: precision medicine takes on drug resistance. *Cancer Discov*. 2017;7(2):137-155. doi:10.1158/2159-8290.CD-16-1123
- Gainor JF, Dardai L, Yoda S, et al. Molecular mechanisms of resistance to first- and second-generation ALK inhibitors in ALK-rearranged lung cancer. *Cancer Discov*. 2016;6(10):1118-1133. doi:10.1158/2159-8290.CD-16-0596
- Choi YL, Soda M, Yamashita Y, et al. EML4-ALK mutations in lung cancer that confer resistance to ALK inhibitors. *N Engl J Med*. 2010;363(18):1734-1739. doi:10.1056/NEJMoa1007478
- Mori M, Kaneko N, Ueno Y, et al. Gilteritinib, a FLT3/AXL inhibitor, shows antileukemic activity in mouse models of FLT3 mutated acute myeloid leukemia. *Invest New Drugs*. 2017;35(5):556-565. doi:10.1007/s10637-017-0470-z
- Lee LY, Hernandez D, Rajkhowa T, et al. Preclinical studies of gilteritinib, a next-generation FLT3 inhibitor. *Blood*. 2017;129(2):257-260. doi:10.1182/blood-2016-10-745133
- Kuravi S, Cheng J, Fangman G, et al. Preclinical evaluation of Gilteritinib on NPM1-ALK-driven anaplastic large cell lymphoma cells. *Mol Cancer Res*. 2021;19(5):913-920. doi:10.1158/1541-7786.MCR-20-0738
- Mizuta H, Okada K, Araki M, et al. Gilteritinib overcomes lorlatinib resistance in ALK-rearranged cancer. *Nat Commun*. 2021;12(1):1261. doi:10.1038/s41467-021-21396-w
- Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods*. 1983;65(1-2):55-63. doi:10.1016/0022-1759(83)90303-4
- Isozaki H, Ichihara E, Takigawa N, et al. Non-Small cell lung cancer cells acquire resistance to the ALK inhibitor Alectinib by activating alternative receptor tyrosine kinases. *Cancer Res*. 2016;76(6):1506-1516. doi:10.1158/0008-5472.CAN-15-1010
- Zhang Z, Hasegawa Y, Hashimoto D, et al. Gilteritinib enhances graft-versus-leukemia effects against FLT3-ITD mutant leukemia after allogeneic hematopoietic stem cell transplantation. *Bone Marrow Transplant*. 2022;57(5):775-780. doi:10.1038/s41409-022-01619-4
- Ohteki T, Yoshida H, Matsuyama T, Duncan GS, Mak TW, Ohashi PS. The transcription factor interferon regulatory factor 1 (IRF-1) is important during the maturation of natural killer 1.1+ T cell

- receptor-alpha/beta+ (NK1+ T) cells, natural killer cells, and intestinal intraepithelial T cells. *J Exp Med*. 1998;187(6):967-972. doi:10.1084/JEM.187.6.967
23. Mok T, Camidge DR, Gadgeel SM, et al. Updated overall survival and final progression-free survival data for patients with treatment-naive advanced ALK-positive non-small-cell lung cancer in the ALEX study. *Ann Oncol*. 2020;31(8):1056-1064. doi:10.1016/j.annonc.2020.04.478
24. Perl AE, Martinelli G, Cortes JE, et al. Gilteritinib or chemotherapy for relapsed or refractory FLT3-mutated AML. *N Engl J Med*. 2019;381(18):1728-1740. doi:10.1056/NEJMOA1902688
25. Mok TS, Wu YL, Ahn MJ, et al. Osimertinib or platinum-Pemetrexed in EGFR T790M-positive lung cancer. *N Engl J Med*. 2017;376(7):629-640. doi:10.1056/NEJMoa1612674
26. Ramalingam SS, Vansteenkiste J, Planchard D, et al. Overall survival with Osimertinib in untreated, EGFR-mutated advanced NSCLC. *N Engl J Med*. 2020;382(1):41-50. doi:10.1056/NEJMoa1913662
27. Piotrowska Z, Niederst MJ, Karlovich CA, et al. Heterogeneity underlies the emergence of EGFR T790 wild-type clones following treatment of T790M-positive cancers with a third-generation EGFR inhibitor. *Cancer Discov*. 2015;5(7):713-722. doi:10.1158/2159-8290.CD-15-0399
28. Taniguchi H, Yamada T, Wang R, et al. AXL confers intrinsic resistance to osimertinib and advances the emergence of tolerant cells. *Nat Commun*. 2019;10(1):259. doi:10.1038/s41467-018-08074-0
29. Alam MW, Borenäs M, Lind DE, et al. Alectinib, an anaplastic lymphoma kinase inhibitor, abolishes ALK activity and growth in ALK-positive Neuroblastoma cells. *Front Oncologia*. 2019;9:9. doi:10.3389/FONC.2019.00579
30. Cheng M, Chen Y, Xiao W, Sun R, Tian Z. NK cell-based immunotherapy for malignant diseases. *Cell Mol Immunol*. 2013;10(3):230-252. doi:10.1038/cmi.2013.10

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Ando C, Ichihara E, Nishi T, et al. Efficacy of gilteritinib in comparison with alectinib for the treatment of ALK-rearranged non-small cell lung cancer. *Cancer Sci*. 2023;114:4343-4354. doi:10.1111/cas.15958