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ORIGINAL ARTICLE

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

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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; IC_{50}, 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.

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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

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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 ($3–5\times10^6$) 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 $12–14\times10^5$ 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 μM. 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,

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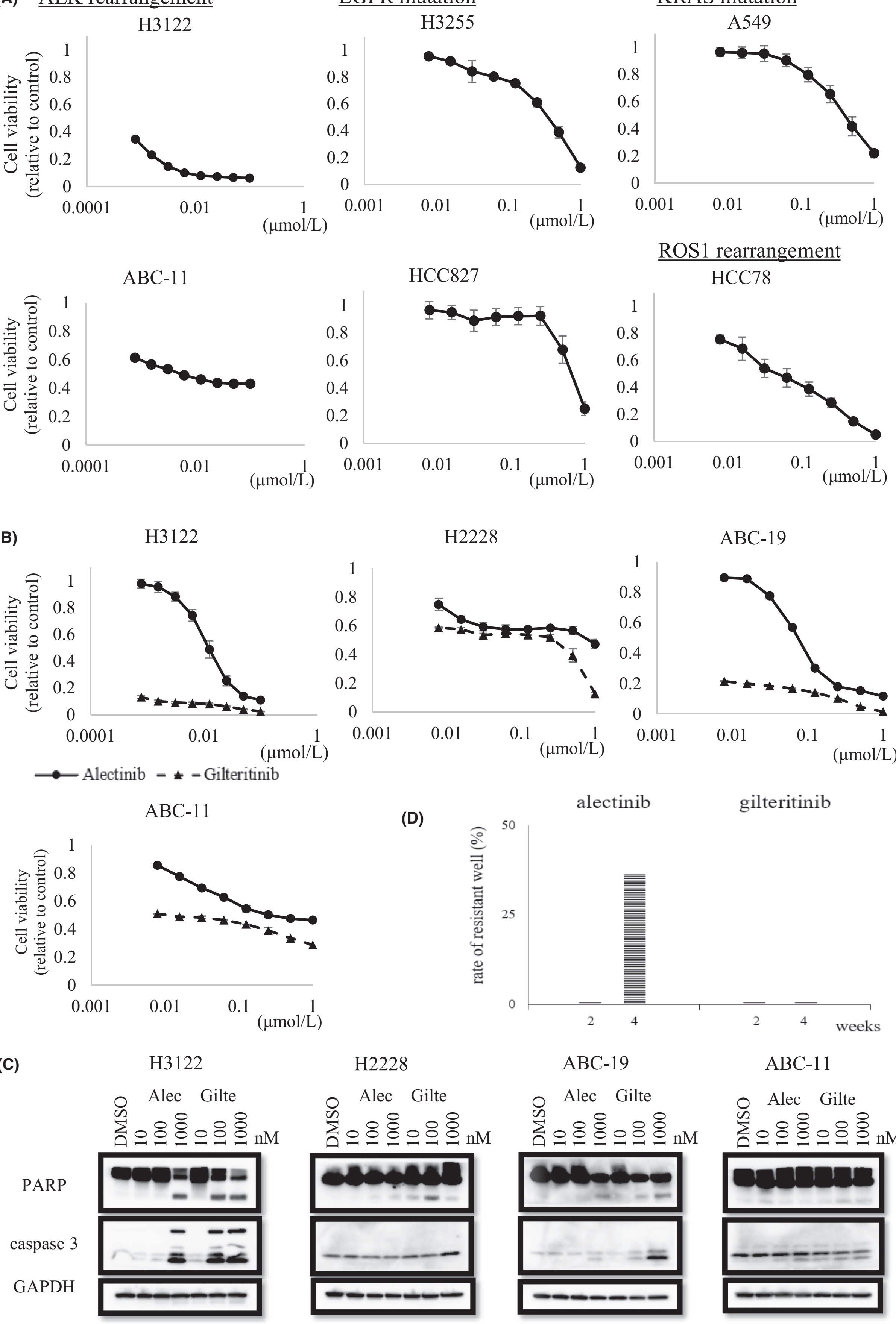

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FIGURE 1 Efficacy of gilteritinib on multiple oncogene-driven NSCLC cell lines. (A) Cell proliferation assay using gilteritinib in cell lines with different gene mutations. Error bars indicate SD. (B) Cell proliferation assay with alectinib and gilteritinib 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 72h. (D) H3122 cells were seeded in 96-well plates at 5000 cells/well and treated with 100nM alectinib or 100nM 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 gilteritinib[16] and monitored the effect of gilteritinib on the phosphoactivation 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 4months 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

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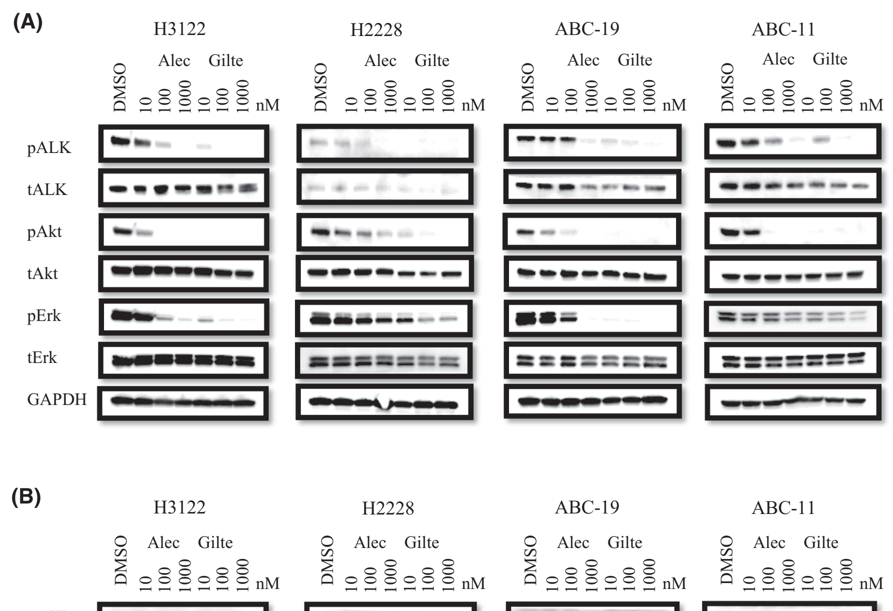

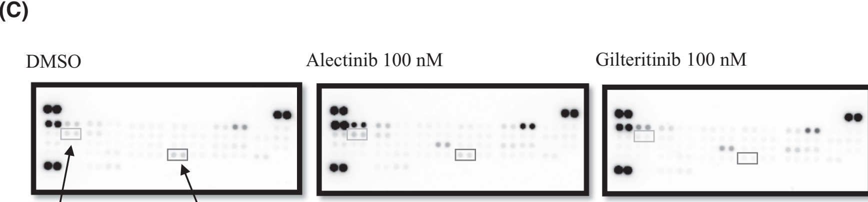

(C)

DMSO Alectinib 100 nM Gilteritinib 100 nM

MET ALK

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

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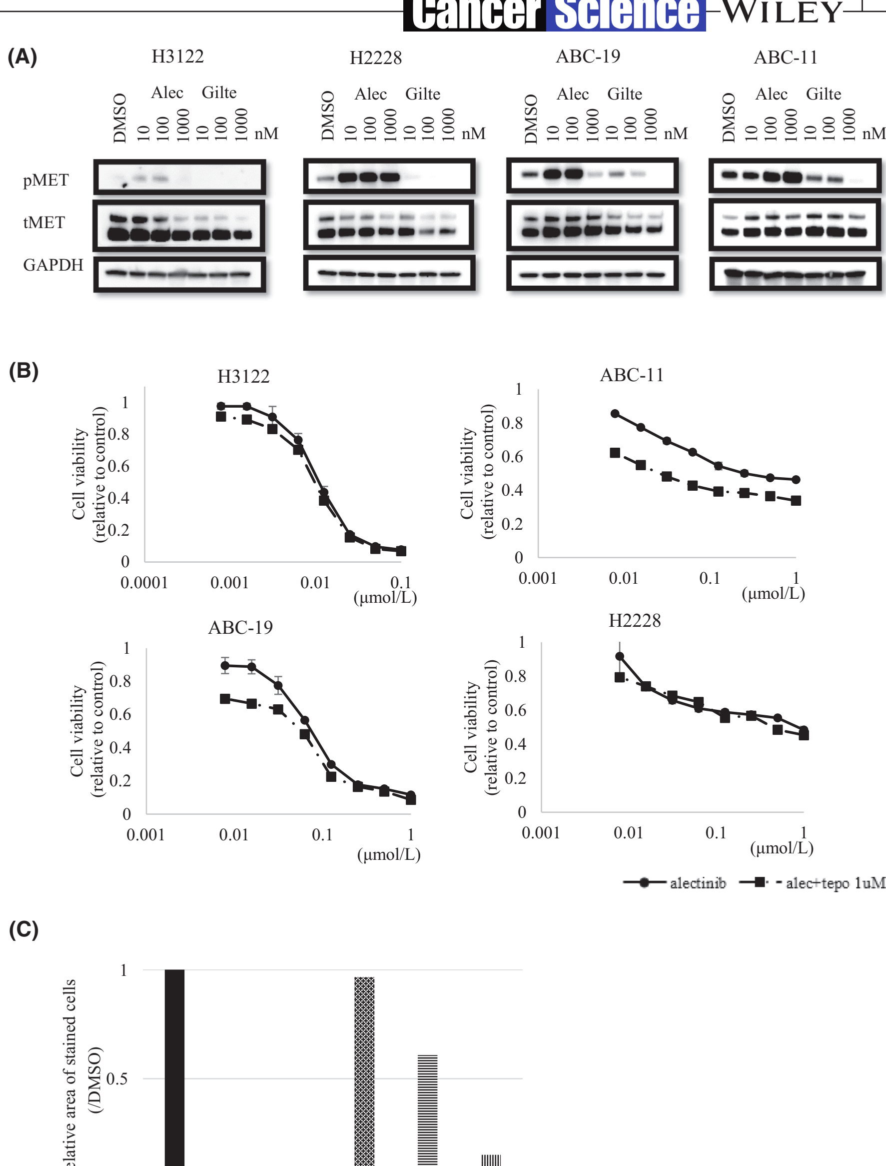

FIGURE 3 Effects of gilteritinib on MET signaling in *ALK*-rearranged cell lines. (A) Phospho-MET levels are evaluated by Western blotting. Cells were treated with the indicated concentrations of alectinib or gilteritinib for 24 h. All experiments were performed in triplicate. (B) Cell proliferation 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 IC_{50} 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

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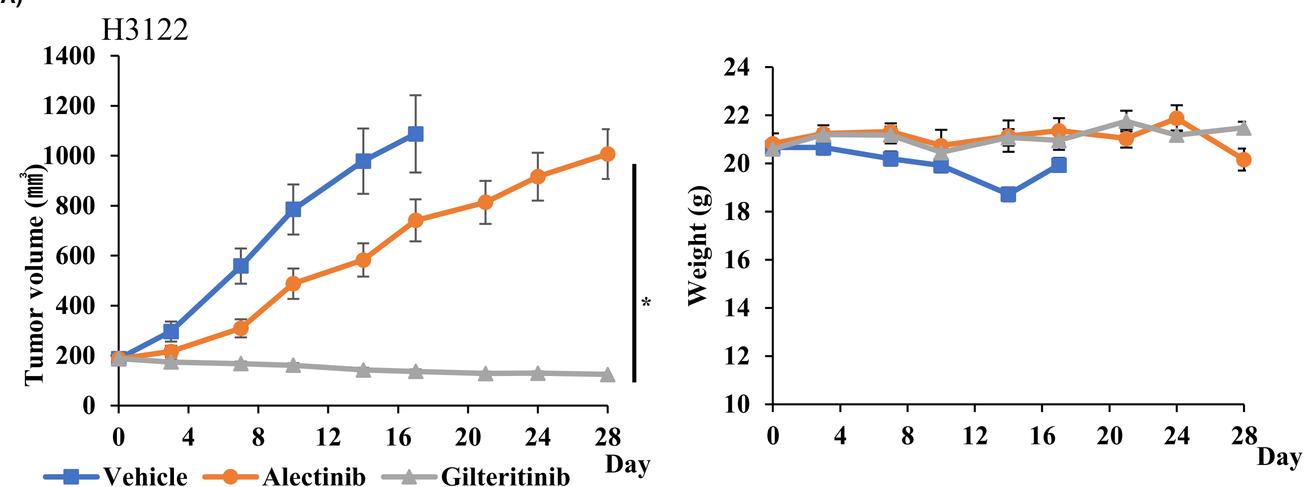

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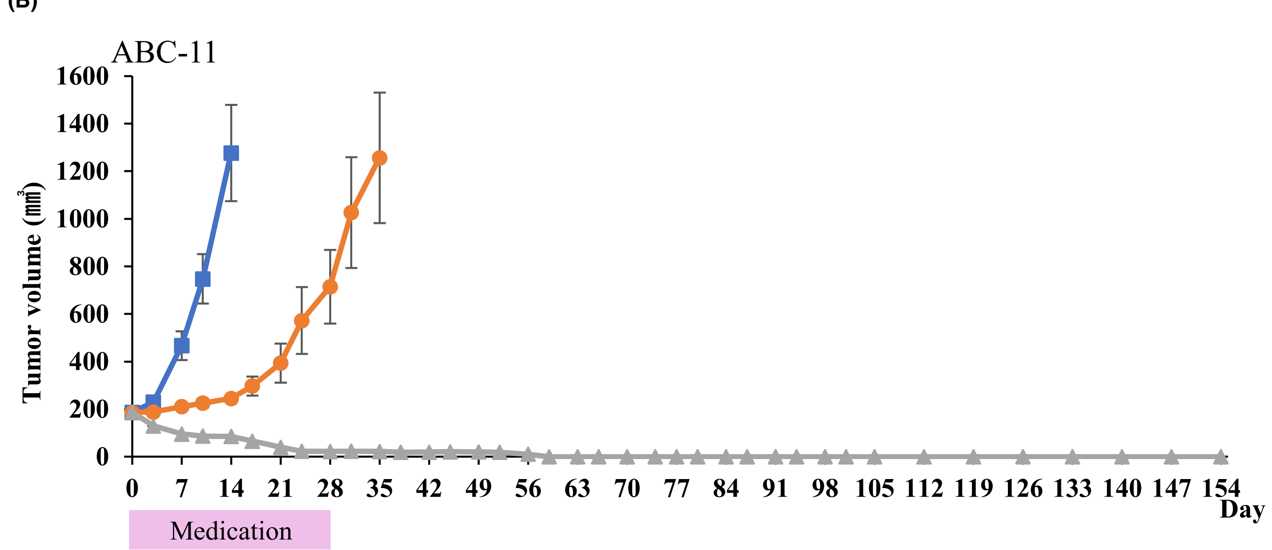

(C) (D)

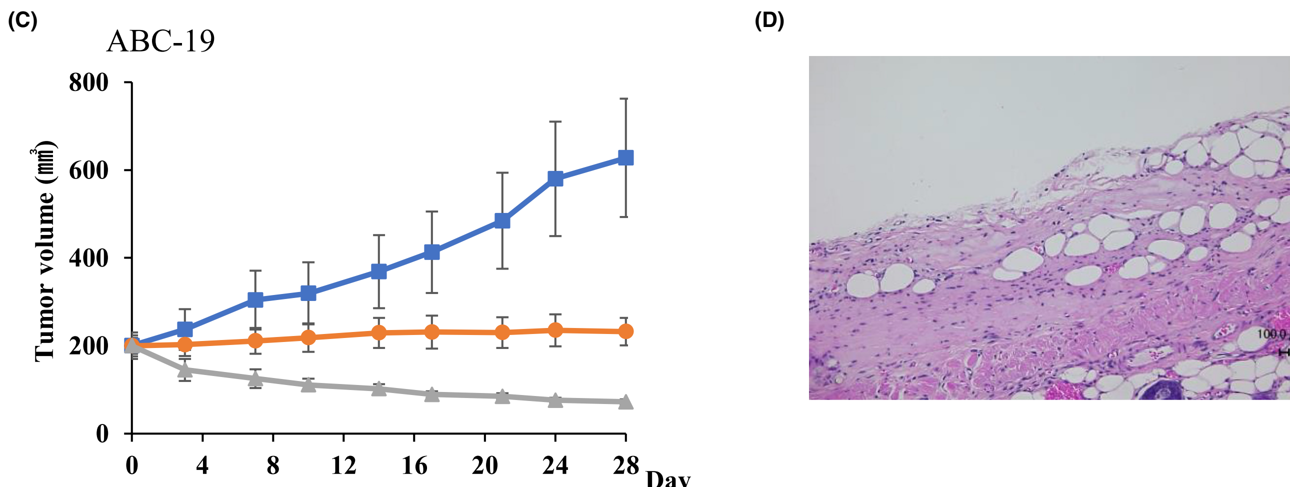

FIGURE 4 Effects of gilteritinib on *ALK*-rearranged xenograft tumors. (A–C) Mice injected with H3122, ABC-11, or ABC-19 cells for tumor growth. The TKI agents (alectinib or gilteritinib, 10 mg/kg/day) were orally administered 5 days per week for 28 days. No body weight loss was observed. Error bars 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 μm.

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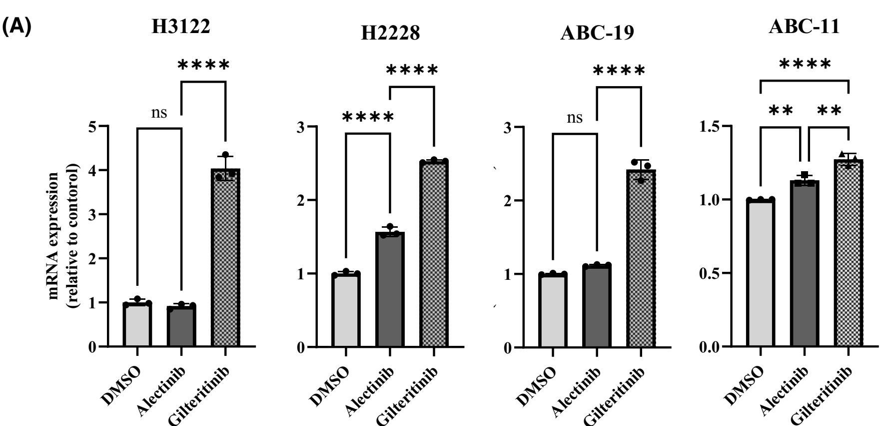

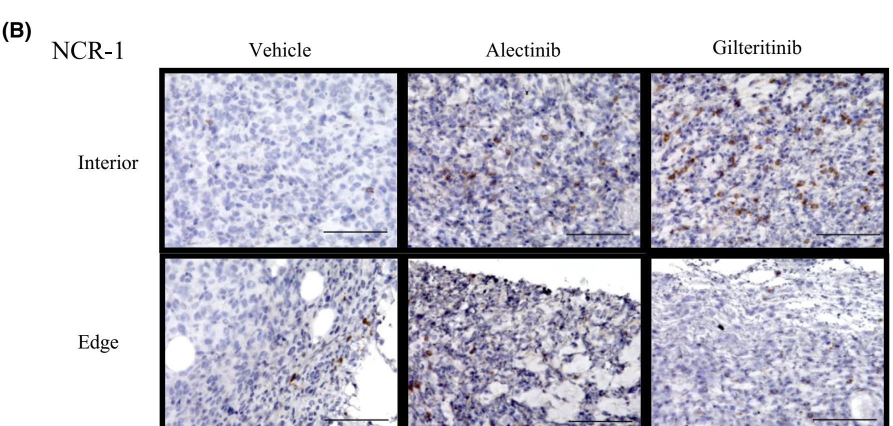

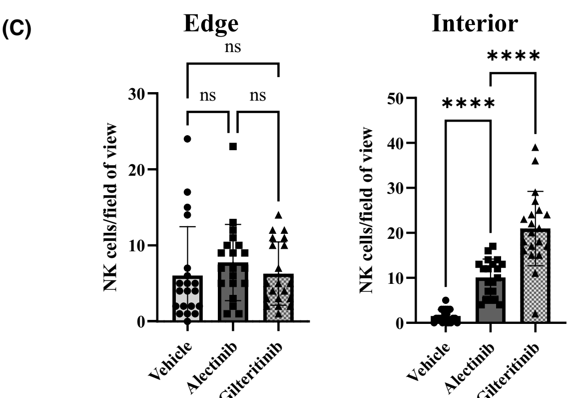

FIGURE 5 Interleukin (IL)-15 production and natural killer (NK) cell recruitment in gilteritinib-treated *ALK*-rearranged NSCLC cells. (A) qPCR quantification of IL-15 mRNA levels relative to GAPDH in *ALK*-rearranged NSCLC cell lines. The cells were treated with DMSO, 100 nM alectinib, or 100 nM gilteritinib 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 10 mg/kg/day on day 4), assessed by immunohistochemical analysis. Scale bar, 100 µm. 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 antitumor 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

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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 refence 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.

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CONFLICT OF INTEREST STATEMENT

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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).

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