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Xiaochaihu decoction induces Bel-7402/5-FU cell apoptosis and autophagy via PI3K/AKT/mTOR pathway

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ABSTRACT

Objective: This study aimed to observe the inhibitory effects of xiaochaihu decoction (XCHD) on human hepatocellular carcinoma (HCC) cells resistant to 5-fluorouracil (5-FU) (Bel-7402/5-FU) *in vitro* and *in vivo* and investigate its possible mechanisms.

Methods: Bel-7402 cells and their resistant cells to 5-FU (Bel-7402/5-FU) were cultured, and a xenograft was established in nude mice. MTT assays were used to detect the cell viability after XCHD treatment, and an inverted phase contrast microscope was used to observe the morphology and flow cytometry and TUNEL assays were used to determine XCHD-induced apoptosis. Western blot was used to detect Bax and Bcl-2 expressions. Cyto-ID staining was used to assess XCHD-induced autophagy, and the autophagy-related protein (LC3, p62, and beclin) was determined. Finally, the PI3K/AKT/mTOR pathway was detected.

Results: Bel-7402/5-FU cells were more resistant to 5-FU compared with Bel-7402 cells ($P < 0.05$), thus XCHD could inhibit the viability of Bel-7402/5-FU cells. Further, XCHD promoted Bel-7402/5-FU cell apoptosis via inducing Bax expression and deducing Bcl-2 expression *in vitro* and *in vivo*. Similarly, XCHD promoted autophagy of Bel-7402/5-FU cells by regulating related protein expression. Finally, XCHD blocked the PI3K/AKT/mTOR pathway.

Conclusion: XCHD induces Bel-7402/5-FU cell apoptosis and autophagy via blocking the PI3K/AKT/mTOR pathway which is one of the important mechanisms by which XCHD reverses the multidrug resistance of HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies globally, with increasing morbidity and associated mortality.[1,2] However, multidrug resistance (MDR) as one of the major challenges in HCC treatment limits chemotherapy drug efficacy.[3] Therefore, developing new therapeutic methods and drugs to overcome MDR is crucial for treating patients with HCC.

Xiaochaihu decoction (XCHD), which is a traditional Chinese medicine (TCM) formula, consists of Bupleuri Radix (Chai-Hu), PinelliaeRhizoma (Ban-Xia), Scutellariae Radix (Huang-Qin), Jujubae Fructus (Da-Zao), Ginseng Radix Et Rhizoma (Ren-Shen), Ginseng Radix Et Rhizoma (Gan-Cao), and Zingiberis Rhizoma (Sheng-Jiang). XCHD was widely used in TCM for liver disease treatment.[4–6] Previous studies revealed that XCHD can prevent HCC by inhibiting oxidative stress.[7] The mechanism by which XCHD regulates MDR in HCC is not fully understood. Therefore, this study aimed to observe the mechanism of action of XCHD as a potential therapeutic agent to overcome MDR in HCC and explore its mechanism through *in vitro* and *in vivo* experiments.

2. Materials and methods

2.1. Cell lines and reagents

KeyGene Biotech Co., Ltd. (Nanjing, China) provided Bel-7402 cells (KG022) and Bel-7402/5-fluorouracil (5-FU) cells (KG125). Invitrogen (Carlsbad, CA, USA) provided Roswell Park Memorial Institute (RPMI) 1640 (C11875500BT), fetal bovine serum (FBS, 10099141), penicillin–streptomycin (15140122), trypsin-ethylenediaminetetraacetic acid (25200–072). Becton Dickinson (San Jose, CA, USA) provided an Annexin V-FITC/propidium iodide (PI) apoptosis detection kit. Enzo Life Sciences, Inc (Farmingdale, ME, USA) provided the Cyto-ID autophagy

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detection kit (ENZ-51031-K200). Takara Bio Inc. (Dalian, China) provided the RNAiso for RNA (9109) and PrimeScript™ 1st Strand cDNA Synthesis Kit (6110B). Cell Signaling Technology (Danvers, MA, USA) provided PI3K (60225-1), AKT (60203-2), *p*-AKT (66444-1), mTOR (2983), and *p*-mTOR (5536). Proteintech (Rosemount, IL, USA) provided Bax (50599-2), Bcl-2 (12789-1), LC3 (14600-1-AP), p62 (18420-1-AP), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (60004), β-actin (66009-1-Ig), and beclin (66665-1-Ig).

2.2. Cell culture

Bel-7402 cells were cultured in RPMI 1640 medium supplemented with 10% (*v/v*) FBS, 100 Units/mL of penicillin, and 100 μg/mL of streptomycin at 37 °C humidified incubator with 5% CO_2 and 95% air. Bel-7402/5-FU cells were cultured in the above-mentioned medium with the addition of 20 μg/mL of 5-FU.

2.3. Animals

The Model Animal Center of Fujian University of TCM provided 20 male mice (BALB/c-nu, 20–22 g) which were housed in a special pathogen-free animal facility where the mice were allowed to eat and drink ad libitum. All of the experimental procedures were performed under the protocols approved by the Institutional Animal Care and the Experimental Animal Ethics Committee of Fujian University of TCM (approval number: FJTCM IACUC 2019083).

2.4. Xenograft model and treatment

Twenty mice were randomly selected and subcutaneously injected with 4×10^7 Bel-7402/5-FU cells in the left flank. Subsequently, mice were randomly divided into four groups, including the control, 5-FU (20 mg/kg), combination (5-FU + XCHD), and XCHD (14.2 g/kg). When tumor size reached 100 mm^3, 5-FU was intraperitoneally injected once every 3 days, XCHD was gavaged for 6 days every week, and the control group was given the same physiological saline for 3 weeks. Vernier calipers were used to record tumor volumes every 2 days using the following formula:

$$V = \pi\ (\text{length} \times \text{width}^2)/6$$

The animals were euthanized after the end of the treatment period at 24 h, and the tumors were removed and weighed rapidly. Tumors were collected after weighing and are frozen at −80 °C or preserved with paraformaldehyde for subsequent study.

2.5. MTT assay

Bel-7402 cells and Bel-7402/5-FU cells were respectively seeded into 96-well plates at a density of 5×10^5 cells per well and incubated with various concentrations of 5-FU or XCHD for 48 h. Each well was added with 100 μL of MTT solution (0.5 mg/mL)solution and incubated at 37 °C for 4 h. Then, 100 μL of dimethyl sulfoxide was added to each well, and the optical density was read at 570 nm of the microplate reader.

2.6. Observation of morphologic changes

Bel-7402/5-FU cells were seeded into 6-well plates at a density of 2×10^5 cells per well. The morphological changes of cells were observed by phase contrast microscope (Olympus, Japan) 48 h after intervention with different XCHD concentrations (0, 0.25, 0.5, and 1.0 mg/mL).

2.7. Hoechst 33258 staining

Bel-7402/5-FU cells were treated with different XCHD concentrations for 48 h, then washed with PBS 3 times and fixed with 4% paraformaldehyde for 10 min, then washed with PBS 3 times. Finally, 1 mL of Hoechst 33258 staining solution was added for 5 min at 37 °C in the dark and imaged with a fluorescence microscope (LEICA, German).

2.8. Reverse-transcription-polymerase chain reaction (RT-PCR)

The total RNA was extracted using the RNAiso for RNA (9109) reagent and PrimeScript™ 1st Strand cDNA Synthesis Kit (6110B) was used to reverse-transcribe total RNA into cDNA (Takara, Dalian, China). The obtained cDNA was used to determine the mRNA amount of Bcl-2 or Bax by PCR. GAPDH was used as an internal control. The following primers were used for the PCR. Bcl-2 forward: 5′-CAG CTG CAC CTG ACG CCC TT-3′ and reverse: 5′-GCC TCC GTT ATC CTG GAT CC-3′; Bax forward: 5′-TGCTTC AGG GTT TCA TCC AGG-3′ and reverse: 5′-TGG CAAAGT AGA AAA GGG CGA-3′; GAPDH forward: 5′-GT CATCCA TGA CAA CTT TGG-3′ and reverse: 5′-GA GCT TGACAA AGT GGT CGT-3′. The reaction condition was as follows: 94 °C for 10 min, 30 cycles of 94 °C for 40 s, 55 °C for 40 s, 72 °C for 40 s, and 72 °C for 10 min.

2.9. Detection of apoptosis

The Annexin V-fluorescein isothiocyanate (FITC)/PI apoptosis detection kit was used. In brief, Bel-7402/5-FU cells were plated in six-well plates at the density of 2×10^5 cells per well and treated with XCHD (0, 0.25, 0.5, and 1.0 mg/mL) for 48 h, and 1×10^6 cells were collected and washed twice with cold PBS and resuspended in 500 μL of 1 × binding buffer. Annexin V-FITC (5 μL) and PI (5 μL) were added to the suspension and incubated for an additional 15 min in the dark. Each analysis collected and analyzed 10000 events.

2.10. Cyto-ID autophagy detection

Bel-7402/5-FU cells were treated with XCHD (0, 0.25, 0.5, and 1.0 mg/mL) for 48 h, and the cells were collected and resuspended in 0.5 mL of freshly diluted Cyto-ID green detection reagent (1 μL of Cyto-ID green detection reagent to a final volume of 2 mL of RPMI-1640 medium). The cells were determined by flow cytometry after incubation for 30 min at 37 °C in the dark.

2.11. Western blot analysis

The proteins of Bel-7402/5-FU cells or tissues were extracted using radioimmunoassay buffer with an inhibitor cocktail (Thermo Fisher Scientific, USA), and determined the protein concentrations by BCA protein assay kit. Proteins (30 μg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred to PVDF membranes, followed by incubation with 5% milk and with primary antibodies (1:1000) at 4 °C overnight. The membranes were incubated with the corresponding secondary antibodies (HRP-conjugated 1:5000) for 1 h at room temperature. Finally, the Image Lab (Bio-Rad Laboratories, Inc., Berkley, California, USA) was used to detect proteins, and three independent experiments were performed to obtain representative data.

2.12. TUNEL assay

Tumor tissues were fixed in 10% formaldehyde for 48 h, paraffin-embedded, and then sectioned into 4-μm-thick slides. TUNEL staining was used for sample analysis. Apoptotic cells were counted as DAB-positive cells (brown staining) in five arbitrarily selected microscopic fields at a magnification of 400×. TUNEL-positive cells were counted as a percentage of the total cells.

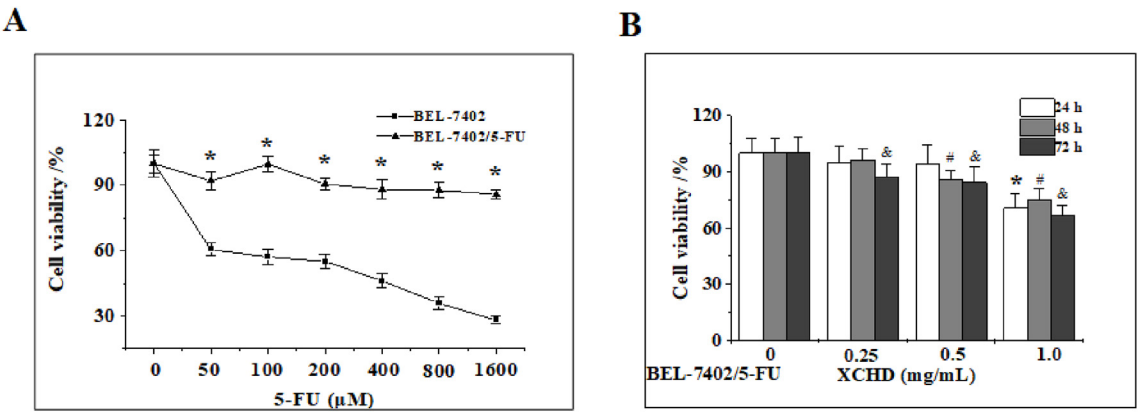

Fig. 1. Effects of XCHD on the viability of Bel-7402/5-FU cells ($n = 8$).
A. The cell viability of Bel-7402 cells and Bel-7402/5-FU cells were incubated with various 5-FU concentrations for 48h; B. The cell viability of Bel-7402/5-FU cells after the XCHD intervention were determined by the MTT assay. $^{*}P < 0.05$, compared with the control group for 24h, $^{\#}P < 0.05$, compared with the control group for 48h, $^{\&}P < 0.05$, compared with the control group for 72 h.

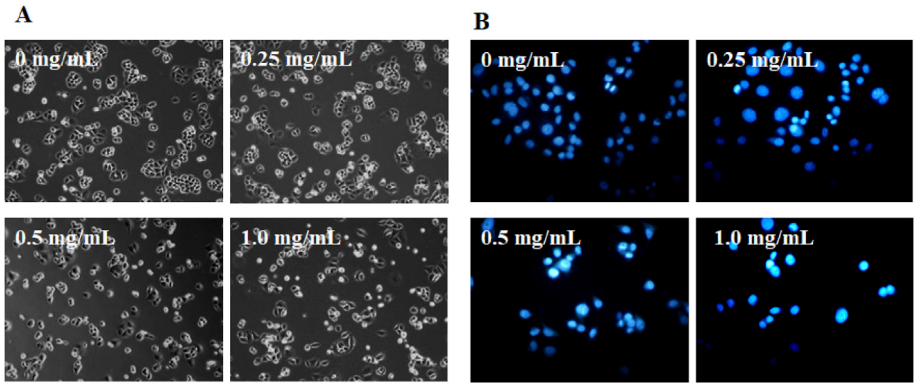

Fig. 2. Effects of XCHD on Bel-7402/5-FU cell morphology and apoptosis ($n = 3$).
A. The morphological changes of Bel-7402/5-FU cells after treatment with different concentrations of XCHD for 48 h were observed by phase contrast microscopy, 100 × ; B. The apoptosis of Bel-7402/5-FU cells was determined by Hoechst 33258 staining, 200 × .

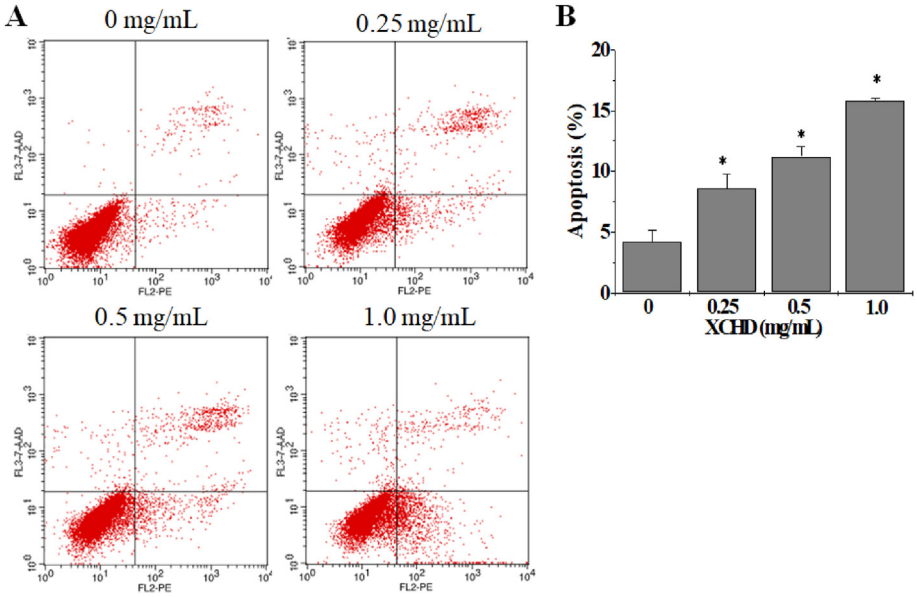

Fig. 3. Effects of XCHD on Bel-7402/5-FU cell apoptosis ($n = 3$).
A. Representative figure of Flow Cytometry (FCM); B. Statistic analysis. $^{*}P < 0.05$, compared with the control group.

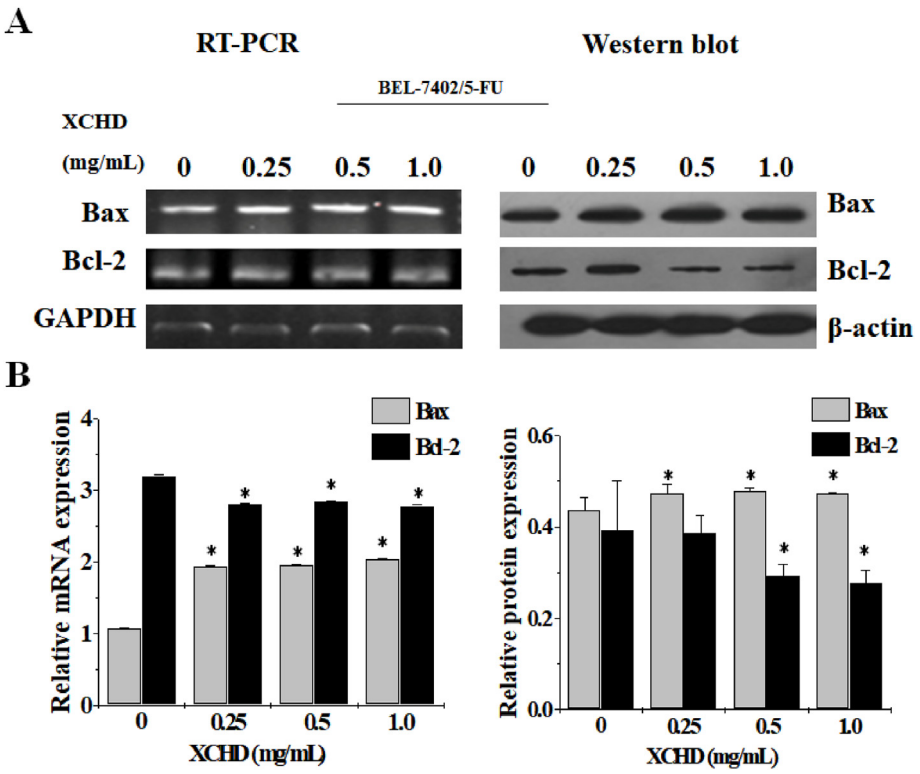

Fig. 4. Effects of XCHD on the mRNA and protein expression of Bax and Blc-2 *in vitro* ($n = 3$).
A. Representative figure of RT-PCR and Western blot; B. Statistical analysis of RT-PCR and Western blot. *$P < 0.05$, compared with the control group.

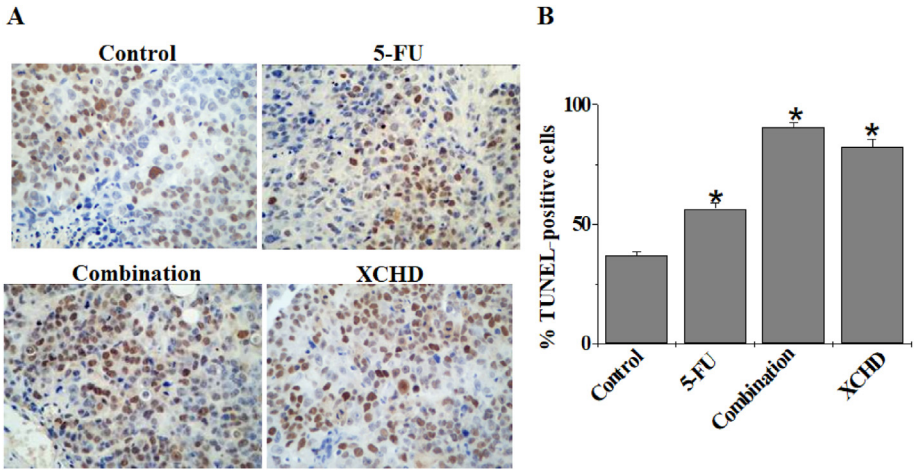

Fig. 5. Effects of XCHD on tumor tissue apoptosis ($n = 3$).
A. Representative figure of TUNEL assay; B. Statistic analysis *$P < 0.05$, compared with the control group.

2.13. Statistical analysis

LSD or Tamhane's T2 tests were used when appropriate for comparisons between experimental groups or experiments. A *P*-value of<0.05 was regarded as statistically significant for both tests. All the statistical analyses were performed using Statistical Package for the Social Sciences version 19.0.

3. Results and discussion

3.1. XCHD inhibited the growth of Bel-7402/5-FU cells

The cell viability of Bel-7402/5-FU cells was significantly higher than BEL-7402 cells after 48 h of 5-FU intervention, indicating that Bel-7402/5-FU cells have stronger drug resistance (Fig. 1A). Moreover, treatment with 0.25–1.0 mg/mL of XCHD for 24, 48, or 72 h reduced the viability of Bel-7402/5-FU cells by 5.2%–12.7%, 6.2%–15.7%, and 29.5%–33.5% ($P < 0.05$) compared to untreated controls, respectively. These indicate that XCHD inhibited the growth of Bel-7402/5-FU cells in both dose- and time-dependent manners (Fig. 1B). We evaluated the effect of XCHD on Bel-7402/5-FU cell morphology using phase contrast microscopy to verify these results. Untreated Bel-7402/5-FU cells appeared as densely packed and disorganized multilayers, as shown in Fig. 2A. In contrast, many of the XCHD-treated cells were rounded, shrunken, and detached from adjacent cells, or floating in the medium. Taken together, these data demonstrate that XCHD inhibited the

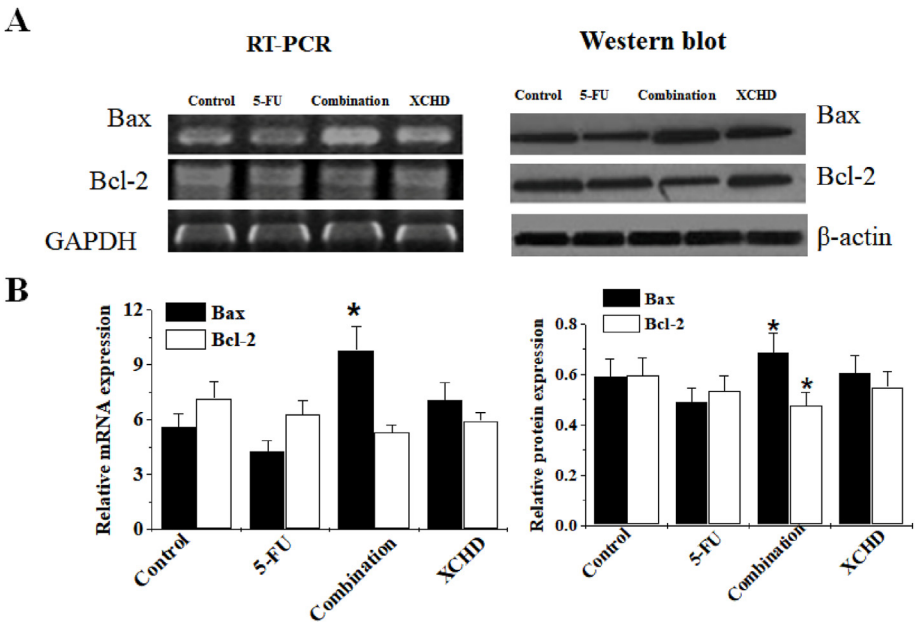

Fig. 6. Effects of XCHD on the mRNA and protein expression of Bax and Blc-2 *in vivo* ($n = 3$).
A. Representative figure of RT-PCR and Western blot; B. Statistic analysis. *$P < 0.05$, compared with the control group.

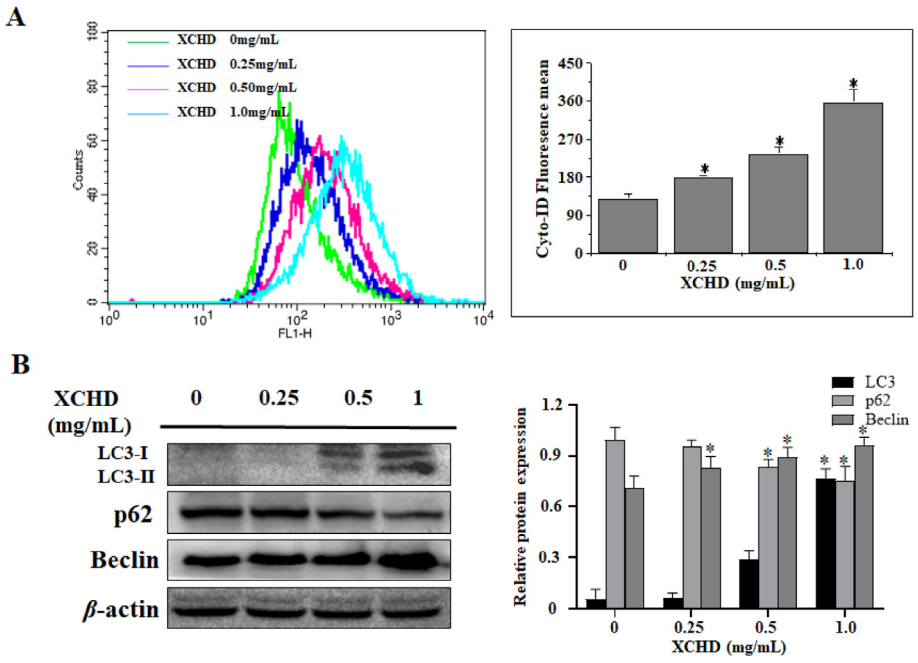

Fig. 7. Effects of XCHD on Bel-7402/5-FU cell autophagy ($n = 3$).
A. Cyto-ID staining and statistical analysis; B. Autophagy-related protein expression and its statistical analysis. *$P < 0.05$, compared with the control group.

growth of Bel-7402/5-FU cells.

3.2. XCHD-induced Bel-7402/5-FU cell apoptosis in vitro and in vivo

Apoptosis is the main mechanism of cancer cell death.[8] Hoechst 33258 staining were performed to investigate the effects of XCHD on apoptosis of Bel-7402/5-FU cells. XCHD increased Bel-7402/5-FU cell apoptosis as the nuclear bright staining cells decreased, as shown in Fig. 2B. Moreover, we measured the apoptotic cell ratio through Annexin V-FITC/PI staining with flow cytometry as shown in Fig. 3, and the results demonstrated the XCHD increased the Bel-7402/5-FU cell apoptotic rate ($P < 0.05$). The Bcl-2 family plays an important role in apoptosis during cancer development (including MDR).[9] The apoptotic biomarkers Bax and Bcl-2 were detected by RT-PCR and Western blot, respectively. The results revealed that XCHD treatment elevated Bax expression, while suppressed Bcl-2 expression (Fig. 4).

Moreover, we further explore the effect of XCHD on Bel-7402/5-FU cell apoptosis in xenograft mice. The results of the TUNEL assay

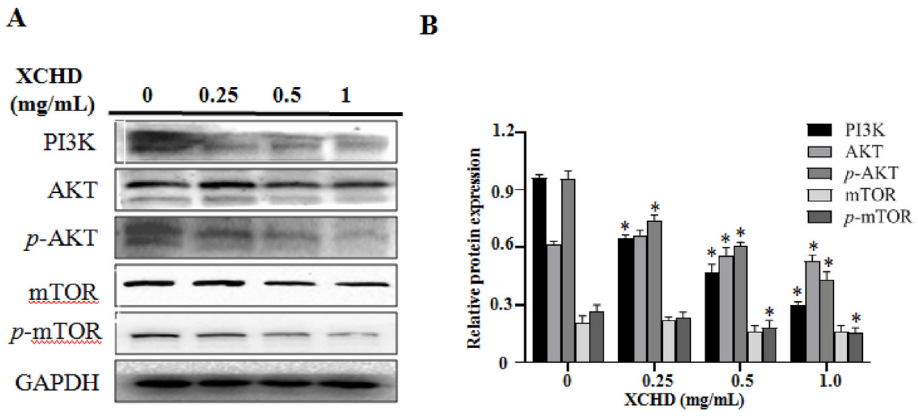

Fig. 8. Effects of XCHD on the PI3K/AKT/mTOR pathway ($n = 3$).
A. Representative figure of Western blot; B. Statistic analysis. *$P < 0.05$, compared with the control group.

revealed that XCHD induced apoptosis in xenograft tissues, and the combination of XCHD and 5-FU is more effective in apoptosis (Fig. 5). Similarly, Bax and Bcl-2 expression coincides with the results *in vitro* (Fig. 6). Altogether, XCHD-induced Bel-7402/5-FU cell apoptosis *in vitro* and *in vivo*.

3.3. XCHD promoted Bel-7402/5-FU cell autophagy

Autophagy plays an important role in tumor occurrence and deterioration, MDR generation, and metastasis recurrence.[10–13] Autophagy can promote oncogene degradation and apoptosis, maintain genome stability and intracellular protein homeostasis, and form "autophagy death" after radiotherapy and chemotherapy to control tumor progression and metastasis.[14–16] Here, Bel-7402/5-FU cell autophagy was detected by Cyto-ID autophagy detection kit. The excitable green fluorescence detection reagent fluoresces brightly when mixed into autophagy precursor and autophagosome. The results revealed that XCHD promoted Bel-7402/5-FU cell autophagy (Fig. 7A). Moreover, the autophagy-related protein was determined. The results revealed that XCHD up-regulated LC-II and Beclin expression, but down-regulated p62 expression (Fig. 7B).

3.4. XCHD blocked the PI3K/AKT/mTOR pathway

One of the most common signaling pathways in tumor cells is PI3K/AKT signaling pathway, which is involved in regulating cell proliferation, growth, survival, migration, and metabolism.[17–20] It shows abnormally high expression in a variety of tumor cells and is a key pathway related to MDR.[21] XCHD-induced phosphorylation of AKT and mTOR, indicating that XCHD prevented the growth of Bel-7402/5-FU cells by blocking the PI3K/AKT/mTOR pathway, as shown in Fig. 8.

4. Conclusion

In conclusion, XCHD induces Bel-7402/5-FU cell apoptosis and autophagy by regulating related proteins via blocking the PI3K/AKT/mTOR pathway, which is proposed to be one of the important mechanisms by which XCHD reverses the MDR of HCC.

Author contributions statement

ZHAO Jinyan: Conceptualization, Methodology, Software. ZHANG Xuejun: Data curation, Writing-Original draft preparation. CHEN Shilan: Visualization, Investigation. WANG Xuejiao: Supervision. PENG Jiao: Software, Validation. LIN Jiumao: Writing-Reviewing and Editing.

Declaration of competing interest

All authors have no conflict of interest to declare.

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