Genomics

Molecular Cancer Research

# Activation of AXL as a Preclinical Acquired Resistance Mechanism Against Osimertinib Treatment in *EGFR*-Mutant Non–Small Cell Lung Cancer Cells

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## Abstract

Osimertinib (AZD9291) has an efficacy superior to that of standard EGFR-tyrosine kinase inhibitors for the first-line treatment of patients with *EGFR*-mutant advanced non–small cell lung cancer (NSCLC). However, patients treated with osimertinib eventually acquire drug resistance, and novel therapeutic strategies to overcome acquired resistance are needed. In clinical or preclinical models, several mechanisms of acquired resistance to osimertinib have been elucidated. However, the acquired resistance mechanisms when osimertinib is initially used for *EGFR*-mutant NSCLC remain unclear. In this study, we experimentally established acquired osimertinib-resistant cell lines from *EGFR*-mutant NSCLC cell lines and investigated the molecular profiles of resistant cells to uncover the mechanisms of acquired resistance. Various resistance mechanisms were identified, including the acquisition of *MET* amplification, EMT induction, and the upregulation of AXL. Using targeted next-generation sequencing with a multigene panel, no secondary mutations were detected in our resistant cell lines. Among three *MET*-amplified cell lines, one cell line was sensitive to a combination of osimertinib and crizotinib. Acquired resistance cell lines derived from H1975 harboring the T790M mutation showed AXL upregulation, and the cell growth of these cell lines was suppressed by a combination of osimertinib and cabozantinib, an inhibitor of multiple tyrosine kinases including AXL, both *in vitro* and *in vivo*. Our results suggest that AXL might be a therapeutic target for overcoming acquired resistance to osimertinib.

**Implications:** Upregulation of AXL is one of the mechanisms of acquired resistance to osimertinib, and combination of osimertinib and cabozantinib might be a key treatment for overcoming osimertinib resistance.

## Introduction

Lung cancer remains the leading cause of cancer mortality worldwide, and non–small cell lung cancer (NSCLC) accounts for more than 85% of all lung cancers, with 50% of these being adenocarcinomas (1–3). *EGFR* mutations, such as L858R point mutations and exon 19 deletions, occur in approximately 10% to 15% and 40% of NSCLC cases in Western and Asian populations, respectively (4). Among patients with *EGFR* mutations, EGFR tyrosine-kinase inhibitors (EGFR-TKIs: gefitinib, erlotinib, and afatinib) are recommended as standard treatments for patients with advanced NSCLC (5, 6). However, acquired resistance develops within about a year in most cases (7). Secondary *EGFR* T790M mutation, detected in about half of such cases, is the most common mechanism of TKI resistance (8, 9).

Osimertinib (AZD9291) is an oral, irreversible, mutant-selective EGFR-TKI designed to inhibit *EGFR*-activating mutations (exon 19 deletion and L858R) in the presence of the T790M mutation (10–12), and it has a high anticancer activity against *EGFR* mutations but a low activity against wild-type *EGFR* (12). On the basis of the positive results of the AURA clinical program (13–15), osimertinib has been approved worldwide for the second-line treatment of patients with T790M-positive NSCLC who experience disease progression during or after treatment with an EGFR-TKI. Furthermore, the FDA recently approved osimertinib for the first-line treatment of patients with metastatic NSCLC whose tumors carry *EGFR* exon 19 deletions or L858R mutations, based on the results of the phase III FLAURA trial (16). In the FLAURA trial, the efficacy of osimertinib versus first-generation EGFR-TKI (either erlotinib or gefitinib) in previously untreated patients with locally advanced or metastatic *EGFR*-mutant–positive NSCLC was compared. Osimertinib showed efficacy superior to that of first-generation EGFR-TKIs with a similar safety profile and lower rates of serious adverse events. However, knowledge of the resistance mechanisms against osimertinib when it is

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**Note:** Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

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**doi:** 10.1158/1541-7786.MCR-18-0628

used as a first-line treatment for EGFR-positive NSCLC, including those with non-T790M mutations, remains insufficient.

In clinical or preclinical models, several mechanisms of acquired resistance to osimertinib have been elucidated, such as *EGFR* C797S mutation (17–19), *MET* amplification (20, 21), and an increased dependence on RAS signaling (22). These resistance mechanisms are mostly caused by genetic alterations, but non-genetic resistance mechanisms are also involved. Therefore, the ability to predict acquired resistance to *EGFR*-mutant NSCLC not only in cases with the T790M mutation, but also in cases without T790M mutation would be useful.

In this study, we established various NSCLC cell lines with acquired resistance to osimertinib and investigated the molecular profiles of resistant cells to uncover the mechanisms of resistance.

## Materials and Methods

### Cell lines and reagents

*EGFR*-mutant HCC827 (exon 19 del. E746-A750), HCC4006 (exon 19 del. L747-A750, P ins), PC-9 (exon 19 del. E746-A750), HCC4011 (L858R), and H1975 (L858R and T790M) cells were used in this study. HCC827, HCC4006, and H1975 were purchased from the ATCC. PC-9 was purchased from the RIKEN cell bank (Wako). HCC4011 cells were provided by Dr. Adi F. Gazdar (The University of Texas Southwestern Medical Center at Dallas, Dallas, TX), who established this cell line in collaboration with Dr. John D. Minna (The University of Texas Southwestern Medical Center at Dallas, Dallas, TX). For cell lines with long-term preservation in liquid nitrogen, a DNA fingerprinting analysis using short tandem repeat profiling and the Cell ID System (Promega) was performed for cell authentication. All cell lines were cultured in RPMI1640 medium supplemented with 10% FBS and grown in a humidified incubator with 5% $CO_2$ at 37°C. Acquired osimertinib-resistant cell lines were established using the following two different procedures: parental cells were exposed to osimertinib with a stepwise escalation from 10 nmol/L to 2 μmol/L over 6 months (stepwise escalation method) or were intermittently and briefly exposed to the drug at 2 μmol/L over 6 months (high-concentration method). On the basis of the example of cisplatin resistance study in which the resistant cells were established using two methods (23), we previously reported that these methods of drug exposure in cell culture provide the different mechanisms of acquired resistance to first- and second-generation EGFR-TKIs (24, 25). Therefore, we also adopted both stepwise escalation method and high-concentration method in this study. A concentration of 2 μmol/L is higher than the physiologic blood concentration described in the attached document. Osimertinib (AZD9291; ChemScene), gefitinib (ChemScene), afatinib (SYNkinase), and cabozantinib (AXL inhibitor; ChemScene) were obtained from the designated sources.

### Western blot analysis

Cells were harvested at 80% to 90% confluence, and cellular proteins were extracted with a lysis buffer [RIPA buffer, phosphokinase inhibitor cocktails 2 and 3 (Sigma-Aldrich)] and Complete Mini (Roche). The primary antibodies used for the Western blot analyses were as follows: anti-EGFR, phosphor- (p-) EGFR (Tyr1068), MET, p-MET (Tyr1234/1235), AKT, p-AKT (Ser473), p44/p42 MAPK, p-p44/p42 MAPK, cleaved (c-) PARP, E-cadherin, vimentin, and ALDH1A1 (Cell Signaling Technology); AXL (R&D Systems); and β-actin (used as the loading control; Merck Millipore). The following secondary antibodies were used: goat anti-rabbit, goat anti-mouse, or donkey anti-goat immunoglobulin G (IgG)-conjugated horseradish peroxidase (Santa Cruz Biotechnology). To detect specific signals, the membranes were examined using the ECL Prime Western Blotting Detection System (GE Healthcare) and LAS-3000 (Fujifilm). The relative band intensity was assessed by densitometric analysis using ImageJ (NIH, Bethesda, MD). Regarding the expression ratio of AXL and Actin, we defined as "upregulated" at a concentration of 4-fold or more compared with a parental cell line.

### DNA and RNA extraction

Genomic DNAs were extracted from cell lines using a DNeasy Blood and Tissue Kit (Qiagen). Total RNAs were extracted from cell lines using a RNeasy Mini Kit (Qiagen). The complementary DNA (cDNA) was synthesized from total RNA using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific).

### DNA analysis

*EGFR* exon 20 mutation was examined using direct sequencing, as reported previously (26). The copy number gains (CNG) of *EGFR* and *MET* were determined using a qRT-PCR assay with TaqMan copy number assays (Thermo Fisher Scientific). TaqMan RNase P Control (Thermo Fisher Scientific) was used as the reference gene. The relative copy number of each sample was determined by comparing the ratio of the expression level of the target gene to that of the reference gene in each sample with the ratio for standard genomic DNA (Merck). On the basis of our previous studies, we defined high-level amplification as values greater than four in cell lines (24, 27).

### Targeted next-generation sequencing

Targeted next-generation sequencing (NGS) was performed for all parental and resistant cell lines. The library was generated using the HaloPlex$^{HS}$ System (Agilent Technologies) and 100 ng of genomic DNA. We applied the ClearSeq Cancer Panel (Agilent Technologies), which was designed to identify somatic variants in 47 cancer-related genes (Supplementary Table S1) targeting known COSMIC hotspots found to be associated with a broad range of cancer types as well as published drug targets. Sequencing data were generated from the MiSeq Sequencer (Illumina), and a mutation analysis was performed using SureCall (Agilent Technologies) according to the manufacturer's recommendations.

### mRNA and miRNA expression analysis using quantitative reverse transcription PCR

The gene expression of ALDH1A1 and ABCB1 was analyzed using quantitative reverse transcription-PCR using cDNAs, TaqMan Gene Expression Assays, and the ABI StepOnePlus Real-Time PCR Instrument (Thermo Fisher Scientific). mRNA expression was calculated using the $\Delta\Delta C_t$ method. The *GAPDH* gene was used as the endogenous control for the mRNA expression analysis.

### siRNA transfection

NSCLC cells were transfected with 5 nmol/L of Silencer Select siRNA against AXL (si-AXL#1 and si-AXL#2) or scrambled negative control siRNA (si-Scramble; Thermo Fisher Scientific) using Lipofectamine RNAiMAX and were incubated for 72 hours.

**Table 1.** Osimertinib-resistant cell lines and resistant mechanisms

| Cell line | Osimertinib exposure | *EGFR* Mutation | Osimertinib $IC_{50}$ (μmol/L) | T790M Mutation | C797S Mutation | *MET* Amplification | EMT Phenotypes | AXL Upregulation |
|---|---|---|---|---|---|---|---|---|
| HCC827 Parental | N/A | | 0.019 | - | - | | | |
| HCC827-ORS | Stepwise | 19 Del | 3.9 | - | - | - | + | + |
| HCC827-ORH | High | | 4.9 | - | - | + | + | - |
| HCC4006 Parental | N/A | | 0.022 | - | - | | | |
| HCC4006-ORS | Stepwise | 19 Del | 4.6 | - | - | - | + | + |
| HCC4006-ORH | High | | 4.6 | - | - | - | + | + |
| PC9 Parental | N/A | | 0.036 | - | - | | | |
| PC9-ORS | Stepwise | 19 Del | 3.9 | - | - | - | - | + |
| PC9-ORH | High | | 3.9 | - | - | + | - | + |
| H1975 Parental | N/A | L858R | 0.036 | + | - | | | |
| H1975-ORS | Stepwise | + | 5.2 | + | - | - | + | + |
| H1975-ORH | High | T790M | 5.2 | + | - | - | + | + |
| HCC4011 Parental | N/A | L858R | 0.031 | - | - | | | |
| HCC4011-ORH | High | | 5.3 | - | - | + | - | - |

Abbreviations: EMT, epithelial to mesenchymal transition; N/A, not applicable

### Cell proliferation assay

Cell proliferation was determined using a modified MTS assay with CellTiter 96 AQueous One Solution Reagent (Promega), as reported previously (24). The antiproliferative effects were described as the 50% inhibitory concentration ($IC_{50}$). For experiments testing the effect of the knockdown of siRNA on cell proliferation and treatment with a combination of osimertinib with cabozantinib, an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; Sigma-Aldrich) assay was used. Cells were cultured at 37°C with 5% $CO_2$, in 6-well plates at a concentration of $1 \times 10^5$ cells/mL for 72 hours. MTT was dissolved in RPMI1640 medium, and 100 μL of the MTT solution were added to each well; the plates were then incubated at 37°C with 5% $CO_2$ for 2 hours. Subsequently, 100 μL of DMSO was added to each well. The cell viability was assessed by measuring the optical densities at 570 nm and at 690 nm on a plate reader. Three independent experiments consisting of triplicate runs (at least) were performed.

### Xenograft model

The protocol was approved by the Animal Care and Use Committee of Okayama University (Okayama, Japan; permit number: OKU-2016398). Six-week-old BALB/c nu/nu female mice were purchased from Japan SLC). H1975, H1975-ORS, and H1975-GRH cells ($2 \times 10^6$) were suspended in 50 μL of RPMI1640 media mixed with 50 μL of Matrigel Basement Membrane Matrix (Corning) and subcutaneously injected into the backs of the mice. When the tumors had reached approximately 50 to 100 $mm^3$ in size, the mice were randomly divided into three groups: an osimertinib (5 mg/kg/day) group, a combined treatment group (osimertinib, 5 mg/kg/day; cabozantinib, 30 mg/kg/day), and a control group ($n = 5$ for each group). Tumor growth was monitored, and individual tumor volumes were measured using a digital caliper and approximated according to the formula $V = 1/2\ ab^2$ ($a$, long diameter; $b$, short diameter). Osimertinib and cabozantinib were prepared in 0.5% (w/v) methyl cellulose. Vehicles and these drugs were administered orally by gavage 5 days per week for 3 weeks. At the end of the experiment, the mice were sacrificed and their tumors were harvested, measured, and photographed.

### Statistical analyses

All the statistical analyses were performed using GraphPad Prism 7 (GraphPad Software). $P < 0.05$ was considered statistically significant. All the tests were two sided.

## Results

### EGFR-mutant cell lines that acquired resistance to osimertinib

Five cell lines (HCC827, HCC4006, PC-9, H1975, and HCC4011) with TKI-sensitive *EGFR* mutations were exposed to osimertinib using two different methods: stepwise escalation (ORS series) and high-concentration exposure (ORH series). As a result, nine cell lines resistant to osimertinib were established: HCC827-ORS, HCC827-ORH, HCC4006-ORS, HCC4006-ORH, PC-9-ORS, PC-9-ORH, H1975-ORH, H1975-ORH, and HCC4011-ORH. We could not establish resistant HCC4011-derived cell lines using the stepwise method within this experimental period.

The characteristics of the resistant cell lines including the $IC_{50}$ values for osimertinib are shown in Table 1. The $IC_{50}$ values against osimertinib of these nine resistant cell lines exceeded 100 times or more, compared with the values of the parental cell lines, and these values were higher than the maximum drug concentration in clinical use. The osimertinib-resistant cell lines also showed resistance to first- and second-generation EGFR-TKIs.

### Genetic alterations in osimertinib-resistant cell lines

We investigated genetic alterations such as point mutations (including *EGFR* T790M and C797S), *MET* amplification, and gains or losses in *EGFR* copy number. First, we examined the mutational status of the tyrosine kinase domain of *EGFR* using direct sequencing and targeted NGS. The T790M mutation was not detected in any of the HCC827, HCC4006, HCC4011, or PC-9 resistant cell lines. Furthermore, the disappearance of T790M was not detected in the H1975 resistant cell lines. The C797S mutation was not detected in the H1975 resistant cell lines as well as other osimertinib-resistant cell lines. In addition, none of the resistant cell lines harbored secondary mutations in the targeted 47 genes including *EGFR, KRAS, NRAS, BRAF,* and *TP53*.

Next, we examined the copy number of several genes, a gain of which is considered to be related to acquired resistance to EGFR-TKIs. A decrease in the *EGFR* copy number was detected in

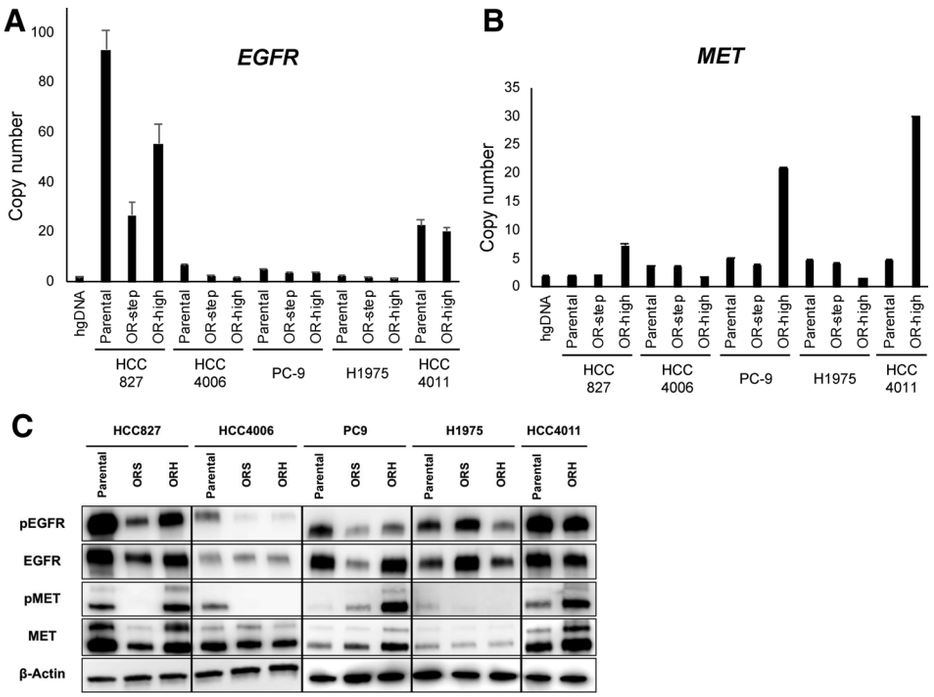


**Figure 1.**
Genetic analysis of NSCLC EGFR–mutant cell lines and their corresponding osimertinib-resistant cell lines. The copy numbers of *EGFR* (**A**) and *MET* (**B**)were determined using a quantitative reverse-transcription PCR assay. An *EGFR* copy number loss was observed in the H827-ORS and H827-ORH cells. The copy number of *MET* was amplified in the HCC827-ORH, PC9-ORH, and HCC4011-ORH cells. **C,** Expressions of EGFR and MET proteins as detected using Western blot analysis. The expressions of phospho-EGFR and EGFR were downregulated in HCC827-ORS and HCC827-ORH, whereas the expressions of phospho-MET and MET were upregulated in HCC827-ORH, PC9-ORH, and HCC4011-ORH.

HCC827-ORS and HCC827-ORH (Fig. 1A). Copy number gains in *MET* were detected in HCC827-ORH, PC9-ORH, and HCC4011-ORH (Fig. 1B). No significant change in the copy number of *YES1* was seen (Supplementary Fig. S1). We also examined the expression levels of EGFR and MET protein and the phosphorylation levels of these proteins using Western blot analysis (Fig. 1C). Consistent with the copy number analysis, the expressions of phospho-EGFR and EGFR were downregulated in HCC827-ORS and HCC827-ORH, whereas the expressions of phospho-MET and MET were upregulated in HCC827-ORH, PC9-ORH, and HCC4011-ORH. HCC4011-ORH with *MET* amplification was sensitive to treatment with a combination of osimertinib and crizotinib, which is a MET inhibitor, but the combined treatment did not have any effect on HCC827-ORH and PC9-ORH (Table 2; Supplementary Fig. S2). Indeed, these two resistant cell lines exhibited *MET* amplification, but this feature is likely attributable to other resistance mechanisms.

### Acquisition of EMT phenotypes in osimertinib-resistant cell lines

To investigate the phenotypic changes following the development of acquired resistance to osimertinib, we comparatively examined the expression levels of an epithelial marker (E-cadherin) and a mesenchymal marker (vimentin) in parental and resistant cell lines. When examined using Western blotting analysis, HCC827-ORS, HCC827-ORH, HCC4006-ORS, and HCC4006-ORH cell lines displayed the downregulation of E-cadherin and the upregulation of vimentin (Fig. 2A). In the H1975-ORS and H1975-ORH cell lines, a loss of E-cadherin expression was clearly observed, compared with the parental cell lines, whereas no clear alterations in vimentin expression were seen. Microscopically, each of the six resistant cell lines (HCC827-ORS, HCC827-ORH, HCC4006-ORS, HCC4006-ORH, H1975-ORS, and H1975-ORH) exhibited a spindle cell–like morphology that was different from that of the parental cell lines (Fig. 2B). These findings suggest the occurrence of an epithelial-to-mesenchymal transition in these cell lines, resulting in acquired resistance to osimertinib. We also checked the expression levels of *ALDH1A1* and *ABCB1*. We have previously reported that these markers were upregulated in first- or second-generation EGFR-TKI resistant cell lines (24, 25). On the basis of the previous study, we also examined these markers in osimertinib-resistant cell lines. The upregulation of *ALDH1A1* was observed in HCC827-ORH using Western blotting analysis (Fig. 2A) and qRT-PCR (Supplementary Fig. S3A). *ABCB1* was upregulated in HCC827-ORH, HCC4006-ORS, and HCC4006-ORH (Supplementary Fig. S3B).

**Table 2.** $IC_{50}$ values (μmol/L) against osimertinib with crizotinib in MET-amplified osimertinib-resistant cell lines

| | | MET Inhibitor | |
|---|---|---|---|
| Cell lines | EGFR-TKI Osimertinib | Crizotinib | Osimertinib with crizotinib (0.2 μmol/L) |
| HCC827-ORH | 4.9 | 4.5 | 4.4 |
| PC9-ORH | 3.5 | 2.3 | 2.2 |
| HCC4011-ORH | 5.3 | 4.4 | 0.042 |

### AXL kinase activation in osimertinib-resistant cell lines

AXL, a member of the receptor tyrosine kinase family (28), has been demonstrated to be an important factor associated with the EMT in certain tumors including NSCLC, breast cancer, and pancreatic cancer (29–32). Although it is becoming increasingly clear that AXL may have an intricate role in cellular migration, its precise role in the EMT remains unknown (32). We investigated AXL expression and confirmed whether AXL is associated with cell viability. Using Western blotting analysis, the expression of AXL was upregulated in HCC827-ORS, HCC4006ORS, HCC4006ORH, PC9-ORS, PC9-ORH, H1975-ORS, and H1975ORH (Supplementary Fig. S4). On the other

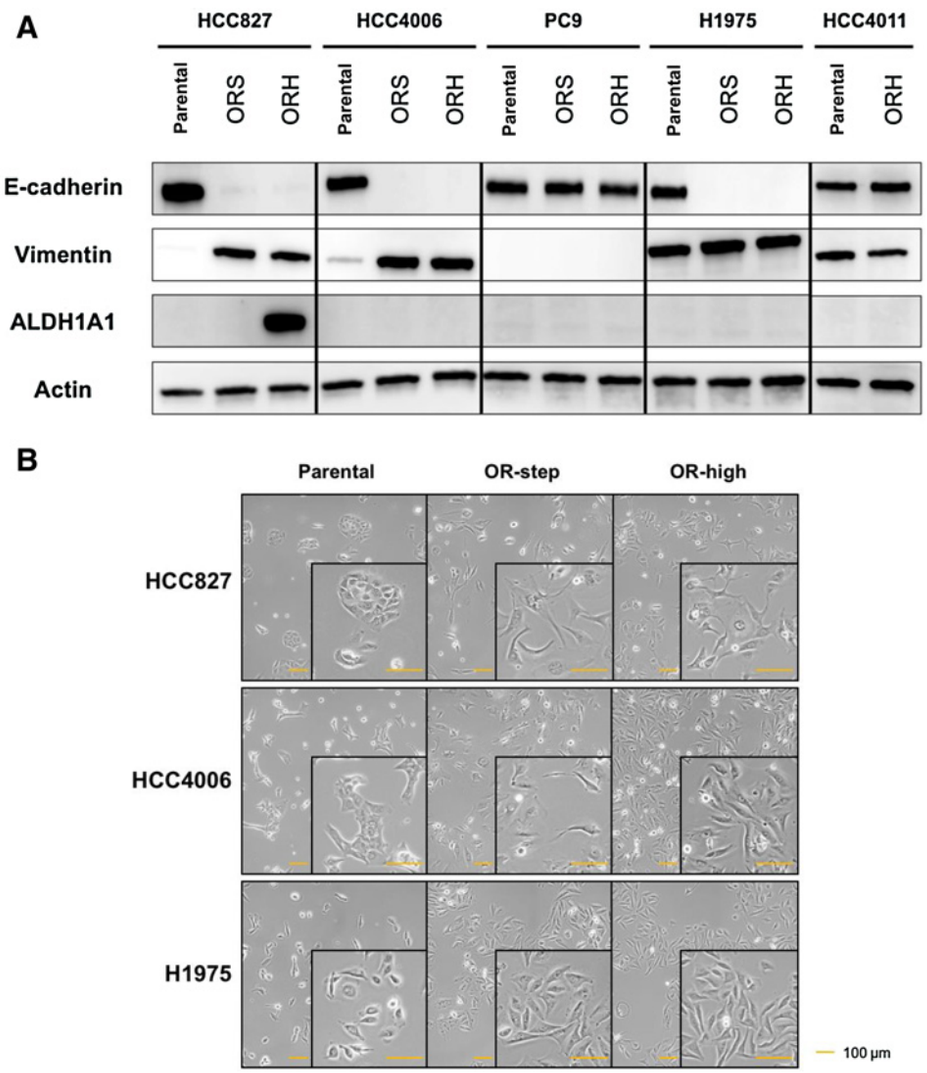


**Figure 2.**
Acquisition of EMT phenotypes in NSCLC EGFR–mutant cell lines and their corresponding osimertinib-resistant cell lines. **A,** Western blot analysis for EMT markers showed that the HCC827-ORS, HCC827-ORH, HCC4006-ORS, and HCC4006-ORH cell lines exhibited the downregulation of E-cadherin and the upregulation of vimentin. H827-ORH cells exhibited the upregulation of ALDH1A1. **B,** Microscopically, each of the six resistant cell lines (HCC827-ORS, HCC827-ORH, HCC4006-ORS, HCC4006-ORH, H1975-ORS, and H1975-ORH) exhibited a spindle cell–like morphology that differed from that of their parental cell lines.

hand, no significant changes in the copy numbers of AXL were seen in osimertinib-resistant cell lines, compared with those in the parental cell lines (Supplementary Fig. S5).

Thus, we focused on the resistant cell lines derived from H1975 and HCC4006 cells to overcome acquired resistance mechanisms related to AXL activation. First, we suppressed the expression of AXL using siRNAs. AXL knockdown had no significant effect on cell viability in the parental H1975 cells. On the other hand, in the H1975-ORS and ORH cells, cell growth was suppressed by AXL siRNAs, compared with nontargeting siRNA (Fig. 3). In the HCC4006 parental and resistant cell lines, like H1975 series, cell growth was suppressed by AXL siRNAs (Supplementary Fig. S6A). These results suggest that the survival of these resistant cell lines depends on AXL signaling. To gain insight into the intracellular signaling events involved in the growth suppression caused by AXL knockdown, we examined the alterations in protein expression by Western blotting analysis. The results are shown in Supplementary Fig. S7. Consistent with the results of MTT assay, cleaved PARP was overexpressed in AXL-knockdown resistant cell lines. We could not detect significant difference in signal pathway.

Next, we examined the effect of cabozantinib monotherapy and combined treatment with osimertinib and cabozantinib. Cabozantinib is an inhibitor of multiple tyrosine kinases, including AXL (33, 34), and has received FDA approval for the treatment of progressive metastatic medullary thyroid cancer and advanced renal cell carcinoma (35–38). In an MTT assay, cabozantinib monotherapy did not provide the sufficient inhibition of cell growth in both H1975 and HCC4006 resistant cell lines, but the sensitivity of the resistant cells to osimertinib was improved with cabozantinib treatment (Fig. 4A; Supplementary Fig S6B). To gain insight into the intracellular signaling events involved in the growth suppression caused by the combined treatment with osimertinib and cabozantinib, we examined the alterations in protein expression. As shown in Fig. 4B, cabozantinib monotherapy slightly downregulated the expression of AXL. The phosphorylation of MAPK was inhibited by osimertinib monotherapy. On the other hand, the phosphorylation of AKT was only

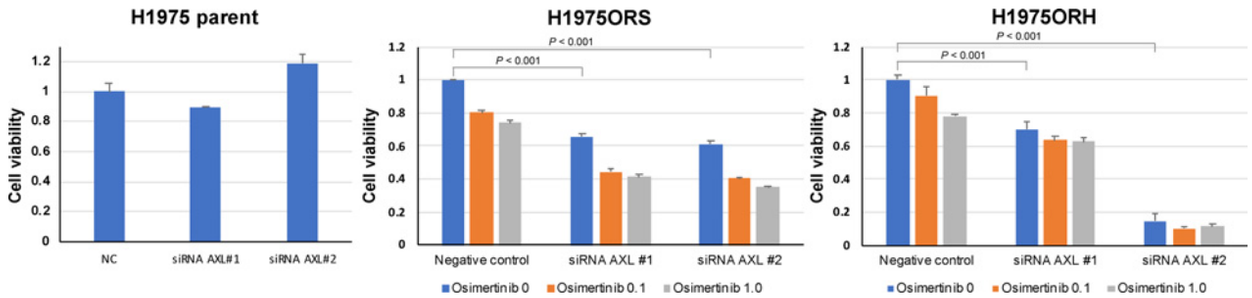


**Figure 3.**

Antitumor effect of AXL knockdown in H1975 parental and osimertinib-resistant cells as determined using an MTT assay. Cells were seeded after treatment with nontargeting siRNA or AXL siRNAs for 72 hours, then treated with or without osimertinib for 48 hours. The cell viability of cells treated with nontargeting siRNA and without osimertinib treatment was set as 1. AXL knockdown had no significant effect on cell viability in the parental H1975 cells. In the H1975-ORS and ORH cells, however, cell growth was suppressed by the AXL siRNAs, compared with nontargeting siRNA.

inhibited by the combined treatment with osimertinib and cabozantinib. The combined treatment was associated with the expression of cleaved PARP (a marker of apoptosis) in both H1975-ORS and H1975-ORH cells. These results indicate that osimertinib or cabozantinib monotherapy was not sufficient to suppress cell proliferation in resistant cell lines, but that combined treatment was effective in overcoming acquired resistance to osimertinib.

### Combined treatment using osimertinib and cabozantinib inhibits tumor growth in a mouse xenograft model of osimertinib-resistant NSCLC

We investigated the antitumor effects of osimertinib monotherapy and the combination of osimertinib and cabozantinib on the growth of H1975-ORS and H1975-ORH cells *in vivo*. As shown in Fig 4C, the tumor growth in the combined treatment group was significantly suppressed during the observation period, compared with that in animals treated with the standard vehicle PBS or the osimertinib monotherapy group. No apparent toxicity, such as weight loss or behavioral changes, was seen in any of the groups.

## Discussion

In this study, we established multiple cell lines that acquired resistance to the third-generation EGFR-TKI osimertinib using five *EGFR*-mutant NSCLC cell lines and examined the various resistance mechanisms. First, we investigated genetic alterations in the resistant cell lines. The *EGFR* C797S mutation is the most common mechanism of resistance to third-generation EGFR-TKIs clinically. In addition to *EGFR* C797S mutation, there are reports of genomic alterations in patient samples that have been sequenced after progression. For instance, *BRAF* V600E mutation (39, 40), *KRAS* mutations (22, 41, 42), *PIK3CA* mutations (41, 42), *ALK* gene fusion (43), etc. are reported. In this study, resistant cell lines were established using two different drug exposure methods for each cell line. However, targeted NGS using a multigene panel did not reveal either *EGFR* C797S mutation or any other secondary mutations in our resistant cell lines. The drug exposure methods for cell lines might be different from the actual conditions *in vivo*. Furthermore, studies using *in vivo* samples are necessary to elucidate the difference in these exposure conditions.

We also investigated copy number alterations for *EGFR* and *MET*. An *EGFR* copy number loss was detected in two HCC827-resistant cell lines, while *MET* amplification occurred in HCC827-ORH, PC9-ORH, and HCC4011-ORH. Among these three *MET*-amplified resistant cell lines, combined treatment with osimertinib and crizotinib was only effective in one of the cell lines. The detailed mechanisms underlying these results remain unknown, but they are consistent with a previous report that *MET* gene amplification and MET receptor activation are insufficient to predict a positive response of NSCLC cells to combined treatment with MET and EGFR inhibitors (44).

Next, we investigated nongenetic alterations. Several resistant cell lines displayed EMT features, which we previously reported as mechanisms of acquired resistance to first- and second-generation EGFR-TKIs. In addition, focusing on AXL as an associated marker of EMT, the expression of total AXL protein was upregulated in several resistant cell lines. Among these AXL-overexpressed resistant cell lines, we showed a decrease in cell viability by AXL knockdown in H1975- and HCC4006-resistant cell lines. As determined using a Western blotting analysis, apoptosis was not induced in the AXL-knockdown H1975 parental cell, but it was induced in H1975-resistant cell lines. Zhang and colleagues reported that the activation of AXL kinase causes resistance to the first-line EGFR-TKI erlotinib in HCC827 cells (45). There is no report describing AXL as a cause of acquired resistance to third-generation EGFR-TKIs. In our study, we first observed that the activation of AXL kinase caused resistance to a third-generation EGFR-TKI. We also showed that cabozantinib improved the sensitivity of osimertinib in H1975-derived acquired resistant cell lines, and combined treatment with osimertinib and cabozantinib suppressed the phosphorylation of AKT. Furthermore, this combined treatment inhibited tumor growth in a xenograft model of osimertinib-resistant NSCLC. These results suggest that the activation of multiple pathways, including AKT, may promote resistance to EGFR-TKIs downstream of AXL upregulation (32). This hypothesis is consistent with previous reports suggesting that AXL drives the growth of cancer cells through the activation of each of these pathways (45–47). Because cabozantinib is a multikinase inhibitor, it might suppress not only AXL, but also other kinases involved in the acquisition of osimertinib resistance. Thus, cabozantinib, an FDA-approved drug, could be a key drug in overcoming acquired resistance to osimertinib.

We believe that the totality of data in this study is meaningful to design the clinical trial with osimertinib and cabozantinib for osimertinib-resistant patients. Although several clinical trials which evaluate the first or third-generation EGFR-TKIs with

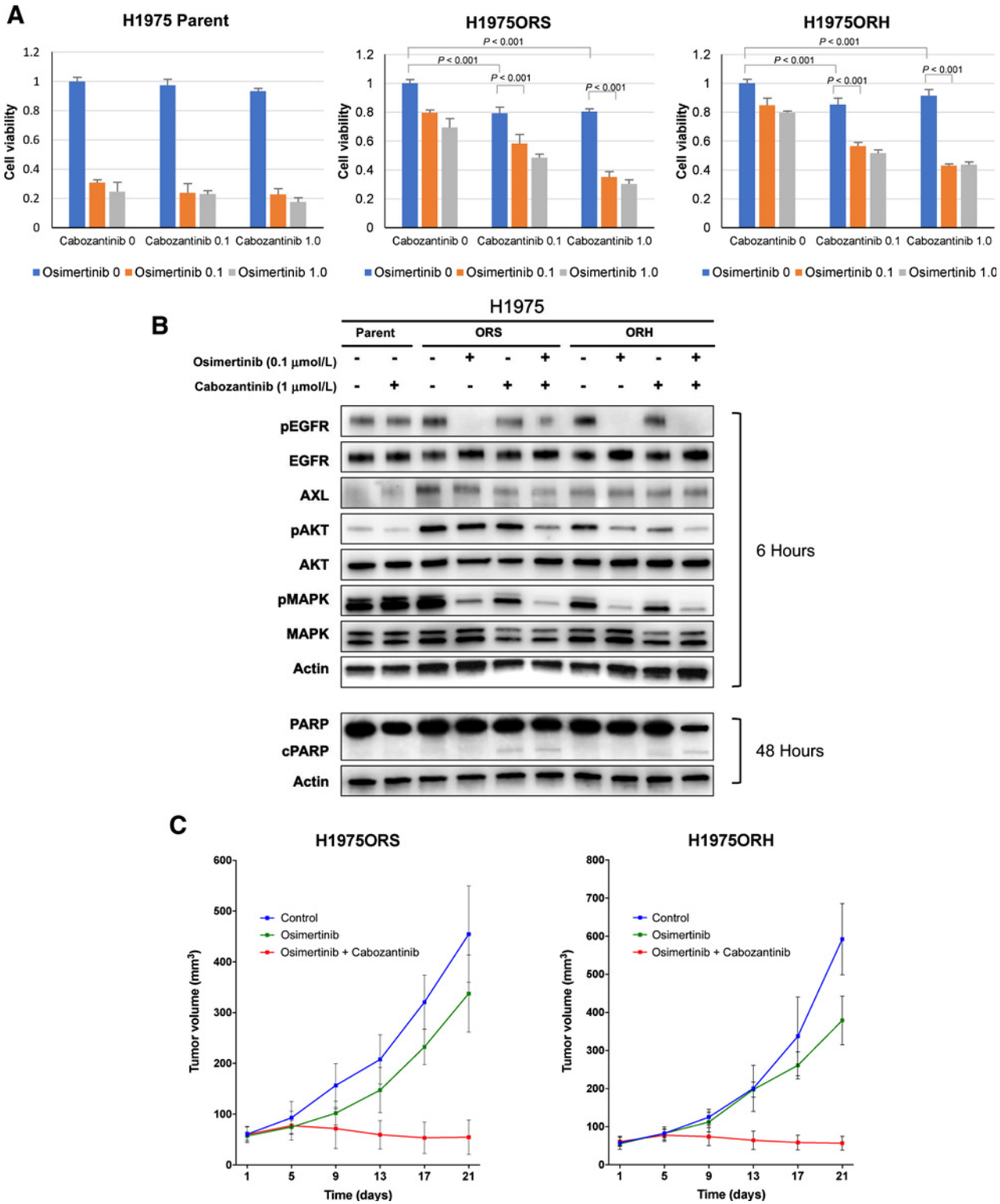


**Figure 4.**

Combined treatment with osimertinib and cabozantinib in H1975 and H1975-resistant cells. **A,** Cell viability after combined treatment with osimertinib and cabozantinib in H1975 and H1975-resistant cells as determined using an MTT assay. **B,** Alterations in protein expression caused by combined treatment with osimertinib and cabozantinib. **C,** Therapeutic effect of combined treatment using osimertinib and cabozantinib on tumor growth *in vivo*. The mean volume of the subcutaneous xenograft tumors was calculated for 5 tumors in each group. The combined treatment significantly inhibited tumor growth in mouse xenograft models of H1975ORS and H1975ORH. Time-dependent changes in tumor volume are shown on the left, and the appearance of the tumor at the time of sacrifice is shown on the right.

selective AXL inhibitors for EGFR-TKI–resistant patients are currently ongoing (NCT02424617, NCT03255083, NCT03599518), the clinical trial with osimertinib and cabozantinib, a multikinase inhibitor suppressing MET in addition to AXL, may bring benefits compared with these selective AXL inhibitors. We have not examined the clinical samples of osimertinib-resistant patients this time, which is the limitation of this study. The number of osimertinib-resistant patients will increase as osimertinib was approved by FDA for the first-line treatment of patients with advanced NSCLC. Further studies for AXL expression in the samples of postprogression patient samples are needed.

In conclusion, we established nine cell lines with acquired resistance to osimertinib from five parental *EGFR*-mutant NSCLC cells. The observed resistance mechanisms varied, including the acquisition of *MET* amplification, EMT induction, and the upregulation of AXL. AXL might be a therapeutic target for overcoming osimertinib resistance.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Authors' Contributions

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## Acknowledgments

The authors thank Dr. Takehiro Matsubara, Ms. Yuko Hanafusa (Okayama University Hospital Biobank, Okayama University Hospital, Okayama, Japan), and Ms. Fumiko Isobe (Department of Thoracic, Breast and Endocrinological Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan) for their technical support. This work was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS KAKENHI grant nos. 17K16608, to K. Shien; 16H05431, to S. Toyooka).

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Received June 13, 2018; revised October 2, 2018; accepted November 14, 2018; published first November 21, 2018.

## References

1. Miller KD, Siegel RL, Lin CC, Mariotto AB, Kramer JL, Rowland JH, et al. Cancer treatment and survivorship statistics, 2016. CA Cancer J Clin 2016;66:271–89.
2. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. CA Cancer J Clin 2015;65:5–29.
3. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin 2015;65:87–108.
4. Pao W, Chmielecki J. Rational, biologically based treatment of EGFR-mutant non-small-cell lung cancer. Nat Rev Cancer 2010;10:760–74.
5. Hanna N, Johnson D, Temin S, Baker S Jr, Brahmer J, Ellis PM, et al. Systemic therapy for stage IV non-small-cell lung cancer: American Society of Clinical Oncology clinical practice guideline update. J Clin Oncol 2017; 35:3484–515.
6. Ettinger DS, Wood DE, Aisner DL, Akerley W, Bauman J, Chirieac LR, et al. Non-small cell lung cancer, version 5.2017, NCCN clinical practice guidelines in oncology. J Natl Compr Canc Netw 2017;15:504–35.
7. Mitsudomi T, Yatabe Y. Mutations of the epidermal growth factor receptor gene and related genes as determinants of epidermal growth factor receptor tyrosine kinase inhibitors sensitivity in lung cancer. Cancer Sci 2007;98: 1817–24.
8. Arcila ME, Oxnard GR, Nafa K, Riely GJ, Solomon SB, Zakowski MF, et al. Rebiopsy of lung cancer patients with acquired resistance to EGFR inhibitors and enhanced detection of the T790M mutation using a locked nucleic acid-based assay. Clin Cancer Res 2011;17:1169–80.
9. Sequist LV, Waltman BA, Dias-Santagata D, Digumarthy S, Turke AB, Fidias P, et al. Genotypic and histological evolution of lung cancers acquiring resistance to EGFR inhibitors. Sci Transl Med 2011;3:75ra26.
10. Zhou W, Ercan D, Chen L, Yun CH, Li D, Capelletti M, et al. Novel mutant-selective EGFR kinase inhibitors against EGFR T790M. Nature 2009;462: 1070–4.
11. Ward RA, Anderton MJ, Ashton S, Bethel PA, Box M, Butterworth S, et al. Structure- and reactivity-based development of covalent inhibitors of the activating and gatekeeper mutant forms of the epidermal growth factor receptor (EGFR). J Med Chem 2013;56:7025–48.
12. Cross DA, Ashton SE, Ghiorghiu S, Eberlein C, Nebhan CA, Spitzler PJ, et al. AZD9291, an irreversible EGFR TKI, overcomes T790M-mediated resistance to EGFR inhibitors in lung cancer. Cancer Discov 2014;4:1046–61.
13. Goss G, Tsai CM, Shepherd FA, Bazhenova L, Lee JS, Chang GC, et al. Osimertinib for pretreated EGFR Thr790Met-positive advanced non-small-cell lung cancer (AURA2): a multicentre, open-label, single-arm, phase 2 study. Lancet Oncol 2016;17:1643–52.
14. Mok TS, Wu YL, Ahn MJ, Garassino MC, Kim HR, Ramalingam SS, et al. Osimertinib or platinum-pemetrexed in EGFR T790M-positive lung cancer. N Engl J Med 2017;376:629–40.
15. Yang JC, Ahn MJ, Kim DW, Ramalingam SS, Sequist LV, Su WC, et al. Osimertinib in pretreated T790M-positive advanced non-small-cell lung cancer: AURA study phase II extension component. J Clin Oncol 2017; 35:1288–96.
16. Soria JC, Ohe Y, Vansteenkiste J, Reungwetwattana T, Chewaskulyong B, Lee KH, et al. Osimertinib in untreated EGFR-mutated advanced non-small-cell lung cancer. N Engl J Med 2018;378:113–25.
17. Niederst MJ, Hu H, Mulvey HE, Lockerman EL, Garcia AR, Piotrowska Z, et al. The allelic context of the C797S mutation acquired upon treatment with third-generation EGFR inhibitors impacts sensitivity to subsequent treatment strategies. Clin Cancer Res 2015;21:3924–33.
18. Thress KS, Paweletz CP, Felip E, Cho BC, Stetson D, Dougherty B, et al. Acquired EGFR C797S mutation mediates resistance to AZD9291 in non-small cell lung cancer harboring EGFR T790M. Nat Med 2015; 21:560–2.
19. Yu HA, Tian SK, Drilon AE, Borsu L, Riely GJ, Arcila ME, et al. Acquired resistance of EGFR-mutant lung cancer to a T790M-specific EGFR inhibitor: emergence of a third mutation (C797S) in the EGFR tyrosine kinase domain. JAMA Oncol 2015;1:982–4.
20. Planchard D, Loriot Y, Andre F, Gobert A, Auger N, Lacroix L, et al. EGFR-independent mechanisms of acquired resistance to AZD9291 in EGFR T790M-positive NSCLC patients. Ann Oncol 2015;26: 2073–8.
21. Ou SH, Agarwal N, Ali SM. High MET amplification level as a resistance mechanism to osimertinib (AZD9291) in a patient that symptomatically responded to crizotinib treatment post-osimertinib progression. Lung Cancer 2016;98:59–61.
22. Eberlein CA, Stetson D, Markovets AA, Al-Kadhimi KJ, Lai Z, Fisher PR, et al. Acquired resistance to the mutant-selective EGFR inhibitor AZD9291 is associated with increased dependence on RAS signaling in preclinical models. Cancer Res 2015;75:2489–500.
23. Sagawa Y, Fujitoh A, Nishi H, Ito H, Yudate T, Isaka K. Establishment of three cisplatin-resistant endometrial cancer cell lines using two methods of cisplatin exposure. Tumour Biol 2011;32:399–408.

24. Shien K, Toyooka S, Yamamoto H, Soh J, Jida M, Thu KL, et al. Acquired resistance to EGFR inhibitors is associated with a manifestation of stem cell-like properties in cancer cells. Cancer Res 2013;73:3051–61.
25. Hashida S, Yamamoto H, Shien K, Miyoshi Y, Ohtsuka T, Suzawa K, et al. Acquisition of cancer stem cell-like properties in non-small cell lung cancer with acquired resistance to afatinib. Cancer Sci 2015;106: 1377–84.
26. Tokumo M, Toyooka S, Kiura K, Shigematsu H, Tomii K, Aoe M, et al. The relationship between epidermal growth factor receptor mutations and clinicopathologic features in non-small cell lung cancers. Clin Cancer Res 2005;11:1167–73.
27. Kubo T, Yamamoto H, Lockwood WW, Valencia I, Soh J, Peyton M, et al. MET gene amplification or EGFR mutation activate MET in lung cancers untreated with EGFR tyrosine kinase inhibitors. Int J Cancer 2009;124:1778–84.
28. Robinson DR, Wu YM, Lin SF. The protein tyrosine kinase family of the human genome. Oncogene 2000;19:5548–57.
29. Vuoriluoto K, Haugen H, Kiviluoto S, Mpindi JP, Nevo J, Gjerdrum C, et al. Vimentin regulates EMT induction by Slug and oncogenic H-Ras and migration by governing Axl expression in breast cancer. Oncogene 2011;30:1436–48.
30. Gjerdrum C, Tiron C, Hoiby T, Stefansson I, Haugen H, Sandal T, et al. Axl is an essential epithelial-to-mesenchymal transition-induced regulator of breast cancer metastasis and patient survival. Proc Natl Acad Sci USA 2010;107:1124–9.
31. Koorstra JB, Karikari CA, Feldmann G, Bisht S, Rojas PL, Offerhaus GJ, et al. The Axl receptor tyrosine kinase confers an adverse prognostic influence in pancreatic cancer and represents a new therapeutic target. Cancer Biol Ther 2009;8:618–26.
32. Okimoto RA, Bivona TG. AXL receptor tyrosine kinase as a therapeutic target in NSCLC. Lung Cancer 2015;6:27–34.
33. Drilon A, Somwar R, Wagner JP, Vellore NA, Eide CA, Zabriskie MS, et al. A novel crizotinib-resistant solvent-front mutation responsive to cabozantinib therapy in a patient with ROS1-rearranged lung cancer. Clin Cancer Res 2016;22:2351–8.
34. Yakes FM, Chen J, Tan J, Yamaguchi K, Shi Y, Yu P, et al. Cabozantinib (XL184), a novel MET and VEGFR2 inhibitor, simultaneously suppresses metastasis, angiogenesis, and tumor growth. Mol Cancer Ther 2011;10: 2298–308.
35. Viola D, Cappagli V, Elisei R. Cabozantinib (XL184) for the treatment of locally advanced or metastatic progressive medullary thyroid cancer. Future Oncol 2013;9:1083–92.
36. Bentzien F, Zuzow M, Heald N, Gibson A, Shi Y, Goon L, et al. *In vitro* and *in vivo* activity of cabozantinib (XL184), an inhibitor of RET, MET, and VEGFR2, in a model of medullary thyroid cancer. Thyroid 2013;23: 1569–77.
37. Choueiri TK, Escudier B, Powles T, Mainwaring PN, Rini BI, Donskov F, et al. Cabozantinib versus everolimus in advanced renal-cell carcinoma. N Engl J Med 2015;373:1814–23.
38. Choueiri TK, Escudier B, Powles T, Tannir NM, Mainwaring PN, Rini BI, et al. Cabozantinib versus everolimus in advanced renal cell carcinoma (METEOR): final results from a randomised, open-label, phase 3 trial. Lancet Oncol 2016;17:917–27.
39. Ho CC, Liao WY, Lin CA, Shih JY, Yu CJ, Chih-Hsin Yang J. Acquired BRAF V600E mutation as resistant mechanism after treatment with osimertinib. J Thorac Oncol 2017;12:567–72.
40. Minari R, Bordi P, La Monica S, Squadrilli A, Leonetti A, Bottarelli L, et al. Concurrent acquired BRAF V600E mutation and MET amplification as resistance mechanism of first-line osimertinib treatment in a patient with EGFR-mutated NSCLC. J Thorac Oncol 2018;13:e89–91.
41. Ramalingam SS, Yang JC, Lee CK, Kurata T, Kim DW, John T, et al. Osimertinib as first-line treatment of EGFR mutation-positive advanced non-small-cell lung cancer. J Clin Oncol 2018;36:841–9.
42. Yang Z, Yang N, Ou Q, Xiang Y, Jiang T, Wu X, et al. Investigating novel resistance mechanisms to third-generation EGFR tyrosine kinase inhibitor osimertinib in non-small cell lung cancer patients. Clin Cancer Res 2018;24:3097–107.
43. Nie K, Jiang H, Zhang C, Geng C, Xu X, Zhang L, et al. Mutational profiling of non-small-cell lung cancer resistant to osimertinib using next-generation sequencing in Chinese patients. Biomed Res Int 2018;2018:9010353.
44. Presutti D, Santini S, Cardinali B, Papoff G, Lalli C, Samperna S, et al. MET gene amplification and MET receptor activation are not sufficient to predict efficacy of combined MET and EGFR inhibitors in EGFR TKI-resistant NSCLC cells. PLoS One 2015;10:e0143333.
45. Zhang Z, Lee JC, Lin L, Olivas V, Au V, LaFramboise T, et al. Activation of the AXL kinase causes resistance to EGFR-targeted therapy in lung cancer. Nat Genet 2012;44:852–60.
46. Linger RM, Cohen RA, Cummings CT, Sather S, Migdall-Wilson J, Middleton DH, et al. Mer or Axl receptor tyrosine kinase inhibition promotes apoptosis, blocks growth and enhances chemosensitivity of human non-small cell lung cancer. Oncogene 2013;32:3420–31.
47. Linger RM, Keating AK, Earp HS, Graham DK. Taking aim at Mer and Axl receptor tyrosine kinases as novel therapeutic targets in solid tumors. Expert Opin Ther Targets 2010;14:1073–90.