Title: Activation of AXL as a preclinical acquired resistance mechanism against osimertinib treatment in EGFR-mutant non-small cell lung cancer cells

Authors: Kei Namba¹, Kazuhiko Shien¹*, Yuta Takahashi¹, Hidejirō Torigoe¹, Hiroki Sato¹, Takahiro Yoshioka², Tatsuaki Takeda³, Eisuke Kurihara¹, Yusuke Ogoshi¹, Hiromasa Yamamoto¹, Junichi Soh¹, Shuta Tomida⁴, and Shinichi Toyooka¹

Affiliations: Departments of ¹Thoracic, Breast and Endocrinological Surgery, ²Gastroenterological Surgery, ³Clinical Pharmacy and ⁴Biobank, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama 700-8558, Japan

Running title: Resistance mechanisms against osimertinib in NSCLC cells

Key words: osimertinib; lung cancer; NSCLC; AXL, acquired resistance; EGFR

Additional information

Financial 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 number: 17K16608 to K. S. and 16H05431 to S. Toyooka).

Address correspondence to:
Abstract

Osimertinib (AZD9291) has an efficacy superior to that of standard EGFR-tyrosine kinase inhibitors for the first-line treatment of EGFR-mutant advanced non-small cell lung cancer (NSCLC) patients. 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-resistance 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 multi-gene 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.

Implication: 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). Epidermal growth factor receptor (EGFR) mutations, such as L858R point mutations and exon 19 deletions, occur in approximately 10%-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 advanced NSCLC patients (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). Based on the positive results of the AURA clinical program (13-15), osimertinib has been approved worldwide for the second-line treatment of T790M-positive NSCLC patients who experience disease progression during or after treatment with an EGFR-TKI. Furthermore, the Food and Drug Administration (FDA) recently approved osimertinib for the first-line treatment of patients with metastatic non-small cell lung cancer (NSCLC) whose tumors carry EGFR exon 19 deletions or L858R mutations, based on the results of the phase 3 FLAURA trial (16). In the FLAURA trial, the efficacy of osimertinib vs. first-generation EGFR-TKI (either erlotinib or gefitinib) in previously-untreated patients with locally-advanced or metastatic EGFR mutant-positive NSCLC were 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 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 American Type Culture Collection (ATCC) (Manassas, VA, USA). PC-9 was purchased from the RIKEN cell bank (Wako, Saitama, Japan). HCC4011 cells were provided by Dr. Adi F. Gazdar (The University of Texas Southwestern Medical Center at Dallas, Dallas, TX, USA), who established this cell line in collaboration with Dr. John D. Minna. 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, Madison, WI, USA) was performed for cell authentication. All cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and grown in a humidified incubator with 5% CO₂ 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). Based on 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 physiological blood concentration described in the attached document. Osimertinib (AZD-9291) (ChemScene, Monmouth Junction, NJ, USA), gefitinib (ChemScene), afatinib (SYNkinase, San Diego, CA, USA), and cabozantinib (AXL inhibitor) (ChemScene) were obtained from the designated sources.
Western blot analysis

Cells were harvested at 80%-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, Basel, Switzerland). 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, Danvers, MA, USA); AXL (R&D Systems, Minneapolis, MN, USA); and β-actin (used as the loading control; Merck Millipore, Billerica, MA, USA). 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, Dallas, TX, USA). To detect specific signals, the membranes were examined using the ECL Prime Western Blotting Detection System (GE Healthcare, Amersham, UK) and LAS-3000 (Fujifilm, Tokyo, Japan). The relative band intensity was assessed by densitometric analysis using ImageJ (National Institute of Health, Bethesda, Maryland, USA). Regarding the expression ratio of AXL and Actin, we defined as “upregulated” at a concentration of four-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, Venlo, the Netherlands). 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 Kits (Thermo Fisher Scientific, Waltham, MA, USA).

**DNA analysis**

*EGFR* exon 20 mutation was examined using direct sequencing, as previously reported (26). The copy number gains (CNGs) of *EGFR* and *MET* were determined using a quantitative real-time PCR (qPCR) 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, Darmstadt, Germany). 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, Santa Clara, CA) 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 microRNA expression analysis using quantitative reverse transcription-PCR

The gene expression of ALDH1A1 and ABCB1 were 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-CT method. The glyceraldehyde-3-phosphate dehydrogenase (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.

**Cell proliferation assay**

Cell proliferation was determined using a modified MTS assay with CellTiter 96 AQueous One Solution Reagent (Promega, Fitchburg, WI, USA), as previously reported (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, St. Louis, MO) 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 h. MTT was dissolved in
RPMI-1640, and 100 μL of the MTT solution were added to each well; the plates were then incubated at 37°C with 5% CO₂ for 2 h. Subsequently, 100 μL of dimethyl sulfoxide were 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 (Permit Number: OKU-2016398). Six-week-old BALB/c nu/nu female mice were purchased from Japan SLC (Shizuoka, Japan). H1975, H1975-ORS and H1975-GRH cells (2 × 10⁶) were suspended in 50 μL of RPMI-1640 media mixed with 50 μL of Matrigel Basement Membrane Matrix (Corning, NY, USA) and subcutaneously injected into the backs of the mice. When the tumors had reached approximately 50–100 mm³ 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 \( V = \frac{1}{2} ab^2 \) (\( a \), 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 9 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. Additionally, none of the resistant cell lines...
harbored secondary mutations in the targeted 47 genes including \textit{EGFR, KRAS, NRAS, BRAF,} and \textit{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 \textit{EGFR} copy number was detected in HCC827-ORS and HCC827-ORH (Figure 1A). Copy number gains in \textit{MET} were detected in HCC827-ORH, PC9-ORH, and HCC4011-ORH (Figure 1B). No significant change in the copy number of \textit{YES1} was seen (Supplementary Fig. S1). We also examined the expression levels of \textit{EGFR} and \textit{MET} protein and the phosphorylation levels of these proteins using western blotting (Figure 1C). Consistent with the copy number analysis, the expressions of phospho-\textit{EGFR} and \textit{EGFR} were downregulated in HCC827-ORS and HCC827-ORH, whereas the expressions of phospho-\textit{MET} and \textit{MET} were upregulated in HCC827-ORH, PC9-ORH and HCC4011-ORH. HCC4011-ORH with \textit{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 \textit{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, HCC827-ORS, HCC827-ORH, HCC4006-ORS and HCC4006-ORH cell lines displayed the downregulation of E-cadherin and the upregulation of vimentin (Figure 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 (Figure 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 \textit{ALDH1A1} and \textit{ABCB1}. We have previously reported that these markers were upregulated in first or second-generation EGFR-TKI resistant cell lines (24, 25). Based on the
previous study we also examined these markers in osimertinib-resistant cell lines. The upregulation of \textit{ALDH1A1} was observed in HCC827-ORH using western blotting (Figure 2A) and qRT-PCR (Supplementary Fig. S3A). \textit{ABCB1} was upregulated in HCC827-ORH, HCC4006-ORS and HCC4006-ORH (Supplementary Fig. S3B).

\textbf{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). While 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, 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 hand, no significant changes in the copy numbers of \textit{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 non-targeting siRNA (Figure 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. 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 (Figure 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 Figure 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 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 Figure 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 (phosphate buffered saline, PBS) or the osimertinib monotherapy group. No apparent toxicity, such as weight loss or behavioral changes, were 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 the present study, resistant cell lines were established using two different drug exposure methods for each cell line. However, targeted NGS using a
multi-gene panel did not reveal not only \textit{EGFR} C797S mutation but also any other secondary mutations in our resistant cell lines. The drug exposure methods for cell lines might be different from the actual conditions \textit{in vivo}. Further studies using \textit{in vivo} samples are necessary to elucidate the difference in these exposure conditions.

We also investigated copy number alterations for \textit{EGFR} and \textit{MET}. An \textit{EGFR} copy number loss was detected in two HCC827 resistant cell lines, while \textit{MET} amplification occurred in HCC827-ORH, PC9-ORH and HCC4011-ORH. Among these three \textit{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 \textit{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 non-genetic alterations. Several resistant cell lines displayed EMT features that 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 et al. 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). Since cabozantinib is a multi-kinase 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 the current 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 selective AXL inhibitors for EGFR-TKI resistant patients are currently ongoing (NCT02424617, NCT03255083, NCT03599518), the clinical trial with osimertinib and cabozantinib, a multi-kinase 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 at the present 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 post progression 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.
Acknowledgements

We 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.

References


Figure legends

Figure 1. Genetic analysis of NSCLC EGFR-mutant cell lines and their corresponding osimertinib-resistant cell lines. The copy numbers of (A) EGFR and (B) MET 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 blotting. 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.

Figure 2. Acquisition of EMT phenotypes in NSCLC EGFR-mutant cell lines and their corresponding osimertinib-resistant cell lines. (A) Western blotting 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.

**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 non-targeting siRNA or AXL siRNAs for 72 hours, then treated with or without osimertinib for 48 hours. The cell viability of cells treated with non-targeting 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 non-targeting siRNA.

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

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Osimertinib exposure</th>
<th>EGFR mutation</th>
<th>Osimertinib IC₅₀ (μM)</th>
<th>T790M mutation</th>
<th>C797S mutation</th>
<th>MET amplification</th>
<th>EMT phenotypes</th>
<th>AXL upregulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCC827 parental</td>
<td>N/A</td>
<td></td>
<td>0.019</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HCC827-ORS</td>
<td>Stepwise</td>
<td>19 del</td>
<td>3.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HCC827-ORH</td>
<td>High</td>
<td></td>
<td>4.9</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>HCC4006 parental</td>
<td>N/A</td>
<td></td>
<td>0.022</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HCC4006-ORS</td>
<td>Stepwise</td>
<td>19 del</td>
<td>4.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HCC4006-ORH</td>
<td>High</td>
<td></td>
<td>4.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PC9 parental</td>
<td>N/A</td>
<td></td>
<td>0.036</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PC9-ORS</td>
<td>Stepwise</td>
<td>19 del</td>
<td>3.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>PC9-ORH</td>
<td>High</td>
<td></td>
<td>3.9</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>H1975 parental</td>
<td>N/A</td>
<td>L858R</td>
<td>0.036</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H1975-ORS</td>
<td>Stepwise</td>
<td>+</td>
<td>5.2</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>H1975-ORH</td>
<td>High</td>
<td>T790M</td>
<td>5.2</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HCC4011 parental</td>
<td>N/A</td>
<td>L858R</td>
<td>0.031</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HCC4011-ORH</td>
<td>High</td>
<td></td>
<td>5.3</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

EGFR, epidermal growth factor receptor; EMT, epithelial to mesenchymal transition; N/A, not applicable

---

# Table 2. IC₅₀ values (μmol/L) against osimertinib with crizotinib in MET amplified osimertinib-resistant cell lines.

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>EGFR-TKI Osimertinib</th>
<th>Crizotinib</th>
<th>Osimertinib with Crizotinib(0.2μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCC827-ORH</td>
<td>4.9</td>
<td>4.5</td>
<td>4.4</td>
</tr>
<tr>
<td>PC9-ORH</td>
<td>3.5</td>
<td>2.3</td>
<td>2.2</td>
</tr>
<tr>
<td>HCC4011-ORH</td>
<td>5.3</td>
<td>4.4</td>
<td>0.042</td>
</tr>
</tbody>
</table>
Figure 1. Genetic analysis of NSCLC EGFR-mutant cell lines and their corresponding osimertinib-resistant cell lines.
Figure 2. Acquisition of EMT phenotypes in NSCLC EGFR-mutant cell lines and their corresponding osimertinib-resistant cell lines.

A

<table>
<thead>
<tr>
<th></th>
<th>HCC827</th>
<th>HCC4006</th>
<th>PC9</th>
<th>H1975</th>
<th>HCC4011</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>parental</td>
<td>ORS</td>
<td>ORH</td>
<td>parental</td>
<td>ORS</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>![image]</td>
<td>![image]</td>
<td>![image]</td>
<td>![image]</td>
<td>![image]</td>
</tr>
<tr>
<td>Vimentin</td>
<td>![image]</td>
<td>![image]</td>
<td>![image]</td>
<td>![image]</td>
<td>![image]</td>
</tr>
<tr>
<td>ALDH1A1</td>
<td>![image]</td>
<td>![image]</td>
<td>![image]</td>
<td>![image]</td>
<td>![image]</td>
</tr>
<tr>
<td>Actin</td>
<td>![image]</td>
<td>![image]</td>
<td>![image]</td>
<td>![image]</td>
<td>![image]</td>
</tr>
</tbody>
</table>

B

- **HCC827**
  - Parental
  - OR-step
  - OR-high

- **HCC4006**
  - Parental
  - OR-step
  - OR-high

- **H1975**
  - Parental
  - OR-step
  - OR-high

100μm
Figure 3. Antitumor effect of AXL knockdown in H1975 parental and osimertinib-resistant cells as determined using an MTT assay.
Figure 4. Combined treatment with osimertinib and cabozantinib in H1975 and H1975 resistant cells.

A

B

H1975

<table>
<thead>
<tr>
<th></th>
<th>parent</th>
<th>ORS</th>
<th>ORH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osimertinib (0.1μM)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cabozantinib (1μM)</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>pEGFR</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EGFR</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AXL</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pAKT</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AKT</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pMAPK</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MAPK</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Actin</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PARP</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>cPARP</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

6 hours

48 hours

C

H1975ORS

H1975ORH
Figure S1. Copy numbers of YES1 in osimertinib-resistant cell lines.

![Graph showing copy numbers of YES1 in osimertinib-resistant cell lines.](image)

Figure S2. PARP and MET expressions in MET-amplified osimertinib-resistant cell lines.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>HCC4011 ORH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osimertinib (1 μM)</td>
<td>- + - +</td>
</tr>
<tr>
<td>Crizotinib (0.2 μM)</td>
<td>- - + +</td>
</tr>
</tbody>
</table>

![Western blot images showing PARP, cleaved-PARP, pMET, MET, and Actin expressions.](image)
Figure S3. Relative ALDH1A1 and ABCB1 expression levels using qRT-PCR in NSCLC EGFR-mutant cell lines and their corresponding osimertinib-resistant cell lines.

![Graph showing ALDH1A1 and ABCB1 expression levels](image)

Figure S4. Expressions of AXL protein in NSCLC EGFR-mutant cell lines and their corresponding osimertinib-resistant cell lines.

<table>
<thead>
<tr>
<th></th>
<th>HCC827</th>
<th>HCC4006</th>
<th>PC9</th>
<th>H1975</th>
<th>HCC4011</th>
</tr>
</thead>
<tbody>
<tr>
<td>parental</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ORS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ORH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Ax1 expression ratio: 1 12.6 0.55 1 7.2 7.4 1 5.9 6.9 1 6.5 5.2 1 0.86

![Graph showing AXL expression](image)

Figure S5. Copy numbers of AXL in osimertinib-resistant cell lines.

![Graph showing AXL copy numbers](image)
Figure S6. siRNA and combined drug treatment studies in HCC4006 and HCC4006 resistant cells.

Figure S7. Combined treatment with AXL knockdown and osimertinib in H1975 and H1975 resistant cells.