Curcumin inhibits the development of non-small cell lung cancer by inhibiting autophagy and apoptosis

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Abstract. Among patients with primary lung cancer, 75-80% present with non-small cell lung cancer (NSCLC). However, there is a lack of studies into the potential preventive effects of curcumin against the activation of autophagy in NSCLC. Therefore, the present study primarily focused on the protective role of curcumin in NSCLC. It was demonstrated that curcumin decreased the viability of the human lung cancer cells lines, A549 and H1299, in a time-and dose-dependent manner (P<0.05). Treatment with curcumin also suppressed the colony formation capacities of A549 and H1299 cells. Following incubation with 10 μM curcumin for 48 h, cell apoptosis was significantly increased by 2.35- and 3.02-fold in A549 and H1299 cells, respectively, when compared with controls (P<0.01). Furthermore, curcumin treatment markedly increased the number and volume of autophagosomes in A549 and H1299 cells when compared with controls. Treatment with 10 μM curcumin for 48 h also significantly reduced the phosphorylation levels of mechanistic target of rapamycin (mTOR), ribosomal protein S6, phosphoinositide 3-kinase and AKT (protein kinase B) in A549 and H1299 cells (P<0.05). These data indicated that curcumin enhanced autophagy and apoptosis in NSCLC cells by acting as an mTOR complex1/2 inhibitor.

Introduction

As the most common cause of cancer-related mortality, lung cancer is divided into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) (1). It is estimated that 75-80% of patients with primary lung cancer present with NSCLC (2,3). At present, surgery is the most effective treatment method for NSCLC. However, the postoperative prognosis remains poor and the 5-year survival rate for NSCLC is reported to be <20% (4). Thus, it is necessary to develop novel prevention and treatment strategies that effectively manage NSCLC.

More recently, autophagy has become established as an important cellular process involved in the development of NSCLC (5,6). Autophagy primarily refers to degradation of cytoplasmic content, superfluous or damaged organelles and engulfment of pathogens (7-9). The mechanism by which autophagy regulates cancer is complex and principally depends on the tumor type and stage (10). Under homeostatic conditions, autophagy generally suppresses the progression of cancer, and abnormal activation of autophagy may lead to malignancy, as observed for NSCLC (11-13). Therefore, insight into the regulation of homeostasis may aid to identify effective therapeutic methods that prevent the progression of NSCLC.

Curcumin is a natural polyphenol that is extracted from the spice turmeric, and is characterized by anti-inflammatory, anti-oxidative, anti-carcinogenic and immuno-regulatory activities (14). In mice, it has been observed that treatment with curcumin decreased *Helicobacter pylori* infection and suppressed infection-induced gastric damage (15). In a clinical trial, an average curcumin dose of 500 mg for 7 days markedly decreased the level of serum lipid peroxide, as a biomarker of oxidative stress (16). Furthermore, an enhanced therapeutic effect against cancer has been observed when curcumin was used alone or in combination with chemotherapeutic agents (17,18). However, to the best of our knowledge, no previous studies have investigated the potential protective effects of curcumin against the activation of autophagy in NSCLC. This was the focus of the present study.

The present study explored the effects of curcumin on the proliferation and migration of lung cancer cells. The findings indicated that curcumin reduced cell growth and suppressed colony formation capacity in human NSCLC cells. Furthermore, abnormal activation of autophagy was inhibited following curcumin treatment, indicating that curcumin may be a useful anticancer agent for the treatment of NSCLC.

Materials and methods

Lung cancer cell lines and cell culture. The human lung cancer cells lines, A549 and H1299 (American Type Culture Collection, Manassas, VA, USA) were cultured in RPMI-1640

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medium and Dulbecco's modified Eagle's medium (DMEM) both supplemented with 10% fetal bovine serum (FBS) (all from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), respectively. The cells were incubated under humidified conditions at 37°C and 5% CO_2.

Cell proliferation assay. Curcumin was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). To determine the effects of curcumin on cell proliferation, A549 and H1299 cells were seeded into 96-well tissue culture plates at a density of $5x10^3$ cells/well. Cells were administered with medium only (containing 0.01% dimethyl sulfoxide as a negative control) or incubated with 0.5, 1, 5, 10 and 20 μM curcumin. Following incubation for 24, 48 and 72 h at 37°C, cell viability was determined with an 3-(4,5-dimeth-ylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide (MTT) assay (Sigma-Aldrich; Merck KGaA; Darmstadt, Germany). Following treatment, the cells were cultured in fresh media containing 0.5 mg/ml MTT for 4 h. Dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA) was then added to the wells to dissolve the formazan products and the absorbance was measured spectrophotometrically at a wavelength of 550 nm. Three replicates were performed and analyzed.

Cell apoptosis assay. Following 10 μM curcumin or DMSO control treatment for 48 h, the cells were washed three times with cold phosphate-buffered saline (PBS). An Annexin V-fluorescein isothiocyanate (FITC)-propidium iodide (PI) Apoptosis kit (Invitrogen; Thermo Fisher Scientific, Inc.) was used to measure cell apoptosis. Briefly, cells were washed three times with 1X PBS and suspended at a density of $2-3x10^6$ cells/ml in 1X Annexin V-binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Annexin V-FITC and PI buffer was administered to the cells, which were then incubated for 15 min at room temperature in the dark. Cells lacking treatment with curcumin were used as an internal control. Following incubation, the cells were filtered with a 200-mesh filter screen and analyzed with a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA) within 1 h of staining. Cell apoptosis was analyzed using BD CellQuest Pro software (BD Biosciences). A total of 10,000 cells were evaluated in each sample.

Western blotting. Following 10 μM curcumin or DMSO control treatment for 48 h, cell protein was extracted using radioimmunoprecipitation assay buffer (Solarbio Science & Technology Co., Ltd., Beijing, China) and was collected after centrifugation at 10,000 x g at 4°C for 20 min. A bicinchoninic protein assay kit (Pierce; Thermo Fisher Scientific, Inc.) was used to determine the protein concentration. A total of 15 μg protein was loaded per lane and separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 8% nonfat dry milk at 4°C overnight. Following three washes with PBS with Tween-20 (5 min/wash), the membranes were incubated with primary antibodies at 4°C overnight. The blots were then incubated with horseradish peroxidase (HRP)-conjugated anti-immunoglobulin G (all 1:5,000; Zhongshan Gold Bridge Biological Technology Co., Beijing, China) for 2 h at room temperature and then washed. Protien

detection was performed with enhanced chemiluminescent substrate (EMD Millipore). Primary antibodies against microtubule-associated protein 1 light chain 3 II/I (LC3II/I; L8918, 1:1,000, Sigma-Aldrich; Merck KGaA), beclin-1 (cat no. 3495; 1:1,000), mechanistic target of rapamycin (mTOR; cat no. 2983; 1:1,000), phosphorylated (p)-mTOR (cat no. 5536; 1:1,000), ribosomal protein S6 (cat no. 2217; 1:1,000), p-S6 (cat no. 4858; 1:1,000), phosphoinositide 3-kinase (PI3K; cat no. 4249; 1:1,000), p-PI3K (cat no. 4228; 1:1,000), AKT (protein kinase B; cat no. 9840; 1:1,000), p-AKT (cat no. 8200; 1:1,000) and β-actin (cat no. 4970; 1:1,000) (all from Cell Signaling Technology, Inc., Boston, MA, USA). β-actin was used as an internal control. ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used for density analysis.

Colony formation assay. Cells were suspended in 0.3% agar (Sigma-Aldrich; Merck KGaA) in RPMI-1640 or DMEM with or without 10 μM curcumin treatment for 48 h and plated at a density of $1x10^5$ cells/dish into a 10-cm dish, which was preloaded with a thin layer of 1.0% agar. Cells were maintained in RPMI-1640 or DMEM supplemented with 10% FBS during the assay and monitored for colony formation. After culturing for 7 days, the colony formation was observed. The clones were stained with trypan blue (Sigma-Aldrich; Merck KGaA) at room temperature for 15 min to evaluate colony formation.

Electron microscopy. Following 10 μM curcumin or DMSO control treatment for 48 h, cells were centrifuged at 800 x g for 10 min at room temperature and the cell pellets were fixed at room temperature in 2.3% glutaraldehyde for 1 h, postfixed in 2% osmium tetroxide (O5500; Sigma-Aldrich; Merck KGaA) at 4°C for 30 min and 0.5% uranyl acetate (EMD Millipore) at room temperature for 15 min, dehydrated and embedded in Spurr epoxy resin (Shanghai Huake Co., Ltd., Shanghai, China) at 4°C overnight. Ultrathin sections (90 nm) were cut and double-stained with 3% uranyl acetate and lead citrate (Solarbio, Science & Technology Co., Ltd.) at room temperature for 20 min, and viewed with a Philips CM10 transmission electron microscope (Phillips Electronics, Amsterdam, The Netherlands).

Statistical analysis. Data are presented as the mean ± standard deviation for the indicated number of separate experiments. Three independent experiments were performed for each study. Data were statistically evaluated with an unpaired Student's t-test using GraphPad Prism software (version 6.0; GraphPad Software, Inc., LaJolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Curcumin decreases human lung cancer cell viability in a time- and dose-dependent manner. A549 and H1299 cells were treated with 0.5, 1, 5, 10 and 20 μM curcumin for 48 h and cell viability was determined using an MTT assay. As depicted in Fig. 1A and B, treatment with 5, 10 and 20 μM curcumin significantly suppressed cell proliferation when compared with control cells (for A549 cells: P<0.05 for 5 μM curcumin and P<0.01 for 10 and 20 μM curcumin; for
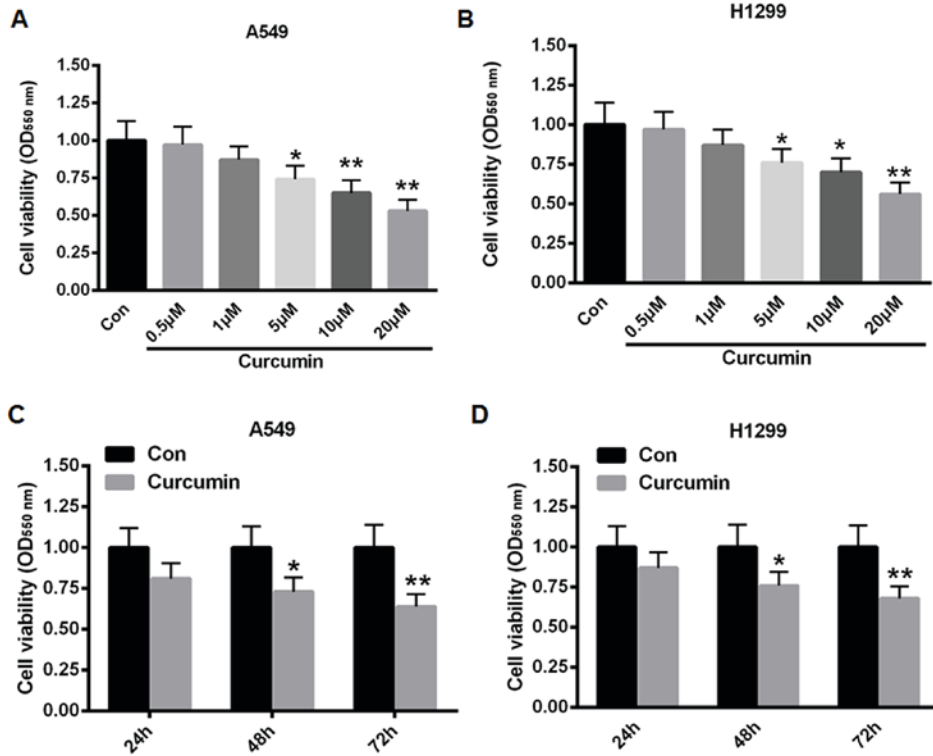
Figure 1. Curcumin decreases A549 and H1299 cell viability in a time- and dose-dependent manner. Treatment of (A) A549 and (B) H1299 cells with 0.5, 1, 5, 10 and 20 μM curcumin for 48 h decreased cell viability. Incubation with 10 μM curcumin for 24, 48 and 72 h also reduced the viability of (C) A549 and (D) H1299 cells. *P<0.05 and **P<0.01 vs. Con. OD, optical density; Con, control.
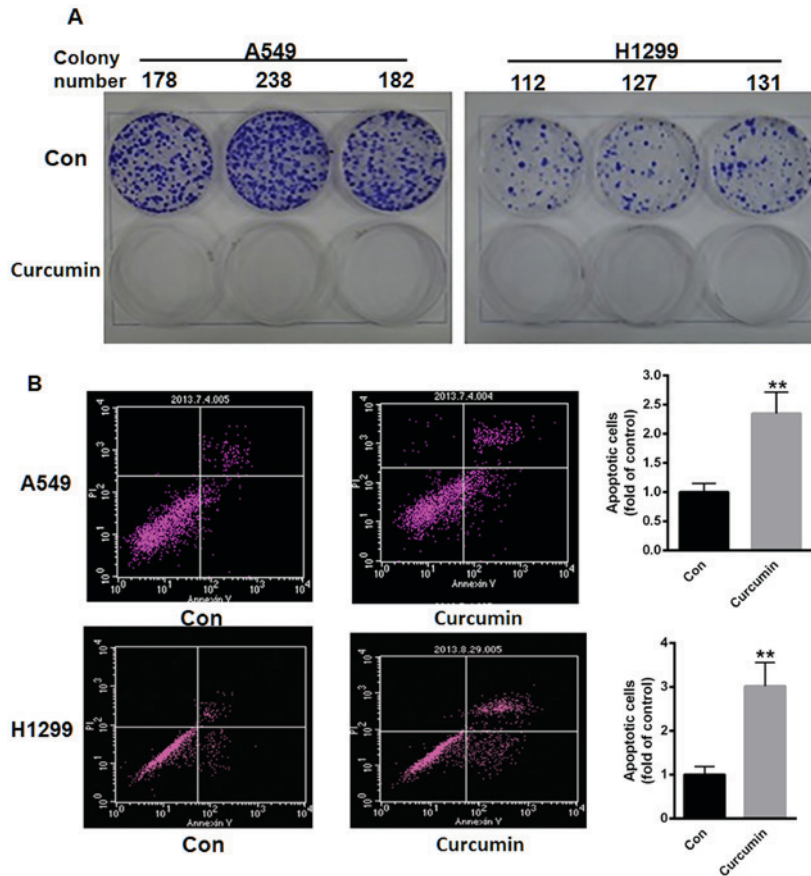

Figure 2. Curcumin inhibits colony formation and promoted cell apoptosis. (A) Curcumin treatment suppressed the colony formation capacity of both A549 and H1299 cells. (B) Incubation with 10 μM curcumin for 48 h increased cell apoptosis in A549 and H1299 cells. **P<0.01 vs. Con. PI, propidium iodide; Con, control.
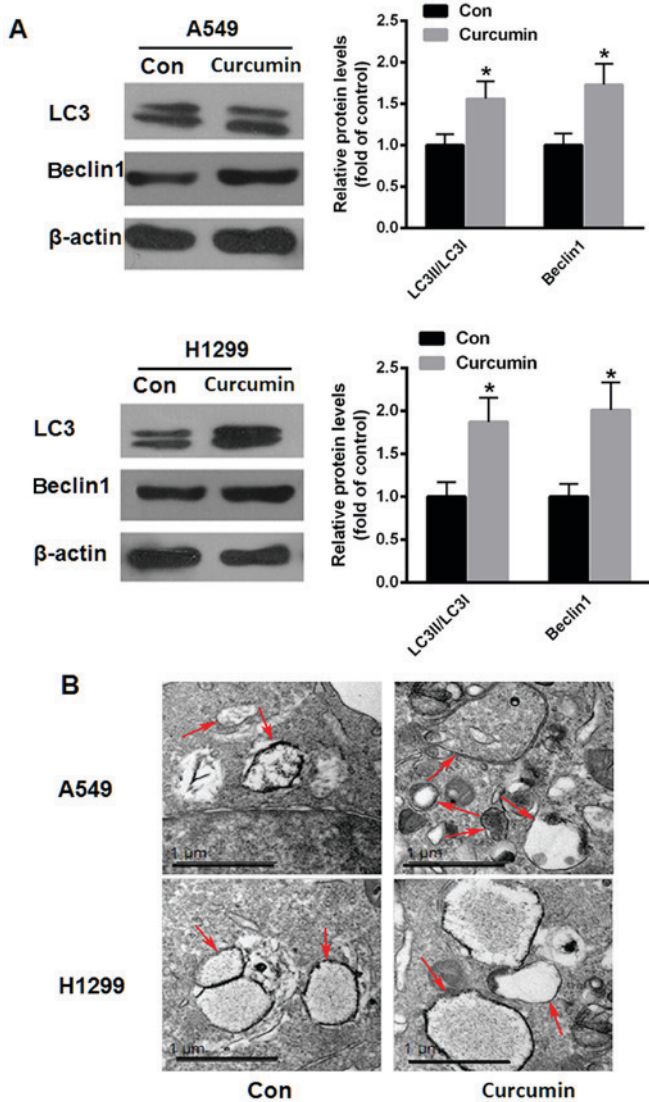
Figure 3. Curcumin enhances autophagy in human lung cancer cells. (A) Western blot analysis of the LC3-II/LC3-I ratio and protein level of beclin-1 in A549 and H1299 cells treated with 10 μM curcumin for 48 h. (B) Electronic microphotography demonstrated that curcumin markedly increased the number and volume of autophagosomes (red arrows). Scale bar length represents 1 μm; magnification, x400. *P<0.05 vs. Con. LC3, microtubule-associated protein 1 light chain 3; Con, control.
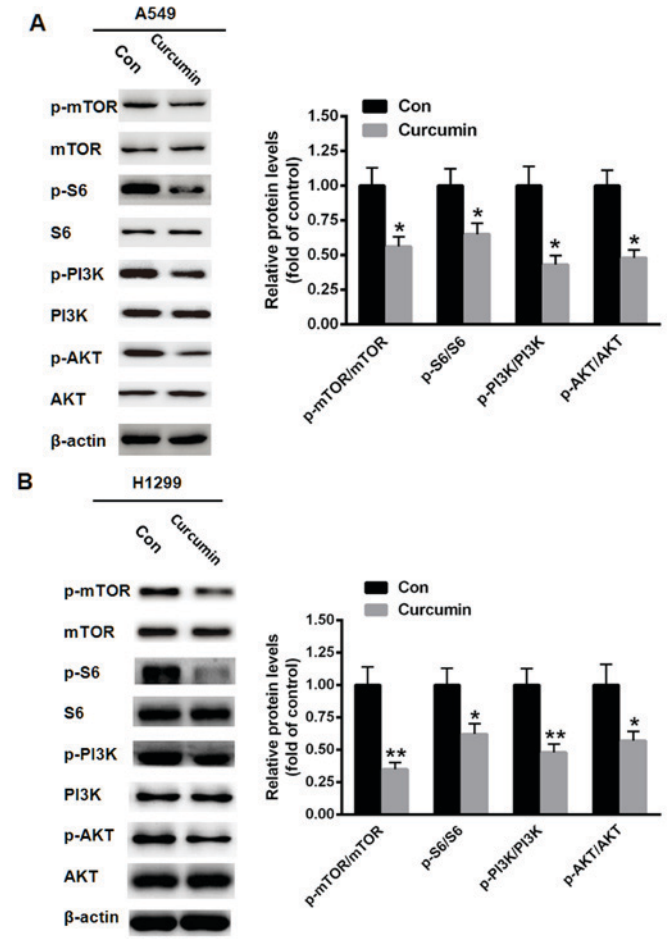

Figure 4. Curcumin suppresses PI3K/mTOR activation. Treatment with 10 μM curcumin reduced the activation of PI3K/mTOR signaling in (A) A549 and (B) H1299 cells. *P<0.05 and **P<0.01 vs. Con. PI3K, phosphoinositide 3-kinase; mTOR, mechanistic target of rapamycin; p-, phosphorylated.

H1299 cells: P<0.05 for 5 and 10 μM curcumin and P<0.01 for 20 μM curcumin). Furthermore, when A549 and H1299 cells were incubated with 10 μM curcumin for 24, 48, and 72 h, cell proliferation was significantly reduced by 27 and 36% for A549 cells, and 24 and 32% for H1299 cells at 48 and 72 h, respectively (Fig. 1C and D). These data suggested that curcumin decreased the viability of A549 and H1299 cells in a time- and dose-dependent manner.

Curcumin inhibits colony formation and promotes apoptosis. A549 and H1299 cells were subsequently treated with 10 μM curcumin for 48 h and the colony formation capacity of cells was determined. As depicted in Fig. 2A, treatment with curcumin suppressed the colony formation capacities of both A549 and H1299 cells. Cell apoptosis was also measured using flow cytometry. Following incubation with 10 μM curcumin

for 48 h, cell apoptosis was significantly increased by 2.35- and 3.02-fold in A549 and H1299 cells, respectively, when compared with controls (P<0.01; Fig. 2B).

Curcumin enhances autophagy in human lung cancer cells. It was also determined whether curcumin induced cell autophagy in lung cancer cells. Following treatment with 10 μM curcumin for 48 h, western bolt analysis demonstrated that the ratio of LC3-II to LC3-I and protein level of beclin-1 were significantly increased in A549 and H1299 cells (P<0.05; Fig. 3A). Electronic microphotography was also used to assess the autophagosomes in each group. As depicted in Fig. 3B, curcumin markedly increased the number and volume of autophagosomes in curcumin-treated cells when compared with controls, indicating that curcumin may induce cell autophagy in human lung cancer cells.

Curcumin suppresses PI3K/mTOR activation. It has been documented that PI3K/mTOR signaling serves a key inhibitory role in the autophagy of various types of human cancer (19). Therefore, the activation of PI3K/mTOR signaling following curcumin treatment was evaluated. As depicted in Fig. 4, treatment with 10 μM curcumin for 48 h markedly reduced the phosphorylation levels of mTOR, S6, PI3K and AKT in A549

(all P<0.05) and H1299 (P<0.05 for S6 and AKT, P<0.01 for mTOR and PI3K) cells.

Discussion

Lung cancer has been reported as the most common cause of cancer-related mortality (20). Therefore, detailed studies into the oncological mechanisms of lung cancer are required for the development of improved therapeutics. The majority of lung carcinomas present as NSCLC (20). In signaling pathways related to cell growth and proliferation, the PI3K/AKT/mTOR pathway serves a key role (21,22). Aberrant regulation of the PI3K/AKT/mTOR pathway has been identified in various types of tumor, including prostate, breast, lung and liver cancer (22,23). Recent studies have investigated the potential of mTOR targeting as a molecular-targeting therapy for the treatment of human cancers (24,25).

The present study focused on curcumin, as a natural poly-phenol isolated from the spice turmeric (26). Previous studies have demonstrated that curcumin suppresses cytotoxicity and DNA injury by suppressing oxidative damage caused by reactive oxygen species (27,28). The current study observed that curcumin decreased the viability of human lung cancer cells in an apparent dose- and time-dependent manner. Furthermore, cell apoptosis was increased and colony formation capacity was inhibited by curcumin treatment, as determined by annexin V-PI staining and evaluation of colony numbers, respectively. These results indicated that curcumin reduced the viability of lung cancer cells by promoting cell apoptosis and reducing colony formation capacity.

Autophagy is a complex process and is under fine regulation, which depends upon the physiological and pathological conditions of the cellular environment (29). As such, autophagy has become established as a potential anti-cancer therapeutic (24). The process of autophagy may stimulate the degradation of cytoplasmic content in the lysosomal compartment of cells at a cellular level (30). The maintenance of autophagy at a steady level is a possible therapeutic target for anti-cancer therapy. In the process of autophagy, the conversion of LC3-I into LC3-II form is regarded as a hallmark of autophagy, and prompts the formation of the autophagosome (29). In the present study, it was observed that curcumin enhanced autophagy in lung cancer cells, potentially by acting as an mTOR complex 1/2 inhibitor.

In conclusion, the present findings demonstrated novel data that curcumin suppressed mTOR/PI3K/AKT signaling, thereby inducing lung cancer cell apoptosis and autophagy. Thus, curcumin is a potential therapeutic for the treatment of human NSCLC. However, the present study lacks *in vivo* experiments and further clinical investigation is required.

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