# Erlotinib-induced autophagy in epidermal growth factor receptor mutated non-small cell lung cancer

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**Abbreviations:** AMPK, 5' adenosine monophosphate-activated protein kinase; Atg, autophagy-related protein; LC3, microtubule-associated protein light chain 3; mTOR, mammalian target of rapamycin; NFκB, nuclear factor kappa beta; PARP, poly (ADP-ribose) polymerase.

## Abstract

**Purpose:** Erlotinib is a commonly used tyrosine kinase inhibitor (TKI) in non-small cell lung cancer (NSCLC). Autophagy is a catabolic process in response to stress and deprivation of nutrients. This study aims to investigate whether autophagy confers acquired resistance to erlotinib treatment in NSCLC.

**Methods:** Four NSCLC cell lines (HCC827, HCC4006, H358 and H1975) with different epidermal growth factor receptor (EGFR) mutation status (exon 19 deletion, exon 19 deletion, wild-type and L858R/T790M respectively) were selected. MTT assay, crystal violet staining and Annexin-V assay were performed to determine cell viability and apoptosis. Autophagic proteins were detected by Western blot. Acidic vesicular organelle (AVO) formation was determined by acridine orange staining. Autophagy inhibitor (chloroquine) and RNA interference were used to demonstrate the biological effect of erlotinib-induced autophagy.

**Results:** In line with EGFR mutation status, it was shown that both HCC827 and HCC4006 cells were sensitive to erlotinib, while H358 and H1975 cell lines were resistant. Erlotinib treatment at clinically relevant concentrations induced autophagy (increased LC3II expression, Atg-5/Atg12 conjugation, formation of AVO and p62 degradation) in sensitive NSCLC cell lines, via p53 nuclear translocation, AMPK activation and mTOR suppression. Addition of chloroquine, as an autophagy inhibitor, enhanced erlotinib sensitivity in sensitive cells. Similarly, silencing of Atg5 or Beclin-1 significantly increased sensitivity to erlotinib in both sensitive cell lines. In contrast, there was no induction of autophagy in resistant H358 and H1975 cell lines upon erlotinib exposure.

**Conclusions:** Erlotinib can induce both apoptosis and autophagy in sensitive NSCLC cell lines with activating EGFR mutation (exon 19 del). Inhibition of autophagy can further enhance sensitivity to erlotinib in EGFR-mutated NSCLC, suggesting that autophagy may serve as a protective mechanism.

(Word count of abstract: 274)

## Introduction

Non-small cell lung cancer (NSCLC) is one of the leading causes of cancer deaths worldwide [1]. The clinical benefit of platinum-based chemotherapy in advanced NSCLC has reached a plateau [2, 3]. Epidermal growth factor receptor (EGFR) is frequently deregulated [4, 5] or overexpressed [6] in NSCLC. The EGFR downstream signaling pathways are pivotal in the initiation and progression of cancers, serving as one of the key therapeutic targets in NSCLC [7]. Erlotinib, as one of the prototypical EGFR tyrosine kinase inhibitors (TKIs), has been approved by U.S. Food and Drug Administration (FDA) for treatment of NSCLC [8-10], however, development of acquired resistance (mostly secondary EGFR mutation (T790M in exon 20) [11, 12] and MET amplification [13]) has almost been the rule during treatment. Better understanding of additional resistance mechanisms, with corresponding therapeutic interventions, could potentially overcome or delay the development of acquired resistance to EGFR TKI treatment.

Autophagy is characterized by the formation of double-membrane vacuoles (autophagosomes) in the cytoplasm. The key steps of autophagic process include the induction and nucleation of autophagic vesicles, followed by fusion with lysosomes and subsequent degradation of their contents [14, 15]. During autophagy, Beclin-1 complex recruits type III PI3K VPS34 to generate phosphatidylinositol 3-phosphate. Autophagy-related protein 12 (Atg12) and microtubule-associated protein light chain 3 (LC3) are involved in the expansion of autophagosome membranes. The E1-like Atg7 and E2-like Atg10 covalently link Atg12 with Atg5, which together bind Atg16 to form pre-autophagosomal structures. LC3 is cleaved by Atg 4 and lipidated to form LC3II, which is commonly used to monitor autophagy [16]. The catabolic cellular self-degradation serves to produce building blocks for molecular synthesis and maintain energy homeostasis during periods of nutrient deprivation [16].

It is still controversial whether autophagy causes cancer cell death or sustains cancer survival under stressful conditions. Abrogation of autophagy, with pharmacological inhibitors (e.g. chloroquine and bafilomycin A1) or shRNA knockdown of autophagic proteins, has been shown to re-sensitize cancer cells to chemotherapy or radiation [17, 18]. However, others suggested synergistic or additive effect in combining chemotherapy with autophagy inducer like rapamycin in a lung cancer cell line model [19]. The effect of autophagy on EGFR TKI treatment in NSCLC is less well-known. In a recent study, autophagy was induced in a relatively resistant NSCLC cell line model upon exposure to EGFR TKI at high concentrations [20].

Therapeutic exposure of cancer cells to an anti-cancer agent would be considered as a form of stress only if they are prone to the growth inhibitory or cytotoxic effects. Since EGFR TKI preferentially works on NSCLC with activating EGFR mutations, we postulate that autophagy would only be induced among the sensitive EGFR-mutated NSCLC treated with clinically relevant concentrations of EGFR TKI, which could serve as a cancer survival mechanism. In this study, we investigated the effect of erlotinib treatment on autophagy in 4 NSCLC cell lines with different EGFR mutation status, in order to understand the possible mechanisms involved and therapeutic implications.

## Materials and Methods

### *Cell lines and cultures*

Four human NSCLC cell lines, HCC827 (EGFR exon 19 del), HCC4006 (EGFR exon 19 del), H358 (EGFR WT) and H1975 (EGFR exon 21 L858R/exon 20 T790M) were obtained from

American Type Culture Collection (Manassas, VA, USA). All cells were maintained in RPMI-1640 medium (Gibco®, Life Technologies, Carlsbad, California, USA) with 10% fetal bovine serum (FBS) (Gibco®, Life Technologies) and cultured in a humidified atmosphere of 5% $CO_2$ at 37°C.

***Drugs and reagents***

Erlotinib (Selleck, Houston, USA) and bafilomycin A1 (Sigma-Aldrich, St. Louis, Missouri, USA) were diluted in dimethyl sulfoxide (DMSO) (Sigma-Aldrich). Chloroquine (Sigma-Aldrich) was diluted in phosphate buffered saline (PBS). Acridine orange hydrochloride was purchased from Sigma-Aldrich. Anti-LC3B and anti-beta-actin were obtained from Sigma-Aldrich. Anti-Beclin-1, Atg5/12, p62, p53, p-AMPK(Thr172), AMPK, p-NFkB (s536), NFkB, p-mTOR(ser2448), and mTOR were obtained from Cell Signaling Technology (Danvers, Massachusetts, USA). siRNA targeting Beclin-1 and Atg5 were purchased from Ambion Life Technologies (Carlsbad, California, USA). Control siRNA was purchased from Santa Cruz (California, USA).

***Cell viability assay***

Cells were cultured in 96-well plates (5,000 cells in 0.2 ml culture medium/well). After drug treatment, 20 μl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (0.5 mg/ml) (Sigma-Aldrich) was added for 2 h, and followed by lysis with DMSO. The absorbance at 570 nm was measured using a microplate reader Fluo Star Optima (Bmg Labtec GmbH, Ortenberg, Germany). For experiments using chloroquine, cells were fixed with 4% formaldehyde (Sigma-Aldrich) for 10 min and then stained with 0.05% crystal violet (Sigma-

Aldrich) in 30% ethanol for 10 min at room temperature. Plates were washed four times with tap water. Cells were lysed with 1% SDS solution (Sigma-Aldrich), and dye uptake was measured at 550 nm using a microplate reader Fluo Star Optima. The absorbance in treatment group was normalized to those of the untreated cells (control). All experiments were done in triplicates.

***Western blot analysis***

Cells were lysed on ice with RIPA lysis buffer [10 mM Tris, 150 mM NaCl, 1 mM methylenediaminetetra-acetic acid, 1% Triton X-100, 0.5% NP40, freshly added 0.2 mM PMSF in isopropanol, 1:50 phosphatase inhibitor cocktail 2 (Sigma-Aldrich), 1:50 protease inhibitor cocktail (Sigma-Aldrich)] for 1 h. The supernatants were collected after centrifugation. Nuclear and cytoplasmic proteins were extracted using NE-PER nuclear and cytoplasmic reagents from Thermo Scientific according to instructions (Rockford, IL, USA). The protein concentration in the supernatants was quantified with Bradford Protein Assay (Bio-Rad, Berkeley, California, USA). For each supernatant, 30-50 μg protein was loaded on 7.5-15% of sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto polyvinylidene fluoride membranes (GE Healthcare, Buckinghamshire, UK). The membranes were incubated with primary antibodies overnight at $4^{o}$C. Further incubation with the corresponding secondary antibody (Cell Signaling technology) for 90 min at $4^{o}$C was then carried out. Detection was performed using an enhanced chemiluminescence (ECL) kit (GE Healthcare). Quantification was done with Image J (National Institute of Health, USA). Results were obtained from at least 3 independent experiments.

***Small interfering RNA (siRNA) transfection***

Knockdown of specific RNA was performed, including *beclin1* (siRNA ID: s16539; sense sequence: CAGAUACUCUUUUAGACCATT; antisense sequence: UGGUCUAAAAGAGUAUCGTG), *ATG5* (siRNA ID: s18160; sense sequence: GCUAUAUCAGGAUGAGAUATT; antisense sequence: UAUCUCAUCCUGAUAUAGCGT) and a scrambled siRNA (sc-37007). Briefly, a mixture of siRNA and transfection reagent (Santa Cruz) in culture medium was incubated with cells for 6 h. Then 5% FBS in culture medium was added and incubated for 48 h. Corresponding protein downregulation was confirmed with Western blot. Drug treatment experiments were conducted for 48 h, cell viability and apoptotic events were then measured.

***Apoptotic assay***

Cells were resuspended in binding buffer at 1-5 x $10^6$/mL. Five microliter of fluorochrome-conjugated Annexin-V and 5 μl of 7-AAD Viability Staining Solution (BD biosciences, New Jersey, USA) were added to 100 μl of the cell suspension, and incubated 15 min at room temperature. Flow cytometry **(**Beckman Coulter, Inc., USA) was performed to detect cells undergoing apoptosis.

***Acridine orange staining***

Autophagy is the process of sequestrating cytoplasmic proteins into the lytic compartment, being characterized by the formation of acidophilic vesicular organelles (AVO) [21]. Cells were stained by 1 μg/ml AO in PBS at 37°C for 15 min, then washed and visualized with fluorescence microscope (Carl Zeiss, Axioskop 2 plus, New York, USA) equipped with a mercury 100-W

lamp, 490-nm band-pass blue excitation filters, a 500 nm dichroic mirror and a 515 nm long pass-barrier filter.

***Statistical analysis***

Data from triplicate experiments were presented in mean ± standard deviation (SD). Comparison between groups was performed using Student's two-tailed t-test by Prism (GraphPad Software, La Jolla, Southern California, USA). A p-value $< 0.05$ was taken as statistically significant.

## Results

### *Autophagy induced in sensitive NSCLC cells after erlotinib treatment*

Four NSCLC cell lines with different EGFR mutation status were chosen: HCC827 (exon 19 del), HCC4006 (exon 19 del), H358 (wild-type) and H1975 (exon 21 L858R, exon 20 T790M). Using MTT assay, HCC827 and HCC4006 cells were relatively sensitive to erlotinib with $IC_{50}$ values 22 ± 5.1 and 46.2 ± 6.4 nM respectively after 72 h treatment, while H358 and H1975 cells were relatively resistant with $IC_{50}$ values 7.4 ± 2.9 and 18.6 ± 8.8 μM respectively. Upon treatment with erlotinib, autophagy was induced only in the 2 sensitive NSCLC cell lines. Accordingly, the conversion of LC3/Atg8 from the cytoplasmic form (LC3-I (18 kDa)) to the autophagosomic form (LC3-II (16 kDa)) after 24 h exposure to low concentration of erlotinib (0.1 and 0.2 μM) was only evident in HCC827 and HCC4006 cells (sensitive) (Fig. 1A), but not in H358 and H1975 cells (resistant) (Fig. 1B). Similarly, autophagic flux was observed in sensitive NSCLC cells after adding bafilomycin A1 (BFA), suggesting induction of autophagy (Fig. 1C). BFA further increased LC3-II expression compared with erlotinib treatment alone, which supported that the increase of LC3-II with erlotinib treatment was due to increased production rather than decreased degradation. After 48 h of erlotinib treatment, acidic vesicular organelles (AVOs), as markers for late stage of autophagy, were detected with acridine orange staining. Fluorescence microscopy demonstrated that cells stained positive for AVOs were more frequently seen with erlotinib treatment compared to control in HCC827 and HCC4006 cells, but not in H358 and H1975 cells (Fig. 1D, E, F, G). The conjugation of Atg5/Atg12 increased in a time-dependent manner when HCC827 and HCC4006 cells were treated with erlotinib (Fig. 1H). Finally, p62 was degraded in a time-dependent manner after erlotinib exposure in HCC827 and HCC4006

cells (Fig. 1I). Taken together, these findings provided strong evidence that erlotinib induced autophagy in sensitive NSCLC cells.

***Pathways involved in autophagy induction in sensitive NSCLC cells***

Previous studies have shown that p53/AMPK/mTOR axis plays an important role in the regulation of autophagy. The appearance of autophagic p62 degradation (Fig. 1I) was accompanied by the p53 translocation from cytosol to nucleus (Fig. 2A and 2B), AMPK activation through up-regulation of p-AMPK (Thr172) (Fig. 2C), p-NFkB activation (Fig. 2D) and down-regulation of p-mTOR (ser2448) (Fig. 2E) after erlotinib treatment (0.2 μM) in both HCC827 and HCC4006 cells.

***Inhibition of autophagy enhanced pro-apoptotic effects of erlotinib in sensitive NSCLC cells***

To investigate whether inhibition of autophagy would affect the cytotoxicity of erlotinib, NSCLC cells were treated with erlotinib in the presence of 10 μM chloroquine (autophagy inhibitor). The concentration of chloroquine was chosen that did not cause significant cell death (data not shown), but was sufficient to inhibit autophagy as shown by accumulation of LC3-II (Fig. 3A). Based on cell viability assay using crystal violet staining, the combination of chloroquine and erlotinib significantly decreased cell viability compared to erlotinib alone in sensitive NSCLC cells, but not in the resistant cell lines H358 and H1975 (Fig. 3B). Similarly, addition of chloroquine to erlotinib increased the percentage of apoptotic cells in sensitive NSCLC cells, but not in resistant cells (Fig. 3C and Table 1). Furthermore, the cleavage of PARP was also enhanced in the presence of chloroquine in the sensitive cells (Fig. 3D).

***Knockdown of ATG5 and Beclin-1 increased sensitivity to erlotinib***

To further confirm the effect of autophagy inhibition on erlotinib-induced apoptosis, a genetic approach through RNA interference to target autophagy-related genes, Atg 5 and Beclin-1, was used. The transfection efficiency of siRNA against Atg5 and Beclin-1 was demonstrated with Western blot showing significant suppression of Atg5 and Beclin-1 protein expression (Fig. 4A), without significant loss of cell viability (data not shown). Treatment with siRNA against Atg5 (6 nM) or Beclin-1 (4 nM) did not result in significant cell death. The cell viability in erlotinib combined with ATG-silencing (HCC827: 37.5 ± 10.5%; HCC4006: 46 ± 3.6%) or beclin-silencing (HCC827: 37.7 ± 3.2%; HCC4006: 25.4 ± 13.0%) was significantly lower than erlotinib combined with control-silencing (HCC827: 62.9 ± 8.9%; HCC4006: 63.6 ± 4.1%, $p<0.01$) (Figure 4B). Apoptotic cell death in erlotinib combined with ATG-silencing (51.8 ± 1.9%) or beclin-silencing (53.4 ± 2.0%) was increased comparing with erlotinib combined with control-silencing (26.7 ± 2.9%, $p<0.01$) in HCC827 cells. Similarly, in HCC4006, apoptotic cell death was significantly enhanced in erlotinib combined with ATG-silencing (53.1 ± 3.4%) or beclin-silencing (61.9 ± 5.1%), comparing with erlotinib combined with control-silencing (31.2 ± 10.6%, $p<0.01$) (Figure 4C).

## Discussion

We have reported *in vitro* evidence of induction of autophagy with erlotinib treatment in NSCLC cell lines carrying activating EGFR mutations. More importantly, our findings supported the role of autophagy in enhancing NSCLC survival in the presence of life-threatening insult from EGFR TKI, serving as a potentially novel mechanism of acquired resistance. Our original hypothesis that this phenomenon would only occur in sensitive EGFR-mutated NSCLC cell lines exposed to

EGFR TKI (erlotinib), as demonstrated in this study, is in line with autophagy as a secondary reaction to the pro-apoptotic effects of erlotinib.

In autophagy, lysosomes fuse with autophagosomes to form autophagolysosomes, responsible for breakdown of membrane-bound cytoplasmic contents. LC3 exists in two forms (LC3-I and its lipidated derivative LC3-II), which is located in autophagosomal membranes prior to their fusion with lysosomes [22]. Autophagy can be detected as early as 24 h by the conversion of LC3-I to LC3-II, which is further supported by demonstration of autophagic flux [23]. In brief, LC3-II protein accumulation can be due to either increase in LC3-II production (i.e. enhanced autophagy) or decrease in its autophagolysosomal breakdown (i.e. suppressed autophagy). Further increase in LC3-II protein expression after treatment with an inhibitor of late-stage autophagy like BFA would support increased LC3-II production. During early induction of autophagy, ubiquitin-mediated association of Atg5 and Atg12 is required to recruit other proteins to the autophagosomal membrane and form the autophagic vacuole [24]. At a later stage of autophagy, acidic vesicular organelles (AVO) would be formed, serving as another marker of autophagy [25]. p62/sequestosome 1 is an ubiquitin-binding scaffold protein that can be specifically degraded by autophagy [26]. Thus, degradation of p62, as a cargo of autophagosome, can also provide indirect evidence of autophagy. Therefore, based on increased LC3-II protein expression (with autophagic flux), Atg 5/Atg 12 and AVO, as well as decreased p62 protein expression, we have demonstrated ample evidence that autophagy was induced with erlotinib treatment in sensitive (EGFR-mutated), but not in resistant NSCLC cells. Furthermore, using both pharmacological blockade (chloroquine, a specific lysosome inhibitor blocking the fusion of autophagosomes and lysosomes [27]) and gene silencing approach (siRNA knockdown of Atg5

and Beclin-1), inhibition of autophagy would enhance EGFR TKI (erlotinib) sensitivity among EGFR-mutated NSCLC cell lines.

Many conventional cytotoxic drugs have been shown to induce autophagy, however, the role of autophagy in chemotherapy and targeted cancer therapy remains controversial. The anti-cancer drugs that can induce autophagy include cetuximab (anti-EGFR antibody) [25], imatinib (BCR-ABL tyrosine kinase inhibitor) [28], TNF-related apoptosis inducing ligand (TRAIL) [29], among many others. Some studies have suggested combining autophagy inducers with anti-cancer therapy, including combination of TKI with autophagy inducing drugs (rapamycin and imatinib) for treating NSCLC [19]. The combination effect was evaluated in EGFR wild-type NSCLC cell lines, suggesting potential benefit of combining TKI with rapamycin or imatinib [19]. On the other hand, other studies have shown beneficial effect of autophagy inhibition in enhancing tumor cell death. Inhibition of autophagy in pre-clinical models improved response to TRAIL-mediated apoptosis in leukemic and colon cancer cell lines [9], and enhanced apoptosis caused by anti-EGFR antibody cetuximab [30]. Recently, autophagy was shown to be induced with EGFR TKI (gefitinib and erlotinib) treatment in a resistant (but not in sensitive) NSCLC cell line model (A549 and NCI-H1299 cells), which was suggested as a possible resistance mechanism to erlotinib [20]. The apparent discrepancy from our findings could be due to several reasons. The concentrations of EGFR TKI that led to significant features of autophagy were relatively high (25 μM erlotinib or gefitinib), in contrast to the use of clinically relevant concentrations in our study ($\leq 0.2$ μM erlotinib). Though erlotinib is relatively specific for EGFR, there could still be possible off-target effects especially at excessive concentrations. Notably, EGFR knockdown with specific siRNA did not abrogate erlotinib-induced autophagy in that

study of resistant cell line model, suggesting possible EGFR-independent actions of erlotinib. Therefore, we believe that our EGFR-mutated NSCLC cell line model with appropriate concentrations of erlotinib should be more clinically relevant.

The mammalian target of rapamycin (mTOR) acts as a major anti-autophagy protein which is regulated by upstream signaling pathway PtdIns3K/Akt, p53-AMP-activated protein kinase and several other proteins [31]. Regarding the mechanisms involved in autophagy induction, recent studies have indicated that both inhibition of PI3K/Akt/mTOR signaling and translocation of p53 into the nucleus could trigger autophagy in cancer cell [32]. It has been reported that many drugs could induce autophagy through inhibition of PI3K/Akt/mTOR pathway [19, 20, 33]. Recently, more attention has been paid on the autophagy modulation function of the tumor suppressor p53 protein. Nuclear p53 can transcriptionally activate autophagy inducers, while cytoplasmic p53 inhibits autophagy [34-36]. Following EGFR inhibition, its downstream signals including Akt and mTOR would be effectively abolished resulting in both apoptosis and autophagy. In addition, our findings also confirmed p53 nuclear translocation and APMK activation upon erlotinib treatment in EGFR-mutated NSCLC cell lines. These concerted efforts could eventually lead to effective blockade of mTOR, and release of its negative control on autophagy.

There are several limitations of our study. Due to limited number of NSCLC cell lines in our model, our findings could be cell line-specific biological behavior rather than a generalizable phenomenon. The measurement of autophagy was limited to within 72 h in our system, which might not reflect the situation of prolonged exposure to erlotinib as in clinical application. Also,

the influence of tumor microenvironment on autophagic response to erlotinib could not be assessed in our model. Future studies of autophagy in acquired resistant cell lines derived from parental lines (HCC827 and HCC4006) or clinical tumor samples obtained from lung cancer patients with acquired resistance to EGFR TKI will help to further elucidate the clinical significance of autophagy as a resistance mechanism to EGFR TKI.

In conclusion, autophagy can be induced upon exposure to clinically relevant concentrations of erlotinib in a NSCLC cell line model with activating EGFR mutations. Strategies to inhibit autophagy may enhance sensitivity to EGFR TKI and potentially overcome acquired resistance.

## CONFLICT OF INTEREST STATEMENT

All authors in this manuscript have no financial and personal relationships with other people or organizations that could inappropriately influence our work. None of the authors have conflict of interest to declare.

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# Legends of figures

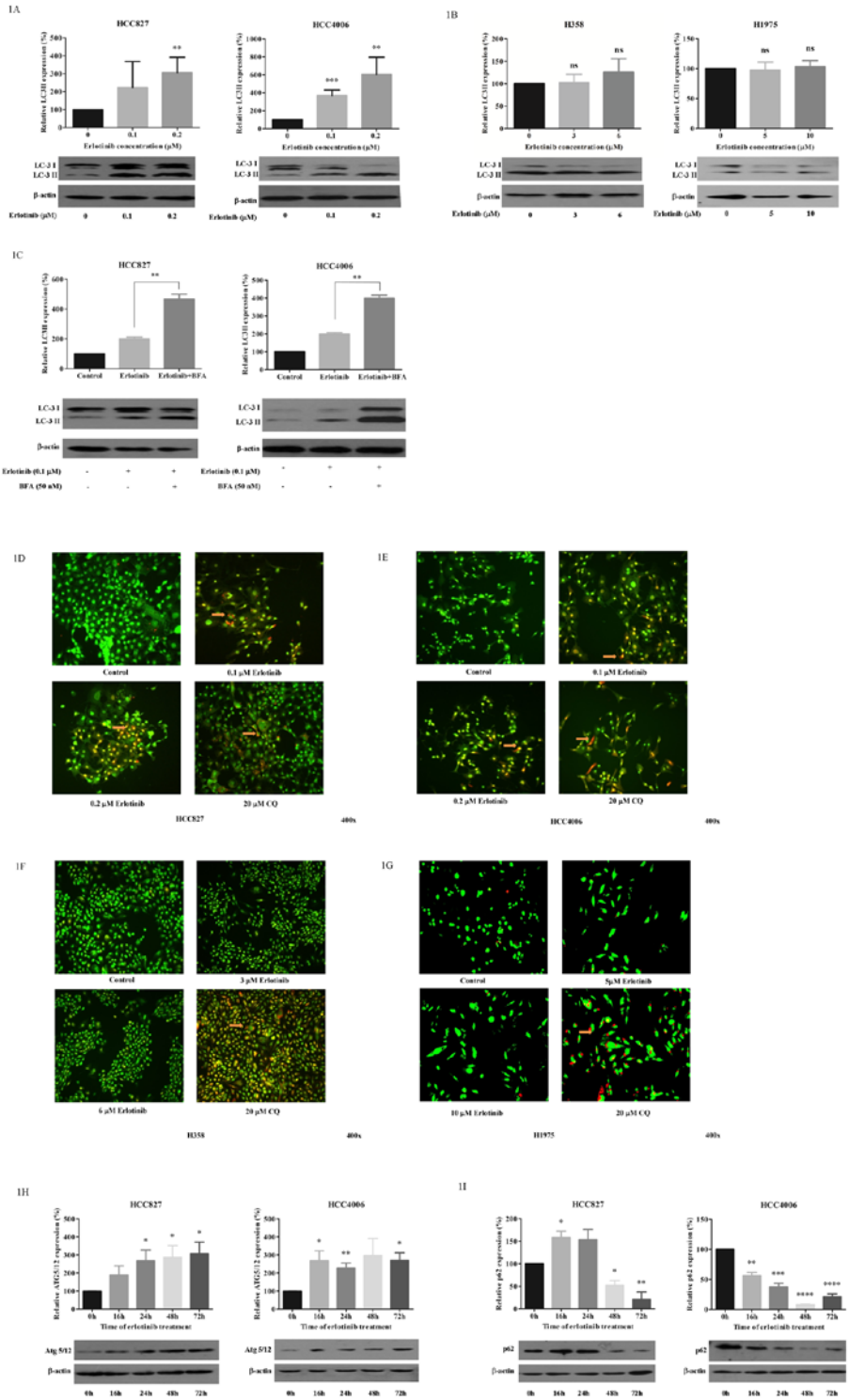


**Figure 1.** Autophagy induction in HCC827, HCC4006, H358 and H1975 cells after erlotinib treatment. (A) LC3-II expression was increased in HCC827 and HCC4006 cells after 24h erlotinib (0.1 μM, 0.2 μM) treatment. (B) LC3-II expression was unchanged in H358 and H1975 cells after 24h erlotinib (up to 10 μM) treatment. (C) Autophagic flux with bafilomycin A1 treatment was shown in both HCC827 and HCC4006 cells. Acidic vesicular organelle (AVO) formation detected by acridine orange staining in (D) HCC827 and (E) HCC4006 cells, but not in (F) H358 and (G) H1975 cells after 48h erlotinib treatment. Chloroquine (CQ) treatment served as a positive control. Arrows indicate the AVO positive cells. (H) Atg5/12 conjugation and (I) p62 degradation with erlotinib treatment (0.2 μM) in HCC827 and HCC4006 cells. Data are shown as mean ± SD (error bars) of 3 independent experiments, depicted with representative immunoblots. Statistical comparison was made between treatment vs control groups, or different time points vs baseline (0h), unless otherwise indicated. *, $p< 0.05$; **, $p< 0.01$; ***, $p < 0.001$; ns, not significant.

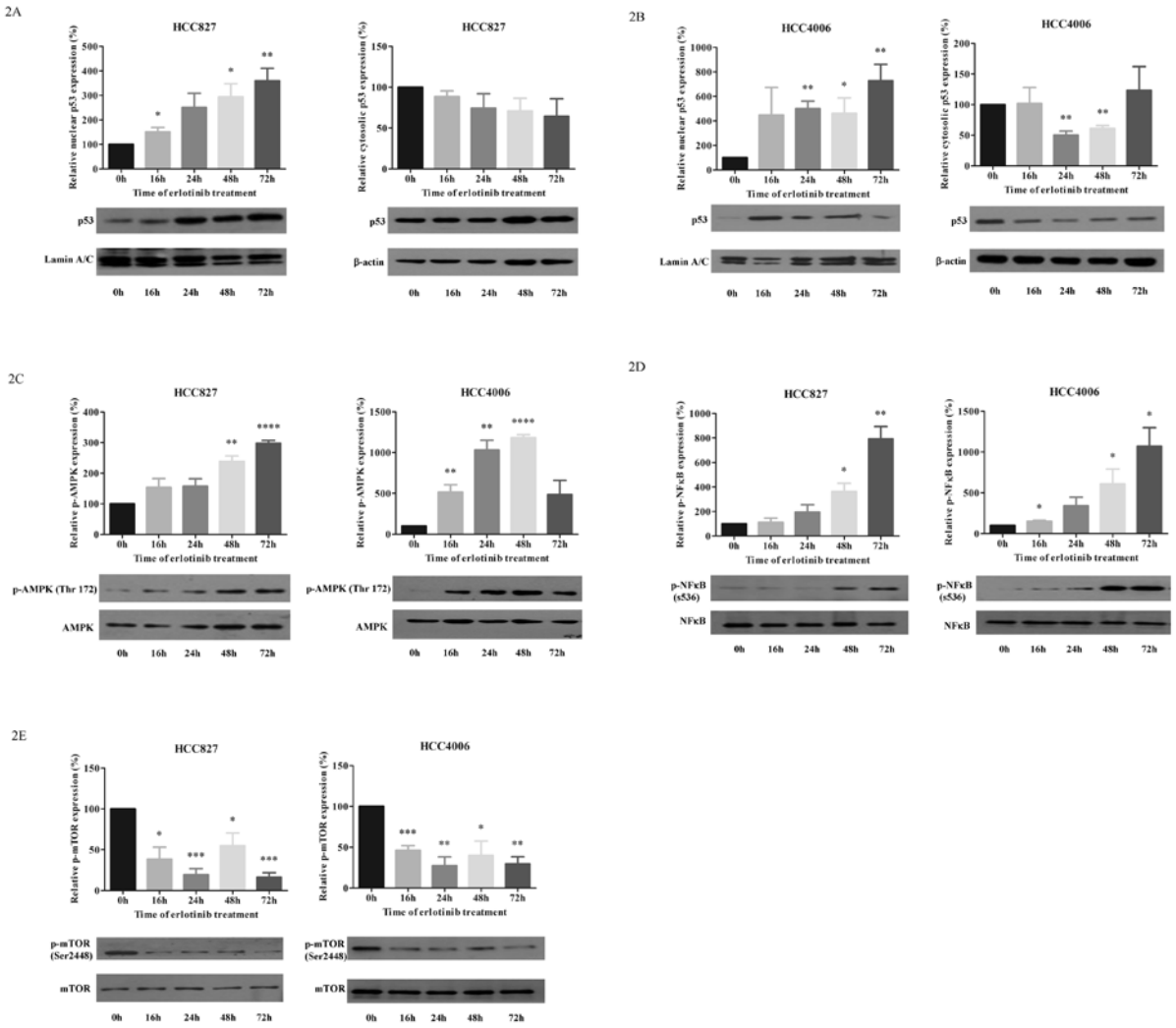
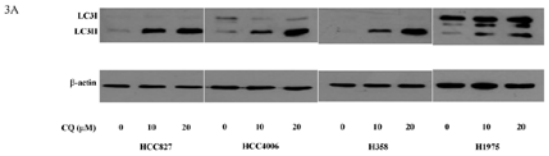
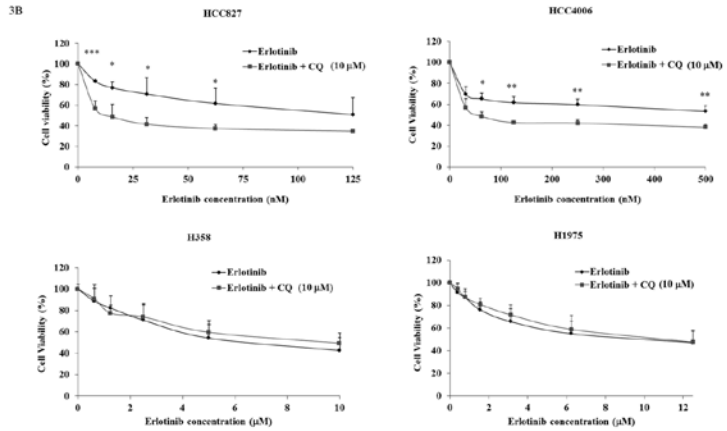
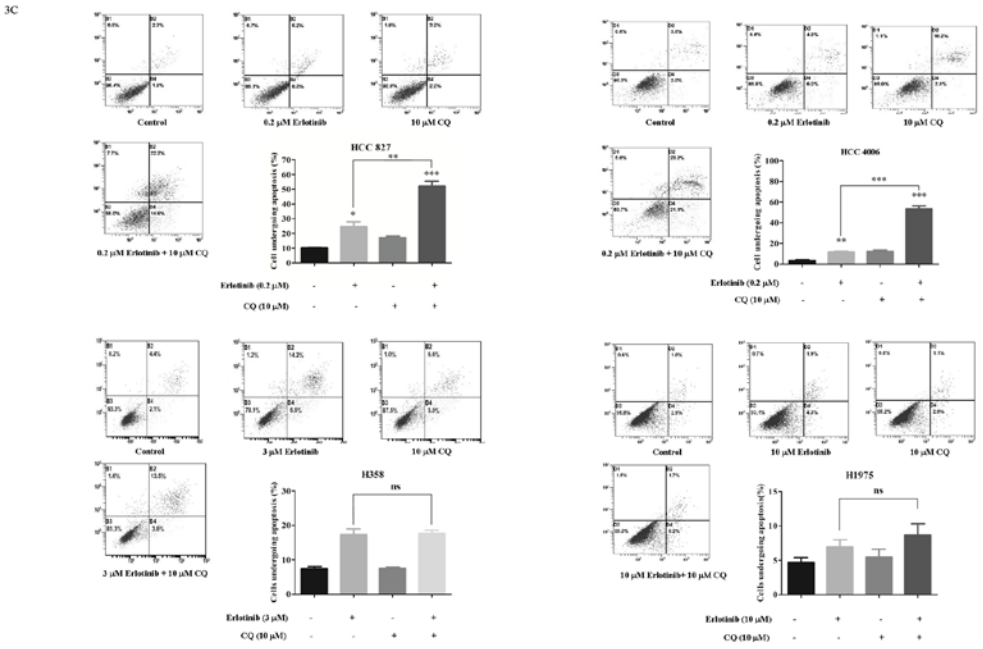



**Figure 2.** Pathways involved in autophagy induction with erlotinib treatment (0.2 μM). Nuclear translocation of p53 was detected in (A) HCC827 and (B) HCC4006 cells. (C) AMP-activated protein kinase and (D) p-NFκB pathways were activated. (E) mTOR pathway was inhibited. Data are shown as mean ± SD (error bars) of 3 independent experiments, depicted with representative immunoblots. Statistical comparison was made between different time points vs baseline (0h). *, $p< 0.05$; **, $p< 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.


3A
LC3I
LC3II
β-actin
CQ (μM) 0 10 20 0 10 20 0 10 20 0 10 20
HCC827
HCC4006
H358
H1975


3B
HCC827
HCC4006
H358
H1975
Erlotinib
Erlotinib + CQ (10 μM)
Cell viability (%)
Cell Viability (%)
Erlotinib concentration (nM)
Erlotinib concentration (μM)


3C
Control
0.2 μM Erlotinib
10 μM CQ
0.2 μM Erlotinib + 10 μM CQ
HCC 827
HCC 4006
Cell undergoing apoptosis (%)
Erlotinib (0.2 μM)
CQ (10 μM)
3 μM Erlotinib
10 μM Erlotinib
3 μM Erlotinib + 10 μM CQ
10 μM Erlotinib+ 10 μM CQ
H358
H1975
ns
Erlotinib (3 μM)
Erlotinib (10 μM)

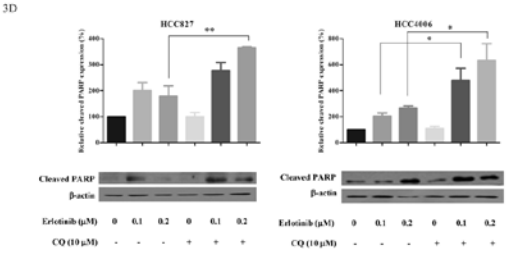

3D
HCC827
HCC4006
Cleaved PARP
β-actin
Erlotinib (μM) 0 0.1 0.2 0 0.1 0.2
CQ (10 μM)

**Figure 3.** Inhibition of autophagy with chloroquine (CQ) enhanced the effect of erlotinib treatment in EGFR-mutated, but not in wild-type, NSCLC cells. (A) LC3II accumulated after 24h of chloroquine exposure in HCC827, HCC4006, H358 and H1975 cells. (B) Cell viability by crystal violet staining in HCC827, HCC4006, H358 and H1975 cells after erlotinib ± chloroquine treatment. (C) Apoptotic cell death detected by Annexin V/7-AAD flow cytometry in HCC827, HCC4006, H358 and H1975 cells after erlotinib ± chloroquine treatment. (D) Cleaved PARP detected by Western blot after erlotinib, chloroqine, or combined treatment in sensitive NSCLC cells. Data are shown as mean ± SD (error bars) of 3 independent experiments, depicted with representative immunoblots. Statistical comparison was made between treatment vs control groups, unless otherwise indicated. *, $p< 0.05$; **, $p< 0.01$; ***, $p < 0.001$; ns, not significant.

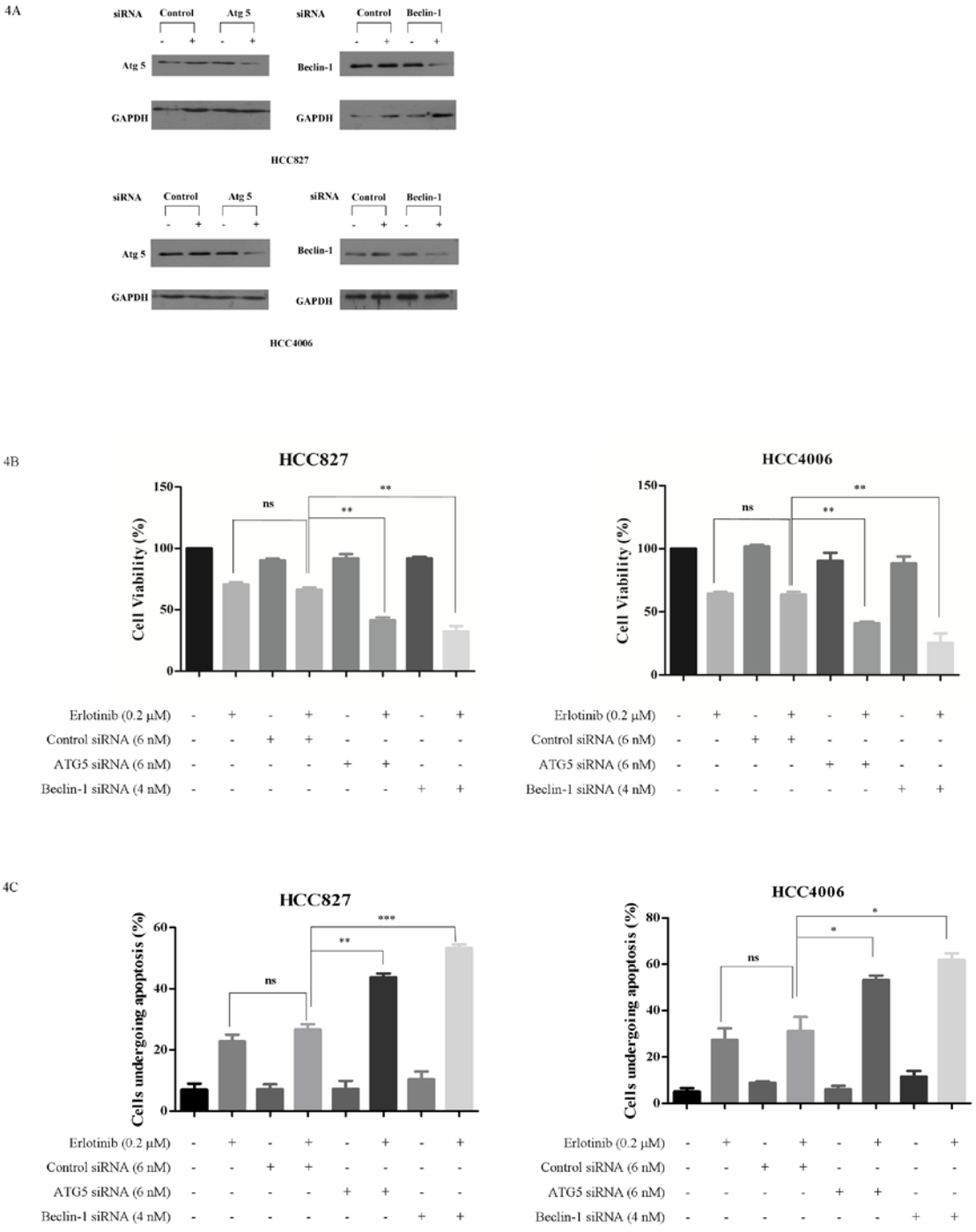



**Figure 4.** Combination of erlotinib with siRNA targeting autophagy related genes Atg5 and Beclin-1 in sensitive NSCLC cells. (A) Atg 5 and Beclin-1 were efficiently downregulated in

HCC827 and HCC4006 cells, with representative immunoblots shown. GAPDH served as the loading control. (B) Cell viability determined by MTT. (C) Apoptotic cell death determined by flow cytometry. Data are shown as mean ± SD (error bars) of 3 independent experiments. Statistical comparison was made between treatment groups as indicated. *, $p< 0.05$; **, $p< 0.01$; ***, $p < 0.001$; ns, not significant.

**Table 1**. The percentage of apoptotic cells (Annexin-V/7-AAD assay) in different cell lines treated with erlotinib, chloroquine or their combination.

| Cell line | Erlotinib | Chloroquine | Chloroquine + Erlotinib |
|---|---|---|---|
| HCC827 | 21.2 ± 4.2% | 13.0 ± 2.5% | 52.3 ± 2.8% |
| HCC4006 | 9.4 ± 2.4% | 9.0 ± 1.8% | 49.3 ± 4.8% |
| H358 | 17.4 ± 2.8% | 7.5 ± 0.7% | 17.7 ± 1.7% |
| H1975 | 6.9± 1.8% | 5.5 ± 2.0% | 8.7 ± 2.8% |