




Therapeutic strategies for afatinib-resistant lung cancer harboring *HER2* alterations

Hidejiro Torigoe^{1,2} | Kazuhiko Shien¹  | Tatsuaki Takeda³ | Takahiro Yoshioka² | Kei Namba¹ | Hiroki Sato¹ | Ken Suzawa¹ | Hiromasa Yamamoto¹  | Junichi Soh¹ | Masakiyo Sakaguchi⁴ | Shuta Tomida⁵ | Kazunori Tsukuda¹ | Shinichiro Miyoshi¹ | Shinichi Toyooka^{1,2} 

¹Departments of Thoracic, Breast and Endocrinological Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

²Clinical Genomic Medicine, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

³Clinical Pharmacy, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

⁴Cell Biology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

⁵Biobank, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

Correspondence

Kazuhiko Shien, Department of Thoracic, Breast and Endocrinological Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan.

Email: k.shien@okayama-u.ac.jp

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Human epidermal growth factor receptor 2 (*HER2*) plays an important role in the pathogenesis of various cancers. *HER2* alterations have been suggested to be a therapeutic target in non-small-cell lung cancer (NSCLC), just as in breast and gastric cancers. We previously reported that the pan-HER inhibitor afatinib could be a useful therapeutic agent as *HER2*-targeted therapy for patients with NSCLC harboring *HER2* alterations. However, acquired resistance to afatinib was observed in the clinical setting, similar to the case for other HER inhibitors. Thus, elucidation of the mechanisms underlying the development of acquired drug resistance and exploring means to overcome acquired drug resistance are important issues in the treatment of NSCLC. In this study, we experimentally established afatinib-resistant cell lines from NSCLC cell lines harboring *HER2* alterations, and investigated the mechanisms underlying the acquisition of drug resistance. The established cell lines showed several unique afatinib-resistance mechanisms, including *MET* amplification, loss of *HER2* amplification and gene expression, epithelial-to-mesenchymal transition (EMT) and acquisition of cancer stem cell (CSC)-like features. The afatinib-resistant cell lines showing *MET* amplification were sensitive to the combination of afatinib plus crizotinib (a *MET* inhibitor), both in vitro and in vivo. The resistant cell lines which showed EMT or had acquired CSC-like features remained sensitive to docetaxel, like the parental cells. These findings may provide clues to countering the resistance to afatinib in NSCLC patients with *HER2* alterations.

KEYWORDS

acquired resistance, afatinib, human epidermal growth factor receptor 2, lung cancer, non-small cell lung cancer

1 | INTRODUCTION

Recent developments in the genomic characterization of tumors have contributed to the development of novel therapeutic

approaches, and improved patient survival has been obtained with some molecular-targeted therapies developed on the basis of the genetic profiles of tumors. For example, EGFR tyrosine kinase inhibitors (TKI), such as gefitinib and erlotinib, have been shown to exhibit

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marked antitumor activity in patients with non-small cell lung cancer (NSCLC) carrying activating *EGFR* mutations.¹ Other oncogenic alterations, such as *ALK*, *KRAS*, *NRAS*, *BRAF*, *MET* and *FGFR*, have also been identified in some subsets of NSCLC patients as possible treatment targets.²⁻⁴

Human epidermal growth factor receptor 2 (HER2) is a member of the HER family, which is composed of 4 receptor tyrosine kinases (RTK). It is frequently overexpressed in various human cancers, and many preclinical studies have demonstrated that overexpression of HER2 or mutations of the *HER2* kinase domain play an important role in oncogenic transformation and tumorigenesis.⁵⁻⁸ In the case of breast and gastric cancers, targeted therapies in patients with HER2-positive tumors have been clinically proven to be effective.^{9,10} Among NSCLC patients, the reported frequency of HER2 overexpression and *HER2* amplification are 11%-32% and 2%-23%, respectively.¹¹⁻¹⁴ *HER2* mutations are identified in approximately 2%-4% of NSCLC and are usually mutually exclusive of other driver mutations.^{15,16} However, it remains to be established whether HER2-targeted therapy is of clinical benefit in patients with *HER2*-altered NSCLC. Previous clinical trials have failed to show any clinical benefit of trastuzumab monotherapy or any additional effect of trastuzumab over conventional first-line chemotherapy in HER2-positive NSCLC patients.¹⁷⁻¹⁹ However, subsets of patients with high HER2 expression levels, *HER2* gene amplification or *HER2* mutations have been demonstrated to show a good response to HER2-targeted treatment.^{17,20,21} In addition, a recent retrospective study showed that HER2-targeted therapy was beneficial in combination with chemotherapy for patients with *HER2*-mutant NSCLC.²² These findings suggest the potential usefulness of HER2-targeted therapy in *HER2*-altered NSCLC patients.

Afatinib, also known as BIBW2992, is an irreversible pan-HER (EGFR, HER2, HER3 and HER4) inhibitor, and has recently been approved for the treatment of NSCLC patients carrying *EGFR* mutations in the tumor. We previously reported that afatinib could inhibit cell growth in both *HER2*-amplified and *HER2*-mutant NSCLC cells, just as in *EGFR*-mutant NSCLC cells, through G1 arrest and apoptosis.²³ In clinical settings, afatinib monotherapy was effective in 3 out of 3 assessable patients with an *HER2*-mutated adenocarcinoma in an exploratory study of 5 patients.²¹ In addition, the recent retrospective studies showed the potential benefit of afatinib for patients with *HER2*-mutant lung cancers.^{22,24} While further prospective studies including randomized trials are required, afatinib could be approved as one of the therapeutic options for *HER2*-altered NSCLC patients.

However, development of acquired resistance to afatinib, just like the case for other HER inhibitors, has been reported in most *EGFR*-mutant NSCLC patients treated with afatinib,^{25,26} and the situation is considered to be similar for *HER2*-altered NSCLC patients. Therefore, overcoming acquired resistance to afatinib is a critical clinical issue.

In this study, we experimentally established various afatinib-resistant cell lines from *HER2*-altered NSCLC cell lines as the parental cell lines, and examined the molecular profiles of these cells to uncover the mechanisms of acquisition of the drug resistance, and investigated the treatment strategies for these cells with acquired resistance to afatinib.

2 | MATERIALS AND METHODS

2.1 | Reagents and cell cultures

Three NSCLC cell lines (Calu3, NCI-H2170 [H2170] and NCI-H1781 [H1781]), purchased from ATCC (Manassas, VA, USA), were used as the patent cell lines for this study. These cell lines were authenticated by ATCC using short-tandem-repeat polymorphism analysis, and used within 6 months of receipt. All the lung cancer cells were cultured in RPMI-1640 media supplemented with 10% FBS, in a humidified incubator with 5% CO₂ at 37°C. Afatinib-resistant sublines were established by different methods: the parental cells were cultured with stepwise escalation of the concentration of afatinib in the culture medium (Calu3 and H2170: from 5 nmol/L to 2 μmol/L, H1781: from 5 nmol/L to 0.4 μmol/L) over a period of 6 months (stepwise escalation method), or use of a high concentration of afatinib from the start of culture (Calu3 and H2170: 2 μmol/L, H1781: 0.4 μmol/L) until 6 months (high concentration method). Finally, afatinib-resistant sublines designated as H2170-ARS, Calu3-ARS and H1781-ARS were established by the stepwise escalation method, and those designated as H2170-ARH, Calu3-ARH and H1781-ARH were established by the high concentration method (Table 1). Acquired resistance was defined as an IC50 value of over a 100-fold higher than that for the parental cell line. Afatinib and docetaxel were purchased from Synkinase (San Diego, CA, USA) and Wako Pure Chemical Industries (Osaka, Japan), respectively. Crizotinib and elacridar were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Details of the phospho-receptor tyrosine kinase array and the cell proliferation assay are included in Document S1.

2.2 | DNA and RNA extraction

Genomic DNA were extracted from the cell lines using the DNeasy Blood and Tissue Kit (Qiagen, Venlo, the Netherlands), and total RNA were extracted using the RNeasy Mini Kit (Qiagen). Complementary DNA (cDNA) was synthesized from total RNA using a

TABLE 1 Afatinib-resistant cell lines and the resistance mechanisms

Cell lines	Afatinib exposure	<i>HER2</i> amp/mut	<i>MET</i> amp	EMT	CSC markers
Calu3	N/A	amp	-	-	-
Calu3-ARS	Stepwise	amp	+	-	-
Calu3-ARH	High	amp	-	+	-
H2170	N/A	amp	-	-	-
H2170-ARS	Stepwise	Loss of amp	-	-	-
H2170-ARH	High	Loss of amp	-	-	-
H1781	N/A	mut (G776VC)	-	-	-
H1781-ARS	Stepwise	mut (G776VC)	-	-	+
H1781-ARH	High	mut (G776VC)	-	-	+

amp, amplification; CSC, cancer stem cell; EMT, epithelial-to-mesenchymal transition; mut, mutation; N/A, not applicable.

High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA), in accordance with the manufacturer's instructions.

2.3 | Copy number analysis by quantitative real-time PCR

Copy number gains (CNG) of *MET*, *HER2* and *EGFR* were determined by quantitative real-time (qRT)-PCR using 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 in standard genomic DNA (Merck, Darmstadt, Germany). The catalog numbers of the TaqMan assays are shown in Table S1A. Based on the results of our previous study, we defined amplification as a value of greater than 4.^{27,28}

2.4 | Fluorescence in situ hybridization assay

A dual-color FISH assay was performed using the PathVysion HER-2 DNA Probe Kit (Abbott Molecular, Des Plaines, IL, USA), in accordance with the manufacturer's instructions. Twenty metaphase spreads and 200 interphase nuclei were analyzed in each slide.

2.5 | Direct sequencing

We determined the mutational status of the tyrosine kinase domain of *HER2* by direct sequencing; the PCR conditions employed are shown in Table S1B.

2.6 | Western blot analysis

The total cell lysate was extracted with lysis buffer, a mixture of RIPA buffer, phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich) and Complete Mini (Roche, Basel, Switzerland). Western blot analysis was carried out using the conventional method using the following primary antibodies: anti-EGFR, phospho- (p-) EGFR (Tyr1068), *HER2*, p-*HER2* (Tyr1221/1222), *MET*, p-*MET* (Tyr1234/1235), *AKT*, p-*AKT* (Ser473), p44/p42 *MAPK*, p-p44/p42 *MAPK*, E-cadherin, N-cadherin, vimentin, *ALDH1* (Cell Signaling Technology, Danvers, MA, USA) and b-actin (used as the loading control) (Merck Millipore, Billerica, MA, USA). The secondary antibody was HRP-conjugated anti-mouse or anti-rabbit IgG (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).

2.7 | mRNA expression analysis by quantitative reverse-transcription PCR

The gene expressions of the putative cancer stem cell (CSC) markers *ALDH1A1*, *ABCB1*, *CD44*, *Oct-4* and *CD133* were analyzed by

qRT-PCR using the cDNA, TaqMan Gene Expression Assays, and the ABI StepOnePlus Real-Time PCR Instrument (Thermo Fisher Scientific). The mRNA expression was calculated using the delta-delta-CT method. The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used as the endogenous control. The catalog numbers of the TaqMan assays are shown in Table S1C.

2.8 | Cell growth inhibition assay

Cells were cultured with or without afatinib for 72 hours and the sensitivity to the drug was determined by a modified MTS assay using CellTiter 96 Aqueous One Solution Reagent (Promega, Fitchburg, WI, USA), as previously described.²⁸ The antiproliferative effect of each drug was expressed in terms of the IC50 value, which refers to the concentration of the drug required to inhibit cell proliferation by 50%.

2.9 | Xenograft model

Six-week-old BALB/c-nu/nu female mice were purchased from Charles River Laboratories (Yokohama, Japan). All mice were provided with sterilized food and water, and housed in a barrier facility under a 12:12-h light: dark cycle. The Calu3-ARS cells (5×10^6) were suspended in 100 μ L of RPMI-1640 medium with the Matrigel Basement Membrane Matrix (Corning, Corning, NY, USA) and injected subcutaneously into the backs of the mice. The volumes of the tumors on the backs of the mice were calculated using the empirical formula $V = \frac{1}{2} \times [(\text{shortest tumor diameter})^2 \times (\text{longest tumor diameter})^2]$. One week after the injection, the mice were randomly allocated to 4 groups that received vehicle, 20 mg/kg/d of afatinib, 10 mg/kg/d of crizotinib, or 20 mg/kg/d of afatinib plus 10 mg/kg/d of crizotinib. The drug solutions were prepared in 0.5 w/v (%) methyl cellulose. The vehicle/drugs were given orally as a suspension by gavage once daily, on 6 days per week. The tumor volumes were measured 3 times a week with calipers. After 3 weeks of treatment, the mice were killed, and the tumors were harvested and photographed.

All the animal experiments were carried out in accordance with protocols approved by the Animal Care and Use Committee, Okayama University (permission number: OKU-2017353).

2.10 | Statistical analysis

All data were analyzed using the JMP v 9.0.0 software (SAS Institute, Cary, NC, USA). Differences among groups were compared by *t* test. $P < .05$ was considered as denoting statistical significance. All tests were 2-sided.

3 | RESULTS

3.1 | Genotypic mechanisms underlying the development of acquired resistance to afatinib

We established 6 afatinib-resistant cell lines from the 3 parental NSCLC cell lines harboring *HER2* alterations, including 2

HER2-amplified cell lines (Calu3 and H2170) and 1 *HER2*-mutant cell line (H1781). To investigate the genotypic changes in the cells with acquired resistance to afatinib, we examined the copy number of *HER2*, *EGFR* and *MET*, gain of which is well known as a mechanism underlying the resistance to HER inhibitors.²⁹ Significant copy number gains of *MET* were detected in the Calu3-ARS cells (Figure 1A). In addition, the *HER2* copy number was almost lost in the H2170-ARS and H2170-ARH cells. Copy number analysis also revealed progressive decrease of the *HER2* copy number of H2170-ARS cells with increasing number of passages, eventually leading to the disappearance of the amplified *HER2* alleles (Figure 1B). To investigate the mechanism underlying the progressive decrease of the copy number of *HER2*, we performed the FISH assay and found that all of H2170 parental cells harbored amplified *HER2* alleles (Figure 1C). Furthermore, the degree of amplification was less in all H2170-derived resistant subline cells than in H2170 parental cells. These findings suggested that the decrease in *HER2* alleles occurred at the individual cell level. Next, we investigated the mutational status of the tyrosine kinase domain of the *HER2* gene; this investigation, however, revealed no additional mutations in any of the afatinib-resistant cell lines (Table 1). We also examined the expression levels of *HER2*, *EGFR* and *MET* protein and the phosphorylation levels of these proteins by western blotting (Figure 1D). Consistent with the results of the copy number analysis, the expressions of p-MET and MET were upregulated in the Calu3-ARS cells as compared to the corresponding parental cells. In the H2170-ARS and H2170-ARH cells, the expressions of p-*HER2* and *HER2* were almost completely lost. In addition, downstream signals, such as p-AKT and p-MAPK activation, in the H2170-ARS and H2170-ARH cells were much weaker as compared to those in the corresponding parental cells. Furthermore, the phospho-RTK array revealed that the activation of almost all the RTK was downregulated in the afatinib-resistant cells as compared to the corresponding parental cells (Figure S1A), whereas the proliferative capability of the cells was maintained at a level similar to that of the corresponding parental cells (Figure S1B).

3.2 | Phenotypic changes in the cells with acquired resistance to afatinib

To investigate the phenotypic changes following the development of acquired resistance to afatinib, we comparatively examined the expression levels of an epithelial marker (E-cadherin) and mesenchymal marker (N-cadherin and vimentin) in the resistant and corresponding parental cells. Western blot analysis revealed downregulation of E-cadherin and upregulation of N-cadherin and vimentin in the Calu3-ARH cells (Figure 2A); furthermore, the resistant cells had acquired a spindle-like shape in contrast to the morphology of the corresponding parental Calu3 cells (Figure 2B). These findings suggest the occurrence of epithelial-to-mesenchymal transition (EMT) in the Calu3-ARH, which was considered as being the mechanism underlying the resistance to afatinib.

Next, we investigated the expression levels of *ALDH1A1*, *ABCB1*, *CD44*, *Oct-4* and *CD133*, which are putative CSC markers in NSCLC

(Figure 2C and Figure S2A). *ALDH1A1* was found to be overexpressed in the H1781-ARS and H1781-ARH cells as compared to the parental H1781 cells. Western blot analysis also revealed overexpression of *ALDH1A1* in the H1781-ARS and H1781-ARH cells (Figure 2A). These cell lines with acquired resistance of afatinib were non-adhesive and capable of forming sphere-like clusters (Figure 2B). We conducted a sphere formation assay to examine the cellular functional features of CSC-like properties,²⁸ and found that H1781-ARS and H1781-ARH cells acquired higher ability to form spheres in suspension culture compared with parental H1781 cells (Figure S2B). To examine the CSC-like properties in vivo, we conducted tumor transplantation experiments.^{28,30,31} We subcutaneously implanted H1781, H1781-ARS and H1781-ARH cells to examine the tumor-forming capability in NOD/SCID mice. We found that H1781-ARS and H1781-ARH cells exhibited higher tumorigenicity than parental H1781 cells. The H1781-ARS and H1781-ARH cells established larger tumors with shorter latencies than the parental H1781 cells (Figure S2C). These findings suggest that the H1781-ARS and H1781-ARH cells had acquired CSC-like properties, which could be considered as the mechanism underlying the resistance to afatinib. *ABCB1* was overexpressed in the H2170-ARS and H2170-ARH cells, but not in either the H1781-ARS or H1781-ARH cells (Figure 2C). *CD44*, *Oct-4* and *CD133* overexpression was not detected in any of the resistant cell lines.

3.3 | Drug sensitivities of the afatinib-resistant cells showing MET amplification

The sensitivities of the cells to various drugs are shown in Table 2. The cell growth inhibition assay showed that the Calu3-ARS cells showing *MET* amplification were insensitive to monotherapy with crizotinib, which is a *MET* inhibitor, but sensitive to combined treatment with afatinib plus crizotinib (Figure 3A). Western blot analysis revealed that afatinib inhibited the expression of p-*HER2* and crizotinib inhibited the expression of p-*MET* in both the parental Calu3 and Calu3-ARS cells. Both *HER2* and *MET* were activated in the Calu3-ARS cells; therefore, crizotinib monotherapy was not effective for inhibiting their downstream signals (Figure 3B).

On the basis of our in vitro data, we investigated the antitumor effect of crizotinib in a xenograft mouse model of afatinib-resistant cells showing *MET* amplification. As shown in Figure 3C,D, combined treatment with afatinib plus crizotinib significantly inhibited the tumor growth of the Calu3-ARS xenografts as compared to vehicle control or either afatinib or crizotinib alone ($P < .01$).

3.4 | Sensitivity of the afatinib-resistant cells to chemotherapeutic agents

The H2170-ARS and H2170-ARH cells, which had lost *HER2* amplification, also acquired resistance to docetaxel (Table 2). To investigate whether their resistance mechanism was associated with overexpression of ATP-binding Cassette sub-family B Member 1 (*ABCB1*), which is well known to induce resistance to chemotherapy, we

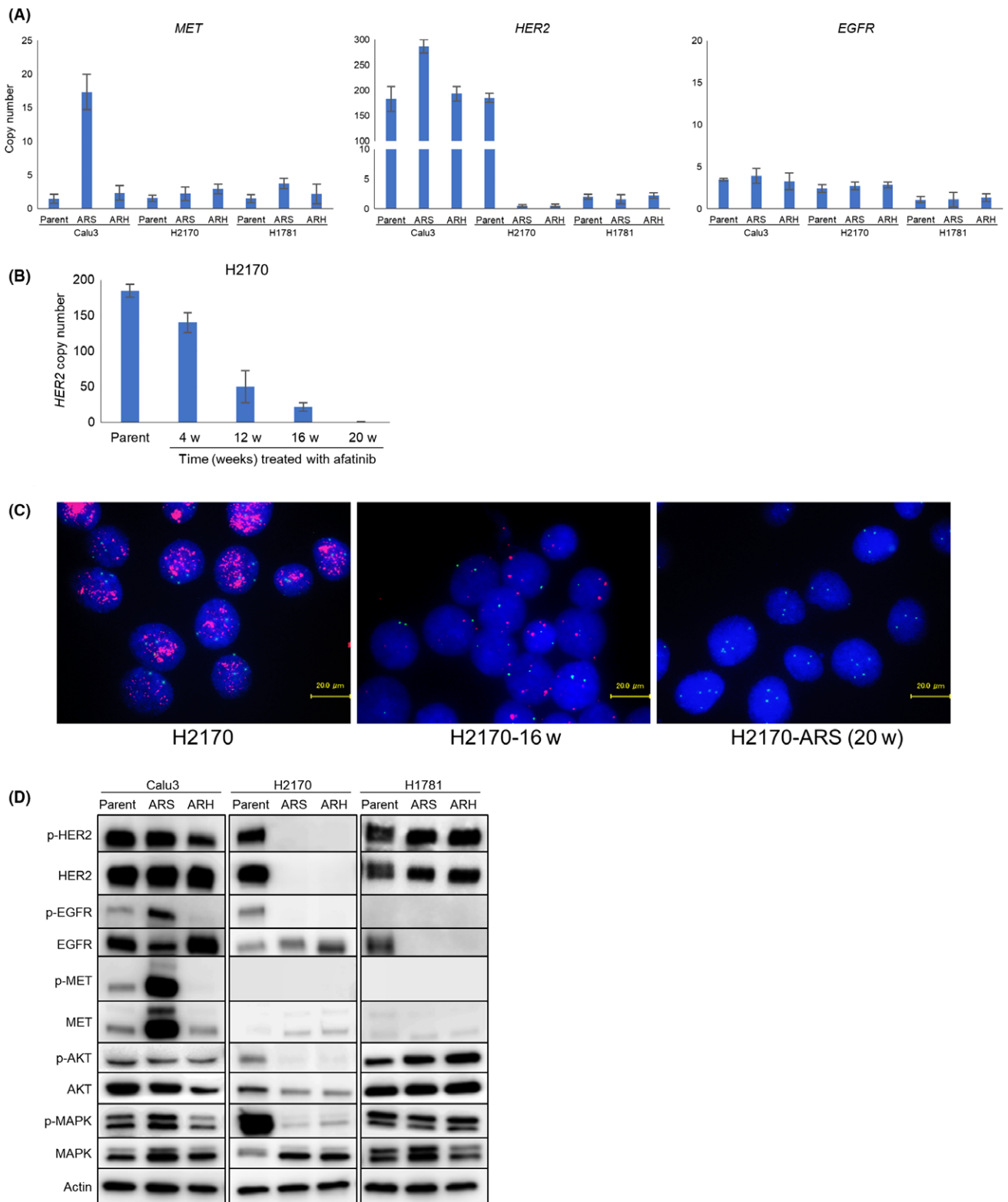


FIGURE 1 Genotypic analysis of the lung cancer cell lines harboring *HER2* alterations and their afatinib-resistant sublines. A, The copy numbers of *MET*, *HER2* and *EGFR* were determined by a quantitative reverse-transcription PCR assay. The *MET* copy number was significantly amplified in the Calu3-ARS cells. The *HER2* copy number was almost lost in the H2170-ARS and H2170-ARH cells. B, Copy number analysis using the H2170-derived resistant subline showed progressive decrease of the *HER2* copy number with an increasing number of passages, with eventual disappearance of amplified *HER2* from the cells. w, weeks. C, FISH assay (red, *HER2*; green, CEP 17; blue, nucleus) showed that the progressive decrease of the *HER2* copy number occurred at the individual cell level. H2170-16w, H2170 cells treated with afatinib for 16 weeks. D, Western blot analysis showed that the expressions of p-MET and MET were upregulated in the Calu3-ARS cells, while the p-HER2 and HER2 expressions were almost lost in the H2170-ARS and H2170-ARH cells

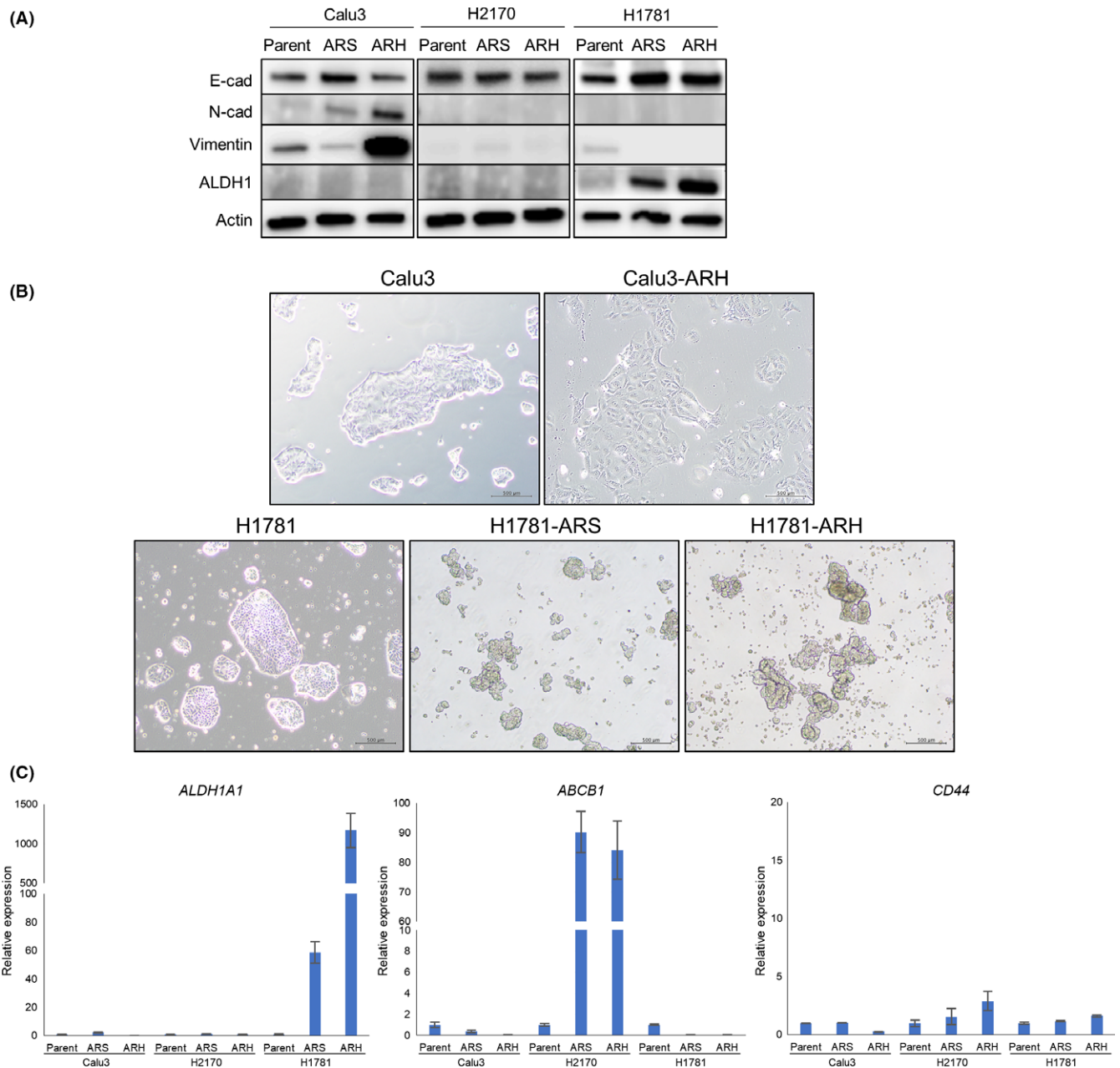


FIGURE 2 Phenotypic analysis of lung cancer cell lines harboring *HER2* alterations and their afatinib-resistant sublines. A, Western blot analysis showed that the Calu3-ARH cells exhibited downregulation of E-cadherin and upregulation of N-cadherin and vimentin, while the H1781-ARS and H1781-ARH cells exhibited upregulation of ALDH1. B, The images of the cells show that the Calu3-ARH cells exhibited a spindle-like shape. The H1781-ARS and H1781-ARH cells were non-adhesive and capable of forming sphere-like clusters. C, mRNA expressions of the putative cancer stem cell markers *ALDH1A1*, *ABCB1* and *CD44* were examined by quantitative reverse-transcription PCR. *ALDH1A1* was overexpressed in the H1781-ARS and H1781-ARH cells, while *ABCB1* was overexpressed in the H2170-ARS and H2170-ARH cells

conducted the cell growth inhibition assay with or without ABCB1 inhibitor elacridar (Figure S3A,B). We found that elacridar recovered the sensitivity to docetaxel, but not to afatinib in H2170-ARS and H2170-ARH cells. These findings were similar to those in our previous report using docetaxel-resistant NSCLC cells with *EGFR*-mutations.³² In contrast, the other afatinib-resistant cell lines, including Calu3-ARH, H1781-ARS and H1781-ARH, which showed EMT or had acquired CSC-like features, showed maintained sensitivity to

docetaxel, because ABCB1 was not upregulated in these acquired resistance cell lines.

4 | DISCUSSION

In this study, we established multiple cell lines that exhibited acquired resistance to the irreversible pan-HER inhibitor afatinib,

TABLE 2 IC50 values ($\mu\text{mol/L}$) against various agents in *HER2*-altered NSCLC cell lines

Cell lines	MET inhibitor			Docetaxel
	Afatinib	Crizotinib	Afatinib plus crizotinib (0.2 $\mu\text{mol/L}$)	
Calu3	0.0029	4	0.033	0.0018
Calu3-ARS	>10 ^a	1.2	0.0088	0.00058
Calu3-ARH	1.8 ^a	3.2	2.2 ^a	0.00013
H2170	0.019	N/A	N/A	0.000048
H2170-ARS	3.8 ^a	N/A	N/A	0.015 ^a
H2170-ARH	2.6 ^a	N/A	N/A	0.015 ^a
H1781	0.0075	N/A	N/A	0.0011
H1781-ARS	0.82 ^a	N/A	N/A	0.0032
H1781-ARH	1.1 ^a	N/A	N/A	0.0029

^aThe IC50 value in each resistant cell line to that in the parental cell line is higher than 5-fold. N/A, not applicable; NSCLC, non-small-cell lung cancer.

from 3 parental *HER2*-altered NSCLC cell lines, and examined the mechanisms underlying the development of acquired resistance to afatinib. The resistance mechanisms varied, and included *MET* amplification and overexpression, loss of *HER2* amplification and expression, EMT and acquisition of CSC-like features. From our previous study using *EGFR*-mutant NSCLC cells, we reported that the method of exposure of cell cultures to drugs influences the mechanisms of acquired resistance to HER inhibitors (gefitinib and afatinib).^{28,33} Consistent with that report, in this study also, conducted using *HER2*-altered NSCLC cells, the resistance mechanisms were affected by the manner of drug exposure. The resistance mechanism in the Calu3-ARS cells established by the stepwise escalation method appeared to be *MET* amplification, whereas that in the Calu3-ARH cells established by the high drug concentration method was considered to be possibly attributable to the acquisition of EMT features.

MET amplification is one of the major mechanisms underlying the acquisition of resistance to HER inhibitors in *EGFR*-mutant NSCLC.^{28,29,33,34} *MET* amplification has also been reported to contribute to trastuzumab resistance in *HER2*-positive breast cancer cells.³⁵ In this study, we identified *MET* amplification as one of the mechanisms underlying the acquisition of resistance of *HER2*-altered NSCLC to the pan-HER inhibitor afatinib. Importantly, we clarified that combined treatment with afatinib plus crizotinib, a *MET* inhibitor, was highly effective against *HER2*-altered NSCLC showing acquired resistance to afatinib, both in vitro and in vivo. These results suggest the importance of reassessment of tumor cells after the acquisition of resistance to afatinib; if such reassessment in clinical cases reveals *MET* amplification, then combined treatment with afatinib plus crizotinib should be considered.

In afatinib-resistant cell lines established from H2170 cells showing *HER2* amplification, the copy number of *HER2* decreased gradually during the course of exposure to afatinib. We previously

reported loss of the *EGFR*-mutant allele and progressive decrease of the *EGFR* copy number in an *EGFR*-mutant NSCLC cell line during gefitinib exposure.²⁸ However, to the best of our knowledge, loss of *HER2* amplification during afatinib treatment has never been reported before in in vitro examinations. However, loss of *HER2* amplification has sometimes been reported after trastuzumab treatment in clinical *HER2*-positive breast or gastric cancer patients.³⁶⁻³⁹ Our finding seems to be consistent with these latter clinical reports. Loss of *HER2* amplification in the residual disease following neoadjuvant trastuzumab-based therapy has been reported to be associated with a poorer relapse-free survival when compared with maintained *HER2* expression.⁴⁰ The biological basis of these genetic alterations has not been clarified yet, and might include tumor heterogeneity, genetic drift toward a more aggressive tumor phenotype, or selective pressure of adjuvant therapy.³⁸ Our in vitro experiments revealed that the decrease in *HER2* alleles occurred at the individual cell level, suggesting that the tumor heterogeneity could not be the reason for acquired afatinib resistance.

The characteristics of the afatinib-resistant cell lines with loss of *HER2* amplification were very interesting. In these resistant cells, the activation of not only *HER2* but also other RTK, and their downstream signals were much lower than those in the corresponding parental cell lines, although their proliferative ability was maintained. However, we have yet to clarify the resistance mechanisms in detail, and continued research into the resistance mechanisms and investigation of the appropriate treatment strategy for afatinib-resistant tumors with loss of *HER2* amplification is necessary.

As for the other afatinib-resistant cell lines, Calu3-ARH showed the features of EMT as the possible mechanism underlying afatinib resistance, and the afatinib-resistant H1781-ARS and H1781-ARH cells had acquired CSC-like properties. Although EMT and acquisition of CSC-like features are well known as mechanisms underlying the acquisition of resistance to various drugs, this is the first report of their occurrence during the course of exposure to afatinib in *HER2*-altered NSCLC cells. Interestingly, the Calu3-ARS, H1781-ARS and H1781-ARH showing EMT or CSC-like features remained sensitive to docetaxel, whereas we previously reported that *EGFR*-mutant NSCLC cells with acquired resistance to *EGFR*-TKI caused by EMT or acquisition of CSC-like features exhibited resistance to docetaxel.^{28,33} None of the Calu3-ARS, H1781-ARS or H1781-ARH cells showed upregulation of ABCB1, which was considered as the reason for the afatinib-resistant cells' maintained sensitivity to docetaxel. Overexpression of ABCB1 has been reported to be strongly associated with EMT or the acquisition of CSC-like features;⁴¹ therefore, the characteristics of the Calu3-ARH, H1781-ARS and H1781-ARH may differ somewhat from those of *EGFR*-TKI-resistant cells showing EMT or CSC-like features reported previously.^{28,33} Moreover, the precise molecular mechanisms underlying how EMT or CSC-like features lead to afatinib resistance remain unclear, and thorough investigation using resistance models established by us could yield clues to uncover these mechanisms.

In conclusion, we established 6 cell lines with acquired resistance to afatinib from 3 parental *HER2*-altered NSCLC cell lines.

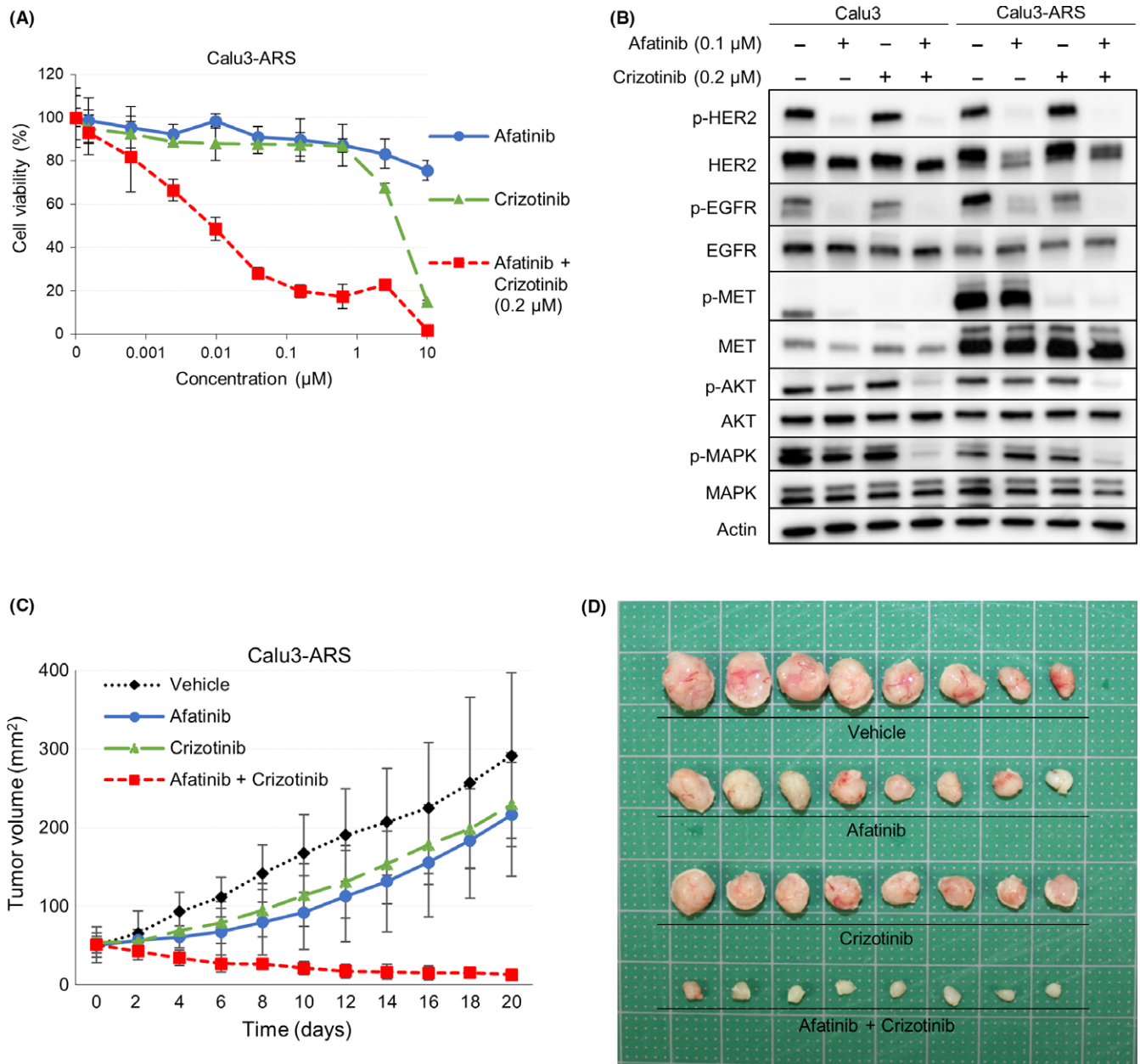


FIGURE 3 The combination of afatinib plus crizotinib (a MET inhibitor) was effective against the afatinib-resistant cell line showing *MET* amplification. A, Cell growth inhibition assay revealed that the combination of afatinib plus crizotinib was highly effective against the Calu3-ARS cells showing *MET* amplification. B, Western blot analysis showed that afatinib inhibited the expression of p-HER2 and crizotinib inhibited the expression of p-MET in both the parental Calu3 and Calu3-ARS cells. C, Mice with Calu3-ARS tumors were given vehicle, afatinib, crizotinib, or afatinib plus crizotinib. The tumor volumes were determined on the indicated days after the start of treatment. Data represent the mean \pm SE ($n = 8$). * $P < .05$ vs vehicle control by the t test. D, Appearance of the Calu3-ARS tumors after treatment at the time of sacrifice of the mice

The mechanisms of afatinib resistance in the established cell lines varied, and included *MET* amplification, loss of *HER2* amplification, and EMT or acquisition of CSC-like features, and the cells showed specific sensitivity patterns to various drugs, such as crizotinib and docetaxel. These findings underscore the importance of reassessment of cancer tumors after treatment and personalized medicine choices made based on such reassessment for cancer treatment.

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CONFLICT OF INTEREST

There are no potential conflicts of interest to declare.

ORCID

Kazuhiko Shien  <http://orcid.org/0000-0002-4959-4220>

Hiromasu Yamamoto  <http://orcid.org/0000-0002-5330-5460>

Shinichi Toyooka  <http://orcid.org/0000-0002-7588-6745>

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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