


# Influence of Autophagy Inhibition on Lung Adenocarcinoma Cell Migration and Invasion Ability, and Efficacy of Gefitinib

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## Abstract

An increasing number of studies have emphasized the role of autophagy in cancer cell metastasis and treatment of malignant tumors. Autophagy inhibitors have been widely used in combination therapies to treat advanced malignancies. Several lung adenocarcinoma cells harbor epidermal growth factor receptor (EGFR) gene mutations, and EGFR tyrosine kinase inhibitors (TKIs) are routinely used in the treatment of lung adenocarcinoma. However, a number of lung adenocarcinoma tumors do not respond or develop resistance to EGFR TKIs. The aim of the present study was to explore the effect of autophagy inhibition on the biological behavior of lung adenocarcinoma cells. In addition, whether autophagy inhibition increases the efficacy of gefitinib in lung adenocarcinoma was investigated. The activation of autophagy was inhibited via the reduction of the expression of ATG5 in A549, H1975 and HCC827 cells. ATG5 knockdown using ATG5 siRNA partially suppressed the LC3B-II expression, decreased the LC3B-I/II conversion rate and enhanced the P62 expression. Cell scratch test and Transwell assay showed that the inhibition of autophagy could impair the migration and invasion ability of cells. These studies suggested that autophagy may play a pro-survival role in lung adenocarcinoma.

## Keywords

autophagy, EGFR, lung cancer

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## Introduction

Lung cancer still killed more patients than colorectal, prostate, breast and brain cancers combined in 2017.<sup>1,2</sup> Epidermal growth factor receptor (EGFR) is a member of the ErbB/HER tyrosine kinase receptor family, and its molecular weight is ~170 kD. Mutations in the EGFR tyrosine kinase region are considered an important cause of carcinogenesis in non-small-cell lung cancer (NSCLC).<sup>3,4</sup> The most common carcinogenic mutations are deletion of exon 19 in EGFR and point mutation in L858R. These mutations lead to constitutive kinase activation by changing their own conformation, which enables EGFR to remain active without ligand stimulation.<sup>5,6</sup> At the same time, patients with EGFR exon 19 deletions and L858R point mutations were found to be more sensitive to EGFR-TKIs.<sup>7-11</sup> As compared with wild-type EGFR, exon 19 deletions and L858R mutant kinases bind with tyrosine kinase inhibitors more closely, while significantly reducing the affinity of the kinases to adenosine triphosphate.<sup>12,13</sup>

Recent studies have shown that the inhibition of autophagy may be an effective way to improve drug resistance in lung

adenocarcinoma. Multiple studies have shown that autophagy is induced in a dose-dependent manner in the treatment of multiple tumors with EGFR-TKIs.<sup>14-18</sup> In addition, it has been recently reported that lung cancer cells treated with EGFR-TKI may activate autophagy, which is a self-protective response of cells, and inhibition of autophagy may enhance the cytotoxic effect of EGFR-TKIs.

The dysregulation of autophagy has been commonly related to cancer progression, and is known to play a key role in the resistance and reduction response to chemotherapeutic agents.<sup>15</sup> Autophagy has been considered as a mechanism for tumor-suppressing and a tumor-promoting. Autophagy is a

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very highly evolutionary conserved catabolic process that degrades isolated organelles and proteins in lysosomes in order to maintain proper homeostasis.<sup>19,20</sup> Autophagy plays an important role in normal development, and impaired autophagy has been associated with several pathologies, including cancer.<sup>21,22</sup> In addition to regulating cell resistance or sensitivity to therapy, autophagy also plays a key role in the migration and invasion of tumor cells. As invasive behavior is a hallmark of lung adenocarcinoma aggressiveness, targeting autophagy could help develop novel therapeutic approaches in the future.<sup>23</sup> We plan to investigate the precise contribution of autophagy to tumor cell migration and invasion and the underlying mechanisms.

The HCC827 cells used in this study is an EGFR L858R mutant cell line, which is sensitive to EGFR-TKIs. The H1975 cells is an EGFR-TKI-resistant cell line coexisting with EGFR L858R and T790M mutations. The H1975 is an EGFR-TKI-resistant cell line with EGFR L858R and T790M mutations. The A549 cell line is a wild type EGFR cell line that is insensitive to EGFR-TKIs. In the present study, 3 types of lung adenocarcinoma cells are selected to explore the influence of autophagy inhibition on the migration and invasion of lung adenocarcinoma cells, and explore the effect of autophagy inhibition on gefitinib cytotoxicity *in vitro*.

## Materials and Methods

### Cell Culture

A549 (Catalog Number:SCSP-503), H1975 (Catalog Number:SCSP-597) and HCC827 (Catalog Number:SCSP-538) cells purchased from Chinese Academy of Sciences cell Bank were cultured in DMEM (cat. no. C11995500BT, Gibco) supplemented with 10% FBS (cat. no. FB15015, Clark) and antibiotics. All cells were kept at 37 °C and 5% CO<sub>2</sub>.

### Drug Treatment

Gefitinib (cat. no. 13166, Cayman chemical) was obtained from Cayman Chemical and dissolved in dimethyl sulfoxide, which were reserved at concentrations of 200 mmol/L.

### Transfection of siRNA

H1975, HCC827 and A549 cells were inoculated in 6-well plates at a density of  $16 \times 10^5$ /well, and the Lipofectamine 2000 reagent was used to transfect ATG5 siRNA or non-targeted siRNA (Guangzhou RiboBio Co., LTD) at a final concentration of 125 nM. After transfection for 48 h, the cells were detected by Western blotting.

### Western Blotting

Cell lysates were standardized for protein content, loaded onto SDS-PAGE gel and transferred to PVDF membrane (EMD Millipore). Membranes were probed with rabbit anti-LC3-B

(cat. no. GTX127375, GeneTex Inc.,1:2000), anti-p62 (cat. no. A7758, ABclonal Biotech Co., Ltd,1:1000), anti-Atg5 (cat. no. DF6010, Biosciences, 1:1000), anti-MMP7 (cat. no. A0695, ABclonal Biotech Co., Ltd,1:1000), anti-Vimentin (cat. no. A2584, ABclonal Biotech Co., Ltd,1:1000), anti-E-Cadherin (cat. no. A3044, ABclonal Biotech Co., Ltd,1:1000), anti-N-Cadherin (cat. no. A0433, ABclonal Biotech Co., Ltd,1:1000), anti-S-nail (cat. no. A5544, ABclonal Biotech Co., Ltd,1:1000), anti- $\beta$ -actin (cat. no. AC026, ABclonal Biotech Co., Ltd,1:10 000) antibodies and secondary antibody (cat. no. AS014, ABclonal Biotech Co., Ltd, 1:5000). Antibody binding was detected using an ECL Chemiluminescence Kit (Beijing Solarbio Science & Technology Co., Ltd).

### RNA Isolation and RT-qPCR

The total RNA was isolated using the TRIzol®Plus RNA purification system according to the manufacturer's instructions (Invitrogen; Thermo Fisher Scientific). cDNA was amplified using a cDNA synthesis kit (Generay). The results were analyzed using ABI PRISM 7000 SDS software. CT values of related genes in all samples were normalized according to the abundance of 18S transcripts. SYBR Green I Master Mix kit (Invitrogen; Thermo Fisher Scientific, Inc.) was applied to perform the RT-qPCR. The cDNAs were analysed by RT-PCR using the following 5'-3' primer sets:

ATG5: Forward, 5'-AAAGATGTGCTTCGAGATGTGT-3';

Reverse, 5'-CACTTTGTCAGTTACCAACGTCA-3';

$\beta$ -actin: Forward, 5'-GCTACAGCTTCACCACCACAG-3';

Reverse, 5'-GGTCTTTACGGATGTCAACGTC-3'.

The fold difference (relative abundance) was calculated by using the  $2^{-\Delta\Delta CT}$  method and was plotted as the mean.

### Migration Assay

Ten thousand tumor cells were injected into the superior Transwell lumen. A medium (600  $\mu$ L) containing 10% fetal bovine serum was added to the inferior cavity. Cells were incubated at 37°C in 5% CO<sub>2</sub> for the corresponding time. At room temperature, the cells were fixed with 4% paraformaldehyde for 20 min, washed with PBS and stained with crystal violet for 30 min. The membrane on the cell was randomly divided into 5 fields and the submembrane migration was observed under an inverted fluorescence microscope.

### Invasion

The Matrigel was coated to the upper 24-well chemotaxis chamber, which was coagulate into Matrigel basement membrane after 3 h at 37°C. The cells ( $5 \times 10^4$ ) were then suspended in a serum-free medium, and 200  $\mu$ l cell suspension was added into the upper chamber. 10% fetal bovine serum (600 L) was added to the lower chamber. The cells were incubated at 37°C with 5% CO<sub>2</sub> for 24 h and stained with crystal violet.

Cells that did not migrate from the membrane were taken and randomly divided into 5 fields under inverted fluorescence microscope to observe the submembrane migration.

### Cell Counting Kit-8 (CCK-8) Assay

The CCK-8 kit (LiankeBio Co., Ltd) was used for cell proliferation and cytotoxicity analysis. In the cell proliferation experiment, 100 tumor cells transfected 48 h after transfection were placed in a 96-well plate for incubation at 37°C with 5% CO<sub>2</sub> for 0, 24, 48, and 72 h, and the optical density (OD) value was determined at the wavelength of 450 nm. In cytotoxicity experiments, the optical density (OD) values of the transfected tumor cells were measured at 450 nm for 48 h after transfection with different doses of gefitinib. The inhibition rate of gefitinib on lung adenocarcinoma cells was calculated as follows: inhibition rate (%) = (OD control - OD drug) / OD control × 100%.

### Statistical Analysis

Statistical Product and Service Solutions (SPSS) 21.0 (IBM Corp.) software was used for statistical analysis. Measurement data were presented as mean ± standard deviation.  $P < .05$  was considered statistically significant. Comparison between the two groups were performed using a two-sided, unpaired Student's t-test.

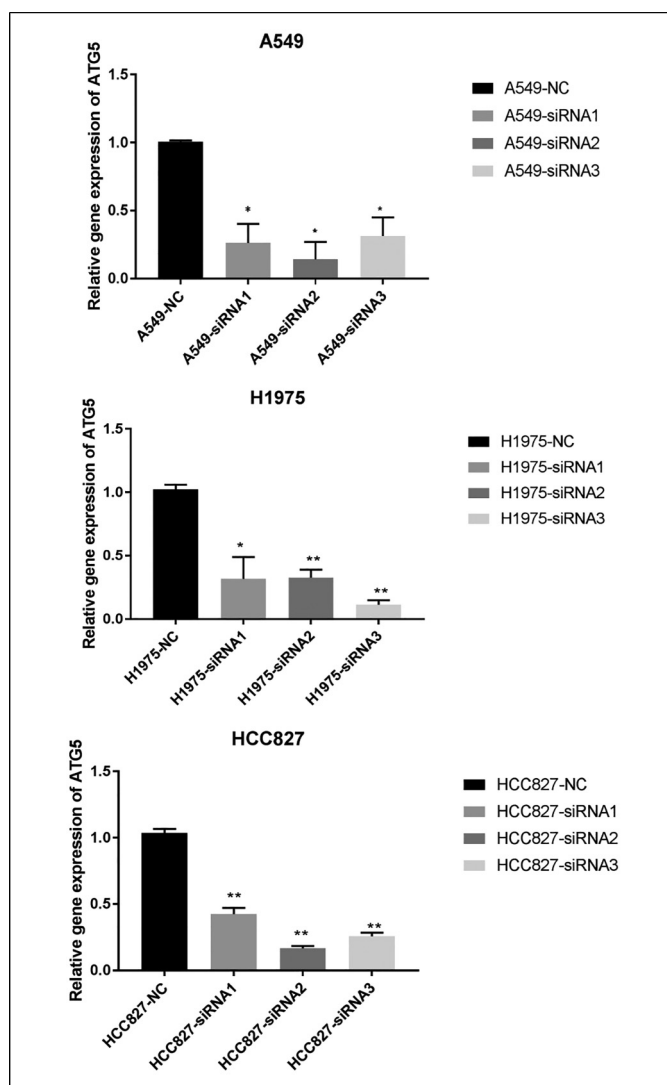
## Results

### Autophagy Is Suppressed by ATG5 siRNA

To confirm the promoting role of autophagy on cell migration and invasion, it was decided to transiently block autophagy by silencing ATG5. ATG5 is one of the main autophagy regulators involved in autophagosome formation. A549, H1975 and HCC827 cells were transfected with ATG5 siRNA and an unrelated siRNA. The effect of ATG5 siRNA on autophagy was evaluated by western blotting for p62 and LC3-I/II. ATG5 downregulation resulted in p62 accumulation and LC3-I/II increase in A549, H1975 and HCC827 cells, demonstrating an impairment of basal autophagy (Figures 1 and 2). Out of the 3 types of siRNA, it was found that ATG5 siRNA-2 was the most effective in decreasing the levels of ATG5 in cells; the protein levels of LC3-II were considerably depleted by the intervention.

### Autophagy Inhibition Suppressed Cell Migration and Invasion in Lung Adenocarcinoma Cells

Next, we analyzed the migration capability of lung adenocarcinoma cell lines in autophagy-blocking conditions by means of wound healing (Figure 3), and Transwell migration and invasion assays (Figure 4). A strong inhibition of chemokinesis was observed in ATG5 siRNA-treated cells, as compared with control cells. When subjected to wound healing assay, ATG5 knockdown cells repaired the wound much slower than control cells. In the Transwell experiment, the number of

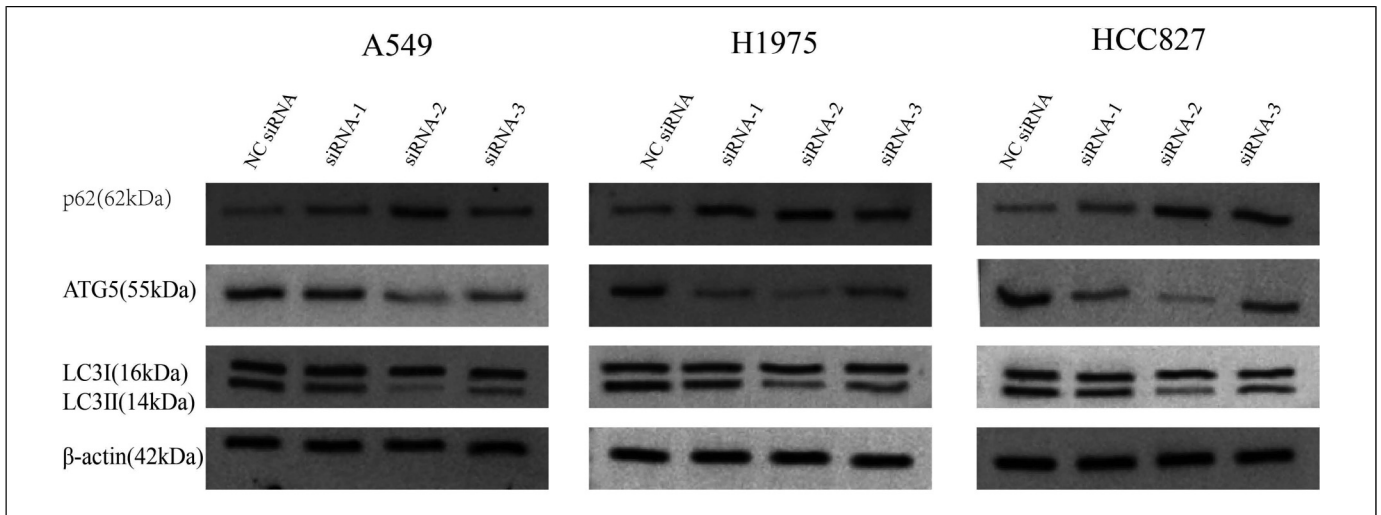


**Figure 1.** The relative levels of ATG5 mRNA were detected by RT-qPCR in A549, H1975 and HCC827 cells. \* $P < .05$  and \*\* $< .005$  versus NC-siRNA group.

cells passing through the ependymal was lower in the ATG5 knockout group than that in the control group. By means of a Transwell-based assay, an evident reduction in cell migration was observed in all 3 cell lines when ATG5 was silenced by siRNA-2. In addition, in the presence of Matrigel, it was found that the invasion capability of all 3 cell lines was also reduced when ATG5 was silenced by siRNA-2, thus confirming that autophagy is inversely correlated with chemokinesis in A549, H1975 and HCC827 cell lines.

### Autophagy Inhibition Impairs the Epithelial-Mesenchymal Transition Process in Lung Adenocarcinoma Cells

EMT (epithelial-mesenchymal transformation) refers to the transformation of epithelial cells into mesenchymal cells in

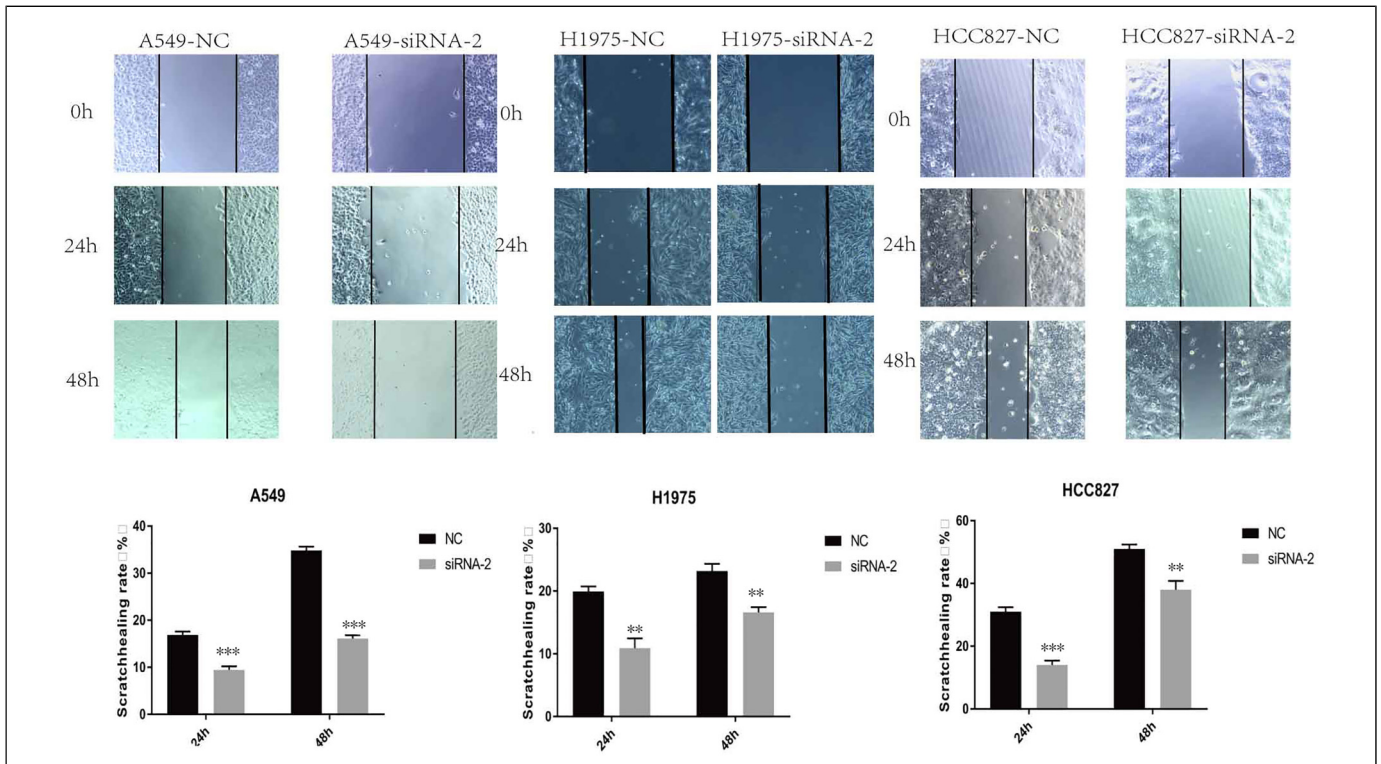


**Figure 2.** The protein expression of ATG5, p62 and LC3-B in A549, H1975 and HCC827 cells was detected by western blotting. \* $P < .05$  versus NC-siRNA group.

morphology, initiating metastasis of epithelial tumor cells.<sup>24</sup> In order to investigate the role of autophagy in the process of EMT in lung adenocarcinoma cells, Western blot were used to detect the expression of EMT key proteins. ATG5-knockdown downregulated the expression of N-cadherin, Snail, MMP7 and Vimentin, whereas E-cadherin was increased compared with the NC-siRNA groups (Figure 5).

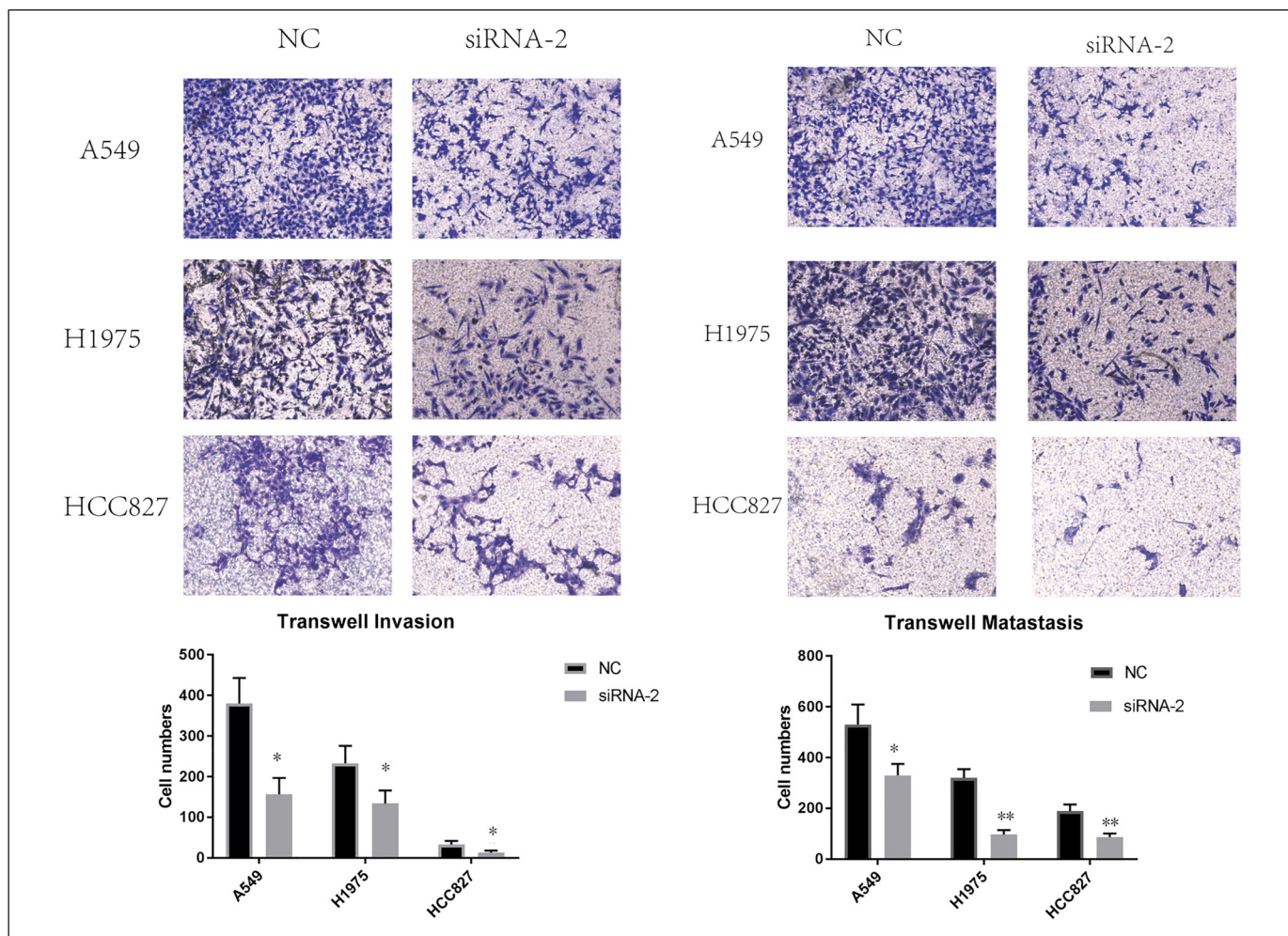
### *Influence of Autophagy Inhibition on the Proliferation of Lung Adenocarcinoma Cells*

As shown in Figure 6, the results of the CCK-8 cell proliferation test showed that the cell increment curve of the ATG siRNA-2 group was not significantly higher than that of the NC-siRNA group. This demonstrated that the inhibition of autophagy had no effect on the proliferation of the 3 types of lung adenocarcinoma cells.



**Figure 3.** The cell migration ability of lung cancer A549, H1975 and HCC827 cell lines was detected by cell scratch test following ATG5.





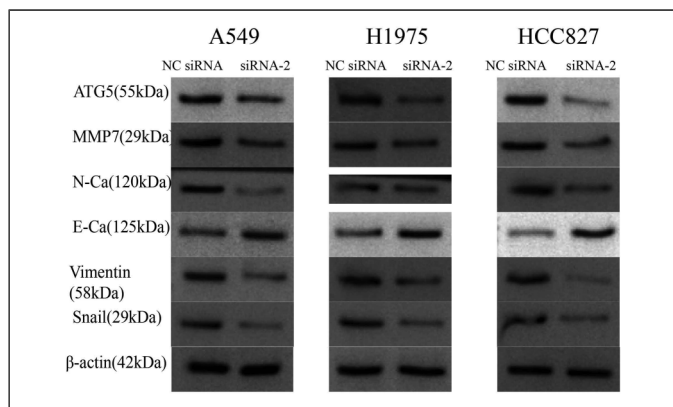
**Figure 4.** The migration and invasion ability of lung cancer cells was detected by Transwell cell migration and invasion assays. \* $P < .05$  versus NC-siRNA group.

### Inhibition of Autophagy Sensitizes NSCLC Cells to Gefitinib

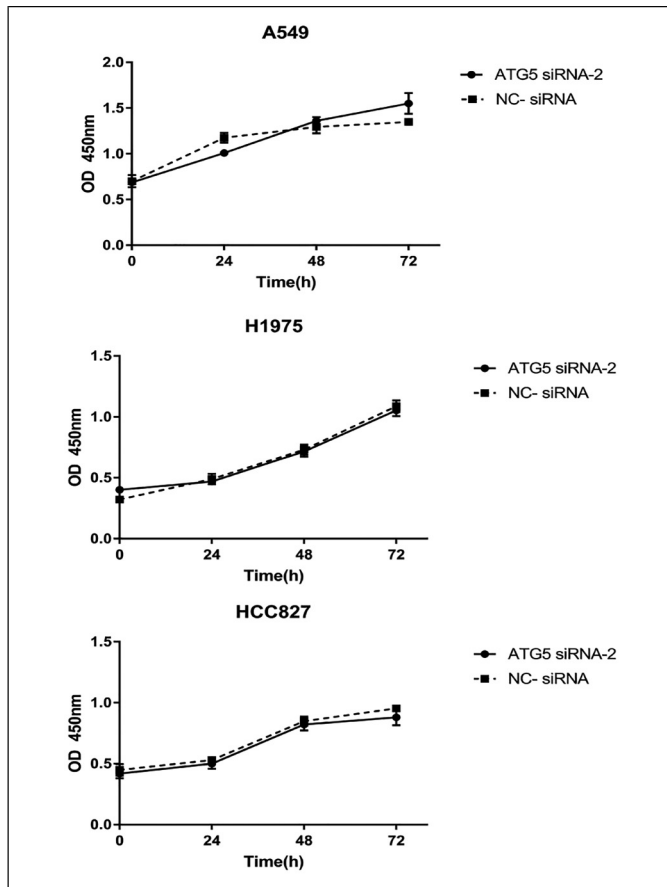
In order to address whether the inhibition of autophagy could sensitize NSCLC cells to gefitinib, the CCK-8 assay was performed (Figure 7). The increase in the concentration of gefitinib led to a marked decline in cell proliferation. ATG5 siRNA knockout increased the sensitivity of A549, H1975 and HCC827 cells to gefitinib. These results suggested that gefitinib-induced autophagy is a protective mechanism, and inhibition of autophagy may improve the efficacy to gefitinib *in vitro*. In combination, the present data suggested that autophagy plays a key role in gefitinib sensitivity. These results further indicated that the inhibition of autophagy enhanced the efficacy of gefitinib.

### Discussion

NSCLC is one of the leading causes of cancer-related mortality worldwide. NSCLC is closely associated with the mutation of EGFR. EGFR-TKIs can effectively inhibit the growth of



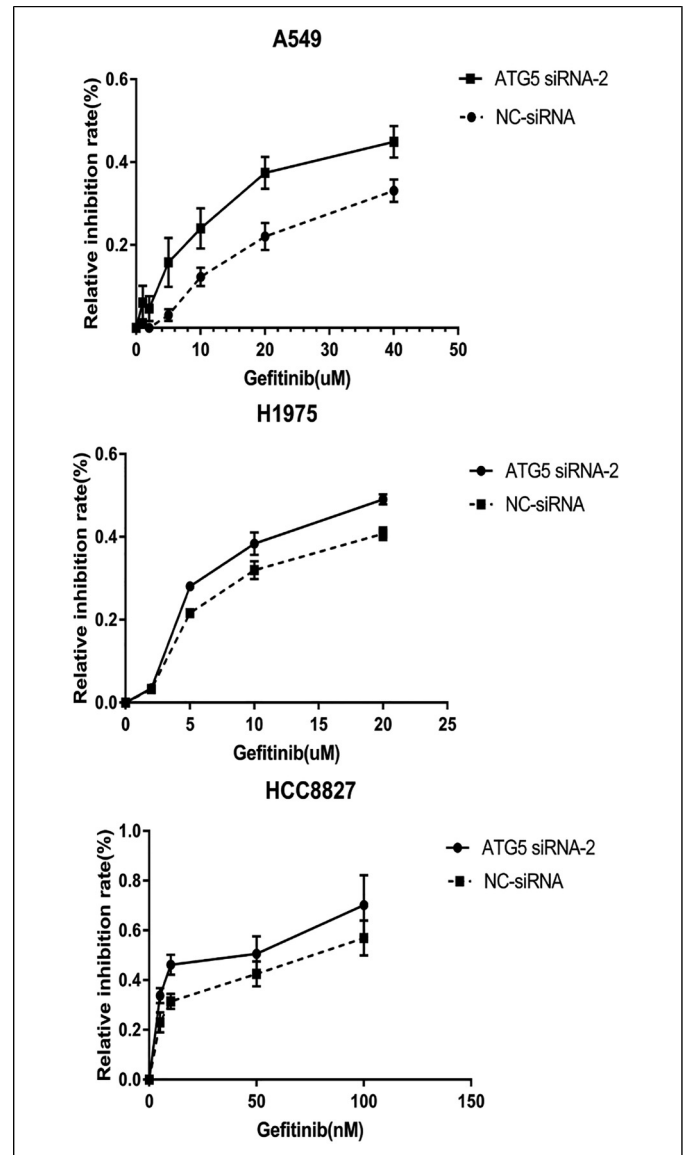
**Figure 5.** Knockdown of ATG5 downregulated the expression of N-cadherin, Snail, Vimentin and MMP7, whereas upregulated the expression of E-cadherin in lung adenocarcinoma.



**Figure 6.** Effect of ATG5 knockdown on the proliferation of lung adenocarcinoma cells. \* $P > .05$  versus NC-siRNA group.

tumor cells harboring EGFR-TKI sensitive mutation gene, which is a type of tumor-targeting drug that aims at the abnormal activation of EGFR in tumor cells.<sup>25,26</sup> Since EGFR mutations are often detected in patients with NSCLC, EGFR-TKIs are often used in those patients with good curative effects. However, acquired resistance occurred after an average of 9.2 to 14.7 months of TKI treatment.<sup>27</sup> Several molecular mechanisms of EGFR-TKIs resistance have been reported. Tan et al.<sup>28</sup> reported that autophagy provided a cytoprotective effect in EGFR-TKI treatment and the autophagy induced by EGFR TKIs protected tumor cells during cancer treatment with EGFR-TKI. Wang et al.<sup>15</sup> suggested that the inhibition of autophagy could significantly enhance the cytotoxic effects of erlotinib on TKI-resistant cancer cells through regulating endoplasmic reticulum stress-mediated apoptosis. Their view has been confirmed in other neutralizing antibodies and TKIs, as well as in a variety of cancer cell lines.<sup>16,17</sup> Therefore, the regulation of autophagy may be a promising way to improve the efficacy of EGFR inhibitors in cancer patients.

Autophagy plays an important role in maintaining intracellular homeostasis. In healthy cells, this homeostasis constitutes a powerful barrier against carcinogenesis. Several oncoproteins inhibit autophagy, while several anticancer proteins promote it. This means that, at least in some cases, the occurrence of



**Figure 7.** Inhibition of gefitinib on the cell growth of NC-siRNA and siAtg5 groups of cancer cells by CCK-8 assay in A549, H1975 and HCC827 cells. \* $P < .05$  versus NC-siRNA group.

cancer is accompanied by a temporary inhibition of autophagy or an increase in some molecular functions that antagonize its antitumor activity. However, in tumorigenesis and development, the metabolic promotion of autophagy is usually activated, and certain cancer cells exhibit an abnormally high level of basic autophagy, even under the conditions of abundant nutrition.<sup>20</sup> In tumor cell lines, reducing or deleting the expression of essential autophagy key genes can reduce the survival and tumorigenesis of tumor cells, thus establishing the important role of autophagy in tumor promotion. Autophagy is also enhanced in the anoxic tumor area needed for the survival of tumor cells. Autophagy has become a means for cells to cope with the pressure of the intracellular and extracellular environment, which is beneficial to the progression of tumors. The activation of EGFR can regulate autophagy through a variety of

signaling pathways. At present, the subtle relationship between autophagy and tumor cell survival is not fully understood, but there is speculation that autophagy may be a protector or predator, depending on its extent or duration.

Recently, several studies have shown that autophagy is induced in a dose-dependent manner during radiotherapy and chemotherapy for a variety of tumors. The main effect of EGFR-TKI therapy is to induce apoptosis, but EGFR-TKIs also activate autophagy. In addition, it has been recently reported that autophagy, as a cellular self-protective response, may be activated in the treatment of NSCLC cell line with EGFR-TKIs, and the inhibition of autophagy enhances the efficacy of EGFR-TKIs.<sup>14,29,30</sup> Zhao et al.<sup>29</sup> suggested that gefitinib could promote autophagy and apoptosis of lung cancer cells by blocking PI3 K/AKT/mTOR pathway, leading to the death of lung cancer cells. Sugita et al.<sup>30</sup> presented that autophagy inhibitor clarithromycin could enhance the cytotoxicity of gefitinib by inhibiting gefitinib-induced cytoprotective autophagy, which is consistent with our study. However, Wei et al.<sup>31</sup> reported that the induction of autophagy may contribute to EGFR-TKIs therapy in NSCLCs with active EGFR mutations, and autophagy inhibitors may play an adverse role in EGFR-TKI treatment. Other studies also reported the killing effect of autophagy, and Fan et al. indicated that autophagic cell death replaced protective autophagy induced by apoptosis.<sup>17</sup> Our study suggested that ATG5 siRNA knockout increased the gefitinib sensitivity in A549, H1975 and HCC827 NSCLC cells, which means inhibition of autophagy enhanced the efficacy of gefitinib.

Autophagy, as an important self-balanced cell recovery mechanism, has become a potential target for the acquired drug resistance phenotype.<sup>32,33</sup> In order to elucidate the effectiveness of both the pro-death and pro-survival roles of autophagy in lung adenocarcinoma cells, the key autophagic gene ATG5 was transiently silenced by siRNA. In the present study, the RNA interference technique was used to inhibit autophagy in 3 types of NSCLC cells, and the therapeutic effect of gefitinib was observed. The results suggested that autophagy was suppressed in all 3 types of lung cancer cells and promoted gefitinib sensitivity. An increasing number of studies suggest that autophagy may be a mechanism of survival for tumor cells during treatment, and its activation may promote resistance to standard chemotherapy. Therefore, targeting autophagy to regulate tumor resistance may serve as a new treatment strategy. Besides modulating cell resistance or sensitivity to therapy, autophagy has also been reported to affect the migration and invasion capabilities of cancer cells.<sup>34</sup> In the current study, following the inhibition of autophagy, the invasion and migration ability of 3 types of lung cancer cells were significantly inhibited, as compared with the control group, suggesting that autophagy may have the ability to promote tumor cell invasion and migration. At the same time, CCK-8 assay was used to determine the cell growth curve of the NC-siRNA and siATG5 groups following siRNA transfection. The results showed that there was no significant difference in cell proliferation between the three groups. This may suggest that the effect of

autophagy on tumor cell biology is mainly the promotion of cell migration and invasion; it has no effect on cell proliferation, which suggested that autophagy may protect cancer cells by inducing EMT. The western-blotting results (Figure 5) showed that the expression of MMP7, Vimentin, N-cadherin and Snail in siATG5 groups were down-regulated and the expression of E-cadherin was up-regulated compared with NC-siRNA groups. We suggest that the contribution of autophagy to cancer cell migration and invasion can be due to EMT. Recently, increased attention has been focused on studying the involvement of autophagy in EMT-like processes in cancer. Autophagy has also been reported to play a survival-promoting role in gliomas by inducing EMT.<sup>35</sup> Su et al.<sup>36</sup> indicated that the inhibition of autophagy impairs the EMT process and enhances cisplatin sensitivity in nasopharyngeal carcinoma, which is consistent with our findings. However, Hill et al.<sup>37</sup> suggested that decreased autophagy induces EMT in alveolar epithelial cells and promotes fibrosis via p62/SQSTM1-NFκB-Snail2 pathway. Wang et al.<sup>38</sup> presented that autophagy inhibition specifically promotes EMT and invasion of RAS-mutated tumor cells by triggering the NFκB/NF-κB pathway through SQSTM1/p62. Our study demonstrated that blockade of autophagy by ATG5 knockdown promoted the reversal of EMT progress and increased the efficacy of EGFR-TKIs in lung adenocarcinoma cells. Combining autophagy inhibitors and EGFR-TKIs may therefore be available to treat lung adenocarcinoma. Autophagy induced by the EGFR inhibitor plays a protective role in tumor cells, and autophagy inhibitor enhances the cytotoxicity of the EGFR inhibitor.

## Conclusion

Autophagy inhibition decreases the migration and invasion ability of lung adenocarcinoma cells, but has no obvious effect on the proliferation of lung adenocarcinoma cells. Autophagy inhibition can improve the cytotoxic effect of gefitinib on lung adenocarcinoma cells.

## Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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## Ethical Approval

Our study involves only cells.

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