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# Curcumin Induces Autophagy via Activating the AMPK Signaling Pathway in Lung Adenocarcinoma Cells

Kui Xiao[1,2], Jiehan Jiang[3], Chaxiang Guan[4], Chunling Dong[2], Guifang Wang[2], Li Bai[2], Jiayuan Sun[2], Chengping Hu[5,*a], and Chunxue Bai[2,*b]

[1]*Department of Respiratory Medicine, The Second Xiangya Hospital, Central South University, Changsha 410011, Hunan, China*
[2]*Department of Pulmonary Medicine, Zhongshan Hospital, Fudan University, Shanghai 200032, China*
[3]*Department of Respiratory Medicine, Changsha Central Hospital, Changsha 410004, Hunan, China*
[4]*Department of Physiology, Xiangya School of Medicine, Central South University, Changsha 410013, Hunan, China*
[5]*Department of Respiratory Medicine, Xiangya Hospital, Central South University, Changsha 410008, Hunan, China*

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**Abstract.** Curcumin is a major yellow pigment and active component of turmeric widely used as dietary spice and herbal medicine. This compound has been reported to be a promising antitumor agent, although the underlying molecular mechanisms are not fully understood yet. In this study, we reported that curcumin inhibited growth of lung adenocarcinoma cells, but had no cytotoxic activity to IMR-90 normal lung fibroblast cells. Curcumin induced autophagy in the A549 human lung adenocarcinoma cell line, evidenced by LC3 immunofluorescence analysis and immunoblotting assays on LC3 and SQSTM1. Moreover, the autophagy inhibitor 3-MA partly blocked the inhibitory effect of curcumin on the growth of A549 cells. Curcumin markedly increased the phosphorylation of AMP-activated protein kinase (AMPK) and acetylCoA carboxylase in A549 cells. At last, pharmacological blockade of the AMPK signaling pathway by compound C and genetic disruption of the AMPK signaling pathway with siRNA-mediated AMPK$\alpha$1 knockdown impaired the autophagy-inducing effect of curcumin. Collectively, our data suggests that curcumin induces autophagy via activating the AMPK signaling pathway and the autophagy is important for the inhibiting effect of curcumin in lung adenocarcinoma cells.

***Keywords***: curcumin, lung adenocarcinoma, autophagy, AMP-activated protein kinase (AMPK)

## Introduction

Lung cancer is the leading cause of cancer-related deaths among both developed and developing countries (1 – 2). Non-small cell lung cancer accounts for at least 80% of all lung cancer cases, and lung adenocarcinoma is the most common type of non-small cell lung cancer (1). Efforts to characterize the features of lung adenocarcinoma by genetic and biochemical methods have increased our understanding of this disease and offered opportunities for development of targeted therapies. Recent breakthroughs have demonstrated that some lung adenocarcinomas carry somatic mutations (3 – 4), which make the tumors more vulnerable to treatment with small molecules or antibodies that interfere with the mutated target proteins. However, the mechanisms of lung adenocarcinoma development and progression are as yet not fully clarified (5 – 6).

Curcumin is a natural compound in the spice turmeric that has been used for dietary and medicinal purposes for thousands of years among many regions including China, India, Japan, Korea, Turkey, the United States, South Africa, Nepal, and Pakistan (7). This compound was found to interact with multiple targets and has been used for centuries to treat a large number of disease conditions, including different varieties of tumors (8 – 9). There is accumulating evidence showing that curcumin is cytotoxic to a variety of tumor cells (8). It has also been found to be safe in human clinical trials (10 – 11). Oral administration of curcumin at a concentration of 200 nmol/kg body weight was found to significantly inhibit lung metastasis as evidenced by the reduction in

Corresponding authors. *[a]huchengp28@126.com, *[b]bai.chunxue@zs-hospital.sh.cn
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the number of lung tumor nodules (80%) and an increase in life span (12). Curcumin derivatives were found to exert strong inhibition on farnesyl protein transferase in lung adenocarcinoma cells (13). In addition, there were a large number of reports on the direct apoptosis-inducing effect of curcumin in lung adenocarcinoma cells (14 – 17). Many molecular mechanisms, including inhibition of ERK1/2 activity (14), inhibiting effect on apoptosis signal-regulating kinase 1 (ASK1) (15), reactive oxygen species (ROS)-dependent mitochondrial signaling pathway (16), and upregulation of $\alpha$1-antitrypsin (17), were proposed to be involved in the tumor-suppressive effect of curcumin.

Autophagy, an evolutionarily conserved process that occurs in all eukaryotic cells from yeast to mammals, is a complex program for lysosomal degradation of subcellular proteins and other constituents, in responding to a broad range of stress, especially nutrient deprivation (18 – 21). In cells, autophagy is responsible for degradation of the superfluous proteins and organelles, which are sequestered into double-membrane autophagosomes (18 – 20). The autophagosomes subsequently fuse with lysosomes for degradation. A large number of publications have shown that autophagy is a non-apoptotic form of programmed cell death (18, 22 – 25). In recent years, some evidence argued that in some conditions, cancer cells could use autophagy as a survival mechanism to avoid death (26). Although it is still a topic of debate whether autophagy is a mechanism of cell survival or cell death, the importance of autophagy in cancer is widely accepted (27). Aoki et al. firstly showed that curcumin suppressed the growth of malignant gliomas through induction of autophagy (28). In recent two years, this effect of curcumin was confirmed in melanoma cells (29), colon cancer cells (30), and hepatocellular carcinoma cells (31).

Until now, whether there is autophagy-inducing effect of curcumin in lung adenocarcinoma cells is unclear. In addition, the molecular mechanisms underlying the autophagy-inducing effect of curcumin in cancer cells were poorly understood. In the present study, we studied the effect of curcumin on autophagy in A549 lung adenocarcinoma cells and showed that the AMP-activated protein kinase (AMPK) signaling pathway critically contributes to the autophagy-inducing effect of curcumin.

## Materials and Methods

*Reagents*

Curcumin and compound C were purchased from Sigma Aldrich (St. Louis, MO, USA). Antibodies against LC3A/B and SQSTM1 were purchased from Millipore Chemicon International (Temecula, CA, USA). Antibodies against phosphor-AMPK and total AMPK were purchased from Cell Signaling Biotechnology (Danvers, MA, USA). Antibody against actin was from Sigma. DAPI was purchased from Invitrogen (Carlsbad, CA, USA). Enhanced chemiluminescence and protease/phosphatase inhibitors were purchased from Pierce (Rockford, IL, USA).

*Cell culture*

A549 human lung carcinoma cell line and IMR-90 human lung fibroblast cell line were obtained from ATCC (Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) in 95% $O_2$ and 5% $CO_2$ (32).

*siRNA-mediated RNA interference*

We knocked down AMPK using siRNAs targeting AMPK-$\alpha$1 (sc-270142; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) in A549 lung adenocarcinoma cells with Lipofectamine-LTX with Plus Reagent (Invitrogen) as described previously (33). The transfection was performed according to the manufacturer's instructions. Western blotting was used to confirm the efficiency of knockdown.

*Cell viability assay*

Cell viability was evaluated by a non-radioactive cell counting kit-8 (CCK-8) assay as described previously (34 – 35). A549 cells ($1 \times 10^4$) were seeded into 48-well plates and cultured overnight to allow attachment. After being serum-starved (0.1% FBS) for 8 h, cells were treated with vehicle (DMSO) or curcumin (50 and 100 $\mu$M) or other agents with 10% FBS. At different time-points post treatment, the medium was removed and 10 $\mu$L of CCK-8 solution was added and incubated for 3 h at 37°C. The optical density at 450 nm was analyzed using a microplate reader (Tecan, Männedorf, Switzerland). Experiments were performed in duplicate.

*Lactate dehydrogenase (LDH) release assay*

LDH release analysis was performed with a colorimetric LDH cytotoxicity assay (Promega, Madison, WI, USA) as described previously (36). A549 cells ($1 \times 10^4$) were seeded into 48-well plates and cultured overnight to allow attachment. After being serum-starved (0.1% FBS) for 8 h, cells were treated with vehicle (DMSO) or 50/100 $\mu$M curcumin or other agents. At different time-points after treatment, the medium was collected for LDH assay. Experiments were performed in duplicate.

*Immunofluorescence*

Immunofluorescence was performed as described

previously (36 – 37). Briefly, the A549 cells were seeded onto coverslips for 12 h. At 24 h after transfection, cells were washed twice with PBS and then fixed with buffer containing 4% paraformaldehyde and 0.1% Triton X-100 at room temperature for 20 min, followed by incubation with antibody against LC3 at 37°C for 2 h and incubation with Alexa Fluor 488–conjugated secondary antibody. DAPI was used to stain the nucleus.

*Immunoblotting*

Immunoblotting analyses of cell-extracts were performed as described previously (34, 38). Cells were lysed with RIPA buffer with protease inhibitor / protein phosphatase inhibitors. Samples were subjected to 10% SDS-PAGE and transferred onto PVDF membranes at 100 V for 1 – 2 h. After being blocked in blocking buffer for 4 h, the membrane was incubated with specific primary antibody and then followed by HRP-labeled secondary antibody. The membranes were then detected using the enhanced chemiluminescence system (Pierce).

*Statistical analyses*

Data are expressed as the mean ± S.E.M. Differences were evaluated by the two-tailed Student's *t*-test or ANOVA followed by Tukey's post-hoc test. Statistical significance was set at $P < 0.05$.

## Results

*Curcumin inhibits lung adenocarcinoma cell growth but does not affect growth of normal lung cells*

As shown in Fig. 1A, incubation with low concentration (50 $\mu$M) and high concentration (100 $\mu$M) of curcumin inhibited the growth of A549 lung adenocarcinoma cells. LDH assay showed that both of the two concentrations of curcumin induced cell injury in A549 lung adenocarcinoma cells (Fig. 1B). We also tested the influence of curcumin on normal lung fibroblast IMR-90 cells. Both of the two concentrations of curcumin did not inhibit the growth of IMR-90 cells (Fig. 1C) and had no toxicity on IMR-90 cells (Fig. 1D).

*Curcumin induces autophagy in lung adenocarcinoma cells*

First, we used immunofluorescence staining with LC3, a marker protein for autophagy, to assess the effect of curcumin on autophagy in A549 cells. We observed that curcumin induced obvious LC3 puncta in A549 cells (Fig. 2A). Next, we measured the protein levels of LC3-II and SQSTM1, two autophagic protein markers, using immunoblotting assay. Curcumin treatment significantly promoted LC3-II level (Fig. 2B) and reduced SQSTM1 level (Fig. 2C) in A549 cells.

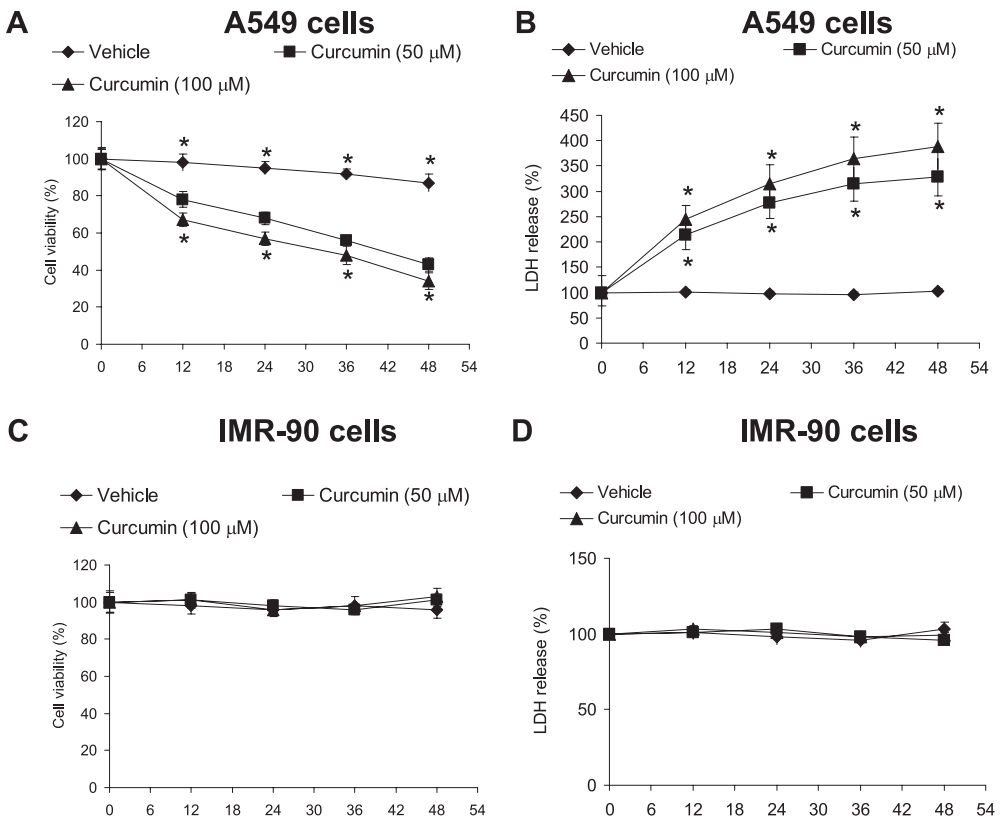


**Fig. 1.** Curcumin inhibits growth of A549 lung adenocarcinoma cells. A) Cell viability assay showing the growth of A549 lung adenocarcinoma cells under low concentration (50 $\mu$M) and high concentration (100 $\mu$M) of curcumin. *$P < 0.05$ vs. vehicle, N = 8. B) LDH release assay showing the cell injury of A549 lung adenocarcinoma cells under low concentration (50 $\mu$M) and high concentration (100 $\mu$M) of curcumin. *$P < 0.05$ vs. vehicle, N = 8. C) Cell viability assay showing the growth of IMR-90 normal lung cells under low concentration (50 $\mu$M) and high concentration (100 $\mu$M) of curcumin, N = 8. D) LDH release assay showing the cell injury of IMR-90 normal lung cells under low concentration (50 $\mu$M) and high concentration (100 $\mu$M) of curcumin, N = 8.

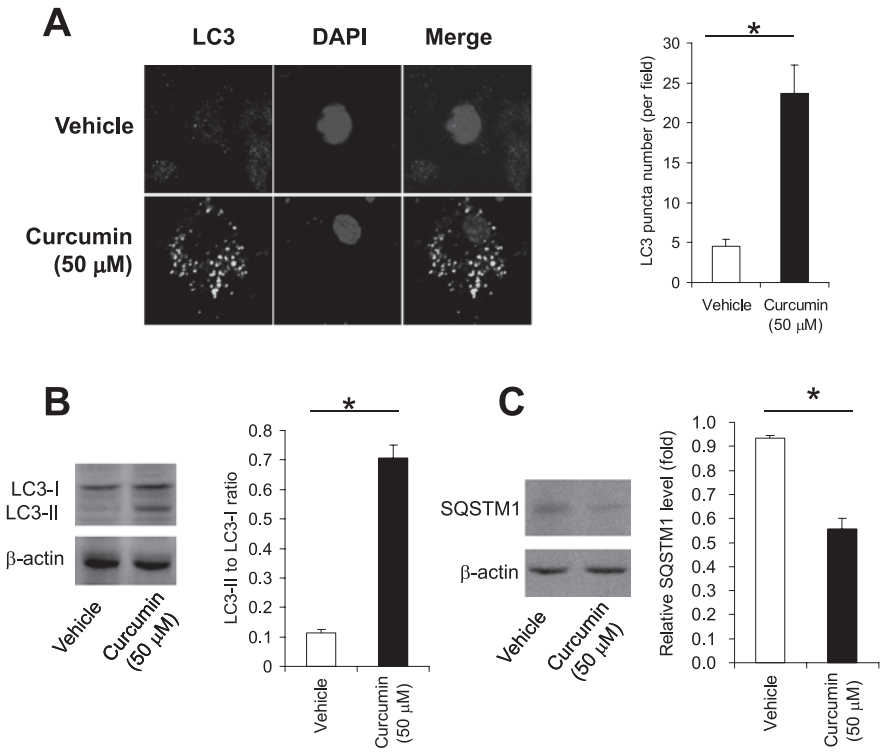


**Fig. 2.** Curcumin induces autophagy in A549 lung adenocarcinoma cells. A) Immunofluorescent staining and quantitative analysis of the of LC3-positive puncta-like structures in curcumin-treated A549 lung adenocarcinoma cells (24 h). DAPI was used to stain the nucleus. *$P < 0.05$ vs. vehicle, N = 6. B) Immunoblotting analysis of LC3-II in A549 lung adenocarcinoma cells treated by curcumin (24 h). *$P < 0.05$ vs. vehicle, N = 6. C) Immunoblotting analysis of SQSTM1 in A549 lung adenocarcinoma cells treated by curcumin (24 h). *$P < 0.05$ vs. vehicle, N = 6.

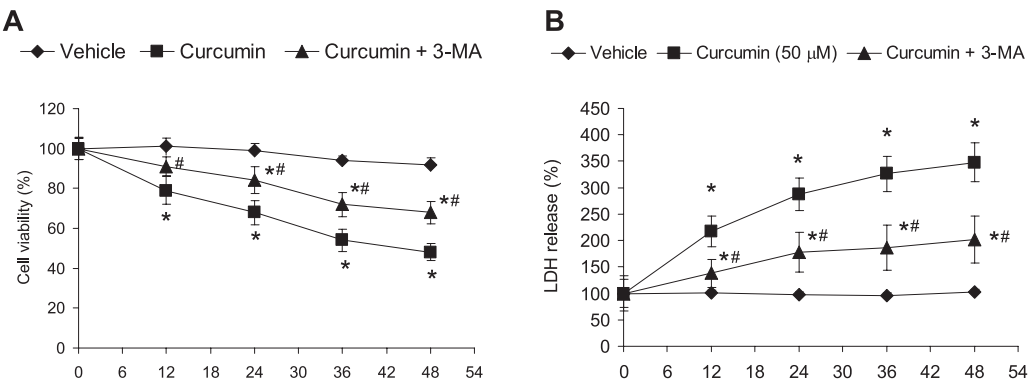


**Fig. 3.** Autophagy inhibitor 3-MA partly blocked the inhibitory effect of curcumin on A549 lung adenocarcinoma cell growth. A) Cell viability assay showing the growth of A549 lung adenocarcinoma cells under curcumin (50 $\mu$M) and 3-MA (10 $\mu$M) treatment. *$P < 0.05$ vs. vehicle. $^{\#}P < 0.05$ vs. curcumin, N = 8. B) LDH release assay showing the cell injury of A549 lung adenocarcinoma cells under curcumin (50 $\mu$M) and 3-MA (10 $\mu$M) treatment. *$P < 0.05$ vs. vehicle, $^{\#}P < 0.05$ vs. curcumin, N = 8.

*Autophagy-inducing effect contributes to the tumor suppression of curcumin in lung adenocarcinoma cells*

We next asked whether the autophagy-inducing effect of curcumin played an important role in its tumor-suppressive effect. 3-MA is a potent chemical inhibitor of autophagy. As shown in Fig. 3A, the 3-MA treatment partly blocked the inhibitory effect of curcumin on the growth of A549 cells. Similarly, 3-MA treatment attenuated the LDH release by curcumin (Fig. 3B).

*Curcumin activates the AMPK signaling pathway in lung adenocarcinoma cells*

We next explored the potential mechanisms underlying the autophagy-inducing effect of curcumin in lung adenocarcinoma cells. We found that curcumin increased the phosphorylation of AMPK (Fig. 4A). Accordingly, the downstream factor of AMPK, ACC, was also phosphorylated by curcumin (Fig. 4B).

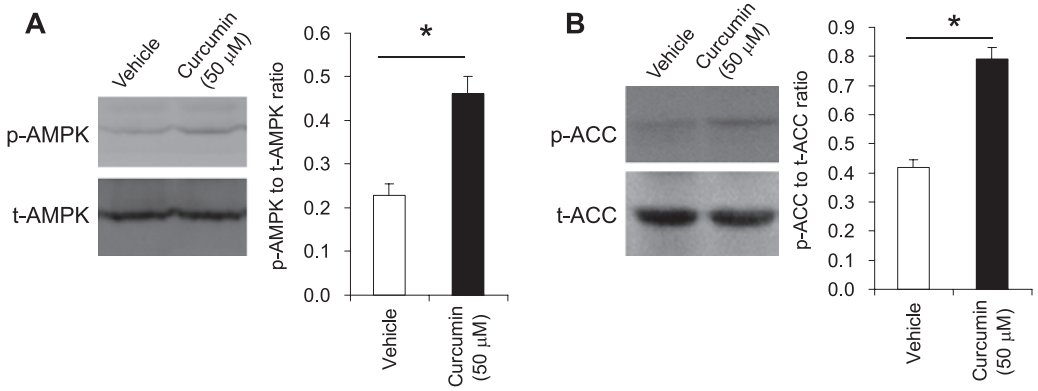


**Fig. 4.** Curcumin activates the AMPK signaling pathway in lung adenocarcinoma cells. A – B) Representative immunoblotting images and quantitative analysis of phosphorylation of AMPK (A) and ACC (B) in lung adenocarcinoma cells treated by curcumin (50 $\mu$M) for 24 h. *$P$ < 0.05 vs. vehicle, N = 4.

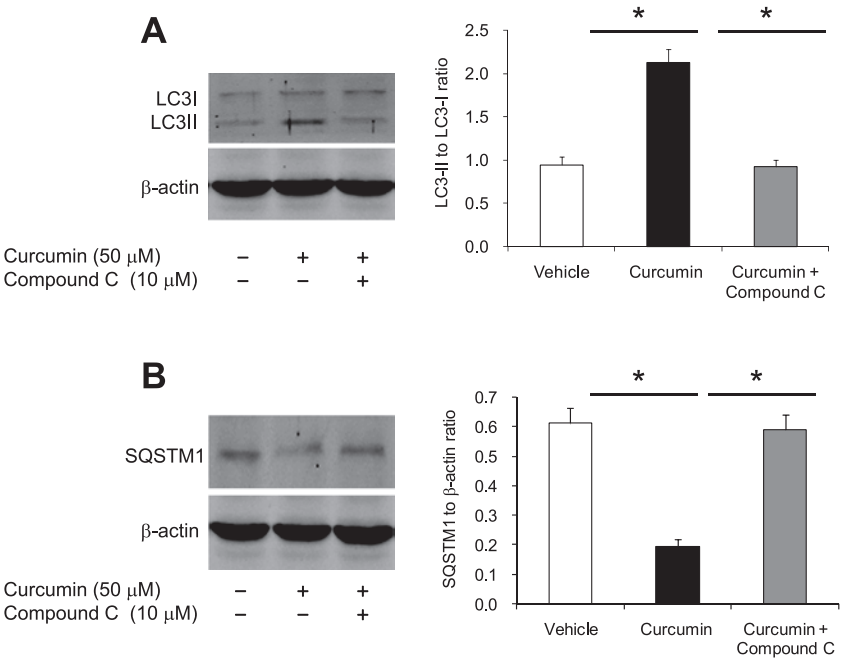


**Fig. 5.** Pharmacological blocking of AMPK signaling abolishes the autophagy-inducing effect of curcumin in lung adenocarcinoma cells. A) Immunoblotting analysis of LC3-II in A549 lung adenocarcinoma cells treated by curcumin and compound C for 24 h. *$P$ < 0.05, N = 4. B) Immunoblotting analysis of SQSTM1 in A549 lung adenocarcinoma cells treated by curcumin and compound C for 24 h. *$P$ < 0.05, N = 4.

*AMPK signaling pathway is critical for the autophagy-inducing effect of curcumin in lung adenocarcinoma cells*

Given that the AMPK is an important regulator of autophagy, we next studied whether the AMPK activation by curcumin is important for its autophagy-inducing effect in lung adenocarcinoma cells. We used compound C to block AMPK signaling activation and found that blocking AMPK signaling activation by compound C abolished the increased LC3-II (Fig. 5A) and decreased SQSTM1 (Fig. 5B) protein levels by curcumin.

To more specifically illustrate the essential role of the AMPK signaling pathway in the autophagy-inducing effect of curcumin, we used siRNA-mediated knockdown of AMPK$\alpha$1, an indispensible catalytic subunit of AMPK, to block the AMPK pathway. As shown in Fig. 6A, siRNA-AMPK$\alpha$1 (si-AMPK$\alpha$1) transfected cells displayed reduction of AMPK$\alpha$1 expression. This knockdown blocked the upregulation of LC3-II (Fig. 6B) and downregulation of SQSTM1 (Fig. 6C).

## Discussion

In the present study, we initially confirmed that curcumin had a cytotoxic effect in A549 lung adenocarcinoma cells but not in IMR-90 normal lung cells. Next, with the results including immunofluorescence

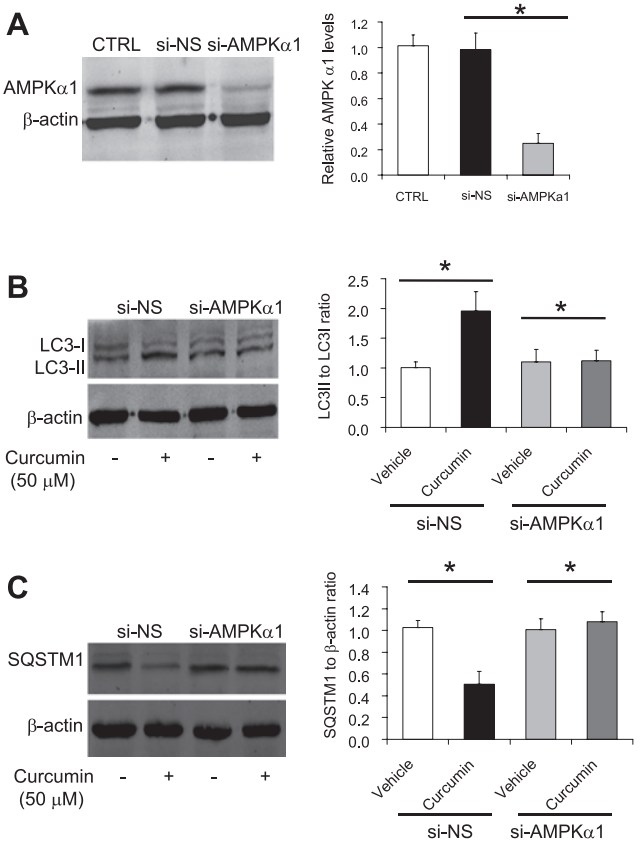


**Fig. 6.** Genetic blocking of AMPK signaling with siRNA-mediated knockdown provokes the autophagy-inducing effect of curcumin in lung adenocarcinoma cells. A) Confirmation of the efficiency of siRNA duplexes-mediated AMPK$\alpha$1 knockdown in A549 lung adenocarcinoma cells. *$P < 0.05$, N = 4. B) Immunoblotting analysis of LC3-II in siRNA-non-silencing (si-NS) or siRNA-AMPK$\alpha$1 transfected A549 lung adenocarcinoma cells treated by curcumin for 24 h. *$P < 0.05$, N = 4. C) Immunoblotting analysis of SQSTM1 in siRNA-non-silencing (si-NS) or siRNA-AMPK$\alpha$1 transfected A549 lung adenocarcinoma cells treated by curcumin for 24 h. *$P < 0.05$, N = 4.

staining of LC3, immunoblotting of LC3-II/I, and immunoblotting of SQSTM1, we found the autophagy-inducing effect of curcumin on A549 lung adenocarcinoma cells. Furthermore, blocking of autophagy using a chemical inhibitor, 3-MA, partly inhibited the inhibiting effect of curcumin on A549 cells, suggesting that the autophagy-inducing effect might contribute to the inhibiting effect of curcumin in lung adenocarcinoma cells. Interestingly, we found that the AMPK signaling pathway is important for this autophagy-inducing effect because AMPK signaling inhibitor compound C and siRNA-mediated knockdown of AMPK$\alpha$1 pronouncedly abolished the influence of curcumin on LC3-II/I and SQSTM1, two protein markers for autophagy.

Curcumin is a major yellow pigment and active component of turmeric widely used for centuries. We found that curcumin did not lead to cell injury on lung normal cells. These results are in line with a previous report showing that curcumin induced apoptosis in MCF-7 breast cancer cells, MDAMB breast carcinoma cells, and HepG2 hepatocarcinoma cells, but not in primary normal rat hepatocytes (39). In fact, this compound has been reported to be a promising tumor suppressive agent both in animals and in humans, although the underlying molecular mechanisms are not fully understood yet. Here, we provide evidence that curcumin inhibits cancer cell growth, at least partly, via inducing the autophagy process. As an ancient and conserved intracellular bulk degradation system which exists in all eukaryotes, autophagy plays important roles in many pathophysiological processes, including development/differentiation (40), immunity/inflammation (41), metabolism (42), obesity/diabetes (43 – 44), and aging (45). Recent data has revealed the critical role of autophagy in cancer (46 – 47). Curcumin was found to be able to activate autophagy in some types of cancer cells, including melanoma cells (29), colon cancer cells (30), and hepatocellular carcinoma cells (31). We noted that there was one previous report showing that H441 lung cancer cells displayed autophagic phenotype upon treatment of 3,5-bis(benzylidene)-4-piperidones, synthetic analogs of curcumin, under observation with electron microscopy (48). However, autophagy is a very complex process that requires multiple molecular methods for monitoring according to the guideline of autophagy study community (49). Therefore, the immunofluorescence and immunoblotting results from our study strongly support the finding from Lagisetty et al. (48), that is, curcumin indeed induces autophagy in lung adenocarcinoma cells.

We found a critical role of the AMPK signaling pathway in the autophagy-inducing effect of curcumin. Curcumin (50 $\mu$M) triggered pronounced phosphorylations of AMPK and its downstream factor ACC in A549 lung adenocarcinoma cells. AMPK, a member of the SNF1/AMPK protein kinase family, is a central energy sensor and metabolic switch found in all eukaryotes (50). In response to the alterations of nutrients and intracellular energy levels, AMPK switches anabolic pathways to catabolic pathways for energy reservation (50). Recent reports have indicated that AMPK mediates acute autophagic response through the interaction between AMPK and ULK1 (51 – 53), implying the essential role of AMPK in the autophagy process. In fact, Yu et al. have demonstrated that curcumin activated the AMPK pathway in prostate cancer cells (54). The activation of AMPK signaling by curcumin was found to be involved in the anti-differentiation (55), anti-gluconeogenic (56), and anti-hepatic steatosis effect (57) of curcumin. Nevertheless, whether AMPK signaling is important for the biological effects of curcumin in cancer cells is

not well documented. In our study, we found that blocking of AMPK signaling with the pharmacological inhibitor compound C or genetic knockdown of AMPK$\alpha$1 successfully abolished the enhancement of LC3-II/I and reduction of SQSTM1, two protein markers of autophagy. These results indicate that the activation of AMPK signaling is required for the autophagy-inducing effect of curcumin in lung adenocarcinoma cells.

In summary, we demonstrate that curcumin inhibited A549 lung adenocarcinoma cell proliferation and induced cell injury in these cells. Curcumin induced autophagy in A549 cells, which was partly inhibited by 3-MA, an autophagy inhibitor. Finally, curcumin activated the AMPK signaling pathway, while blocking of AMPK signaling with compound C or AMPK$\alpha$1 knockdown abolished the autophagy-inducing effect of curcumin. All these findings suggest that curcumin induces autophagy via activating the AMPK signaling pathway in lung adenocarcinoma cells, which might add to the understanding of the biological effects of curcumin on cancer cells.

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