Triple therapy with osimertinib, bevacizumab and cetuximab in EGFR-mutant lung cancer with HIF-1α/TGF-α expression

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Abstract. Osimertinib, a third generation epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, is the standard treatment for patients with lung cancer harboring EGFR T790M; however, acquired resistance is inevitable due to genetic and epigenetic changes in cancer cells. In addition, a recent randomized clinical trial revealed that the combination of osimertinib and bevacizumab failed to exhibit superior progression-free survival compared with osimertinib alone. The present study aimed to investigate the effect of triple therapy with osimertinib, bevacizumab and cetuximab in xenograft tumors with different initial tumor volumes (conventional model, 200 mm^3 and large model, 500 mm^3). The results demonstrated that osimertinib significantly inhibited tumor growth in both the conventional and large models; however, maximum tumor regression was attenuated in the large model in which hypoxia-inducible factor-1α (HIF-1α) and transforming growth factor-α (TGF-α) expression levels increased. Although the combination of osimertinib and bevacizumab exerted a greater inhibitory effect on tumor growth compared with osimertinib in the conventional model, the effect of this combination therapy was attenuated in the large model. TGF-α attenuated sensitivity to osimertinib *in vitro*; however, this negative effect was counteracted by the combination of osimertinib and cetuximab, but not osimertinib and bevacizumab. In the large xenograft tumor model, the triple therapy induced the greatest inhibitory effect on tumor growth compared with osimertinib alone and its combination with bevacizumab. Clinical trials of the triple therapy are required for patients with lung cancer with EGFR mutations and HIF-1α/TGF-α.

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Introduction

Non-small cell lung cancer (NSCLC) is one of the deadliest diseases worldwide; however, its prognosis has been improved by the discovery of driver oncogenes and the development of corresponding molecular targeted therapies. In particular, epidermal growth factor receptor (*EGFR*) gene mutations are the most frequent driver mutations in never-smokers or individuals with Asian ethnicity. EGFR tyrosine kinase inhibitors (TKIs) are standard therapies for patients with lung cancer harboring EGFR mutations (1,2); however, their inhibitory effects are insufficient to achieve complete remission and acquired resistance usually develops within two years (2). The third-generation EGFR-TKI osimertinib is the standard of care for patients with NSCLC harboring EGFR T790M, which is the most common mechanism of resistance for first- or second-generation EGFR-TKIs. Although osimertinib has been approved to treat patients with untreated EGFR-mutant lung cancers (3,4), resistance is ultimately inevitable (5).

Multiple factors have been reported to negatively impact the progression-free survival or time-to-treatment failure of EGFR-TKIs, such as co-occurring gene mutations, tumor mutation burden, pre-existing clonal MET amplification, or HER2 expression (6-9). Studies have also demonstrated that clinical characteristics like tumor volume (10) or cavity wall thickness (11) correlate negatively with EGFR-TKI efficacy. Consequently, therapies combining EGFR-TKIs and other agents could be more effective than EGFR-TKI monotherapy in EGFR-mutant lung cancer with negative predictive factors.

Combination therapies aiming to achieve the deep remission of EGFR-mutant lung cancers have been an active area of investigation, with clinical trials demonstrating the benefits of EGFR-TKIs combined with antiangiogenetic agents such as the anti-vascular endothelial growth factor (VEGF) antibody bevacizumab (12,13) or the anti-VEGF receptor (VEGFR) antibody ramucirumab (14). In addition, studies have investigated intensive EGFR inhibition using

EGFR-TKIs with anti-EGFR antibodies such as cetuximab or necitumumab (15,16); however, the optimal combination therapy remains unclear.

In this study, we investigated the effect of tumor volume on osimertinib efficacy in a preclinical *in vivo* model and assessed the potential of combining osimertinib with bevacizumab and/or cetuximab to produce greater remission in lung tumors harboring EGFR T790M mutations.

Materials and methods

Cell lines. RPC-9 gefitinib-resistant lung adenocarcinoma cells harboring EGFR exon 19 deletion mutation and T790M were established in our laboratory (17). H1975 pulmonary adenocarcinoma cells harboring L858R and T790M were purchased from the American Type Culture Collection.

Cell culture and growth inhibition in vitro. Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum in a 37˚C incubator with a humidified 5% CO_2 atmosphere, where the oxygen levels were maintained at either 21% (normoxia) or 1% (hypoxia). Growth inhibition was determined using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as described previously (17). Each assay was performed in triplicate.

Crystal violet assay. Cells were seeded in 6-well plates at a density of $5x10^4$ cells/well, grown under normoxic or hypoxic conditions for 48 h, and then grown with or without various concentrations of osimertinib. After three days, the cells were fixed with 10% formalin for 10 min, stained with crystal violet solution (Sigma-Aldrich) for 10 min, and then washed with H_2O. After the plates had been dried overnight, stained cells were quantified using ImageJ software (version 1.52a, National Institute of Health).

Immunoblot analysis. Cells and frozen tissues were lysed using radioimmunoprecipitation assay buffer [1% Triton X-100, 0.1% SDS, 50 mmol·L-1 Tris/HCl (pH 7.4), 150 mmol·L-1 NaCl, 1 mmol·L-1 ethylenediaminetetraacetic acid, 1 mmol·L-1 ethylene glycol tetraacetic acid, 10 mmol·L-1 β-glycerol phosphate, 10 mmol·L-1 NaF, 1 mmol·L-1 sodium orthovanadate-containing protease inhibitor tablets (Roche Applied Sciences)]. Proteins were separated by electrophoresis on polyacrylamide gels, transferred onto nitrocellulose membranes, and probed with specific antibodies that were detected using Enhanced Chemiluminescence Plus (GE Healthcare Biosciences). Bands were detected using an ImageQuant LAS-4000 imager (GE Healthcare Biosciences).

Reagents and antibodies. Gefitinib, cetuximab, and bevacizumab were purchased from EVERLTH. Osimertinib was purchased from Selleck Chemicals. Antibodies against phospho-EGFR (#3777), EGFR (#2232), phospho-ERK (#9101), ERK (#9102), phospho-AKT (#9271), AKT (#9272), hypoxia inducible factor-1α (HIF-1α; #36169), GAPDH (#2118), and CD31 (#77699) were purchased from Cell Signaling Technology. Anti-TGF-α antibodies (#ab9585) were purchased from Abcam.

Phospho-RTK array. A Human Phospho-RTK Array Kit (R&D Systems) was used according to the manufacturer's instructions. Bands and dots were detected using an ImageQuant LAS-4000 imager (GE Healthcare Biosciences). Mean pixel density was measured using ImageJ (version 1.52a, National Institute of Health).

mRNA expression analysis. RNA was extracted from cells using an RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Total cDNA was synthesized and amplified using a PrimeScript RT Reagent Kit (Perfect Real Time; TaKaRa). RNA expression was analyzed using real-time quantitative reverse transcription-PCR (qRT-PCR) with SYBR Premix Ex Taq II (Tli RNase H Plus; TaKaRa), according to the manufacturer's protocol. PCR amplification was performed using a LightCycler Real-Time PCR System (Roche Applied Science), and gene dosage was calculated using a standard curve analysis. PCR was carried out using primers (forward, 5'-AGATTCCCACACTCAGTTCTGCTTC-3'; reverse, 5'-ACA GCGTGCACCAACGTACC-3').

Xenograft model. Female 5-7-week-old athymic mice were purchased from Charles River Laboratories. All mice were provided with sterile food and water and were housed in a barrier facility under a 12 h light/dark cycle. Cells ($5x10^6$) were injected bilaterally into the back of each mouse. After 7-21 days, the mice were randomly divided into groups and then treated either with a mono-, double, or triple therapy (3-4 mice per group) consisting of a vehicle, osimertinib (per os 5 mg/kg, five times a week), cetuximab [intraperitoneal (i.p.) 1 mg/mouse, twice a week], and bevacizumab (i.p. 5 mg/kg, twice a week). Each drug was administered for 28 days, with a 28 day follow-up period. Tumor volume ($width^2$ x length/2) was measured twice a week. Euthanasia was then induced via administration of 5% isoflurane via an anesthesia machine. In addition to the assessment of inhibitory effect, the mice were treated with each drug similar to above mentioned regimen for 3 days, following which the tumor samples were collected for immunohistochemical analysis after euthanasia. Experimental protocols were approved by the Animal Care and Use Committee of Okayama University, Okayama, Japan (OKU-2017084, OKU-2020152).

Immunohistochemical analysis. Tissue samples were fixed using formalin, embedded in paraffin, and cut to a thickness of 5 μm before being placed on glass slides and deparaffinized in Hemo-De (FALMA) and graded alcohol. For antigen retrieval, sections were incubated in 10 mmol/l sodium citrate buffer, pH 6.0, for 10 min in a 95˚C water bath, after which the sections were incubated with 0.3% hydrogen peroxide in methanol to block endogenous peroxidase activity. The slides were rinsed with Tris-buffered saline containing 0.1% Tween-20, and the sections were blocked with goat serum for 60 min. The sections were incubated with anti-CD31, anti-HIF-1α, or anti-TGF-α antibodies overnight at 4˚C (dilution factors described in the data sheets) and amplified using biotinylated anti-rabbit antibodies and avidin-biotinylated horseradish peroxidase (HRP) conjugate for 30 min (SignalStain Boost IHC Detection Reagent (HRP, rabbit) #8114, Cell Signaling Technology, Inc.), reacted with 3,3-diaminobenzidine, and counterstained with hematoxylin.

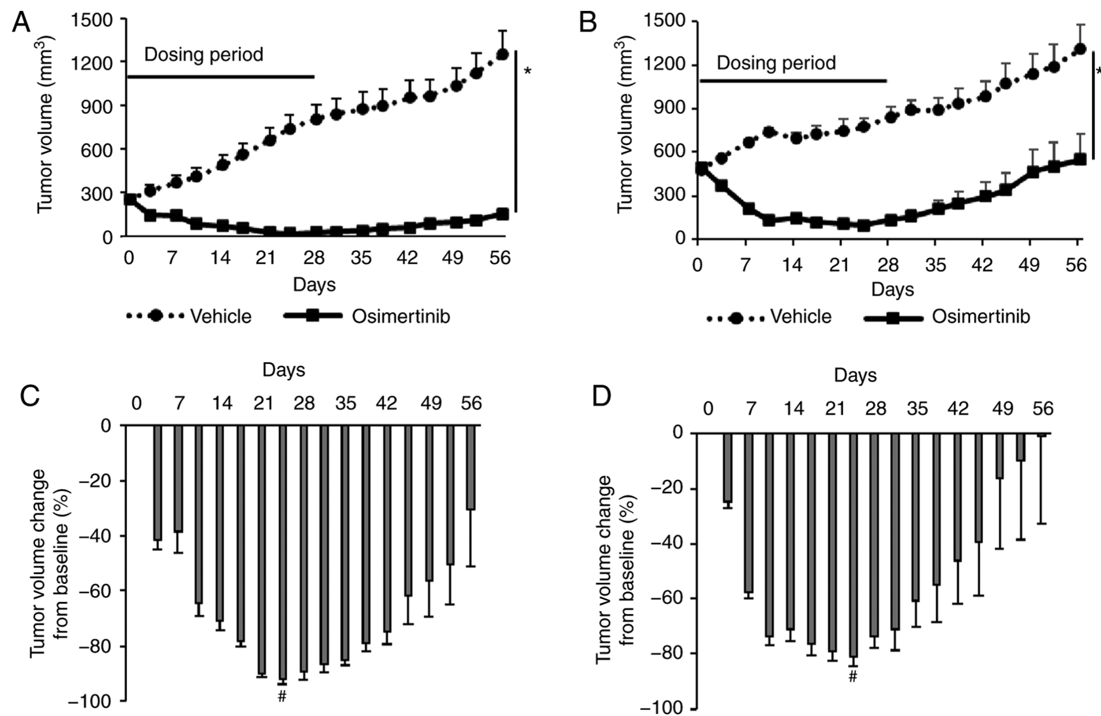

Figure 1. Effect of tumor volume on the efficacy of osimertinib monotherapy in RPC-9 cells harboring epidermal growth factor receptor mutations. The inhibitory effect of osimertinib on tumor growth in (A) the conventional tumor model (starting tumor volume, 200 mm^3; n=8) or (B) the large tumor model (starting tumor volume, 500 mm^3; n=8). Osimertinib (5 mg/kg, 5 times/week) was orally administered to mice with RPC-9 cell xenograft tumors for 28 days, with a 28-day observation period. Osimertinib monotherapy significantly inhibited tumor growth at day 56 in both models. Data are presented as the mean ± SEM. The effect of osimertinib on the regression rate of xenograft tumors in (C) the conventional and (D) large models. The maximum tumor regression at day 24 was -89.3 and -73.8% in the convention and large models, respectively. *P<0.05; $^{\#}$P<0.05. Data are presented as the mean ± SEM.

Statistical analysis. Statistical analyses were performed using STATA software version 15.1 (StataCorp.). Differences between two groups were compared using two-tailed paired Student's t-tests. Differences between three or more groups were compared using one-way ANOVA followed by Bonferroni's test. P-values of <0.05 were considered statistically significant. The Pearson correlation coefficient was calculated using STATA software version 15.1 (StataCorp.).

Results

Effect of tumor volume on osimertinib monotherapy in lung cancer harboring EGFR mutations in vivo. First, we assessed the inhibitory effect of osimertinib monotherapy on tumor growth in a mouse xenograft model derived from RPC-9 cells harboring EGFR exon 19 deletion and T790M mutation. Two types of xenograft lung cancer models were prepared using the RPC-9 cells to achieve different starting tumor volumes (conventional: 200 mm^3 or large: 500 mm^3). Both models were administered with osimertinib (5 mg/kg, 5 times/week by gavage) for 28 days and then observed for another 28 days. Consistent with the *in vitro* data (Fig. S1A), osimertinib monotherapy significantly inhibited tumor growth compared to the vehicle in both the conventional (maximum tumor diameter at day 56; vehicle: 15.4 mm ± 0.67 vs. osimertinib: 6.9 mm ± 0.74, mean ± SE) and large models (maximum tumor diameter at day 56; vehicle: 15.0 mm ± 0.97 vs. osimertinib: 8.7 mm ± 2.2, mean ± SE) (Fig. 1A and B); however, maximum tumor regression was significantly lower in the large model than in the conventional model (-73.8% vs. -89.3% on day 24, P=0.015, t-test; Fig. 1C and D). These results suggest that although osimertinib monotherapy is effective, the magnitude of its antitumor effect in the xenograft model is affected by tumor volume.

Increased HIF-1α and TGF-α expression may attenuate the efficacy of osimertinib. To explore the cause of distinct tumor inhibition between the conventional and large models, we performed pathological examinations on tumors from both models. Since we suspected that hypoxia may have been induced by the increase in tumor volume, we measured HIF-1α expression. As expected, we observed higher HIF-1α expression in tumors from the large model than in the conventional model (Fig. 2A). HIF-1α regulates the transcription of various growth factors (18); therefore, we investigated the phosphorylation status of receptor tyrosine kinase (RTK) using a Phospho-RTK array (Fig. S1B). The phosphorylation of most RTKs, including EGFR, was lower in tumors from the conventional model treated with osimertinib for 7 days (Fig. 2B), whereas the inhibition of RTK phosphorylation (other than EGFR) was generally limited in the large model (Fig. 2C). In addition, the inhibitory effect of osimertinib on EGFR phosphorylation was lower in tumors from the large model than the conventional model. Due to the observed increase in HIF-1α expression and the modest inhibition of EGFR phosphorylation, we also measured TGF-α expression, finding that TGF-α expression was higher in tumors from the large model than the conventional model (Fig. 2A). As expected, a significant correlation was observed between HIF-1α and TGF-α expression levels in these tumors (Fig. S1C).

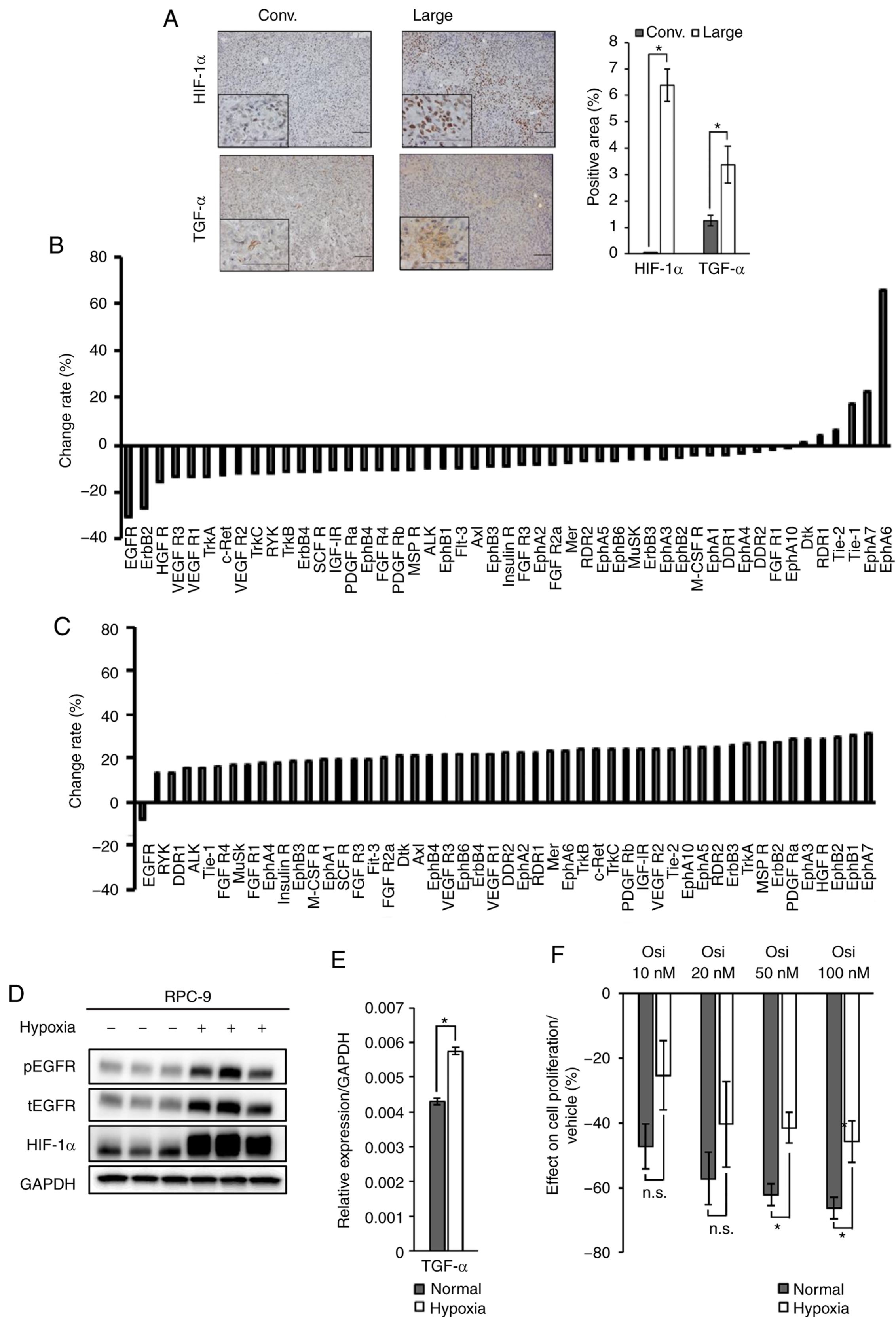

Figure 2. HIF-1α/TGF-α expression attenuates sensitivity to osimertinib in RPC-9 cells harboring EGFR mutations. (A) Immunohistochemical analysis of HIF-1α and TGF-α expression in xenograft tumors from the large and conventional models. Scale bar, 100 μm. Magnification of the zoomed in squares on the bottom left, 800 fold. Positive cells were quantified using ImageJ software. Data are presented as the mean ± SEM. Effect of osimertinib (5 mg/kg/day, day 7) on receptor tyrosine kinase phosphorylation in RPC-9 cell xenograft tumors from the (B) conventional or (C) large models. Mean pixel density was measured using ImageJ software. (D) HIF-1α protein expression and EGFR phosphorylation in RPC-9 cells cultured under hypoxic or normoxic conditions for 48 h. (E) TGF-α RNA expression in RPC-9 cells cultured under hypoxic or normoxic conditions for 48 h. (F) Inhibitory effect of osimertinib (72 h) on the viability of RPC-9 cells pre-incubated under hypoxic or normoxic conditions for 48 h. Crystal violet assay data were quantified using ImageJ software. Data are presented as the mean ± SEM. *P<0.05. HIF-1α, hypoxia-inducible factor-1α; TGF-α, transforming growth factor-α; EGFR, epidermal growth factor receptor; Conv., conventional model; Osi, osimertinib, n.s., not significant; t, total; p, phosphorylated.

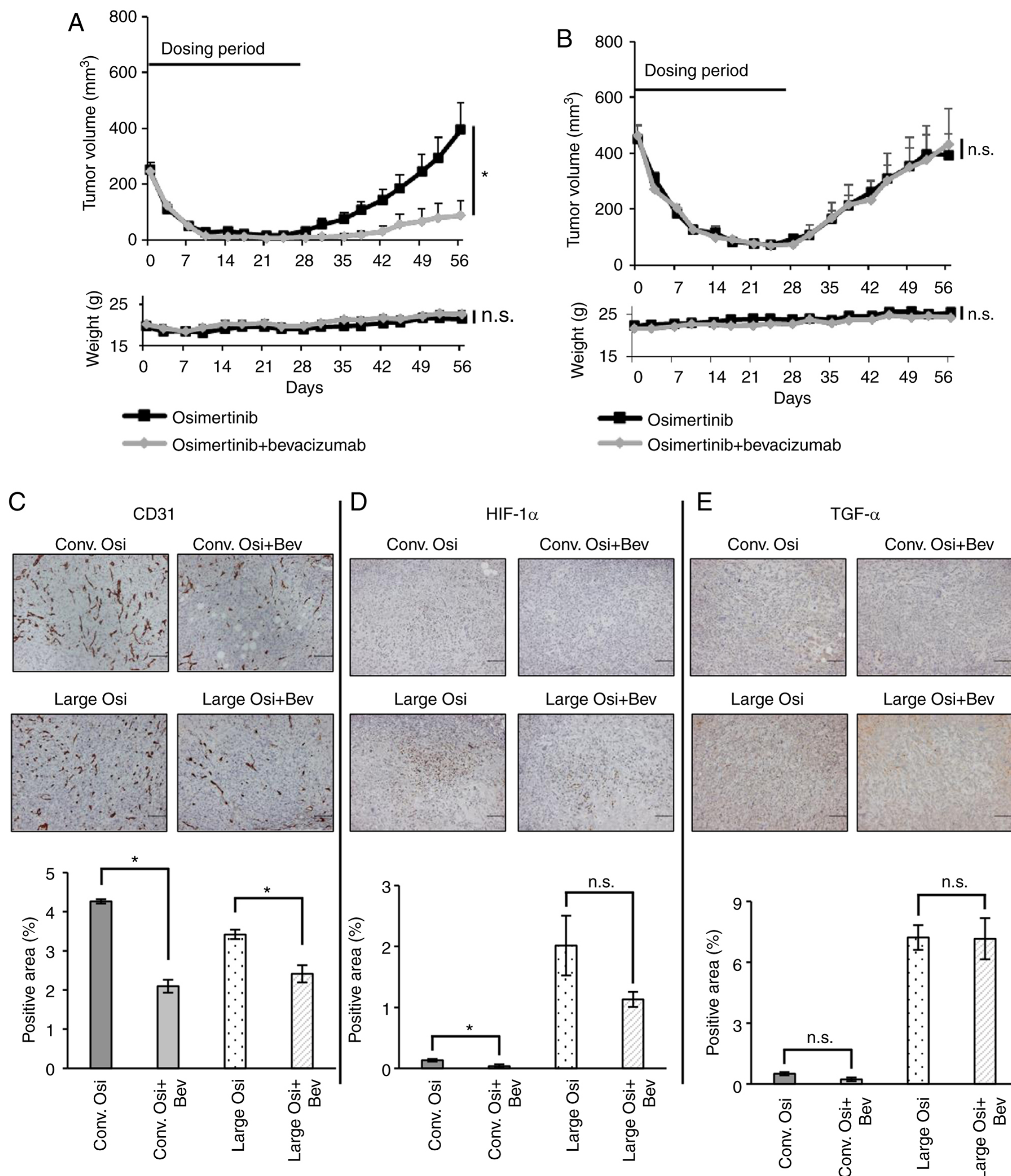

Figure 3. Combination therapy with osimertinib and bevacizumab exhibits limited efficacy in xenograft tumors with HIF-1α/TGF-α expression. Effect of osimertinib monotherapy (5 mg/kg, 5 times/week) or its combination with bevacizumab (5 mg/kg, twice/week) on RPC-9 cell xenograft tumors from the (A) conventional (starting tumor volume, 200 mm^3; n=8) or (B) large (starting tumor volume, 500 mm^3; n=6) models for 28 days, with a 28-day observation period. Body weight loss was not observed in the mice. The combination therapy inhibited tumor growth compared with osimertinib monotherapy at day 56 in the conventional model but not the large model. Immunohistochemical analysis in xenograft tumors from the conventional and large models treated with the combination therapy or osimertinib. Scale bar, 100 μm. Positive cells were quantified using ImageJ software. (C) CD31, (D) HIF-1α and (E) TGF-α expression levels in xenograft tumors from the large model. Data are presented as the mean ± SEM. $^*P<0.05$. HIF-1α, hypoxia-inducible factor-1α; TGF-α, transforming growth factor-α; Osi, osimertinib; Bev, bevacizumab; n.s., not significant.

Consequently, we assessed the effect of HIF-1α expression on sensitivity to osimertinib *in vitro*. As reported previously (19), HIF-1α and TGF-α expression were induced in RPC-9 cells cultured under hypoxic conditions (Fig. 2D and E). In addition, sensitivity to osimertinib was significantly lower in RPC-9 cells pre-incubated under hypoxic conditions than in cells pre-incubated under normoxic conditions (Figs. 2F and S1D). Together, these results suggest that the HIF-1α/TGF-α axis may account for the differing osimertinib sensitivity observed in the conventional and large models.

Combination therapy with osimertinib and bevacizumab exhibits limited efficacy in the large model. Next, we tested the effect of combination therapy with osimertinib and bevacizumab (OsiBev) in both the conventional and large models derived from RPC-9 cells, since EGFR-TKI plus bevacizumab is one of the most clinically relevant combination therapies (12). In the conventional model, combination therapy with OsiBev inhibited tumor growth to the same extent as osimertinib monotherapy during the treatment period (Fig. 3A) and the maximum tumor regression did not differ significantly

between therapies (osimertinib: -93.3% vs. OsiBev: -96.6% at day 24, P=0.16, t-test). However, the combination therapy significantly delayed tumor re-growth compared to osimertinib monotherapy. The regression rate of the tumor treated with OsiBev was significantly higher than that of the tumor treated with osimertinib monotherapy at day 56 (osimertinib: 57.3% vs. OsiBev: -64.0%, P=0.01, t-test) (maximum tumor diameter at day 56; osimertinib: 10.1 mm ± 1.9 vs. OsiBev: 3.5 mm ± 1.5, mean ± SE).

In the large model, no significant difference in the maximum tumor regression rate was observed between the osimertinib monotherapy and OsiBev (osimertinib: -84.4% vs. OsiBev: -83.6% on day 24, P=0.86, t-test; Fig. 3B). Moreover, combination therapy with OsiBev did not delay tumor re-growth compared to osimertinib monotherapy (tumor regression rate at day 56, osimertinib: -7.9% vs. OsiBev: -0.9%, P=0.87, t-test) (maximum tumor diameter at day 56; osimertinib: 9.6 mm ± 0.77 vs. OsiBev: 10.3 mm ± 1.8, mean ± SE) unlike in the conventional model (Fig. 3B).

To evaluate the effects of bevacizumab, we measured the expression of the vascular endothelial marker, CD31. As expected, there were significantly fewer CD31-positive cells in tumors treated with the combination therapy than osimertinib monotherapy in both the conventional and large models (Fig. 3C). Although HIF-1α expression was significantly lower and TGF-α expression did not change in the conventional model treated with the combination therapy (Fig. 3D and E), neither HIF-1α nor TGF-α expression decreased significantly in tumors from the large model treated with the combination therapy (Fig. 3D and E). Therefore, OsiBev only achieved a limited decline in HIF-1α expression and relatively high TGF-α expression was maintained in tumors from the large model compared to the conventional model.

Cetuximab restores the inhibitory effects of osimertinib against EGFR-mutant lung cancer cells stimulated by TGF-α in vitro. Having observed relatively high TGF-α expression in tumors from the large model, we decided to assess the effect of TGF-α on the inhibitory function of osimertinib *in vitro*. As expected, adding TGF-α (100 ng/ml) to the culture medium significantly reduced the inhibitory effect of osimertinib on RPC-9 or H1975 cell viability (Fig. 4A and D). Moreover, western blotting revealed that TGF-α activated the phosphorylation of the EGFR downstream signaling protein ERK and increased HIF-1α expression in RPC-9 and H1975 cells (Fig. 4B, C, E and F). ERK phosphorylation and HIF-1α protein expression were decreased in cells treated with osimertinib and not TGF-α, but were partially restored in RPC-9 or H1975 cells treated with osimertinib and TGF-α.

We also assessed the effect of bevacizumab *in vitro*, finding that bevacizumab alone had little inhibitory effect on cell viability and its combination with osimertinib did not restore sensitivity to osimertinib in RPC-9 or H1975 cells incubated with TGF-α *in vitro* (Fig. 4A and D). Cetuximab, which inhibits EGFR activation by blocking ligand binding, had little inhibitory effect on cell viability and partially inhibited ERK phosphorylation and HIF-1α protein expression in cells treated without TGF-α. Interestingly, the combination of osimertinib plus cetuximab (OsiCet) exerted a similar inhibitory effect on cell vaibility to osimertinib monotherapy in RPC-9 or H1975 cells without TGF-α stimulation, but exhibited a superior inhibitory effect after TGF-α stimulation (Fig. 4A and D). Consistent with this, cetuximab alone had little effect but the combination of osimertinib and cetuximab showed a tendency of superior inhibitory effect on cell viability in RPC-9 cells pre-incubated under hypoxic conditions (Fig. S2). Furthermore, ERK phosphorylation and HIF-1α were lower in RPC-9 or H1975 cells treated with OsiCet and TGF-α compared to osimertinib alone and TGF-α (Fig. 4B, C, E and F). Taken together, these findings suggest that TGF-α plays an important role in mediating the effect of osimertinib, and cetuximab could restore cellular sensitivity to osimertinib.

Triple therapy with osimertinib, bevacizumab, and cetuximab exerts beneficial effects in the large model. Finally, we confirmed the effect of cetuximab in the large model. Although cetuximab monotherapy exhibited a modest effect similar to bevacizumab (Fig. S3A), OsiCet tended to exert a superior inhibitory effect on tumor growth; however, this effect was not significantly higher than for osimertinib monotherapy (Fig. S3B).

Therefore, we examined the effect of triple therapy with osimertinib, bevacizumab, and cetuximab *in vivo*. Although triple therapy did not inhibit tumor growth more than OsiBev in the conventional model (maximum tumor diameter at day 56; osimertinib: 8.4 mm ± 1.7 vs. OsiBev: 3.4 mm ± 1.7 vs. triplet: 3.7 mm ± 1.3, mean ± SE) (Fig. 5A), it exerted a greater tumor inhibitory effect than osimertinib monotherapy, OsiBev, or OsiCet in the large model (maximum tumor diameter at day 56; osimertinib: 11.2 mm ± 1.9 vs. OsiBev: 11.4 mm ± 0.49 vs. triplet: 4.7 mm ± 1.2, mean ± SE) (Figs. 5B and S3B). Importantly, no decrease in body weight was observed in any of the mice (Fig. 5A and B). Pathological assessment of the tumors revealed that the triple therapy tended to reduce CD31, HIF-1α, and TGF-α expression compared to the double therapies (Fig. 5C-E).

Together, the findings of this study suggest that osimertinib or its combination with bevacizumab exerted limited efficacy in lung cancer with EGFR T790M mutation and HIF-1α/TGF-α expression; however, triple therapy with osimertinib, bevacizumab, and cetuximab induces an even greater inhibitory effect (Fig. S4).

Discussion

In this study, we found that: i) TGF-α attenuated sensitivity to osimertinib and increased HIF-1α expression in EGFR mutant NSCLC; ii) OsiCet restored sensitivity to osimertinib and decreased HIF-1α expression, but OsiBev did not; iii) triple therapy with osimertinib, bevacizumab, and cetuximab effectively inhibited the tumor growth of NSCLC with HIF-1α/TGF-α. Previous studies show that HIF-1α expression is an indicator of poor prognosis in patients with NSCLC and HIF-1α overexpression attenuates the effect of bevacizumab, whereas HIF-1α inhibitors improve its effect (20-22). Moreover, cetuximab is reported to decrease HIF-1α protein synthesis (23). Taken together, these findings suggest that cetuximab may be required to counteract the activation loop via the HIF-1α/TGF-α axis (Fig. S4); therefore, combining

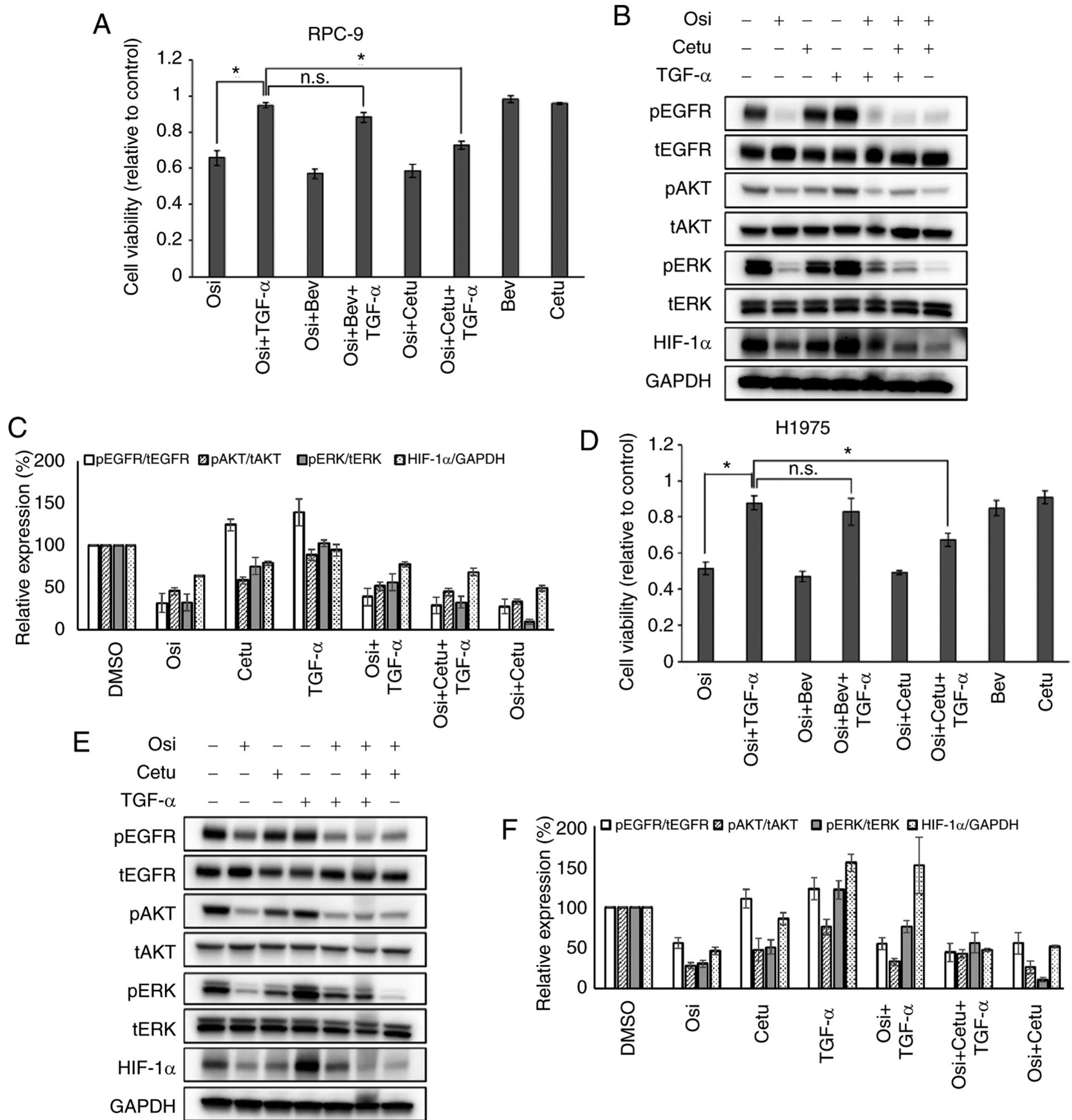

Figure 4. Cetuximab restores osimertinib efficacy in epidermal growth factor receptor-mutant lung cancer cells stimulated with TGF-α *in vitro*. (A) Effect of osimertinib on the viability of RPC-9 cells incubated with the indicated drugs and/or TGF-α (100 ng/ml) for 96 h (Osi, 10 nM; Bev, 150 µg/ml and Cetu, 5 µg/ml). (B) Effect of osimertinib and cetuximab on HIF-1α expression in RPC-9 cells incubated with the indicated drugs for 4 h [Osi, 10 nM; Cetu, 5 µg/ml and TGF-α (50 ng/ml)]. (C) Effect of the osimertinib/cetuximab combination on HIF-1α expression in RPC-9 cells. Data from (B) were quantified to determine the relative values of phosphorylation and protein expression to the corresponding expression of total protein or GAPDH; these values were then graphed for comparison against relative values for the DMSO control group. Mean pixel density was measured using ImageJ software. (D) Effect of osimertinib on the viability of H1975 cells incubated with the indicated drugs and/or TGF-α (100 ng/ml) for 96 h (Osi, 10 nM; Bev, 150 µg/ml and Cetu, 5 µg/ml). (E) Effect of osimertinib and cetuximab on HIF-1α expression in H1975 cells incubated with the indicated drugs for 4 h [Osi, 10 nM; Cetu, 5 µg/ml and TGF-α (50 ng/ml)]. (F) Effect of the osimertinib/cetuximab combination on HIF-1α expression in H1975 cells. Data from (E) were quantified to determine the relative values of phosphorylation and protein expression to the corresponding expression of total protein or GAPDH; these values were then graphed for comparison against relative values for the DMSO control group. Mean pixel density was measured using ImageJ software. Data are presented as the mean ± SEM. *P<0.05. HIF-1α, hypoxia-inducible factor-1α; TGF-α, transforming growth factor-α; Osi, osimertinib; Bev, bevacizumab; Cetu, cetuximab; n.s., not significant; p, phosphorylated; t, total.

cetuximab with osimertinib and bevacizumab may allow osimeritinib or bevacizumab to function effectively.

The efficacy of EGFR-TKI treatment has been found to vary, potentially due to the heterogeneity in the tumor or its microenvironment (24). In fact, the expression of HIF-1α/TGF-α was distinct and heterogenous in central and peripheral areas in the xenograft tumors, and the degree of heterogeneity was greater in the large model than in the small model. A higher dose of osimertinib might be able to provide benefits in cases of EGFR-mutant lung cancer in which the

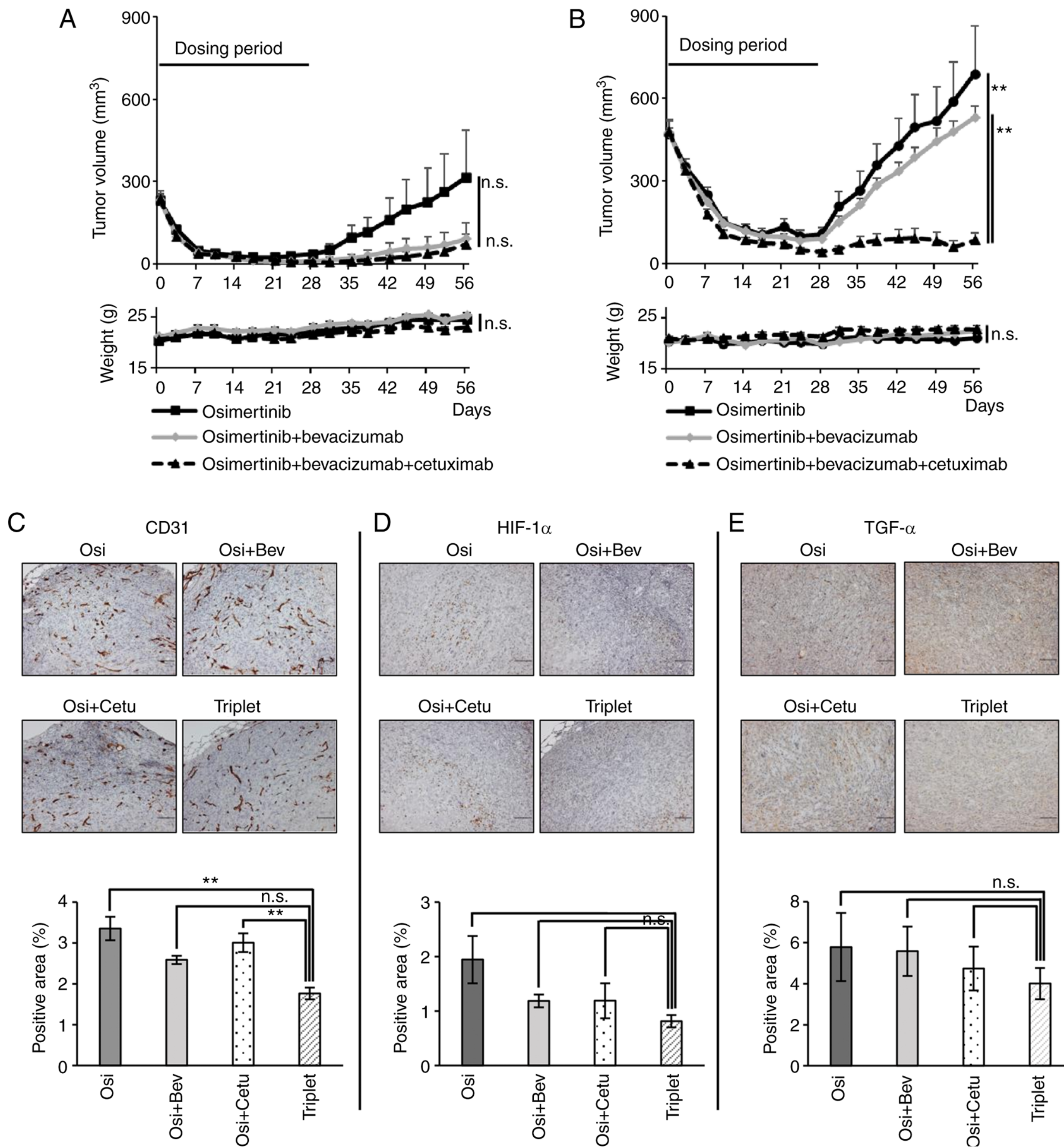

Figure 5. Effect of triple therapy with osimertinib, bevacizumab and cetuximab in xenograft tumors with HIF-1α/TGF-α expression. Effect of osimertinib monotherapy (5 mg/kg, 5 times/week), its combination with bevacizumab (5 mg/kg, twice/week) or triple therapy with bevacizumab (5 mg/kg, twice/week) and cetuximab (1 mg/body, twice/week) for 28 days on RPC-9 cell xenograft tumors from the (A) conventional (starting tumor volume, 200 mm^3; n=8) or (B) large (starting tumor volume, 500 mm^3; n=8) models, over a 28-day observation period. Body weight loss was not observed among the mice. The triple therapy did not inhibit tumor growth more than combination therapy in the conventional model at day 56 (P=1.00, one-way ANOVA with Bonferroni's test), but the triple therapy inhibited tumor growth more than osimertinib or the combination therapy in the large model. Immunohistochemical analysis in xenograft tumors from the large model treated with the triple therapy, osimertinib or osimertinib plus cetuximab. (C) CD31, (D) HIF-1α and (E) TGF-α expression levels in xenograft tumors. Scale bar; 100 μm. Positive cells were quantified using ImageJ software. Data are presented as the mean ± SEM. $^{**}P<0.01$. HIF-1α, hypoxia-inducible factor-1α; TGF-α, transforming growth factor-α; Osi, osimertinib; Bev, bevacizumab; Cetu, cetuximab; n.s., not significant.

standard dose of osimertinib does not confer effective inhibition (25). Although some EGFR-mutant lung cancers may be suitable for EGFR-TKI monotherapy, others may require intensive treatment with combination or triple therapies involving EGFR-TKIs and other agents. The toxicity of combination therapy must also be considered, but recent preclinical studies suggested a beneficial effect of three- or four-drug combination with low-dose therapy for EGFR-mutant lung cancer (26,27).

Tomoshige *et al* reported that TGF-α expression is relatively high in EGFR mutated lung cancer compared to EGFR wild-type lung cancer in the Cancer Genome Atlas (28). In addition, they used preclinical models to demonstrate that TGF-α promoted the progression of EGFR-mutated, but not KRAS-mutated, lung cancer and correlated with poor prognosis in patients with lung cancer harboring EGFR mutations (28). Although these findings suggest that combination therapy

with EGFR-TKI and cetuximab could be a reasonable strategy for treating patients with EGFR-mutant lung cancer, clinical trials have failed to show that the combination of afatinib and cetuximab is superior to afatinib monotherapy (15).

EGFR-TKIs have been successfully combined with anti-angiogenic agents such as bevacizumab or ramucirumab to treat patients with lung cancer harboring EGFR mutations (12,14); however, a recent randomized clinical trial revealed that the combination of osimertinib and bevacizumab failed to show superior progression-free survival compared to osimertinib monotherapy (29). Multiple negative predictive biomarkers have been reported for the effect of EGFR-TKIs (6-11); however, the predictive factor for combination therapies including EGR-TKIs and bevacizumab has not yet been identified. Although the reason for this negative result remains unknown, some negative predictive factors (for example HIF-1α/TGF-α expression) may be unbalanced between the combination and monotherapy groups. These results may indicate that biomarker-driven patient selection is required for clinical trials of combination therapy. The expression of HIF-1α is reported to be associated with T factor of the TNM staging system, lymph node metastasis, and poorly differentiated tumors (30,31). Therefore, a patient with lung cancer harboring such a clinical characteristic might benefit from triple therapy.

HIF-1 inhibitors were actively investigated through clinical trials, but an HIF-1 inhibitor has not been clinically approved yet (32,33). The main obstacle to the development of HIF-1 inhibitors as therapies is their lack of specificity; therefore, identifying more specific inhibitors is warranted. In this study, we used clinically available drugs, such as cetuximab, but the combination of osimertinib and specific HIF1 inhibitors might be a promising therapeutic strategy for EGFR-mutant lung cancer with HIF-1α high expression.

In summary, the HIF-1α/TGF-α axis may be involved in the effect of osimertinib and its combination with bevacizumab in lung cancer harboring EGFR T790M mutation. Furthermore, triple therapy with osimertinib, bevacizumab, and cetuximab may be able to achieve deep remission in EGFR-mutant lung cancers with HIF-1α/TGF-α expression; therefore, clinical trials are required to explore this combination further.

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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 upon reasonable request.

Authors' contributions

KaN and KO had full access to all data and assume responsibility for data integrity and the accuracy of data analysis. KaN and KO contributed to the study design, and manuscript writing. KaN, KO, HW and GM performed the experiments and collected the data. KaN, KO, HW, GM, TN, HH, KiN, YK, TK, KR, EI, KH, MT, YM and KK performed data analysis. KaN and KO confirmed the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were approved by the Animal Care and Use Committee of Okayama University (Okayama, Japan; approval no. OKU-2017084, OKU-2020152).

Patient consent for publication

Not applicable.

Competing interests

Dr Kadoaki Ohashi reports research funding from Boehringer Ingelheim, Novartis, AstraZeneca, Eli Lilly, MSD, and Daiichi-Sankyo outside the submitted work. Dr Kadoaki Ohashi reports personal fees from AstraZeneca, MSD, and Chugai pharmaceutical outside the submitted work. Dr Kiichiro Ninomiya has received honoraria from AstraZeneca, Boehringer Ingelheim, Eli Lilly, MSD, Ono Pharmaceutical, Nippon Kayaku, Taiho pharmaceutical, Kyowa-Kirin, and Chugai pharmaceutical outside the submitted work. Dr Katsuyuki Hotta received honoraria from AstraZeneca and MSD; research funding from Chugai Pharmaceutical, Eli Lilly Japan, Bristol-Myers Squibb, Astellas Pharma and AstraZeneca outside the submitted work. Dr Katsuyuki Kiura received honoraria from MSD; research funding from Ono Pharmaceutical, Boehringer Ingelheim, Taiho Pharmaceutical, Chugai Pharmaceutical, Nippon Kayaku, Bristol-Myers Squibb, and Shionogi & Co., Ltd., outside the submitted work.

References

1. Ninomiya K, Teraoka S, Zenke Y, Kenmotsu H, Nakamura Y, Okuma Y, Tamiya A, Nosaki K, Morise M, Aokage K, *et al*: Japanese Lung Cancer Society Guidelines for Stage IV NSCLC With EGFR Mutations. JTO Clin Res Rep 2: 100107, 2021.
2. Ohashi K, Maruvka YE, Michor F and Pao W: Epidermal growth factor receptor tyrosine kinase inhibitor-resistant disease. J Clin Oncol 31: 1070-1080, 2013.
3. Mok TS, Wu YL, Ahn MJ, Garassino MC, Kim HR, Ramalingam SS, Shepherd FA, He Y, Akamatsu H, Theelen WS, *et al*: Osimertinib or platinum-pemetrexed in EGFR T790M-positive lung cancer. N Engl J Med 376: 629-640, 2017.
4. Ramalingam SS, Vansteenkiste J, Planchard D, Cho BC, Gray JE, Ohe Y, Zhou C, Reungwetwattana T, Cheng Y, Chewaskulyong B, *et al*: Overall survival with osimertinib in untreated, EGFR-mutated advanced NSCLC. N Engl J Med 382: 41-50, 2020.

5. Piper-Vallillo AJ, Sequist LV and Piotrowska Z: Emerging treatment paradigms for EGFR-mutant lung cancers progressing on osimertinib: A review. J Clin Oncol: Jun 18, 2020 (Epub ahead of print). doi: 10.1200/JCO.19.03123.
6. Blakely CM, Watkins TBK, Wu W, Gini B, Chabon JJ, McCoach CE, McGranahan N, Wilson GA, Birkbak NJ, Olivas VR, *et al*: Evolution and clinical impact of co-occurring genetic alterations in advanced-stage EGFR-mutant lung cancers. Nat Genet 49: 1693-1704, 2017.
7. Lai GGY, Lim TH, Lim J, Liew PJR, Kwang XL, Nahar R, Aung ZW, Takano A, Lee YY, Lau DPX, *et al*: Clonal MET amplification as a determinant of tyrosine kinase inhibitor resistance in epidermal growth factor receptor-mutant non-small-cell lung cancer. J Clin Oncol 37: 876-884, 2019.
8. Offin M, Rizvi H, Tenet M, Ni A, Sanchez-Vega F, Li BT, Drilon A, Kris MG, Rudin CM, Schultz N, *et al*: Tumor mutation burden and efficacy of EGFR-tyrosine kinase inhibitors in patients with EGFR-mutant lung cancers. Clin Cancer Res 25: 1063-1069, 2019.
9. Ohashi K, Ninomiya K, Yoshioka H, Bessho A, Shibayama T, Aoe K, Ishikawa N, Kozuki T, Kawai H, Kuyama S, *et al*: Impact of HER2 expression on EGFR-TKI treatment outcomes in lung tumors harboring EGFR mutations: A HER2-CS study subset analysis. Lung Cancer 150: 83-89, 2020.
10. Pan Y, Gao G, Chen X, Tian Q, Wu F, Liu Q, Wang Y, Jiang T, Liu Y, Li X, *et al*: Larger tumors are associated with inferior progression-free survival of first-line EGFR-tyrosine kinase inhibitors and a lower abundance of EGFR mutation in patients with advanced non-small cell lung cancer. Thorac Cancer 10: 686-694, 2019.
11. Zhou F, Ma W, Li W, Ni H, Gao G, Chen X, Zhang J and Shi J: Thick-wall cavity predicts worse progression-free survival in lung adenocarcinoma treated with first-line EGFR-TKIs. BMC Cancer 18: 1033, 2018.
12. Saito H, Fukuhara T, Furuya N, Watanabe K, Sugawara S, Iwasawa S, Tsunezuka Y, Yamaguchi O, Okada M, Yoshimori K, *et al*: Erlotinib plus bevacizumab versus erlotinib alone in patients with EGFR-positive advanced non-squamous non-small-cell lung cancer (NEJ026): Interim analysis of an open-label, randomised, multicentre, phase 3 trial. Lancet Oncol 20: 625-635, 2019.
13. Ninomiya T, Ishikawa N, Inoue K, Kubo T, Yasugi M, Shibayama T, Maeda T, Fujitaka K, Kodani M, Yokoyama T, *et al*: Phase 2 study of afatinib alone or combined with bevacizumab in chemonaive patients with advanced non-small-cell lung cancer harboring EGFR mutations: AfaBev-CS study protocol. Clin Lung Cancer 20: 134-138, 2019.
14. Nakagawa K, Garon EB, Seto T, Nishio M, Ponce Aix S, Paz-Ares L, Chiu CH, Park K, Novello S, Nadal E, *et al*: Ramucirumab plus erlotinib in patients with untreated, EGFR-mutated, advanced non-small-cell lung cancer (RELAY): A randomised, double-blind, placebo-controlled, phase 3 trial. Lancet Oncol 20: 1655-1669, 2019.
15. Goldberg SB, Redman MW, Lilenbaum R, Politi K, Stinchcombe TE, Horn L, Chen EH, Mashru SH, Gettinger SN, Melnick MA, *et al*: Randomized trial of afatinib plus cetuximab versus afatinib alone for first-line treatment of EGFR-mutant non-small-cell lung cancer: Final results from SWOG S1403. J Clin Oncol 38: 4076-4085, 2020.
16. Kudo K, Ohashi K, Makimoto G, Higo H, Kato Y, Kayatani H, Kurata Y, Takami Y, Minami D, Ninomiya T, *et al*: Triplet therapy with afatinib, cetuximab, and bevacizumab induces deep remission in lung cancer cells harboring EGFR T790M in vivo. Mol Oncol 11: 670-681, 2017.
17. Ogino A, Kitao H, Hirano S, Uchida A, Ishiai M, Kozuki T, Takigawa N, Takata M, Kiura K and Tanimoto M: Emergence of epidermal growth factor receptor T790M mutation during chronic exposure to gefitinib in a non small cell lung cancer cell line. Cancer Res 67: 7807-7814, 2007.
18. Semenza GL: Targeting HIF-1 for cancer therapy. Nat Rev Cancer 3: 721-732, 2003.
19. Minakata K, Takahashi F, Nara T, Hashimoto M, Tajima K, Murakami A, Nurwidya F, Yae S, Koizumi F, Moriyama H, *et al*: Hypoxia induces gefitinib resistance in non-small-cell lung cancer with both mutant and wild-type epidermal growth factor receptors. Cancer Sci 103: 1946-1954, 2012.
20. Wang Q, Hu DF, Rui Y, Jiang AB, Liu ZL and Huang LN: Prognosis value of HIF-1α expression in patients with non-small cell lung cancer. Gene 541: 69-74, 2014.
21. Hartwich J, Orr WS, Ng CY, Spence Y, Morton C and Davidoff AM: HIF-1α activation mediates resistance to anti-angiogenic therapy in neuroblastoma xenografts. J Pediatr Surg 48: 39-46, 2013.
22. Rapisarda A, Hollingshead M, Uranchimeg B, Bonomi CA, Borgel SD, Carter JP, Gehrs B, Raffeld M, Kinders RJ, Parchment R, *et al*: Increased antitumor activity of bevacizumab in combination with hypoxia inducible factor-1 inhibition. Mol Cancer 8: 1867-1877, 2009.
23. Li X, Lu Y, Liang K, Pan T, Mendelsohn J and Fan Z: Requirement of hypoxia-inducible factor-1alpha down-regulation in mediating the antitumor activity of the anti-epidermal growth factor receptor monoclonal antibody cetuximab. Mol Cancer Ther 7: 1207-1217, 2008.
24. Roper N, Brown AL, Wei JS, Pack S, Trindade C, Kim C, Restifo O, Gao S, Sindiri S, Mehrabadi F, *et al*: Clonal evolution and heterogeneity of osimertinib acquired resistance mechanisms in EGFR mutant lung cancer. Cell Rep Med 1: 100007, 2020.
25. Park S, Lee MH, Seong M, Kim ST, Kang JH, Cho BC, Lee KH, Cho EK, Sun JM, Lee SH, *et al*: A phase II, multicenter, two cohort study of 160 mg osimertinib in EGFR T790M-positive non-small-cell lung cancer patients with brain metastases or leptomeningeal disease who progressed on prior EGFR TKI therapy. Ann Oncol 31: 1397-1404, 2020.
26. Romaniello D, Mazzeo L, Mancini M, Marrocco I, Noronha A, Kreitman M, Srivastava S, Ghosh S, Lindzen M, Salame TM, *et al*: A Combination of approved antibodies overcomes resistance of lung cancer to osimertinib by blocking bypass pathways. Clin Cancer Res 24: 5610-5621, 2018.
27. Fernandes Neto JM, Nadal E, Bosdriesz E, Ooft SN, Farre L, McLean C, Klarenbeek S, Jurgens A, Hagen H, Wang L, *et al*: Multiple low dose therapy as an effective strategy to treat EGFR inhibitor-resistant NSCLC tumours. Nat Commun 11: 3157, 2020.
28. Tomoshige K, Guo M, Tsuchiya T, Fukazawa T, Fink-Baldauf IM, Stuart WD, Naomoto Y, Nagayasu T and Maeda Y: An EGFR ligand promotes EGFR-mutant but not KRAS-mutant lung cancer in vivo. Oncogene 37: 3894-908, 2018.
29. Akamatsu H, Toi Y, Hayashi H, Fujimoto D, Tachihara M, Furuya N, Otani S, Shimizu J, Katakami N, Azuma K, *et al*: Efficacy of osimertinib plus bevacizumab vs osimertinib in patients with EGFR T790M-mutated non-small cell lung cancer previously treated with epidermal growth factor receptor-tyrosine kinase inhibitor: West Japan oncology Group 8715L Phase 2 randomized clinical trial. JAMA Oncol 7: 386-394, 2021.
30. Swinson DE, Jones JL, Cox G, Richardson D, Harris AL and O'Byrne KJ: Hypoxia-inducible factor-1 alpha in non small cell lung cancer: Relation to growth factor, protease and apoptosis pathways. Int J Cancer 111: 43-50, 2004.
31. Ren W, Mi D, Yang K, Cao N, Tian J, Li Z and Ma B: The expression of hypoxia-inducible factor-1α and its clinical significance in lung cancer: A systematic review and meta-analysis. Swiss Med Wkly 143: w13855, 2013.
32. Fallah J and Rini BI: HIF inhibitors: Status of current clinical development. Curr Oncol Rep 21: 6, 2019.
33. Ma Z, Xiang X, Li S, Xie P, Gong Q, Goh BC and Wang L: Targeting hypoxia-inducible factor-1, for cancer treatment: Recent advances in developing small-molecule inhibitors from natural compounds. Semin Cancer Biol: Sep 28, 2020 (Epub ahead of print). doi: 10.1016/j.semcancer.2020.09.011.

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