

## Original Article

# Ursodeoxycholic acid induces autophagy via LC3B to suppress hepatocellular carcinoma in vivo and in vitro

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**Abstract:** Background and aims: Hepatocellular carcinoma (HCC) is a common cancer worldwide. Researchers have found that UDCA can be used to inhibit the growth of tumors. Microtubule-associated protein light chain 3B (LC3B) is an important regulator of autophagosomes. No researches have been published on the relationship of UDCA and LC3B. Methods: A Cell Counting Kit-8 cell viability assay, cell migration assay, quantitative reverse transcription PCR (qRT-PCR) and western blot were conducted for the SMMC-7721 and HepG2 cell lines. Hematoxylin-eosin (HE) staining and immunohistochemistry (IHC) were used to analyze nude mice with 7721 xenografts. The expression of LC3B was evaluated both in vivo and in vitro. Results: Our studies demonstrated that UDCA reduced the viability of the primary HCC cell lines 7721 and HepG2 (Student's t-test,  $P < 0.05$ ) and inhibited the migration of 7721 cells (Student's t-test,  $P < 0.05$ ). UDCA also increased the expression of LC3B and p53 in vitro (Student's t-test,  $P < 0.05$ ). Additionally, UDCA inhibited the growth of tumors (Student's t-test,  $P < 0.05$ ) and promoted the expression of LC3B in nude mice. Conclusion: Our data showed that UDCA promoted the expression of LC3B, with suppressed HCC in vivo and in vitro.

**Keywords:** Ursodeoxycholic acid, hepatocellular carcinoma, LC3B, p53, autophagy

## Introduction

Liver cancer is the sixth most common cancer (782,451 new cases), the second cause of cancer related death (745,517 cases), and accounts for 9.1% of all cancers [1]. Hepatocellular carcinoma (HCC) represents more than 90% of primary liver cancers and is a major global health problem. The incidence of HCC increases progressively with advancing age in all populations, and reaches a peak at 70 years of age. In Chinese and black African populations, the mean age of patients with HCC is appreciably younger [2].

Ursodeoxycholic acid (UDCA), as a hydrophilic bile acid, is widely used to treat hepatobiliary diseases in a clinical setting. UDCA can promote the secretion of bile and inhibit bile reabsorption from the intestines. Therefore, UDCA is

used to treat cholestasis, and it is also known to exert direct cytoprotective effects on hepatocytes [3, 4]. Researchers have found that UDCA can be used to inhibit the growth of tumors. Lim et al. [5] demonstrated that UDCA killed drug-resistant gastric cancer cells via the induction of autophagic death. Chung et al. [6] found that UDCA suppressed HCC cell growth through the inhibition of the degradation of DLC1, and Zhu et al. showed that UDCA induced apoptosis of HCC cells [7]. However, the mechanism by which UDCA inhibits tumors is not well defined.

Autophagy is an evolutionarily conserved process that functions in the degradation of long-lived proteins, aggregated proteins, damaged organelles and certain pathogens [8]. However, the functions of autophagy with respect to tumor cells are still controversial. Guo et al. [9] showed that the autophagy process was

**Table 1.** Primers used for qRT-PCR

Gene	Sequences
LC3B	Forward: 5'-AAACGCATTTGCCATCACACA-3' Reverse: 5'-GGACCTTCAGCAGTTTACAGTCAG-3'
p53	Forward: 5'-ACATGACGGAGGTTGTGAGG-3' Reverse: 5'-GTAGTGGATGGTGGTACAGTCAGAG-3'
$\beta$ -actin	Forward: 5'-TGGCACCCAGCACAATGAA-3' Reverse: 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'

required by activated Ras in order to maintain oxidative metabolism and tumorigenesis, while, Elgendy et al. [10] suggested that Ras-induced autophagy may kill tumor cells that are on the verge of oncogenic transformation. Lim and Han [5] demonstrated that ursodexocholic acid effectively killed drug-resistant gastric cancer cells through induction of autophagic death. In our research, we showed an incremental increase in the expression level of microtubule-associated protein light chain 3B (LC3B), which is an important regulator of autophagosomes. This demonstrates that UDCA may inhibit HCC through the induction of autophagy.

## Methods and materials

### Reagents

UDCA (from Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO) (from Amresco 0231) to achieve the final concentrations of 0.3, 0.6, 0.8, 1.0 and 1.2 mmol/l. UDCA at varied doses was used to treat the HCC cells for 24 h, 48 h or 72 h to identify its effect on cell viability. Cells treated with UDCA at 0.8 and 1.2 mmol/l were further investigated to determine the expression of autophagy associated factors.

UDCA was suspended in the drinking water of the mice at concentrations of 90 mg/kg/day and 270 mg/kg/day.

### Cell lines and culture

The human hepatoma cell line HepG2 was obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). SMMC-7221 (7721) cells were a generous gift from the Central Laboratory of Shandong Province Hospital (Shandong, China). Both cell lines were maintained in high-glucose Dulbecco's Modified Eagle's Medium (DMEM)

supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin G and 100  $\mu$ g/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

To passage the cells, the cell monolayer was rinsed briefly with 1×phosphate-buffered saline (PBS) twice, 1 ml pre-warmed (37°C) 0.05% Trypsin-EDTA solution was added and the cells were incubated with this solution for 3-7 minutes (different cells were incubated for different lengths of time). Once the cell layer was dispersed, Trypsin was deactivated by the addition of 2-3 ml complete growth medium. After all the cells detached, we centrifuged the cell suspension for 4 minutes (1000 rpm/min). The supernatant was discarded and the cell pellet was resuspended in an appropriate volume of medium for cell counting by Trypan Blue. The cells were split according to their confluence rate. The culture medium was changed once every two days.

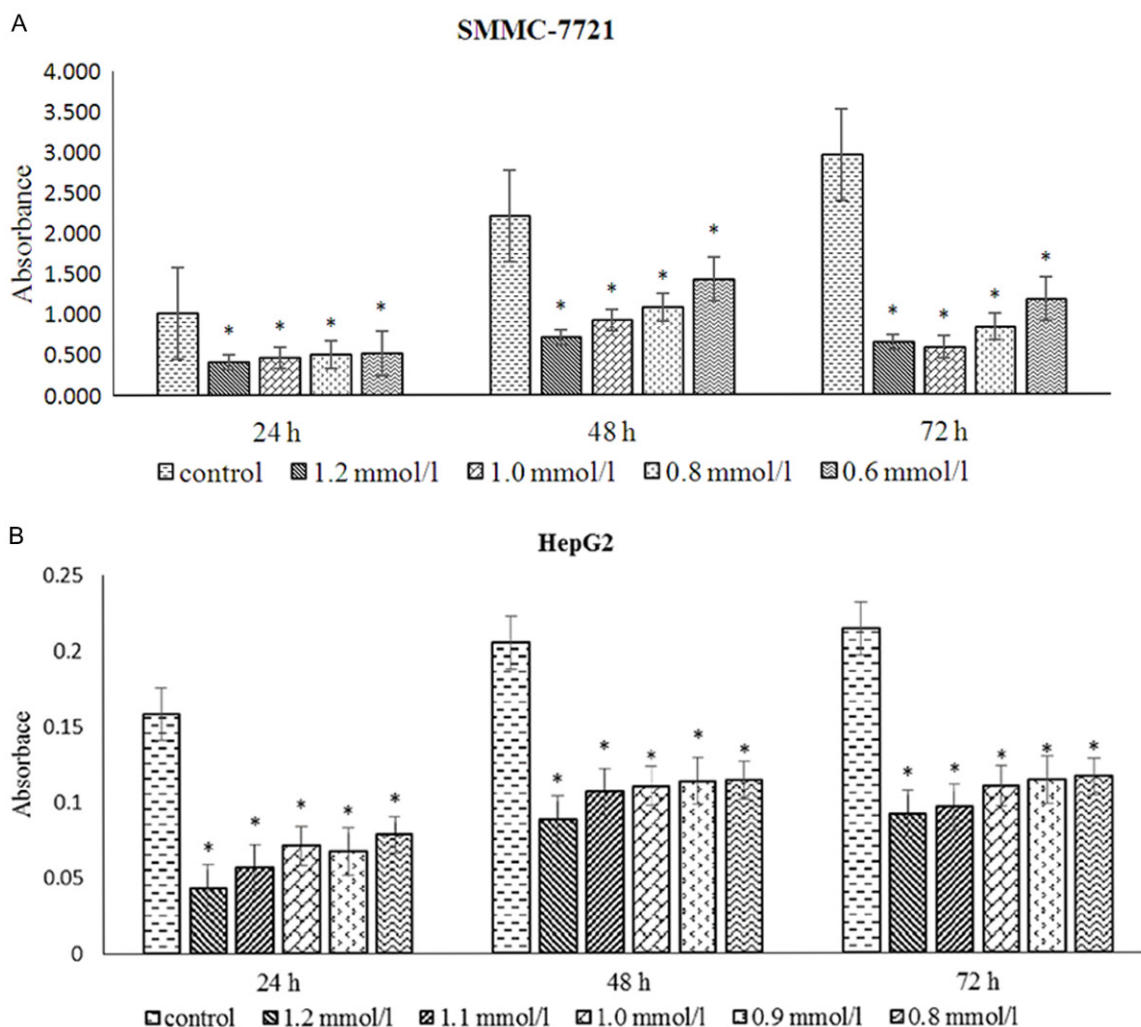
### Treatment of nude mice

In all, 5×10<sup>6</sup> cells (resuspended in 100  $\mu$ l of DMEM) were injected subcutaneously into the posterior axillary region of BALB/c (nu/nu) nude mice (Vital River, Beijing, China). Tumor tissues were excised from the nude mice after the tumors were of sufficient size. Tumor cells were obtained from tumor tissues by grinding. Approximately 2×10<sup>6</sup> cells (resuspended in 100  $\mu$ l of DMEM) were injected subcutaneously into the posterior axillary of BALB/c (nu/nu) nude mice. Three to 4 weeks later, the mice were assigned to different treatment groups. The mice were used in experiments 7 to 8 weeks after the inoculation of the tumor cells. UDCA was dissolved in the drinking water of the mice. Nude mice bearing tumors derived from 7721 cells received the water ad libitum. At the end of the experiment, the mice were sacrificed by cervical dislocation, and the tumors were excised. All animal care and experimentation conformed to the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health, and were approved by the animal ethics committee of Shandong University.

### Cell treatment for time-course and dose-response studies

7721 and HepG2 cells were grown in DMEM complete growth medium followed by a 24 h

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**Figure 1.** CCK-8 analysis of the reduction of cell viability after UDCA treatment at different time points and different concentrations of UDCA. A. UDCA treatment of 7721 cells. B. UDCA treatment of HepG2 cells. All results are expressed as the mean  $\pm$  S.D. from three independent experiments. \* $P < 0.05$  indicates a statistically significant difference compared with the control group.

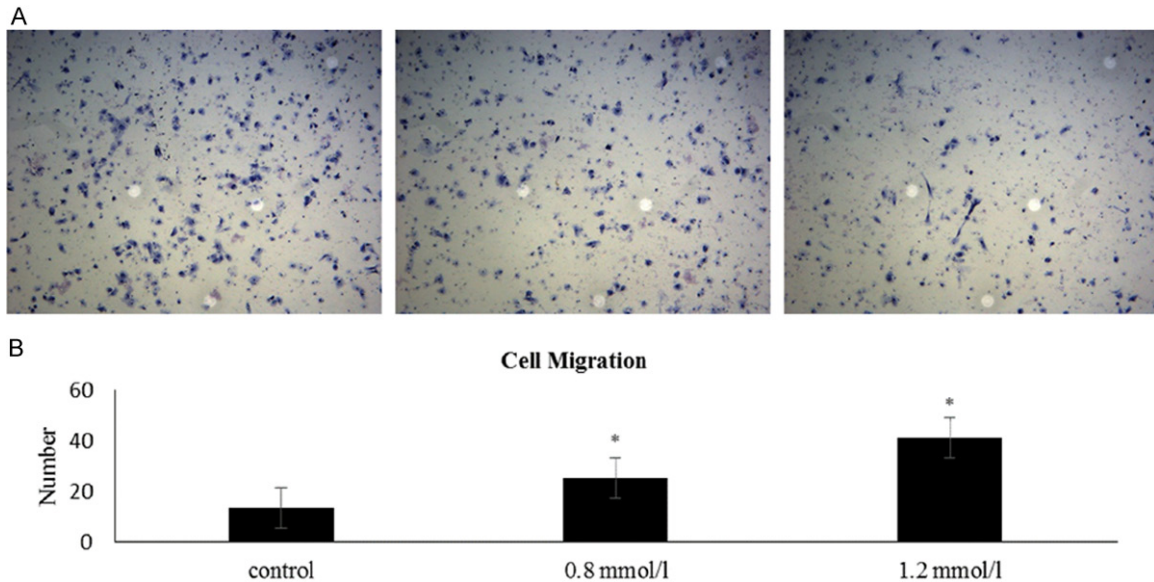
incubation to allow the cells to attach. Afterwards, the medium was withdrawn and replaced with fresh medium and UDCA (UDCA stock solution was dissolved in the medium). We detected the absorbance at 24 h, 48 h and 72 h after UDCA treatment using a Cell Counting Kit-8 (CCK-8) cell viability assay (Dojindo, Japan) according to the manufacturer's instructions.

### Cell migration assays

The migration of HCC cells was assessed using the 24-well polycarbonate membrane cell migration assay kit (Corning Incorporated Costar, Shanghai, China) according to the manufacturer's instructions. Briefly, the HCC cell lines

were incubated in serum-free medium for 24 h. The cells were then transferred to the upper chamber of a Transwell plate by seeding  $5 \times 10^5$  cells per well in 200  $\mu$ l serum-free medium with different concentrations UDCA. Next, 0.7 ml of 10% FBS containing medium was added to the lower chamber as a chemoattractant. The cells were then incubated for 24 h at 37°C. Non-migrating cells on the upper membrane surface were scraped off with cotton swabs. Cells that migrated to the bottom of the membrane were stained with 0.1% crystal violet for 30 min, which was, followed by washes in water for 30 s to remove residual dye. Four views were examined per Transwell plate and the cells/view were counted at 200 $\times$  magnification. Each experiment was performed in triplicate.

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**Figure 2.** UDCA inhibits the migration of 7721. A. The control group without UDCA (left). The group treated with 0.8 mmol/l UDCA (middle). The group treated with 1.2 mmol/l UDCA (right). B. Histogram of cell migration. All results are expressed as the mean  $\pm$  S.D. from three independent experiments. \* $P < 0.05$  indicates a statistically significant difference compared with the control group.

### Quantitative reverse transcription PCR (qRT-PCR)

Total RNA was isolated using TRIzol<sup>®</sup> Reagent (TaKaRa Bio Inc., Dalian, China) according to manufacturers' instructions. Subsequently, cDNA was generated using a PrimeScript<sup>™</sup> RT Reagent Kit with gDNA eraser (Takara Bio Inc., Dalian, China). RNA samples were extracted from both 7721 and HepG2 cells. cDNA was synthesized using a Superscript III First-Strand cDNA Synthesis kit (Takara Bio Inc., Dalian, China). qRT-PCR was performed using SYBR Green PCR Master Mix. The primers used in this experiment are shown in **Table 1**. The relative gene expression quantification method was used to calculate the fold changes in mRNA expression.

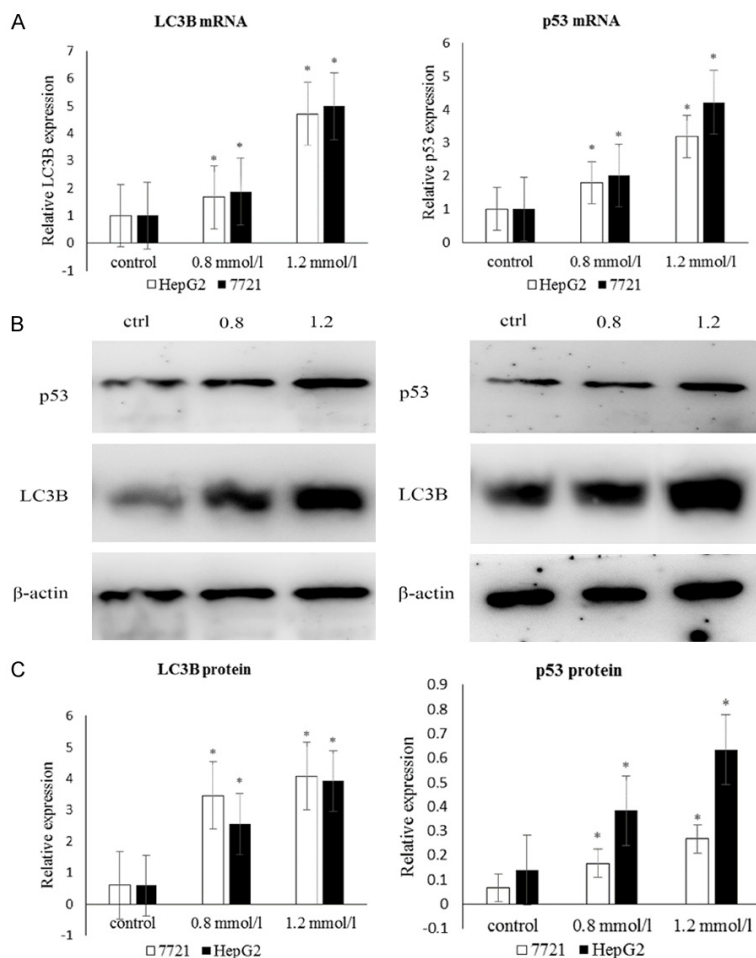
### Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

This protocol was approved by the Institutional Review Board of the Shandong Province Hospital, Shandong University, Shandong, China. Total protein was extracted from cells in RIPA lysis buffer (Beyotime, Shanghai, China) and was quantified by Bicinchoninic acid assay. In total, 30  $\mu$ g of protein was separated using 12% SDS-PAGE and then transferred to a poly-

vinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). The membrane was blocked in a 5% powdered milk solution and incubated with the primary antibody overnight at 4°C. After washing, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (ZSGB-BIO, Beijing, China) at 37°C for 1 h. Protein bands were visualized using Western Bright ECL (Thermo, Shanghai, China) and were detected using an Amersham Imager 600 (General Electric, USA). Antibodies included those against p53 (Cell Signaling Technology, Boston, MA, USA),  $\beta$ -actin (ZSGB-BIO, Beijing, China), and LC3B (Abcam, Shanghai, China).

### Immunohistochemistry (IHC)

This protocol was approved by the Institutional Review Board of Shandong Province Hospital, Shandong University, Shandong, China. The tumors were evaluated by immunohistochemical staining. Briefly, the tumor specimens were fixed in 4% paraformaldehyde for 24 h, after which they were embedded in paraffin for subsequent immunohistochemical examination of LC3B expression. After deparaffinization and rehydration in turpentine and alcohol, respectively, the slides were incubated with hydrogen peroxide to quench endogenous peroxidase activ-



**Figure 3.** UDCA improves the level of LC3B and p53 in 7721 and HepG2 cells. A. The mRNA expression levels of LC3B and p53 increased as the dosage of UDCA increased. The relative levels of LC3B and p53 were detected by qRT-PCR. B, C. The band and gray level analysis of protein expression of LC3B and p53. All results are expressed as the mean  $\pm$  S.D. from three independent experiments. \* $P < 0.05$  indicates a statistically significant difference compared with the control group.

ity. The tumor sections were then blocked with normal serum, and were treated sequentially with normal goat serum, anti-rabbit LC3B antibody (1:200), and biotin-labeled goat anti-rabbit IgG (1:400). Finally, the sections were dehydrated in alcohol and mounted with neutral resins.

#### Statistical analysis

Data were collected in an MS-Excel spreadsheet. Group comparisons for continuous data were made using Student's t-test for independent means, which was performed by IBM SPSS Statistics 22. All experiments were performed in triplicate. Statistical significance was set at  $P < 0.05$ .

## Results

### *UDCA reduces the viability of the primary HCC cell lines 7721 and HepG2*

To investigate the effect of UDCA on cell proliferation, in vitro studies were performed in 7721 and HepG2 cell lines.

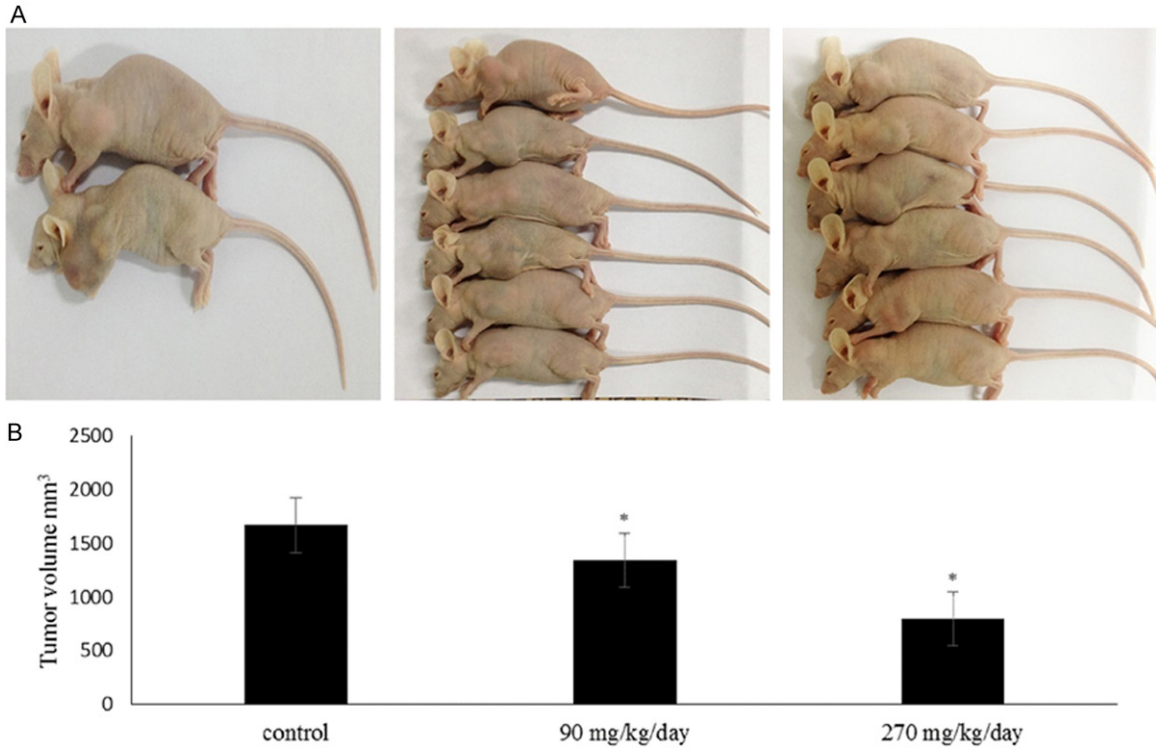
A CCK-8 assay is based on the measurement of activity of a marker associated with the number of viable cell. Our results showed different decreases in OD (450 nm) value in cells treated with UDCA (0.6, 0.8, 0.9, 1.0, 1.1, 1.2 mmol/l) for 24 h, 48 h and 72 h (**Figure 1**). A significant difference was observed between the control groups and the experimental groups of 7721 cells that were treated with different dosages UDCA at 24 h (Student's t-test,  $P < 0.05$ ). A statistically significant difference was also observed between the experimental groups and the control groups of 7721 cells 48 h/72 h after UDCA treatment (Student's t-test,  $P < 0.05$ ). The viability of HepG2 cells was significantly reduced after-treatment with UDCA compared with cells that were not treated with UDCA (Student's t-test,  $P < 0.05$ ).

compared with cells that were not treated with UDCA (Student's t-test,  $P < 0.05$ ).

### *UDCA inhibits the migration of 7721 cells*

According to the results of the CCK-8 cell viability assay, HepG2 cells treated with UDCA showed an obvious reduction in number at 24 h (Student's t-test,  $P < 0.05$ ), which would affect their migration. Although a statistically significant difference was observed in 7721 cells treated with UDCA at 24 h (Student's t-test,  $P < 0.05$ ), the change was very small. Therefore, 7721 cells were selected to test the effect of UDCA on cell migrations. UDCA significantly inhibited the migration of 7721 cells (Student's t-test,  $P < 0.05$ ). A significant reduction in migra-

## UDCA for HCC



**Figure 4.** UDCA inhibits in vivo growth of xenograft tumors. A. Images of xenografts from mice treated with UDCA (control group (left) n=2, 90 mg/kg/day (middle): n=6, 270 mg/kg/day (right): n=6). B. The tumor volumes were difference among the various groups. All results are expressed as the mean  $\pm$  S.D. from three independent experiments. \* $P < 0.05$  indicates a statistically significant difference compared with the control group.

tion was found when 7721 and HepG2 cells were treated with UDCA at 48 h and 72 h (Student's t-test,  $P < 0.05$ ), but we treated only 7721 cells with UDCA and assessed cell migration after 24 h. We observed that UDCA could inhibit the migration of 7721 cells, as shown in **Figure 2**. Finally, this inhibition was intensified as the dosage increased.

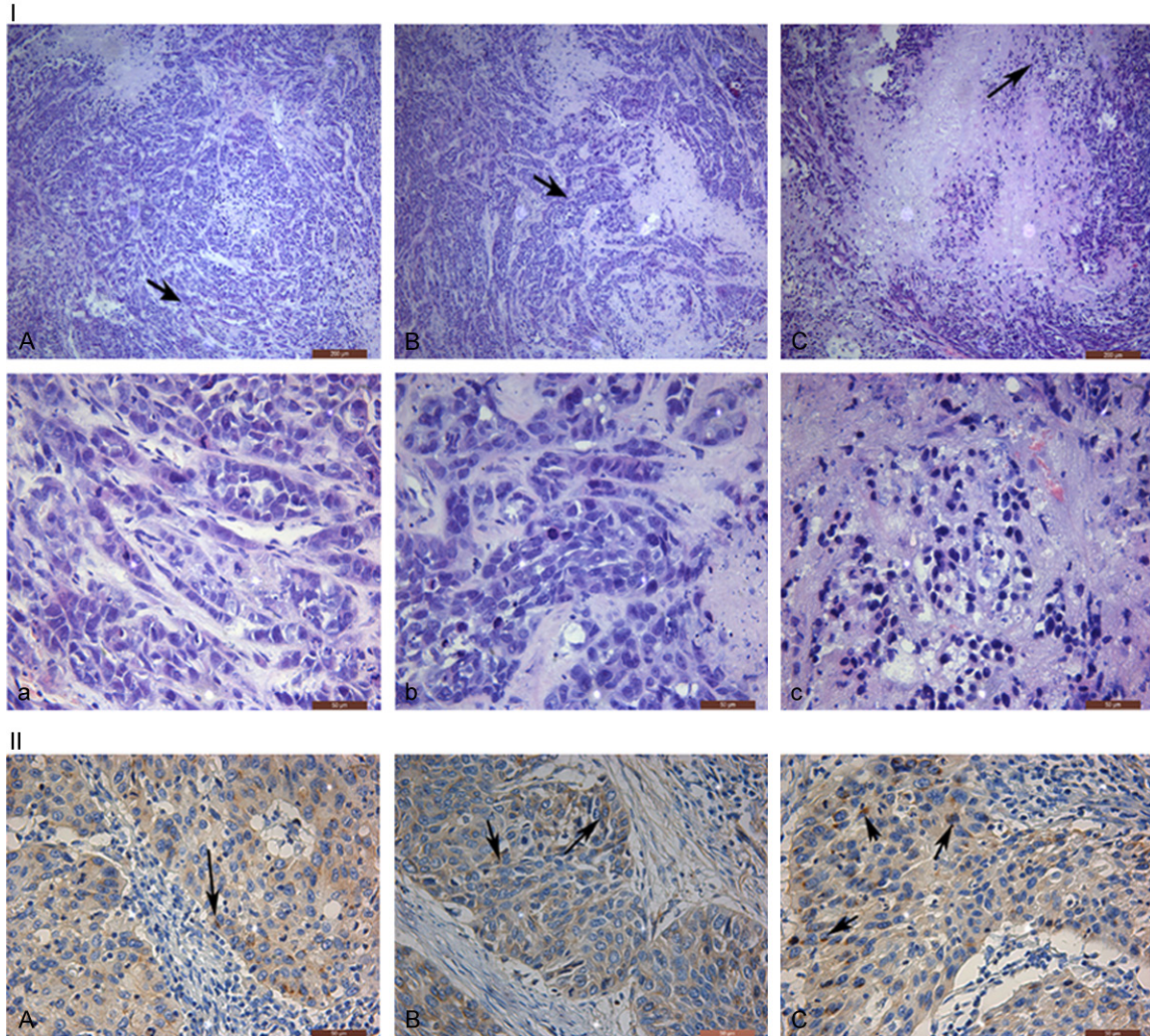
### *UDCA improves the level of LC3B and p53 in vitro*

We used 0.8 and 1.2 mmol/l UDCA to treat 7721 and HepG2 cells. As revealed by qRT-PCR, LC3B mRNA levels were significantly up-regulated in the HCC cell lines 7721 and HepG2 (Student's t-test,  $P < 0.05$ ), and their mRNA level increased as the dosage increased. The western blot analysis showed the same changes. The expression of LC3B protein increased as the dosage of UDCA increased, and this change in expression was also statistically significant (Student's t-test,  $P < 0.05$ ). In order to investigate the reasons for the increase in LC3B protein, we examined whether other proteins were

involved in UDCA-induced autophagy. We then found that the levels of p53 mRNA and protein were greatly increased in 7721 and HepG2 cells, upon treatment with UDCA. Additionally, these differences in expression were statistically significant (Student's t-test,  $P < 0.05$ ). The results are shown in **Figure 3**.

### *UDCA inhibits tumor growth and promotes the expression of LC3B in nude mice*

To determine whether we were able to recapitulate the molecular events we observed in 7721 and HepG2 cells, an HCC xenograft model was established in BALB/c nude mice. BALB/c nude mice that were inoculated with 7721 cells were treated with UDCA suspended in the drinking water. We monitored the tumor growth over an 8-week period. As shown in **Figure 4**, 8 weeks after transplantation, tumor growth in nude mice that were treated with UDCA was significantly slower than that in control mice that were not treated with UDCA (Student's t-test,  $P < 0.05$ ). Hematoxylin-eosin staining of tumors from nude mice showed that the percentage of



**Figure 5.** I. Hematoxylin-eosin staining of tumor sections obtained from nude mice. A. Xenograft tumors from mice treated with 270 mg/kg/day UDCA. B. Xenograft tumors from mice treated with 90 mg/kg/day UDCA. C. Control group without UDCA. A-C. Low ( $\times 200$ ) magnifications. a-c. High ( $\times 400$ ) magnifications (a-c each show amplified areas indicated by the arrows in A-C). II. IHC staining of paraffin-embedded xenograft tumors. A. Control group without UDCA treatment. B. Xenograft tumors from mice that were treated with 90 mg/kg/day UDCA. C. Xenograft tumors from mice that were treated with 270 mg/kg/day UDCA. The arrow indicates LC3B staining.

cells that underwent cell death increased as the dosages of UDCA increased (**Figure 5I**). IHC staining (**Figure 5II**) of sections from paraffin-embedded xenograft tumors showed an increase in LC3B expression in tumors from mice treated with UDCA.

### Discussion

Autophagy is a type of self degradative and autophagosome lysosome fusion. Autophagy is characterized by a set of autophagy-related (ATG) proteins [13], such as, Beclin-1 [14] and a lipid-conjugated form of microtubule-associat-

ed protein 1 light chain 3 (LC3) [15]. This process primarily acts as a pro-survival mechanism to overcome stressful conditions, but it ultimately commits the cell to death during prolonged states of stress or in severely stressful environments [16]. LC3 has three isoforms (LC3A, LC3B and LC3C) [17]. LC3B is used as a marker of autophagy, because it is the key regulator of autophagosome formation. Lim et al. [5] found that UDCA could increase the ATG5 level to induce autophagy. However, since no studies have been conducted on the relationship of UDCA and LC3B, we examined this relationship in current study.

Although UDCA is widely used to treat hepatobiliary diseases, many studies have shown that it has antitumor effects. Tsagarakis et al. [18] found that UDCA exerted a concentration-dependent effect on apoptosis of HepG2 cells. Tarao K et al. [19] considered that UDCA may prevent the development of HCC in individuals with hepatitis C virus-associated liver cirrhosis.

In the current study, we demonstrated that UDCA reduced the viability of HCC cells in both a dose-dependent and a time-dependent manner. In addition, UDCA could suppress cell migration. Both of these results demonstrate that UDCA can inhibit HCC.

To test the mechanism through which UDCA inhibits HCC, we examined the relationship between UDCA and LC3B. Our research showed that UDCA could increase the levels of LC3B mRNA and protein both in vivo and in vitro.

Rosenfeldt et al. [20] demonstrated that p53 status determined the role of autophagy in the development of pancreatic tumors. They found that p53 played an important role in autophagy. Therefore, we studied the changes in p53 expression in 7721 and HepG2 cells upon treatment with UDCA. We found that the expression of p53 increased with UDCA treatment, which suggests that UDCA promotes the expression of LC3B via an increase in the expression of p53.

All of the results discussed above indicated that UDCA plays an important role in autophagy in HCC cell lines and that UDCA may be used to treat or prevent HCC.

Future studies will involve verification of the relationship of LC3B and p53. We also plan to test other mechanisms of autophagy in the UDCA-mediated inhibition of HCC. For example, Du et al. [21] demonstrated that microRNA-182 was up-regulated in HCC tissues. We could therefore study the relationship between microRNA-182 and UDCA.

## Conclusion

In conclusion, our results showed that LC3B is frequently up-regulated in HCC cell lines and tumor xenografts of nude mice treated with UDCA. This may provide a direction for future studies on the molecular mechanisms of UDCA on HCC. Finally, UDCA is a potential drug candidate for HCC.

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## Disclosure of conflict of interest

None.

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