



CDK4/6 signaling attenuates the effect of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors in *EGFR*-mutant non-small cell lung cancer

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Background: Epidermal growth factor receptor (*EGFR*) mutations, such as exon 19 deletion and exon 21 L858R, are driver oncogenes of non-small cell lung cancer (NSCLC), with *EGFR* tyrosine kinase inhibitors (TKIs) being effective against *EGFR*-mutant NSCLC. However, the efficacy of *EGFR*-TKIs is transient and eventually leads to acquired resistance. Herein, we focused on the significance of cell cycle factors as a mechanism to attenuate the effect of *EGFR*-TKIs in *EGFR*-mutant NSCLC before the emergence of acquired resistance.

Methods: Using several *EGFR*-mutant cell lines, we investigated the significance of cell cycle factors to attenuate the effect of *EGFR*-TKIs in *EGFR*-mutant NSCLC.

Results: In several *EGFR*-mutant cell lines, certain cancer cells continued to proliferate without *EGFR* signaling, and the cell cycle regulator retinoblastoma protein (RB) was not completely dephosphorylated. Further inhibition of phosphorylated RB with cyclin-dependent kinase (CDK) 4/6 inhibitors, combined with the *EGFR*-TKI osimertinib, enhanced G0/G1 cell cycle accumulation and growth inhibition of the *EGFR*-mutant NSCLC in both *in vitro* and *in vivo* models. Furthermore, residual RB phosphorylation without *EGFR* signaling was maintained by extracellular signal-regulated kinase (ERK) signaling, and the ERK inhibition pathway showed further RB dephosphorylation.

Conclusions: Our study demonstrated that the CDK4/6-RB signal axis, maintained by the MAPK pathway, attenuates the efficacy of *EGFR*-TKIs in *EGFR*-mutant NSCLC, and targeting CDK4/6 enhances this efficacy. Thus, combining CDK4/6 inhibitors and *EGFR*-TKI could be a novel treatment strategy for TKI-naïve *EGFR*-mutant NSCLC.

Keywords: Epidermal growth factor receptor (*EGFR*); non-small cell lung cancer (NSCLC); cell cycle; CDK4/6 inhibitor

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Introduction

Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) are used to treat *EGFR*-mutant non-small cell lung cancer (NSCLC). Osimertinib, a third-generation EGFR TKI, has been shown to prolong survival in patients with *EGFR*-mutant NSCLC compared with first-generation EGFR-TKIs (1). However, even with this effective agent, few tumors respond completely, leading to acquired resistance and suggesting the existence of mechanisms that attenuate the effects of EGFR-TKIs before resistance is acquired and allow some *EGFR*-mutant cancer cells to survive treatment. Heterogeneity may be one of the factors responsible for the non-curative effects of EGFR-TKIs. *EGFR*-mutant NSCLC is heterogeneous even before treatment, and this heterogeneity progresses with EGFR-TKI treatment (2-4). However, even with relatively homogeneous cell line models *in vitro*, *EGFR*-mutant NSCLC cells are not curatively inhibited by EGFR-TKI (5), implying the existence of other mechanisms by which *EGFR*-mutant cells survive under EGFR-TKI exposure. A potential mechanism is the adaptation of cancer cells to EGFR-TKIs where *EGFR*-mutant cancer cells can survive without EGFR signaling (5-7).

Cyclin-dependent kinase 4/6 (CDK4/6) inhibitors have been approved for breast cancer treatment (8,9) and they target and inhibit CDK4/6, a cell cycle regulator, during cell division. A CDK4/6 inhibitor combined with endocrine therapy for hormone receptor-positive breast cancer has been shown to prolong progression-free survival (10) compared with endocrine therapy alone. CDK4/6 inhibitors are also effective against lung cancer (11). Abemaciclib,

a CDK4/6 inhibitor, was shown to overcome acquired resistance to EGFR-TKIs in a previous study (12). In that study, a combination therapy of abemaciclib and EGFR-TKI effectively inhibited *EGFR*-mutant cancer cells that had acquired resistance to EGFR-TKIs through various resistance mechanisms, suggesting that CDK4/6 signaling may act as a hub of acquired resistance to EGFR-TKIs. Thus, CDK4/6 signaling may attenuate the effect of EGFR-TKI before and after the acquisition of resistance.

In this study, we focused on the impact of CDK4/6 signaling on the effect of EGFR-TKI before the acquisition of resistance. We aimed to elucidate the role of cell cycle factors in *EGFR*-mutant NSCLC cells and verify whether CDK4/6 inhibitors combined with EGFR-TKI exert a more profound inhibitory effect on cancer cells before the acquisition of resistance. We present this article in accordance with the ARRIVE reporting checklist (available at <https://tlcr.amegroups.com/article/view/10.21037/tlcr-23-99/rc>).

Methods

Cell lines and reagents

PC-9 cells (exon 19 del E746_A750) and NCI-H1975 cells (exon 21 L858R) were purchased from the European Collection of Authenticated Cell Culture (Salisbury, UK). HCC827 (exon19 del E746_A750) cells were provided by Dr. William Pao (Vanderbilt University). H3255 cells (exon21 L858R) were provided by Fujimoto and Kurie (MD Anderson Cancer Center, Houston, TX, USA). The 11-18 cells (exon 21 L858R) were provided by Koichi Hagiwara (Jichi Medical University, Japan). H820 (exon19 del and MET amplification) cells were provided by Pro Shinichi Toyooka (Okayama University). We cultured all cell lines in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin added and kept them at 37 °C in a humidified atmosphere of 5% CO₂. Osimertinib and palbociclib were purchased from Selleck Chemicals (Houston, TX, USA). Abemaciclib was provided by Eli Lilly (Indianapolis, IN, USA). All compounds were dissolved in dimethyl sulfoxide for *in vitro* studies.

Cell proliferation assay

Growth inhibition was measured using a modified MTT assay. Briefly, the cells were seeded in 96-well plates at a

Highlight box

Key findings

- Cyclin-dependent kinase 4/6 (CDK4/6)-RB signaling attenuated the effects of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) in *EGFR*-mutant cancer cells.
- Targeting CDK4/6 enhanced the efficacy of EGFR-TKIs.

What is known and what is new?

- EGFR-TKI efficacy in *EGFR*-mutant non-small cell lung cancer (NSCLC) is transient; more effective therapy should be developed.
- Combining CDK4/6 inhibitors and *EGFR*-TKI exerted a more profound inhibitory effect on *EGFR*-mutant NSCLC cells.

What is the implication, and what should change now?

- Combining CDK4/6 inhibitors and EGFR-TKI is a potential novel treatment strategy for TKI-naïve *EGFR*-mutant NSCLC.

density of 1,500–3,000 cells/well (PC-9: 1,500 cells/well; HCC827, 11-18, H1975, H3255: 3,000 cells/well) and treated with each drug for 96 h.

Cell staining assay

We plated the cells in 6-well plates with a density of 25,000–100,000 cells per well (PC-9: 50,000 cells/well; HCC827: 100,000 cells/well; 11-18: 100,000 cells/well; H1975: 25,000 cells/well; H3255: 100,000 cells/well) and treated the following day. Three days following the initial treatment, we changed the media and drugs. We fixed the cells in 10% formalin for 10 min and then stained them with a crystal violet solution (Sigma-Aldrich) for another 10 min. The plates were washed with tap water and then dried overnight.

Combination index (CI)

We used a constant-ratio design for the combination assay to analyze the data efficiently. We calculated the combined drug effect using the multiple-drug effect analysis based on the median-effect principle in this study. We treated cells seeded in triplicate in 96-well plates with TKIs and/or CDK 4/6 inhibitors for 96 h at the concentrations shown. We used an MTT assay to measure cell viability. We used CompuSyn software (version 1.0) to calculate CI. In the plots made by CompuSyn, the y-axis shows the CI, with CI <1, 1, or >1 meaning synergistic, additive, or antagonistic effects, respectively. The x-axis shows fractional activity (Fa), which is how much the treatment inhibited the cells compared to the vehicle control.

Immunoblotting

We used the following antibodies from Cell Signaling Technology (Danvers, MA, USA) to do immunoblotting: phosphorylated (p)-EGFR Tyr1068 (No. 3777), EGFR (No. 2232), p-retinoblastoma protein (p-RB) (No. 8516), RB (No. 9313), CDK4 (No. 12790), p-ERK1/2 Thr202/Tyr204 (No. 9101), ERK1/2 (No. 9102), p-AKT Ser473 (No. 9271), AKT (No. 9272), and GAPDH (No. 2118). We bought anti-rabbit IgG horseradish peroxidase-linked whole antibody (donkey; NA934) from GE Healthcare Biosciences (Piscataway, NJ, USA). For immunoblotting, harvested cells were washed in phosphate buffered saline (PBS) and lysed in radioimmunoprecipitation assay buffer [1% Triton X-100, 0.1% sodium dodecyl sulfate, 50 mmol/L Tris-HCl, pH 7.4,

150 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 1 mmol/L EGTA, 10 mmol/L β -glycerolphosphate, 10 mmol/L NaF, and 1 mmol/L sodium orthovanadate] containing a protease inhibitor cocktail (Roche Applied Sciences, Indianapolis, IN, USA). We used sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to separate the proteins and electrotransferred them to membranes. We then incubated them with the primary and secondary antibodies shown. We used the Enhanced Chemiluminescence Plus reagent (GE Healthcare Biosciences) to do chemiluminescence. We used an ImageQuant LAS-4000 imager (GE Healthcare Biosciences) to see the bands.

Flow cytometry analysis of cell cycle

The cells were seeded in 10-cm dishes, incubated at 37 °C for 12 h, synchronized by starvation in a serum-free RPMI medium for 24 h, and then incubated at 37 °C with or without osimertinib and/or a CDK4 inhibitor for 24 h. Fifty thousand cells per sample were harvested with 0.25% trypsin, washed in PBS, fixed in 4% formaldehyde/PBS for 5 min, and permeabilized with 0.1% TritonX-100 (Sigma-Aldrich)/PBS for 5 min. After washing, the cells were resuspended in a staining buffer containing 1 μ g/mL 4',6-diamino-2-phenylindole (DAPI) (BD Biosciences Pharmingen, San Diego, CA, USA). All analyses were performed using MACSQuant (Miltenyi Biotec, San Jose, CA, USA), and data were analyzed using FlowJo software (BD Biosciences, San Jose, CA, USA).

Small interfering RNA (siRNA) transfection

We transfected siRNA to knock down genes using optimal conditions. We used CDK4 and ERK1 siRNAs (Dharmacon) and Lipofectamine Transfection Reagent (Thermo Fisher Scientific, MA, USA) to transfect cells in 6-well plates (PC-9: 25,000 cells/well). For each candidate gene, we tested two gene-specific siRNAs that were pre-designed, as well as the negative control (Dharmacon Non-targeting Control siRNA).

Xenograft mouse models

We bought female BALB/c nu/nu mice (6 weeks old) from Charles River Laboratories Japan (Yokohama, Japan). We gave all mice sterilized food and water and kept them in a barrier facility with a 12-hour light/dark cycle. We injected

cancer cells (PC-9: 2×10^6 cells/tumor) under the skin of the mice's backs on both sides. We treated them with abemaciclib and osimertinib together when the average tumor size reached 150 mm^3 . We randomly put the mice into four groups (four mice each): vehicle, osimertinib (5 mg/kg/day), abemaciclib (50 mg/kg/day), or both drugs. We gave osimertinib and abemaciclib by mouth once a day and five times a week. To minimize potential confounders, the order of treatments was randomly determined each time. We measured the tumor size ($\text{width}^2 \times \text{length}/2$) regularly. We did statistical analyses based on tumor sizes on day 28. The mice were observed every day for signs of humane endpoints, including weight reduction, decreased mobility, and tumor sizes exceeding $2,000 \text{ mm}^3$, among others. Once these endpoints were identified, the mice were anesthetized with isoflurane and then euthanized through cervical dislocation. The Institutional Animal Care and Research Advisory Committee of the Department of Animal Resources, Okayama University Advanced Science Research Center (Okayama, Japan) oversaw all animal experiments. We followed the Policy on the Care and Use of Laboratory Animals, Okayama University, and Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions, Ministry of Education, Culture, Sports, Science, and Technology of Japan. A protocol was prepared before the study without registration and the Animal Care and Use Committee of Okayama University (No. OKU-2021772) approved the experimental protocol.

Statistical analysis

Differences between groups were statistically analyzed using the Student's *t*-test in this study.

Results

EGFR-mutant cells continue proliferating under EGFR-TKI exposure

To investigate the cell cycle status in *EGFR*-mutant NSCLC treated with *EGFR*-TKIs, we treated several *EGFR*-mutant cell lines with the *EGFR*-TKI osimertinib and determined their cell cycle status using flow cytometry (Figure 1A, and Figure S1). Osimertinib treatment expectedly decreased the S/G2/M phase ratio with increased G0/G1 cell cycle accumulation, consistent with previous reports (13). However, the S/G2/M phase cells were persistent even after osimertinib exposure, indicating that some cells had

proliferated. We immunoblotted cell cycle proteins RB, CDK4, and CDK6 (Figure 1B). CDK4 expression was unaltered by osimertinib in PC-9, HCC827, and 11-18 cells; incompletely inhibited in H1975 cells; and completely inhibited in H3255 cells. Consistently, phosphorylated RB was not completely inhibited by osimertinib in PC-9, HCC827, 11-18, and H1975 cells, but it was completely inhibited in H3255 cells. CDK6 was not inhibited in PC-9 and HCC827 cells, incompletely inhibited in 11-18, and H1975 cells, and completely inhibited in H3255 cells. These data suggest that osimertinib does not completely inhibit cell cycle factors in *EGFR*-mutant NSCLC cells, except for H3255.

CDK4/6 inhibitor further inhibited cell cycling of osimertinib-treated NSCLC cells

We investigated the effects of the CDK4/6 inhibitor, abemaciclib, on phosphorylated RB (Figure 2A). Abemaciclib and osimertinib suppressed phosphorylated RB compared with osimertinib or abemaciclib monotherapy in PC-9, HCC827, 11-18 and H1975 cells. H3255 cells did not show these observations. There was no obvious effect of abemaciclib on CDK4/6 expression in the investigated cells; abemaciclib did not affect the intracellular signaling of AKT or ERK. To further investigate the impact of CDK inhibition, we knocked down CDK4 using two individual siRNAs (Figure S2A). Compared to controlled siRNA, siCDK4 more effectively inhibited pRB in the presence of osimertinib, suggesting the residual pRB was maintained through CDK. Next, we investigated the effects of abemaciclib on the osimertinib-induced persistent cell cycle (Figure 2B). Compared to each agent alone, combining osimertinib with abemaciclib led to increased cell cycle arrest at the G0/G1 phase and reduced cell proliferation at the S/G2/M phase in PC-9 and H1975 cells. Although the overall effect was consistent with data from PC-9 and H1975 cells, the increase in cell cycle arrest at the G0/G1 phase in both HCC827 and 11-18 cells was subtle when osimertinib was combined with abemaciclib. In contrast, the combination did not lead to increased cell cycle arrest in H3255 cells.

Remaining cell cycling and phosphorylated RB was maintained by ERK pathway

We and others have shown that *EGFR*-TKIs do not completely inhibit the ERK pathway in *EGFR*-mutant

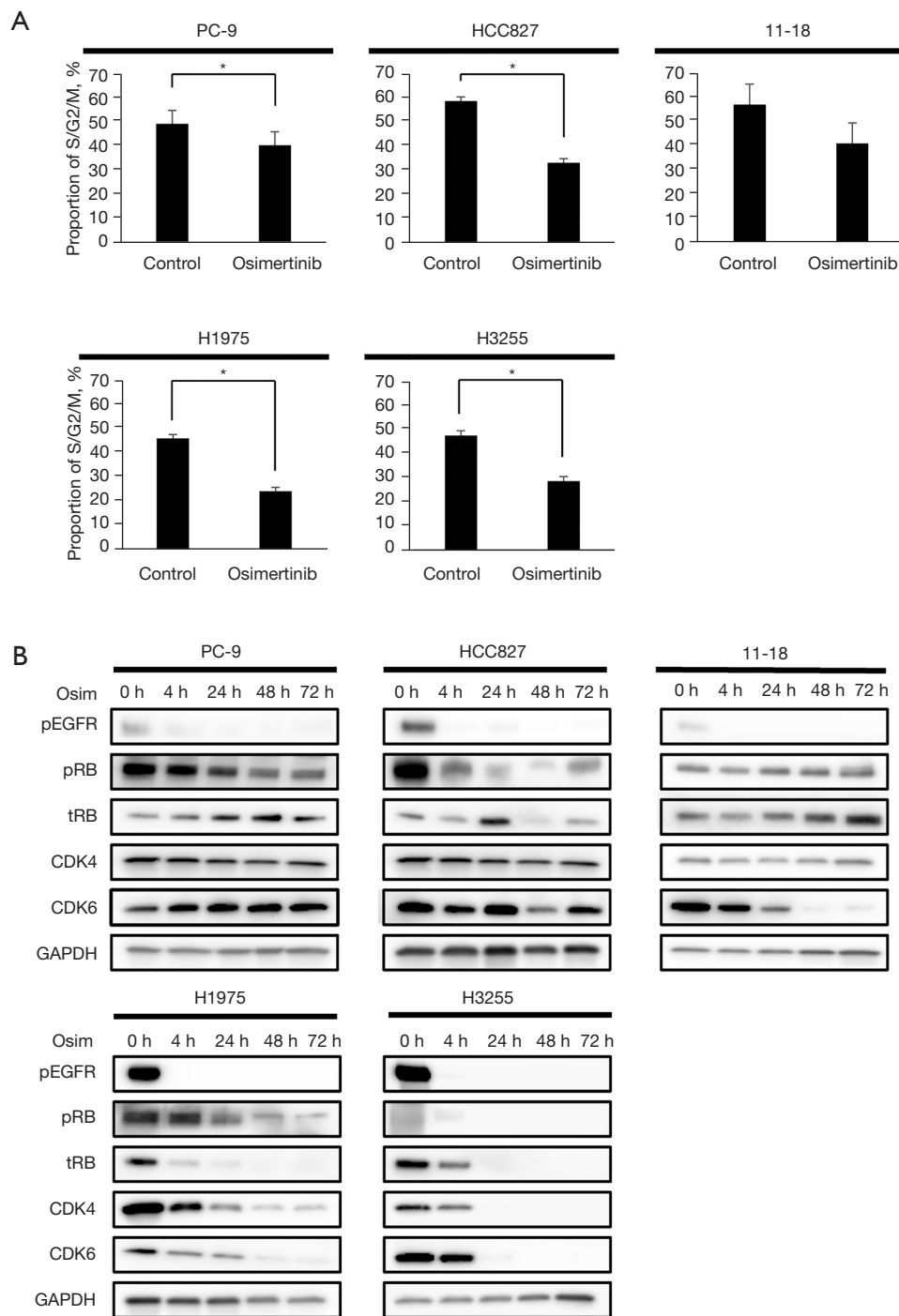


Figure 1 Changes in the cell cycle and related protein activity due to Osim loading. (A) Cell cycle analysis was performed using DAPI staining, followed by flow cytometry. The cells were treated for 24 h with dimethyl sulfoxide or 100 nM Osim. Error bars represent standard error. *, $P < 0.05$ (Student's t -test). (B) Immunoblots of the indicated proteins in PC-9, HCC827, 11-18, H1975, and H3255 cells treated with DMSO or 100 nmol/L Osim for 4, 24, 48, or 72 h. Osim, osimertinib; EGFR, epidermal growth factor receptor; RB, retinoblastoma protein; p, phosphorylated; t, total; CDK, cyclin-dependent kinase; DAPI, 4',6-diamidino-2-phenylindole, dihydrochloride; DMSO, dimethyl sulfoxide.

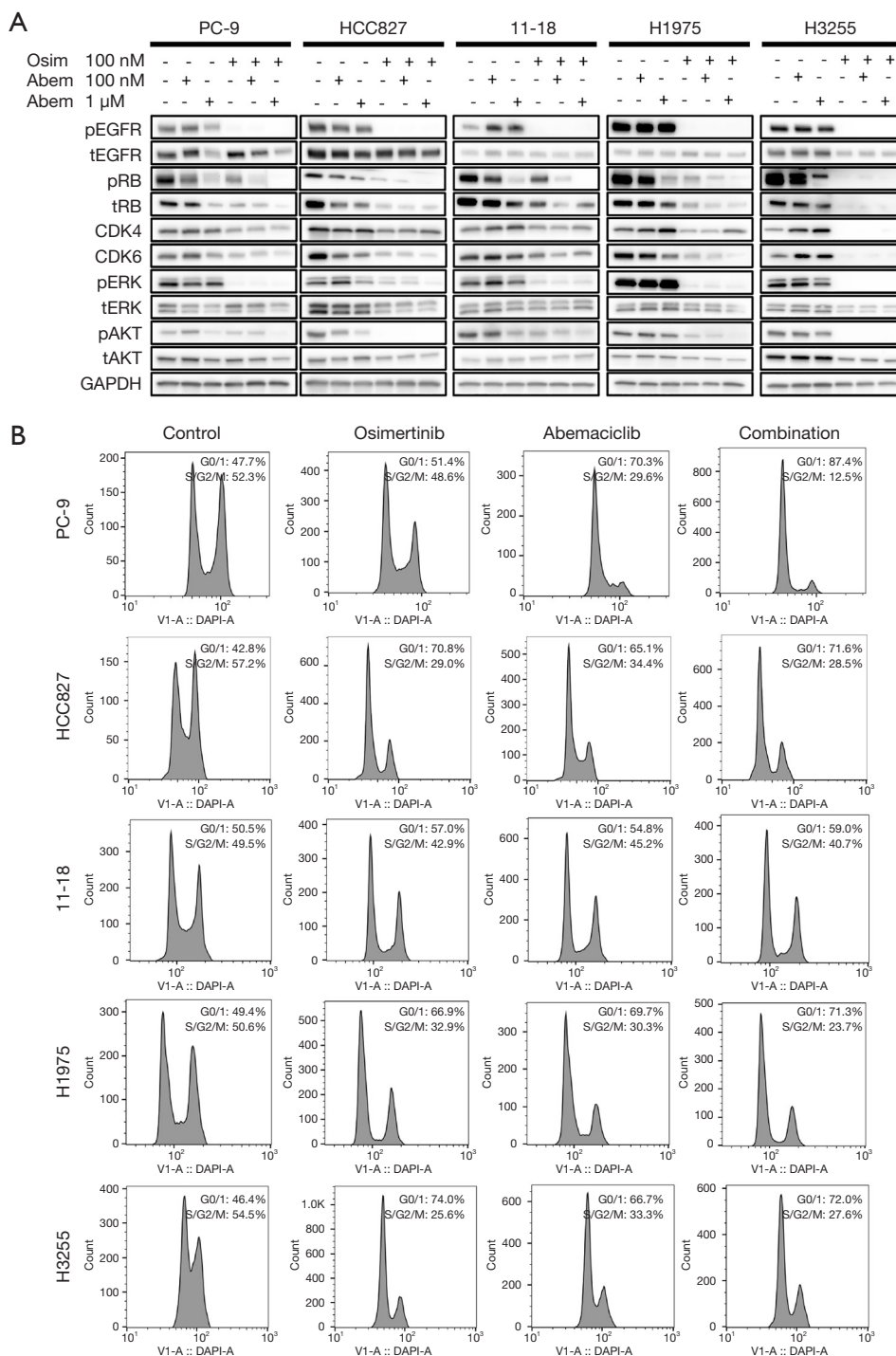


Figure 2 Combined effect of Osim and Abem on the cell cycle and related protein activity. (A) Immunoblots of the indicated proteins in PC-9, HCC827, 11-18, H1975, and H3255 cells treated with 100 nmol/L or 1 μmol/L Abem combined with 100 nmol/L Osim for 48 h. (B) Cell cycle changes in PC-9, HCC827, 11-18, H1975, and H3255 cells treated with DMSO or Osim (100 nM) for 24 h. All cell lines were treated with Abem (1 μmol/L) or a combination of both for 24 h. Cell cycle analysis was performed using DAPI staining, followed by flow cytometry. Osim, osimertinib; Abem, abemaciclib; p, phosphorylated; t, total; EGFR, epidermal growth factor receptor; RB, retinoblastoma protein; CDK, cyclin-dependent kinase; DMSO, dimethyl sulfoxide; DAPI, 4',6-diamino-2-phenylindole.

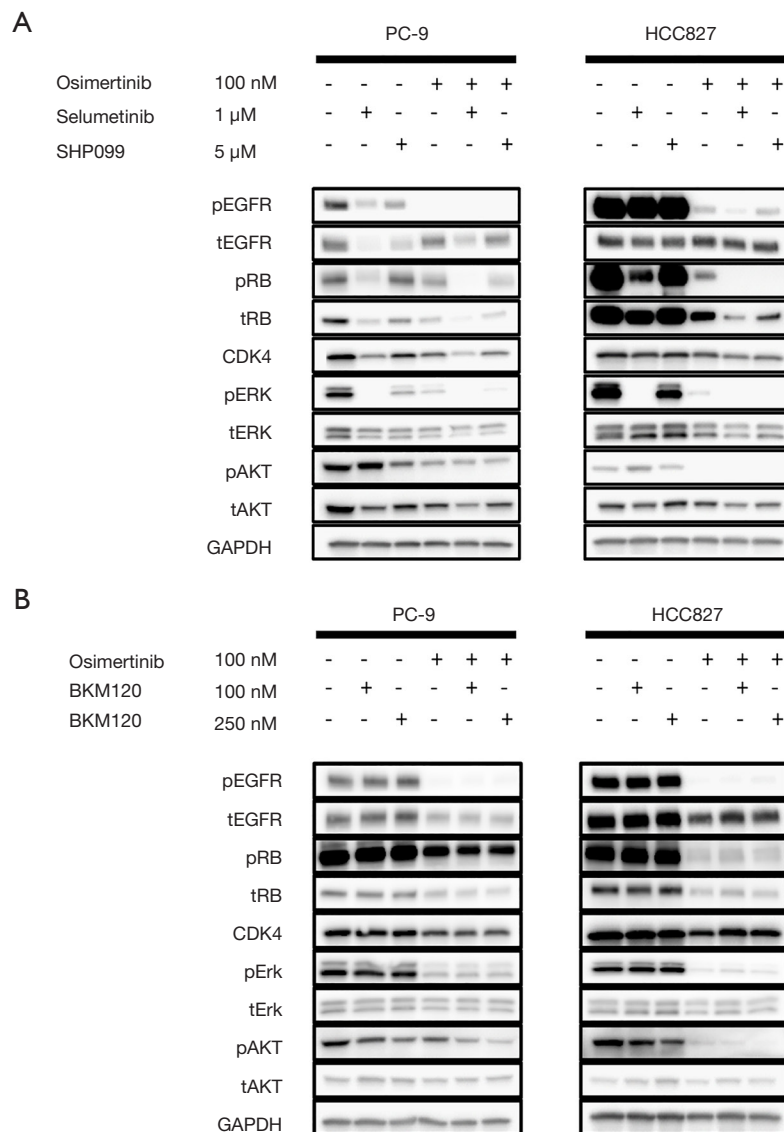


Figure 3 Inhibition of RB phosphorylation by MEK inhibitor, SHP2 inhibitor, or PI3K inhibitor combined with Osim. (A) Immunoblots of the indicated proteins in PC-9 and HCC827 cells treated with Osim (100 nmol/L) for 48 h. All cell lines were also treated with selumetinib (1 μ mol/L), SHP099 (5 μ mol/L), or combination for 48 h. (B) Immunoblots of the indicated proteins in PC-9 and HCC827 cells treated with Osim (100 nM) for 48 h. All cell lines were treated with BKM120 (100 or 250 nmol/L) or a combination of both for 48 h. p, phosphorylated; t, total; EGFR, epidermal growth factor receptor; RB, retinoblastoma protein; CDK, cyclin-dependent kinase; MEK, MAPK-ERK kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase.

NSCLC cells, and the activity of this pathway attenuates the effects of EGFR-TKIs (5-7). We investigated the remaining ERK signal activity on pRB and the cell cycle after osimertinib exposure (Figure 3A). To further inhibit the remaining ERK signaling, the mitogen-activated protein kinase (MAPK)-ERK kinase (MEK) inhibitor selumetinib was used in combination with osimertinib. Consistent

with previous findings (5,6), osimertinib monotherapy did not completely inhibit pERK but selumetinib completely inhibited the remaining ERK phosphorylation when combined with osimertinib; selumetinib did not alter AKT phosphorylation. Furthermore, phosphorylated RB was inhibited by complete ERK inhibition, suggesting that the remaining phosphorylated RB resulted from ERK signaling.

We also investigated the SHP2 inhibitor SHP099, which inhibits ERK upstream of SHP2. Consistent with selumetinib data, the SHP2 inhibitor combined with osimertinib further inhibited pERK; the inhibition was incomplete, leading to more profound pRB inhibition. To further investigate the impact of ERK signaling, we knocked down ERK1 using two individual siRNAs (Figure S2B). Compared to controlled siRNA, siERK1 more effectively inhibited pRB in the presence of osimertinib. Furthermore, compared to controlled siRNA, siERK1 in the presence of osimertinib more effectively inhibited the cell growth (Figure S2B). These data suggest that the remaining pRB was maintained by ERK signaling. Then, we investigated the effects of an AKT pathway inhibitor on RB signaling (Figure 3B). Phosphorylated AKT was further inhibited by the PI3K inhibitor BKM120 when combined with osimertinib, whereas ERK phosphorylation remained unaltered. In contrast to ERK pathway inhibitors, phosphorylated RB was not inhibited by the combination of BKM120 and osimertinib.

Taken together, these data demonstrate that the remaining RB activation under EGFR inhibition was maintained by ERK signaling.

CDK4/6 inhibitor enhanced inhibition of cell proliferation with osimertinib

The effects of the CDK4/6 inhibitor abemaciclib on cell proliferation were investigated using crystal violet and MTT assays. The crystal violet assay showed that osimertinib inhibited *EGFR*-mutant cell lines, with some remaining cells (Figure 4A). Abemaciclib monotherapy inhibited the investigated cells to various degrees with little effect at 100 nM, and the combination of osimertinib and abemaciclib showed more profound cell growth inhibition than either agent alone. There were few residual cells in the combination group treated with 1 μ M abemaciclib among PC-9, HCC827, 11-18 and H1975 cells, whereas substantial residual cells remained in the osimertinib monotherapy group. In H3255 cells, there were few residual cells with osimertinib irrespective of the combination of abemaciclib. The MTT assay showed similar results (Figure 4B). Osimertinib in the presence of abemaciclib inhibited cell proliferation more effectively than osimertinib alone in cell lines other than H3255 cells. To determine whether the enhanced effects of abemaciclib were synergistic or additive, we calculated the CI using the MTT assay. In all cell lines tested, the fraction affected (Fa) had

a CI of <1 , which means that osimertinib and abemaciclib work together better in *EGFR*-mutant cell lines except for H3255 (Figure 4C).

Next, we investigated the efficacy of the combination of abemaciclib and osimertinib on *EGFR*-TKI-resistant cells (H820 and PC-9/OsiR). The H820 cell line, which harbors the *EGFR* ex19 deletion and *MET* amplification, is intrinsically resistant to *EGFR*-TKIs. The PC-9/OsiR cell line was derived from PC-9 cells that were chronically exposed to osimertinib and thus acquired resistance to the agent. The combination of osimertinib and abemaciclib inhibited H820 cells effectively, whereas little effect was observed when either agent was used alone on the resistant cell lines. The combination was not effective against PC-9/OsiR cells, which were the acquired resistance model for osimertinib (Figure S3).

Enhanced efficacy by CDK4/6 inhibition was confirmed with another CDK4/6 inhibitor

To confirm that the enhanced effects of osimertinib with abemaciclib were not off-target effects but were exerted through CDK4/6 inhibition, we investigated another CDK4/6 inhibitor, palbociclib (Figure S4). Consistent with the data for abemaciclib, palbociclib combined with osimertinib further inhibited phosphorylated RB (Figure S4A), and synergistically enhanced cell growth inhibition (Figure S4B-S4D). These data suggest that the enhanced effects of osimertinib by abemaciclib or palbociclib are exerted through CDK4/6 inhibition.

CDK4/6 inhibitor showed durable efficacy when combined with osimertinib

To determine the long-term efficacy of the CDK4/6 inhibitor against *EGFR*-mutant NSCLC cells, we investigated cell viability after treatment with osimertinib alone or in combination for 2 weeks compared to that at the start of the treatment (Figure 5A). Cancer cells treated with osimertinib alone showed increased proliferation in 2 weeks, whereas the combination remarkably inhibited proliferation at 2 weeks of treatment.

The *in vivo* effects of CDK4/6 inhibitor were also investigated using a xenograft tumor model. PC-9 xenograft tumors were treated with osimertinib, abemaciclib, or a combination of both (Figure 5B). Osimertinib monotherapy significantly inhibited the tumors, and the combination therapy showed more effective tumor inhibition than

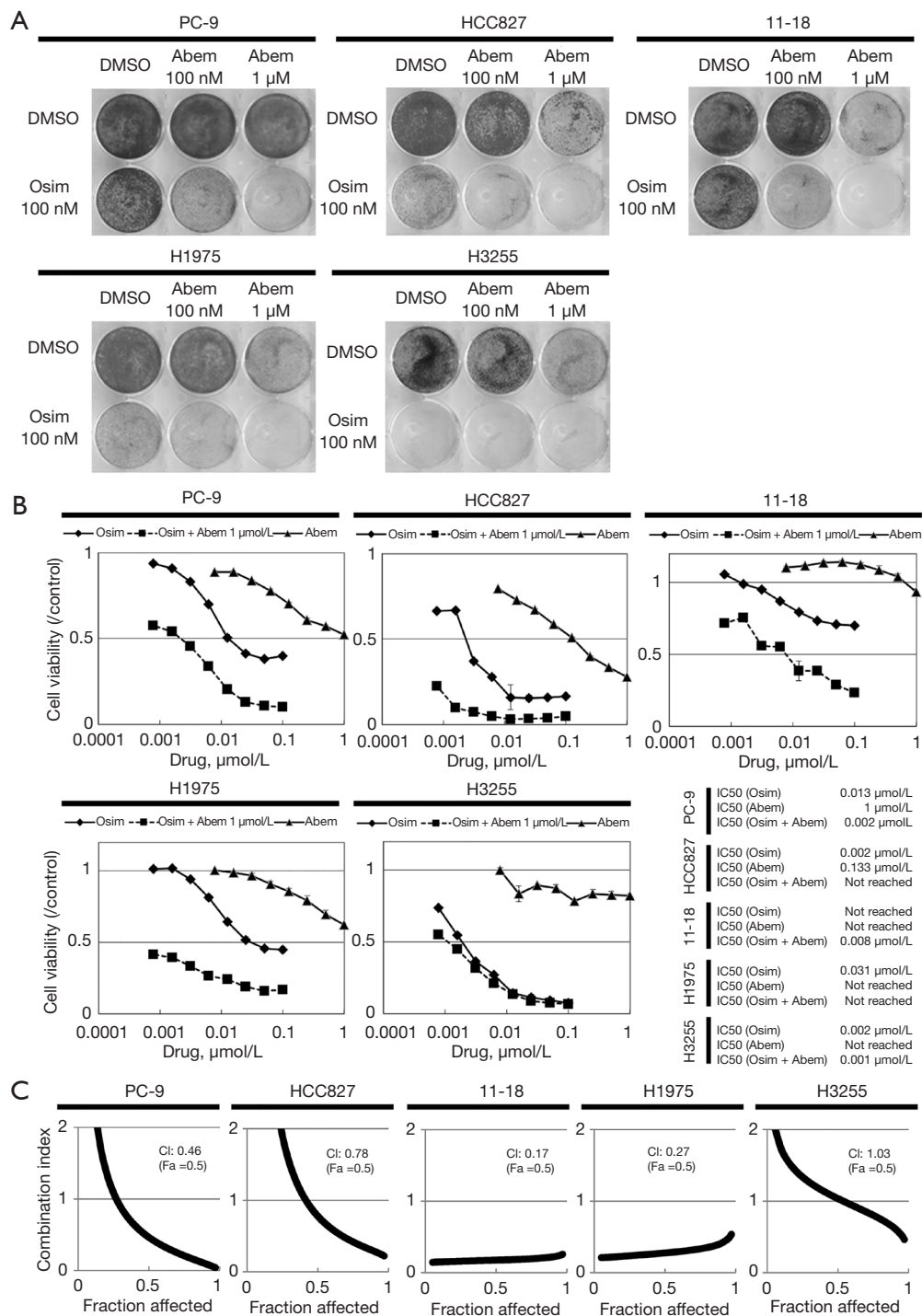


Figure 4 Inhibition of cancer cell proliferation and viability by Abem combined with Osim. (A) Crystal violet-stained cells after drug loading for 6 days (magnification, 1 \times). Cancer cells were treated with 100 nmol/L or 1 μ mol/L Abem combined with 100 nmol/L Osim for 6 days. (B) Viability curves for cell lines treated with Osim alone (rhombuses), Abem alone (triangles), or Osim and Abem (1 μ mol/L; squares). All drugs were loaded for 96 h. Error bars represent standard error. (C) The Osim to Abem ratio was 1:10. DMSO, dimethyl sulfoxide; Osim, osimertinib; Abem, abemaciclib; IC₅₀, 50% inhibition concentration; CI, combination index; Fa, fraction affected.

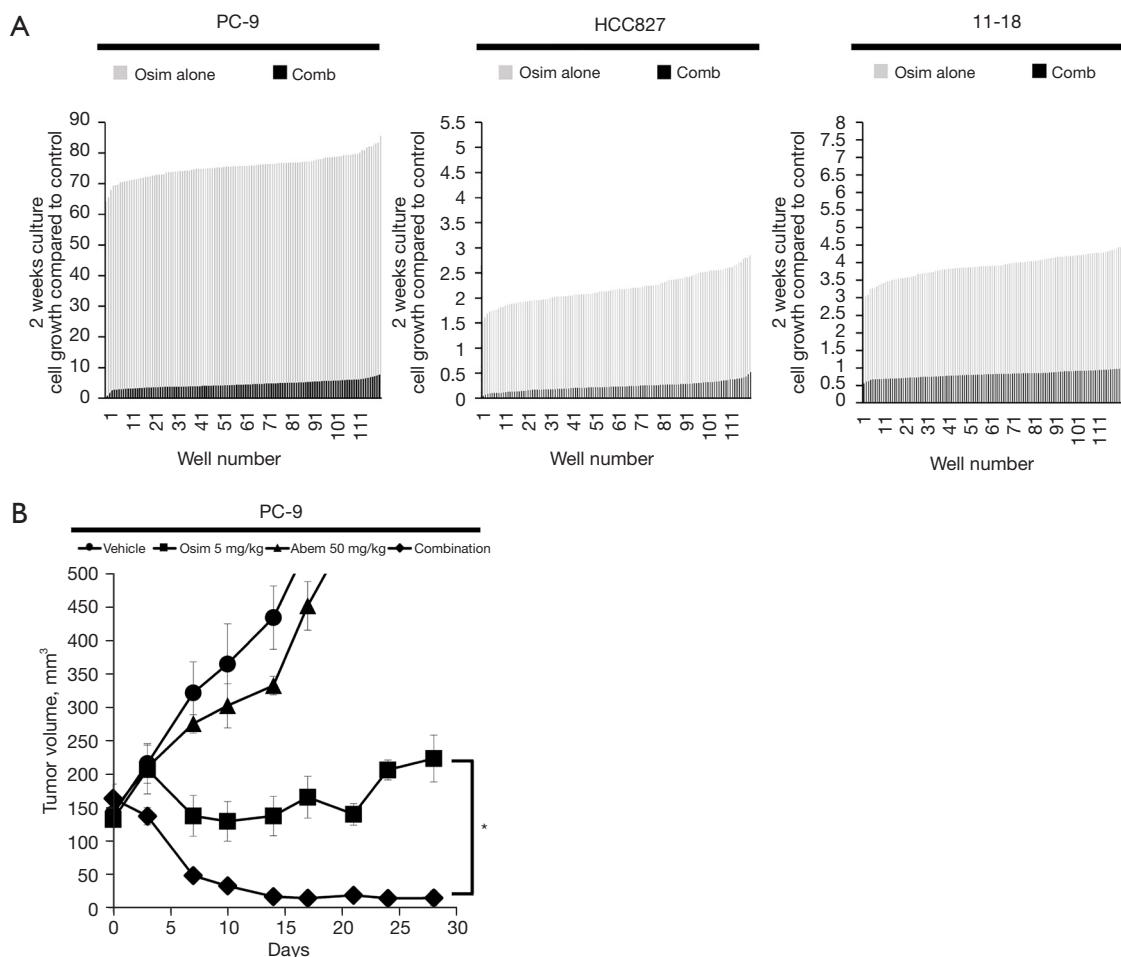


Figure 5 Long-term effects of CDK4/6 inhibitor combined with EGFR-TKI *in vivo* and *in vitro*. (A) Suppressive effect of cell proliferation by the long-term loading of Abem. Each cell line was seeded in 96-well plates and treated with Osim (100 nmol/L) or a combination of Osim (100 nmol/L) and Abem (1 μmol/L) for 2 weeks. Cell proliferation per well at each time point was compared to day 1 values using the MTT assay. (B) Combination of Osim and CDK4/6 inhibitors in a xenograft mouse model. Mice transplanted with PC-9 cells were orally administered Osim (5 mg/kg/day) and Abem (50 mg/kg/day) five times a week. Error bars represent standard error. *, P<0.01 (Student's *t*-test). Osim, osimertinib; Abem, abemaciclib; Comb, combination with Osim and Abem; CDK, cyclin-dependent kinase; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor.

osimertinib alone. Notably, tumors treated with osimertinib monotherapy started re-growing after 3 weeks of treatment, whereas those treated with the combination therapy did not; six of eight tumors treated with the combination were undetectable after 4 weeks of treatment. There was no apparent weight loss in each group.

Combination with CDK4/6 inhibitor was not effective in slow-proliferating NSCLC cells with less RB activation

The enhanced effects of osimertinib with a CDK4/6

inhibitor were not observed in H3255 cells, in contrast to the other investigated cell lines (Figure 4). To determine the potential predictive markers of the efficacy of combination therapy, we first investigated the proliferation rate of each cell line (Figure 6A). In the cell lines that showed high sensitivity to the combination therapy, such as PC-9, HCC827 and 11-18 cells, cell proliferation was relatively high, with a doubling time of less than 20 h. In H3255, where the CDK4/6 inhibitor did not enhance the effects of osimertinib, the proliferation rate was the lowest, with a doubling time of more than 60 h. In H1975 cells, which

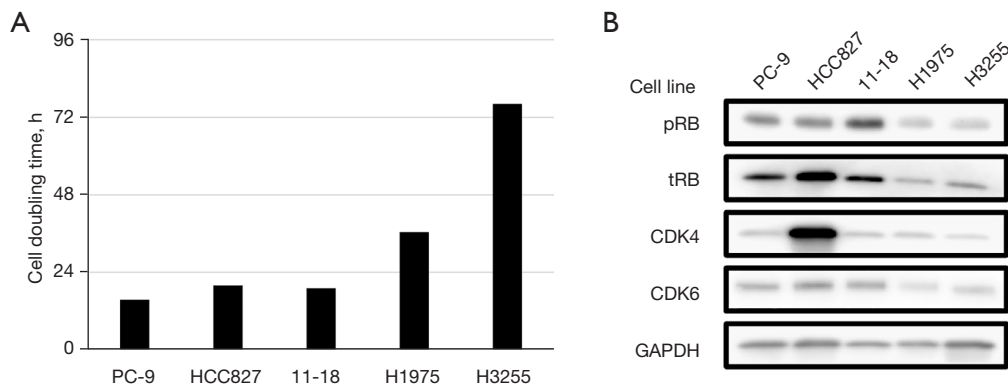


Figure 6 Differences in cell cycle-related proteins and cell growth proliferation time depending on cell line. (A) Cell doubling time of each cell line. The cells were seeded in 10 cm dishes and cultured for 24, 48, 72, and 96 h. We counted the cells and drew the cell proliferation curves. The doubling time of each cell line was calculated from the curves. (B) Immunoblots of the indicated proteins in PC-9, HCC827, 11-18, H1975, and H3255 cells in drug-free medium. p, phosphorylated; t, total; RB, retinoblastoma protein; CDK, cyclin-dependent kinase.

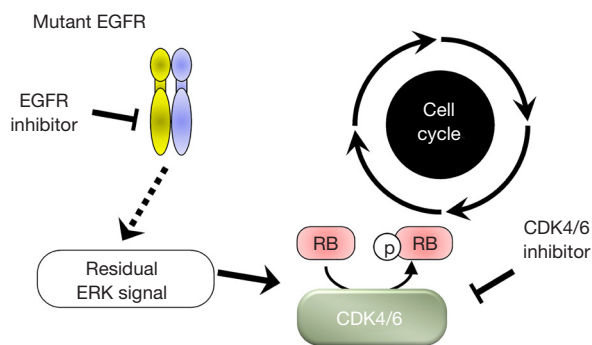


Figure 7 Graphical abstract. Remaining ERK signaling under EGFR inhibition maintains the CDK4/6-RB signal axis in EGFR-mutant lung cancer cells. The CDK4/6-RB signal axis attenuates the effects of EGFR inhibition by maintaining the cell cycle. EGFR, epidermal growth factor receptor; RB, retinoblastoma protein; CDK, cyclin-dependent kinase; ERK, extracellular signal-regulated kinase.

showed moderate sensitivity to combination therapy, the proliferation rate was intermediate, with a doubling time of approximately 40 h. We also determined cell cycle status using immunoblotting (Figure 6B). Cell lines sensitive to the combination (PC-9, HCC827 and 11-18) showed more phosphorylated RB and higher expression of RB and CDK6 than H1975 or H3255. CDK6 expression was not consistent between cell lines. These data suggest that rapidly growing tumors with more activated cell cycle signaling, including

RB and CDK6, are more sensitive to combination therapy.

Discussion

Our findings indicate that EGFR-TKI therapy did not completely halt the cell cycle in EGFR-mutant NSCLC (Figure 7). Residual ERK signaling, even under the influence of EGFR-TKI, sustained the CDK4/6-RB signaling axis, thereby maintaining the cell cycle and attenuating the effects of the EGFR-TKI. The use of CDK4/6 inhibitors enhanced the efficacy of EGFR-TKI.

EGFR-TKIs inhibit EGFR-mutant NSCLC tumors; however, acquired resistance inevitably develops in a relatively short period. Preclinical and clinical studies have revealed numerous acquired resistance mechanisms and suggested potential strategies to overcome them (2,7,14,15). However, the resistance mechanisms are diverse, and the frequency of each mechanism is relatively low, leading to difficulties in establishing clinically effective treatments corresponding to each resistance mechanism. Moreover, growing evidence has shown that tumors have different resistance mechanisms, and heterogeneous resistance mechanisms exist within one patient, making it difficult to overcome acquired resistance (16,17). Therefore, we believe that enhanced initial treatment with upfront combination therapy to prevent or delay acquired resistance and its heterogeneity is more important.

Cancer cells showing tolerance to TKI therapy survive TKI exposure in a dormant state without proliferation (18).

However, our study found that cell proliferation was not completely stopped without EGFR signaling, with the remaining CDK4/6-RB signaling axis. We, along with other groups, previously found that the MAPK pathway was not completely inhibited under continuous EGFR inhibition, and the remaining MAPK signaling attenuated the effects of EGFR-TKIs, hindering curative efficacy in *EGFR*-mutant NSCLC (6,7). In the current study, we revealed that the CDK4/6-RB signaling axis, which was maintained by the remaining ERK signaling, was one of the underlying mechanisms. However, the precise mechanisms that maintain ERK and CDK4/6-RB signaling remain unclear and need to be further investigated.

CDK4/6 inhibitors have been reported to overcome acquired resistance to osimertinib with various resistance mechanisms, such as C797X secondary mutation, MET amplification, and BRAF secondary mutation (12,19). These data suggest that CDK4/6 signaling affects EGFR-TKIs sensitivity in *EGFR*-mutant NSCLC. In contrast, we revealed that upfront use of CDK4/6 inhibitors enhanced EGFR-TKI efficacy before the acquisition of resistance. Although the upfront use of CDK4/6 inhibitors combined with EGFR-TKI has not been well investigated in *EGFR*-mutant NSCLC, its efficacy has been reported in an esophageal cancer preclinical model, showing that the combination of palbociclib and erlotinib prevented the acquisition of resistance in erlotinib-sensitive esophageal cancer cells (20). Similarly, our data showed the long-term inhibition of cell proliferation in *EGFR*-mutant NSCLC. In addition, CDK4/6 gene amplification is associated with *de novo* EGFR-TKI resistance in *EGFR*-mutant NSCLC patients with shorter progression-free survival (21). These data indicate that CDK4/6 signaling is associated with EGFR-TKI sensitivity before the emergence of acquired resistance.

CDK4/6 inhibitor enhanced the efficacy of EGFR-TKI in *EGFR*-mutant NSCLC cells. The efficacy of osimertinib was not enhanced in H3255 cells (Figure 4). In contrast to other *EGFR*-mutant cells, H3255 cells showed slower cell proliferation with a longer doubling time and less phosphorylated RB (Figure 6). Supporting our findings, it has been reported that the loss of RB is associated with resistance to CDK4/6 inhibitors in breast cancer cells (22). An aggressive disease phenotype and highly phosphorylated RB using immunohistochemistry might predict the combination of CDK4/6 inhibitors and EGFR-TKI in clinical settings.

Efficacy of combination with EGFR-TKI and a CDK/6 inhibitor

Our preclinical data suggest potential for clinical application. However, our study has limitations due to its nature as preclinical research. For instance, the efficacy of the combination therapy in the xenograft model doesn't necessarily guarantee its efficacy in a clinical setting. This is because the tumor microenvironment can vary significantly between the mouse xenograft model and human lung tissue. Therefore, caution is needed in interpretation, and validation in clinical studies is warranted.

Conclusions

Our study demonstrated that the CDK4/6-RB signal axis, maintained by the MAPK pathway, plays an important role in attenuating the efficacy of EGFR-TKIs in *EGFR*-mutant NSCLC and that targeting CDK4/6 enhances efficacy. A new way to treat TKI-naïve *EGFR*-mutant NSCLC could be to use CDK4/6 inhibitors and EGFR-TKI together.

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Footnote

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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. The experiments were performed in accordance with the Policy on the Care and Use of Laboratory Animals, Okayama University, and Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions, Ministry of Education, Culture, Sports, Science, and Technology of Japan. The experimental protocol was approved by the Animal Care and Use Committee of Okayama University (No. OKU-2021772).

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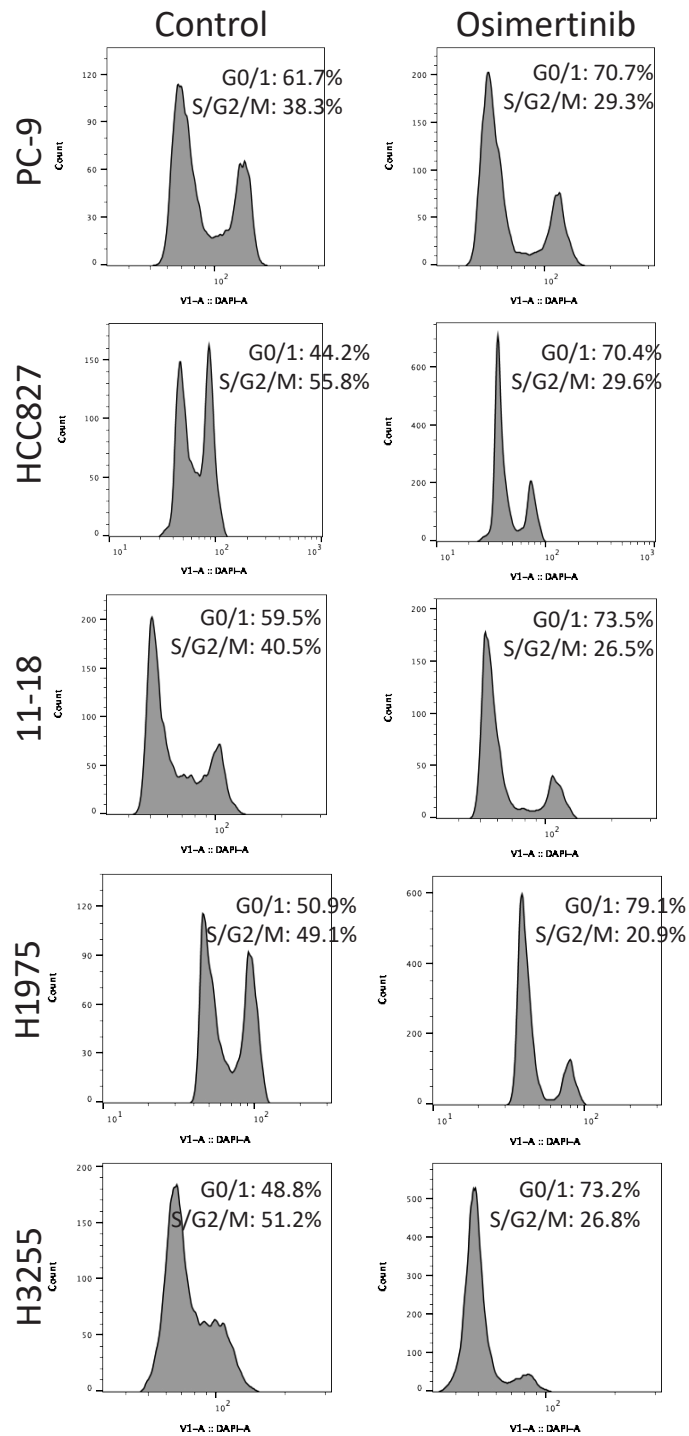


Figure S1 Representative cell cycle histogram in *Figure 1A*. Cell cycle analysis was performed using DAPI staining, followed by flow cytometry. The cells were treated for 24 h with dimethyl sulfoxide or 100 nM osimertinib. DAPI, 4',6-diamidino-2-phenylindole, dihydrochloride.

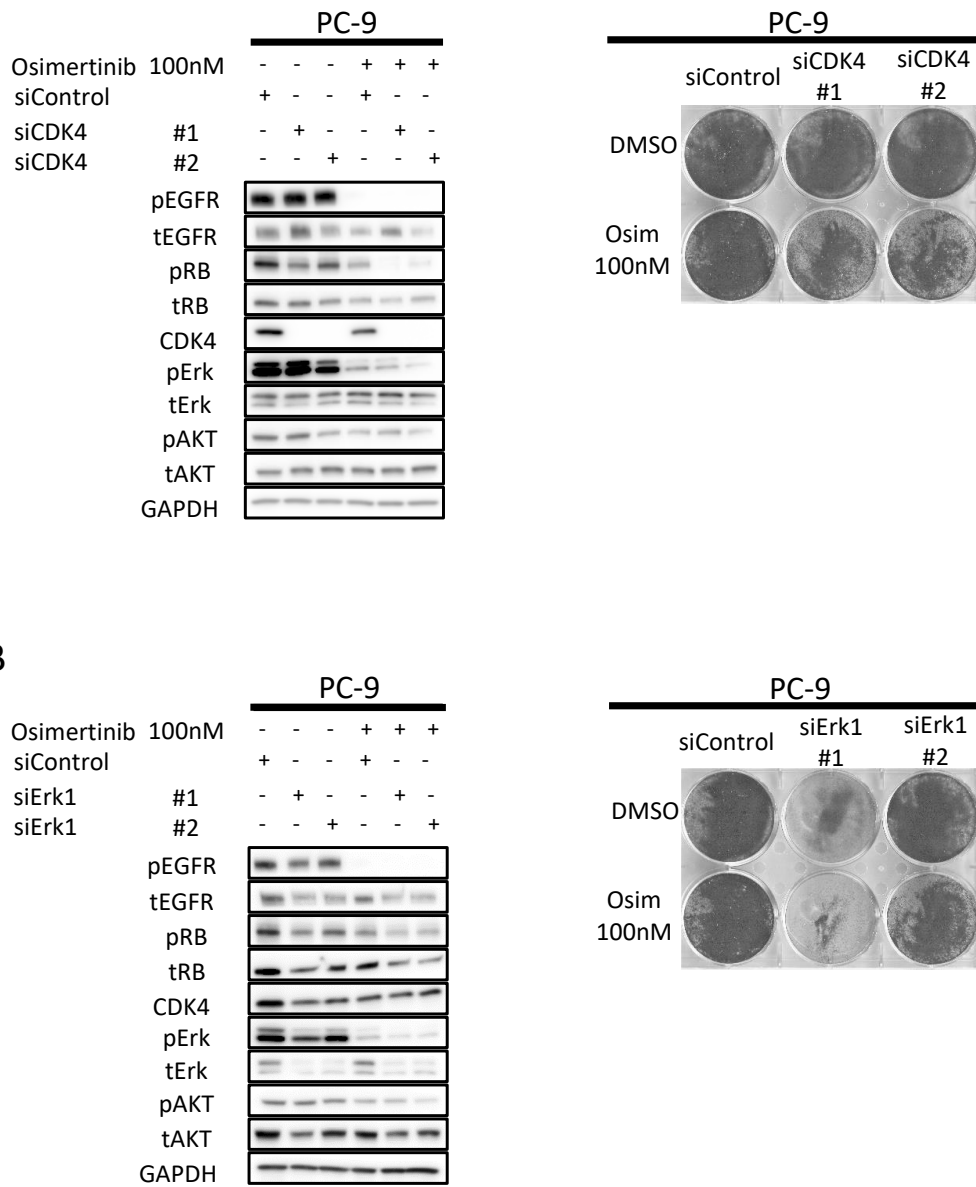


Figure S2 Effects of CDK4 and ERK1 silencing combined with Osim and CDK4/6 inhibitor on oncogenic signaling pathways and cell growth. (A) Two different CDK4-targeting siRNAs were used. Immunoblots of the indicated proteins in PC-9 cells treated with Osim (100 nmol/L) for 24 h. Crystal violet-stained cells after drug loading for 6 days. PC-9 cells were treated with 100 nmol/L Osim (magnification, 1×). (B) Two different ERK1-targeting siRNAs were used. Immunoblots of the indicated proteins in PC-9 cells treated with Osim (100 nmol/L) for 24 h. Crystal violet-stained cells after drug loading for 6 days (magnification, 1×). PC-9 cells were treated with 100 nmol/L Osim. Osim, osimertinib; CDK, cyclin-dependent kinase; p, phosphorylated; t, total; EGFR, epidermal growth factor receptor; RB, retinoblastoma protein; DMSO, dimethyl sulfoxide; siRNA, small interfering RNA.

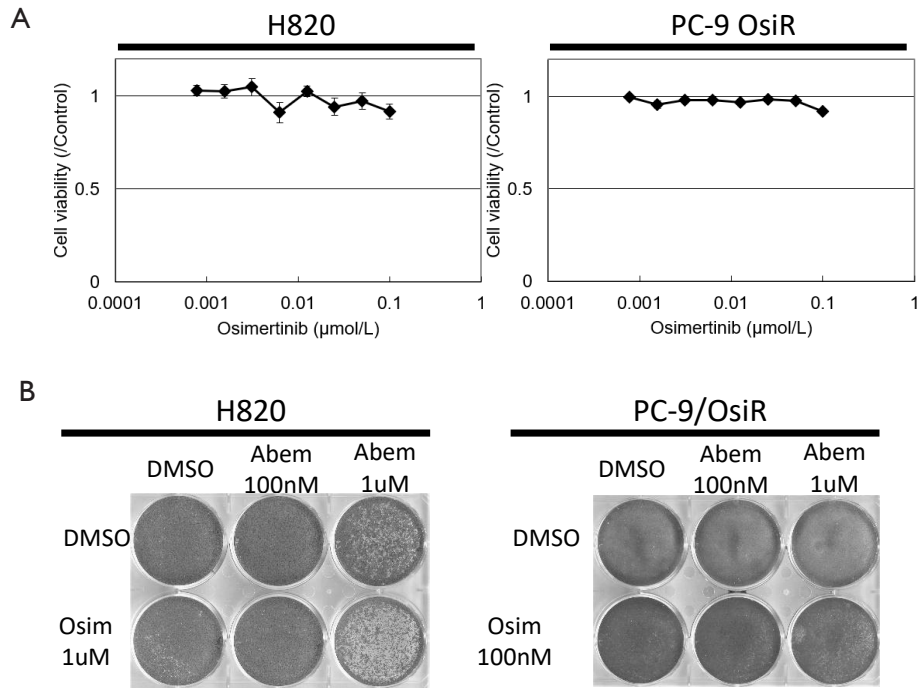


Figure S3 Effect of combination therapy with Abem on cell proliferation in Osim-resistant cell lines. (A) Viability curves for cell lines treated with Osim. Osim was loaded for 96 h. Error bars represent standard error. (B) Crystal violet-stained cells after drug loading for 6 days (magnification, 1 \times). Cancer cells were treated with 100 nmol/L or 1 μ mol/L Abem combined with 100 nmol/L Osim for 6 days. DMSO, dimethyl sulfoxide; Osim, osimertinib; Abem, abemaciclib.

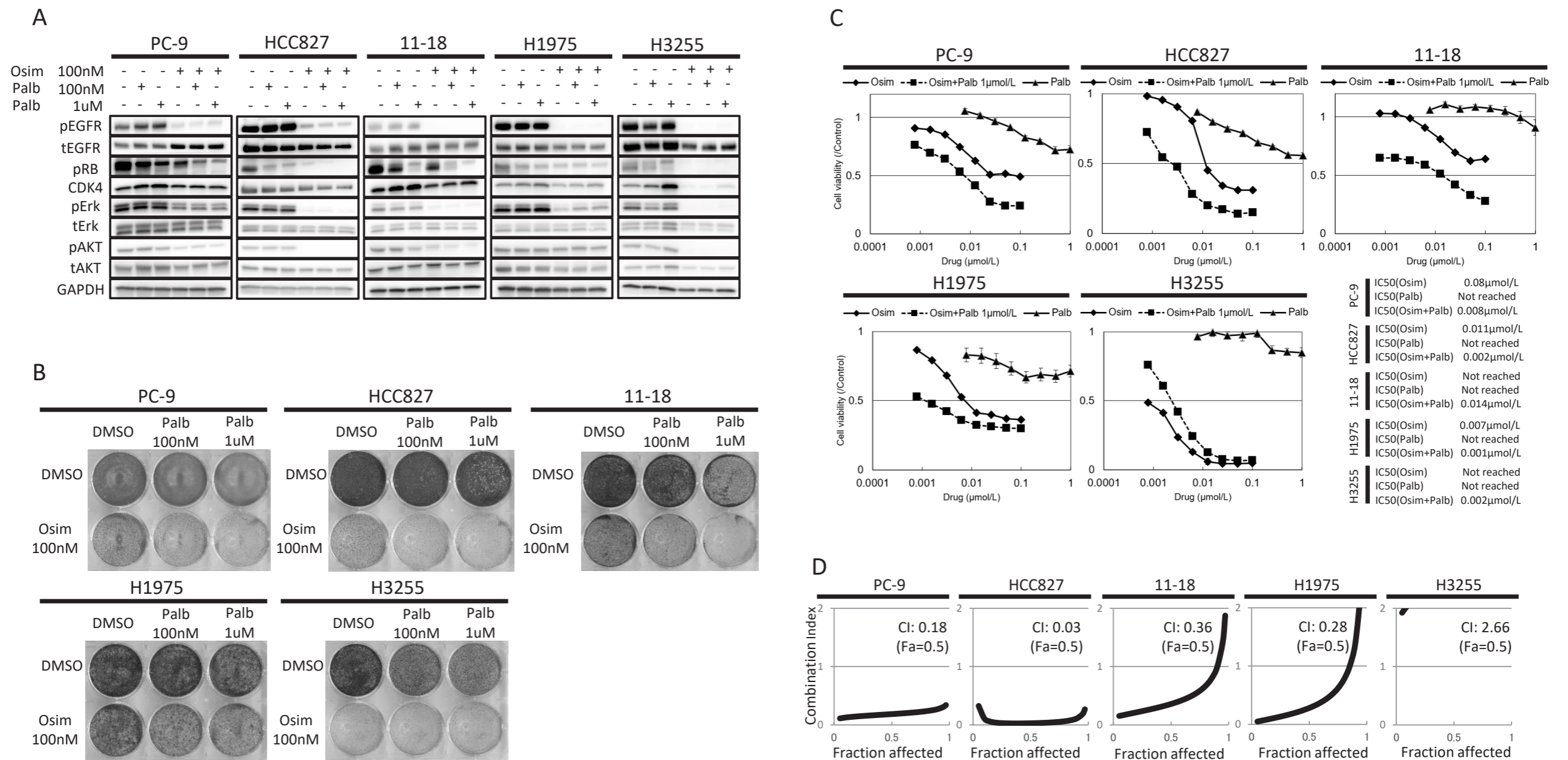


Figure S4 Combined effect of Osim and Palb on the cell cycle, related protein activity, and cell proliferation. (A) Immunoblots of the indicated proteins in PC-9, HCC827, 11-18, H1975, and H3255 cells treated with 100 nmol/L or 1 µmol/L Palb combined with 100 nmol/L Osim for 48 h. (B) Crystal violet-stained cells after drug loading for 6 days (magnification, 1×). Cancer cells were treated with 100 nmol/L or 1 µmol/L Palb combined with 100 nmol/L Osim for 6 days. DMSO was used at a calculated concentration of 0.1%. (C) Cell viability curves for cell lines treated with the Osim alone (rhombuses), Palb alone (triangles), or Osim and Palb (1 µmol/L; squares). All drugs were loaded for 96 h. Error bars represent the standard error. (D) The ratio of the Osim and Palb concentrations was 1:10. Osim, osimertinib; Palb, palbociclib; IC50, 50% inhibition concentration; CI, combination index; Fa, fraction affected; DMSO, dimethyl sulfoxide.