


ORIGINAL ARTICLE

Fibroblast growth factor receptor 3 overexpression mediates ALK inhibitor resistance in ALK-rearranged non-small cell lung cancer

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Abstract

The rearrangement of *anaplastic lymphoma kinase* (ALK) occurs in 3%–5% of patients with non-small cell lung cancer (NSCLC) and confers sensitivity to ALK-tyrosine kinase inhibitors (TKIs). For the treatment of patients with ALK-rearranged NSCLC, various additional ALK-TKIs have been developed. Ceritinib is a second-generation ALK-TKI and has shown great efficacy in the treatment of patients with both newly diagnosed and crizotinib (a first-generation ALK-TKI)-refractory ALK-rearranged NSCLC. However, tumors can also develop ceritinib resistance. This may result from secondary ALK mutations, but other mechanisms responsible for this have not been fully elucidated. In this study, we explored the mechanisms of ceritinib resistance by establishing ceritinib-resistant, echinoderm microtubule-associated protein-like 4 (EML4)-ALK-positive H3122 cells and ceritinib-resistant patient-derived cells. We identified a mechanism of ceritinib resistance induced by bypass signals that is mediated by the overexpression and activation of fibroblast growth factor receptor 3 (FGFR3). FGFR3 knockdown by small hairpin RNA or treatment with FGFR inhibitors was found to resensitize the resistant cells to ceritinib in vitro and in vivo. FGFR ligands

Abbreviations: ALK, anaplastic lymphoma kinase; EGFR, epidermal growth factor receptors; ERK, extracellular signal-regulated kinases; FGFR, fibroblast growth factor receptor; FISH, fluorescence in situ hybridization; RTK, receptor tyrosine kinase; TKI, tyrosine kinase inhibitors.

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from either human serum or fetal bovine serum were able to activate FGFR3 and induce ceritinib resistance. A detailed analysis of ceritinib-resistant patient-derived specimens confirmed that tyrosine-protein kinase Met (cMET) amplification induces ceritinib resistance. Amplified cMET counteractivated EGFR and/or Her3 and induced ceritinib resistance. These results reveal multiple ceritinib resistance mechanisms and suggest that ceritinib resistance might be overcome by identifying precise resistance mechanisms.

KEYWORDS

ALK kinase, ceritinib, drug resistance, FGFR3, lung cancer

1 | INTRODUCTION

Worldwide, approximately 1.8 million people die of lung cancer annually. This makes it the leading cause of cancer deaths.¹ As sequencing technologies have developed, multiple oncogenic driver mutations and genetic alterations in patients with lung cancer have been identified. Among these is anaplastic lymphoma kinase (ALK) rearrangement, which occurs in 3%-5% of those with lung adenocarcinoma.² ALK rearrangement is most frequently seen in the echinoderm microtubule-associated protein-like 4 (EML4)-ALK fusion gene of individuals with non-small cell lung cancer (NSCLC). As this was first identified in 2007, numerous other ALK fusion genes with various fusion partners have been identified in patients with lung cancer.^{3,4} In cases of ALK-rearranged lung cancer, the patients tend to be relatively young and nonsmokers or light smokers. Their tumors are usually adenocarcinomas.^{2,5} Crizotinib is a first-generation ALK inhibitor that was approved for the treatment of patients with ALK-rearranged NSCLC in 2011 by the Food and Drug Administration (FDA) in the United States and in 2012 by the Pharmaceuticals and Medical Devices Agency (PMDA) in Japan.⁶⁻⁸

Crizotinib often produced drastic regression of ALK-rearranged NSCLC.⁷ However, more than 50% of patients relapsed within a year due to the development of crizotinib-resistant tumors.⁷⁻⁹ The main causes of crizotinib resistance are mutations in the ALK kinase domain, activation of bypass pathways such as epidermal growth factor receptors (EGFRs), and amplification of c-Kit tyrosine kinase receptors with upregulation of its stem cell factor ligand.⁹⁻¹⁵ To overcome crizotinib-resistant mutations, multiple next-generation ALK inhibitors have been developed, including alectinib, brigatinib, lorlatinib, and ceritinib.¹⁶⁻¹⁹ Ceritinib was approved by the FDA in April 2014 and by the PMDA in March 2016. In clinical trials, patients with ALK-rearranged NSCLC, including post-crizotinib treatment patients, responded well to ceritinib.¹⁷ The potency of ceritinib is >10-fold higher than that of crizotinib and has been found efficacious against multiple crizotinib-resistant mutations both in vitro and in vivo.²⁰ However, tumors also eventually develop ceritinib resistance due to ceritinib-resistant mutations in the ALK kinase domain such as G1202R or F1174C/V.²⁰ Unfortunately, little is known about the ceritinib resistance mechanisms especially bypass pathway-mediated resistance.

We established ceritinib-resistant H3122 (EML4-ALK-harboring cell line) cells in vitro by treating them with increasing concentrations of ceritinib over 6 months. In addition to assessing the ceritinib resistance of these cell lines, we examined the clinical specimens taken from ceritinib-resistant patients. After comprehensive analyses, we identified a novel ceritinib resistance mechanism mediated by FGFR3 activation. This finding highlights the importance of determining the resistance mechanisms in the ALK-TKI resistant patients to ensure an appropriate treatment strategy.

2 | MATERIAL AND METHODS

Detailed information is shown in Appendix S2.

2.1 | Patients

Clinical specimens were collected from patients with ALK-rearranged NSCLC who acquired ceritinib resistance. The patients submitted written informed consent for all genetic and cell biological analyses, which were performed in accordance with the protocols approved by the institutional review board (IRB) of the Japanese Foundation for Cancer Research (#2013-1093).

2.2 | Cell lines

H3122 human NSCLC cell line (harboring EML4-ALK variant 1) was obtained in 2010, which was originally established from the lung cancer patient as previously described.²¹ Ba/F3, immortalized murine bone marrow-derived pro-B cells were obtained from the RIKEN BRC Cell Bank (RIKEN BioResource Center) in 2012.

2.3 | Reagents

Ceritinib, alectinib, lorlatinib, cabozantinib, and zoligratinib were purchased from ActiveBiochem. Crizotinib, brigatinib, and infigratinib were purchased from Biochempartner. AZD4547 was

purchased from Selleck, and PHA665752 was purchased from Tocris Bioscience.

2.4 | Cell growth assay

Cell viability was measured using the CellTiter-Glo assay reagent (Promega) and Centro LB 960 microplate luminometer (Berthold Technologies).

2.5 | Immunoblotting

Cell lysis and immunoblotting were performed as previously described.^{9,22}

2.6 | Sequencing and qRT-PCR

Sequencing was bidirectionally conducted using Sanger sequencing, and qRT-PCR was performed using FastStart Essential DNA Green Master (Roche) according to the manufacturer's protocol.

2.7 | Lentivirus transduction

Viruses were produced in 293FT cells as previously described.¹³

2.8 | Phospho-receptor tyrosine kinase (RTK) array

The RTK array was performed using a human phospho-RTK array kit (R&D Systems) according to the manufacturer's protocol.

2.9 | Mouse experiments

Female BALB/*c-nu/nu* (nude) mice were purchased from Charles River Laboratories, Yokohama, Japan. All animal procedures were performed in accordance with protocols approved by the JFCR Animal Care and Use Committee.

2.10 | FISH analysis

FISH analyses to detect *ALK*, *CEP2*, and *MET* were conducted using formalin-fixed, paraffin-embedded tissues with in-house probes made from BAC clones (the exact clone names are available upon request).

2.11 | Microarray analysis

RNA was purified using the RNeasy mini kit (Qiagen). A total of 100ng of extracted RNA was labeled and hybridized onto the

GeneChip PrimeView human gene expression array (Affymetrix Inc.). Microarray data have been deposited in the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo) under accession numbers GSE77764.

2.12 | Statistical analysis

All data are presented as mean \pm standard deviation. Statistical analysis was performed using the two-tailed Student's *t* test or Mann-Whitney U test (for mice experiments). Significant *p* values are defined as **p* < 0.05.

3 | RESULTS

3.1 | Establishment of ceritinib-resistant H3122 cells

H3122 cells from the patient with NSCLC harbored *EML4-ALK* fusion genes and were highly sensitive to ALK inhibitors (Figure 1A). Ceritinib has shown remarkable clinical effects as a first, second, and later line of treatment.^{17,23,24} However, the tumors eventually develop ceritinib resistance mediated by mutations, such as ALK-G1202R, ALK-F1174C/V, or P-glycoprotein upregulation.^{20,25} To identify the ceritinib resistance mechanisms, we first treated H3122 cells with increasing concentrations of ceritinib for 6-12 months and established four ceritinib-resistant H3122 LR cell lines (Figure 1B). The IC₅₀ of ceritinib for the H3122 cells after 3 days of treatment was approximately 8nM. However, the H3122 LR1 and LR5 cells survived after continuous exposure to 100nM ceritinib, and the H3122 LR2 and LR3 cells even grew after exposure to ceritinib concentrations of 1 μ M. The H3122 LR1 and LR5 cells showed moderate resistance to ceritinib, crizotinib, and alectinib (Figure 1C,D). Neither exhibited ALK tyrosine kinase mutations. We performed an immunoblotting analysis and found *EML4-ALK* overexpression and phospho-ALK upregulation in the H3122 LR1 and LR5 cells (Figure 1E). We quantified the number of *ALK* copies using qRT-PCR with genomic DNA and identified *ALK* gene amplification in both the H3122 LR1 and LR5 cells (Figure 1F). In contrast, *ALK* amplification was not observed in the H3122 LR2 and LR3 cells. FISH analysis clearly showed that *ALK* gene amplification was caused by gene amplification of the *EML4-ALK* locus within chromosome 2 in H3122 LR1 and H3122 LR5 cells because we detected more ALK 3' split signals to *CEP2* signals in H3122 LR1 and H3122 LR5 cells than in H3122 and H3122 LR3 cells (Figure S1). These results suggest that *ALK* gene amplification induced moderate resistance to ceritinib.

3.2 | Identification of L1198F in the H3122 LR2 cells

The H3122 LR2 cells demonstrated much higher ceritinib resistance than the H3122 cells (Figure S2A), and the phospho-ALK

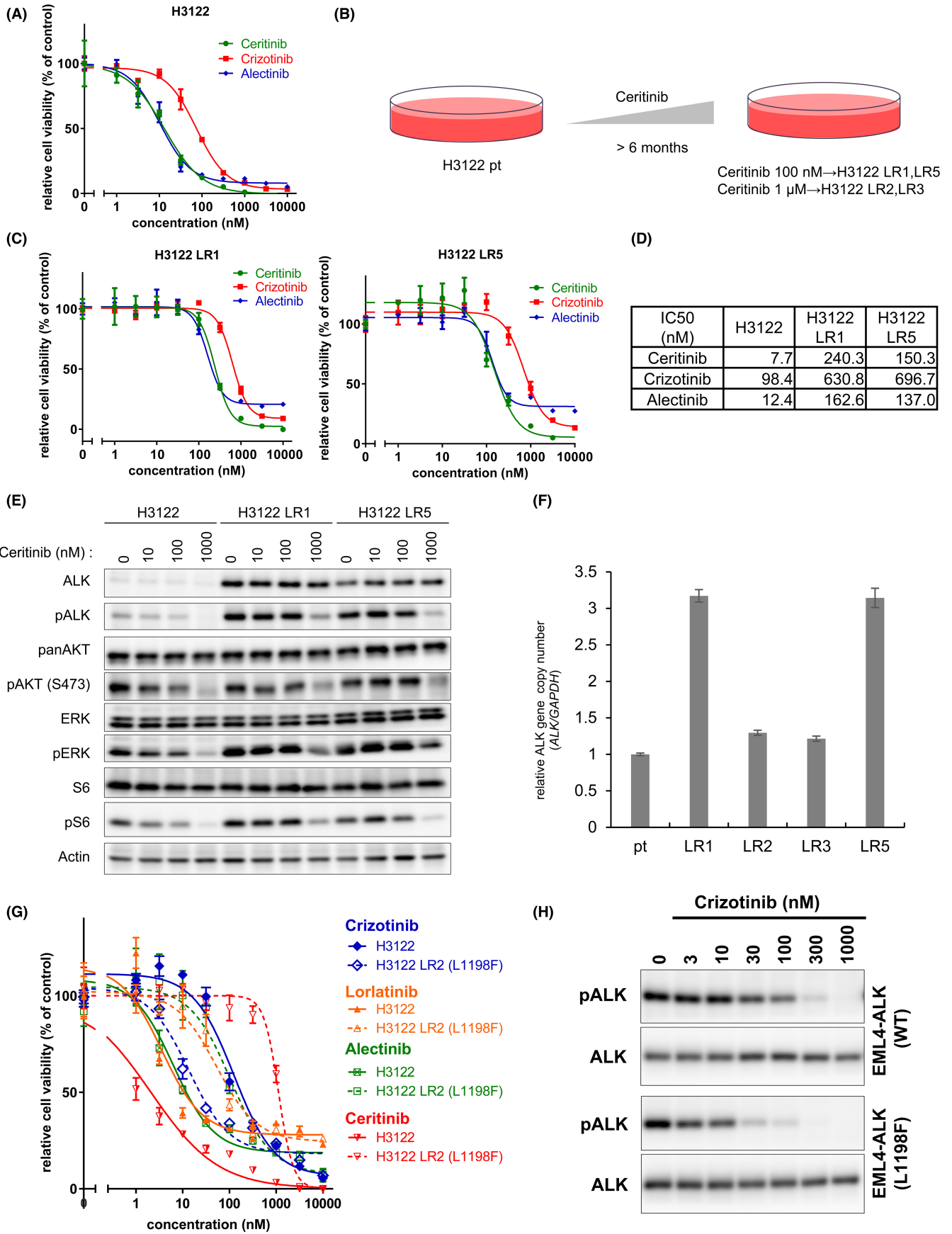


FIGURE 1 Anaplastic lymphoma kinase (ALK) amplification in the H3122 LR1 and LR5 and ALK L1198F mutation in the H3122 LR2 conferred intermediate and high resistance to ceritinib, respectively. A, C, G, The H3122 and H3122 LR cells were treated with the indicated concentrations of ALK inhibitors for 72 h. Calculated IC₅₀ values are shown in (D). B, The ceritinib (LDK378)-resistant H3122 cells were established by treating the parental H3122 (H3122 pt) cells with increasing concentrations of ceritinib over 6 months. E, The H3122 and H3122 LR1 and LR5 cells were treated with the indicated concentrations of ceritinib or crizotinib for 6 h. After incubation, the cells were lysed and analyzed by immunoblotting. F, Genomic DNA was extracted from five cell lines, and each relative ALK gene copy number was analyzed by qRT-PCR. H, The Ba/F3 expressing EML4-ALK-WT or -L1198F cells were treated with the indicated concentrations of ceritinib for 4 h. After incubation, the cells were lysed and analyzed by immunoblotting.

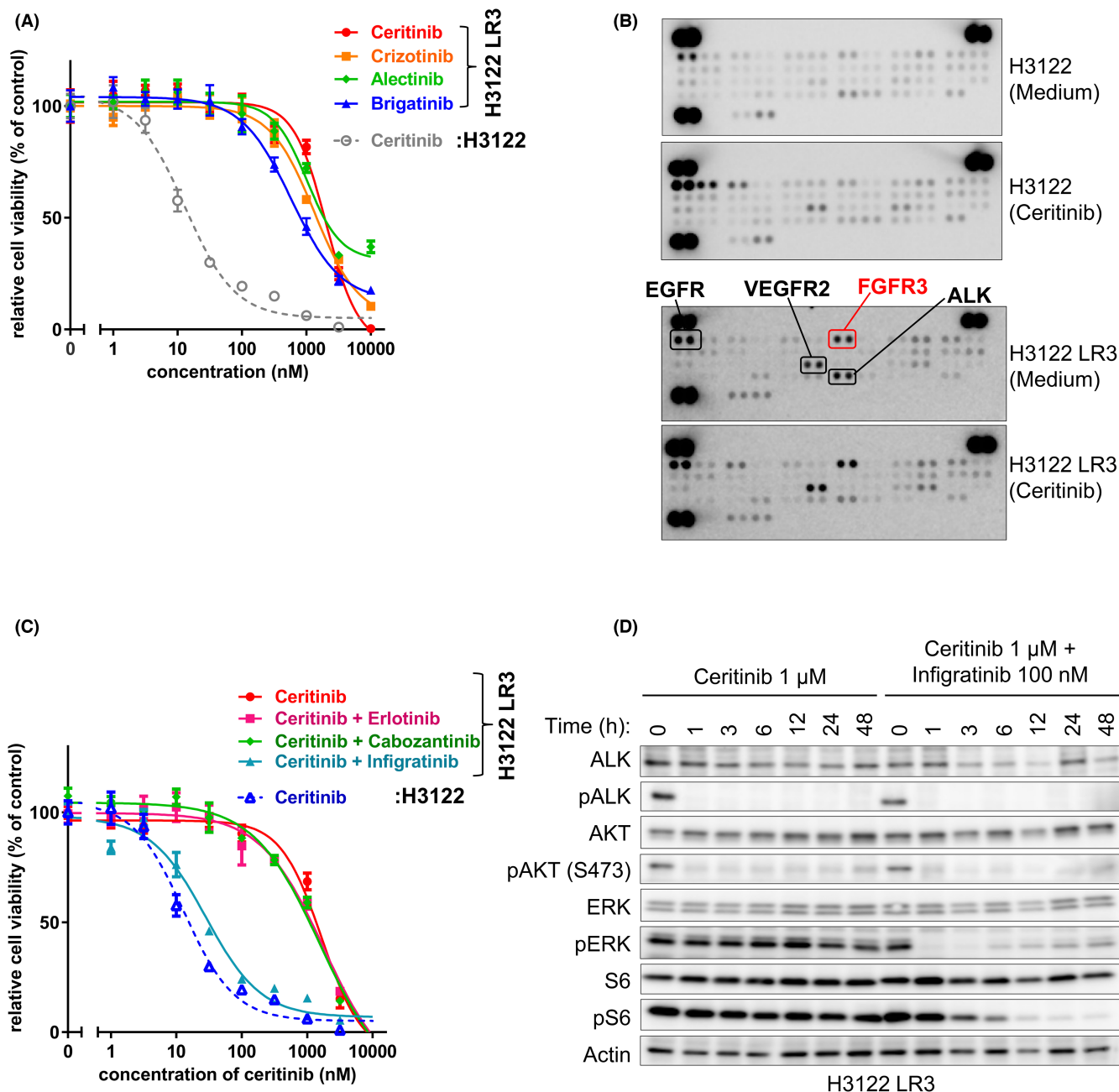


FIGURE 2 Ceritinib resistance was induced by ligand-dependent activation of FGFR3 in the H3122 LR3 cells. A, C, The H3122 and H3122 LR3 cells were treated with various concentrations of ceritinib with or without erlotinib (1 μ M), cabozantinib (1 μ M), or infigratinib (100 nM) for 72 h. B, The phosphorylation levels of 49 RTKs in the H3122 and H3122 LR3 cells were measured using an RTK array with cell lysates treated with or without 1 μ M of ceritinib for 8 h. D, The H3122 LR3 cells were treated with ceritinib (1 μ M) with or without infigratinib (100 nM) for 1–48 h. After incubation, the cells were lysed and analyzed by immunoblotting.

levels of the H3122 LR2 cells were maintained even at a ceritinib concentration of $1\ \mu\text{M}$ (Figure S2B). This suggested that the H3122 LR2 cells harbored a ceritinib resistance mutation. Thus, we sequenced the ALK gene from the H3122 LR2 cells and 10 clones isolated from ceritinib-resistant H3122 LR2 cells and found a c3592t (L1198F) mutation in the ALK (Figure S2C). Several studies have reported that the L1198F mutation confers resistance to various other ALK inhibitors, but not crizotinib.^{26–28} The H3122 LR2 cells were found to be resistant to lorlatinib and alectinib as well as ceritinib but sensitive to crizotinib (Figure S2D). Similarly, Ba/F3 cells expressing EML4–ALK–L1198F showed high ceritinib resistance (Figure S3A,B). The IC₅₀ of all next-generation ALK inhibitors (ceritinib, alectinib, lorlatinib, and brigatinib) were higher in Ba/F3–EML4–ALK–L1198F than in Ba/F3–EML4–ALK–WT, but the IC₅₀ of crizotinib was lower in Ba/F3–EML4–ALK–L1198F than in Ba/F3–EML4–ALK–WT (Figure S3C–E).

3.3 | Induction of H3122 LR3 ceritinib resistance by FGFR3 activation

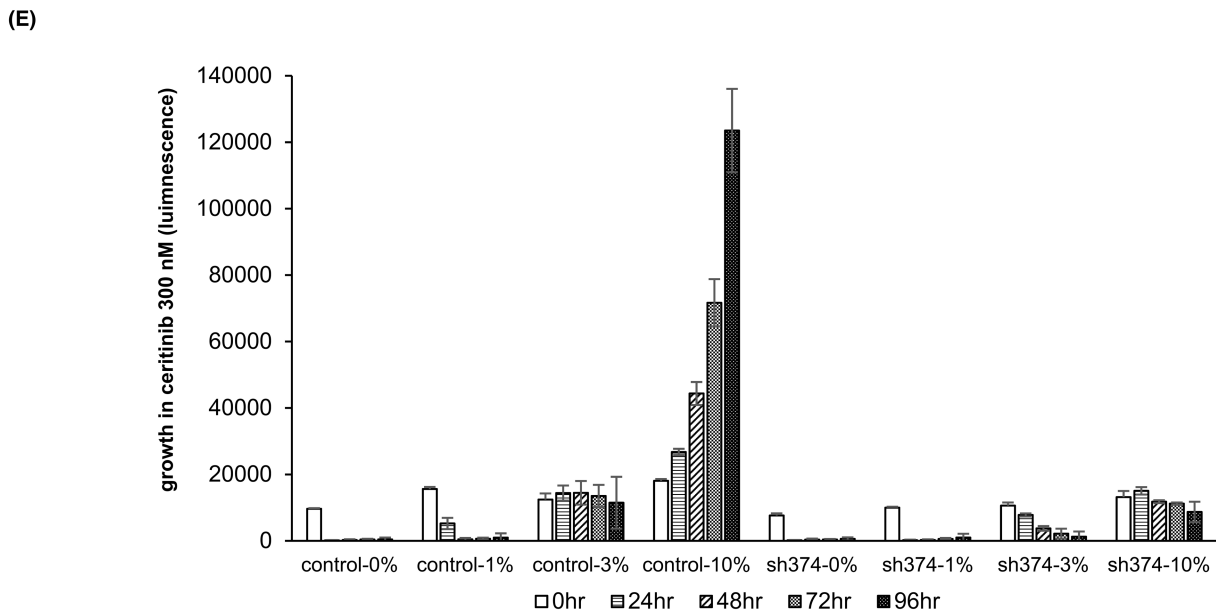
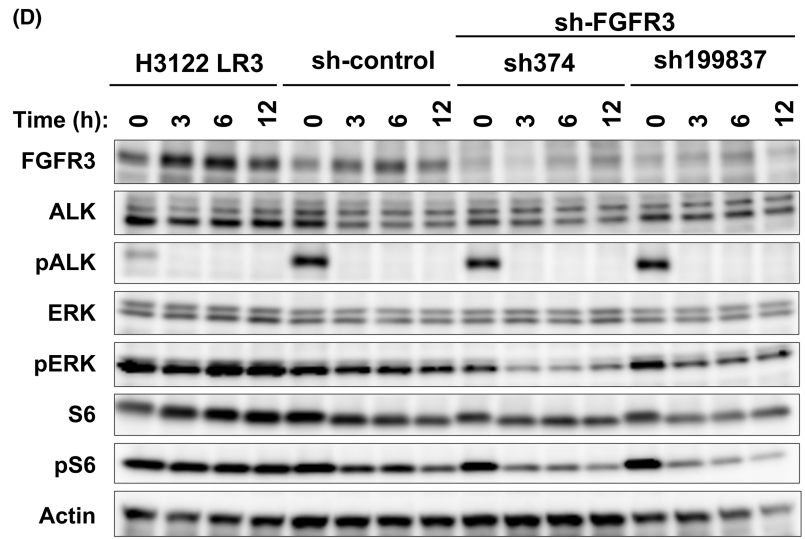
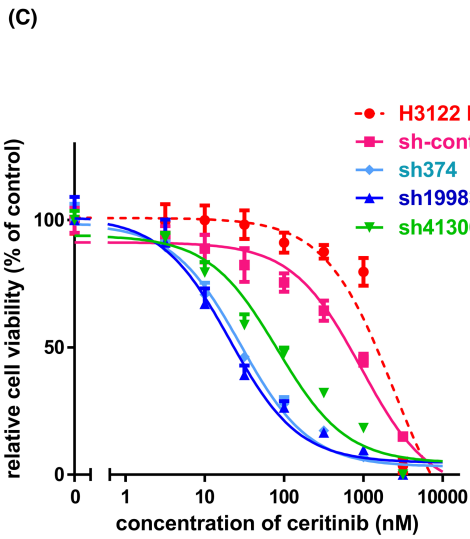
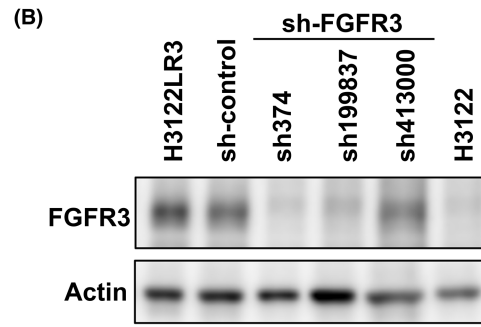
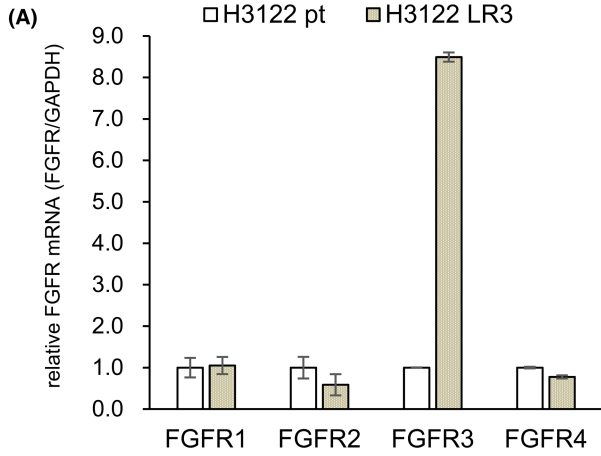
We next evaluated the ceritinib-resistant H3122 LR3 cells and found that they were also highly resistant to other ALK inhibitors (Figure 2A). The H3122 LR3 cells did not show ALK amplification or an EML4–ALK mutation. Therefore, we inferred that a different resistance mechanism such as activation of another RTK had induced the ceritinib resistance in these cells. To identify the activation of other RTKs, we performed a phospho-RTK array to compare the H3122 cells and the H3122 LR3 cells with and without ceritinib exposure. In the H3122 LR3 cells, we detected more phospho-EGFR, vascular endothelial growth factor receptor (VEGFR)2, and FGFR3 than in the H3122 cells (Figure 2B). On the basis of this result, we treated the H3122 LR3 cells with ceritinib and several RTK inhibitors corresponding to specific RTK (erlotinib, EGFR inhibitor; cabozantinib, VEGFR inhibitor; and infgratinib, FGFR inhibitor). Interestingly, only combined treatment with ceritinib and infgratinib resensitized the cells to ceritinib (Figure 2C). Immunoblotting analysis showed that the downstream signaling of ALK was maintained during ceritinib treatment alone but decreased when ceritinib was combined with infgratinib (Figure 2D). AZD4547, another FGFR inhibitor, also resensitized the H3122 LR3 cells to ceritinib (Figure S4A). Additionally, the combination of other ALK inhibitors with infgratinib was effective against the H3122 LR3 cells but did not affect the parental H3122 cells (Figure S4B–D). Infgratinib alone did not

suppress the growth of either the H3122 or H3122 LR3 cells even at concentrations as high as $3\ \mu\text{M}$ (Figure S3E). All 10 single clones of the H3122 LR3 cells were also resensitized to ceritinib when it was combined with infgratinib (Figure S4F). Additionally, the H3122 LR3 cells grew faster with higher fetal bovine serum (FBS) concentrations than the H3122 cells, and the induction of ceritinib resistance was found to be dependent on FBS (Figure S5A,B). The dependence on serum was similarly observed when we used human serum instead of FBS (Figure S6). These results suggested that ligand-dependent overactivation of FGFR was a significant factor in the ALK inhibitor resistance of the H3122 LR3 cells.

3.4 | Resensitization of the H3122 LR3 cells to ceritinib by FGFR3 small hairpin RNA (shRNA) knockdown

The FGFR family comprises five variants, and all of these but FGFR5 have tyrosine kinase domains.^{29,30} We quantified the mRNA expression of *FGFR1–4* in the parental H3122 and H3122 LR3 cells using quantitative PCR and found overexpression of FGFR3 in the H3122 LR3 cells (Figure 3A), whereas *FGFR3* gene amplification was not observed (Figure S7). The mRNA expression of FGFR3 ligands (FGF1, FGF2, FGF4, FGF9, and FGF18) was also similar between the parental H3122 and H3122 LR3 cells (Figure S8). Additionally, we performed gene expression analysis using cDNA microarray to confirm whether other molecules or pathways were related to the ceritinib resistance of the H3122 LR3 cells (Figure S9A–C). In the microarray analysis, *FGFR3* was the gene with the greatest increase in expression. Next, to examine whether the overexpressed FGFR3 was responsible for the ceritinib resistance in the H3122 LR3 cells, we performed an shRNA knockdown of FGFR3 in the H3122 LR3 cells (Figure 3B). The FGFR3 expression level was much lower in the H3122 LR3–sh374 and H3122 LR3–sh199837 and slightly lower in the H3122 LR3–sh413000 than in the H3122 LR3 and the H3122 LR3–sh-control cells. We found that the FGFR3 knockdown resensitized the cells to ceritinib according to the decrease in FGFR3 expression levels of the shFGFR3 (Figure 3C). Immunoblotting analysis showed the downstream signaling in the FGFR3 knockdown cells treated with $1\ \mu\text{M}$ of ceritinib to be reduced more than those of the H3122 LR3 and the sh-control cells (Figure 3D). Also, ceritinib resistance was not induced in the H3122 LR3–sh374 even in the medium containing 10% human serum (Figure 3E). Therefore, we

FIGURE 3 FGFR3 knockdown in H3122 LR3 cells restored sensitivity to ceritinib. A, Quantitative real-time PCR was used to measure FGFR (*FGFR1–4*) mRNA expression levels in the H3122 and H3122 LR3 cells. The H3122 LR3 mRNA expression levels (*FGFRs/GAPDH*) relative to those in the H3122 parental cells are shown in the bar graph. B, FGFR3 protein expression in the indicated shRNA-infected H3122 LR3 cells was analyzed by immunoblotting. C, H3122 LR3 and shFGFR3 H3122 LR3 cells were treated with the indicated concentrations of ceritinib for 72 h. D, The H3122 LR3 and shFGFR3 H3122 LR3 cells were treated with $1\ \mu\text{M}$ ceritinib for the indicated amount of time (0–2 h). After incubation, the cells were lysed and analyzed by immunoblotting. E, H3122 LR3 control cells and H3122 LR3–sh374 cells were plated in RPMI-1640 containing the indicated concentrations of human AB serum (Mediatech). Beginning the next day, they were cultured with 300 nM ceritinib for 0–96 h.



concluded that FGFR3 overexpression induces serum-derived FGFR ligand-dependent ceritinib resistance.

3.5 | Resistance of H3122 LR3 cells suppressed by ALK inhibitor combined with FGFR inhibitor

We next performed an experiment to confirm whether combined treatment with ALK inhibitor and FGFR inhibitor was also effective in vivo. However, we found that treatment with ceritinib and infogatinib markedly decreased the body weight of mice (data not shown), so we adopted another combination strategy: alectinib and zoligratinib (an FGFR inhibitor).³¹ The combined treatment with

alectinib and zoligratinib also effectively inhibited the growth of the H3122 LR3 cells in vitro (Figure 4A), whereas zoligratinib treatment alone did not affected cell growth (Figure S10). We inoculated the H3122 LR3 cells into nude mice and administered the combined drug treatment daily over 6 days (Figure 4B). An RTK array on the tumor cells found greater phosphorylation of FGFR3 in the H3122 LR3 tumors than in the H3122 tumors (Figure S11A). As with the in vitro results, the combined treatment with alectinib and zoligratinib was also effective in vivo (Figure 4C). Similarly, an H3122 LR3-sh-control xenograft tumor was resistant to ceritinib, but an H3122 LR3-sh374 xenograft was sensitive to ceritinib in vivo (Figure S11B). In conclusion, combined treatment with ALK inhibitors and FGFR inhibitors can overcome FGFR3-mediated resistance mechanisms.

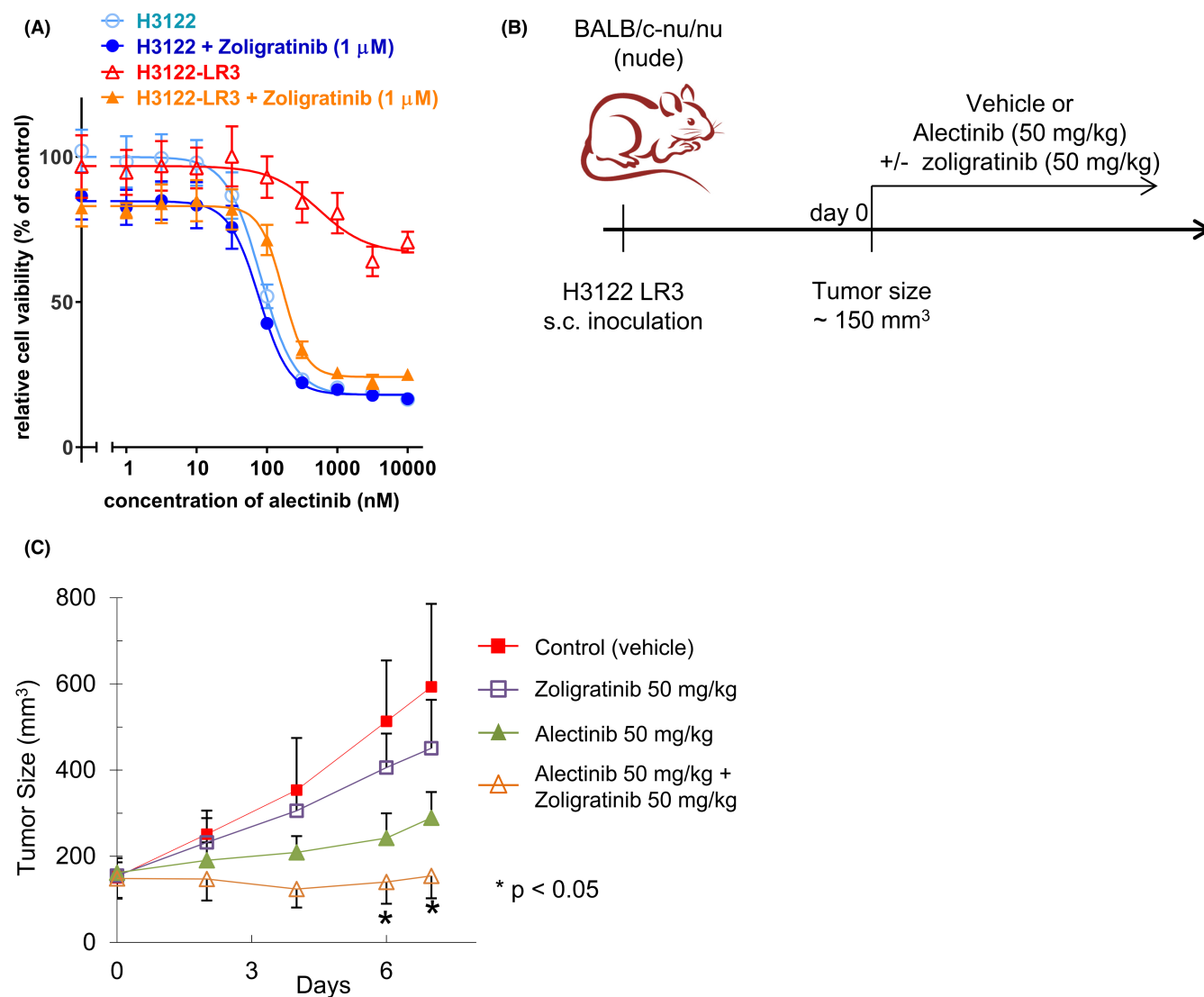


FIGURE 4 Combined treatment with alectinib and the FGFR inhibitor, zoligratinib, effectively resensitized H3122 LR3 cells to ceritinib in vivo. A, The H3122 LR3 cells were treated with various concentrations of alectinib with or without 1 μM of zoligratinib for 72 h. B, Schematic representation of the treatment schedule. After the tumors reached sizes of approximately 150 mm³, the mice were randomized by tumor size, and daily treatment with 50 mg/kg of alectinib with or without 50 mg/kg of zoligratinib was initiated. The tumor volumes were measured as 0.5 × length × width × height. The mean tumor volumes are shown in (C). *p < 0.05.

3.6 | Ceritinib resistance mechanisms other than ALK mutations in the clinical specimens

In patients treated with crizotinib, only one-third developed resistance due to ALK secondary mutations or ALK fusion gene amplification.³² We have analyzed the clinical specimens treated with ceritinib, and reported several ceritinib resistance mechanisms (Table S1). Of the tumor samples, 42% (5/12) carried ALK resistance mutations such as F1174C, F1174V, and G1202R. This is consistent with a previous report that analyzed biopsy specimens from ceritinib-resistant patients.³³ Of the 58% (7/12) of our samples without ALK mutations, we found P-glycoprotein overexpression in three samples and tyrosine-protein kinase *Met* (cMET) gene amplification in one sample (JFCR-059-2).³⁴ In two of the remaining three samples, we were unable to establish cell lines, but we successfully established a cell line in the third sample, JFCR-093. In our recent study, we found that treatment with GSK3 inhibitors or Src inhibitors sensitizes the resistant cells established from JFCR-093 to ALK inhibitors.³⁵ JFCR-059-2 patient had been treated with first-line crizotinib and had shown a marked partial response, as measured by the Response Evaluation Criteria in Solid Tumors, lasting 14 months (Figure S12A). After relapsing on crizotinib, the patient received chemotherapy (cisplatin, pemetrexed plus bevacizumab) for 3 months, followed by bevacizumab for 4 months, and then with docetaxel for 10 weeks. The patient was then enrolled in a phase II trial for ceritinib and showed a significant response (Figure S12B, left and middle). However, 8 months later, the disease progressed because of ceritinib resistance (Figure S12B, right). To identify the ceritinib resistance mechanism, we established the JFCR-059-2 using the malignant fluid cell line. Through FISH analysis, we confirmed the presence of ALK rearrangement (Figure S12C, left). However, we did not observe any secondary mutations in ALK in the ceritinib-resistant tumor sample. FISH analysis of EGFR and cMET revealed cMET amplification in the JFCR-059-2 cells (Figure S12C, right). Additionally, strong phospho-cMET and phospho-EGFR, and intermediate phospho-human HER3 signals were observed in the JFCR-059-2 cells in a phospho-RTK array (Figure S12D). We found that treatment with PHA665752 (cMET inhibitor) but not with erlotinib (EGFR inhibitor) sensitized the JFCR-059-2 cells to ceritinib (Figure S12E). Additionally, PHA665752 treatment inhibited phospho-cMET and phospho-EGFR, suggesting that the phosphorylation of EGFR was mediated by cMET (Figure S12F). Because crizotinib can inhibit both ALK and cMET,³⁶ we treated the cells with crizotinib, other ALK-tyrosine kinase inhibitors (TKIs; ceritinib, alectinib, or lorlatinib), or alectinib with PHA665752 and examined the downstream signaling by immunoblotting. This revealed that the downstream phospho-AKT, -ERK, and -S6 were inhibited according to the extent of the cMET inhibition by crizotinib or PHA-665752 and ALK inhibition. However, a high concentration of crizotinib was required to inhibit both the ALK- and cMET-mediated downstream signaling (Figure S12F). Next, we subcutaneously inoculated JFCR-059-2 cells into nude mice and treated them with ALK inhibitors in combination with or without crizotinib. As expected, alectinib or lorlatinib single

treatment did not induce tumor shrinkage, but crizotinib single treatment did induce tumor regression by inhibiting both ALK and cMET; however, the tumors were not completely diminished by the single treatment. On the other hand, half dose of alectinib or lorlatinib combined with half dose of crizotinib almost completely diminished the tumor (Figure S13). These results suggested that crizotinib with other ALK-inhibitor combination might be effective for the treatment of cMET amplification-mediated ALK-TKI resistance. The analysis of clinically developed ceritinib-resistant specimens suggested that cell-line establishment or tumor xenograft establishment were deemed effective means of identifying the resistance mechanisms resulting from bypass pathway activation.

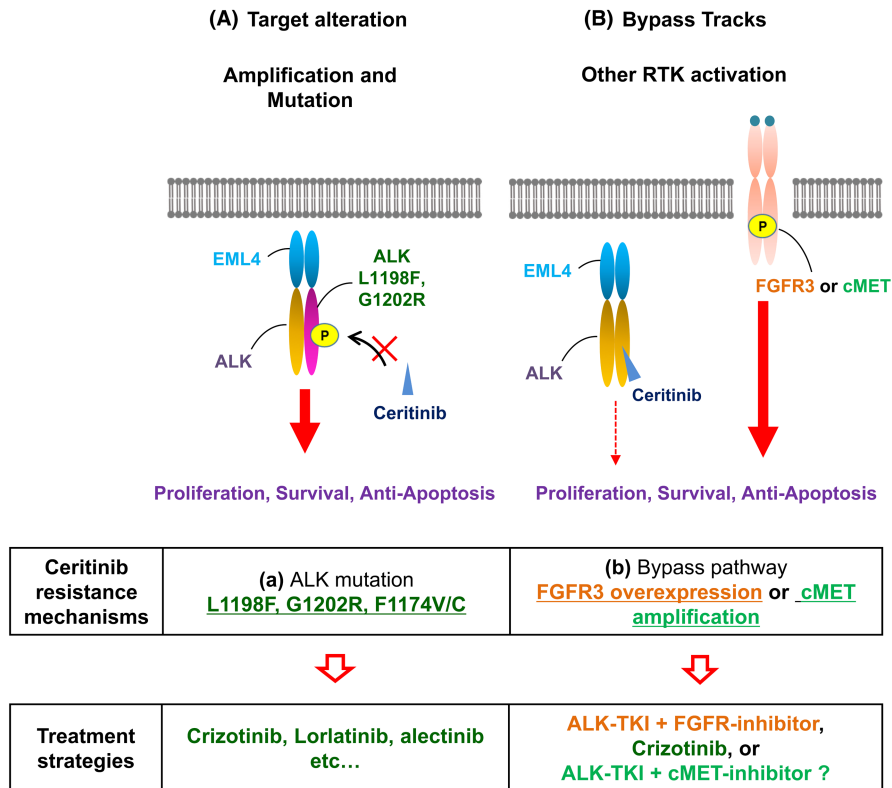
4 | DISCUSSION

Anaplastic lymphoma kinase inhibitors have been successfully developed following the identification of ALK-rearranged NSCLC in 2007.^{3,37} Today, five ALK inhibitors (crizotinib, ceritinib, alectinib, brigatinib, and lorlatinib) are available for clinical use. Ceritinib has shown great clinical efficacy against ALK-rearranged NSCLC, regardless of treatment history of crizotinib.¹⁷ However, as with other ALK inhibitors, ceritinib resistance has been observed in patients treated with ceritinib, so it is important to identify all such mechanisms and to determine how they can be overcome. In this study, we conducted comprehensive analyses of ceritinib resistance using cell line models and clinical specimens from ceritinib-refractory patients with advanced ALK-positive NSCLC and identified FGFR3 activation-mediated ceritinib resistance mechanism (Figure 5).

First, we found that ALK amplification and L1198F mutation conferred intermediate and high ceritinib resistance, respectively (Figure 1). An L1198F mutation, with an additional C1156Y mutation, has previously been identified in a lorlatinib-refractory patient. In our previous research, we have used MP-CAFE to create computational simulations that revealed the free binding of ceritinib to L1198F mutations is higher than that of crizotinib or gilteritinib (a multikinase inhibitor approved for FLT3-mutated acute myeloid leukemia).^{28,38} Our results indicate that crizotinib can be a treatment option for patients with a treatment history of second- or third-generation ALK inhibitors with L1198F mutation in ALK.

Second, we found that FGFR3 bypass pathway activation caused ceritinib resistance in the H3122 LR3 cells (Figure 2). Bypass pathway activation is one of the most common resistance mechanisms against crizotinib, in which it is mediated by EGFR or cKIT activation.^{9,10,14} However, there have been few reports on ceritinib resistance mediated by bypass pathway activation. FGFR1-4 have tyrosine kinase domains, and many reports have noted that their abnormality is related to various types of cancer development, including breast, gastric, and colorectal cancers.³⁹⁻⁴¹ Additionally, other groups have used cell line models to demonstrate that FGFR1 contributes to resistance against EGFR-TKIs in EGFR-mutated lung cancer.^{42,43} Because FGFR abnormalities have been observed in many types of cancer, multiple FGFR

FIGURE 5 New ceritinib resistance mechanisms and therapeutic strategies to overcome them were identified. A diagrammatic representation of the identified ceritinib resistance mechanisms mediated by anaplastic lymphoma kinase (ALK) mutation (left) and bypass pathway activation resulting from FGFR3 or cMET overexpression.



inhibitors have been developed, and several of these are currently available for clinical use.^{44,45} In the present study, FGFR3 overexpression conferred ligand-dependent high resistance to ALK inhibitors, and FGFR3 shRNA knockdown resensitized the H3122 LR3 cells to ceritinib (Figure 3). As ceritinib and infigratinib treatment caused drastic weight loss, we chose alectinib with zoligratinib. This combination treatment was tolerable for the mice and induced marked shrinkage of H3122 LR3 tumor (Figure 4C). In addition to FGFR abnormalities described above, recent studies revealed that FGFR fusion proteins can induce resistance to EGFR TKIs.^{46,47} Therefore, we analyzed the existence of *FGFR3* fusion protein in H3122 LR3 cells, but no *FGFR3* fusion proteins were detected. However, this result does not preclude the possibility that *FGFR3* fusion can induce resistance to ALK TKIs. Further studies and development of appropriate combination therapy from the viewpoints of both efficacy and safety will be required.

Third, we summarized 12 clinically developed ceritinib-resistant specimens and realized that over half had ceritinib resistance mechanisms other than ALK mutations or amplification (Table S1). One tumor sample carried *MET* gene amplification (Figure S12). cMET has been reported to induce alectinib resistance. Also, autocrine activation of cMET by HGF upregulation causes resistance to alectinib.⁴⁸⁻⁵⁰ Additionally, *MET* amplification has been observed in a few alectinib-resistant clinical specimens.⁵¹⁻⁵³ Interestingly, these reports suggest that crizotinib cannot completely overcome this resistance despite its ability to inhibit cMET activation. Indeed, a result of phase II trial showed limited efficacy of crizotinib to patients with ALK-positive NSCLC treated with alectinib immediately before crizotinib monotherapy.⁵⁴ In our study, cMET

activation was observed in JFCR-059-2 and was found to induce EGFR and HER3 tyrosine phosphorylation, as previously reported in *MET* amplification-mediated EGFR-TKI gefitinib resistance.⁵⁵ However, in JFCR-059-2 cells, combination of alectinib or lorlatinib with crizotinib (as a cMET inhibitor) could induce almost complete eradication of tumor in an in vivo model. Thus, at least in JFCR-059-2 cells, EGFR activation is one of the downstream of cMET-mediated growth signaling activation, and cMET and ALK inhibition can overcome the resistance. To overcome these bypass pathway-mediated resistances, combination therapies will be required, and further studies are needed to test the efficacy and toxicity of combination therapy in vivo and in clinical trials.

In the present study, we analyzed ceritinib resistance mechanisms using ceritinib-resistant H3122 cells and ceritinib-resistant patient-derived cells and showed that over half of patients harbor non-ALK alteration resistance mechanisms. We also found FGFR3 activation to be one of the bypass pathway resistance mechanisms. Because ceritinib has been approved for all lines of therapy, we expect increasing research attention will be paid to ceritinib resistance in the near future. As FGFR3 overexpression induced the resistance to all the current ALK inhibitors, this resistance mechanism might be found not only in ceritinib-refractory patients, but also other ALK-TKI-resistant patients. Further studies are needed to comprehensively illustrate the diverse resistance mechanisms and to identify therapeutic strategies for overcoming this resistance.

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ETHICS STATEMENT

Approval of the research protocol by an Institutional Review Board: Japanese Foundation for Cancer Research (#2013-1093). Informed consent: Written informed consent for all genetic and cell biological analyses was obtained from the patients. Registry name and the registration no. of the study/trial: N/A. Animal studies: All animal procedures were performed in accordance with protocols approved by the JFCR Animal Care and Use Committee.

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SUPPORTING INFORMATION

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

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