Dual blockade of EGFR tyrosine kinase using osimertinib and afatinib eradicates EGFR-mutant Ba/F3 cells

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Abstract. Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) are efficacious drugs for non-small cell lung cancers (NSCLCs) with EGFR-activating mutations. Afatinib, a second-generation EGFR-TKI and osimertinib, a third-generation EGFR-TKI, are both standard therapies for patients with these types of cancer. Each drug possesses distinct binding sites for the tyrosine kinase domain of EGFR. The present study examined the efficacy of single and combination TKI therapy using *in vitro* growth inhibition assays of Ba/F3 cells with an EGFR-activating Del19 mutation. Afatinib or osimertinib treatment alone markedly inhibited cell proliferation in Ba/F3 cells, although drug-resistant cells eventually appeared with secondary EGFR mutations (either T790M or C797S, respectively) as determined by direct sequencing. Notably a combination of afatinib and osimertinib eradicated Ba/F3 cells with no development of resistance. We also evaluated the efficacy of afatinib, osimertinib, and a combination of the two, using drug-resistant cells with T790M or C797S mutations. Osimertinib was effective for treating Ba/F3 cells with the T790M mutation, whereas afatinib was moderately effective against C797S Ba/F3 cells. However, subsequent treatment, even when both drugs were used in combination, could not completely eradicate the Ba/F3 population and doubly resistant cells with a variety of triple mutations were generated, including Del19/T790M/C797S. In conclusion, an initial treatment with a combination of osimertinib and afatinib is potentially more effective for eradicating mutant EGFR-dependent cells than sequential drug use. This should be tested in future clinical trials to establish whether such a combination would be effective for the treatment of NSCLC.

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Introduction

Non-small cell lung cancer (NSCLC) is the most common form of lung cancer and is notable for being resistant to chemotherapy. Epidermal growth factor receptor (EGFR) is a molecular oncotherapeutic target for many types of cancer, particularly NSCLC (1). In particular, EGFR tyrosine kinase inhibitors (EGFR-TKIs) have shown excellent efficacy for NSCLCs with EGFR-activating mutations, such as exon 19 deletions (Del19) or L858R substitutions (2,3). These mutations alter EGFR, leading to preferential binding between ATP and the kinase domain, causing spontaneous activation (1). Furthermore, EGFR-TKI combinations, such as those involving VEGF antibody or chemotherapy, have recently improved treatment efficacy compared to that with EGFR-TKI alone (4,5). However, EGFR-linked cancers ultimately become refractory to EGFR-TKI treatment. Previous research has elucidated several mechanisms that underlie resistance, revealing that approximately 50% of EGFR-TKI-resistant NSCLCs with EGFR-activating mutations possess a T790M secondary mutation (6). It is therefore critical that further investigation be undertaken to identify new strategies to overcome EGFR-TKI resistance, particularly those involving T790M.

Afatinib is a second-generation EGFR-TKI that irreversibly binds to the ATP-binding pocket of EGFR tyrosine kinase more strongly than first-generation reversible EGFR-TKIs, leading to efficient inhibition (7). In phase II clinical trials, afatinib was shown to improve progression-free survival in patients with advanced NSCLC compared to first-generation EGFR-TKI gefitinib (8). Additionally, second-generation EGFR-TKI dacomitinib was more effective than first-generation EGFR-TKIs for patients with advanced NSCLC with EGFR-activating mutations (9). Therefore, second-generation EGFR TKIs, particularly afatinib, are now recommended for NSCLCs with EGFR-activating mutations. Pre-clinical studies suggest that second-generation EGFR-TKIs are also effective at treating cancer cells with T790M. This substitution has been shown to associate with the development of resistance to first-generation EGFR-TKIs (6). However, clinical observations found little efficacy against NSCLC with the T790M mutation (10). Specifically, afatinib had an 8% response rate for patients who progressed from prior treatment with first-generation EGFR-TKIs. This suggests that afatinib is insufficient to overcome T790M-induced resistance.

In contrast, third-generation EGFR-TKI osimertinib irreversibly binds EGFR with T790M, in addition to EGFR with L858R and Del19 (11). Importantly, osimertinib does not bind wild-type EGFR as strongly as these mutants so is highly specific for NSCLC with EGFR-activating mutations and is consequently much less toxic. Finally, osimertinib is more effective against NSCLCs with T790M compared to other cytotoxic chemotherapies (12). Although first- and second-generation EGFR-TKIs only marginally reduced tumor sizes (if at all), osimertinib demonstrated a 61% response rate for NSCLCs with T790M (12). Furthermore, osimertinib improved progression-free survival in patients with EGFR-TKI-naïve NSCLC harboring EGFR-activating mutations compared to other TKIs (13). Osimertinib is therefore a standard therapy for NSCLC with EGFR-activating mutations, particularly those with T790M.

Despite the unique properties of osimertinib, including its ability to inhibit the tyrosine kinase activity of EGFR with T790M, cells still eventually acquire resistance. To investigate how this occurs in more detail, and develop new treatment regimens for NSCLC with EGFR-activating mutations, we investigated the underlying mechanisms of afatinib and osimertinib resistance using Ba/F3 cells with Del19. Using these data, we explored potential new therapeutic strategies for overcoming resistance to EGFR-TKI therapy that will contribute to improving patient outcomes.

Materials and methods

Cell culture and reagents. Murine pro-B Ba/F3 cells were purchased from the Riken BioResource Center (Tsukuba, Japan) and cultured in RPMI-1640 medium (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin-amphotericin B (Wako Pure Chemical Industries, Ltd., Osaka, Japan) at 37˚C in a humidified atmosphere with 5% CO_2. Cells were analyzed using a previously validated short-tandem repeat method (14). Ba/F3 cells with Del19 were stably transfected with a full-length cDNA fragment encoding the human *EGFR* Del19 mutant (del E746_A750), as previously described (15). Afatinib and the osimertinib were purchased from Selleck Chemicals (Houston, TX, USA). Stock solutions of 10 mM afatinib and osimertinib were prepared in dimethyl sulfoxide (DMSO) and stored at -20˚C.

Establishment of afatinib and osimertinib-resistant clones through N-ethyl-N-nitrosourea (ENU) mutagenesis. Ba/F3 cells with *EGFR* Del19 were exposed to 100 mg/ml ENU (Sigma-Aldrich; Merck KGaA) for 24 h and then washed and cultured in RPMI-1640 (Sigma-Aldrich; Merck KGaA) with 10% FBS for 24 h. Similar to previous studies (14,15), $5x10^4$ cells were plated on 96-well plates. The concentrations of afatinib and osimertinib used were 100 nM when used singularly, or 50 nM when used in combination, to mimic plasma concentrations achieved in-clinic (15). Media were changed weekly and cell growth was observed visually until confluence achieved.

EGFR mutation analyses. Total RNA from resistant cells was isolated using an RNeasy Mini kit (Qiagen, Hilden, Germany). cDNA was transcribed from total RNA using a High Capacity RNA-to-cDNA kit (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The tyrosine kinase domains of *EGFR* (exons 18 to 21) were amplified with previously reported primers (15). Sanger sequencing was performed using a Genetic Analyzer 3,130 or 3,500 xl (Applied Biosystems; Thermo Fisher Scientific, Inc.).

Cell growth inhibition assays. Afatinib and osimertinib sensitivity was evaluated using CellTiter-Glo Luminescent Cell Viability Assays (Promega Corporation, Madison, WI, USA). Briefly, cells were resuspended in medium containing 10% FBS in 96-well plates at $2x10^3$ cells/well. After overnight incubation at 37˚C, afatinib or osimertinib were added at concentrations ranging from 0.0033 to 1 μM. Cells were left for 72 h and viability was quantified based on luminescence after the addition of CellTiter-Glo reagent (Promega Corporation). Experimental sampling was performed for 6-wells for each treatment.

Antibodies and western blotting. Cells were seeded at a density of $1x10^6$ cells/plate in 60-mm plates and allowed to grow overnight in medium containing 0.5% FBS before the addition of afatinib or osimertinib. Cells were incubated for 3 h, harvested, and then lysed in buffer containing 25 mM Tris (pH 8.3), 192 mM glycine, 0.1% sodium dodecyl sulfate and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cell lysates were centrifuged at 15,000 x g for 10 min at 4˚C, and the supernatant was collected for subsequent procedures. Western blotting was performed following a standard protocol; a total of 30 μg sample was resolved by 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes, which were probed with antibodies against phospho-EGFR (cat. no. 2234; Cell Signaling Technology, Inc., Danvers, MA, USA), EGFR (cat. no. 4267; Cell Signaling Technology, Inc.), phospho-ERK1/2 (cat. no. 16982; Santa Cruz Biotechnology, Santa Cruz, CA, USA), ERK1/2 (cat. no. 9102; Cell Signaling Technology, Inc.) and β-actin (cat. no. 4970; Cell Signaling Technology, Inc.) diluted at 1:1,000 at 4˚C overnight. Secondarily those membranes were probed with anti-rabbit antibody (cat. no. NA934V; GE Healthcare Life Sciences, Little Chalfont, UK) diluted at 1:2,500. Immune complexes were detected with ECL detection reagents (GE Healthcare). Protein bands were quantified using ImageJ version 1.52 software (NIH; National Institutes of Health, Bethesda, MD, USA) and normalized against β-actin.

Statistical analyses. Statistical analyses were performed using SPSS 22.0 (IBM Corp., Armonk, NY, USA). For statistical hypothesis testing, Fisher's exact test was used. One-way analysis of variance (ANOVA) with Dunnett's post hoc test was applied for analysis of western blotting results. All statistical tests were two-sided. P-values <0.05 were considered to indicate statistically significant results. Data are graphically displayed using GraphPad Prism 5.0 for Windows (GraphPad Software, Inc., La Jolla, CA, USA).

Results

An afatinib and osimertinib combination prevents the appearance of drug resistant clones in Ba/F3 cells with Del19. We

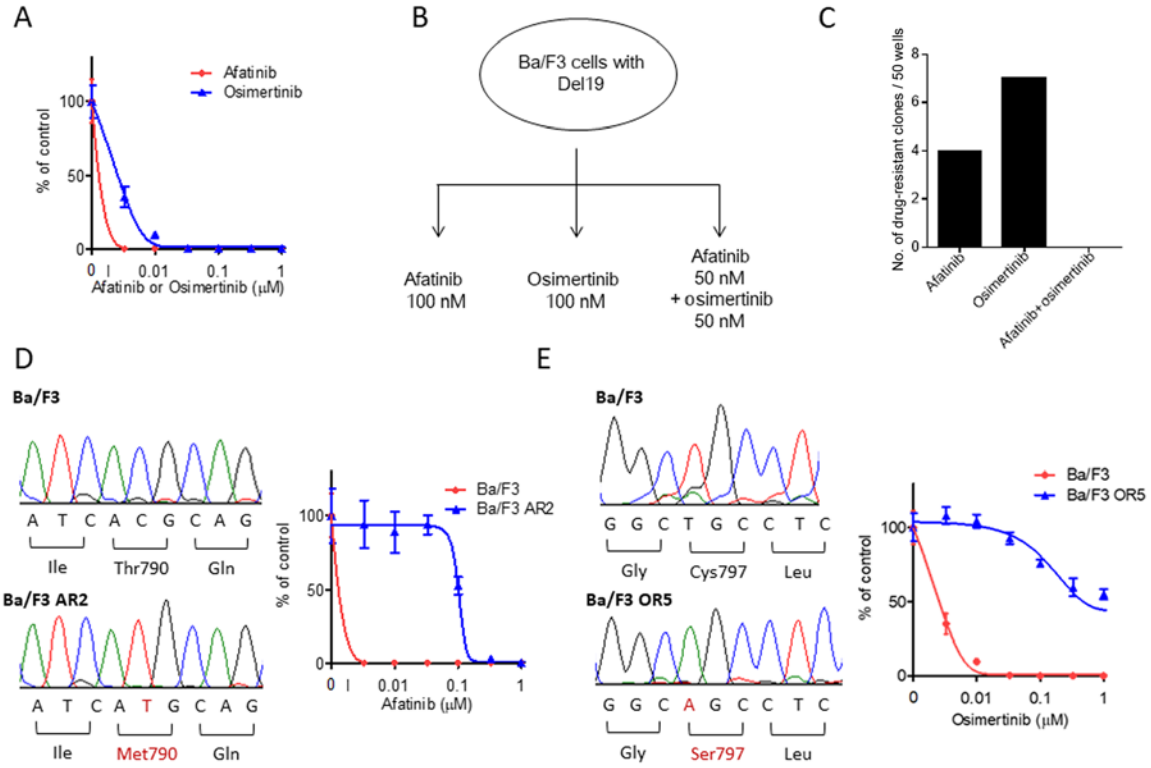

Figure 1. Afatinib and osimertinib combination therapy eradicates Ba/F3 cells with the EGFR-activating mutation Del19. (A) Ba/F3 cells with Del19 were treated with the indicated concentrations of afatinib or osimertinib and cell viability was measured 3 days later. Values are plotted relative to untreated control cells (means ± SD). (B) An overview of the experimental methods used for establishing resistant clones. (C) The numbers of resistant clones per 50 wells were counted for each treatment. The groups were treated with 100 nM afatinib, 100 nM osimertinib, or 50 nM afatinib with 50 nM osimertinib for 14 days. (D) Chromatograms of the *EGFR* sequences from Ba/F3 cells with Del19 and afatinib-resistant Ba/F3 AR2 cells. These cells were also treated with the indicated concentrations of afatinib and cell viability was measured 3 days later. Values are plotted relative to untreated control cells (means ± SD). (E) Chromatograms of the *EGFR* sequences from Ba/F3 cells with Del19 and osimertinib-resistant Ba/F3 OR5 cells. These cells were also treated with the indicated concentrations of osimertinib and cell viability was measured 3 days later. Values are plotted relative to untreated control cells (means ± SD). EGFR, epidermal growth factor receptor.

first evaluated the efficacy of afatinib and osimertinib for treating Ba/F3 cells with Del19, revealing that both strongly inhibited cell proliferation. The afatinib and osimertinib IC_{50} values were both <10 nM (Fig. 1A), markedly lower than values that would be clinically available (15). We next examined whether Ba/F3 cells with Del19 acquired resistance to afatinib or osimertinib. Ba/F3 cells with Del19 were exposed to 100 mg/ml of ENU mutagen for 24 h and then treated with 100 nM afatinib, 100 nM osimertinib, or 50 nM afatinib and 50 nM osimertinib in combination for 14 days (Fig. 1B). For each group, 50 wells containing $5x10^4$ cells/well were examined for resistant clones. For cells treated with afatinib alone, we observed cell confluency (suggesting drug resistance) in 4 of the 50 wells (Fig. 1C). Osimertinib alone led to confluency in 7 of the 50 wells (afatinib vs. osimertinib, P=0.52; Fig. 1C). Notably, treatment with afatinib and osimertinib did not lead to any resistant clones among the 50 wells (Fig. 1C). We repeated these observations across 500 wells but were unable to find any resistant clones for the afatinib and osimertinib combination.

As we expected that secondary mutations in EGFR lead to EGFR-TKI resistance, we sequenced the kinase domains of *EGFR* exons 18 and 21. All four of the afatinib-resistant clones, including Ba/F3 AR2, had the secondary EGFR mutation T790M (Fig. 1D). *In vitro* growth inhibition assays showed that the IC_{50} values for these resistant cells were >30-fold higher than Ba/F3 cells with only Del19 (Fig. 1D). We also examined the seven osimertinib-resistant clones, including Ba/F3 OR5, finding that they all had the secondary EGFR mutation C797S (Fig. 1E). *In vitro* growth inhibition assays showed that the IC_{50} values for these cells were >100-fold higher than Ba/F3 cells with only Del19 (Fig. 1E). These results suggest that secondary EGFR mutations, such as T790M or C797S, can cause resistance to afatinib or osimertinib, whereas both drugs used in combination prevented resistant clones.

Afatinib- or osimertinib-resistant clones maintain sensitivity to other EGFR-TKIs. Based on our previous experiment, we hypothesized that afatinib prevents the appearance of cells with C797S in a Ba/F3 cell population with Del19, whereas osimertinib prevents the appearance of cells with T790M. To test this hypothesis, we examined the efficacy of osimertinib against T790M afatinib-resistant cells. In contrast to afatinib treatment, osimertinib dose-dependently inhibited cell-proliferation in Del19/T790M cells, almost completely eradicating viable cells at 50 nM (Fig. 2A). Conversely, we examined the efficacy of afatinib in osimertinib-resistant cells with C797S. In contrast to osimertinib treatment, afatinib moderately inhibited proliferation, almost completely eradicating viable cells at 50 nM (Fig. 2B). Then, we examined signaling molecules in cells treated with afatinib or osimertinib. Parental Ba/F3 cells exhibited significant decreases in the phosphorylation of EGFR upon afatinib and osimertinib treatment (Fig. 2C and D).

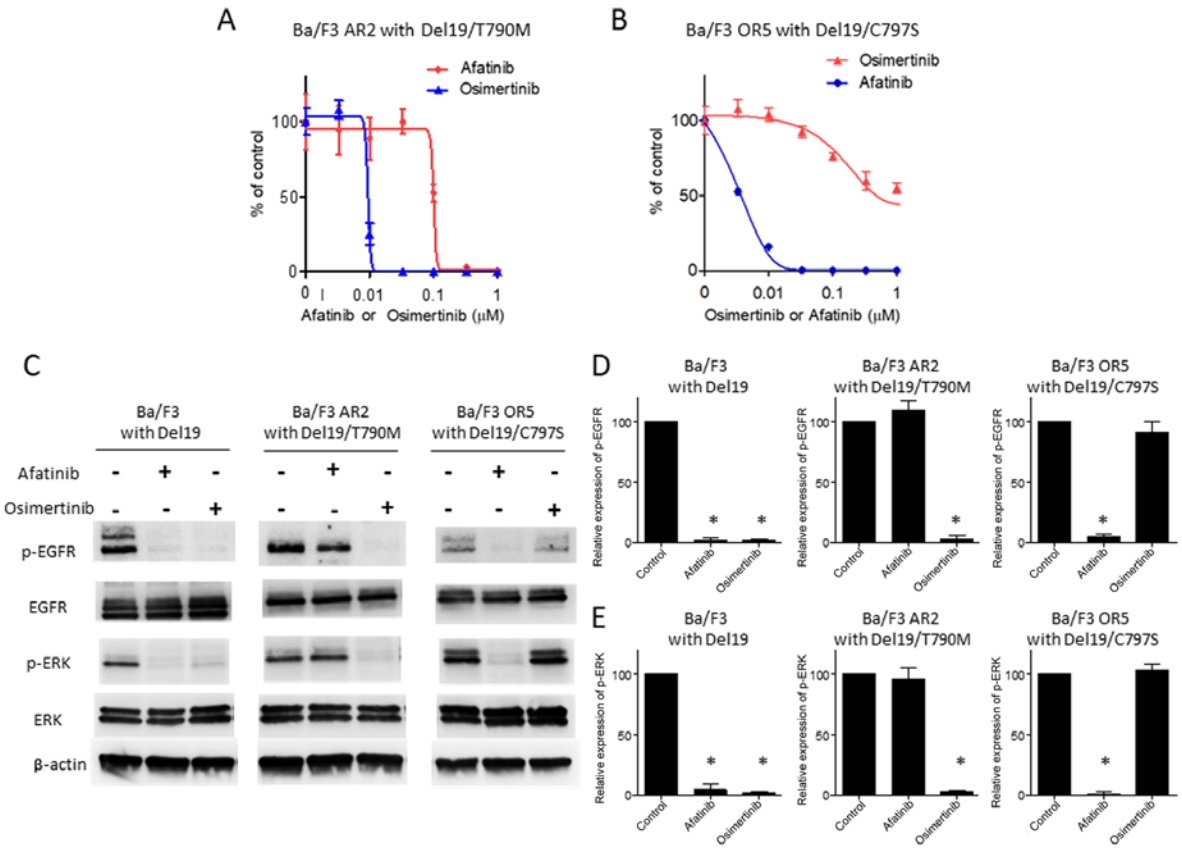

Figure 2. Afatinib- or osimertinib-resistant clones maintain sensitivity to other EGFR-TKIs. (A) Ba/F3 AR2 cells with the Del19/T790M double mutation were treated with the indicated concentrations of afatinib or osimertinib. Cell viability was measured 3 days later and values are plotted relative to untreated control cells (means ± SD). (B) Ba/F3 OR5 cells with Del19/C797S were treated with the indicated concentrations of afatinib or osimertinib and cell viability was measured 3 days later. (C) Ba/F3 cells, Ba/F3 AR2 cells with Del19/T790M, and Ba/F3 OR5 cells with Del19/C797S were treated with 50 nM afatinib or osimertinib for 3 h and then were probed for the indicated protein. The ratios of phospho-EGFR/β-actin to total EGFR/β-actin (D), or phospho-ERK/β-actin to total ERK/β-actin (E) are shown. Values are plotted relative to those in untreated control cells (mean ± SD; n=6 for A and B; n=3 for D and E). *P<0.05, one-way ANOVA with Dunnett's post hoc test. EGFR-TKIs, EGFR tyrosine kinase inhibitors; EGFR, epidermal growth factor receptor.

Additionally, these cells exhibited significant decreases in the phosphorylation of ERK, downstream of EGFR, induced by either drug (Fig. 2C and E). In contrast to parental Ba/F3 cells, Ba/F3 cells with Del19/T790M did not exhibit downregulation of EGFR or ERK phosphorylation upon afatinib treatment (Fig. 2C-E). Furthermore, Ba/F3 cells with Del19/C797S did not exhibit decreases in the phosphorylation of EGFR or ERK upon osimertinib treatment (Fig. 2C-E). Combined, these results suggest that osimertinib and afatinib may block EGFR-ERK signaling in Ba/F3 cells with Del19/T790M and Del19/C797S, respectively, eradicating those cells. Therefore, the combination of osimertinib and afatinib prevents the appearance of resistant clones.

Treatment with an afatinib and osimertinib combination does not eradicate resistant clones after the generation of C797S mutations in Ba/F3 cells with Del19. Then, we speculated that afatinib-resistant clones with T790M (generated after afatinib monotherapy) may be eradicated by osimertinib or an osimertinib and afatinib combination. We therefore treated afatinib-resistant cells that possessed T790M with either 100 nM osimertinib or 50 nM osimertinib with 50 nM afatinib for 14 days (Fig. 3A). However, contrary to expectations, treatment with 100 nM osimertinib led to 4 of the 50 wells having resistant cells, including Ba/F3 AR2 OR2 (Fig. 3B). Furthermore, treatment with 50 nM osimertinib and 50 nM afatinib led to 3 of the 50 wells having resistant cells (P>0.05; Fig. 3B). To investigate the underlying mechanisms, we sequenced the kinase domain of *EGFR*. All the resistant clones that emerged after treatment with osimertinib or the osimertinib with afatinib combination had a Del19/T790M/C797S triple mutation in the EGFR kinase domain (Fig. 3C). *In vitro* growth inhibition assays revealed that the resistant Ba/F3 AR2 OR2 cells maintained strong growth in 100 nM osimertinib, 100 nM afatinib, and the 50 nM osimertinib and 50 nM afatinib combination (Fig. 3D). These observations suggested that afatinib-resistant cells with T790M were more sensitive to sequential osimertinib treatment or an osimertinib with afatinib combination. However, a small population of cells with Del19/T790M/C797S survived and proliferated despite drug treatment.

Treatment with an afatinib and osimertinib combination does not eradicate resistant clones after the generation of C797S mutations in Ba/F3 cells with Del19. Finally, we examined whether osimertinib-resistant clones with C797S could be eradicated by treatment with afatinib or an afatinib and osimertinib combination. To assess this, we treated C797S osimertinib-resistant cells with either 100 nM afatinib or a 50 nM afatinib with 50 nM osimertinib combination for 14 days (Fig. 4A). This revealed that treatment with 100 nM

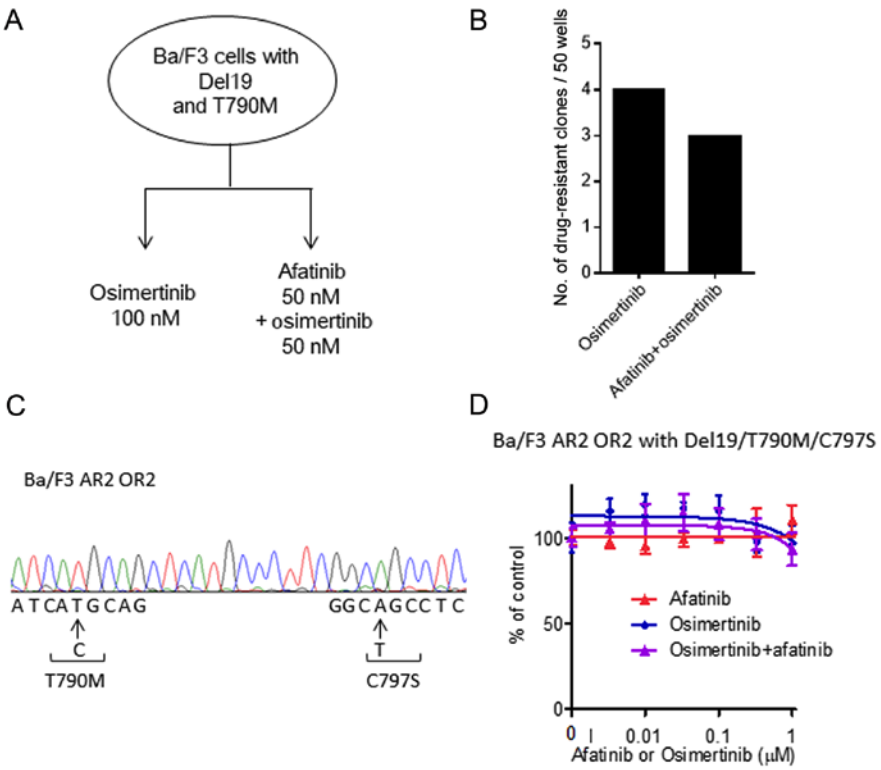

Figure 3. Afatinib and osimertinib combination therapy does not eradicate Ba/F3 cells with Del19 and T790M mutations after the development of afatinib resistance. (A) An overview of the experimental methods used for establishing resistant clones. (B) The numbers of resistant clones per 50 wells were counted for each treatment. The groups were treated with 100 nM osimertinib or 50 nM afatinib with 50 nM osimertinib for 14 days. (C) Chromatograms of the *EGFR* sequences from afatinib and osimertinib-resistant Ba/F3 AR2 OR2 cells. (D) Ba/F3 AR2 OR2 cells were treated with the indicated concentrations of afatinib, osimertinib, or a combination of both. Cell viability was measured 3 days later and values plotted relative to untreated control cells (means ± SD). EGFR, epidermal growth factor receptor.

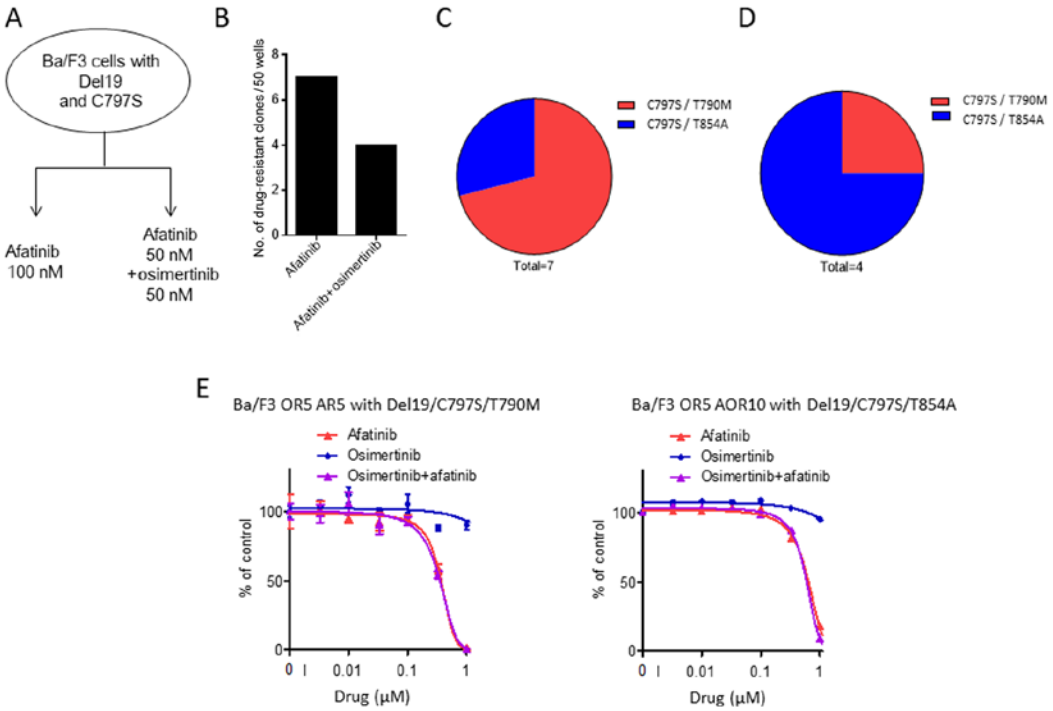

Figure 4. Afatinib and osimertinib combination therapy could not eradicate Ba/F3 cells with Del19 and the C797S mutations following the development of osimertinib resistance. (A) An overview of the experimental methods used for establishing resistant clones. (B) The numbers of resistant clones per 50 wells were counted. The groups were treated with 100 nM afatinib or 50 nM afatinib with 50 nM osimertinib for 14 days. (C) A pie chart showing the secondary EGFR mutations in subsequent afatinib-resistant clones derived from osimertinib-resistant Ba/F3 OR5 cells. (D) A pie chart showing the proportions of the different secondary EGFR mutations that emerged from osimertinib-resistant Ba/F3 OR5 cells treated with a 50 nM afatinib and 50 nM osimertinib combination. (E) Ba/F3 OR5 AR5 cells and Ba/F3 OR5 AOR10 cells were treated with the indicated concentrations of afatinib, osimertinib, or a combination of both. Cell viability was measured 3 days later and values plotted relative to untreated control cells (means ± SD). EGFR, epidermal growth factor receptor.

Table I. EGFR tyrosine kinase inhibitor combination therapy.

Combination therapy	Resistance mechanism	Resistance mutation	Cell line model	(Refs.)
Afatinib + cetuximab	EGFR secondary mutation	EGFR T790M	H1975	(20)
Brigatinib + cetuximab		EGFR T790M/C797S	PC9, MGH121, Ba/F3	(21)
EAI045 + cetuximab		EGFR T790M/C797S	H1975, Ba/F3	(22)
Gefitinib + osimertinib		EGFR T790M/C797S	Ba/F3	(23)
Gefitinib + WZ4002		EGFR T790M/C797S	Ba/F3	(24)
Gefitinib + osimertinib		EGFR T790M/C797S	Ba/F3	(25)
Afatinib + osimertinib		EGFR T790M/C797S	Ba/F3	Present study
EGFR-TKI + sonidegib	Others	Hedgehog signal activation	HCC827	(26)
EGFR-TKI + MET inhibitor		MET amplification	HCC827	(27)
EGFR-TKI + MEK inhibitor		MAPK1 amplification	PC9	(28)

EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor.

afatinib led to 7 of 50 wells with resistant cells (Fig. 4B). In addition, the 50 nM afatinib with 50 nM osimertinib combination generated a similar number, with 4 resistant wells from the total 50 (Fig. 4B). We again sequenced *EGFR* exons 18 and 21, showing that two distinct triple mutations had emerged in cells resistant to sequential afatinib single therapy, Del19/C797S/T790M and Del19/C797S/T854A (Fig. 4C). In cells resistant to the afatinib and osimertinib combination, we observed two triple mutations, including Del19/C797S/T790M and Del19/C797S/T854A (Fig. 4D). *In vitro* growth inhibition assays indicated that cells with the triple mutation (Ba/F3 OR5 AR5 with Del19/C797S/T790M and Ba/F3 OR5 AOR10 with Del19/C797S/T854A) proliferated in 100 nM osimertinib, 100 nM afatinib, and a combination of 50 nM osimertinib and 50 nM afatinib (Fig. 4E). These data suggest that these mutations lead to EGFR-TKI resistance in cancer cells, in addition to the mutations identified in previous reports (16,17). In summary, our observations suggest that osimertinib-resistant cells with C797S are sensitive to sequential afatinib treatment and afatinib with osimertinib combination therapy. However, a small population acquire additional mutations (typically T790M or T854A) that allows them to proliferate despite drug treatment.

Discussion

The present study found that epidermal growth factor receptor (EGFR) dual-blockade using afatinib and osimertinib eradicated Ba/F3 cancer cells with Del19, indicating that this combination prevents the generation of resistance to these two drugs. In contrast to combination treatment, osimertinib alone primarily generated resistant clones with the C797S mutation, and afatinib alone generated resistant clones with T790M. In fact, tissue samples from tumors resistant to afatinib are reported to frequently carry secondary T790M EGFR mutations at a rate of 50% (18). In addition, 20-30% of patients treated with osimertinib acquire resistance with an accompanying C797S mutation (19). Considering these results, EGFR dual-blockade using afatinib and osimertinib may be a clinically relevant solution for preventing secondary EGFR mutation-dependent resistance.

Previously, several combination therapies have been reported to overcome the resistance to epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs), which depends on EGFR secondary mutations or other mechanisms such as bypass signaling (Table I) (20-28). Another EGFR-TKI, brigatinib, or allosteric EGFR inhibitor EAI045 could overcome both T790M- and C797S-positive triple-mutant resistant clones, particularly when combined with the anti-EGFR antibody cetuximab (21,22). Alternatively, we and others have suggested that a combination of two different types of EGFR-TKIs may prevent the appearance of secondary EGFR mutation-dependent resistant clones (23-25). Ercan *et al* and Uchibori *et al* also reported that a gefitinib and osimertinib combination prevented the appearance of resistant Ba/F3 clones with the EGFR mutation L858R (23,25). Third-generation EGFR-TKIs generate covalent bonds with cysteine 797 in EGFR; therefore, this could prevent the increased ATP affinity mediated by the T790M mutation (11). Alternatively, first-generation or second-generation EGFR-TKIs may form hydrogen bonds with methionine 793 in EGFR and competitively inhibit ATP binding in the ATP binding pocket, regardless of the presence of the C797S mutation (15). The second-generation EGFR-TKI afatinib and third-generation EGFR-TKI osimertinib used in the present study may competitively bind to the ATP binding pockets of the mutant EGFR in the combination treatment, enabling these drugs to complement each other in terms of their disadvantages mediated by secondary EGFR mutation.

In contrast to combination therapy with osimertinib and afatinib, sequential therapy with osimertinib after resistance to afatinib did not completely eliminate Ba/F3 cells, nor reduce development of resistant clones. This also occurred after sequential therapy with afatinib following the emergence of osimertinib resistance. Clinical observation found that afatinib followed by osimertinib achieved long-term overall survival in the case of secondary T790M-positive non-small cell lung cancers (NSCLC) (29,30). However, another report indicated that 20-30% of patients treated secondarily with osimertinib acquired resistance with an accompanying C797S mutation (19). Therefore, to totally eradicate mutant

EGFR-dependent cells, the present study suggests initial treatment with an osimertinib and afatinib combination would have greater efficacy than sequential usage, and this should be tested in future clinical trials.

The present study also revealed that cells with the Del19/T790M/C797S triple mutation were resistant to even osimertinib and afatinib in combination. Niederst *et al* previously reported that a combination of first- and third-generation EGFR-TKIs can prevent proliferation of NSCLC cells with a double T790M/C797S mutation when T790M and C797S are located on different alleles (i.e., a trans position) (24). However, in the case of T790M and C797S located on the same allele (i.e., a *cis* position), a first- and third-generation EGFR-TKI combination had limited efficacy (24). Piotrowska *et al* reported that T790M and C797S were on the same allele in 44/46 of evaluable patients (98%), indicating that the resistant *cis* position is more common (31). Based on these observations, we hypothesize that both the afatinib- and osimertinib-resistant cells in the present study had T790M and C797S mutations in a *cis* location. This would mean that an afatinib and osimertinib combination could not eradicate resistant cells following the development of afatinib-resistance and the T790M mutation.

Ba/F3 cells are a limited model for investigating EGFR secondary mutation-dependent resistance to EGFR-TKIs. In actual clinical settings, other resistance mechanisms, such as bypass signaling, epithelial-mesenchymal transition, or EGFR-downstream activation, may occur. Therefore, the EGFR-dual blockade strategy may be less useful in these situations, and other combination strategies may be required.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

KY, YK and HH were involved in the conception and design of the study, the performance of the experiments, the data analysis and interpretation and the manuscript writing. YC was involved in the data analysis and their interpretation. TM and KN were involved in the conception and the design of the study and the interpretation of the date. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

KY, HH, TM and KN received research funding from Boehringer Ingelheim Co., Ltd. The other authors declare that they have no competing interests.

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