Overexpression of Ulk2 inhibits proliferation and enhances chemosensitivity to cisplatin in non-small cell lung cancer

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Abstract. The aim of the present study was to examine the function of unc-51 like autophagy activating kinase 2 (Ulk2) in non-small cell lung cancer (NSCLC). Western blotting was used to analyze the protein expression of Ulk2 in seven pairs of cancerous and adjacent non-cancerous NSCLC specimens. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis was used to determine the mRNA expression of Ulk2 in 20 pairs of tumor and adjacent normal tissues. Two NSCLC cell lines, A549 and H460, were transfected with an Ulk2 overexpression plasmid or empty vector; cell proliferation and chemosensitivity were measured using an MTT assay, and flow cytometry and western blotting were used to evaluate apoptosis. A nude mouse tumorigenesis experiment was used to assess tumor volume *in vivo*, using A549 cells stably overexpressing Ulk2 and control cells. The protein expression levels of Ulk2 were significantly lower in 6/7 (85.7%) cases of NSCLC compared with in non-cancerous tissues, as determined by western blotting ($P<0.05$). The mRNA expression levels of Ulk2 were significantly lower in 16/20 (70.0%) NSCLC specimens compared with in non-cancerous tissues, as revealed by RT-qPCR ($P<0.05$). Overexpression of Ulk2 significantly inhibited the proliferation of A549 and H460 cells ($P<0.05$) and sensitized the NSCLC cell lines to cisplatin- and etoposide-induced inhibition of proliferation, and to cisplatin-induced apoptosis, with a significant difference identified compared with the control group ($P<0.05$). Overexpression of Ulk2 significantly increased basal autophagy levels in A549 and H460 cells ($P<0.05$). Thus, Ulk2-induced enhanced chemosensitivity was suggested to be partly mediated through increased autophagy. The overexpression of Ulk2 significantly suppressed tumor volume *in vivo* ($P<0.05$). Overexpression of Ulk2 inhibits cancer cell proliferation and enhances chemosensitivity to cisplatin in NSCLC.

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Introduction

Lung cancer is a leading cause of cancer-associated mortality worldwide, and non-small cell lung cancer (NSCLC) accounts for >80% of all lung cancer cases (1). Despite recent improvements in treatment, the prognosis for patients with NSCLC remains poor (2). The current challenge is to identify new therapeutic targets and strategies and to incorporate them into existing treatment regimens with the goal of improving therapeutic gain. Autophagy is a process in eukaryotic cells that involves the degradation and recycling of cell proteins and organelles by autophagic lysosomes (3). Throughout this process, damaged cellular proteins and organelles may be recycled and reused, which is crucial for maintaining cellular homeostasis and metabolic balance (4). Under normal physiological conditions, autophagy is necessary for stabilizing the internal cellular environment, during stress, autophagy may reduce the accumulation of damaged proteins and organelles and inhibit oncogenesis (5). Autophagy may also enhance or inhibit the sensitivity of cancer cells to cytotoxic chemotherapy in different cancer models (6). Autophagy may contribute to chemoresistance in hepatocellular carcinoma cells and chemosensitivity in H460 cells (7,8). Unc-51 like autophagy activating kinase 1/2 (Ulk1/2) are homologous mammalian autophagy-associated proteins that are crucial for the initiation of autophagy (9). Ulk1 is closely associated with tumor development, but little is known regarding the function of Ulk2 in tumorigenesis (10-12). One previous study has confirmed that there are functional differences between Ulk1 and Ulk2 (13). The aim of the present study was to determine the function of Ulk2 in NSCLC.

Patients and methods

Patients and specimens. A total of 27 pairs of samples were obtained from patients with NSCLC who were treated at the Department of Thoracic and Cardiovascular Surgery, Zhongnan Hospital of Wuhan University (Wuhan, China). The mean patient age was 63 years (range, 35-78 years), and 66.6% of the patients were male. All non-cancerous tissue samples

were collected from areas >5 cm away from the tumor. The patients underwent surgical resection and were pathologically diagnosed with NSCLC between June 2016 and June 2017. None of the patients received chemotherapy or radiotherapy prior to surgery. The Human Research Ethics Committee of Wuhan University ethically approved the study protocol. Written informed consent was obtained from all patients.

Cell culture. A549 and H460 human lung cancer cell lines were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) or the Shanghai Institute of Pharmaceutical Industry (Shanghai, China). RPMI-1640, fetal bovine serum, penicillin/streptomycin solution, trypsin and PBS were purchased from HyClone, GE Healthcare Life Sciences (Logan, UT, USA). A549 and H460 lung cancer cell lines were maintained at 37˚C in a humidified atmosphere containing 5% CO_2 in RPMI-1640 supplemented with 10% fetal bovine serum, 1% penicillin and 1% streptomycin.

Cell transfection. The Ulk2 overexpression plasmid and blank plasmid were purchased from Addgene, Inc. (Cambridge, MA, USA). TurboFect transfection reagent and puromycin were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). A549 and H460 human lung cancer cells were seeded in 6-well cell culture dishes at 30% confluence and cultured at 37˚C in a humidified atmosphere containing 5% CO_2 in RPMI-1640 supplemented with 10% fetal bovine serum to reach a confluence of 70-80%. The cells were then transfected with the plasmid DNA using a cationic lipid (0.2 μg/μl) (10 μg of plasmid DNA/50 μl TurboFect transfection reagent/6-well dish) for 6 h at 37˚C. Further analysis was performed after 48 h.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from normal cancer tissues using TRIzol® (Life Technologies; Thermo Fisher Scientific, Inc.). Total RNA preparation was performed in accordance with the manufacturer's protocol. Following DNase I (Takara Biotechnology Co., Ltd., Dalian, China) treatment at 37˚C for 30 min, 2 μg of RNA was reverse transcribed using a Takara RNA LA PCR kit (Takara Biotechnology Co., Ltd.). The 25 μl standard reaction system included 12.5 μl of Real-Time PCR Master Mix SYBR-Green I, 0.5 μl of forward primer (10 μmol/l), 0.5 μl of reverse primer (10 μmol/l), 1 μl of cDNA and 10.5 μl of ddHO. The reaction conditions were as follows: Initial denaturation at 95˚C for 3 min, then denaturation at 95˚C for 4.5 min, annealing at 60˚C for 40 sec and extension at 72˚C for 40 sec. The reactions were performed for 40 cycles. The data were analyzed using iQ5 Gene expression software (version 1.0; Bio-Rad Laboratories, Inc., Hercules CA, USA). C_q values were determined by using the 7500 System SDS software (version.1.2.3; Applied Biosystems; Thermo Fisher Scientific, Inc.). The primer sequences were as follows: Ulk2 sense, 5'-CTCCTCAGGTTCTCCAGT GC-3' and antisense, 5'-TTGGTGGGAGAAGTTCCAAG-3'; GAPDH sense, 5'-TCAAGAAGGTGGTGAAGCAGG-3' and antisense, 5'-TCAAAGGTGGAGGAGTGGGT-3'. The relative mRNA expression levels of Ulk2 were calculated using the $2^{-\Delta\Delta Cq}$ method (14), with GAPDH as the housekeeping gene.

Cell proliferation assay. Cisplatin and etoposide were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Chloroquine was purchased from Sangon Biotech Co., Ltd. (Shanghai, China). MTT reagent was purchased from BioSharp (Hefei, China). To determine the effect of Ulk2 on cell proliferation, 3,000 transfected cells per well with the Ulk2 overexpression plasmid and blank plasmid were seeded in 96-well plates and incubated at 37˚C for 1, 2, 3, 4 and 5 days. Following incubation, 20 μl MTT solution was added to each well (5 mg/ml). After 4 h at 37˚C, 150 μl DMSO was added to the well to dissolve the purple formazan. The number of viable cells was determined by measuring the absorbance at 490 nm using a FlexStation 3 plate reader (Molecular Devices, LLC, Sunnyvale, CA, USA). For chemosensitivity experiments, cisplatin and etoposide were added 12 h after cell seeding at 37˚C, and MTT assays were performed 24 h later at 37˚C.

Cell apoptosis assay. Annexin V-fluorescein isothiocyanate/propidium iodide detection kits were purchased from BD Biosciences (Franklin Lakes, NJ, USA). A549 and H460 lung cancer cells in the logarithmic growth phase were collected and evenly distributed in 6-well plates. Adherent cells were transfected upon reaching 70% confluence, and 6 h later. The cells were treated with cisplatin 36 h at 37˚C following transfection. Subsequently, the cells were digested with 0.1% trypsin. Next, the cell suspension was centrifuged at 150 x g for 5 min at 4˚C and the cells were harvested. The supernatant was removed and the precipitate was washed twice with PBS at 37˚C for 5 min. The cells were resuspended in PBS and adjusted to a concentration of $1x10^6$ cells/ml. A total of 100 μl Annexin V-FITC reagent was added to the cells for 10-15 min at room temperature in the dark. Cells were then centrifuged at 150 x g for 5 min at 4˚C and washed with PBS once at 37˚C for 5 min. Cell apoptosis was then detected using a flow cytometer. The data were analyzed using CellQuest 3.0 software (BD Biosciences, Franklin Lakes, NJ, USA).

Western blotting. Antibodies were purchased from various commercial sources, as follows: Ulk2 antibody was sourced from GeneTex, Inc. (Irvine, CA, USA); microtubule associated protein 1 light chain 3 A and B (LC3 I and II), cleaved poly (ADP-ribose) polymerase (PARP), mouse anti-human cleaved caspase-3 and GAPDH antibodies were all sourced from Cell Signaling Technology, Inc. (Danvers, MA, USA); nucleoporin p62 (p62) antibody was purchased from Abcam (Cambridge, UK); and Goat anti-mouse IgG antibody was obtained from ProteinTech Group, Inc. (Chicago, IL, USA). The electrophoresis system was purchased from Bio-Rad Laboratories, Inc. To begin, ~100 mg of A549 cancer cells were lysed with 1 ml of pre-cooled radioimmunoprecipitation assay buffer containing 150 mM NaCl, 1.0% NP-40 or 0.1%Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 8.0) and protease inhibitors (cat. no. ab65621; Abcam) at 4˚C for 15 sec and then in an ice bath for 10 min. The lysate was then centrifuged at 12,000 x g at 4˚C for 10 min and the supernatant was harvested. The concentration of the total protein was quantified using the Bradford method. A total of 50 μg protein per lane was separated by 12.5% SDS-PAGE and electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Roche Applied Science, Penzberg, Germany). The membrane was then blocked

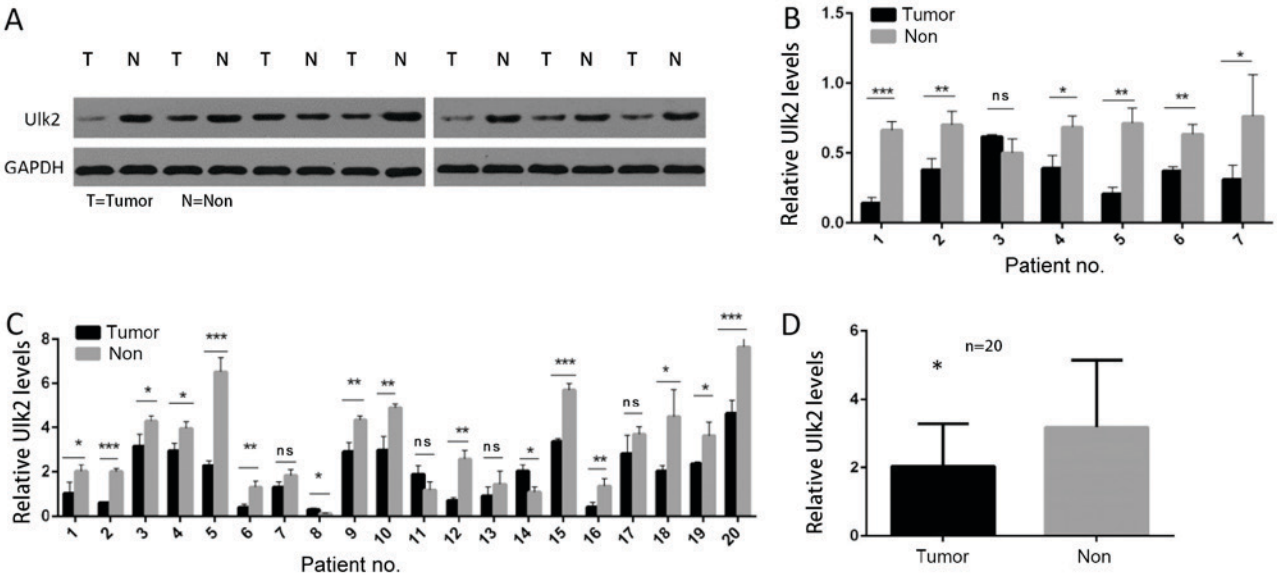

Figure 1. Expression of Ulk2 is lower in tumor tissues compared with normal tissues. (A) Ulk2 protein expression is lower in tumor tissues compared with adjacent normal tissues, as revealed by western blotting. (B) Quantified results of western blotting (n=7). *P<0.05, **P<0.01 and ***P<0.001 with comparisons shown by lines. (C) Ulk2 mRNA expression levels were significantly lower in 16/20 tumor tissues compared with adjacent normal tissues, as revealed by a reverse transcription-quantitative polymerase chain reaction. *P<0.05, **P<0.01 and ***P<0.001 with comparisons shown by lines. (D) mRNA expression levels of Ulk2 were significantly lower in tumor tissues compared with non-tumor tissues. *P<0.05 vs. non-tumor tissues. Ulk2, unc-51 like autophagy activating kinase 2; Non, non-tumor tissues; NS, not significant.

with 5% skim milk for 2 h at 37˚C. Membranes were incubated with antibodies against Ulk2 (1:1,000; cat. no. GTX111476), LC3 I and II (1:1,000; cat. no. 4180S), cleaved PARP (1:1,000; cat. no. 5625T), mouse anti-human cleaved caspase-3 (1:1,000; cat. no. 9661T), p62 (1:2,000; cat. no. ab56416) and GAPDH (1:6,000; cat. no. 5174T) at 4˚C overnight. The membranes were washed three times with TBS for 10 min each. Goat anti-mouse Immunoglobulin G (1:10,000; cat. no. SA00001-1) was used as a secondary antibody at room temperature for 2 h. Chemiluminescence on PVDF membranes was detected using an ECL Plus kit (Pierce; Thermo Fisher Scientific, Inc.). Quantity One® 1-D analysis software (version.4.62; Bio-Rad Laboratories, Inc.) was used to quantitatively analyze the density of the bands.

Acridine orange (AO) staining. AO (Sigma-Aldrich; Merck KGaA) was used to evaluate the presence of autophagy via fluorescence microscopy. AO is a fluorescent molecule used to identify lysosomal vacuolation, autophagy or apoptosis. AO interacts with DNA, emitting green fluorescence. Following transfection with plasmids for 24 h, the A549 human lung cancer cells were treated with AO (1 μg/ml) in a serum-free RPMI-1640 medium for 15 min at 37˚C in the dark. The cells were then washed four times with PBS, and fluorescent micrographs were obtained using an inverted fluorescent microscope (magnification, x400; Olympus Corporation, Tokyo, Japan).

Nude mice tumorigenesis experiment. Animal experiments were performed in accordance with the Animal Biosafety Level 3 Laboratory of Wuhan University guidelines, with the ethical approval of the Institutional Animal Care and Use Committee of Wuhan University. Puromycin was used to obtain A549 cells stably overexpressing Ulk2 (2 μg/ml). A total of 8 BALB/c-nu mice (weight, 16-18 g; 4 males and 4 females; aged 4 weeks) were obtained from the Animal Biosafety Level 3 Laboratory of Wuhan University, The mice had free access to food and water, and were maintained in a room at 20-22˚C, 40-70% humidity and with a 12 h light/dark cycle. Ulk2-overexpressing and control A549 cells ($5x10^6$) diluted in 0.1 ml PBS were injected subcutaneously into both flanks in the nude mice (n=8). Tumors appeared after 12 days, and their dimensions were measured every 4 days using digital calipers. The tumor volume was calculated as follows: $V=(width^2 \times length)/2$. After 36 days, the mice were sacrificed through carbon dioxide euthanasia, and the tumor weights were recorded.

Statistical analysis. All the data are expressed as mean ± standard deviation. SPSS software (version 17.0; SPSS, Inc., Chicago, IL, USA) was used to analyze differences. A paired Student's t-test was used to compare the difference among and between groups, respectively. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of Ulk2 is lower in tumor tissues compared with normal tissues. Ulk2 protein expression was lower in tumor tissues compared with in adjacent normal tissues, as determined by western blotting (Fig. 1A), and the differences in the expression levels of Ulk2 between the seven pairs of tumor and normal tissues were revealed to be significant in all but one patient (P<0.05; Fig. 1B). Ulk2 mRNA expression levels were also revealed to be lower in tumor tissues compared with in adjacent normal tissues by RT-qPCR in 14/20 patients used (P<0.05; Fig. 1C and D).

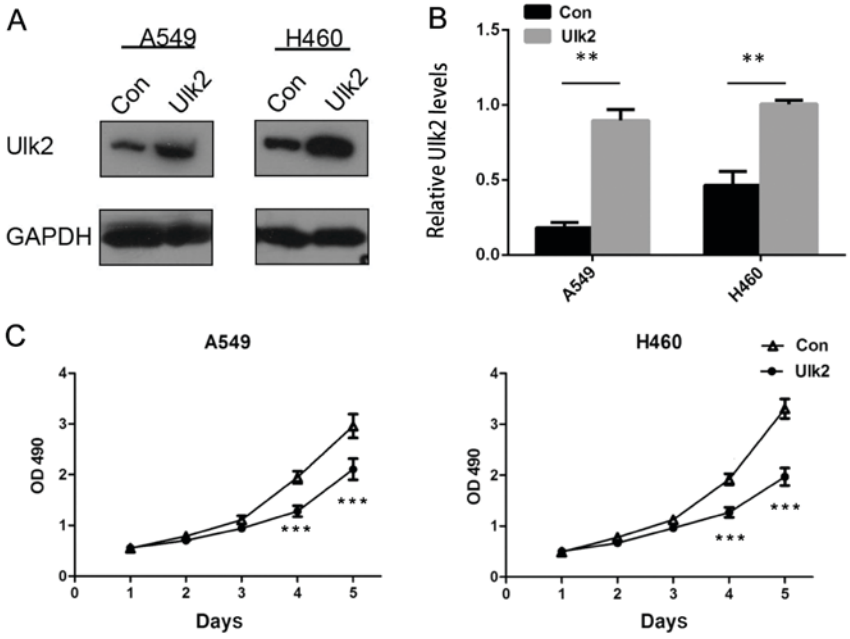

Figure 2. Overexpression of Ulk2 inhibits cell proliferation. A549 and H460 cells were transfected with Ulk2 overexpression plasmids or empty vectors (control group). (A) Whole cell lysates were subjected to western blotting for Ulk2 and GAPDH. (B) Quantified results of western blotting. **P<0.01 with comparisons shown by lines. (C) Transfected cells were incubated for 1, 2, 3, 4 or 5 days. Cell proliferation was then determined using an MTT assay. ***P<0.001 vs. the control. Ulk2, unc-51 like autophagy activating kinase 2; Con, control.

Overexpression of Ulk2 inhibits cell proliferation. A549 and H460 lung cancer cells were transfected with an Ulk2 overexpression plasmid or an empty vector, and the protein lysate was collected. Western blotting revealed that the Ulk2 overexpression plasmid significantly increased the Ulk2 protein levels in the two cell lines compared with those in the control group (P<0.01; Fig. 2A and B). Based on the results of MTT assays, it was observed that the overexpression of Ulk2 significantly suppressed cell proliferation at 4 and 5 days compared with that in the control group (P<0.001; Fig. 2C).

Overexpression of Ulk2 enhances chemosensitivity. To determine the effect of Ulk2 overexpression on chemosensitivity, A549 and H460 cells were divided into two groups (Ulk2 overexpression and control groups), and the cells were treated with cisplatin and etoposide 24 h after plasmid transfection. The cells were incubated for 24 h, and cell viability was assessed using an MTT assay. The results demonstrated that chemosensitivity to cisplatin and etoposide was significantly enhanced in the Ulk2 overexpression group compared with that in the control group (P<0.001; Fig. 3A).

Overexpression of Ulk2 promotes cisplatin-induced apoptosis. Subsequently, protein lysates were collected from the two groups and subjected to western blotting. It was revealed that cleaved caspase-3 and PARP were significantly upregulated in the Ulk2 overexpression groups compared with the control groups (P<0.01; Fig. 3B and C), suggesting that the overexpression of Ulk2 promoted cisplatin-induced apoptosis. To study the effect of Ulk2 overexpression on apoptosis induced by cisplatin, A549 and H460 cells were collected during the logarithmic growth phase and dispersed in a 6-well plate. The cells were divided into Ulk2 overexpression and control groups, treated with 33 μM cisplatin 24 h after transfection, and incubated for 48 h prior to FACS analysis. The results demonstrated that overexpression of Ulk2 promoted apoptosis in the NSCLC cell lines A549 and H460 compared with the control cells (Fig. 3D).

Overexpression of Ulk2 enhances autophagy. LC3 II and p62 were selected as markers of autophagy (15). Following transfection with an Ulk2 overexpression plasmid, the protein levels of LC3 II significantly increased and that of the autophagy substrate protein p62 significantly decreased compared with the control cells (P<0.05; Fig. 4A and B). The change in the expression of LC3 II and p62 revealed that overexpression of Ulk2 enhances autophagy. An increased number of apoptotic bodies were observed in the Ulk2 overexpression group compared with the control group with AO staining (Fig. 4C). Chloroquine is an inhibitor of cell proliferation and a known inhibitor of autophagy (16). The two groups of cancer cells were treated with chloroquine and cell viability was evaluated 24 h later. Notably, a significant reduction in cell viability was observed following chloroquine treatment (P<0.05; Fig. 4D). This result demonstrated that enhanced chemosensitivity caused by Ulk2 may be partly mediated through the autophagy signaling pathway.

Overexpression of Ulk2 suppresses tumor volume in vivo. Western blotting verified that the A549 cells stably significantly overexpressed Ulk2 protein levels compared with the control (P<0.05; Fig. 5A and B). Tumors were resected from tumor-bearing mice and measured (Fig. 5C), and their weight and volume were revealed to be significantly lower in the group overexpressing Ulk2 compared with in the control group (P<0.001; Fig. 5D). Overexpression of Ulk2 exerted a significant inhibitory effect on tumor volume *in vivo* compared with the control group (P<0.001; Fig. 5E).

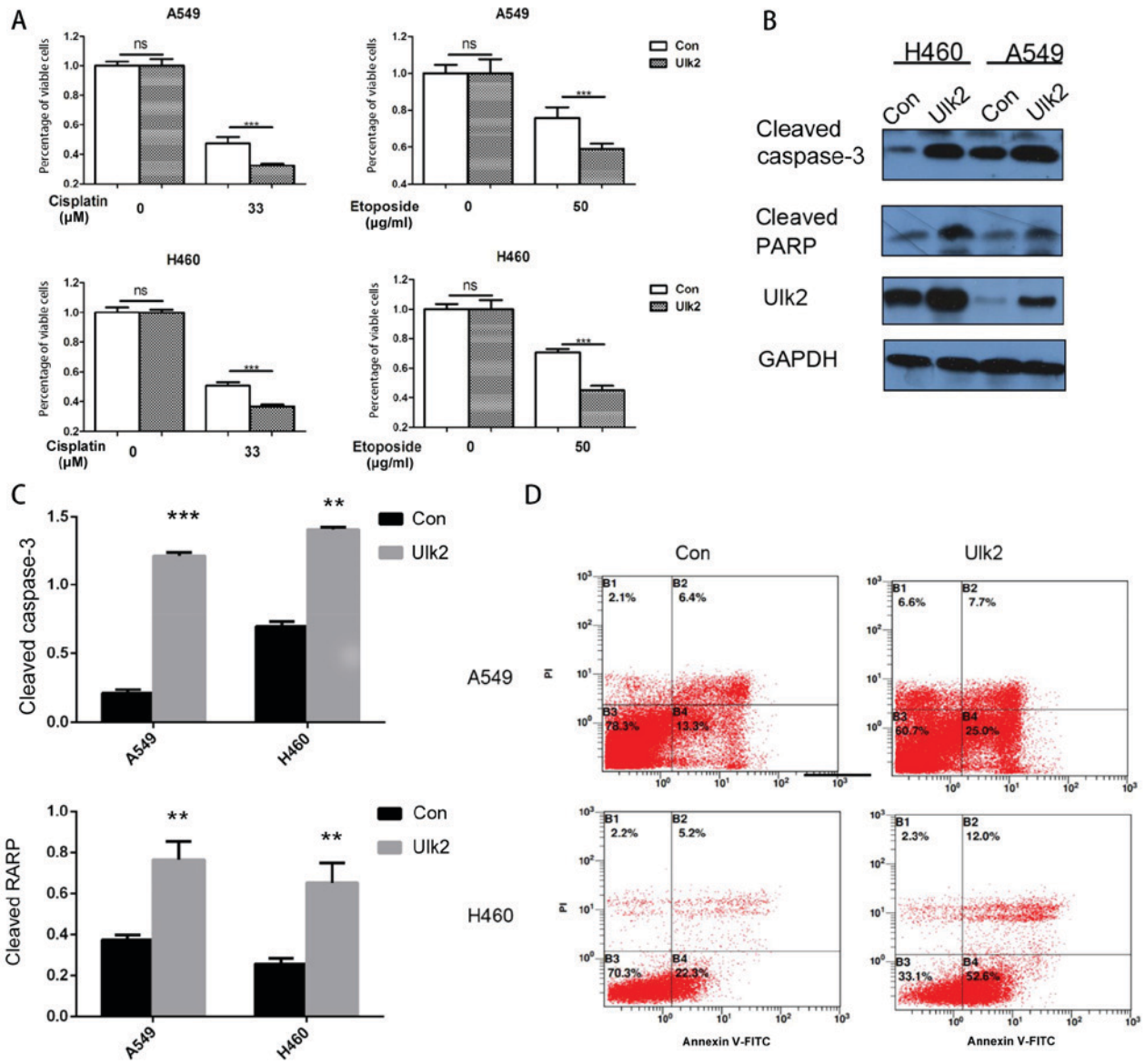

Figure 3. Overexpression of Ulk2 enhances chemosensitivity and promotes apoptosis induced by cisplatin in A549 and H460 cells. (A) Ulk2 overexpression cells and control cells were treated with cisplatin (33 μM) and etoposide (50 μg/ml) at 37˚C, and then incubated for 24 h. Cell viability was determined using an MTT assay. ***P<0.001 with comparisons shown by lines. A549 and H460 cells were divided into Ulk2 overexpression and control groups. Whole cells were treated with cisplatin (33 μM) for 48 h. (B) Whole cell lysates were subjected to western blot analysis for cleaved caspase-3, cleaved PARP, Ulk2 and GAPDH and (C) quantified. **P<0.01 and ***P<0.001 with comparisons shown by lines. (D) Apoptosis was determined using flow cytometric analysis following Annexin V fluorescein isothiocyanate/propidium iodide staining. Ulk2, unc-51 like autophagy activating kinase 2; Con, control; NS, not significant; PARP, poly (ADP-ribose) polymerase.

Discussion

Autophagy is a highly conserved catabolic process that ensures cell survival and homeostasis under unfavorable conditions (17). Using double-membrane vesicles, referred to as autophagosomes, toxic and misfolded proteins and malfunctioning organelles are degraded to supply cells with the building blocks necessary for energy processing and protein synthesis (18). It has been confirmed that autophagy and cancer are closely correlated (19), as the basal autophagy status was revealed to be altered in a variety of human solid tumor types (20).

The formation of the Ulk1/2 complex is key to autophagy. A number of studies have confirmed a close correlation between Ulk1 and cancer (21-23); however, the results of studies into its function in different tumor types appear to be conflicting (10-12). Ro *et al* (13) reported a difference between Ulk1 and Ulk2 in terms of intracellular function. Due to a lack of studies examining Ulk2 in cancer, the present study focused on the function of Ulk2 in NSCLC. It was revealed that the expression of Ulk2 was lower in NSCLC specimens compared with that in non-cancerous tissues, using western blotting and RT-qPCR. To the best of our knowledge, the present study was the first to confirm that Ulk2 overexpression may significantly inhibit proliferation in NSCLC cell lines *in vitro* and *in vivo*, and enhanced chemosensitivity in the Ulk2 overexpression group was verified through cell proliferation and cell apoptosis assays. In addition, it was observed that Ulk2 overexpression promoted autophagy in these cell lines. By comparing cell proliferation during treatment with cisplatin and etoposide,

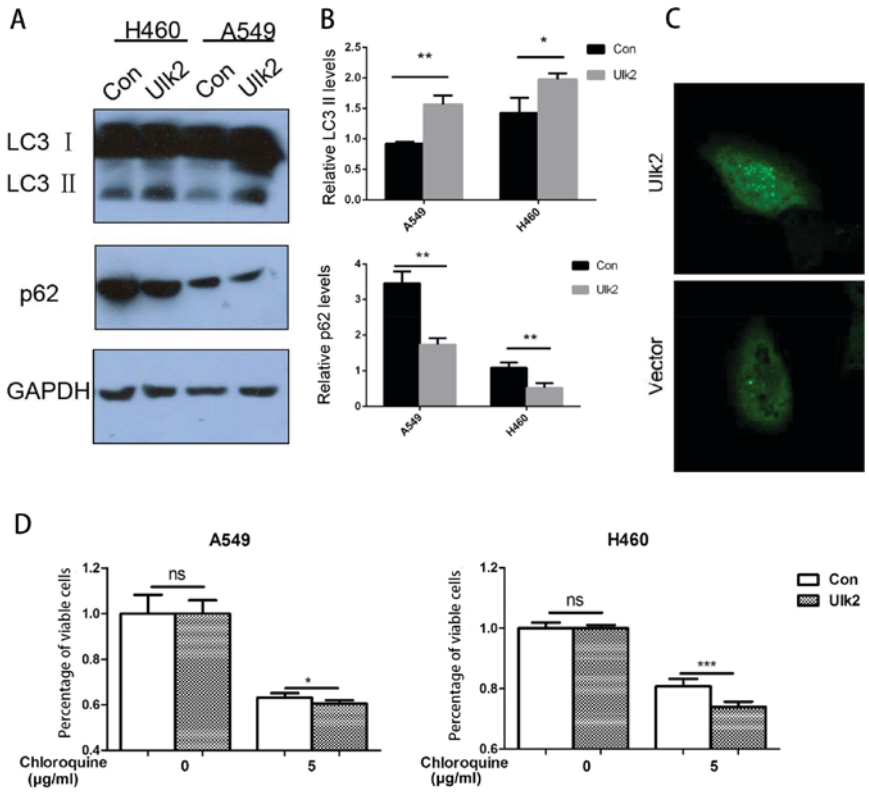

Figure 4. Overexpression of Ulk2 enhances autophagy in A549 and H460 cells. A549 and H460 cells were transfected with Ulk2 overexpression plasmids or an empty vector. (A) Whole cell lysates were subjected to western blot analysis for LC3 I, LC3 II, p62, and GAPDH and (B) protein levels were quantified. *P<0.05 and **P<0.01 with comparisons shown by lines. (C) An increased number of apoptotic bodies were observed in the Ulk2 overexpression group compared with the control group with alcidine orange staining. (D) Cells were treated with or without chloroquine (5 μg/ml) for 24 h. Cell viability was determined using an MTT assay. *P<0.05 and ***P<0.001 with comparisons shown by lines. Ulk2, unc-51 like autophagy activating kinase 2; Con, control; NS, not significant; LC3, microtubule associated protein 1 light chain 3 α; p62, nucleoporin p62.

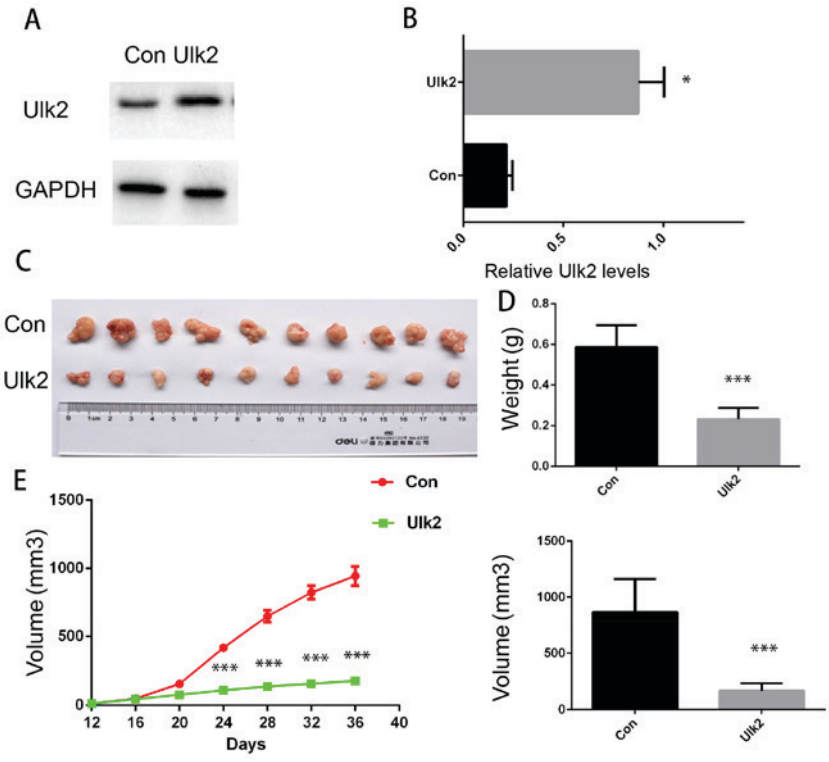

Figure 5. Overexpression of Ulk2 suppresses tumor volume *in vivo*. A549 cell lines stably overexpressing Ulk2 were established, confirmed using (A) western blotting and (B) quantified. *P<0.05 vs. the control. (C) Tumor graft sizes from nude mice 36 days following an injection of Ulk2-overexpressing and control A549 cells. (D) Tumor volumes and mean tumor weights in nude mice (n=8). ***P<0.001 vs. the control. (E) Tumor volumes were measured and recorded every 4 days, and tumor growth curves were created for each group (n=8). ***P<0.001 vs. the control. Ulk2, unc-51 like autophagy activating kinase 2; Con, control.

it was observed that the enhancement of chemosensitivity by Ulk2 may be partly mediated through autophagy signaling pathways. However, the lack of immunohistochemical staining in the *in vivo* tumor tissues is a limitation of the present study in comprehensively validating the *in vitro* results. The aim of future studies will be to gather a greater number of clinical specimens in order to investigate the expression of Ulk2, determine its association with clinical and pathological characteristics and prognosis, and elucidate the underlying intercellular molecular mechanisms and the involvement of Ulk2 in autophagy and other signaling pathways.

Researchers have previously discovered that the absence or downregulation of a wide variety of cancer-associated autophagy genes is closely associated with tumorigenesis (24). Although progress has been made in identifying the molecular mechanism of autophagy and its association with cancer in previous years (24-26), the effect of autophagy on cancer remains a subject of debate. It is possible that there is a dual association between autophagy and cancer, and that autophagy may have opposing effects at different stages of tumor development (27). Further research is required to elucidate the function of autophagy in cancer (28). Considering its involvement in cancer development, the regulation of autophagy may prove to be an effective method for targeting a tumor.

To conclude, the overexpression of Ulk2 inhibited cell proliferation and promoted chemosensitivity in NSCLC cell lines. The expression of Ulk2 was demonstrated to be lower in tumor tissues compared with that in normal tissues, and the overexpression of Ulk2 significantly inhibited the proliferation and enhanced the chemosensitivity of NSCLC cells *in vivo* and *in vitro*. In addition, the Ulk2-induced increase in chemosensitivity may be partly mediated through autophagy signaling pathways. However, the clinical significance of Ulk2 and the specific underlying mechanism require further investigation.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JPZ designed all experiments, HC and ZTY performed all experiments, and YQB and YFC analyzed the experimental results.

Ethics approval and consent to participate

The clinical and experimental studies were approved by the Human Research Ethics Committee of Wuhan University and the Institutional Animal Care and Use Committee of Wuhan University and all patients provided written informed consent.

Patient consent for publication

All patients provided written informed consent for the publication of their data.

Competing interests

The authors declare that they have no competing interests.

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