

Curcumin enhances the antitumor effect of ABT-737 via activation of the ROS-ASK1-JNK pathway in hepatocellular carcinoma cells

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Abstract. At present, the therapeutic treatment strategies for patients with hepatocellular carcinoma (HCC) remain unsatisfactory, and novel methods are urgently required to treat this disease. Members of the B cell lymphoma (Bcl)-2 family are anti-apoptotic proteins, which are commonly expressed at high levels in certain HCC tissues and positively correlate with the treatment resistance of patients with HCC. ABT-737, an inhibitor of Bcl-2 anti-apoptotic proteins, has been demonstrated to exhibit potent antitumor effects in several types of tumor, including HCC. However, treatment with ABT-737 alone also activates certain pro-survival signaling pathways, which attenuate the antitumor validity of ABT-737. Curcumin, which is obtained from *Curcuma longa*, is also an antitumor potentiator in multiple types of cancer. In the present study, the synergistic effect of curcumin and ABT-737 on HCC cells was investigated for the first time, to the best of our knowledge. It was found that curcumin markedly enhanced the antitumor effects of ABT-737 on HepG2 cells, which was partially dependent on the induction of apoptosis, according to western blot analysis and flow cytometric apoptosis analysis. In addition, the sustained activation of the ROS-ASK1-c-Jun N-terminal kinase pathway may be an important mediator of the synergistic effect of curcumin and ABT-737. Collectively, these results indicated that the combination of curcumin and

ABT-737 can efficaciously induce the death of HCC cells, and may offer a potential treatment strategy for patients with HCC.

Introduction

Hepatocellular carcinoma (HCC) is a common type of cancer worldwide, and is the third most common cause of cancer-associated mortality (1). The therapeutic effects of current treatment strategies for patients with HCC remain unsatisfactory, thus it is important to develop novel methods of treatment for this disease (2,3).

B cell lymphoma (Bcl)-2, Bcl-extra large (xL) and Bcl-2-like-2 (w) are the anti-apoptotic protein members of the Bcl-2 family in mammalian cells, which contain four BH domains (BH1, BH2, BH3 and BH4) (4). The Bcl-2 anti-apoptotic proteins have been reported to prevent mitochondrial outer membrane permeabilization and repress apoptosis under stress (5). The expression of Bcl-2 anti-apoptotic proteins is often increased in numerous types of tumor tissue, which is commonly associated with treatment resistance (5,6). Previous studies have found that the levels of Bcl-2 anti-apoptotic proteins are closely associated with the pathological grade and survival rate of patients with HCC (7-9). Therefore, the targeting of Bcl-2 anti-apoptotic proteins may be a candidate for the treatment of patients with HCC. ABT-737 is an inhibitor of Bcl-2, Bcl-xL and Bcl-w, which is currently in phase I clinical trials for patients with leukemia (10-12). It has been previously reported that ABT-737 also has antitumor effects in HCC cell lines (13-17). However, certain pro-survival signaling pathways in HCC cells are often activated upon ABT-737 treatment, which attenuates the antitumor effect of ABT-737 (13,16,17). Therefore, novel treatment strategies require investigation in order to improve the efficacy of ABT-737.

Curcumin, also known as diferuloylmethane, is obtained from *Curcuma longa*, and is regarded as a potent anticancer drug in various types of tumor, including HCC (18-21). Previous studies have demonstrated that curcumin is able to

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effectively inhibit the proliferation of HCC cells *in vitro* and *in vivo* (22-25). In addition, curcumin significantly enhances the antitumor effects of certain traditional chemotherapeutic drugs and molecular-targeted drugs (26-32). However, the synergistic effect of curcumin and ABT-737 remains to be fully elucidated.

The present study aimed to investigate the antitumor effects of combination therapy of ABT-737 with curcumin on HepG2 cells. Whether curcumin enhances the antitumor effect of ABT-737 via the induction of apoptosis in HepG2 cells was investigated, and the potential involvement of the reactive oxygen species (ROS)-apoptosis signal-regulating kinase 1 (ASK1)-c-Jun N-terminal kinase (JNK) pathway was examined.

Materials and methods

Reagents. HyClone Dulbecco's modified Eagle's medium (DMEM) nutrient mixture and HyClone fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). ABT-737, SP600125 and *N*-acetyl-L-cysteine (NAC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). An Annexin V-Fluorescein Isothiocyanate (FITC)/Propidium Iodide (PI) Apoptosis Detection kit was purchased from Beyotime Institute of Biotechnology (Beijing, China). Cell Counting Kit-8 (CCK-8) reagent was obtained from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Invitrogen Lipofectamine 2000 was obtained from Thermo Fisher Scientific, Inc. Curcumin was purchased from Sigma-Aldrich and dissolved in dimethylsulfoxide (Sigma-Aldrich) to prepare a stock solution, which was used for treating HepG2 cells. Antibodies against Bcl-2 (cat. no. sc-25780), poly(ADP ribose) polymerase 1 (PARP-1; cat. no. sc-25780) and caspase-3 (cat. no. sc-7148) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Rabbit polyclonal antibodies against myeloid cell leukemia-1 (Mcl-1; cat. no. 4572), total-JNK (cat. no. 9252) and phosphorylated (p)-JNK (cat. no. 9255), and against glyceraldehyde 3-phosphate dehydrogenase (GAPDH; cat. no. 2118) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Horseradish peroxidase-conjugated goat anti-rabbit (cat. no. A0208) and anti-mouse IgG (cat. no. A0216) antibodies were purchased from Beyotime Institute of Biotechnology.

Cell culture. The HepG2 human HCC cell line was obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM supplemented with 10% FBS, streptomycin (100 μ g/ml) and penicillin (100 U/ml) (both from Sigma-Aldrich) at 37°C in a humidified atmosphere containing 5% CO₂. All seeded cells were grown to 70-80% confluence prior to treatment. Cells were treated with 2 μ M curcumin, 10 μ M ABT-737 or 10 mM NAC for 24 h and then subjected to further experiments.

Hoechst 33258 staining. The treated cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) for 10 min at room temperature and then washed with phosphate-buffered saline (PBS) three times. Subsequently, the cells were incubated with Hoechst 33258 (Sigma-Aldrich) according to the manufacturer's instructions. The cells were then observed under a

fluorescence microscope (IX81; Olympus Corp., Tokyo, Japan) to identify apoptotic cells.

Trypan blue exclusion assay. Cells were treated with ABT-737 (0, 1, 5 or 10 μ M) and/or curcumin (0 or 2 μ M) for 36 h, harvested and suspended in PBS. The cells were then added with an equal volume of 0.08% Trypan blue solution (Sigma-Aldrich), and were incubated for 5 min at room temperature. Subsequently, the living and dead cells were counted using an optical microscope. The cells failing to exclude the blue dye were defined as dead cells, and the cell death rate was estimated as the percentage of dead cells.

Cytotoxicity assay. The cytotoxicity assay was performed using CCK-8 reagent. In brief, the HepG2 cells were seeded into 96-well plates at a density of 1x10³ cells/well. After 24 h, the cells were incubated with ABT-737 (0, 1, 5 or 10 μ M) and/or curcumin (0 or 2 μ M) for 36 h. Subsequently, CCK-8 reagent was added to each well, according to the manufacturer's protocol. Following incubation in the dark for 50 min at room temperature, the absorption values of the cells were detected at a wavelength of 450 nm using a Multiskan Spectrum microplate reader (Thermo Fisher Scientific, Inc.).

Western blot analysis. The total proteins of the treated cells were extracted from the whole-cell protein lysates prepared with radio-immunoprecipitation assay lysis and extraction buffer (Thermo Fisher Scientific) according to manufacturer's instruction at room temperature with centrifugation at 17,320 x g for 5 min at 4°C, and the concentration of the protein was measured using a Bicinchoninic Acid (BCA) Protein Assay kit (Sigma-Aldrich). Subsequently, 60 μ g protein of each sample was loaded for 10% polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride (PVDF) membranes (Thermo Fisher Scientific). The PVDF membranes were then incubated with 5% albumin in PBS with 1% Tween-20 (TBST) for 2 h at 37°C. Subsequently, the membranes were separately incubated with antibodies against Bcl-2 (1:1,000), Mcl-1 (1:2,000), PARP (1:1,000), cleaved caspase-3 (1:500), total-JNK (1:1,000), phosphorylated (p)-JNK (1:200) and GAPDH (1:3,000) for 12 h at 4°C. Following incubation and washing with PBST three times, the membranes were separately incubated with goat anti-rabbit and anti-mouse IgG for 1 h at 37°C. Immunoreactivity was then visualized by adding chemiluminescent peroxidase substrate (Thermo Fisher Scientific) in the dark. The chemiluminescence signal was recorded using the ChemiDoc XRS imaging system (Bio-Rad Laboratories, Hercules, CA, USA). Data analysis and quantification were performed using Quantity One software (version 4.6; Bio-Rad Laboratories, Inc.).

ROS detection. The HepG2 cells were seeded into 12-well plates and were cultured overnight, following which the cells were treated with the different treatments. Following treatment, the cells were washed with PBS three times, and the 2',7'-dichlorofluorescein diacetate fluorescent probe (Sigma-Aldrich) was added, according to the manufacturer's protocol. Subsequently, the cells were washed and harvested in PBS. The fluorescent value of each sample was then detected at a 488 nm excitation wavelength and a 525 nm emission wavelength using a fluorescent plate reader (VICTOR™ X5

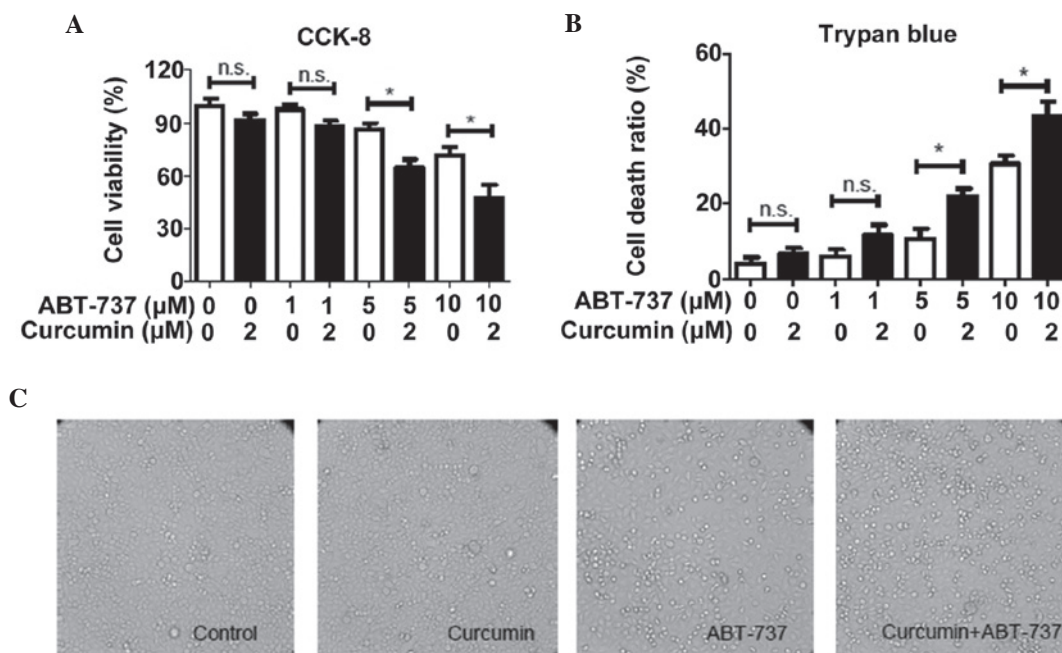


Figure 1. Curcumin enhances the antitumor effects of ABT-737 on HepG2 hepatocellular carcinoma cells. (A) HepG2 cells were seeded into 96-well plates and cultured overnight. The cells were then treated with curcumin and ABT-737 at the indicated concentrations for 36 h. Subsequent to treatment, a CCK-8 assay was used to determine the cell viability. (B) HepG2 cells were treated as in A, following which a Trypan blue exclusion assay was used to measure the total cell death ratio, according to the following equation: Cell death ratio = number of dead cells / (number of living cells+number of dead cells) x 100%. *P<0.05. (C) HepG2 cells were seeded in 6-well plates and cultured overnight. The cells were then separately treated with 1% dimethyl sulfoxide, 2 μ M curcumin, 10 μ M ABT-737 and 2 μ M curcumin+10 μ M ABT-737 for 36 h. Subsequently, the cells were observed under a microscope (magnification, x100) and floating cells were regarded as dead cells. The combination of ABT-737 and curcumin resulted in an increased number of floating cells and therefore cell death compared with single treatments or the control group. CCK-8, Cell Counting Kit-8; n.s., not significant.

Multilabel Plate Reader; Perkin Elmer, Waltham, MA, USA), with the ROS levels presented as the fold induction.

Transfection with small interfering RNA (siRNA). siRNA for ASK1 (5'-AAUUGCAGUCUGCACAGCCUUCGG-3') or control siRNA were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). The HepG2 cells were seeded into six-well plates and were cultured overnight. The cells at 70% confluence were then transfected with siRNA (100 pmol/well) using Lipofectamine 2000, according to the manufacturer's protocol.

Annexin V-FITC/PI staining. The treated cells, including the adherent and floating cells were harvested in PBS. The cells were then incubated with annexin V-FITC and PI for 15 min at room temperature. The stained cells were then analyzed using flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA, USA).

Caspase-3 activity detection. Caspase-3 activity was detected by the specific caspase-3 substrate, Ac-DEVD-AM C (Sigma-Aldrich). Briefly, the treated cells were suspended in radioimmunoprecipitation assay cell lysis buffer. The proteins were extracted according to the abovementioned procedure and the concentration was measured using the BCA kit. Subsequently, Ac-DEVD-AM C was added to each cell lysate containing 200 μ g protein, according to the manufacturer's protocol. Following incubation at 37°C for 1 h, the caspase-3 activity was measured by the fluorescence intensity at an excitation wavelength at 365 nm and an emission wavelength at

435 nm using a Victor™ X5 Multilabel Plate Reader (Perkin Elmer, Waltham, MA, USA).

Statistical analysis. All experiments in the present study were performed a minimum of three times. The data are expressed as the mean \pm standard deviation. Statistical differences were analyzed using two-way analysis of variance. Excel 2010 (Microsoft Corp., Redmond, WA, USA) software was used for all statistical analyses. P<0.05 was considered to indicate a statistically significant difference.

Results

Curcumin enhances the antitumor effect of ABT-737 on HepG2 HCC cells. In the present study, a cytotoxicity assay was used to evaluate the synergistic effect of curcumin and ABT-737 on HepG2 cells. As shown in Fig. 1A, no significant alterations in cell viability were observed when the cells were treated with 2 μ M curcumin alone for 24 h, whereas cell viability was observed to reduce in a dose-dependent manner following co-treatment with ABT-737. In addition, 2 μ M curcumin was observed to significantly enhance the cytotoxic effects of ABT-737 (5 and 10 μ M) on HepG2 cells (Fig. 1A). To determine whether the cytotoxic effects of ABT-737 and curcumin were associated with the increase in the number of dead cells, a Trypan blue exclusion assay was used to calculate the rate of cell death. As shown in Fig. 1B, 5 or 10 μ M ABT-737-induced cell death was significantly increased by the addition of 2 μ M curcumin, which indicated that the increase in cell death may be due to the

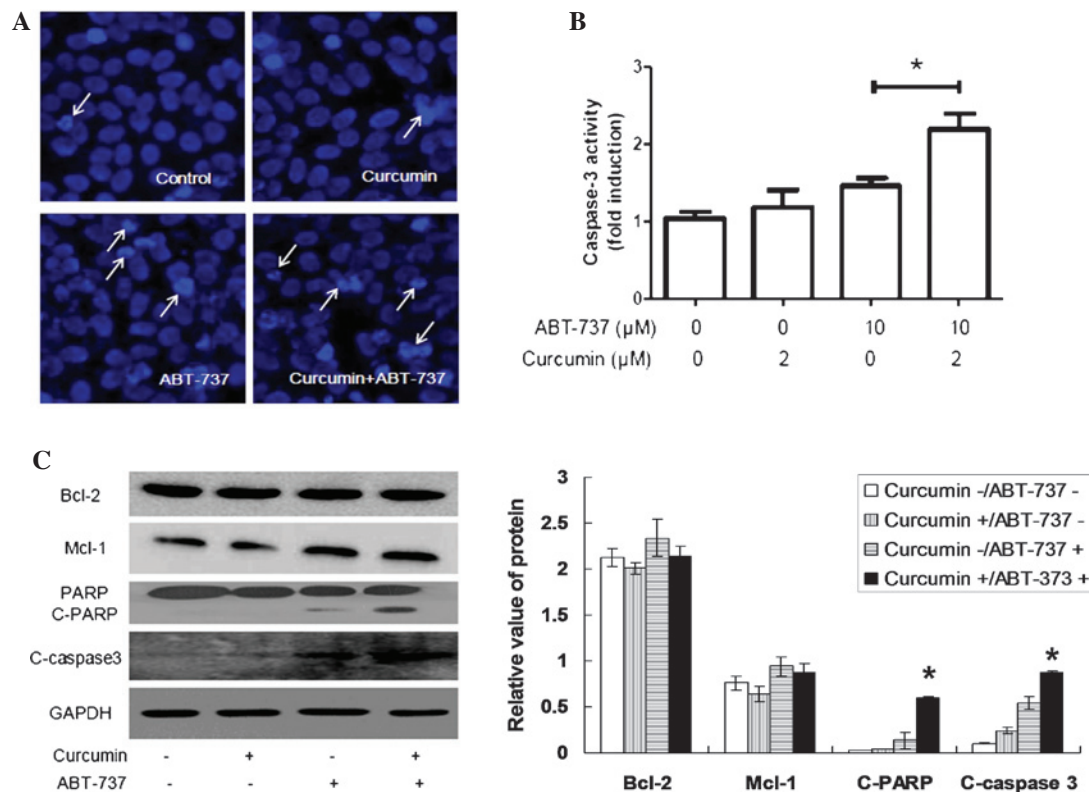


Figure 2. ABT-737 combined with curcumin significantly induces apoptosis in HepG2 cells. (A) HepG2 cells were separately treated with 1% dimethyl sulfoxide, 2 μ M curcumin, 10 μ M ABT-737 and 2 μ M curcumin+10 μ M ABT-737 for 24 h, and were stained with Hoechst 33258. Subsequently, the morphological characteristics of the nuclei were observed under a fluorescence microscope (magnification, x630). The white arrows represent abnormal nuclei with typical characteristics of apoptosis. (B) HepG2 cells were treated as in A, following which caspase activity was detected and presented as fold induction. (C) HepG2 cells were treated as in (A), following which the total cellular proteins were extracted. Subsequently, the proteins were loaded for western blotting and the levels of Bcl-2, Mcl-1, PARP/C-PARP and C-caspase-3 were assessed. Data are expressed as the mean \pm standard deviation. * $P < 0.05$ as indicated or vs. all other groups. Bcl-2, B cell lymphoma-2; Mcl-1, myeloid cell leukemia-1; PARP, poly adenosine diphosphate-ribose polymerase; C-, cleaved; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

synergistic effect of curcumin and ABT-737 on HepG2 cells. Additionally, the number of floating cells, defined as dead cells, in the DMEM of each well were markedly increased in the curcumin and ABT-737 co-treatment group when visually observed under a light microscope (Fig. 1C). These results further confirmed the synergistic effect of curcumin and ABT-737 on HepG2 cells.

ABT-737 combined with curcumin significantly induces apoptosis in HepG2 cells. As apoptosis is a key part of ABT-737-mediated cell death (16,33,34), whether curcumin promotes ABT-737-induced apoptosis in HepG2 cells was further investigated in the present study. Hoechst 33258 staining demonstrated that the number of abnormal nuclei with the typical characteristics of apoptosis was significantly increased in the HepG2 cells co-treated with curcumin and ABT-737, compared with the cells treated with ABT-737 alone (Fig. 2A). In addition, the activity of caspase-3, which is a key executor of typical apoptotic pathway, was markedly increased (Fig. 2B), and the level of cleaved caspase-3 also increased accordingly (Fig. 2C). These data suggested that curcumin and ABT-737 synergistically induce apoptosis of HepG2 cells.

ROS levels are increased in HepG2 cells following co-treatment with ABT-737 and curcumin. A previous study

demonstrated that ROS are important in ABT-737-induced apoptosis (16), therefore, the present study further investigated the role of ROS on the induction of apoptosis in HepG2 cells with the co-treatment of curcumin and ABT-737. As shown in Fig. 3A, co-treatment with curcumin and ABT-737 significantly increased the level of ROS in HepG2 cells. In addition, the administration of the antioxidant NAC attenuated the synergistic effect of curcumin and ABT-737 on the induction of apoptosis (Fig. 3B-D), which indicated that the increase of ROS was an important factor for the antitumor effect of curcumin and ABT-737.

Increased ROS levels promote the sustained activation of JNK, which leads to apoptosis in HepG2 cells. As increased levels of ROS have been reported to activate JNK (35,36), and sustained activation of JNK has been demonstrated as a mechanism for ABT-737-induced apoptosis (16), changes in the activity of JNK in curcumin and ABT-737 co-treated cells were further examined in the present study. As shown in Fig. 4A, JNK was not activated at the 18 h time point in the curcumin (2 μ M)-treated cells or ABT-737 (10 μ M)-treated cells, however, JNK was activated in the curcumin and ABT-737 co-treated cells. In addition, the JNK inhibitor, SP600125, significantly suppressed the apoptosis, which was induced by the combination of curcumin and ABT-737 in the HepG2 cells (Fig. 4B). These results indicated that

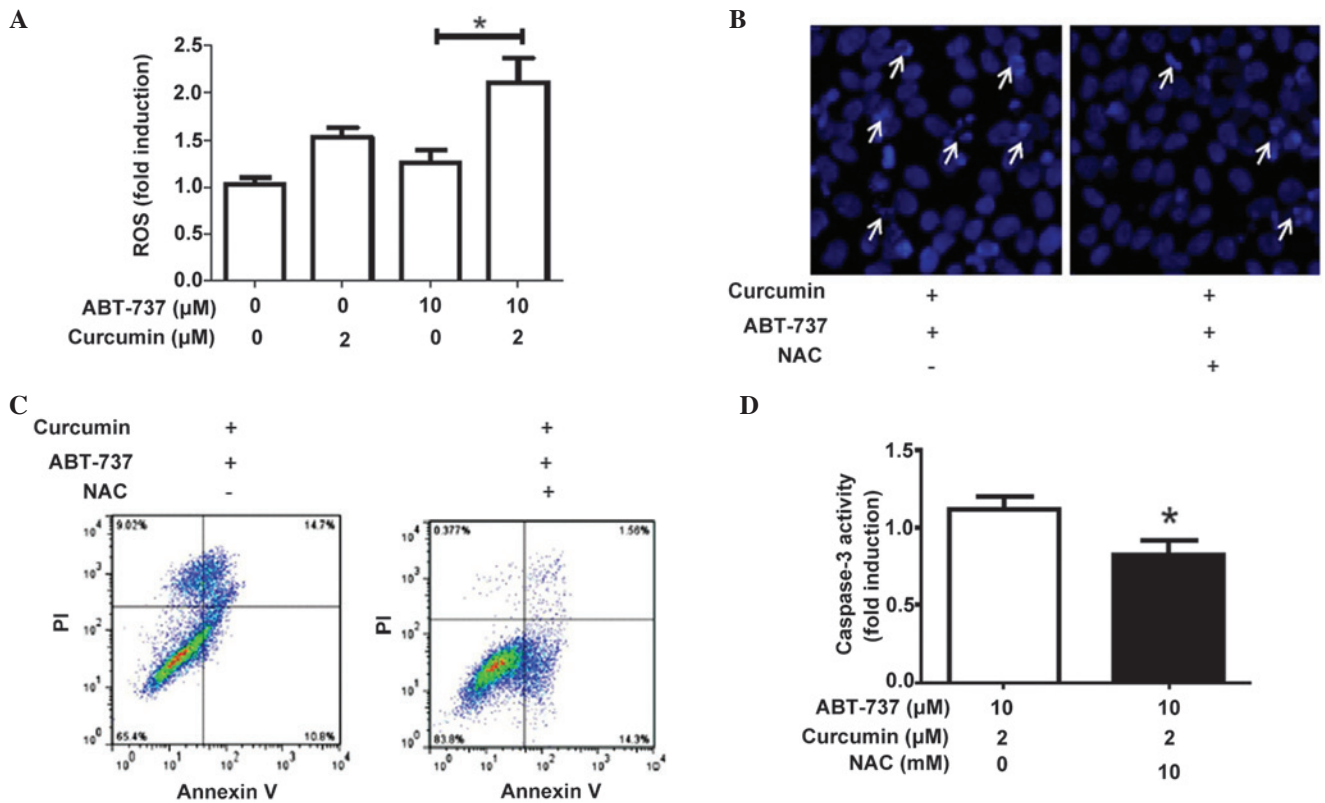


Figure 3. ROS levels are increased in HepG2 cells following co-treatment with ABT-737 and curcumin. (A) HepG2 cells were separately treated with 1% dimethyl sulfoxide, 2 μ M curcumin, 10 μ M ABT-737 and 2 μ M curcumin+10 μ M ABT-737 for 24 h, following which a 2',7'-dichlorofluorescein diacetate probe was used to measure the levels of cellular ROS. HepG2 cells were treated with 2 μ M curcumin and 10 μ M ABT-737 for 24 h in the presence or absence of the antioxidant, NAC (10 mM). Subsequently, the levels of HepG2 cell apoptosis were analyzed using (B) Hoechst 33258 staining (white arrows indicate cells undergoing apoptosis with nuclear fragmentation visualized by Hoechst staining), (C) Annexin V-fluorescein isothiocyanate/PI staining and (D) caspase-3 activity detection. Data are expressed as the mean \pm standard deviation. * P <0.05. ROS, reactive oxygen species; PI, propidium iodide; NAC, N-acetyl-L-cysteine.

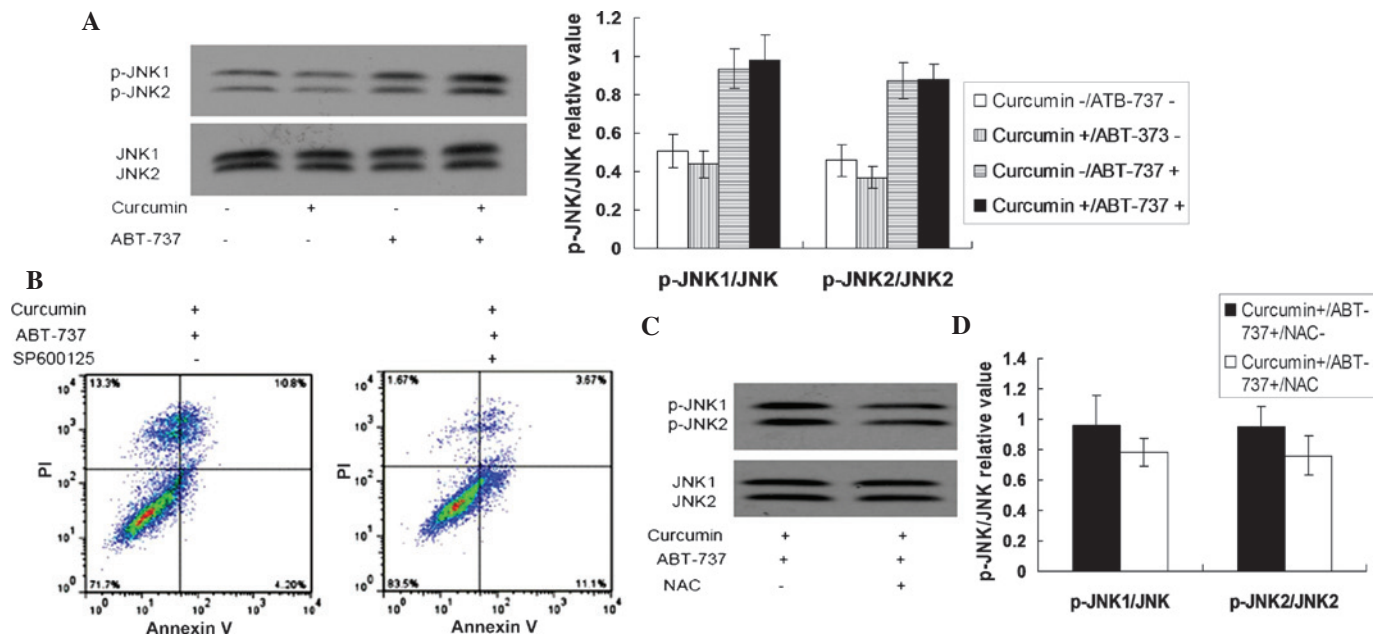


Figure 4. Increased levels of ROS promote the sustained activation of JNK, which leads to the apoptosis of HepG2 cells. (A) HepG2 cells were separately treated with 1% dimethyl sulfoxide, 2 μ M curcumin, 10 μ M ABT-737 and 2 μ M curcumin+10 μ M ABT-737 for 24 h, following which the total cellular proteins were extracted. Subsequently, the protein levels of p-JNK1/JNK2 and total JNK1/JNK2 were detected using western blotting. (B) HepG2 cells were treated with 2 μ M curcumin+10 μ M ABT-737 for 24 h in the presence or absence of 10 μ M SP600125 (a JNK inhibitor). Following treatment, apoptosis of the HepG2 cells was assessed using annexin V-fluorescein isothiocyanate/PI staining. (C) HepG2 cells were treated with 2 μ M curcumin+10 μ M ABT-737 for 24 h in the presence or absence of antioxidant, NAC (10 mM). Subsequently, the levels of p-JNK1/JNK2 and total JNK1/JNK2 were detected using western blotting. (D) HepG2 cells were separately transfected with control siRNA or ASK1 siRNA for 24 h, following which the cells were treated with 2 μ M curcumin+10 μ M ABT-737 for 24 h. A Trypan blue exclusion assay was then used to measure the total cell death ratio. * P <0.05 vs. control. ROS, reactive oxygen species; JNK, c-Jun N-terminal kinase; p-, phosphorylated; siRNA, small interfering RNA; PI, propidium iodide.

curcumin may enhance the cytotoxicity of ABT-737 via the sustained activation of JNK. Additionally, the antioxidant, NAC, was able to reverse the activation of JNK induced by the co-treatment of curcumin and ABT-737 (Fig. 4C), which suggested that the increased ROS levels in the curcumin and ABT-737 co-treated cells activated JNK. Downregulating the level of ASK1, which is often important in the ROS-mediated activation of JNK (16), by siRNA attenuated the synergistic cytotoxicity of curcumin and ABT-737 on the HepG2 cells (Fig. 4D). These data indicated that curcumin enhanced the antitumor effect of ABT-737, which was partially dependent on the ROS-ASK1-JNK pathway.

Discussion

The pathology of HCC is complex, which presents a challenge in the development of effective treatments for HCC (37,38). Certain novel strategies of combination therapy have been investigated previously, and have shown potent antitumor activity in pre-clinical tests (39-44). Combination therapy appears to be a promising strategy for patients with HCC, due to the fact that it can improve the treatment effect and also alleviate the side effects of the drugs (40). Previous studies have confirmed that curcumin and ABT-737 are potential anticancer drugs, and can induce cell death in HCC cells in a dose-dependent manner (13,45,46). In the present study, it was found that 2 μ M curcumin alone had no significant cytotoxic effects on the HepG2 cells, however it enhanced the antitumor effect of ABT-737 on HepG2 cells. The results indicated that curcumin markedly enhanced the level of ROS in ABT-737-treated cells. In addition, the increased ROS levels resulted in the promotion of HepG2 cell apoptosis, which was partially dependent on the ASK1-mediated sustained activation of JNK. Therefore, the combination of curcumin and ABT-737 may serve as a potential therapeutic treatment strategy for patients with HCC.

ROS are important in regulating cell survival and cell death (47). It has been reported that low levels of ROS promote cell survival via multiple signaling pathways, whereas high levels of ROS can be an important mechanism of cell death (47). A previous study confirmed that ABT-737 resulted in low levels of ROS in HepG2 cells, which may have promoted cell survival via cytoprotective autophagy (16). In the present study, curcumin significantly increased the levels of ROS in the ABT-737-treated HepG2 cells. Notably, the increased levels of ROS did not further promote cell survival, however, it markedly induced cell death. The different levels of ROS may have determined the pro-survival or pro-apoptotic roles in HCC cells. In addition, the reason for the marked increase in the levels of ROS may be associated with mitochondrial stress-induced damage following administration of the combination of the two drugs. The associated mechanisms require further investigation.

JNK is a member of the mitogen-activated protein kinase family and has three isoforms, including JNK1, JNK2 and JNK3 (48). JNK can be activated through the phosphorylation of the Thr residue under diverse types of stress, including ROS (35,49,50). JNK is involved in the regulation of various cellular process, including proliferation, differentiation, cell death and survival (51). In addition, the activation of JNK is closely associated with the carcinogenesis, development and treatment of HCC (52). Previous studies have demonstrated

that ABT-737-mediated activation of JNK can either promote cell survival or lead to cell death in different types of HCC cell (13,16,17). In the present study, it was found that JNK was not activated at the 18 h time point in HepG2 cells with ABT-737 treatment alone, however, it was activated when the cells were co-treated with curcumin and ABT-737. In addition, the activation of JNK significantly promoted cell death, which suggested the pro-apoptotic role of JNK under these conditions. The downregulation of ASK1, a key mediator of the ROS-JNK pathway (53), was observed to markedly reduce the total cell death rate in the curcumin and ABT-737 co-treated cells. However, the downstream signaling pathways of activated JNK in the HepG2 cells with the co-treatment of curcumin and ABT-737 are complex, therefore, the detailed mechanisms require further investigation.

Collectively, the present study indicated that the combination of curcumin and ABT-737 can efficaciously induce the death of HCC cells, and may offer a potential treatment strategy for patients with HCC.

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