**Summary**

**Purpose:** To investigate the effects of autophagy on growth inhibition by gefitinib in non-small cell lung cancer (NSCLC) cell lines and its probable mechanism.

**Methods:** The mRNA and protein levels of Beclin 1, autophagy related 5 (Atg5) and Atg7 were assessed. H460 and Calu6 NSCLC cell lines were transfected with plasmids expressing green fluorescent protein (GFP)-LC3 and the formation of autophagosome was monitored under fluorescent microscope. In addition, H460 cells were treated with agonists of autophagy (everolimus and 3-methyladenine/3MA), AMP-activated protein kinase (AMPK) inhibitor (Compound C) and gefitinib, respectively. Cells were stained and studied under microscope. Cell colonies were counted and growth inhibition was calculated. Phosphorylated acetyl-Coenzyme A carboxylase (ACC) and AMPK were detected. Moreover, H460 cells were transfected with small interfering RNA (siRNA) against AMPK2 subunit and AMPK 2 was knocked down.

**Results:** LCII was accumulated to a higher level after treatment with gefitinib than that without addition of gefitinib, and gefitinib increased GFP punctuated cells. Besides, everolimus enhanced the autophagic process induced by gefitinib. Consistent with this, everolimus enhanced the growth inhibition of gefitinib on H460 cells. Also, incubation with gefitinib could significantly increase AMPK phosphorylation and phosphorylated ACC. Compound C AMPK inhibitor could reverse the activation of gefitinib on autophagy, as determined by Beclin 1, Atg5 and Atg7 mRNA levels. Knockdown of AMPK2 also significantly inhibited the activation of autophagy by gefitinib.

**Conclusion:** Inhibition of AMPK by its antagonist (Compound C) or siRNA predominantly blocked the induction of autophagy by gefitinib.

**Key words:** AMPK, autophagy, gefitinib, non small cell lung cancer

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**Introduction**

Lung cancer has become the leading cause of cancer-related deaths worldwide [1]. For patients with advanced NSCLC, chemotherapy is still not effective enough, with a median survival of about 12 months [2,3]. Therefore, targeted therapy or in combination with other therapies is being considered as a promising strategy to fight NSCLC [4].

Epidermal growth factor receptor (EGFR) exists on the cell membrane and is activated by binding of its specific ligands, such as epidermal growth factor (EGF) and transforming growth factor (TGF) [5]. Activation of EGFR elicits downstream activation and signal transduction, including MPK, AKT and JNK pathways, leading to the initiation and progression of cancer [6,7]. Mutations that lead to EGFR up-regulation or activation have been associated with a number of cancers [5,8]. As a result, EGFR was identified as an important targeting molecule in NSCLC [9]. By inhibiting the tyrosine kinase activity of EGFR, two tyrosine kinase inhibitors (TKIs), namely gefitinib and erlotinib, have been developed for the treatment of NSCLC [10,11]. Previous studies have demonstrated that the two drugs could inhibit tumor growth both in vivo and in vitro [12].
Besides, both EGFR-TKIs showed good tolerability and antitumor activity in NSCLC patients.

Autophagy is a self-induced proteolytic process of eukaryotic cells that results in the breakdown of intracellular material within macroautophagosomes or lysosomes. Autophagy is regulated during the phase of tumor growth [13]. Modulation of autophagy could sensitize tumor cells to many cytotoxic drugs or reverse the resistance to chemotherapeutic drugs, representing a promising strategy to improve the efficacy of cancer treatment [14].

Recent studies have shown that gefitinib can induce a high level of autophagy [15], however, its pathological significance and molecular mechanisms remain poorly understood.

Therefore, in the current study we aimed to investigate whether gefitinib could activate the autophagic process in NSCLC cells and to explore its molecular mechanism.

**Methods**

**Cell culture and reagents**

H460 and Calu6 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in DMEM medium (Invitrogen, Shanghai, China) supplemented with 10% fetal bovine serum (Gibco, Shanghai, China) as well as 100U/ml penicillin and 100 μg/ml streptomycin. Gefitinib and everolimus were obtained from SelleckChem (Houston, TX, USA).

**siRNA and transient transfections**

siRNA against Atg5 and AMPKα2 subunit were purchased from Dharmacon (ThermoScientific, Brookfield, Wisconsin, USA). All transient transfections were conducted using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. In brief, cells were seeded into six-well plates. Eighteen hrs later, cells (approximately 70% confluence) were transfected with 20nM siRNA oligos for another 24 hrs before harvest. The siRNA sequences for targeting Atg5 and AMPK α2 were as follows: 5'-AGGGAAGCAGAACCACAUACAU5' (Atg5), 5'-CGUACCGGAAUACUCGA5' (AMPK α2). As negative control, a siRNA sequence targeting luciferase was used: 5'-CGUACCGGAAUACUCGA5'.

**BrdU Assays**

A cell proliferation enzyme-linked immunosorbent assay (BrdU kit; Beyotime, Shanghai, China) was used to measure the incorporation of BrdU during DNA synthesis following the manufacturer’s instructions.

**Western blotting**

Antibodies were purchased from the following manufacturers: anti-Atg7, Beclin 1, Atg5 and β-Actin antibodies from Abgent (San Diego, California, USA); anti-AMPK, β-actin and LC3 antibodies from Santa Cruz Biotechnology (Santa Cruz, California, USA); Cells were lysed in radioimmunoprecipitation (RIPA) buffer containing 50 mM Tris-HCl, 150 mM NaCl, 5 mM MgCl2, 2 mM EDTA, 1% NP40 and 0.1% SDS. The lysates were loaded onto 10% SDS-PAGE gels and transferred to PVDF membranes (Millipore, USA). The membranes were blocked with 10% nonfat milk and then incubated with different primary antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibodies. The proteins were visualized by an ECL chemiluminescence detection kit (Amersham Biosciences, Buckinghamshire, UK).

**In vitro analysis of GFP-LC3 punctuated structures**

GFP-LC3-expressing plasmid is a mammalian expression vector containing the human LC3B gene fused at its 5′ end to the GFP gene. Plasmids were transfected into H460 cells using Lipofectamine 2000. Punctuated GFP-LC3 in each view was quantified by using particle analysis tool of Image J software.

**RNA isolation and real-time PCR**

Total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer’s instructions. cDNA was synthesized from mRNA with the Reverse Transcription System (Promega, Madison, Wisconsin, USA). Real-time quantitative PCR was carried out using SYBR green as the fluorescent reporter (Takara, Shiga, Japan) with an Applied Biosystems 7900 Real-Time PCR machine. Each sample was normalized on the basis of its actin content. A total of 45 cycles (5 secs denaturation at 95 ºC, 31 secs annealing/extension at 55 ºC) were run for each primer. Primer sequences were as follows: for Beclin 1, GGTGTCCTCTGGCAGATTCA (Forward) and TCAGTCCTGCGCTGAGTTCT (Reverse); for Atg5, TTAATACGACTTGTAGGAGGC (Forward) and ATGCCAGTGGAGAAGCAGAG (Reverse); for Atg7, ATGGCTGGGCACCAAGGACAT (Forward) and CATCATTGGAAGATGCAGCCA (Reverse); for AMPKα2, CTGTAAAGCAGAGCGTTGA (Forward) and AAATCGGCTAATCTTTGACATCA (Reverse); for β-actin, CATGTACGTTTGTATCAGGCG (Forward) and CTCTCACAAGCAG (Reverse).

**Statistics**

Values were expressed as mean±SEM (standard error of the mean) from 3 or 4 independent experiments. Statistical differences were analyzed by Student’s t test or one-way ANOVA test. Statistical significance was displayed as *p<0.05, **p<0.01 or ***p<0.001.
Results

**Gefitinib induces autophagy in NSCLC cells**

Firstly, we examined the induction of autophagy by gefitinib in H460 and Calu6 NSCLC cell lines. As shown in Figure 1A, gefitinib treatment resulted in markedly increased expression of several molecular indicators of autophagy, as evidenced by up-regulation of Beclin 1, Atg5 and Atg7 mRNA and protein levels. The transition of LC3 I to LC3 II was also elevated by gefitinib in H460 cells (Figure 1B). Moreover, we transfected the H460 cells with plasmids expressing GFP-LC3 and monitored the formation of autophagosome. GFP-LC3-positive punctuated structures, typical of enhanced autophagy, were detectable in H460 cells treated with gefitinib (Figure 1C and 1D). Altogether, these observations confirmed that gefitinib could induce autophagy in NSCLC cells.

**Supplementary Figure 1.** Protein levels of Beclin 1 were determined by Western blot in H460 cells treated with vehicle control (C), gefitinib (G, 1μM), everolimus (E,1nM) or gefitinib plus everolimus (GE).

**Autophagy enhances the anticancer roles of gefitinib**

To investigate the impact of autophagy on the anticancer role of gefitinib, H460 cells were treated with everolimus, an autophagy agonist. As shown in the supplementary Figure 1, combinatorial treatments enhanced the autophagic process as evidenced by the expression of Beclin 1. In addition, everolimus enhanced the inhibitory effect

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**Figure 1.** Gefitinib induces autophagy in NSCLC cells. (A) mRNA (upper) and protein (down) levels of autophagic markers (Beclin 1, Atg5 and Atg7) in H460 (left) and Calu6 (right) cells were determined by real-time PCR and western blot. Cells were treated with gefitinib (1μM) or vehicle control for 16 hrs or 30 hrs for RNA and protein analysis, respectively. (B) Western blotting analysis of LC3-I and -II in H460 (left) and Calu6 (right) cells with or without gefitinib (1μM) for 30 hrs. β-actin was used as internal control. (C) Representative pictures of GFP-LC3 punctuated structures in H460 cells. Scale bar = 50 M. (D) Quantification of GFP-LC3 punctuated structure per field (**p<0.01, ***p<0.001).
**Figure 2.** Activation of autophagy promotes the anticancer activity of gefitinib. 

**A:** Representative pictures. 

**B:** Quantification of clone formation in H460 cells as indicated. Cells were pretreated with gefitinib for 72-96 hrs, and then treated with everolimus for another 72-96 hrs. 

**C:** Cell proliferative potential was determined by BrdU assay in H460 cells in the absence or presence of everolimus. Cells were pretreated with gefitinib for 72-96 hrs, and then treated with everolimus for another 72-96 hrs. *p< 0.05, **p< 0.01, ***p< 0.001.

**Figure 3.** Inhibition of autophagy reduces the anticancer activity of gefitinib. 

**(A)** Cell proliferative potential was determined by BrdU assays in H460 cells. 

**(B-C)** mRNA (B) and protein (C) levels of Atg5 were analyzed by real-time PCR and Western blotting in H460 cells transfected with Atg5 siRNA oligos or negative control (Ctrl). 

**(D)** BrdU analysis was performed in H460 cells with or without gefitinib, after transfection of Atg5 siRNA oligos or negative control (Ctrl) (*p<0.05, **p<0.01, ***p<0.001).
of gefitinib on tumor cell growth, as examined by clone formation. Besides, this effect was further confirmed by measuring the BrdU incorporation in H460 cells (Figure 2), suggesting that autophagy activation could reinforce the anticancer role of gefitinib.

Inhibition of autophagy reduces the anticancer effects of gefitinib

Next, we determined whether inhibition of autophagy could affect the anticancer effects of gefitinib. As shown in the Supplementary Figure 2A, gefitinib could not upregulate beclin 1 expression in cells pre-treated with 3MA, a pharmacological inhibitor of autophagy. As a result, 3MA significantly reversed the inhibitory role of cell growth by gefitinib (Figure 3A). Besides, a knockdown of the autophagy gene Atg5 in H460 cells using siRNA also blocked the induction of autophagic markers by gefitinib (Supplementary Figure 2B). Consistently, Atg5 deficiency also attenuated the role of gefitinib (Figure 3B-D), suggesting that autophagy is required for the anticancer activity of gefitinib.

Activation of autophagy by gefitinib is mediated by AMPK

Next, we tested whether the activation of autophagy by gefitinib is mediated by AMPK in H460 and Calu6 cells. As shown in Figure 4A and 4B, incubation with gefitinib could significantly increase AMPK phosphorylation. Besides, phosphorylated ACC, a down-stream target of AMPK, was also induced by gefitinib.

Then, the AMPK inhibitor, Compound C, could reverse the activation of gefitinib on autophagy, as determined by Beclin 1, Atg5 and Atg7 mRNA levels (Figure 4C). The protein levels of Beclin 1 were also attenuated by Compound C (Supplementary Figure 3A). Moreover, H460 cells were transfected with siRNA against AMPKα2 subunit (Figure 4D). As a result, knockdown of AMPKα2 also significantly inhibited the activation of autophagy by gefitinib, demonstrating that AMPK plays an
Gefitinib and autophagy

Discussion

The results of the present study showed that gefitinib induced autophagy in H460 and Calu6 cells. The autophagy agonist everolimus could enhance the anticancer role of gefitinib. Moreover, we found that gefitinib induced autophagy through activation of AMPK signaling.

Autophagy is a genetically programmed, evolutionarily conserved process that degrades long-lived cellular proteins and organelles [16]. Autophagy is important in normal development and response to changing environmental stimuli, including bacterial and viral infections, neurodegenerative disorders, and cardiovascular disease [17]. Numerous lines of evidence also suggest an anticancer role for autophagy [18]. For example, the autophagy gene Beclin 1, which is essential for autophagic vesicle formation, is a haploinsufficient tumor suppressor in mice and is monoallelically lost in human tumors [19]. Besides, it was shown that oncogenes (such as Bcl-2) could inhibit autophagy while tumor suppressors (such as PTEN and p53) induce its activation [20], suggesting that autophagy serves as anticancer mechanism.

In the current study, we demonstrated that gefitinib, a selective inhibitor of EGFR, could induce autophagy in NSCLC cells. Autophagy also enhanced the anticancer roles of gefitinib, while inhibition of autophagy affected the activity of gefitinib. Besides, at the molecular level, numerous studies have shown that AMPK plays an important role in autophagy [21,22]. Our data also showed that AMPK activation was required for the autophagy induction by gefitinib. Interestingly, previous studies showed that signaling pathways downstream of EGFR and other RTKs such as PI3K/Akt pathway, were involved in the regulation of autophagy [23]. Another TKI, imatinib, also could activate autophagy in several types of cancer cells [24,25]. Therefore, our study, together with others, demonstrated that activation of autophagy may represent a novel mechanism in the regulation of tumor cell progression by TKI drugs. Interestingly, a previous report by Vucicevic et al. showed that Compound C could induce autophagy in cancer cells [26]. Their results suggested that Compound C treatment led to Beclin 1 induction, p62 decrease and conversion of LC3-I to autophagosome-associated LC3-II in human glioma cells.
However, we observed that Compound C did not activate the transcription of Beclin 1, Atg5 and Atg7, indicating Compound C is not involved in autophagy. Although it is unclear for this inconsistency, the cells were different in the Vucicevic et al. and our study.

It was shown that AMPK promoted autophagy by directly activating Atg1 through phosphorylation of Ser 317 and Ser 777, indicating a signaling mechanism for Atg1 regulation and autophagy induction in response to nutrient signaling [27]. Moreover, AMPK was shown to regulate autophagy at the transcriptional level. For example, FoxO3a transcription factor activation by AMPK induced expression of the autophagy-related proteins LC3-II, Gabarapl1, and Beclin1 in primary mouse skeletal muscle myotubes [28]. Therefore, AMPK, a key sensor of cellular energy status, has an essential role in autophagy induction. Due to its involvement in cellular stress resistance, AMPK has been linked to the regulation of tumorigenesis. Indeed, AMPK was considered as a tumor suppressor in many tumors through multiple mechanisms, including activation of p53, a well-known inhibitor in cell cycle and proliferation [29]. Moreover, recent studies demonstrated that AMPKa deficiency in both transformed and non-transformed cells led to a switch to aerobic glycolysis (Warburg effect) in the absence of energetic crisis. As a result, disruption of AMPK signaling pathway enhances metabolic reprogramming of cancer cells to drive the Warburg effect and promotes tumor development and progression in vivo [30]. Interestingly, several chemical drugs were previously demonstrated to regulate tumor cell progression through activation of AMPK, such as metformin, curcumin and berberine [31-33].

Taken together, we provided a novel insight that gefitinib-AMPK-autophagy signaling axis serves as a functional pathway in NSCLC cells which in turn suggests a novel mechanism for gefitinib in the treatment of NSCLC.

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