



Research Paper

ULK1, a novel therapeutic target to delay drug tolerance to EGFR-TKIs in an *EGFR*-mutant non-small cell lung cancer model

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ABSTRACT

Background: The presence of drug-tolerant persister (DTP) cells reduces the effectiveness of epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs) in *EGFR*-mutated non-small cell lung cancer (NSCLC). Although autophagy is a potential target for eliminating DTP cells, clinical trials targeting autophagy in *EGFR*-mutant NSCLC have been unsuccessful—likely because most trials have utilized chloroquine, non-specifically inhibiting autophagy targeting lysosomes with dose limitations. This study focused on unc-51-like autophagy activating kinase 1 (ULK1), a more specific target for autophagy inhibition, to assess whether ULK1 inhibition delays the emergence of tolerance to EGFR-tyrosine kinase inhibitors in *EGFR*-mutant NSCLC cells.

Methods: DTP cells were generated by treating *EGFR*-mutant NSCLC cell lines with osimertinib. Autophagy status and ULK1 expression were evaluated using immunofluorescence, western blotting, and quantitative real-time PCR. The effects of both pharmacological and genetic inhibition of ULK1 were examined *in vitro* and *in vivo*.

Results: ULK1 and LC3-II, markers of autophagy, were upregulated in DTP cells. Inhibition of ULK1, either pharmacologically or genetically, suppressed autophagy, prevented the formation of DTP cells, and enhanced the antitumor activity of osimertinib in both *in vitro* and *in vivo* models. Furthermore, upregulation of ULK1 expression through serum starvation conferred tolerance to osimertinib, reinforcing ULK1's role in drug tolerance. Notably, ULK1 inhibition showed modest efficacy even after cells transitioned to the DTP or acquired resistance states.

Conclusion: ULK1 plays a critical role in mediating drug tolerance in *EGFR*-mutant NSCLC. Targeting ULK1 represents a promising therapeutic approach to enhance the efficacy of EGFR-TKIs and to delay the development of drug tolerance.

1. Introduction

Over the past two decades, various molecular targeted agents for non-small cell lung cancer (NSCLC), including epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs), have been developed [1–3] and have demonstrated significant efficacy against NSCLC harboring driver oncogenes. Among them, osimertinib is one of the most effective EGFR-TKIs against *EGFR*-mutant NSCLC, offering significantly

improved progression-free survival and overall survival compared with first-generation EGFR-TKIs (median progression-free survival, 18.9 months vs. 10.2 months; hazard ratio, 0.46; 95 % confidence interval, 0.37–0.57, $P < 0.001$ and median overall survival, 38.6 months vs. 31.8 months; hazard ratio, 0.799; 95 % confidence interval, 0.641–0.997; $P = 0.0462$)¹. However, in many clinical cases, *EGFR*-mutant NSCLC initially decreases in size following the initiation of EGFR-TKI therapy and subsequently stabilizes to a residual size before eventually

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progressing due to acquired resistance to EGFR-TKIs. Curative outcomes are rarely achieved with EGFR-TKIs, including osimertinib, because some cancer cells survive despite treatment. These surviving cells—referred to as drug-tolerant persister (DTP) cells—represent a reversible state [4], which is considered a preliminary stage of classical acquired resistance caused by irreversible alterations such as secondary genetic mutations. Following drug cessation, these cells can resume growth and proliferation while remaining sensitive to the same therapy [5].

Although the precise mechanism underlying tolerance to EGFR-TKIs, such as through the DTP state, remains unclear, numerous studies have delineated various mechanisms underlying DTP formation in NSCLC [4,6–9]. For example, epigenetic adaptations that alter H3K27 methylation via KDM6, metabolic reprogramming such as dependence on mitochondrial oxidative phosphorylation, immune evasion via CD70 (which is highly expressed in DTP cells), and survival signaling via TROP2 have all been implicated in the DTP state [4,8,9]. Other identified mechanisms include bypass pathway activation, such as the reactivation of the mitogen-activated protein kinase (MAPK) pathway through Src signaling [6,7,10]. Furthermore, MAPK pathway or Src inhibitors have been shown to enhance the efficacy of osimertinib in reducing the number of DTP cells. However, some DTP cells persist even with combination therapy, suggesting that inhibiting bypass cell signaling pathways alone is insufficient to fully eliminate DTP cells. This indicates the existence of additional mechanisms by which cancer cells evade EGFR signal inhibition in *EGFR*-mutant NSCLC.

Autophagy represents another potential therapeutic target to overcome tolerance to EGFR-TKIs in *EGFR*-mutant NSCLC [11]. Malignant tumors, including *EGFR*-mutant NSCLC, are often subjected to multiple autophagy-inducing stressors such as high metabolic demands due to hypoxia, malnutrition, and increased proliferation [11]. Although such stressors typically induce apoptosis or necrosis, tumor cells with a high apoptotic threshold often rely on autophagy as a survival strategy to evade cell death and necrosis in response to metabolic crises. Chloroquine, an autophagy inhibitor, has been shown to enhance the effects of EGFR-TKIs in many preclinical models [12,13]. However, despite the promising effects of chloroquine, clinical studies investigating the efficacy of combined EGFR-TKIs and chloroquine have yielded disappointing results [14]. This may be because chloroquine is not an autophagy-specific inhibitor but rather a multifunctional lysosome inhibitor [15]. Therefore, owing to its multiple functions, chloroquine cannot be administered at doses sufficient to inhibit autophagy effectively in clinical settings. Furthermore, lysosomal inhibition by chloroquine affects various essential cellular processes beyond autophagy, including endocytosis and intracellular trafficking, potentially leading to narrow therapeutic windows and off-target toxicities that preclude its clinical utility.

Unc-51-like autophagy activating kinase 1 (ULK1) is a serine/threonine kinase that plays a critical role in the initiation of autophagy. ULK1 forms a complex that promotes the formation of an isolation membrane, which develops into an autophagosome. Compared to the non-specific inhibition of lysosomal acidification, targeting the upstream kinase ULK1 offers a more precise approach to blocking the autophagy-specific signaling cascade. While it has been previously reported that autophagy is involved in EGFR-TKI tolerance, lysosomal inhibition of autophagy has not yielded significant data in clinical studies. Given that ULK1 is the only serine/threonine kinase in the autophagy pathway, it represents a highly druggable and selective target that could circumvent the limitations of lysosomotropic agents. Based on reports that ULK1 is involved in the survival of DTPs [16], we focused on ULK1 as a more specific target. As an autophagy-specific factor involved in the early stages of autophagy, ULK1 represents a promising therapeutic target, with ULK1 inhibitors offering autophagy-specific inhibition with potentially fewer off-target effects than chloroquine. In this study, we focused on ULK1, a key regulator of autophagy, as a novel therapeutic target for early-phase intervention. Our aim was to establish

an upfront combination strategy with EGFR-TKIs that yields a superior antitumor response by effectively preventing the emergence and maintenance of DTP cells, thereby blocking the evolutionary path toward acquired resistance.

2. Materials and methods

2.1. Cell lines and drug Sources

PC-9 cells (EGFR Ex19 del E746_A750) were purchased from the European Collection of Authenticated Cell Cultures (Salisbury, UK). HCC827 (EGFR Ex19 del E746_A750), 11–18 (EGFR Ex21 L858R), and H1975 (EGFR Ex21 L858R /Ex20 T790M) cells were kindly provided by Dr. William Pao (Vanderbilt University, Nashville, TN, USA). All cell lines were cultured in the RPMI 1640 medium (Sigma-Aldrich), supplemented with 10 % heat-inactivated FBS and 1 % penicillin/streptomycin, and maintained at 37 % in a humidified atmosphere containing 5 % CO₂. Osimertinib, cisplatin, paclitaxel, ULK1 inhibitors (SBI-0206965 and MRT68921), and hydroxychloroquine (HCQ) were purchased from Selleck Chemicals (Houston, TX, USA). All compounds were dissolved in dimethyl sulfoxide for *in vitro* studies.

2.2. Generation of DTP cells

DTP cells were generated by treating the cells with 1 μ M osimertinib for 9 days. During treatment, the media, including osimertinib, were refreshed every 3 days.

2.3. Establishment of cell lines resistant to osimertinib

The PC-9 and HCC827 cell lines were treated with gradually increasing concentrations of osimertinib, starting at 0.01 μ mol/L. After 4–5 months, we obtained the acquired resistant cell lines—PC-9/OR and HCC827/OR—capable of proliferating in the presence of 1 μ mol/L osimertinib.

2.4. Cell proliferation assay

Cell growth inhibition was measured using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells were seeded in 96-well plates at a density of 1,500–4,000 cells per well (PC-9: 1,500 cells/well; HCC827: 4,000 cells/well; 11–18: 4,000 cells/well; H1975: 1,500 cells/well) and continuously treated with the respective drug for 72 h.

2.5. Flux assay

To confirm the presence of autophagy and measure autophagic flux, we first assessed the expression of LC3 bands by Western blotting. LC3 exists in two forms: LC3-I (cytosolic) and LC3-II (lipid-conjugated). The accumulation or enhancement of the LC3-II band can indicate either the induction of autophagy or the inhibition of autophagic degradation (flux blockade). Therefore, the further enhancement of the LC3-II band upon treatment with the lysosomal inhibitor HCQ (hydroxychloroquine) is necessary to confirm the actual presence of autophagic flux. Western blotting was performed using the PC9, HCC827, 11–18, and H1975 cell lines. For each line, three conditions were tested: parental cells (P), DTP, and DTP cells treated with 25 μ mol/L HCQ for 24 h. Additionally, fluorescent immunostaining was performed on these cells. Changes in the expression of LC3 puncta (dot-like signals) near the cell membrane were observed to further assess autophagosome formation.

2.6. Short interfering RNA transfection

Cells were transfected with short interfering RNAs (siRNAs) to knock down target genes under optimized conditions. Non-targeting and ULK1

siRNAs (Dharmacon, Lafayette, CO, USA) were used along with Lipofectamine Transfection Reagent (Thermo Fisher Scientific) to transfect cells seeded in 6-well plates (PC-9: 25,000 cells per well). ULK1 knockdown was performed using ULK1 siRNA in the two wells on the right, and 100 nM osimertinib was added to the bottom two wells.

2.7. Xenograft mouse models

Six-week-old female BALB/c nu/nu mice were obtained from Charles River Laboratories (Wilmington, MA, USA). All mice were provided with sterilized food and water and maintained in a barrier facility under a 12-hour light/dark cycle. Cancer cells were subcutaneously injected into both the right and left flanks of the mice. Tumor volumes were calculated periodically using the formula: $\text{width}^2 \times \text{length} / 2$. All animal experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Research Advisory Committee at the Department of Animal Resources, Okayama University Advanced Science Research Center (Okayama, Japan). The experiments complied with the Policy on the Care and Use of Laboratory Animals of Okayama University, as well as the Fundamental Guidelines for the Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions issued by the Ministry of Education, Culture, Sports, Science and Technology of Japan. The study protocol was approved by the Animal Care and Use Committee of Okayama University (approval number: OKU-2022745).

2.8. Plasmid transfection

Cells were seeded into a 6-well plate at a density of 25,000 cells per well. The following day, the DNA-reagent complex was prepared. First, a culture medium was prepared by mixing Opti-MEM (150 μL per well) with Lipofectamine 2000 (8 μL per well).

Next, the plasmid DNA was diluted with Opti-MEM to a concentration of 5,500 ng/ μL . The medium and DNA were mixed in a 1:1 ratio and allowed to stand at room temperature for approximately 5 min to form the complex (liposome-DNA complex). Then, 300 μL was added to each well, the plate was gently shaken to mix, and the cells were cultured in a 37 °C, 5 % CO₂ incubator. Drug treatment was then performed the following day.

The plasmid used in this experiment was purchased from GenScript Japan and is listed below: ULK1_OHu109469D_pcDNA3.1+/C-(K)-DYK (Clone ID: OHu109469D), pcDNA3.1+/C-(K)-DYK_empty.

2.9. Experiments using human samples

Experiments involving human tissue were approved by the Ethics Committee of Okayama University Hospital (approval number: K2209-001), and informed consent was obtained from all participants.

Whole-transcriptome analysis was performed on paired formalin-fixed, paraffin-embedded samples of EGFR-mutant lung cancer tissues collected from patients before and during osimertinib treatment. We followed published experimental methods [17] and the manufacturer's instructions for Whole Transcriptome Atlas analysis (NanoString Technologies, Seattle, WA, USA). The following morphological markers were used: 1:10 SYTO13, 1:40 anti-panCK-Alexa Fluor 532, 1:40 anti-CD45-Alexa Fluor 594 (NanoString GeoMx Solid Tumor Kit), and 1:500 anti-CD68-Alexa Fluor 647 (Santa Cruz, SC-20060) in a blocking buffer W (NanoString). These four morphological markers enabled the identification of nuclear, epithelial, immune, and macrophage compartments. Immunofluorescence imaging, region of interest selection, and spatially indexed barcode cleavage and collection were carried out using the GeoMx DSP instrument (NanoString). Library preparation was performed in accordance with the manufacturer's protocol. FASTQ files were processed into count matrices as previously described [17]. In brief, deduplicated sequencing counts were generated based on unique molecular identifiers and molecular target tag sequences. Targets that

consistently fell below the limit of quantitation were excluded, and the data were normalized using upper quartile (Q3) normalization.

2.10. Statistical analysis

Statistical analyses were conducted using STATA software (version 17; StataCorp, College Station, TX, USA). Comparisons between two groups were made using the two-tailed paired Student's *t*-test, while comparisons among three groups were performed using one-way analysis of variance followed by Bonferroni post hoc correction. A *P*-value less than 0.05 was considered statistically significant.

2.11. Data availability

The data generated in this study are available upon request from the corresponding author.

3. Results

3.1. Characteristics of DTP cells to osimertinib

To investigate characteristics of DTP cells in response to osimertinib, we treated PC-9, HCC827, 11–18, and H1975 cells with 1 μM of osimertinib for 9 days and assessed their sensitivity to various anticancer agents using cell proliferation assays. DTP cells derived from each cell line were less sensitive to osimertinib than the corresponding parental cells (Fig. 1A, left), and sensitivity to osimertinib was restored after 10–14 days of drug-free period (Fig. S1A). In contrast, sensitivity to cytotoxic agents such as cisplatin and paclitaxel remained unchanged between DTP and parental cells (Fig. S2A). Interestingly, DTP cells showed increased sensitivity to the ULK1 inhibitor SBI-0206965, although only to a modest degree (Fig. 1A, right). These data suggest that tolerance was reversible and specific to osimertinib and that ULK1 signaling may play a more critical role in DTP cells.

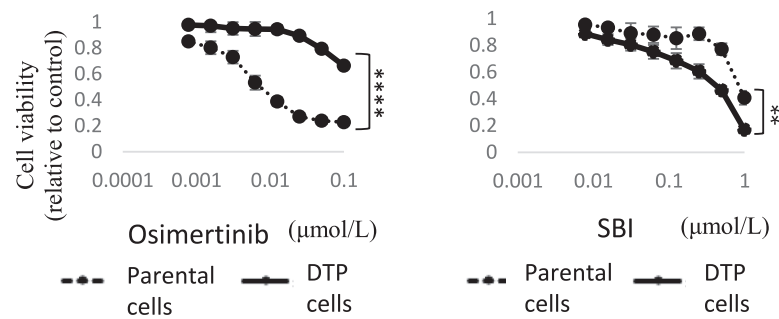
3.2. ULK1 was upregulated in DTP cells

We compared ULK1 expression levels between parental and DTP cells (Fig. 2A). ULK1 expression was more in DTP cells than in parental cells in all investigated cell lines. Consistent with this result, ULK1 mRNA levels were significantly elevated in DTP cells (Fig. 2B). The elevated ULK1 expression returned to baseline levels after drug withdrawal (Fig. S3A).

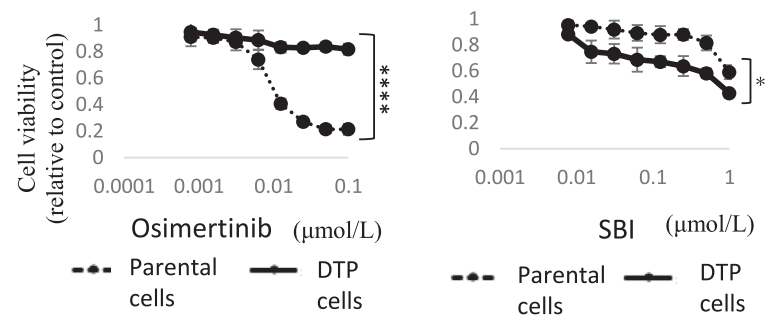
Next, the autophagy status in DTP cells was determined using a flux assay (Fig. 2C and D). While HCQ induced variable responses in LC3-II, a marker of autophagy activity, across parental cell lines, it consistently triggered a more robust accumulation of LC3-II in DTP cells compared to their parental counterparts (Fig. 2C). Consistent with the immunoblotting results, LC3 puncta formation under HCQ exposure was variable across parental cell lines, whereas markedly more robust formation was observed in DTP cells (Fig. 2D). These findings suggest that the augmentation of autophagic flux is a key feature of the transition to the DTP state. Then, we examined the phosphorylation of mTOR and AMPK, key upstream ULK1 regulators (Fig. S3B). No significant differences in mTOR and AMPK phosphorylation were observed between parental and DTP cells. This suggests that ULK1 activation in DTP cells may occur via mTOR/AMPK-independent signaling, although the exact mechanism remains to be elucidated.

Finally, the activation status of ULK1, LC3, AKT, and ERK in parental cells following short-term osimertinib treatment was determined (Fig. S3C). While AKT and ERK signaling was expectedly inhibited and ULK1 expression showed a slight increase in both PC-9 and HCC827 cells, there was no concomitant increase in LC3-II levels, indicating that short-term exposure to osimertinib is insufficient to fully induce autophagic activation in parental cells.

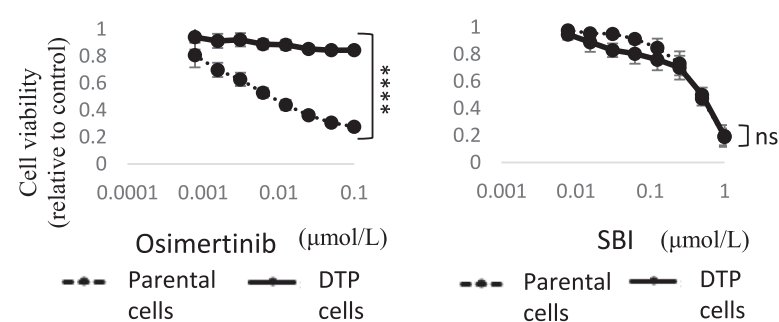
(A) PC-9



HCC827



11-18



H1975

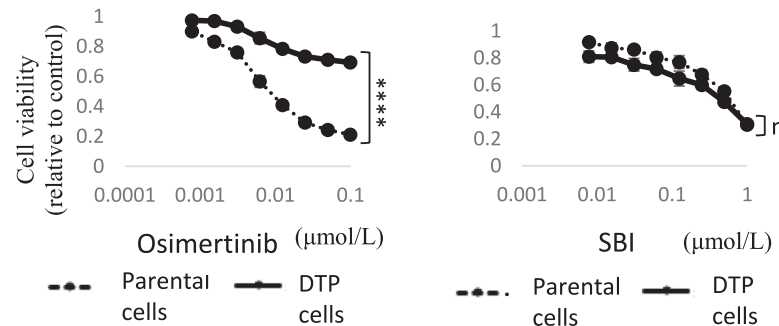


Fig. 1. Sensitivity of DTP Cells to Osimertinib and SBI-0206965. (A) In PC-9, HCC827, 11-18, and H1975 cells, drug-tolerant persister cells (DTP) pretreated with 1 $\mu\text{mol/L}$ osimertinib for 9 days were treated with 100 nmol/L osimertinib or 5 $\mu\text{mol/L}$ ULK1 inhibitor(SBI), and viability was assessed using the MTT assay.

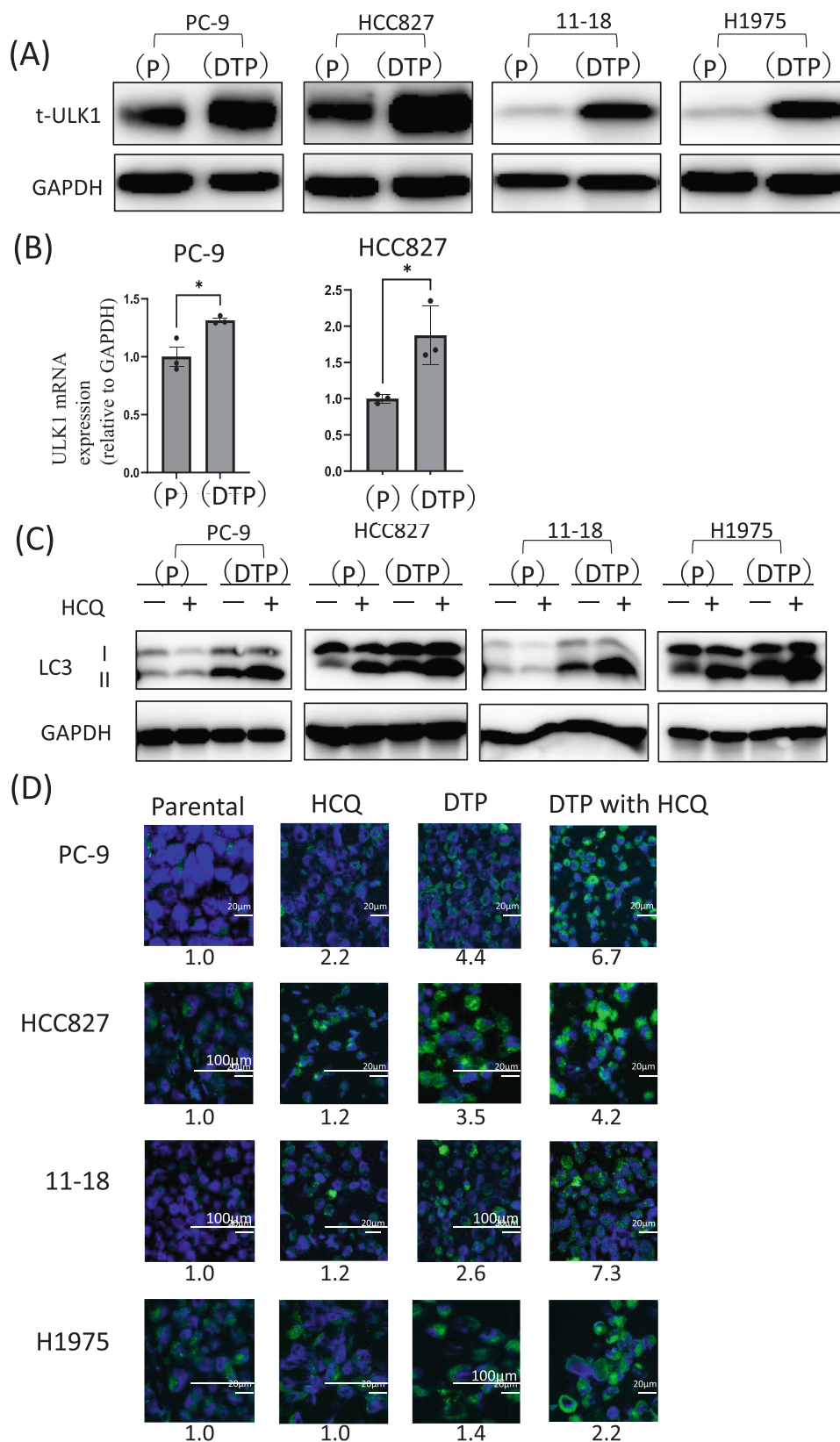


Fig. 2. ULK1 Expression and Autophagy in DTP Cells. (A) Western blot of indicated proteins in PC-9 parental (P) and DTP cells. (B) ULK1 mRNA expression in parental (P) and DTP cells. (C) Western blot of indicated proteins in P, DTP, and each cell line treated with 25 $\mu\text{mol/L}$ hydroxychloroquine (HCQ) for 24 h in PC-9, HCC827, 11-18, and H1975 cells. (D) In PC-9 and HCC827 cells, parental cells, DTP, and each cell line treated with 25 $\mu\text{mol/L}$ HCQ for 24 h were evaluated by immunofluorescence staining. Numbers below each image represent the relative intensity of LC3 puncta, quantified using ImageJ software.

3.3. Targeting ULK1 inhibited the emergence of DTP cells

We investigated the impact of ULK1 inhibition on DTP cells. SBI-0206965, in combination with osimertinib, reduced ULK1 expression to levels comparable to those in parental cells (Fig. 3A). LC3-II expression, elevated in DTP cells, was also reduced by the combined treatment with SBI-0206965 and osimertinib (Fig. 3B). Similar to the observations in DTP cells, SBI-0206965 decreased ULK1 expression to some extent in parental PC-9 and HCC827 cells; however, its inhibitory effect on LC3-II accumulation was less pronounced (Fig. S4A), suggesting that autophagy is more active in DTP cells. Additionally, the ULK1-substrate ATG13 was further phosphorylated in DTP cells, and its phosphorylation returned to baseline levels with SBI-0206965 treatment (Fig. S4B). We also assessed p62, a transporter of proteins to the autophagosome, in DTP cells (Fig. S4C). p62 expression was reduced in DTP cells but was not restored in those treated with SBI-0206965.

Next, we determined whether ULK1 inhibition prevented the emergence of DTP cells (Fig. 3C and S4D). Osimertinib monotherapy effectively inhibited cells, whereas SBI-0206965 showed little to moderate inhibition. Osimertinib combined with SBI-0206965 inhibited DTP cells more effectively than either agent alone in all investigated cell lines. The efficacy of this combination was investigated using another ULK1 inhibitor, MRT68921 (Fig. S4E and F). Osimertinib combined with MRT68921 inhibited cell proliferation compared with either agent alone. Then, we determined the effect of ULK1 inhibition on intracellular signaling pathways, including AKT and ERK (Fig. 3D). No remarkable difference in intracellular signaling was observed with or

without ULK1 inhibition, suggesting that the enhanced efficacy of osimertinib with the ULK1 inhibitor was not mediated by intracellular signal inhibition.

To exclude the possibility of off-target effects of pharmacogenetic ULK1 inhibition, we investigated the efficacy of genetic inhibition of ULK1 using siRNAs. siRNAs targeting ULK1 effectively inhibited ULK1 expression (Fig. 4A) and, in the presence of osimertinib, inhibited cell growth more effectively than non-targeting siRNAs (Fig. 4B, and S4G). Consistent results were obtained using two independent siRNA sequences targeting different regions of ULK1 (Fig. S5A and B), demonstrating that the observed effects were not due to off-target silencing. Furthermore, to investigate whether inhibition of autophagy fully mimics the effect of ULK1 inhibition, or whether other pathways are involved, we performed siRNA-mediated knockdown of ATG7, another essential component of autophagy (Fig. S5C, D, and E). siATG7 enhanced the efficacy of osimertinib in *EGFR*-mutant lung cancer cells, consistent with the results obtained with siULK1. These findings suggest that the efficacy of ULK1 inhibition is primarily mediated through autophagy inhibition.

The *in vivo* efficacy of combining osimertinib with a ULK1 inhibitor was evaluated (Fig. 4C). To assess the efficacy of upfront combination therapy, treatment was initiated immediately after tumor formation was confirmed, reaching an average volume of approximately 150 mm³. ULK1 inhibitor MRT68921 monotherapy had minimal impact on tumor growth. In contrast, osimertinib monotherapy significantly reduced tumor size; however, tumor growth plateaued after five days of treatment, leaving residual tumors, and indicating the development of

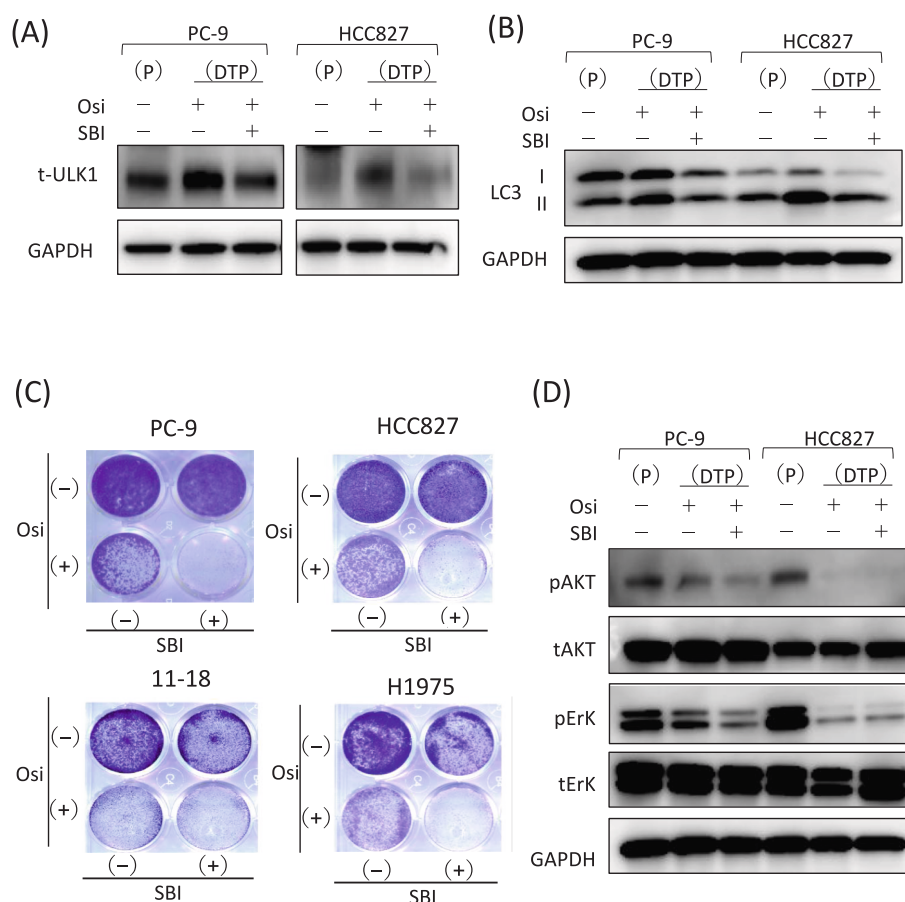


Fig. 3. Combination of ULK1 Inhibition and Osimertinib Suppresses DTP Cells. (A) and (B) Western blot showing ULK1 and LC3 expression in parental (P), DTP, and DTP cells treated with 1 μ mol/L SBI-0206965 (SBI) for 24 h. (C) Crystal violet staining showing the effect of treatment with DMSO, 100 nmol/L osimertinib, 1 μ mol/L SBI, or the combination for 1 week. (D) Western blot of P, DTP, and DTP cells treated with 1 μ mol/L SBI for 9 days in PC-9 and HCC827 cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

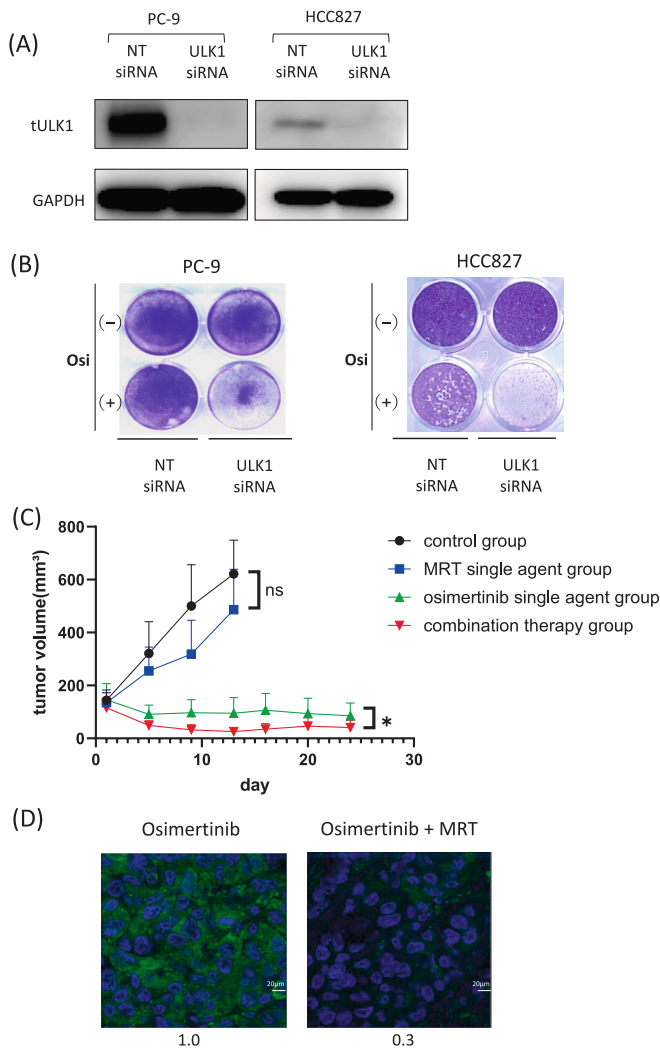


Fig. 4. ULK1 Knockdown and Combination Therapy Efficacy In Vivo. (A) Western blot confirmation of ULK1 knockdown by siRNAs in PC-9 cells. (B) Crystal violet staining showing cell growth inhibition in PC-9 cells treated with siRNAs targeting ULK1 or scrambled siRNAs in the presence of 100 nM osimertinib. (C) Tumor volume changes in mice ($n = 4 - 5$ per group): control, ULK1 inhibitor monotherapy (MRT68921; 20 mg/kg i.p., 3×/week), osimertinib monotherapy (5 mg/kg p.o., 5×/week), and combination groups. (D) PC-9 xenografts in the osimertinib monotherapy group and combination therapy group were immunofluorescently stained. The left side shows the results of osimertinib monotherapy, and the right side shows the results of combination therapy. Numbers below each image represent the relative intensity of LC3 puncta, quantified using ImageJ software. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

tolerance to osimertinib. The combination of osimertinib and MRT68921 resulted in an even more profound tumor inhibition with little residual tumor. We also assessed autophagic activity in the tumors (Fig. 4D). Osimertinib-treated tumors showed strong LC3 staining, whereas those treated with the combination therapy showed reduced LC3 staining, suggesting that autophagic activity in tolerant tumors was suppressed by ULK1 inhibition. Additionally, we investigated whether the combination strategy could prevent tumor relapse. For this purpose, we used a higher dose of osimertinib (15 mg/kg) compared to the initial experiment (5 mg/kg) to achieve near-complete tumor regression during the treatment phase (Fig. S6A). However, tumors rapidly regrew following treatment cessation in both the combination and monotherapy groups. These findings suggest that while ULK1 inhibition significantly augments the initial antitumor effects of osimertinib, it is insufficient to

achieve complete tumor eradication or prevent relapse following drug withdrawal in this EGFR-mutant NSCLC model.

Finally, we investigated whether ULK1 overexpression is sufficient to confer resistance to osimertinib. Unexpectedly, forced overexpression of ULK1 did not induce osimertinib resistance in EGFR-mutant cancer cells (Fig. S6B and C). While the precise underlying mechanisms remain to be elucidated, one possible explanation is that drug tolerance is not driven solely by ULK1 signaling but requires the coordination of ULK1 with other concomitant cellular factors.

3.4. ULK1-mediated autophagy activation via serum starvation induced tolerance to osimertinib

To evaluate whether the activation of the ULK1–autophagy axis induced tolerance to osimertinib, we subjected cells to serum starvation as a stress condition. ULK1 expression was higher in serum-starved cells than in parental cells (Fig. 5A). Serum-starved cells showed increased LC3 expression, which was suppressed by the ULK1 inhibitor SBI-0206965 (Fig. 5B). Then, the impact of serum starvation-induced activation of the ULK1–autophagy axis on the sensitivity of cells to osimertinib was determined (Fig. 5C). The sensitivity of serum-starved cells to osimertinib was less than that of parental cells. Collectively, these data suggest that increased ULK1 expression due to serum starvation induces tolerance to osimertinib by activating autophagy in EGFR-mutant cancer cells.

3.5. Importance of ULK1 in the later stages of osimertinib treatment

Thus far, we have demonstrated that the combination of a ULK1 inhibitor and osimertinib, administered before the emergence of DTP cells, effectively prevents their emergence. Next, we investigated the efficacy of this combination therapy in cells that had already transitioned to the DTP state or had acquired resistance to osimertinib. DTP cells were generated by exposing parental cells to osimertinib for 9 days, as described in our previous experiments. Acquired osimertinib-resistant (OR) cell lines (PC-9/OR and HCC827/OR) were established via chronic exposure to osimertinib for more than 3 months.

Similar to the increased ULK1 expression observed in DTP cells (Fig. 2A), we confirmed that ULK1 levels were also elevated in the acquired resistance models (Fig. S7A). While the combination of osimertinib and a ULK1 inhibitor showed an inhibitory effect on both DTP and acquired resistance cells (Fig. S7B and C), the efficacy was not as pronounced as the marked preventive effect observed in parental cells (Fig. 3C). Taken together, these results suggest that ULK1 signaling plays a more critical role during the initiation of DTP cells rather than in the maintenance of the DTP or acquired resistance states, highlighting the importance of early therapeutic intervention.

3.6. ULK1 expression in human NSCLC tissue samples

Finally, we conducted gene expression analysis using a pair of human EGFR-mutant NSCLC tissue samples obtained before and during osimertinib treatment (prior to emergence of acquired resistance) in clinical practice. The mRNA expression levels of autophagy-related genes, including ULK1, were determined using these samples. After the administration of osimertinib, the expression of factors involved in the formation of the isolation membrane, such as ULK1, Atg13, and Atg101, tended to increase, suggesting that autophagy may be activated in human EGFR-mutant lung cancer-tolerant tissues, although these exploratory findings warrant further validation in larger patient cohorts (Fig. S7D).

4. Discussion

We demonstrated that ULK1 is a promising therapeutic target for addressing drug tolerance to EGFR-TKIs in EGFR-mutant NSCLC. One of

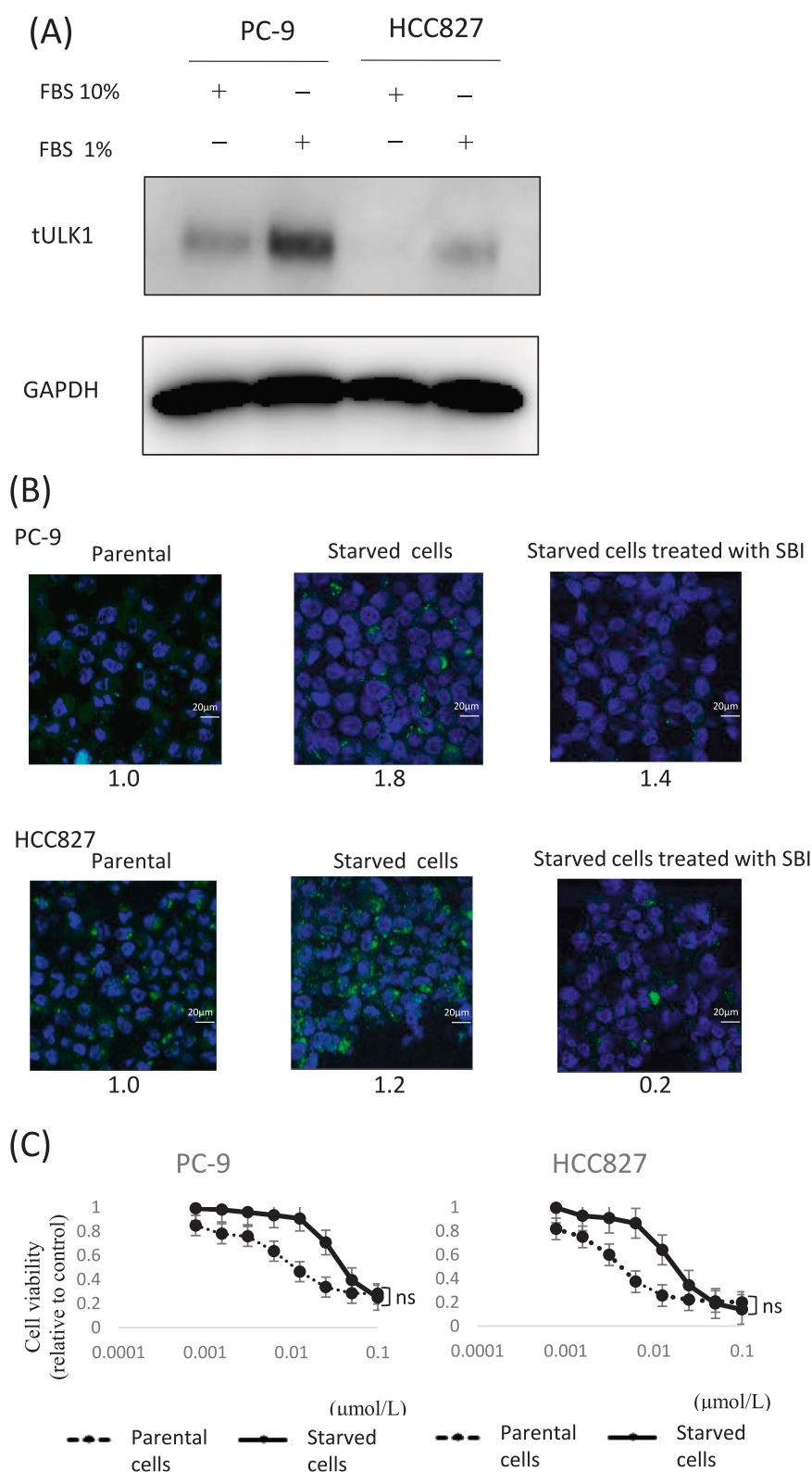


Fig. 5. Starvation-Induced Cells and Drug Sensitivity. Starved cells established by culturing in 1 % FBS for 2 days. (A) ULK1 expression in parental and starved PC-9 and HCC827 cells. (B) In PC-9 and HCC827 cells, parent cells, starved cells, and starved cells treated with 1 $\mu\text{mol/L}$ ULK1 inhibitor (SBI) for 2 days were evaluated by fluorescent immunostaining. Numbers below each image represent the relative intensity of LC3 puncta, quantified using ImageJ software. (C) MTT assay of PC-9 and HCC827 parental and starved cells treated with 100 nmol/L osimertinib for 72 h.

the major findings of this study was the upregulation of ULK1 expression in DTP cells following osimertinib treatment, which was accompanied by enhanced autophagic activity, as reflected by increased LC3-II levels in the flux assays. Notably, the pharmacological inhibition of ULK1 significantly reduced the emergence of DTP cells and enhanced the anti-tumor effects of osimertinib both *in vitro* and *in vivo*. These findings suggest that autophagy plays an essential role in cancer cell survival under therapeutic stress and that ULK1 is a key regulator of this process, although the clinical activation of this axis remains exploratory.

The ULK1–autophagy axis has been reported to exert context-dependent effects in different stages of tumor progression [18,19]. In the initial phase, autophagy suppresses tumor growth by directly inducing cell death. However, once tumors are established, autophagy may promote tumor cell survival, and metastasis by facilitating adaptation to environmental and therapeutic stress. These findings suggest that the significance of ULK1 in drug tolerance differs according to the emerging stage of DTP cells, including their initiation and maintenance stages. In the present study, inhibition of ULK1 effectively prevented the formation and supported the suppression of established DTP cells; nonetheless, combined treatment with osimertinib and SBI-0206965 showed greater efficacy when given early, before tolerance or acquired resistance was established. This suggests that ULK1 signaling is more critical during the emergence of DTP cells rather than during their maintenance or resistance acquisition. Therefore, targeting the “reservoir” DTP cells at the earliest stages of treatment is critical, and upfront combination therapy is a more promising strategy than the sequential addition of ULK1 inhibitors in patients with *EGFR*-mutant NSCLC.

Although limited data exist on the combination of anticancer therapy and ULK1 inhibition, recent studies have shown that ULK1 inhibitors increase the efficacy of drug therapies in cancer treatment. For example, ULK1 inhibition overcame adaptive resistance to drug therapy in acute myeloid leukemia cells by suppressing autophagy and synergizing with chemotherapy and venetoclax [20]. Another study demonstrated that ULK1 inhibition, together with a KRAS inhibitor, further suppressed human KRAS G12C-driven NSCLC cells [21]. These findings, together with our results, demonstrated enhanced efficacy of *EGFR*-TKIs in combination with a ULK1 inhibitor in *EGFR*-mutant NSCLC. Therefore, ULK1 can be a promising novel therapeutic target for enhancing the efficacy of targeted therapies across multiple cancer types.

In this study, drug tolerance to *EGFR*-TKIs was associated with the activation of the ULK1–autophagy axis, whereas DTP cells did not show tolerance to cytotoxic agents. Given that autophagy is generally considered a protective response to cellular stress, including hypoxia, radiation, and cytotoxic chemotherapy [22], our results indicated tolerance specifically in response to *EGFR*-TKIs. However, the basis for this selective resistance remains unclear. One possible explanation is that DTP cells are induced not only by ULK1–autophagy but also by other factors. Furthermore, autophagy had been implicated in resistance to BRAF inhibitors in melanoma cells, often alongside other stress-response pathways [23]. In addition, our findings showed that, although the combination with osimertinib and a ULK1 inhibitor dramatically inhibited the emergence of DTP cells, some DTP cells persisted even under combination therapy, suggesting that ULK1–autophagy is not the sole driver of tolerance. Among the multiple mechanisms that induce tolerance, ULK1–autophagy may play a more significant role in tolerance to *EGFR*-TKIs but not to cytotoxic chemotherapy in *EGFR*-mutant NSCLC cells.

In addition to its established role in autophagy, ULK1 is recognized as a multifunctional kinase involved in various other cellular processes. In hepatocytes, ULK1 is involved in cholesterol biosynthesis, and its action is mediated by AKT signaling [24]. ULK1 possesses a consensus sequence for AKT and has been demonstrated to undergo phosphorylation at Ser774 upon insulin stimulation *in vitro*. Insulin-stimulated AKT phosphorylation was abolished in ULK1 knockout mice, suggesting that ULK1 may play a reciprocal regulatory role in AKT signaling (19). Additionally, there is evidence supporting a feedback loop between

ULK1 and AMPK, in which ULK1 regulates AMPK activity, as indicated by ULK1-dependent phosphorylation sites identified on multiple AMPK isoforms [25]. Therefore, AMPK and AKT are likely to antagonistically regulate autophagy through ULK1. Intriguingly, our data showed that while ULK1 was significantly upregulated in DTP cells, the activity of its canonical upstream regulators, such as AMPK and mTORC1, remained largely unchanged (Fig. S3B). This suggests that the induction of autophagy in DTP cells is independent of the classical pathways. Instead, ULK1 may be specifically and actively recruited as a survival strategy to evade cell death under the unique stress of *EGFR* signaling inhibition.

There are several limitations to this study. First, the precise mechanisms by which ULK1 induces *EGFR*-TKI-specific resistance remain unclear. Specifically, the discrepancy regarding p62 expression—which was reduced in DTP cells but not restored by ULK1 inhibition—suggests that ULK1 might operate partly through non-canonical or autophagy-independent pathways in this context. Second, this study employed only a limited number of cell lines. Further investigation is needed to clarify the precise mechanisms through which ULK1–autophagy signaling contributes to osimertinib resistance.

In conclusion, this study highlights the pivotal role of the ULK1–autophagy axis in the emergence of osimertinib resistance in *EGFR*-mutant NSCLC. Targeting ULK1 with specific inhibitors represents a promising therapeutic strategy to suppress the emergence of drug tolerance and enhance patient outcomes.

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CRediT authorship contribution statement

Ayako Morita: Writing – original draft, Validation, Methodology, Investigation, Data curation, Conceptualization. **Kammei Rai:** Writing – review & editing, Resources, Methodology, Investigation. **Tadahiro Kuribayashi:** Methodology, Investigation. **Shuta Tomida:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Tatsuya Nishi:** Writing – review & editing, Resources, Investigation. **Naofumi Hara:** Writing – review & editing, Resources, Investigation. **Go Makimoto:** Writing – review & editing, Resources. **Kiichiro Ninomiya:** Writing – review & editing, Resources. **Toshio Kubo:** Writing – review & editing, Resources. **Kadoaki Ohashi:** Writing – review & editing, Resources. **Katsuyuki Hotta:** Writing – review & editing, Resources. **Nobuaki Ochi:** Writing – review & editing, Resources. **Yosuke Togashi:** Writing – review & editing, Supervision. **Yoshinobu Maeda:** Writing – review & editing, Supervision. **Eiki Ichihara:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Conceptualization.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author used ChatGPT in order to improve language. After using this tool/service, the authors reviewed and edited the content as needed and takes full responsibility for the content of the publication.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: GM has received honoraria from MSD, Chugai Pharmaceutical, Novartis, Ono Pharmaceutical, Bristol-Myers Squibb, and Taiho pharmaceutical outside of this study. KO has received honoraria from Eli Lilly Japan, Novartis Pharma K.K., Chugai Pharmaceutical Co., Ltd., AstraZeneca K. K., and Amgen K.K; grants from Nippon Boehringer Ingelheim Co., Ltd., Chugai Pharmaceutical Co., Ltd. KH has received honoraria from

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Appendix A. Supplementary data

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