



Acquired resistance mechanisms to afatinib in *HER2*-amplified gastric cancer cells

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Abstract

Cancer treatment, especially that for breast and lung cancer, has entered a new era and continues to evolve, with the development of genome analysis technology and the advent of molecular targeted drugs including tyrosine kinase inhibitors. Nevertheless, acquired drug resistance to molecular targeted drugs is unavoidable, creating a clinically challenging problem. We recently reported the antitumor effect of a pan-HER inhibitor, afatinib, against human epidermal growth factor receptor 2 (*HER2*)-amplified gastric cancer cells. The purpose of the present study was to identify the mechanisms of acquired afatinib resistance and to investigate the treatment strategies for *HER2*-amplified gastric cancer cells. Two afatinib-resistant gastric cancer cell lines were established from 2 *HER2*-amplified cell lines, N87 and SNU216. Subsequently, we investigated the molecular profiles of resistant cells. The activation of the *HER2* pathway was downregulated in N87-derived resistant cells, whereas it was upregulated in SNU216-derived resistant cells. In the N87-derived cell line, both *MET* and *AXL* were activated, and combination treatment with afatinib and cabozantinib, a multikinase inhibitor that inhibits *MET* and *AXL*, suppressed the cell growth of cells with acquired resistance both in vitro and in vivo. In the SNU216-derived cell line, *YES1*, which is a member of the *Src* family, was remarkably activated, and dasatinib, a *Src* inhibitor, exerted a strong antitumor effect in these cells. In conclusion, we identified *MET* and *AXL* activation in addition to *YES1* activation as novel mechanisms of afatinib resistance in *HER2*-driven gastric cancer. Our results also indicated that treatment strategies targeting individual mechanisms of resistance are key to overcoming such resistance.

KEYWORDS

afatinib, gastric cancer, *HER2*, *MET*, *YES1*

1 | INTRODUCTION

Thanks to the dramatic development of genome analysis technology, including next-generation sequencing, cancer treatment has advanced to a new stage. The development of molecularly targeted drugs has enabled us to perform precision medicine. However, molecular targeted therapy for gastric cancer has lagged behind that for lung cancer and breast cancer, despite gastric cancer being a leading cause of death from cancer worldwide.¹

Human epidermal growth factor receptor 2 (HER2) is one of the few therapeutic target genes for gastric cancer, and trastuzumab is the only anti-HER2 drug with established clinical evidence for this cancer type² at present. Lapatinib is the only HER2-targeting small molecular drug for which phase III clinical trials have been performed. Neither the TyTAN trial nor the LOGiC trial demonstrated the superiority of lapatinib against HER2-amplified gastric cancer.^{3,4} Afatinib, or BIBW2992, is a small molecular drug that binds to the kinase domain of epidermal growth factor receptor (EGFR), HER2 and HER4, and is known as a pan-HER inhibitor. Previously, we reported the antitumor effect of afatinib in HER2-amplified gastric cancer.⁵ Although both trastuzumab and afatinib are HER2-targeted drugs, trastuzumab is a humanized IgG1 monoclonal antibody⁶ and afatinib is a receptor tyrosine kinase (RTK).⁷ Because the mechanisms of these 2 drugs are totally different, afatinib could be another treatment option for gastric cancer. However, it is a well-known fact that the emergence of resistant cancer cells during the course of molecular targeted therapy is a limitation of molecularly targeted drugs. Acquired resistance to trastuzumab in gastric cancer has already been reported,⁸ and drug resistance is also anticipated to be a problem for afatinib. In the present study, we established 2 afatinib-resistant gastric cancer cell lines and analyzed the mechanisms of acquired resistance. In addition, we investigated treatment strategies for these afatinib-resistant cell lines so as to contribute to the development of precision medicine for gastric cancer patients.

2 | MATERIALS AND METHODS

2.1 | Cell lines and reagents

Two gastric cancer cell lines, NCI-N87 (N87) and SNU216, were used in the present study. N87 was purchased from ATCC, and SNU216 was obtained from the Korean Cell Line Bank. Both cell lines were cultured in RPMI 1640 media supplemented with 10% FBS. Afatinib, crizotinib and cabozantinib were purchased from Synkinase (San Diego, CA, USA), Sigma-Aldrich (St. Louis, MO, USA), and ChemScene (Monmouth Junction, NJ, USA), respectively. Dasatinib was purchased from Bristol-Myers Squibb (New York, NY, USA).

2.2 | DNA and RNA extraction

Genomic DNAs of the cell lines were extracted using a DNeasy Blood and Tissue Kit. As for the RNAs, extraction was conducted

using an RNeasy Mini Kit (Qiagen, Venlo, the Netherlands), and the RNAs were then reversed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, San Jose, CA, USA). These processes were performed according to the manufacturer's instructions.

2.3 | Copy number and gene expression assay

To determine the copy number variation or the gene expression of various genes, we performed quantitative real-time PCR (qPCR) (StepOnePlus real-time PCR system, Applied Biosystems, Waltham, MA, USA) using the $\Delta\Delta\text{CT}$ method and a Taqman copy number assay and Taqman gene expression assay (Thermo Fisher Scientific), respectively. Both the copy number variation and gene expression analyses were performed in triplicate. We defined the copy number calculated in control human genomic DNA (Promega, Fitchburg, WI, USA) as 2 and amplification as values of greater than 4, in accordance with the protocol of our previous study^{9,10}. As for the relative gene expression, the expression level of each parent cell line was defined as 1.

2.4 | Direct sequencing assay

A direct sequencing assay was performed to identify acquired oncogenic mutations in HER2 or PIK3CA in afatinib-resistant cell lines. Specifically, exons 17 to 24, which include the coding sequence of the transmembrane domain and the kinase domain of HER2, and exons 9 and 20 of PIK3CA were analyzed in the direct sequencing assay. The sequences of the primers used in this study were the same as those previously reported.¹¹⁻¹³

2.5 | Western blot analysis

Total cell lysate extraction and western blotting were performed using a previously described protocol.^{14,15} The extracted protein was quantitated using a protein assay (Bio-Rad Laboratories) and was transferred to a membrane using a transfer system (Bio-Rad Laboratories, Hercules, CA, USA). Then, the membranes were probed with primary antibodies diluted with 5% BSA overnight at 4°C. After washing more than 3 times using PBS-tween, the membranes were incubated with the secondary antibodies for 1 hour at 25°C. To detect specific signals, we examined the membrane using the ECL Prime Western Blotting Detection System (GE Healthcare) and LAS-3000 (Fuji Film). The primary antibodies were as follows: p-HER2 (Tyr1221/1222), HER2, p-EGFR (Tyr1068), EGFR, p-MAPK (Erk1/2) (Thr202/Tyr204), MAPK (Erk1/2), p-AKT (Ser473), AKT, IGF-I receptor (IGF-1R), p-IGF-I receptor (phospho-IGF-1R) (Tyr1135/1136), MET, p-MET (Tyr1234/1235), YES, Src, p-Src (Tyr416), ALDH1A1, ABCB1, E-cadherin, vimentin and p-AXL (Tyr702) (Cell Signaling Technology, Danvers, MA, USA), AXL (R&D Systems, Minneapolis, MN, USA), and actin (Santa Cruz Biotechnology, Dallas, TX, USA). The secondary antibodies used in this study were as follows: goat anti-mouse IgG-HRP, goat anti-rat IgG-HRP and donkey anti-goat IgG-HRP (Santa Cruz Biotechnology).

2.6 | Phospho-receptor tyrosine kinase array

We evaluated the phosphorylation of 42 distinct RTK in the total cell lysate using a Human Phospho-Receptor Tyrosine Kinase (RTK) Array Kit (R&D Systems). The total cell lysates were extracted in the same manner as for western blotting, and the phosphorylation of the RTK was analyzed according to the manufacturer's instructions.

2.7 | Cell viability assay and combination index

The antitumor effects of the drugs were determined using an MTS assay. The IC_{50} value, which is the drug concentration used to inhibit the cell proliferation by 50%, was calculated based on the results of the MTS assay. In the MTS assay, the cells were equally seeded on a 96-well plate (2000 cells/well), and drug dilutions were added 12 hours later. The concentrations of the drugs ranged from 0.16 nmol/L to 10 μ mol/L. After 3 days of medication, the cell proliferation was determined using CellTiter 96 AQueous bromide One Solution Reagent (Promega). In cases where 2 drugs were administered simultaneously as a combination therapy, we used the same concentration for both drugs. The synergism of the 2 drugs was calculated as a combination index (CI) based on the result of the MTS assay using Calcsyn software (Biosoft, Cambridge, UK). Combination therapy effects of $CI < 1$, $CI = 1$ and $CI > 1$ were defined as synergistic effects, additive effects and antagonistic effects, respectively.

2.8 | Statistical analysis

The statistical analysis was conducted using EZR version 1.35 (Jichi Medical University, Saitama, Japan) and the R Commander Graphical User Interface (The R Foundation for Statistical Computing, Vienna, Austria).¹⁶ Differences between 2 groups were evaluated using a *t* test, and differences with a 2-tailed *P*-value less than 0.05 were considered statistically significant.

2.9 | Xenograft mouse model

For the animal experimental procedures, all the mice were handled in accordance with the Policy on the Care and Use of Laboratory Animals, Okayama University. Six-week old mice were purchased from CLEA Japan (Tokyo, Japan). N87-AR (N87-derived afatinib-resistant cell line) and SNU216-AR (SNU216-derived afatinib-resistant cell line) cells (approximately 1.0×10^6) were suspended in 50 μ L of RPMI 1640 and 50 μ L of Corning Matrigel Basement Membrane Matrix (Corning, NY, USA), then subcutaneously transplanted at 2 locations on the backside of each mouse. Because the tumor growth

of SNU216-AR was extremely slow, we continued the experiment using N87-AR only. The experimental mice were randomly divided into 3 groups: an afatinib group (20 mg/kg/day), a combination therapy group (afatinib, 20 mg/kg/day and cabozantinib, 100 mg/kg/day) and a control group (placebo). The drugs were diluted in 0.5 w/v (%) methyl cellulose (Wako Pure Chemical Industries) and were orally administered 5 times a week for 3 weeks. We started drug administration after confirming that the tumor had begun to increase by palpation and tumor diameter measurement. In the control group, mice were treated with 100 μ L of methyl cellulose only, 5 times per week. The tumor volume was measured using calipers and was calculated using the following empirical formula: Volume (mm^3) = length (longest diameter) \times width (shortest diameter)² \times 0.5.

3 | RESULTS

3.1 | Establishment of afatinib-resistant gastric cancer cell lines

Two gastric cancer cell lines, N87 and SNU216, were exposed to a high concentration of afatinib (2 μ mol/L) intermittently for more than 6 months, and afatinib-resistant cell lines (N87-AR and SNU216-AR) were established from each parent cell line. Both parent cell lines are *HER2*-amplified and highly sensitive to afatinib. The drug sensitivities were evaluated using the IC_{50} value calculated from the result of the MTS assay to confirm the successful establishment of resistant cells (Table 1).

3.2 | Gene alterations and activation of *HER2* in afatinib-resistant cell lines

Initially, we compared the gene alterations, expression, and activation of *HER2* in afatinib-resistant cells with those of the parent cells. The copy number assay showed that both resistant cells maintained the amplification of *HER2* (Figure 1A), but the gene expression of *HER2* was significantly downregulated compared with that in the parent cells (Figure 1B). Likewise, western blotting demonstrated the downregulation of total *HER2* in both afatinib-resistant cell lines. In contrast, there was a clear difference in the activation of *HER2* and down-signal pathway molecules between the 2 resistant cell types: phosphorylated *HER2* (p-*HER2*) and signals via *HER2*, p-ERK and p-AKT were downregulated in N87-AR, while p-*HER2* was upregulated in SNU216-AR, and this cell type maintained the activation of AKT and ERK (Figure 1C). No oncogenic mutations were detected in the transmembrane and kinase domains of *HER2* using direct sequencing.

TABLE 1 IC_{50} value for afatinib and *HER2* alteration in parental and afatinib-resistant cell lines

Cell line	Characteristics	<i>HER2</i> amplification	IC_{50} (nmol/L)
N87	Parental cell line	Yes	3.1
N87-AR	Afatinib resistant cell line	Yes	1089.6
SNU216	Parental cell line	Yes	29.0
SNU216-AR	Afatinib resistant cell line	Yes	4431.7

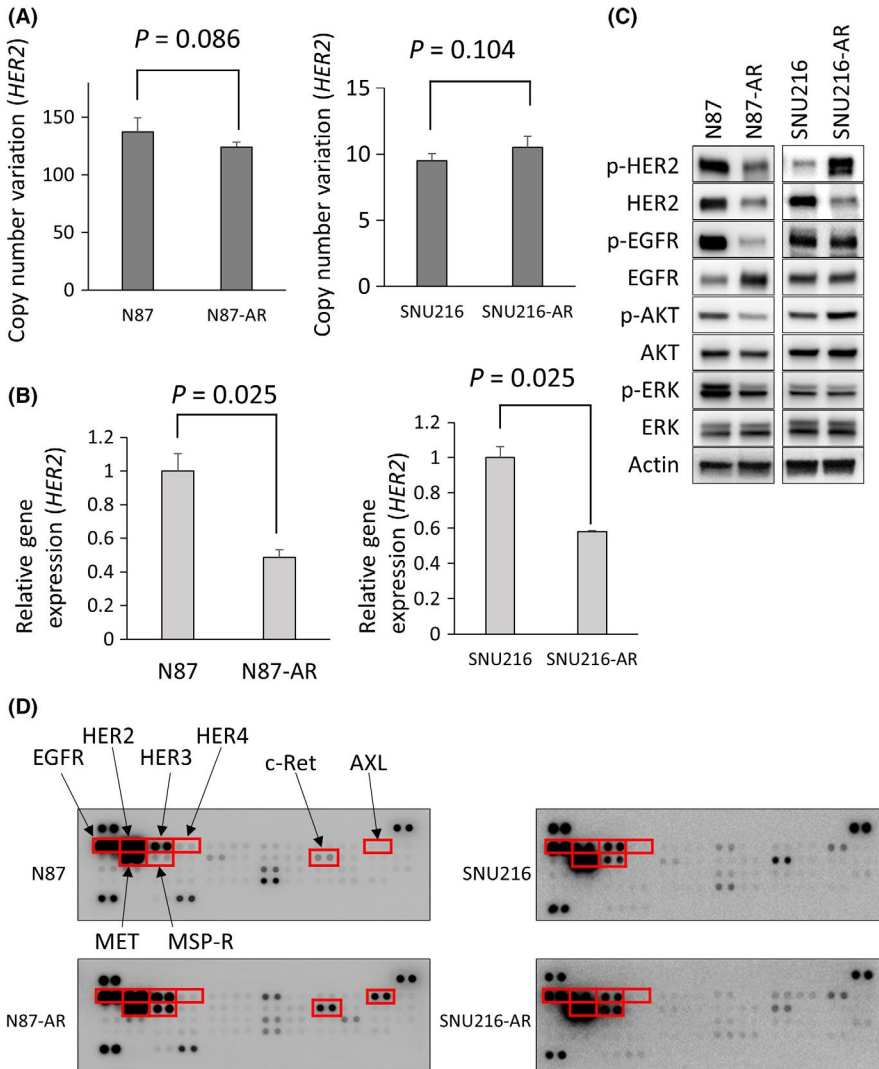


FIGURE 1 Difference in *HER2* status and activation of RTK between parental and afatinib-resistant cells. A, Copy number variation and gene expression of *HER2* in parental and afatinib-resistant cell lines. Both resistant cell lines, N87-AR and SNU216-AR, continued to exhibit *HER2* amplification. B, Relative gene expression of *HER2* in parental and afatinib-resistant cell lines. Gene expression was significantly downregulated in both afatinib-resistant cell lines. C, Activation of *HER2* and down-signal pathway molecules evaluated using western blotting. *HER2* and downstream molecules were downregulated in N87-AR, and p-*HER2* was upregulated in SNU216-AR. D, Phospho-RTK assay of parental and resistant cells. The phosphorylation of MSP-R, c-RET and AXL were upregulated in N87-AR

3.3 | Activation of MSP-R, AXL and c-Ret in resistant cell lines

We evaluated the activation of RTK using a phospho-RTK array to narrow down the candidate mechanisms of resistance. The results of the resistant cells were compared with those of the parental cells. In N87-AR, the activation of MSP-R, which reportedly forms a hetero-dimer with MET,¹⁷ was upregulated. In addition to MSP-R, the activation of AXL and c-RET was also upregulated. In particular, the upregulation of AXL was remarkable. In contrast, no clear upregulation of RTK was observed in SNU216-AR compared with the parental cells (Figure 1D).

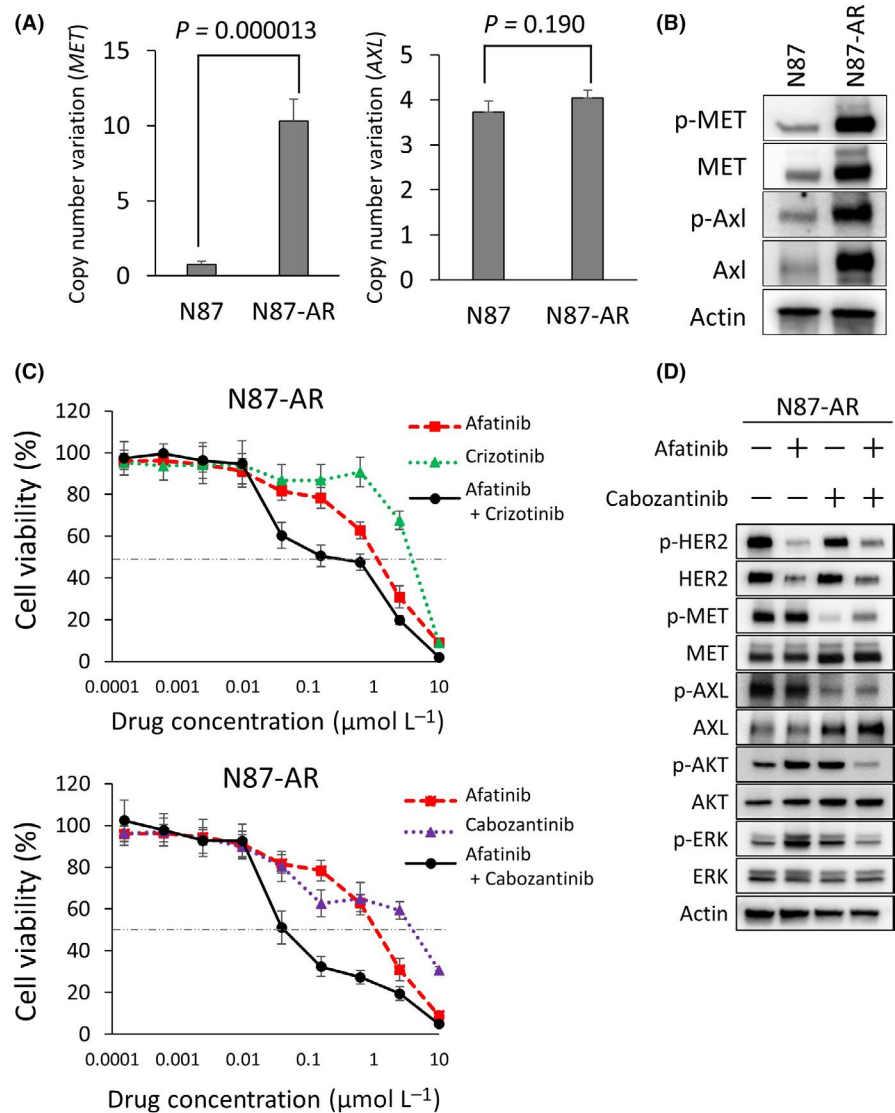
3.4 | Acquisition of MET and AXL amplification in afatinib-resistant cell lines

Based on the results of the RTK array, we performed a copy number assay of *MET* and *AXL* in N87-AR; *MET* was clearly amplified in N87-AR (Figure 2A), and no significant difference was detected in the copy number variation of *AXL*. Western blotting showed that total and phosphorylated *MET* were also dramatically upregulated

in N87-AR. In addition, we confirmed the upregulation of AXL using western blotting (Figure 2B).

Based on the revealed mechanisms of resistance, we tried to establish a therapeutic strategy for afatinib-resistant cells. To overcome the resistance caused by *MET* amplification, we administered a *MET* inhibitor and/or afatinib to N87-AR. The effect of the anti-tumor drugs was estimated using an MTS assay, and we used crizotinib or cabozantinib as *MET* inhibitors. Besides the activation of *MET*, crizotinib reportedly inhibits ALK and cabozantinib inhibits AXL, RET, KIT, FLT3 and VEGFR2. The effect of either crizotinib or cabozantinib alone was insufficient to suppress cell proliferation of N87-AR, but both crizotinib and cabozantinib restored the sensitivity to afatinib (Figure 2C). These combination therapies were judged to have a synergistic effect based on their CI (Figure S1A,B). When the 2 *MET* inhibitors were compared, cabozantinib was more effective than crizotinib against N87-AR, and the IC_{50} value of combination therapy with afatinib and cabozantinib was 42.5 nmol/L. Subsequently, we performed western blotting to examine the effect of afatinib and cabozantinib on the signaling pathway in N87-AR. As expected, afatinib downregulated p-*HER2*, and cabozantinib downregulated p-*MET*

FIGURE 2 *MET* amplification and *AXL* overexpression in the afatinib-resistant cell lines N87-AR. A, Copy number assay of *MET* and *AXL*. *MET* was amplified in N87-AR. B, The activation of *MET* and *AXL* was analyzed using western blotting. Total and phosphorylated *MET* and *AXL* were clearly upregulated in N87-AR. C, Drug sensitivities to afatinib and/or *MET* inhibitors in N87-AR as evaluated using an MTS assay. Combination treatment with afatinib and cabozantinib had a remarkable effect in N87-AR. D, Effects of afatinib and/or cabozantinib on *HER2* and down-signal pathway molecules in N87-AR. The combination therapy suppressed the activation of *HER2*, *MET*, *AXL*, *ERK*, and *AKT*. The concentrations of afatinib and cabozantinib were both 100 nmol/L, and the medication was performed for 24 h



and p-*AXL* (Figure 2D). Neither afatinib nor cabozantinib monotherapy suppressed the activation of *AKT* and *ERK*, but the combination therapy, nonetheless, downregulated both *AKT* and *ERK*.

3.5 | Acquisition of *Yes1* amplification in afatinib-resistant cell lines

Next, we performed a copy number assay of *YES1*, a member of the *Src* family, in N87-AR and SNU216-AR. *Yes1* was amplified in SNU216-AR but not in N87-AR (Figure 3A). The protein levels of total and phosphorylated *Src* and *Yes* were markedly upregulated in SNU216-AR (Figure 3B).

Next, we tried to overcome afatinib-resistance induced by *Yes1* amplification in SNU216-AR. Similar to our strategy for N87-AR, we used the *Src* inhibitor dasatinib and/or afatinib in SNU216-AR. Unlike crizotinib or cabozantinib in the *MET*-amplified resistant cells, dasatinib monotherapy was effective in the *Yes1* amplified cells. Combination therapy with afatinib produced a dramatic effect, and the IC_{50} value was as low as 10.3 nmol/L (Figure 3C). This

combination therapy was also considered to be synergistic (Figure S1C). Regarding the signaling pathway, afatinib downregulated p-*HER2*, and dasatinib downregulated p-*Src*, as expected. Afatinib suppressed the activation of *AKT* in afatinib-resistant cells, and dasatinib also downregulated p-*HER2* unexpectedly. Although neither afatinib nor dasatinib monotherapy suppressed the activation of *ERK*, the combination therapy of these drugs downregulated it (Figure 3D). Because dasatinib downregulated p-*HER2*, we also evaluated the effect of dasatinib in the SNU216 parental cell line and found that dasatinib monotherapy was effective in this cell line as well (Figure 3E).

3.6 | Antitumor effect of cabozantinib in a xenograft mouse model

Based on the results of an in vitro assay, we performed an in vivo assay to examine the effect of combination therapy using afatinib and cabozantinib in N87-AR. The combination therapy had a significant antitumor effect against N87-AR (Figure 4A,B). Afatinib also had

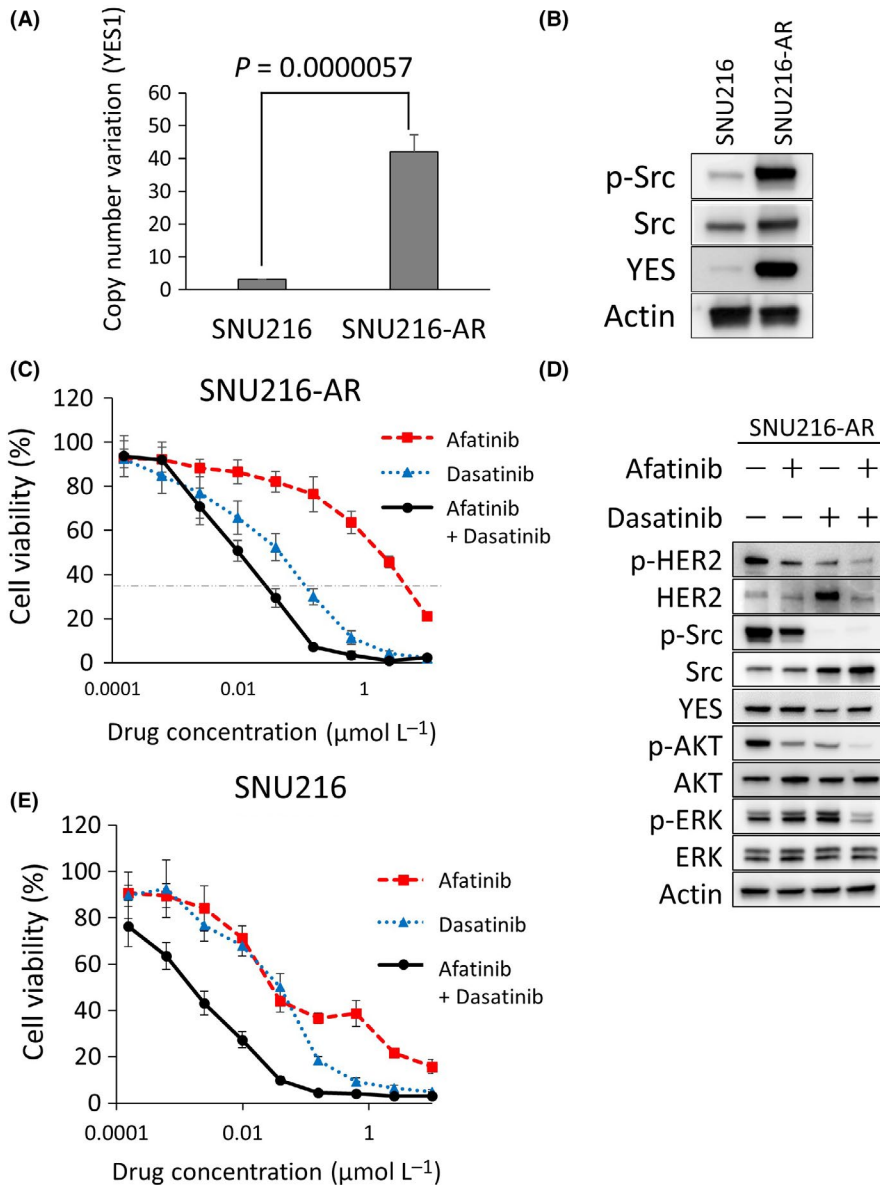


FIGURE 3 YES1 amplification in the afatinib-resistant cell line SNU216-AR. A, Copy number assay of YES1. YES1 was amplified in SNU216-AR. B, Activation of Src and expression of YES1 as evaluated using western blotting. Total and phosphorylated Src and YES1 were upregulated in SNU216-AR. C, Drug sensitivities to afatinib and/or Src inhibitors in SNU216-AR as calculated using an MTS assay. Not only the combination treatment of afatinib and dasatinib, but also dasatinib monotherapy had a remarkable effect in SNU216-AR. D, Effects of afatinib and/or dasatinib on HER2 and down-signal pathway molecules in SNU216-AR. The combination therapy suppressed the activation of HER2, Src, ERK and AKT. The concentrations of afatinib and dasatinib were both 100 nmol/L, and the medication was performed for 24 h. E, Effects of dasatinib and/or afatinib in SNU216 parental cells. The combination therapy was dramatically effective, but both afatinib and dasatinib monotherapy also showed strong antitumor effects

a mild antitumor effect even when administered as a single agent, although the effect was limited compared with its effect when combined with cabozantinib. To evaluate the tolerability of the molecularly targeted drugs, the body weights of the mice were measured before and after treatment. No significant changes in body weight were observed, suggesting that monotherapy with these drugs was tolerable (Figure 4C).

4 | DISCUSSION

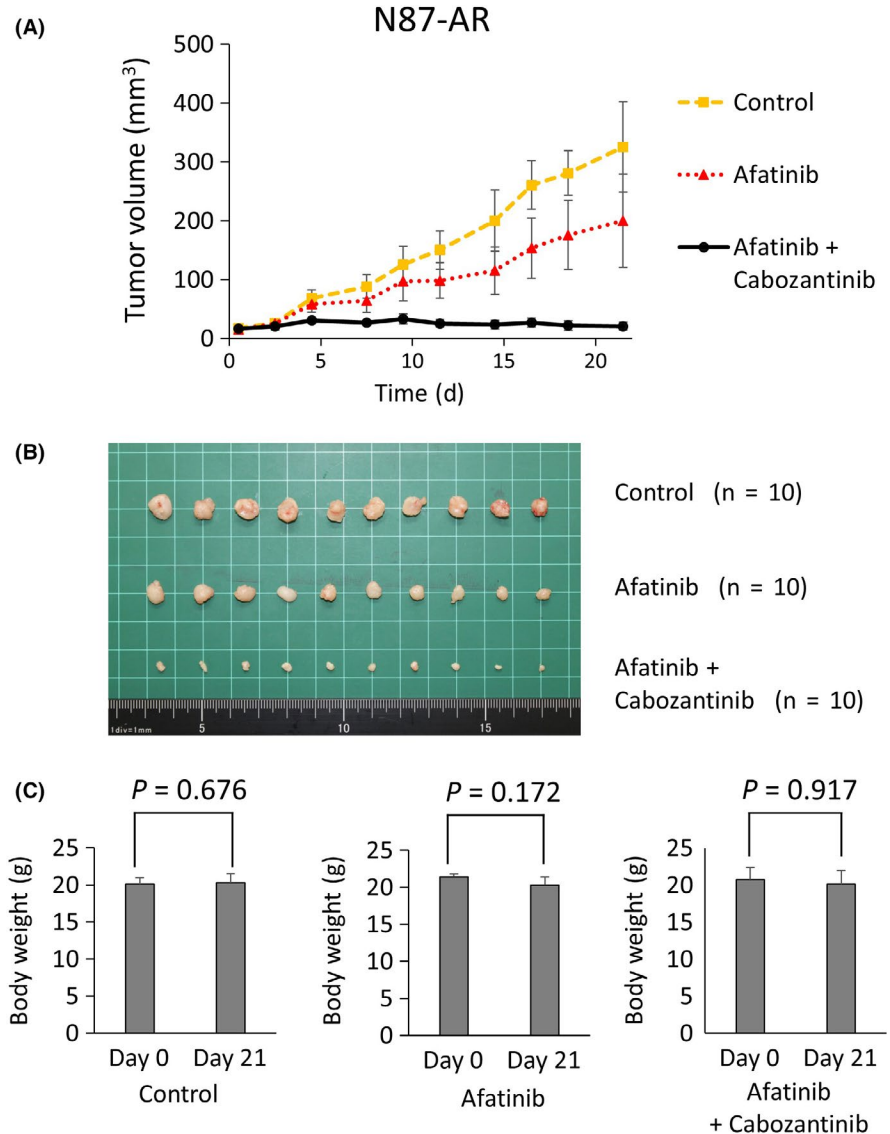
In this study, we established 2 afatinib-resistant cell lines using *HER2*-amplified gastric cancer cell lines and identified: (i) *MET* amplification with *AXL* upregulation and (ii) *YES1* amplification as mechanisms of resistance. Moreover, we investigated treatment strategies for afatinib-resistant cell lines and showed that: (i) combination therapy with afatinib and cabozantinib and (ii) dasatinib monotherapy

were capable of overcoming the abovementioned mechanisms of resistance, respectively.

The gene amplification of *MET* has already been reported as a mechanism of afatinib resistance in lung cancer.¹⁸ *AXL* upregulation has also been reported as a mechanism of resistance to TKI.¹⁹⁻²¹ In N87-AR, a clear difference between the effects of 2 *MET* inhibitors, crizotinib and cabozantinib, was seen when these inhibitors were used in combination with afatinib, as only cabozantinib downregulated the activation of *AXL*. This phenomenon suggests that not only the activation of *MET* but also the activation of *AXL* plays a pivotal role in the acquisition of afatinib resistance in this cell line. In addition, cabozantinib also inhibited the activation of *RET*, which was slightly upregulated in N87-AR. Based on the fact that the heterogeneity of gastric cancer is a challenging and significant issue,^{22,23} the inhibition of multiple targets could be an especially effective strategy for this cancer type.

Previously, we reported that *YES1* amplification mediates resistance to lapatinib, a dual inhibitor of *EGFR* and *HER2*, in

FIGURE 4 Effects of combination therapy with afatinib and cabozantinib in N87-AR in vivo. A, Combination therapy had a dramatic effect in N87-AR. B, Photographs of a tumor resected from a xenograft model, taken on day 21. C, Body weight of the mice. No weight losses were observed in any of the groups



HER2-amplified breast cancer.²⁴ Based on the results of this study, we are convinced that YES1 plays an important role in the acquisition of resistance in HER2-amplified malignant tumors. Indeed, a recent report of clinical genomic sequencing of human lung cancer samples obtained after the acquisition of resistance to EGFR inhibitors has revealed the amplification of YES1.²⁵

Regarding other mechanisms of resistance, we examined whether the resistant cells acquired epithelial-mesenchymal transition (EMT) features, cancer stem cell (CSC)-like features,¹⁰ the upregulation of IGF1R or IGFBP,^{26,27} or oncogenic mutations of PIK3CA²⁸⁻³⁰ based on previously published articles. The acquisition of EMT or CSC-like features was evaluated using western blotting to examine the expressions of E-cadherin, vimentin and ALDH1A1. However, no clear differences between the parental cells and the afatinib-resistant cells were found in these analyses. Taking into consideration the fact that the acquisition of EMT or CSC-like features is the major mechanism of afatinib-resistance in lung cancer, the mechanisms of resistance may vary according to cancer type.³¹

Afatinib monotherapy had a slight antitumor effect against afatinib-resistant N87-AR cells in an in vivo assay, but not in vitro. We supposed that the microenvironment, including the existence of fibroblasts or stromal cells, promoted the efficacy of afatinib treatment, although the detailed mechanism remains unclear. Nevertheless, the effect of afatinib against N87-AR was quite limited, compared with the dramatic effect of afatinib in the parental cell line, N87.⁵ Combination therapy with afatinib and cabozantinib exerted an overwhelmingly superior effect, compared with afatinib monotherapy.

Diarrhea and rash have been reported as the most frequent adverse events associated with afatinib treatment,^{32,33} while diarrhea, hypertension and palmar-plantar erythrodysesthesia syndrome (PPES) have been reported for cabozantinib.^{34,35} In this experiment, the appearance of these adverse events was a concern, because the combination of these 2 agents might have increased the severity of such events. However, severe adverse events were not suggested in our experiment, based on the weight loss of the examined mice, while a strong antitumor effect was observed.

In this study, dasatinib suppressed the activation of HER2 in addition to Src. Some articles have already reported the interaction of Src family proteins and HER2.^{36,37} This phenomenon might explain the reason why dasatinib monotherapy had a remarkable antitumor effect against the YES1 and HER2-amplified resistant cell line SNU216-AR. In contrast, afatinib downregulated not only phosphorylated-HER2, but also total-HER2, unlike dasatinib. Afatinib is thought to inhibit the activation of HER2 more strongly than dasatinib and to kill cells in which the gene expression of HER2 is more strongly activated. Consequently, afatinib was capable of downregulating the total HER2. To confirm that dasatinib inhibited the activation of both HER2 and Src, we examined the antitumor effect of dasatinib against SNU216 parental cells and observed that SNU216 was also highly sensitive to dasatinib. Thus, dasatinib could potentially be another therapeutic option in the treatment of HER2-amplified gastric cancer cells, and not just for afatinib-resistant cells.

In conclusion, we identified: (i) MET amplification with AXL overexpression and (ii) Yes1 amplification as mechanisms of afatinib resistance in gastric cancer cells. Combination therapy with afatinib and cabozantinib or dasatinib monotherapy were capable of overcoming the abovementioned resistance mechanisms, respectively. As precision medicine continues to evolve in cancer treatment, we are convinced that these findings will contribute to the development of treatment strategies for patients with gastric cancer.

DISCLOSURE

The authors have no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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