Original Article

Anlotinib enhances the anti-tumor activity of osimertinib in patients with non-small cell lung cancer by reversing drug resistance

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Background: Osimertinib, a third-generation epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitor (TKI), significantly improves the prognosis of patients with *EGFR*-mutant non-small cell lung cancer (NSCLC). However, subsequently-acquired resistance limits its effectiveness. This study aimed to explore the efficacy of anlotinib, a multitarget inhibitor of angiogenesis, in combination with osimertinib using *in vitro* and *in vivo* EGFR-TKI-sensitive and EGFR-TKI-resistant models.

Methods: We established osimertinib-resistant cell lines (H1975-OR and PC9-OR) and evaluated the effects of osimertinib, anlotinib, and their combination on cell proliferation *in vivo* and *in vitro*. In addition, we used pleural fluid from nine patients with *EGFR*-mutant NSCLC who received osimertinib therapy in the clinic to successfully establish a zebrafish patient-derived xenograft (zPDX) model. The effect of the combined treatment *in vivo* was assessed by quantifying red fluorescent regions representing tumor cell growth in zebrafish embryos to assess tumor proliferation and migration.

Results: Combination osimertinib and anlotinib therapy did not have an obvious synergistic antiproliferative effect in parental H1975 and PC9 cells; however, anlotinib reversed osimertinib resistance in osimertinib-resistant H1975-OR and PC9-OR cells *in vivo* and *in vitro*. A similar phenomenon was observed in the zPDX model.

Conclusions: In conclusion, anlotinib did not significantly enhance the anti-tumor effects of osimertinib in osimertinib-sensitive NSCLC cell lines or a zPDX model. However, it partially reversed osimertinib resistance. This combination therapy may improve the outcomes of patients with advanced NSCLC showing osimertinib-resistance.

Keywords: Lung cancer; osimertinib; anlotinib; zebrafish patient-derived xenograft (zPDX)

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Introduction

Lung cancer contributes significantly to the global burden of disease and is one of the main causes of death worldwide (1). Non-small cell lung cancer (NSCLC) is a major pathological type of lung cancer, and most patients present with advanced-stage NSCLC at the time of diagnosis. The emergence of targeted therapies has significantly improved the prognosis of patients

with NSCLC with oncogenic driver mutations, such as mutations of the epidermal growth factor receptor (*EGFR*), anaplastic lymphoma kinase, and Kirsten rat sarcoma viral oncogene homolog (2-4). Osimertinib, a third-generation EGFR-tyrosine kinase inhibitor (TKI), is effective against multiple mutations, including exon 19, exon 21, and T790M mutations (5), and can effectively cross the blood-brain barrier (6). Osimertinib has been confirmed to provide greater survival benefits for patients with *EGFR* mutations than first- and second-generation EGFR-TKIs and is now widely used as a first-line treatment (7,8). However, acquired resistance to osimertinib presents a significant challenge in the treatment of NSCLC. The mechanisms driving this resistance are intricate and involve both EGFR-dependent and EGFR-independent pathways (9-11). Among the EGFR-dependent mechanisms, the *C797S* mutation is particularly common, occurring in approximately 15% of resistant cases in the second-line osimertinib group in the AURA3 trial (12). This mutation impairs the binding of osimertinib to EGFR, thereby reducing its therapeutic efficacy. Other mutations, such as *G796X*, *L792X*, and *T790M* loss, can also hinder drug binding or modify receptor signaling, complicating treatment outcomes (13,14). In contrast, the EGFR-independent pathway is of increasing importance in the study of molecular mechanisms. These include the activation of bypass pathways (such as *c-Met* amplification, *FGFR1* amplification, *HER2* amplification and alteration), fusion of oncogenes (such as *RET*, *ALK*, and *BRAF*), activation of downstream important signaling pathways such as PI3K/AKT/mTOR and RAS/MAPK/ERK, tissue type conversion to small cell lung cancer or squamous cell carcinoma, and epithelial-mesenchymal transformation (EMT) (15,16). Therefore, targeting molecules involved in the regulation of these important target genes and pathways (e.g., cytokines and non-coding RNAs) and epigenetic modifications (e.g., methylation, ubiquitination, and glycosylation) has also emerged as a new strategy to overcome osimertinib resistance (17-22). Briefly, given the diversity of these mechanisms, developing strategies to overcome osimertinib resistance is crucial. Ongoing research into combination therapies shows promise in addressing these challenges and providing more effective treatment options for patients with *EGFR*-mutant NSCLC.

In patients with *EGFR*-mutant NSCLC, combination therapy, including combination chemotherapy, radiotherapy, immunotherapy, and anti-angiogenic drugs, further enhances the therapeutic effect of EGFR-TKIs (23,24). There is an overlap between the vascular EGFR (VEGFR) and EGFR axes (25). Therefore, combination therapies may exert a synergistic anti-tumor effect through the dual inhibition of these two pathways. Recently, clinical studies of first-generation EGFR-TKIs combined with anti-vascular drugs have shown improved progression-free survival (PFS) (26-28), whereas second-generation EGFR-TKIs combined with anti-vascular drugs do not appear to enhance PFS in individuals with *EGFR*-mutant NSCLC (29,30). The effectiveness of third-generation EGFR-TKIs combined with anti-angiogenic therapy for prolonging survival in patients with *EGFR*-mutant NSCLC remains a contentious issue. The RAMOSE trial revealed a significant improvement in PFS for patients receiving the combination of osimertinib and ramucirumab, with a median PFS of 24.8 months compared with a PFS of 15.6 months achieved with indivdual treatment (31). However, the WJOG9717L study found that the combination of osimertinib and bevacizumab did not demonstrate a similar synergistic effect (32).

Anlotinib is a new anti-angiogenic drug that is administered orally; therefore, it is more convenient to

Highlight box

Key findings

- Anlotinib did not significantly enhance osimertinib's anti-tumor effects in osimertinib-sensitive non-small cell lung cancer (NSCLC) models.
- Anlotinib partially reversed osimertinib resistance in resistant cell lines and a patient-derived xenograft model.

What is known and what is new?

- Acquired resistance to osimertinib is a major challenge in epidermal growth factor receptor (*EGFR*)-mutant NSCLC treatment.
- Combining EGFR-tyrosine kinase inhibitors with anti-angiogenic drugs has shown promise in some studies.
- Anlotinib reverses osimertinib resistance in both *in vitro* and *in vivo* models, including patient-derived xenografts.
- Combination therapy is particularly effective in osimertinib-resistant settings but offers limited benefits in osimertinib-sensitive tumors.
- A zebrafish patient-derived xenograft (zPDX) model effectively mimicked clinical response to osimertinib treatment.

What is the implication, and what should change now?

- Clinical trials should evaluate anlotinib-osimertinib combination in patients with NSCLC showing osimertinib resistance.
- Further research is needed to elucidate resistance reversal mechanisms and identify predictive biomarkers.
- zPDX models should be explored for personalized NSCLC treatment strategies.

use than existing injectable drugs such as bevacizumab. Anlotinib has been licensed in China as a third-line treatment for refractory advanced NSCLC based on research demonstrating its potent anti-angiogenic and anti-tumor properties (33,34). As a multitarget receptor TKI, anlotinib has multiple targets, including VEGFR [1–3], fibroblast growth factor receptor (FGFR; 1–4), platelet-derived growth factor receptor-α/β, and c-Kit (35). Owing to the multitarget inhibitory effect of anlotinib, it may exert synergistic anti-cancer effects when combined with EGFR-TKIs. Recent studies have shown that anlotinib can overcome acquired resistance to gefitinib through the EGFR, FGFR1, and VEGFR2 pathways in patients with NSCLC (36-38). However, few studies have investigated whether the combination of osimertinib and anlotinib can achieve better therapeutic effects than single-agent therapy or delay osimertinib resistance. In our previous research, based on *in vitro* cell lines and a mouse xenotransplantation model, we found that anlotinib reverses osimertinib resistance by inhibiting EMT and angiogenesis in NSCLC (39). However, owing to the limitations of the study model, the heterogeneity and complexity of a single patient's tumor could not be fully captured.

Therefore, this study aimed to compare the anti-tumor impact of anlotinib combined with osimertinib and osimertinib alone using *in vitro* experiments and a zebrafish patient-derived xenograft (zPDX) model to elucidate the anti-tumor activity of osimertinib and the impact of anlotinib in reversing osimertinib resistance. This strategy allowed us to better model the genetic diversity and drug response of actual human tumors, providing a more effective guide for evaluating the efficacy of combination therapy. We present this article in accordance with the ARRIVE and MDAR reporting checklists (available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-24-759/rc).

Methods

Reagents

Osimertinib (S7297) and anlotinib (S8726) were obtained from Selleck Chemicals (Houston, TX, USA). Osimertinib and anlotinib were initially dissolved in dimethyl sulfoxide (DMSO) and water, respectively, and were further diluted to the desired concentrations. 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT; 3580GR005) was purchased from BioFroxx (Einhausen, Germany). Cell Counting Kit-8 (CCK-8) solution (MA0218) was purchased from Meilunbio (Dalian, China).

Primary sample acquisition and processing

Between January 2022 and January 2023, malignant pleural effusion samples were obtained from 11 patients with lung adenocarcinoma from the Respiratory Department at Jinling Hospital, Nanjing, China. These patients had EGFR mutations and were being treated with osimertinib or had been previously treated with osimertinib. Primary tumor cells were isolated from the pleural effusions using centrifugation, Hank's Balanced Salt Solution (HBSS) flushing, and Liberase enzyme digestion and the cells were collected through a 70-μm filter membrane. The study was approved by the Ethics Committee of Jinling Hospital, Affiliated Hospital of Medical School, Nanjing University (No. DBNJ20228) and performed in accordance with the Declaration of Helsinki (as revised in 2013). All patients provided written informed consent.

Cell culture

Lung adenocarcinoma cell lines (H1975 and PC9) were obtained from the cell bank of the Chinese Academy of Science and validated using short tandem repeat profiling. Both cell lines and isolated primary lung cancer cells were cultured in Roswell Park Memorial Institute 1640 supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA). All cells were grown in a 5% CO_2 atmosphere at 37 ℃.

Generation of osimertinib-resistant NSCLC cell lines

For screening, the PC9 and H1975 cell lines were cultured in a medium containing osimertinib solution. The initial concentration of osimertinib for the treatment of H1975 and PC9 cells was 1 nM. The cells were then transferred to drug-free media and the exponential growth cells were screened for drug-resistant cell lines. Gradually, the concentration of osimertinib was increased to 1 μM. The whole process lasted approximately 6 months, and we obtained osimertinib-resistant cell lines H1975-OR and PC9-OR.

Cell viability assay

For the MTT test, 3,000 cancer cells per well were planted in 96-well plates. Following 48 h of incubation at 37 ℃

and a 5% CO_2 atmosphere, the medium was replaced with different concentrations of osimertinib solution, which was dissolved in the complete medium. After incubation in 5% CO_2 at 37 ℃ for 72 h, the MTT (5 mg/mL) solution was added to the sample wells (10 µL/well) and cultivated for 2–4 h. Then, the medium from each well was removed, and 150 µL DMSO solution was added to each well and shaken on a horizontal shaker for 15 min. The absorbance of each well was subsequently measured at 490 nm.

Cell proliferation assay

Cancer cells pre-treated with osimertinib or anlotinib for 72 h were seeded in 96-well plates (2,000 cells/well), and the CCK-8 assay was then performed. After adding the CCK-8 solution (10 µL/well) and incubating the plates in 5% CO_2 at 37 ℃ for 2 h, the absorbance was measured at 450 nm. Following cell culture, the absorbance values were recorded at 450 nm at 0, 24, 48, 72, and 96 h.

Determining the maximum tolerated dose (MTD) of anti-tumor drugs in zebrafish embryos

Wild-type zebrafish embryos were randomly seeded in 6-well plates (15 embryos/well) at 48 h post-fertilization (hpf) and then incubated at 34 ℃ for 24 h. Fresh medium was used as the control group, whereas zebrafish embryos were soaked in different concentrations of osimertinib or anlotinib and cultured at 34 ℃ for 72 h. During this process, the survival rate of the zebrafish embryos and toxicity (such as edema, malformation, and bending) were recorded daily to ascertain the MTD of the anti-tumor drugs in zebrafish. In cases where a suitable drug concentration could not be determined in the first round, the concentration range of the drug test was narrowed, and another round of testing was performed until the drug concentration was determined.

Establishment of a zebrafish xenograft model

Staining of tumor cells

At room temperature, tumor cells obtained from the pleural fluid samples as mentioned above, were stained with moderate fluorochrome CellTracker™ CM-Dil (Invitrogen, Carlsbad, CA, USA), which was pre-dissolved in DMSO (1 µg/µL), for 5 min at 37 ℃, and the cells were subsequently incubated at 4 ℃ for 15 min. Lastly, the stained tumor cells were washed three times with HBSS and resuspended in 5–20 µL of HBSS for subsequent injection.

Injection of tumor cells

At 48 hpf, wild-type zebrafish embryos were fixed on low-melting-point agar plates. Thereafter, the stained tumor cell suspension was injected into the yolk space of the zebrafish at an injection volume of 400 cells/embryo.

Drug soaking

Zebrafish embryos injected with tumor cells were incubated at 34 ℃ for 24 h. The embryos were observed under a microscope at 1 day post-injection (dpi). Zebrafish embryos that were successfully transplanted and had relatively uniform tumor cell size were selected for further drug soaking. Zebrafish embryos were randomly divided into four groups (<25 embryos per group) and soaked in one of the following mediums: a blank control medium, osimertinib, anlotinib, or osimertinib combined with anlotinib. The medium concentration was determined based on the previously determined MTD. Embryos were incubated in 6-well plates at 34 ℃. The culture solution containing the drug was replenished every 24 h and administered continuously for 3 days.

Fluorescence imaging and data processing

At 4 dpi, zebrafish embryos were fixed with low-melting-point glue, and fluorescence imaging was performed. The red fluorescent regions labeled with CM-DiI dye were photographed and recorded using a stereo microscope (MVX10, Olympus, Tokyo, Japan) at a resolution of 1,600×1,200. ImageJ software (version 1.46; National Institutes of Health, Bethesda, MD, USA) was used for image processing and quantifying the red fluorescent region of the yolk and the trunk of the zebrafish embryos (indicating tumor cell proliferation and migration in zebrafish). The animal experiments were performed under a project license (No. DZJSSDWLS240084) granted by the Animal Ethics Committee of Jinling Hospital, Affiliated Hospital of Medical School, Nanjing University, in compliance with national guidelines for the care and use of animals.

Statistical analysis

GraphPad Prism (version 9.0) was used for statistical analyses. Data are represented as the mean ± standard error of the mean. The Student's *t*-test or two-way analysis of variance was employed to compare differences between groups, and nonlinear regression analysis was used to

calculate the half-maximal inhibitory concentration (IC_{50}) values of drugs. Every experiment was independently performed at least three times. Statistical significance was set at *, P<0.05, **, P<0.01, and ***, P<0.001.

Results

Construction of osimertinib-resistant H1975-OR and PC9-OR cell lines

H1975 is a lung adenocarcinoma cell line carrying exon 21 L858R or exon 20 T790M mutations, and PC9 is an NSCLC cell line carrying an exon 19 deletion. Both cell lines are common lung adenocarcinoma cell lines that are sensitive to osimertinib. We generated osimertinib-resistant H1975-OR and PC9-OR cells by continuously treating parental H1975 and PC9 cells with osimertinib. The IC_{50} values of osimertinib in the associated cells were determined using the MTT assay. The results demonstrated that the IC_{50} values of osimertinib in H1975 and H1975-OR were 0.21 and 3.13 μM, respectively (*Figure 1A*). In addition, the IC_{50} values of osimertinib in PC9 and PC9-OR were 0.33 and 3.63 μM, respectively (*Figure 1B*). These results indicate that the cell lines resistant to osimertinib were successfully constructed. A CCK-8 assay was conducted to identify the proliferation capacities of the parental and drug-resistant cells. Proliferation capacities of the drug-resistant H1975-OR and PC9-OR cells were stronger than those of the parental cell lines H1975 and PC9, respectively (*Figure 1C*,*1D*). In addition, we tested the cellular inhibition ability of anlotinib in these cells and found that the IC_{50} values of anlotinib in H1975 and H1975-OR cells were 3.94 and 2.78 μM, respectively (*Figure 1E*), while the IC_{50} values of anlotinib in PC9 and PC9-OR cells were 3.76 and 3.15 μM, respectively (*Figure 1F*). Considering that the IC_{50} values of anlotinib were similar in H1975, H1975-OR, PC9, and PC9-OR, we selected 3 μM anlotinib as the concentration for subsequent *in vitro* experiments.

Combined effect of anlotinib and osimertinib in vitro

To detect the anti-cancer effects of anlotinib and osimertinib combination therapy in H1975, H1975-OR, PC9, and PC9-OR cells, a CCK-8 cell proliferation test was conducted to detect the changes in cell proliferation after treatment with osimertinib alone, anlotinib alone, and osimertinib combined with anlotinib. The results show that in the parent cell lines H1975 (*Figure 2A*) and PC9 (*Figure 2B*), the combination of osimertinib and anlotinib did not produce a more significant anti-tumor effect than osimertinib alone. However, in H1975-OR (*Figure 2C*) and PC9-OR (*Figure 2D*) cells, anlotinib and osimertinib combination therapy inhibited the proliferation of drug-resistant cells more than osimertinib or anlotinib monotherapy.

In addition, in H1975-OR (*Figure 2E*) and PC9-OR (*Figure 2F*) cells, the IC_{50} values of osimertinib were significantly reduced when combined with anlotinib (IC_{50}: 0.77 μM in H1975-OR; IC_{50}: 1.05 μM in PC9-OR). This suggests that anlotinib partially reversed osimertinib resistance in H1975-OR and PC9-OR cells.

Determining the MTD of osimertinib and anlotinib

Based on the results of our previous research, the MTD of osimertinib in zebrafish was set at 1 μM (40). To determine the MTD of anlotinib, we first set five concentration intervals for screening: 0.025, 0.05, 0.1, 0.2, and 0.4 μM (*Figure 3A*,*3B*). The survival rate and toxicity of wild-type zebrafish embryos at these concentrations were observed for 3 days. The results showed that at 0.1 μM, all zebrafish embryos remained viable and did not show toxic manifestations, such as edema and distortion. However, at 0.2 μM, although there were no signs of toxicity, zebrafish embryos began to die, and the survival rate was only 53.3% at the end of the experiment (*Figure 3A*,*3B*). Therefore, we selected 0.1 μM as the MTD of anlotinib for subsequent zebrafish animal experiments. In addition, the combination of 1 μM osimertinib and 0.1 μM anlotinib in zebrafish embryos did not worsen the survival rate or toxicity, demonstrating that the combined treatment was tolerated in zebrafish embryos.

Anlotinib reverses osimertinib resistance in zebrafish xenograft models

In vitro cell experiments showed that anlotinib reversed osimertinib resistance in H1975-OR and PC9-OR cells. Next, we verified these results in zebrafish xenograft models. The above-mentioned cell lines were injected into zebrafish to establish tumor xenotransplantation models. At 1 dpi, zebrafish embryos were treated with osimertinib alone, anlotinib alone, or osimertinib combined with anlotinib and soaked at the MTD determined in the previous screening. The results showed that in the zebrafish xenograft models, osimertinib monotherapy markedly

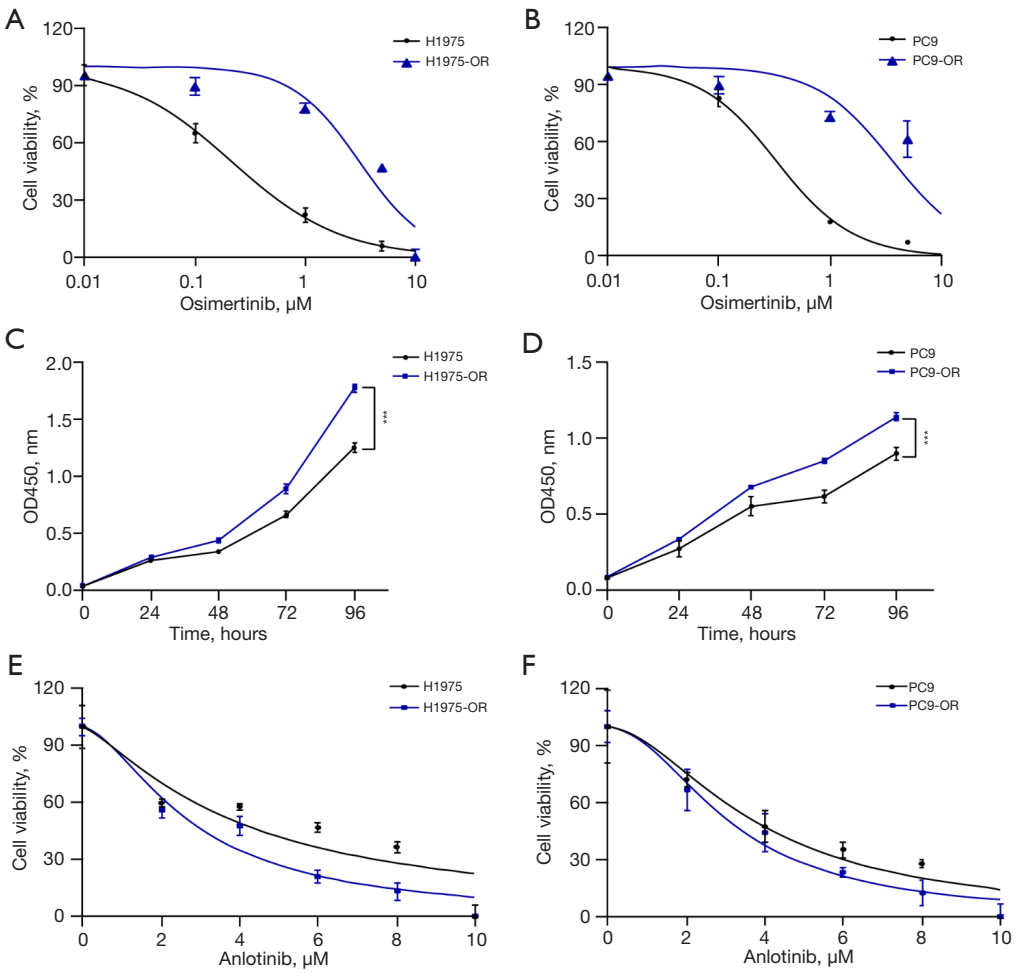


Figure 1 Osimertinib-resistant NSCLC cell lines were established, and the IC_{50} values of osimertinib and anlotinib were determined. An MTT assay was used to explore cell viability in (A) H1975 and H1975-OR cells and (B) PC9 and PC9-OR cells treated with different concentrations of osimertinib. A CCK-8 assay was conducted to explore the proliferative capacities of (C) H1975 and H1975-OR cells and (D) PC9 and PC9-OR cells. An MTT assay was conducted to measure cell viability in (E) H1975 and H1975-OR cells and (F) PC9 and PC9-OR cells treated with different concentrations of anlotinib. P values were calculated using two-way ANOVA analysis; ***, P<0.001. OD, optical density; NSCLC, non-small cell lung cancer; IC_{50}, half-maximal inhibitory concentration; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide; CCK-8, Cell Counting Kit-8; ANOVA, analysis of variance.

suppressed the proliferation of the sensitive cell lines H1975 and PC9, but osimertinib and anlotinib combination therapy did not produce a better anti-cancer effect than osimertinib monotherapy (*Figure 4A*). Consistent with the results of *in vitro* cell experiments, compared with anlotinib or osimertinib monotherapy, anlotinib and osimertinib combination therapy significantly inhibited the proliferation of H1975-OR and PC9-OR cells in the animal models (*Figure 4B*). The corresponding bright-field images of the zebrafish xenograft models are displayed in Figure S1.

In addition, we measured the migration of tumor cells in the zebrafish. The results indicated that osimertinib inhibited cell migration significantly in parental H1975 and PC9 cells. Additionally, anlotinib inhibited the migration of tumor cells in the animal models, but the difference was not statistically significant. However, it can still be concluded that the inhibitory effect of the combination therapy did not exceed that of the osimertinib monotherapy group (*Figure 5A*). Moreover, in H1975-OR cells in the zebrafish, neither osimertinib nor anlotinib alone significantly inhibited tumor cell migration, but the combination therapy had a significant inhibitory effect (*Figure 5B*). In PC9-OR

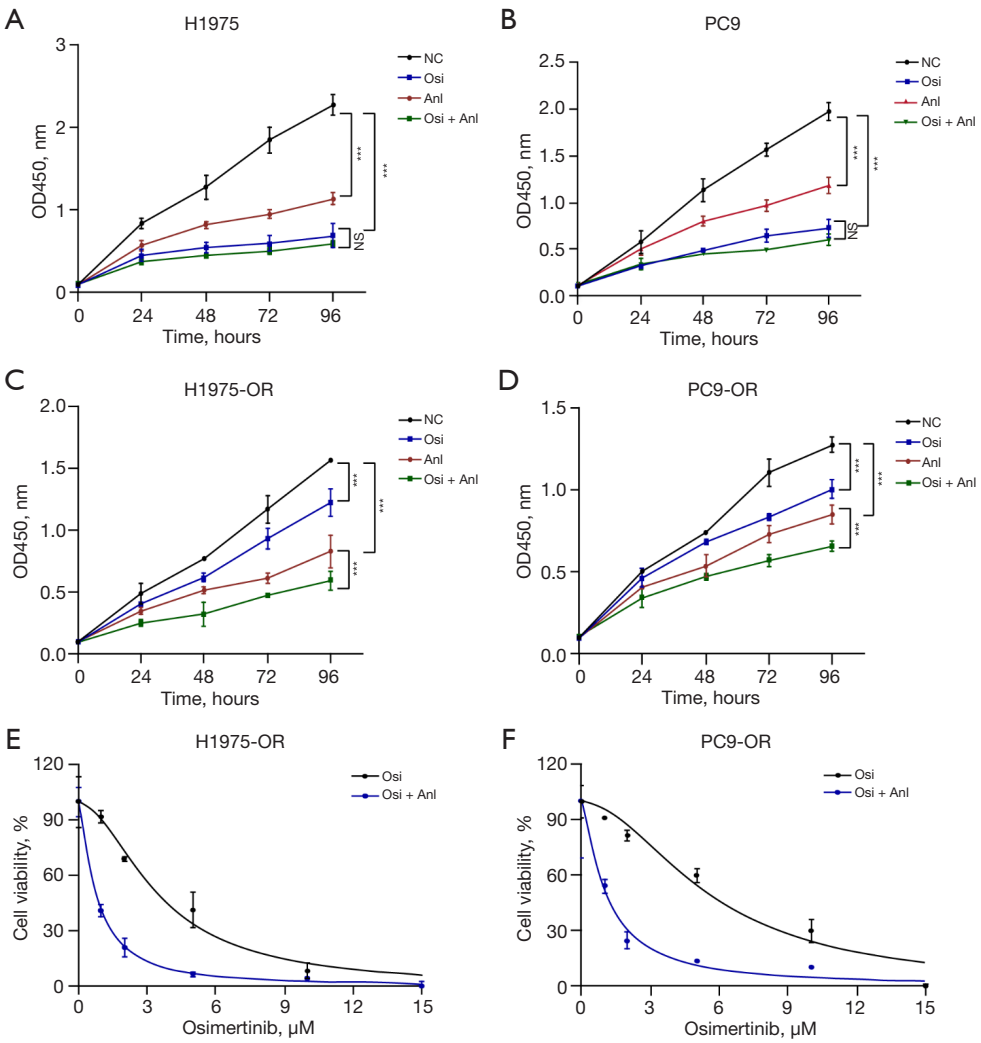


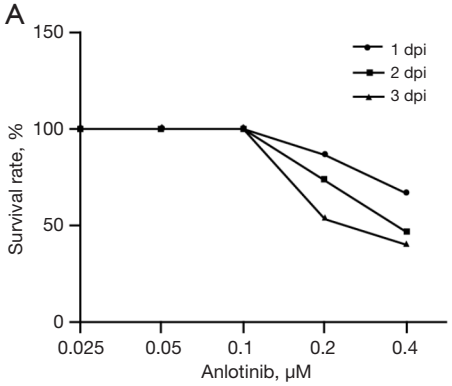
Figure 2 Effects of combination therapy with anlotinib and osimertinib on NSCLC cells *in vitro*. A CCK-8 assay was conducted to explore the proliferative capacities of (A) H1975, (B) PC9, (C) H1975-OR, and (D) PC9-OR cells, pre-treated with the NC, osimertinib monotherapy (1 μM), anlotinib monotherapy (3 μM), and a combination of anlotinib (3 μM) and osimertinib (1 μM) for 72 h. An MTT test was conducted to determine cell viability in (E) H1975-OR and (F) PC9-OR cells treated with different concentrations of osimertinib monotherapy or the combination of anlotinib (3 μM) and osimertinib. P values were calculated by two-way ANOVA analysis; ***, P<0.001; NS, not significant. OD, optical density; NC, negative control; Osi, osimertinib; Anl, anlotinib; Osi + Anl, osimertinib combined with anlotinib; NSCLC, non-small cell lung cancer; CCK-8, Cell Counting Kit-8; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide; ANOVA, analysis of variance.

cells in the zebrafish xenograft model, osimertinib alone did not significantly inhibit cell migration, but both the anlotinib and the combination groups showed significantly inhibited cell migration. However, because few cells migrated in both groups, there was no statistical difference between the two groups (*Figure 5B*). The corresponding bright-field images of the zebrafish xenograft models are displayed in Figure S2. Overall, these results indicate that anlotinib reverses osimertinib resistance *in vivo*.

Clinical data of patients

To further verify drug efficacy in clinical patients, we collected pleural fluid from 11 patients with advanced lung adenocarcinoma to establish a corresponding zPDX model. All included patients had *EGFR* mutations

A



B

Anlotinib, μM	Survival number/total number		
	1 dpi	2 dpi	3 dpi
0.025	15/15	15/15	15/15
0.05	15/15	15/15	15/15
0.1	15/15	15/15	15/15
0.2	13/15	11/15	8/15
0.4	10/15	7/15	6/15

Figure 3 Determination of the MTD of anlotinib in a zebrafish model. (A) Dose-survival rate curves of zebrafish embryos soaked in different concentrations of anlotinib. (B) Summary of the survival number of the zebrafish embryos at each anlotinib concentration after 3 days of anlotinib soaking. dpi, day post-injection; MTD, maximum tolerated dose.

and were previously treated with osimertinib. Basic clinicopathological data are presented in *Table 1*. Based on the Response Evaluation Criteria in Solid Tumors (version 1.1) (41), we assessed the treatment response of the enrolled patients after osimertinib treatment at the time of sample collection and divided the patients into two groups: the sensitive to osimertinib (n=8) and resistant to osimertinib (n=3) groups.

Effects of osimertinib combined with anlotinib in the zPDX model

The pleural effusion samples from the 11 patients were transplanted into zebrafish. After successfully establishing a zPDX model, we first treated the zPDX embryos with osimertinib alone, anlotinib alone, or osimertinib combined with anlotinib and soaked them at the MTD determined in the previous screening. After drug soaking for 3 days, we observed and quantified the red fluorescence expression in zebrafish to measure tumor cell proliferation and migration. Significant differences in cell proliferation and migration in the patient-derived zPDX model indicated that osimertinib was effective and cells were osimertinib-sensitive. If there were no significant differences, osimertinib resistance was concluded. Notably, the migration ability of the patient-derived cells in zebrafish was weak, possibly because the vitality of these tumors *in vitro* was relatively fragile. Therefore, we focused on changes in cell proliferation in the zPDX model. First, we performed consistency analysis between the clinical drug effect in each patient and the corresponding zPDX model drug sensitivity, and the accuracy of the zPDX model for reproducing the anti-cancer effect of osimertinib reached 81.8% (9/11) (*Table 2*). This may be due to the patient's history of taking multiple medications, comorbidities, or the efficacy limitations of the zebrafish model itself. To exclude other interference and more accurately reflect the effect of the combination therapy, we selected nine samples (#1–#9) from patients whose clinical responses were consistent with the therapeutic effect of the zPDX model for follow-up analysis.

In the clinical osimertinib-sensitive group (#1–#6), osimertinib significantly inhibited the proliferation of tumor cells in the zPDX model, while anlotinib monotherapy only showed significant inhibition in three cases, and none of the combined groups achieved anti-cancer effects exceeding that of either osimertinib or anlotinib alone (*Figure 6*, Figure S3, *Table 3*), indicating that anlotinib and osimertinib combination therapy did not have a better anti-cancer effect in the osimertinib-sensitive group than monotherapy. The corresponding bright-field images of the zPDX model are displayed in Figure S4.

Furthermore, in the clinical osimertinib-resistant group (#7–#9), osimertinib had no significant inhibitory effect on tumor cell proliferation. In addition, even though the anlotinib monotherapy group showed a significant anti-tumor effect, a significant anti-tumor effect was demonstrated in the combination therapy group compared with that in the anlotinib monotherapy group (*Figure 7*, Figure S3, *Table 3*). The results indicate that anlotinib somewhat reverses osimertinib resistance in an *in vivo* zPDX model. The corresponding bright-field images of the

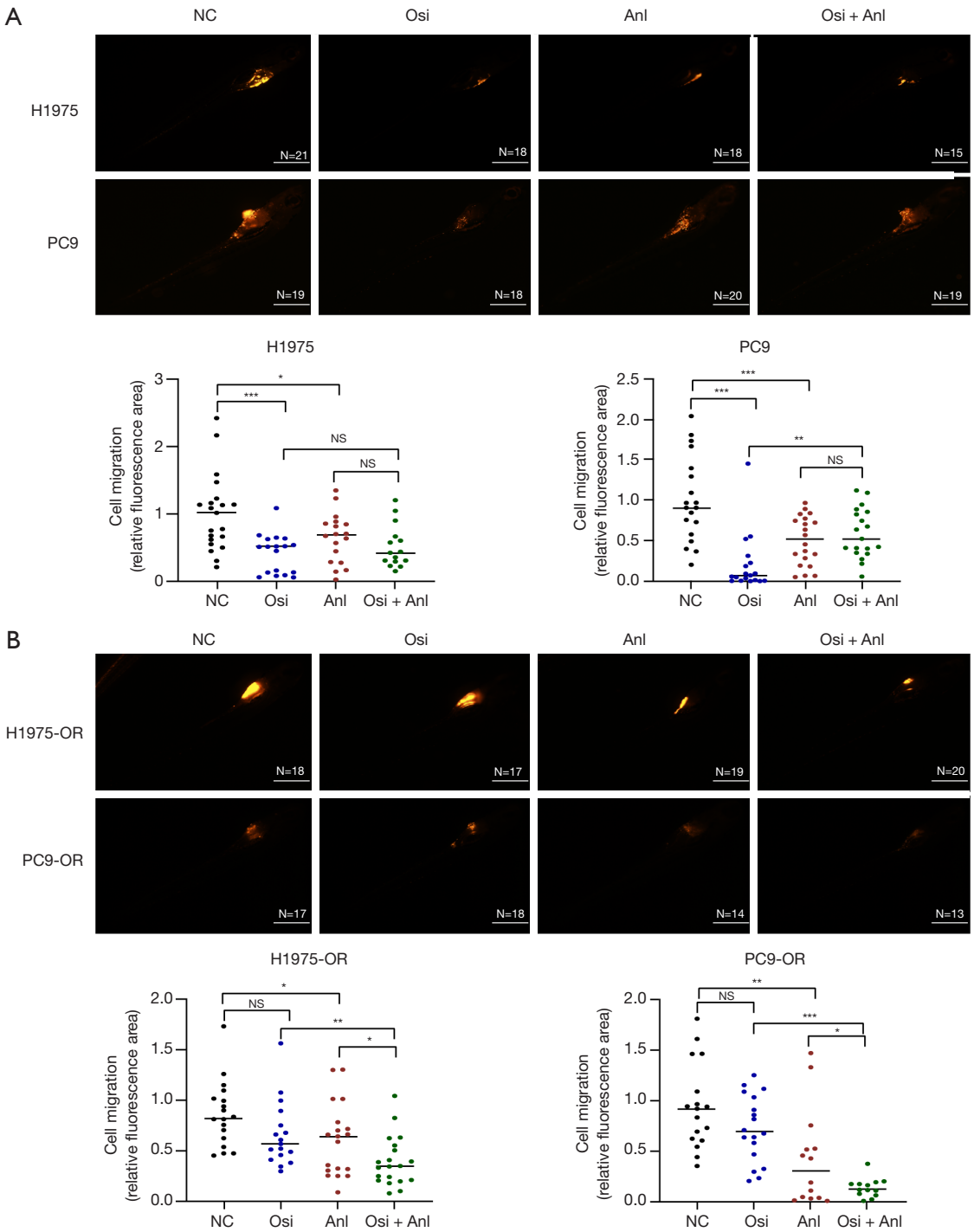


Figure 4 The inhibitory effect of anlotinib combined with osimertinib on NSCLC cell proliferation was investigated *in vivo* in zebrafish xenograft models. (A) Representative images and quantitative analysis of cell proliferation in the H1975 and PC9 zebrafish xenograft models (scale bar, 500 μm). (B) Representative images and quantitative analysis of cell proliferation in the H1975-OR and PC9-OR zebrafish xenograft models (scale bar, 500 μm). The number of xenografts analyzed is indicated in the corresponding images. P values were calculated by unpaired Student's *t*-tests; *, P<0.05; **, P<0.01; ***, P<0.001; NS, not significant. Each data point represents an independent biological replicate. NC, negative control; Osi, osimertinib; Anl, anlotinib; Osi + Anl, osimertinib combined with anlotinib; NSCLC, non-small cell lung cancer.

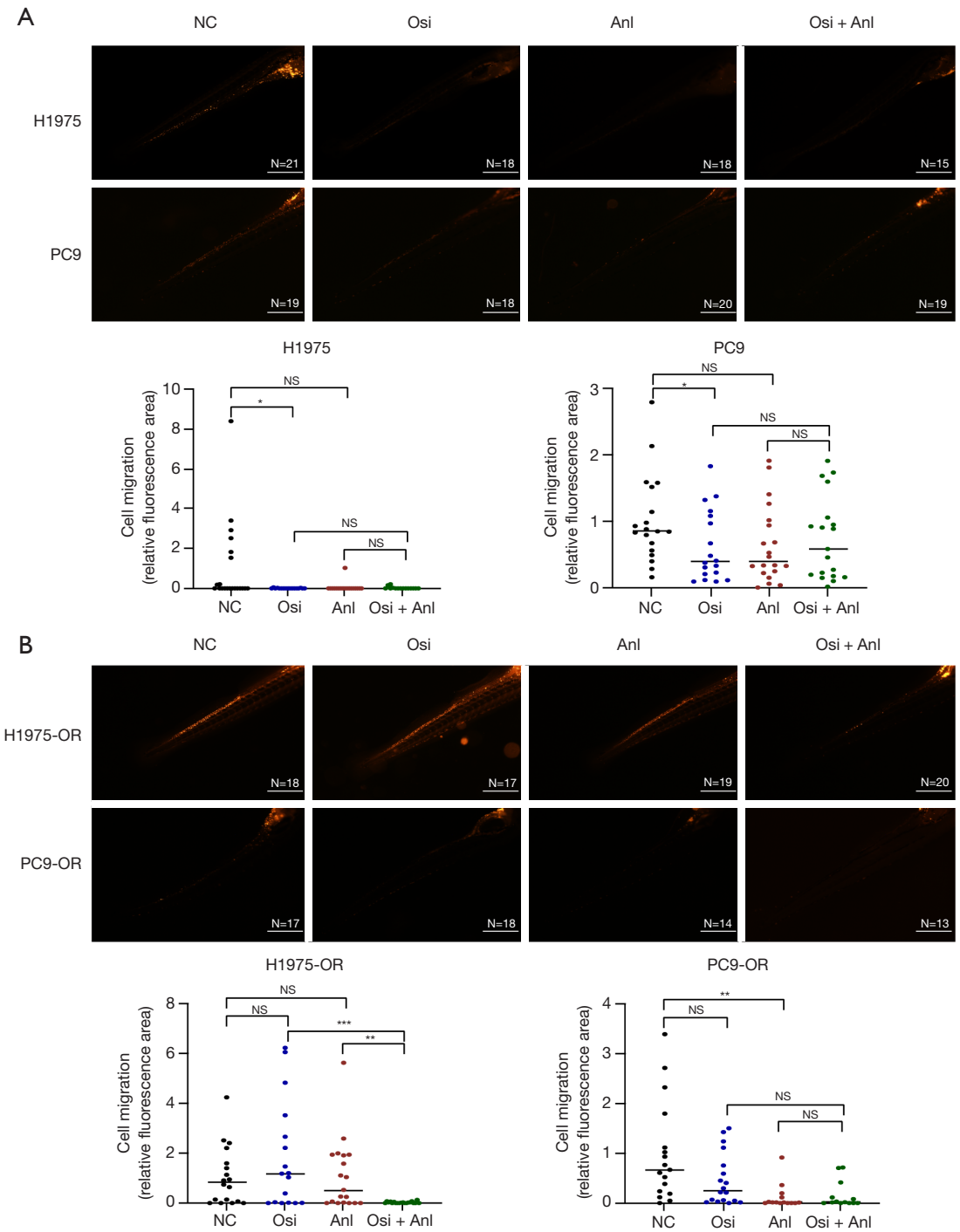


Figure 5 The inhibitory effect of anlotinib combined with osimertinib on NSCLC cell migration was investigated *in vivo* in zebrafish xenograft models. (A) Representative images and quantitative analysis of cell migration in the H1975 and PC9 zebrafish xenograft models (scale bar, 200 μm). (B) Representative images and quantitative analysis of cell migration in the H1975-OR and PC9-OR zebrafish xenograft models (scale bar, 200 μm). The number of xenografts analyzed is indicated in the corresponding images. P values were calculated by unpaired Student's *t*-tests; *, P<0.05; **, P<0.01; ***, P<0.001; NS, not significant. Each data point represents an independent biological replicate. NC, negative control; Osi, osimertinib; Anl, anlotinib; Osi + Anl, osimertinib combined with anlotinib; NSCLC, non-small cell lung cancer.

Table 1 Clinically relevant data of patients

Case No.	Age (years)	Sex	Smoking history	TNM stage	Distant metastasis	EGFR mutation	Co-occurring genetic alterations	Surgical history	Anti-vascular therapy history	Response to osimertinib†
1#	55	Female	No	IVA (T4N2M1a)	Pleural	Exon19 del, Exon20 T790M	–	No	No	S
2#	65	Male	Yes	IVA (T4N0M1a)	Pleural	Exon19 del	TP53	No	No	S
3#	50	Male	Yes	IVA (T2N1M1a)	Pulmonary, pleural	Exon21 L858R	MET amplification	No	No	S
4#	75	Female	No	IVA (T4N3M1b)	Pulmonary, osseous	Exon21 L858R	–	No	No	S
5#	77	Male	Yes	IVB (T4N3M1c)	Pulmonary, pleural, osseous	Exon21 L858R	TP53	No	Yes	S
6#	83	Male	No	IVB (T2N0M1c)	Pleural, osseous	Exon19 del	TP53	Yes	No	S
7#	52	Male	Yes	IVA (T2N2M1a)	Pleural	Exon19 del	MET amplification	No	Yes	R
8#	60	Female	No	IVA (T3N2M1a)	Pleural	Exon19 del, Exon20 T790M	TP53, RB1	No	Yes	R
9#	77	Female	No	IVB (T4N2M1c)	Pleural, osseous, brain	Exon19 del	TP53, MET amplification	Yes	Yes	R
10#	64	Female	No	IVA (T4N3M1a)	Pleural	Exon19 del	MET amplification	Yes	No	S
11#	53	Male	Yes	IVB (T4N0M1c)	Pulmonary, pleural, osseous	Exon21 L858R	MET amplification, ERBB2 amplification	Yes	Yes	S

†, the response status to osimertinib at the time of sample collection. TNM, tumor-node-metastasis; *EGFR*, epidermal growth factor receptor; S, sensitive to osimertinib; R, resistant to osimertinib.

Table 2 Consistency analysis between clinical responses and zPDX responses to osimertinib treatment

zPDX responses	Clinical responses	
	S	R
S	6	0
R	2	3

Data are presented as number. zPDX, zebrafish patient-derived xenograft; S, sensitive to osimertinib; R, resistant to osimertinib.

model are displayed in Figure S5.

Discussion

Acquired resistance to osimertinib poses a significant challenge in clinical practice, with no current standardized treatment for patients with NSCLC and osimertinib resistance. A viable strategy for augmenting treatment efficacy and reducing drug resistance is the combination of anti-vascular drugs. The present study examined the effectiveness of anlotinib coupled with osimertinib in treating patients with NSCLC, particularly those resistant to osimertinib. We conducted *in vitro* cell and *in vivo* zebrafish xenograft experiments. Our results suggested that the synergistic anti-tumor effect of osimertinib and anlotinib combination therapy is limited in osimertinib-sensitive tumors; however, anlotinib appears to reverse osimertinib resistance in tumors.

Compared to first- and second-generation EGFR-TKIs, osimertinib has a stronger anti-cancer effect. However, there is no consensus on whether the combination of osimertinib and anti-vascular drugs can prolong the survival of patients with NSCLC. Liu *et al.* and Yu *et al.* indicated that apatinib and ramucirumab can greatly enhance the anti-tumor effect of osimertinib in patients with NSCLC with *EGFR*

Table 3 Summary of patient-derived zPDX treatment outcomes

Group	Case No.	zPDX (cell proliferation), P value				Whether the combination treatment group achieved better anti-cancer effects than the monotherapy group
		NC *vs.* Osi	NC *vs.* Anl	Osi *vs.* Osi + Anl	Anl *vs.* Osi + Anl	
Clinical Osi-sensitive	1#	0.001***	0.65	0.72	0.06	No
	2#	0.002**	0.39	0.31	0.47	No
	3#	0.001**	0.07	0.45	0.29	No
	4#	0.046*	0.001**	0.10	0.99	No
	5#	0.001***	0.02*	0.04*	0.17	No
	6#	0.03*	0.01*	0.64	0.63	No
Clinical Osi-resistant	7#	0.86	0.80	0.02*	0.047*	Yes
	8#	0.76	0.09	0.10	0.03*	Yes
	9#	0.18	0.044*	0.006**	0.04*	Yes

*, P<0.05; **, P<0.01; ***, P<0.001. zPDX, zebrafish patient-derived xenograft; NC, negative control; Osi, osimertinib; Anl, anlotinib; Osi + Anl, osimertinib combined with anlotinib.

T790M mutations (42,43). Conversely, several other clinical studies have shown that osimertinib plus bevacizumab do not significantly prolong PFS or overall survival (OS) in patients with NSCLC with *EGFR* T790M mutations (44-46). The reasons for this phenomenon remain unclear. Subgroup analysis suggested that smoking history and concomitant mutations (particularly *TP53*) may enhance the efficacy of combination therapy, whereas a history of anti-angiogenic drug therapy may reduce the efficacy of combination therapy (44-46). When osimertinib is used as a first-line treatment for *EGFR*-mutant NSCLC, its combination with ramucirumab has been shown to extend PFS (31). In contrast, the combination of osimertinib with bevacizumab did not demonstrate a similar improvement in PFS (32). Besides the differences in drug combinations, the duration of exposure to anti-angiogenic agents may also contribute to the disparity in outcomes. Unlike bevacizumab and ramucirumab, which are macromolecular monoclonal antibodies, anlotinib is a small-molecule TKI. Anlotinib monotherapy can prolong the OS of patients with NSCLC with *EGFR* mutations (47). As nilotinib has more targets and crosses multiple downstream pathways of *EGFR*, its combination with osimertinib may enhance its efficacy of osimertinib (48,49). A multicenter retrospective analysis by Chen *et al.* of 268 osimertinib-resistant patients with NSCLC with the *EGFR* T790M mutation showed that subsequent anlotinib therapy achieved a more significant survival benefit (median OS: 12.18 months) (50). Another study involving 33 patients with NSCLC with *EGFR* T790M mutations showed that combination therapy resulted in good clinical outcomes (median OS: 23.8 months) (51). Additionally, a retrospective analysis by Wang *et al.*, which included 34 osimertinib-resistant patients with advanced NSCLC, showed that osimertinib combined with anlotinib was effective in treating osimertinib-resistant NSCLC (median OS: 19.0 months) (52). In 2023, Lei *et al.* found that osimertinib and anlotinib combination therapy restored osimertinib sensitivity in osimertinib-resistant cell lines and a mouse model (53). Mechanistically, anlotinib counteracts osimertinib resistance in patients with NSCLC by deactivating the c-MET/MYC/AXL pathway, thereby enhancing the anti-tumor effect of the latter (53).

However, in the aforementioned study, the authors focused on *in vitro* cells and *in vivo* experiments with a mouse xenotransplantation model derived from osimertinib-resistant cells, but the relevant phenomena have not been confirmed in corresponding clinical samples. With this context, in the current study, pleural effusion samples of patients with *EGFR*-mutant NSCLC were collected to establish a zPDX model, and the effect of combined treatment of anlotinib and osimertinib was described more from the perspective of clinical samples. Herein, the combination of anlotinib with osimertinib achieved similar outcomes. In *in vitro* and *in vivo* (zPDX model) studies, anlotinib did not strengthen the anti-tumor effect of osimertinib in parent H1975 and PC9 cells, possibly due

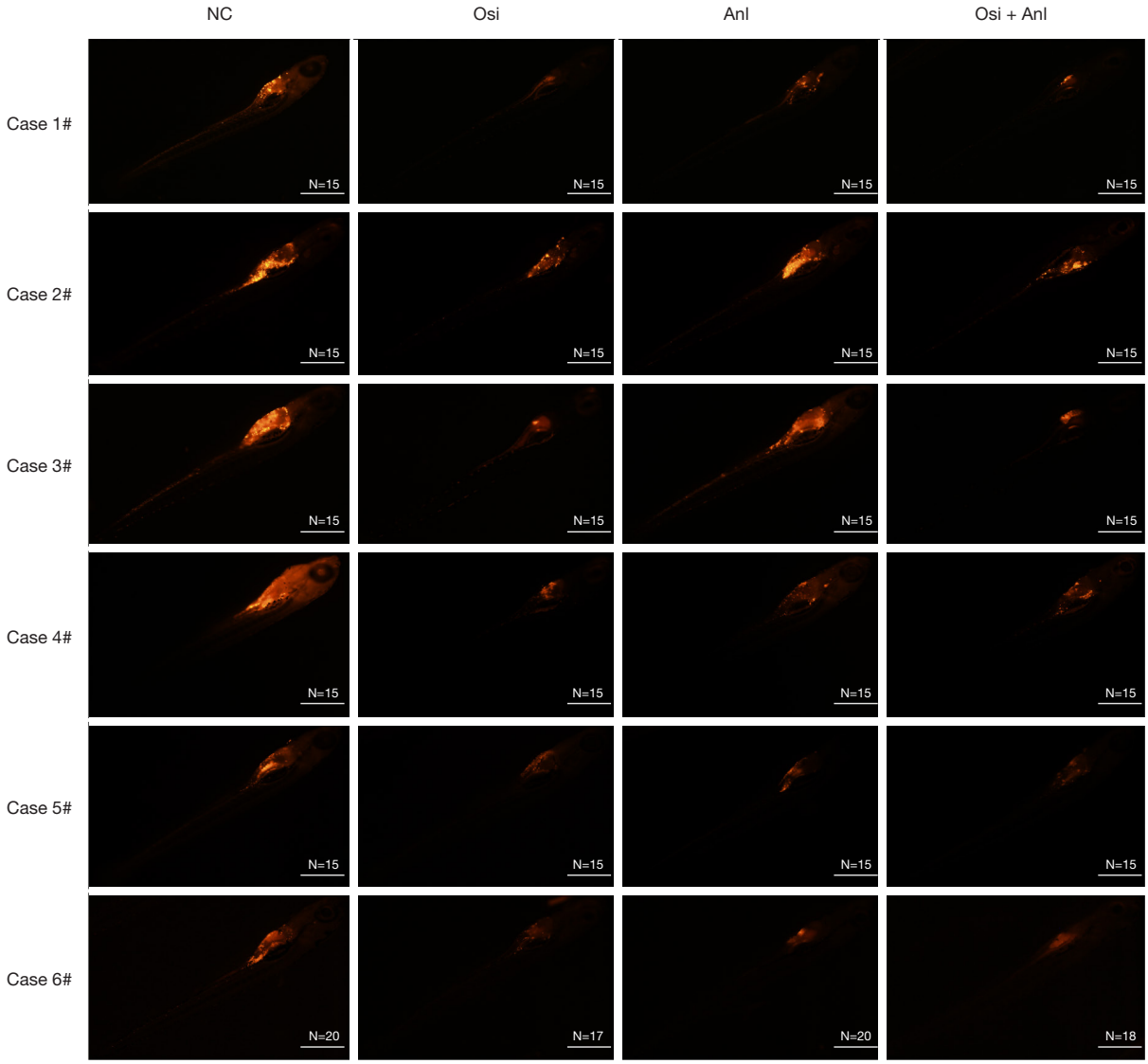


Figure 6 Representative images of cell proliferation in a zPDX model derived from patients who are clinically sensitive to osimertinib (scale bar, 500 μm). The number of xenografts analyzed is indicated in the corresponding images. NC, negative control; Osi, osimertinib; Anl, anlotinib; Osi + Anl, osimertinib combined with anlotinib; zPDX, zebrafish patient-derived xenograft.

to the already potent anti-tumor impact of osimertinib, wherein the additional advantages of combining anti-angiogenic drugs failed to surpass the statistical significance threshold. However, the combination of anlotinib and osimertinib exhibited a good anti-tumor and safety effect in H1975-OR and PC9-OR cell lines, suggesting that anlotinib can partially reverse osimertinib resistance.

Genetic mutations, drug history, and complications in patients with cancer in the real world result in tumors being more complex than those *in vitro*. Therefore, we collected primary tumor cells from patients with *EGFR*-mutant NSCLC who had received osimertinib therapy to establish a xenograft model and further validate our findings. Due to its high efficiency and sensitivity, zPDX is an important animal model for drug screening (54). Therefore, in this study, we investigated the impact of combination treatment in patients with *EGFR*-mutant NSCLC using a zPDX model. According to our research, the accuracy of the zPDX model in reflecting the response of patients to osimertinib was 81.8%, which is consistent with the

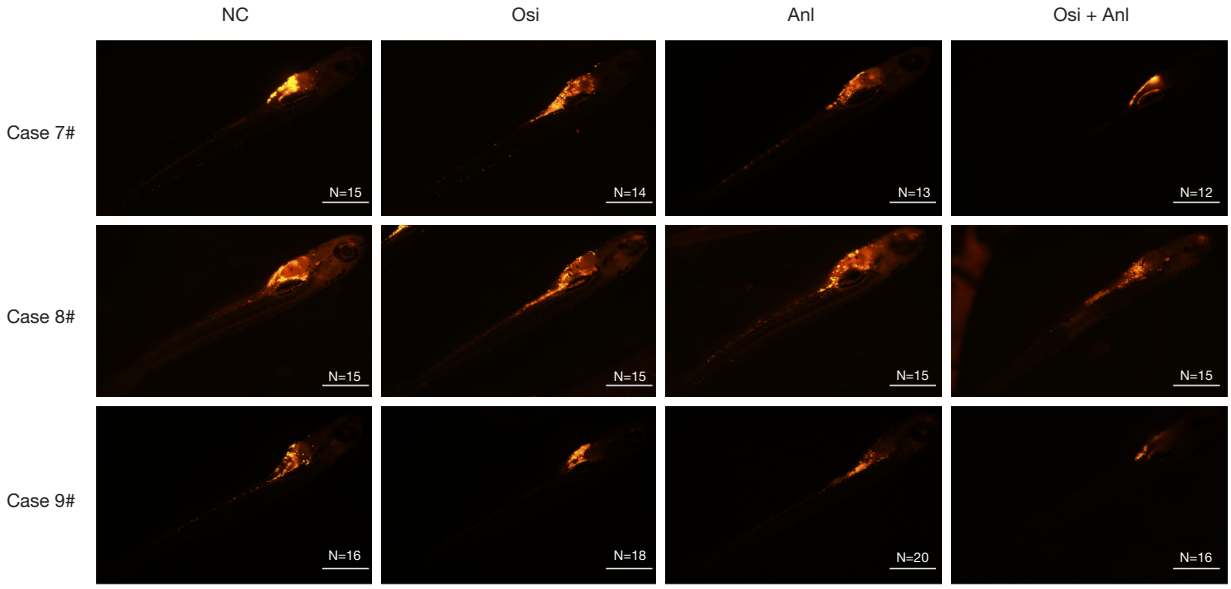


Figure 7 Representative images of cell proliferation in a zPDX model derived from patients who are clinically resistant to osimertinib (scale bar, 500 μm). The number of xenografts analyzed is indicated in the corresponding images. NC, negative control; Osi, osimertinib; Anl, anlotinib; Osi + Anl, osimertinib combined with anlotinib; zPDX, zebrafish patient-derived xenograft.

accuracy observed in previous zPDX studies (75–91%) (40,55,56). Understandably, the zebrafish model cannot fully replicate the drug effect that occurs in clinical patients due to many factors. For example, converting the clinical drug dose to a soaking drug dose is difficult, and there are certain antagonistic or synergistic anti-cancer effects of therapeutic drugs in patients who have received multiple drug therapies. Overall, despite these limitations, the zPDX model is highly reliable and accurately replicates patient treatment responses.

Notably, no synergistic anti-tumor effects of osimertinib combined with anlotinib were observed in the zPDX model derived from patients who were clinically sensitive to osimertinib. Of the patients who were clinically resistant to osimertinib, only one corresponding zebrafish (8#) did not benefit from anlotinib and osimertinib combination therapy over osimertinib monotherapy. The animal was insensitive to both osimertinib and anlotinib; although the combination therapy exhibited better anti-tumor activity than the anlotinib monotherapy group, its effects were not significantly different compared with those of the osimertinib monotherapy. In the future, more zebrafish should be used in each group to improve the reliability of the test. Furthermore, a history of anti-vascular therapy may affect the therapeutic effect of anlotinib because prolonged use of anti-vascular agents can reduce the number of blood vessels in the tumor and affect drug perfusion. In addition, the mechanism underlying osimertinib resistance is complex, and if shared downstream pathways of osimertinib and anlotinib are not involved, the addition of anlotinib may have limited benefits.

There are several limitations in this study. First, the sample size of this study was limited, which made it difficult to conduct further subgroup analyses to determine which sensitive and resistant populations may benefit from combination therapy, especially for patients with complex conditions such as multi-site distant metastasis or multitarget mutations. Therefore, a larger sample size is required to explore the efficacy and safety of this combination therapy. Second, interactions between anlotinib and specific molecular mechanisms underlying osimertinib resistance require further research. Lastly, the osimertinib-resistant H1975 and PC9 cells used in this experiment were gradually screened through prolonged exposure to the drug in culture, and factors such as repeated freeze-thaw cycles and continuous drug exposure may have contributed to the observed increase in their baseline IC_{50} values against osimertinib, which are higher than those reported in some previous studies (57,58). Irrespective of these limitations, the findings of this study provide an assessment of the benefits and limitations associated with combination therapy and a foundation for more treatment

options for patients with lung cancer. More importantly, the current positive results suggest that prophylactic combination therapy with anlotinib may delay the development of resistance in patients with EGFR-mutated NSCLC receiving osimertinib as first-line treatment. Such validation will require additional preclinical and clinical patient data from first-line combination therapy trials. In addition, more future studies are needed to assess whether combination therapy leads to an increase in therapeutic toxicity, and determine the optimal combination dosing (59).

Conclusions

In summary, this study demonstrated that the effectiveness of combination therapy with anlotinib and osimertinib was limited in osimertinib-sensitive tumors; however, anlotinib reversed osimertinib resistance in patients with NSCLC to some extent. This research offers novel ideas and clinically relevant experimental evidence for the reversal of osimertinib resistance.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE and MDAR reporting checklists. Available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-24-759/rc

Data Sharing Statement: Available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-24-759/dss

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-24-759/coif). Y.S. serves as an Editor-in-Chief of *Translational Lung Cancer Research*. The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Our study was performed in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of Jinling Hospital, Affiliated Hospital of Medical School, Nanjing University (No. DBNJ20228) and informed consent was obtained from all patients. The animal experiments were performed under a project license (No. DZJSSDWLS240084) granted by the Animal Ethics Committee of Jinling Hospital, Affiliated Hospital of Medical School, Nanjing University, in compliance with national guidelines for the care and use of animals.

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