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ORIGINAL ARTICLE

Pan-HER inhibitors overcome lorlatinib resistance caused by NRG1/HER3 activation in *ALK*-rearranged lung cancer

Hirokazu Taniguchi[1] | Kazumasa Akagi[1] | Yosuke Dotsu[1] | Tadaaki Yamada[2] | Sawana Ono[1] | Erika Imamura[1] | Hiroshi Gyotoku[1] | Shinnosuke Takemoto[1] | Hiroyuki Yamaguchi[1,3] | Triparna Sen[4] | Seiji Yano[5,6,7] | Hiroshi Mukae[1]

[1]Department of Respiratory Medicine, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

[2]Department of Pulmonary Medicine, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto, Japan

[3]Clinical Oncology Center, Nagasaki University Hospital, Nagasaki, Japan

[4]Department of Oncological Sciences, Icahn School of Medicine at Mount Sinai, New York, New York, USA

[5]Division of Medical Oncology, Cancer Research Institute, Kanazawa University, Kanazawa, Japan

[6]Nano Life Science Institute, Kanazawa University, Kanazawa, Japan

[7]Department of Respiratory Medicine, Faculty of Medicine, Institute of Medical Pharmaceutical and Health Sciences, Kanazawa University, Kanazawa, Japan

Correspondence
Hirokazu Taniguchi, Department of Respiratory Medicine, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan. 1-7-1 Sakamoto, Nagasaki 852-8501, Japan.
Email: hirokazu_pc@nagasaki-u.ac.jp

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Abstract

Lorlatinib, a third-generation anaplastic lymphoma kinase (ALK)-tyrosine kinase inhibitor (TKI) with a broad coverage against *ALK* mutations, has demonstrated dramatic effects in patients with *ALK*-rearranged lung cancer. The mechanisms of acquired resistance to lorlatinib by secondary *ALK* compound mutations have recently been reported; however, resistance mechanisms other than secondary mutations remain unclear. Here, we investigated the molecular mechanisms of the acquired resistance in *ALK*-rearranged lung cancer cells in vitro. We established two different lorlatinib-resistant *ALK*-rearranged lung cancer cell lines (H3122LR and A925LLR) via long-term administration of lorlatinib. These resistant cells did not harbor the secondary *ALK* mutations and showed cross-resistance to the other kinds of ALK-TKIs (crizotinib or alectinib) compared with the parental cells; however, these resistant cells overexpressed the phosphorylated human epidermal growth factor receptor 3 (HER3) protein and the ligand of HER3 (neuregulin 1; NRG1). Pharmacological inhibition of HER3 with pan-HER inhibitors or genetic knockdown of *HER3* with siRNA resensitized H3122LR and A925LLR cells to lorlatinib in vitro, indicating that H3122LR and A925LLR acquired resistance by NRG1/HER3 activation. These findings demonstrated that targeting NRG1/HER3 is a potential novel therapeutic option for lorlatinib-resistant *ALK*-rearranged lung cancer.

KEYWORDS
ALK inhibitor, drug resistance, HER3, lorlatinib, non–small-cell lung cancer

1 | INTRODUCTION

Anaplastic lymphoma kinase (*ALK*) gene rearrangement, which mainly involves echinoderm microtubule-associated protein-like 4 (*EML4*)-*ALK*-positive lung cancer, accounts for 3%–5% of non–small-cell lung cancers (NSCLC).[1,2] It is known to be more prevalent in younger people and in non- or light-smokers. ALK tyrosine kinase inhibitors (TKI) have demonstrated impressive clinical efficacy against *ALK* rearrangement-positive lung cancer. A first-generation ALK-TKI, crizotinib, showed marked responses to *ALK* rearrangement-positive

lung cancer, inducing responses in ~70% of patients and a median progression-free survival (PFS) of ~12 months.[3,4] Moreover, recent clinical trials have shown the second-generation ALK-TKIs alectinib, ceritinib, and brigatinib to be better options as first-line treatments than crizotinib[5–8] and standard treatment for patients with advanced or recurrent *ALK* rearrangement-positive lung cancer has switched from crizotinib to front-line second-generation ALK-TKIs. However, despite the encouraging initial clinical benefits, tumors subsequently acquired resistance and recurred in almost all patients. Regarding the resistance mechanisms, secondary *ALK* mutations, such as G1202R, I1171N, or F1174, are considered the main causes of resistance to second-generation ALK-TKIs and are commonly detected in clinically resistant patients.[9]

Lorlatinib, a third-generation ALK-TKI, has shown a wide spectrum of sensitivity to these *ALK* secondary mutations, including G1202R, I1171N, and F1174, in preclinical studies.[9–11] In addition, recent clinical trials have proven that lorlatinib shows great efficacy in patients with *ALK* mutations who had failed one or more second-generation ALK-TKIs.[12–14] Furthermore, interim analysis of a phase III clinical trial comparing lorlatinib with crizotinib in patients with previously untreated, advanced *ALK* rearrangement-positive lung cancer reported that patients who received lorlatinib had significantly longer PFS, a higher overall and intracranial response, and better quality of life than those who received crizotinib.[15] Whereas there is no doubt that lorlatinib is a superior treatment option for patients with *ALK* rearrangement-positive lung cancer, most patients acquired resistance to lorlatinib after a certain period of treatment. Clinical and preclinical studies have revealed that multiple *ALK* compound mutations have caused resistance to lorlatinib sequential therapy[16–18]; however, the mechanisms of resistance in treatment-naïve patients who received lorlatinib, especially *ALK*-independent resistance mechanisms, have not been fully clarified yet.

In this study, we established lorlatinib-resistant (LR) cell lines H3122LR and A925LLR using the dose-escalation method in vitro to investigate the mechanisms of acquired resistance to lorlatinib. We found that activation of human epidermal growth factor receptor (HER) 3 conferred resistance to lorlatinib in these resistant cells and suggest a therapeutic strategy to overcome lorlatinib resistance in vitro.

2 | MATERIALS AND METHODS

2.1 | Cell cultures and reagents

The human lung adenocarcinoma cell line H3122 (*EML4-ALK* variant 1 E13: A20) was kindly provided by Dr. Jeffrey A. Engelman (Novartis Institutes for BioMedical Research); A925L (*EML4-ALK* variant 5a, E2: A20) was established from a surgical specimen obtained from a male Japanese patient (T2N2M0, stage IIIA) as reported previously.[19] These cell lines were maintained in RPMI 1640 medium (GIBCO) supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (50 g/ml) in a humidified CO_2 incubator at 37°C. Cells were passaged for less than 3 months before being renewed with frozen, early-passage stocks. Cells were regularly screened for mycoplasma using the MycoAlert Mycoplasma Detection Kit (Lonza). Lorlatinib, alectinib, crizotinib, afatinib, and dacomitinib were purchased from Selleckchem. Recombinant human Neuregulin-1 (NRG1) was purchased from R&D Systems.

2.2 | Human phospho-kinase antibody array

The relative levels of phosphorylation of 43 kinases and two related total proteins were measured using the Human Phospho-Kinase Array Kit (R&D Systems) in accordance to the manufacturer's instructions. Briefly, cells were cultured in RPMI-1640 containing 10% FBS and lysed in array buffer before reaching confluence. The arrays were blocked with blocking buffer and incubated with 450 μg of the cell lysate overnight at 4°C. The arrays were washed, incubated with an HRP-conjugated phospho-kinase antibody, and treated with SuperSignal West Dura Extended Duration Substrate Enhanced Chemiluminescent Substrate (Pierce Biotechnology).

2.3 | Antibodies and western blot analysis

The primary antibodies anti-ALK (C26G7), anti-phospho-ALK (Tyr1604), anti-phospho-HER3 (Tyr1289), anti-HER3, anti-phospho-Akt (S473), anti-Akt, anti-phospho-epidermal growth factor receptor (EGFR), anti-phospho-MET (Tyr1234/1235), anti-MET, and anti-β-actin were obtained from Cell Signaling Technology. Anti-ERK1/ERK2, p-ERK1/ERK2 (T202/Y204), and anti-EGFR were obtained from R&D Systems. Western blot analysis was performed as described previously.[20]

2.4 | Cell viability assay

Cell viability was determined using the MTT dye reduction method. Briefly, tumor cells (2–3 $\times 10^3$ cells/100 μl/well) in RPMI-1640 medium supplemented with 10% FBS were plated in 96-well plates and cultured with the indicated compound for 72 h. After culturing, 50 μg of MTT solution (2 mg/ml; Sigma) was added to each well. The plates were incubated for 2 h, the medium was removed, and the dark blue crystals in each well were dissolved in 100 μl of DMSO. Absorbance was measured using a microplate reader at a test wavelength of 550 nm and a reference wavelength of 630 nm. The percentage growth was determined relative to the controls.

2.5 | Transfection of siRNA

For transfection of siRNA, siRNA specific to *ALK* was obtained from Horizon Discovery, siRNA specific to *HER3* and the siRNA negative control were purchased from Thermo Fisher Scientific. Introduction

of siRNA was performed using Lipofectamine iMAX (Thermo Fisher Scientific) in accordance with the manufacturer's instructions.

2.6 | Quantitative PCR

Total RNA was extracted using the NucleoSpin® RNA Plus kit (TaKaRa Bio Inc.) according to the manufacturer's instructions. The SuperScript VILO cDNA synthesis kit and master mix (Invitrogen) were used for cDNA synthesis according to the manufacturer's instructions. TaqMan Gene Expression Assays (Thermo Fisher Scientific) for NRG1 (Hs01101538_m1) and GAPDH (Hs02786624_g1) were performed according to the manufacturer's protocol. qPCR analysis was performed using the ViiA7 Real-Time PCR System (Applied Biosystems). The relative mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.7 | Statistical analysis

Data from the MTT assays were expressed as mean ± SD. Statistical significance of differences was analyzed using a two-way unpaired *t*-test or one-way ANOVA. All statistical analyses were performed using GraphPad Prism ver. 9.0 (GraphPad Software, Inc.), with a two-sided p-value < 0.05 considered statistically significant.

3 | RESULTS

3.1 | Establishment of LR *EML4-ALK* lung cancer cells

To explore the mechanisms of acquired resistance to lorlatinib, we sought to establish LR cell lines from H3122 and A925L cells using dose-escalation methods (Figure 1A). H3122 and A925L cells were cultured with increasing concentrations of lorlatinib, starting from 1 nmol/L and stepwise up to 1 μmol/L, to generate LR cell lines, named H3122LR (LR) and A925LLR, respectively. Both cell lines acquired resistance after exposure to lorlatinib for ~4 months and could grow in the presence of 1 μmol/L lorlatinib. Next, we examined the sensitivity of the generated-resistant cells to selective ALK inhibitors to confirm that resistance to lorlatinib. H3122LR and A925LLR did not acquire resistance only to lorlatinib but also to other generation ALK-TKIs, crizotinib, and alectinib compared with the parental H3122 and A925L cells in vitro, respectively (Figure 1B,C and Figure S1A–D). Next, we examined the protein expression and phosphorylation of ALK and downstream molecules to determine the molecular mechanisms of resistance. Western blotting showed that lorlatinib inhibited the phosphorylation of ALK but not of AKT and ERK, and the downstream molecules of ALK in H3122LR and A925LLR cells. By comparison, lorlatinib inhibited the phosphorylation of ALK, AKT, and ERK in the parental H3122 and A925L cells (Figure 1D,E).

As previous studies had shown that multiple *ALK* compound mutants are responsible for resistance to lorlatinib sequential therapy,[16–18] we next sought to determine the presence of *ALK* mutations in *ALK* exons using whole-exome capture methods; however, *ALK* resistance mutations such as I1171, F1174, R1192, L1196, L1198, G1202, G1206, G1269, or R1275 and other mutations in the exon were not detected (data not shown). Consistently, knockdown of *ALK* by specific siRNA did not inhibit the viability of H31226LR and A925LLR cells, despite the fact that specific siRNA of *ALK* clearly suppressed the phosphorylation of ALK (Figure 2A,B).

These results indicate that H3122LR and A925LLR cells acquired resistance via an *ALK*-independent mechanism.

3.2 | HER3 activation conferred cell survival in lorlatinib-resistant cells

As H3122LR and A925LLR did not have any mutations in the *ALK* gene, and previous studies had reported that bypass signals by the activation of receptor tyrosine kinases (RTKs) could induce resistance to ALK-TKIs, we next evaluated the activation of RTKs using a phospho-RTK array in H3122LR and A925LLR cells compared with the parental cells.

We found that the phosphorylation of HER3 increased in H3122LR and A925LLR cells compared with the parental cells (Figure 3A). Consistently, western blot analysis also showed that the phosphorylation of HER3 increased in H3122LR and A925LLR cells compared with the parental cells (Figure 3B).

To determine the influence of HER3 ligands, we next evaluated the mRNA expression of *neuregulin 1* (*NRG1*), which is a major ligand of HER3.[21] Quantitative PCR revealed that the mRNA levels of *NRG1* significantly increased in H3122LR and A925LLR cells compared with the parental cells (Figure 3C). Moreover, the cell viability of H3122 cells treated with r-NRG1 was significantly increased compared with that of untreated control cells, showing that r-NRG1 affected the cell proliferation in the *ALK*-rearranged lung cancer cells (Figure 3D). Cell viability following treatment with lorlatinib and r-NRG1 showed a slight but statistically significant decrease compared with r-NRG1 alone (decrease rate; 23.1%, r-NRG1 vs. lorlatinib and r-NRG1). As the sensitivity of H3122 cells to lorlatinib was very high (Figure 1B), some population of H3122 cells might respond to lorlatinib even in conditions with r-NRG1. However, the decrease rate was much lower than that of untreated control vs lorlatinib alone (decrease rate; 59.9%, untreated control vs. lorlatinib). Furthermore, combined lorlatinib with r-NRG1 did not decrease the cell viability compared with untreated control cells even when the cells were treated with lorlatinib, indicating that the HER3 activation with r-NRG1 caused the resistance to lorlatinib. Similar results were observed in A925L cells. The cell viability of A925L cells treated with lorlatinib and r-NRG1 did not show a significant difference compared with r-NRG1 alone (Figure 3D). Consistently, recombinant NRG1 enhanced the

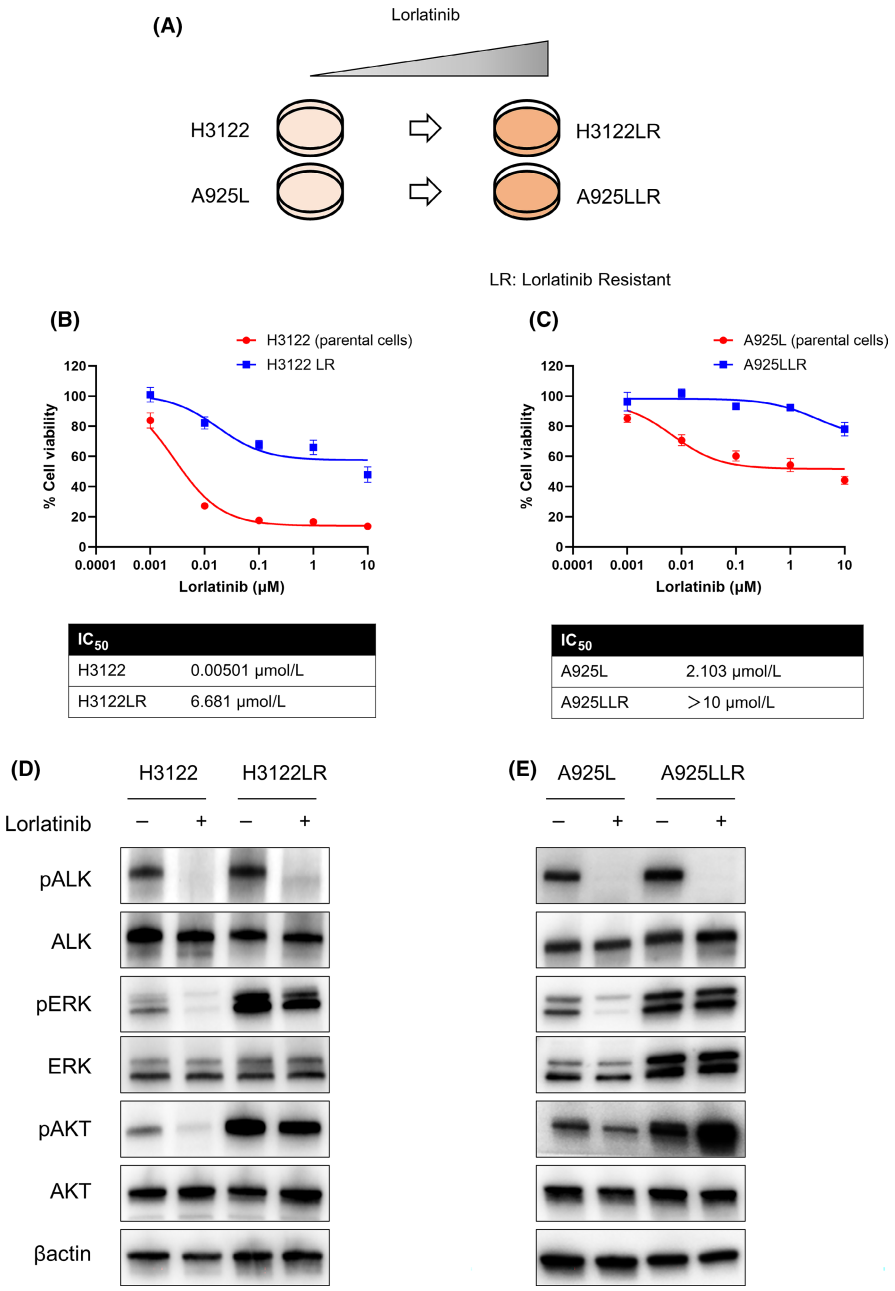


FIGURE 1 Establishing a method for the lorlatinib-resistant cell lines. (A) H3122 and A925L parental cells were exposed to dose escalation of lorlatinib from 1 nmol/L to 1 μmol/L. H3122LR and A925LLR were cultured with 1 μmol/L lorlatinib 48 h before each experiment. (B) H3122 parental and H3122LR cells, and (C) A955L parental and A925LLR cells were incubated with the indicated concentrations of lorlatinib for 72 h. Cell viability was detected using MTT assays. IC_{50} levels were calculated using GraphPad Prism ver. 9. (D) H3122 parental and H3122LR cells, and (E) A955L parental and A925LLR cells incubated with or without 1 μmol/L of lorlatinib for 2 h. The cells were lysed and the indicated proteins were detected by western blotting

phosphorylation of HER3, AKT, and ERK, even in the presence of 1 μM lorlatinib in the parental H3122 and A925L cells (Figure 3E). We examined NRG1 rearrangement presence using whole-exome capture methods, and found that NRG1 rearrangement in the exon was not detected in either H3122LR or A925LLR cells (data not shown).

In our analysis, phospho-RTK array and western blotting showed that phosphorylation of MET was higher in the H3122LR cells than in the parental H3122 cells (Figure 3A and Figure S2A). A recent study demonstrated that MET alternation was detected in 12% and 22% of biopsies from patients who had experienced disease progression against second-generation inhibitors or lorlatinib, respectively.[22] However, crizotinib, a dual ALK and MET inhibitor, did not inhibit cell viability in H3122LR (Figure S1A) and did not suppress the phosphorylation of AKT and ERK compared with lorlatinib in H3122LR cells, although the phosphorylation of MET was markedly inhibited by treatment with crizotinib (Figure S2B), suggesting that MET phosphorylation did not affect cell survival in H3122LR cells.

These results indicated that these resistant cells acquired resistance to lorlatinib and maintained cell survival by activating the NRG1/HER3 pathway.

3.3 | Inhibition of HER3 resensitized the lorlatinib-resistant cells to lorlatinib

To determine the influence of NRG1/HER3 signaling on the survival of H3122LR and A925LLR cells, we next examined the effect of

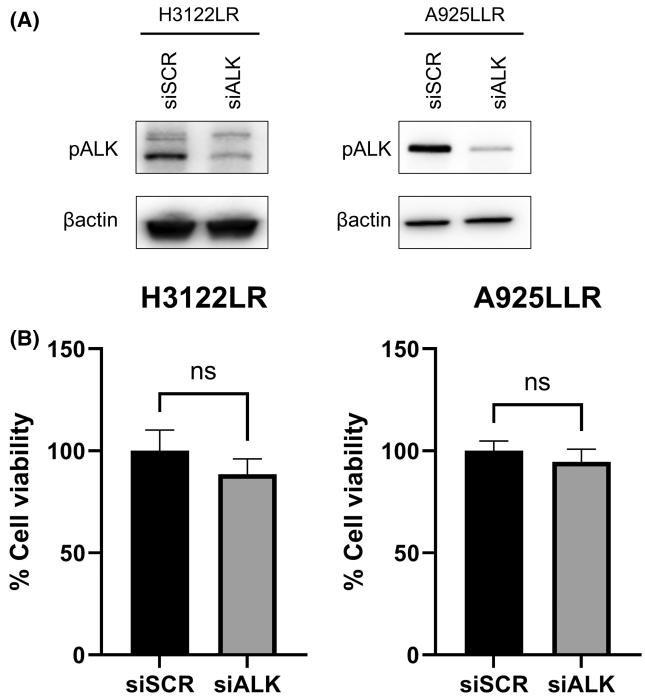


FIGURE 2 Genetic inhibition of *ALK* with specific siRNA. (A) Knockdown of *ALK* (or scramble control [siSCR]) using specific siRNAs in H3122LR and A925LR for 48 h. The cells were lysed and the indicated proteins were detected by western blotting. (B) Knockdown of *ALK* (or scramble control [siSCR]) by specific siRNA in H3122LR and A925LR for 72 h. Cell viability was detected using MTT assays ($N = 4$). *p*-values were calculated using Student's *t*-test; ns, nonsignificant; $p > 0.05$.

HER3 knockdown with specific siRNA on cell survival in the resistant cells. Knockdown LR cells were then treated with lorlatinib. Western blot analysis showed that two clones of specific siRNA successfully knocked down *HER3*, and lorlatinib inhibited ALK phosphorylation (Figure 4A). Regarding cell viability, knockdown of *HER3* with siRNAs significantly inhibited the viability of H3122LR and A925LLR cells mediated by lorlatinib, compared with scramble control plus lorlatinib (Figure 4B). These results suggested that the survival of LR cells predominantly depended on the NRG1/HER3 signal in the presence of lorlatinib.

There was no specific HER3 inhibitor available clinically in the field of lung cancers; however, pan-HER inhibitors afatinib and dacomitinib have been clinically approved for patients with *EGFR*-mutated NSCLC. Therefore, we next assessed the effect of combined treatment with pan-HER inhibitors and lorlatinib in the LR cells. Although single usage of either pan-HER inhibitor hardly affected the viability of H3122LR and A925LLR cells, afatinib or dacomitinib inhibited viability in the presence of lorlatinib, crizotinib, or alectinib (Figures 5A–D and Figure S3A–D).

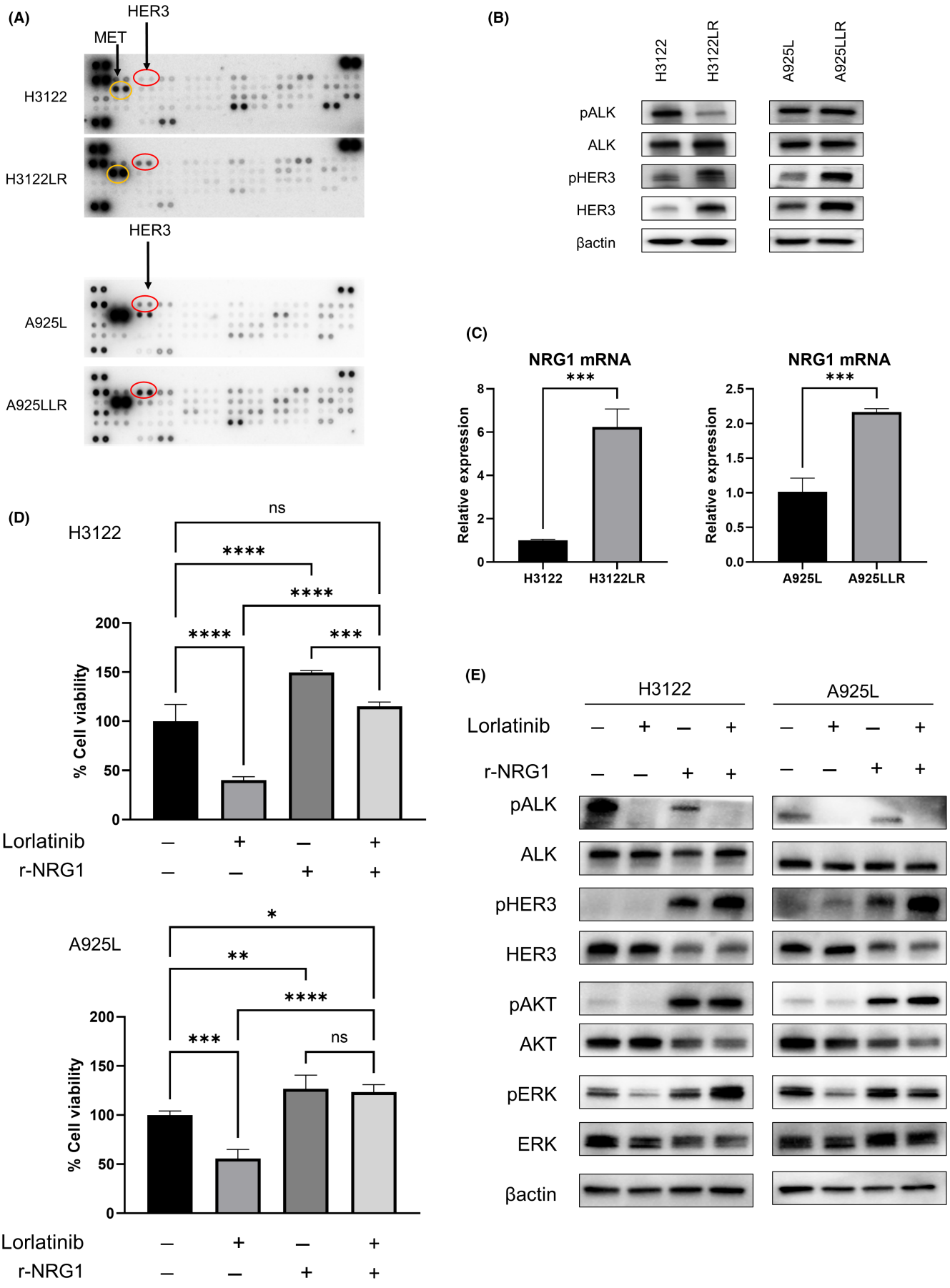
To confirm the combined antitumor effect of lorlatinib and pan-HER inhibitors, we examined the long-term co-culture of the resistant cells with lorlatinib and afatinib or dacomitinib. Continuous co-treatment with lorlatinib and afatinib or dacomitinib for 14 days inhibited cell growth compared with treatment with lorlatinib alone in H3122LR cells (Figure 5E). Western blotting showed that the combined use of lorlatinib and afatinib or dacomitinib markedly inhibited the phosphorylation of ERK and AKT compared with lorlatinib treatment alone (Figure 5F). These findings indicated that the sensitivity of H3122LR and A925LLR cells to lorlatinib was resensitized by combined treatment with a pan-HER inhibitor.

4 | DISCUSSION

In this study, we revealed that *EML4-ALK* lung cancer cells acquired resistance to lorlatinib by activation of the NRG1/HER3 pathway (Figure 6) and that the resistant cells did not have secondary *ALK* mutations, in contrast with a previous study.[18] We further found that the combined use of lorlatinib and the pan-HER inhibitor afatinib or dacomitinib could resensitize and overcome lorlatinib resistance in vitro. To our knowledge, this study is the first to report that HER3 activation promotes lorlatinib resistance.

NRG1/HER3 activation plays important roles in human cancer cell survival. *HER3* mutations and/or overexpression have been reported in a wide variety of cancers, including several types of adenocarcinomas, melanomas, and gliomas.[23] Because of its lack of intrinsic kinase activity, HER3 cannot activate signaling within homodimers; however, HER3 induces its phosphorylation in the presence of HER3 ligands or linking other RTKs.[24] Its activation has also been reported to be a pivotal factor in several types of drug-resistant or drug-tolerant cells. In epithelial-like *KRAS*-mutant cancer cells, MEK inhibition upregulated HER3, which in turn activated PI3K–AKT and MAPK signaling.[25] In *BRAF*-mutant cancers, a BRAF inhibitor or trametinib upregulates HER3 by inducing the expression of FOXD3 or by decreasing the expression of the transcription repressors C-terminal binding proteins 1 and 2 and by autocrine secretion of NRG1.[26,27] We previously reported that HER3 is the key molecule that induces drug tolerance to osimertinib, a third-generation EGFR-TKI, in *EGFR*-mutated lung cancer cells.[20] Regarding *ALK*-rearranged lung cancer cells, a preclinical study showed that alectinib-resistant cells lost the *EML4-ALK* driver oncogene, and activated the NRG1/HER3 pathway to maintain cell survival alternatively.[28] Furthermore, we previously reported that HER3 activation contributes to the emergence of ALK inhibitor-tolerant cells in *ALK*-rearranged lung cancer.[29] Therefore, the NRG1/HER3 pathway causes resistance to molecular-targeted agents as bypassed signals in several types of cancers, and blocking the NRG1/HER3 pathway would suppress tumor progression in several cancers resistant to molecular-targeted agents. In addition, it is promising that we could demonstrate in this study that a pan-HER inhibitor, afatinib or dacomitinib, with lorlatinib resensitized the LR cells in vitro because afatinib and dacomitinib have already been approved in the clinical setting for patients with *EGFR*-mutated NSCLC.

Standard first-line treatments with molecular-targeted therapies for patients with NSCLC harboring driver mutations, such


(A)
HER3
MET
H3122
H3122LR
HER3
A925L
A925LLR
(B)
H3122
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A925L
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pALK
ALK
pHER3
HER3
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(C)
NRG1 mRNA
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Relative expression
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Lorlatinib
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H3122
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ALK
pHER3
HER3
pAKT
AKT
pERK
ERK
βactin

FIGURE 3 Activation of the NRG1/HER3 pathway-mediated lorlatinib resistance. (A) Human phospho-RTK array analysis of H3122 parental and H3122LR cells (upper), and A925L parental and A925LLR cells (bottom). (B) Western blots showing the indicated protein expression of indicated proteins of H3122 parental and H3122LR cells (left), and A925L parental and A925LLR cells (right). (C) Quantitative mRNA expression of NRG1 in H3122 parental and H3122LR cells (left), and A925L parental and A925LLR cells (right). (D) H3122 parental cells (upper) and A925L (bottom) were treated with 1 μmol/L lorlatinib with or without 100 ng/ml of recombinant NRG1 for 72 h. Cell viability was detected using with MTT assays ($N = 4$). (E) H3122 parental and H3122LR cells (left) and A955L parental and A925LLR cells (right) incubated with 1 μmol/L lorlatinib with or without 100 ng/mL recombinant NRG1 for 2 h. Cells were lysed and the indicated proteins were detected by western blotting. *p*-values were calculated using one-way ANOVA. $^{*}p < 0.0332$, $^{**}p < 0.0021$, $^{***}p < 0.0002$, $^{****}p < 0.0001$.

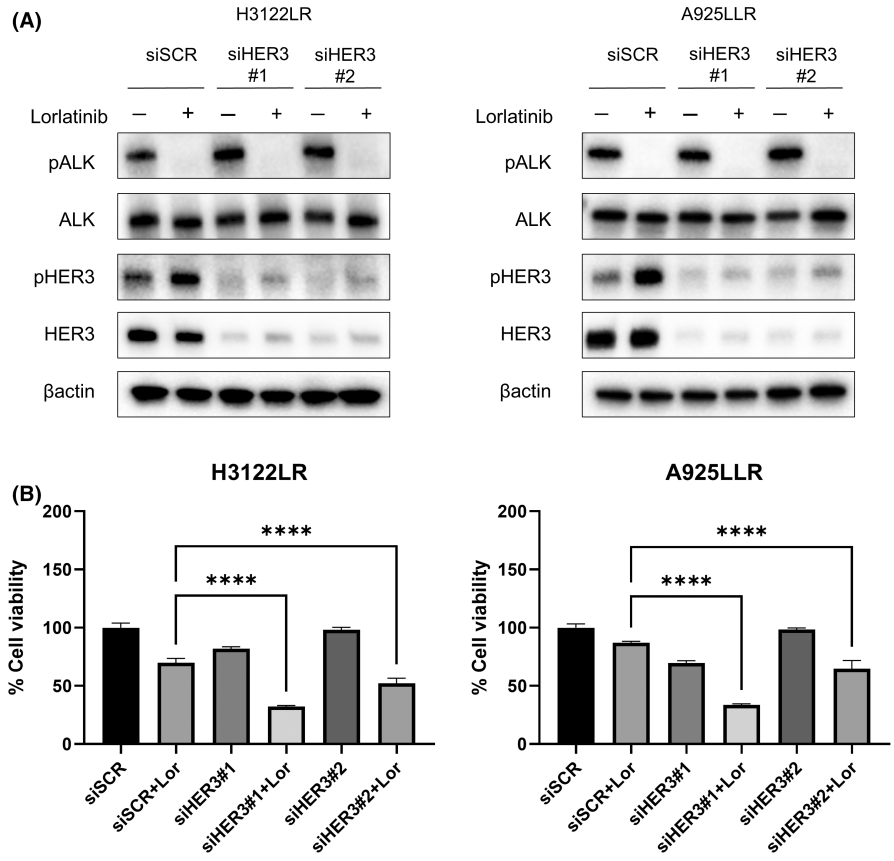

FIGURE 4 Genetic knockdown of *HER3* enhances antitumor of lorlatinib in lorlatinib-resistant cells. (A) Knockdown of *HER3* (or siRNA control [siSCR]) by siRNA followed by treatment with 1 μmol/L of lorlatinib for 2 h in H3122LR and A925LLR. Western blots showing the indicated protein expression of indicated proteins of H3122LR cells and A925LLR cells. (B) Knockdown of *HER3* (or siRNA control [siSCR]) by siRNA followed by treatment with 1 μmol/L lorlatinib for 72 h in H3122LR and A925LLR. Western blots showing the indicated protein expression of indicated proteins of H3122LR cells, and A925L parental and A925LLR cells. Cell viability was detected using MTT assays ($N = 4$). *p*-values were calculated using one-way ANOVA. $^{*}p < 0.0332$, $^{**}p < 0.0021$, $^{***}p < 0.0002$, $^{****}p < 0.0001$. ns, nonsignificance.

as *ALK* rearrangement or *EGFR* mutation, have been dramatically developed in this decade.[30] The interim analysis of clinical trials of first-line treatment comparing lorlatinib with crizotinib revealed that an objective response occurred in 76% of the patients in the lorlatinib group and 58% of those in the crizotinib group. The percentage of patients who were alive without disease progression at 12 months was 78% in the lorlatinib group and 39% in the crizotinib group, showing the significantly higher effect of lorlatinib than that of crizotnib.[15] To date, no study has compared the effect of lorlatinib with second-generation ALK-TKIs and cross-trial comparisons are not recommended intrinsically because of differences in designs and populations in the trials; however, the efficacy of lorlatinib is expected to be equal or higher than that of second-generation ALK-TKIs.[5–8] Several preclinical studies have reported that lorlatinib inhibits the activation of ALK even when secondary mutations of *ALK*, which caused resistance to first- or second-generation ALK-TKIs, are included in the *ALK* gene.[9,11,31]

Our results highlight the antitumor effects of lorlatinib in combination with HER3 inhibition in in vitro models of LR *ALK*-rearranged NSCLC. Further studies are required in the future to clarify whether the combination of lorlatinib and HER3 inhibition suppresses the growth of tumors in in vivo models and patients with *ALK*-rearranged-NSCLC who acquired resistance to lorlatinib.

In conclusion, the bypass signal from NRG1/HER3 causes resistance to lorlatinib in *ALK* rearrangement-positive lung cancer cells. Activation of NRG1/HER3 induces the phosphorylation of ERK and AKT (Figure 6), and genetic or pharmacological inhibition of HER3 resensitizes LR cells to lorlatinib. Our findings demonstrated a pivotal role of HER3 inhibition in LR cells caused by NRG1/HER3 activation.

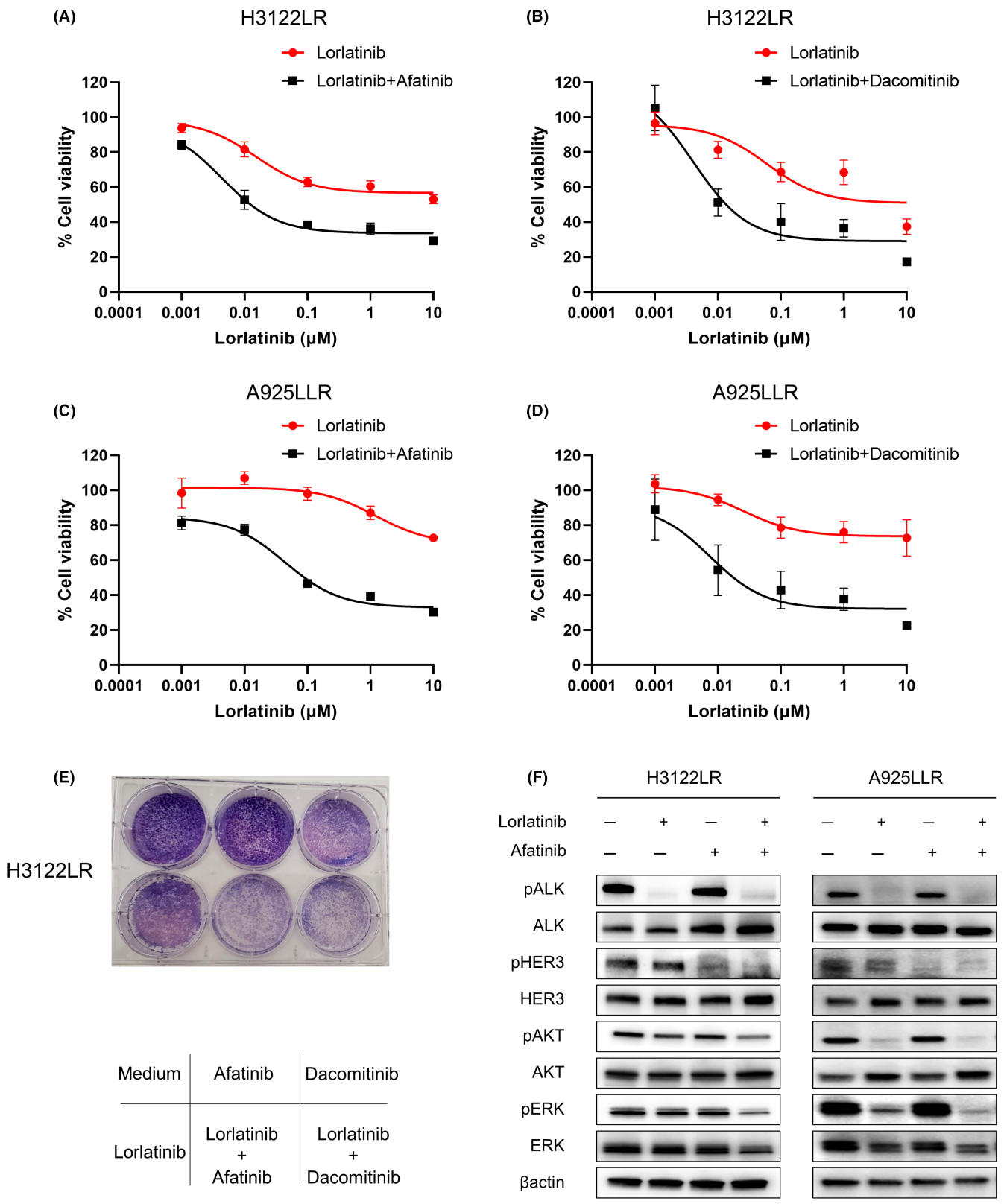

FIGURE 5 HER3 inhibition with pan-HER inhibitor resensitized the lorlatinib-resistant cells to lorlatinib. (A and B) H3122LR cells were treated with the indicated concentrations of lorlatinib with or without (A) 100 nmol/L of afatinib and (B) 100 nmol/L of dacomitinib for 72 h. (C and D) A925LLR cells were treated with the indicated concentrations of lorlatinib with or without (C) 100 nmol/L of afatinib and (D) 100 nmol/L of dacomitinib for 72 h. Cell viability was detected using MTT assays ($N = 4$). (E) H3122LR cells were treated with DMSO, 1 μmol/L lorlatinib, 100 nmol/L afatinib, 100 nmol/L dacomitinib, or a combination of 1 μmol/L lorlatinib for 14 days with the drugs replenished every 3 days. The plates were stained with crystal violet and imaged. A representative plate of three independent experiments is shown. (F) Cells were treated with 1 μmol/L lorlatinib with or without 100 nmol/L of afatinib for 2 h. Western blots showing the indicated protein expression of indicated proteins of H3122LR cells and A925LLR cells.

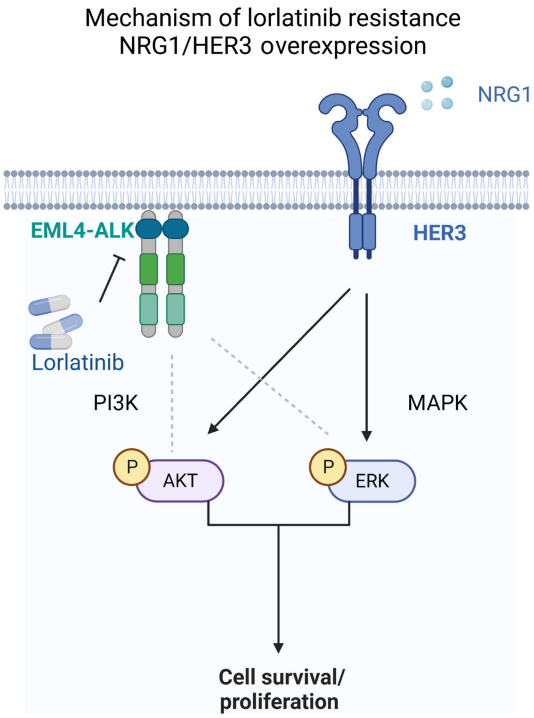

FIGURE 6 Graphical abstract for the mechanism of lorlatinib resistance.

AUTHOR CONTRIBUTIONS

Conceptualization: HT, TY; Methodology: HT, KA, YD, TY, EI, HG, ST (Takemoto), HY; Investigation: HT, KA, YD, TY, SO, EI, HG; Validation: HT, KA, YD, EI, HG, ST (Takemoto), HY; Formal analysis: HT, KA, YD; Writing – Original Draft: HT; Review and Editing: HT, KA, HY, TY, SY, TS (Sen), HM; Supervision: SY, HM; Funding acquisition: HT, SY, HM. All authors read and approved the final version of the manuscript.

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ORCID

Hirokazu Taniguchi https://orcid.org/0000-0003-2414-8344
Tadaaki Yamada https://orcid.org/0000-0002-6945-281X
Seiji Yano https://orcid.org/0000-0002-6151-2988

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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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