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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 1×10^5 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 (2×10^6) 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

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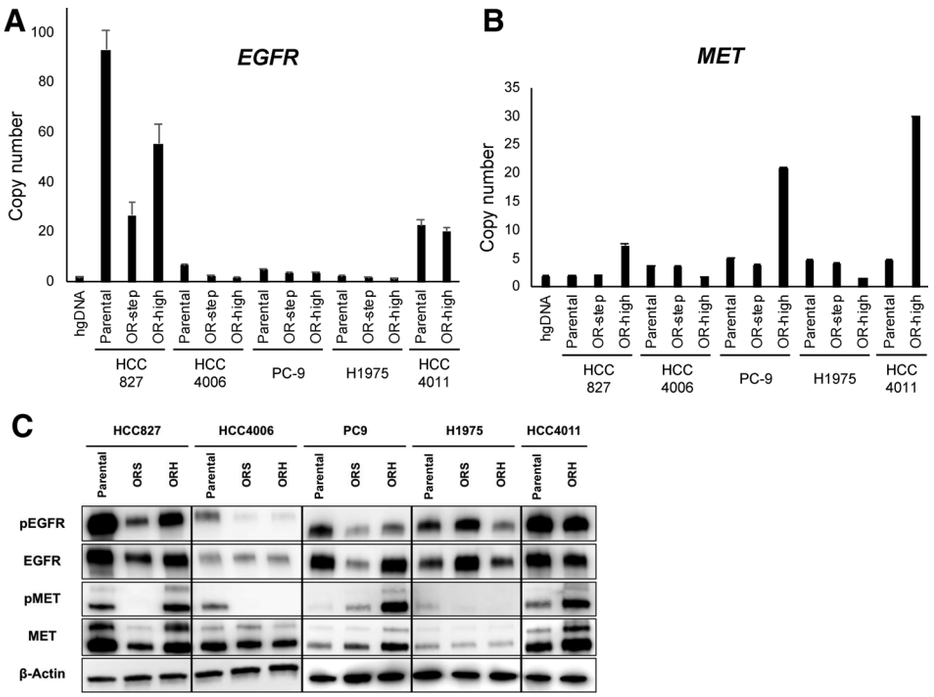

Figure 1.
Genetic analysis of NSCLC EGFR–mutant cell lines and their corresponding osimertinib-resistant cell lines. The copy numbers of *EGFR* (**A**) and *MET* (**B**)were determined using a quantitative reverse-transcription PCR assay. An *EGFR* copy number loss was observed in the H827-ORS and H827-ORH 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

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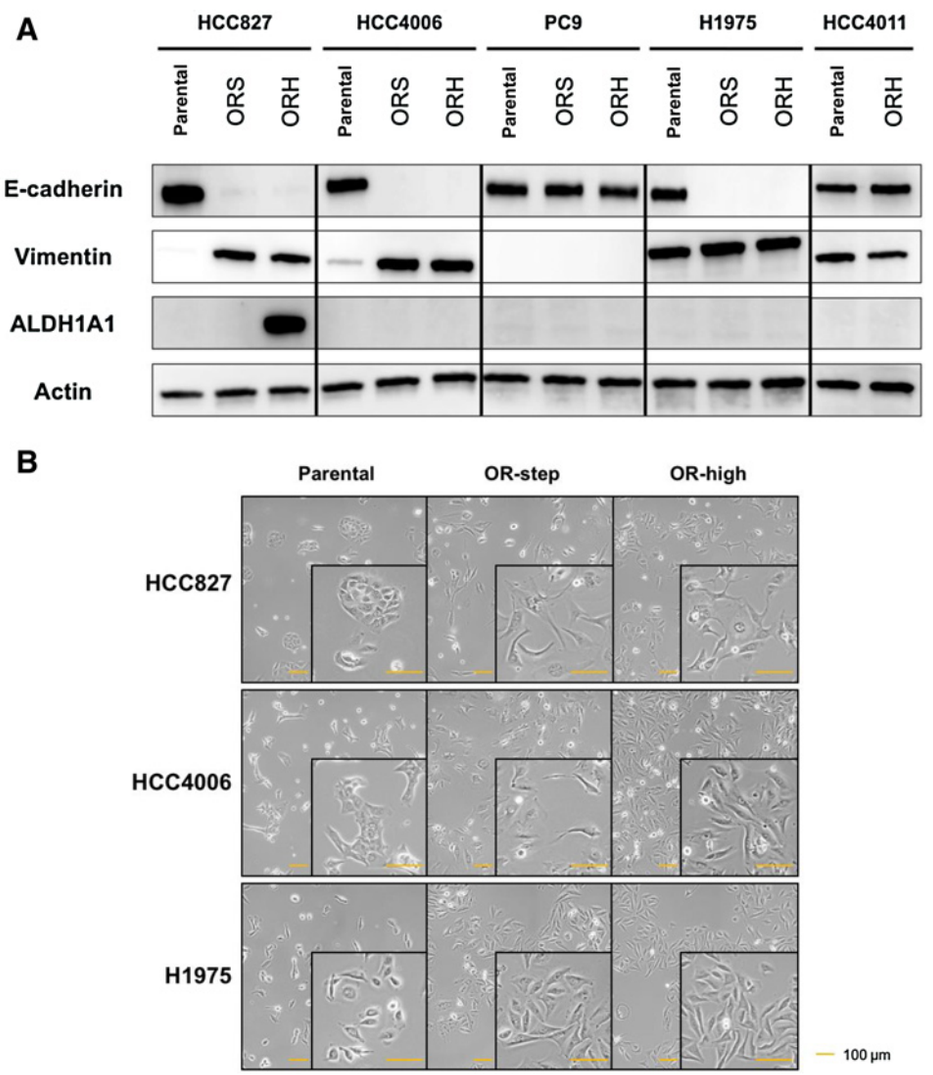

Figure 2.
Acquisition of EMT phenotypes in NSCLC EGFR–mutant cell lines and their corresponding osimertinib-resistant cell lines. **A,** Western blot analysis for EMT markers showed that the HCC827-ORS, HCC827-ORH, HCC4006-ORS, and HCC4006-ORH cell lines exhibited the downregulation of E-cadherin and 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

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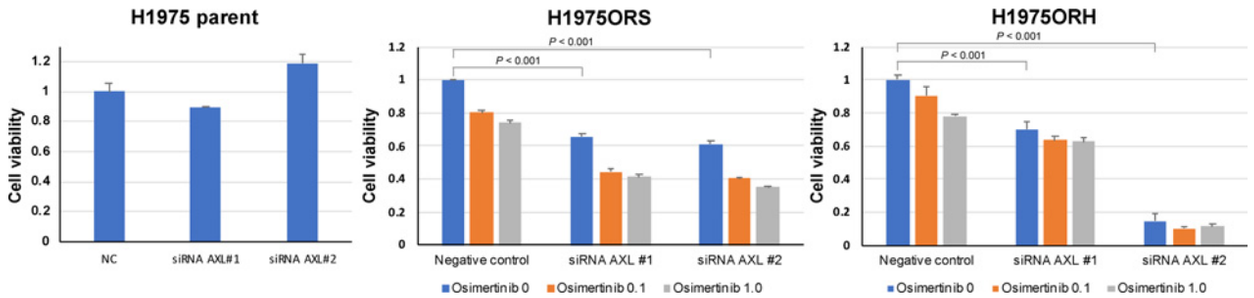

Figure 3.

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

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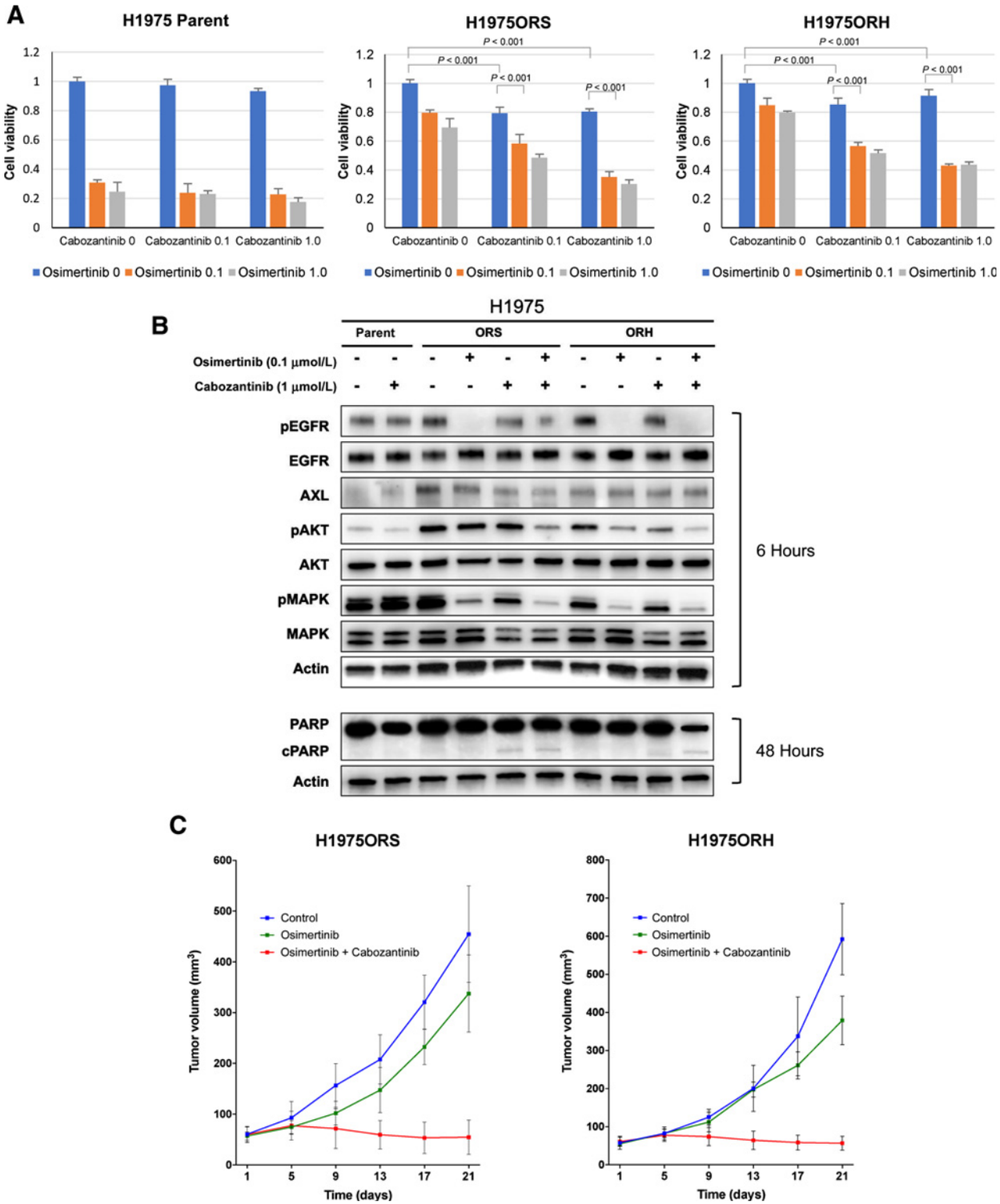

Figure 4.

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.

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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

Conception and design: K. Namba, K. Shien, S. Toyooka
Development of methodology: K. Namba, K. Shien, E. Kurihara
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Namba, Y. Takahashi, H. Torigoe, T. Takeda, E. Kurihara, Y. Ogoshi
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Namba, T. Takeda, S. Tomida, S. Toyooka
Writing, review, and/or revision of the manuscript: K. Namba, K. Shien, H. Sato, T. Yoshioka, H. Yamamoto, S. Toyooka
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Namba, S. Tomida
Study supervision: K. Namba, K. Shien, J. Soh

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