Curcumin induces Mitochondrial Apoptosis in Human Hepatoma Cells through BCLAF1-mediated Modulation of PI3K/AKT/GSK-3β Signaling

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Abstract

Curcumin is a yellow pigment extracted from the rhizome of turmeric, a traditional Chinese medicine. Here, we tested the hypothesis that curcumin-mediated downregulation of BCLAF1 triggers mitochondrial apoptosis in hepatoma cells by inhibiting PI3K/AKT/GSK-3β signaling. Treatment of the human hepatoma cell lines, HepG2 and SK-Hep-1, with various concentrations of curcumin revealed a time-dependent and concentration-dependent inhibition of cell proliferation, increased apoptosis, cell cycle arrest at the G0/G1 phase, reduced mitochondrial membrane potential, and reduced expression levels of PI3K, p-PI3K, AKT, p-AKT, GSK-3β, and p-GSK-3β. Additionally, curcumin suppressed the levels of apoptotic factors after treating the cells with LY294002, a PI3K inhibitor. Curcumin also suppressed the expression of BCLAF1. Treating stable BCLAF1 knockout HepG2 and SK-Hep-1 cells with curcumin further enhanced apoptosis and increased the number of cells in G0/G1 cell cycle arrest, while inhibiting the downregulation of PI3K/AKT/GSK-3β pathway-related proteins. Treatment of a nude mouse xenograft model bearing HepG2 cells with curcumin inhibited tumor growth, disrupted the cellular structure of the tumor tissue, and suppressed the expression of BCLAF1 and PI3K/AKT/GSK-3β proteins. In summary, our in vitro and in vivo analyses show that curcumin downregulates BCLAF1 expression, inhibits the activation of the PI3K/AKT/GSK-3β pathway, and triggers mitochondrial apoptosis in HCC. These findings uncover a potential therapeutic strategy leveraging the antitumor effects of curcumin against HCC.

Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver cancer, with about 840,000 new cases and at least 780,000 HCC deaths recorded each year [1]. However, due to the lack of effective biomarkers, HCC is often detected at an advanced, inoperable stage [2] and traditional chemoradiotherapy is associated with severe adverse reactions. Thus, effective therapeutic strategies are urgently needed for improved HCC outcomes.

Turmeric has numerous biological effects, including anti-inflammatory [3], antioxidant [4], osteoarthritis relief [5], and wound healing effects [6-7]. Curcumin, the main active component of turmeric, is reported to have pharmacological activity [8]and has numerous potential applications due to its low toxicity and easy availability [9]. Curcumin is reported to have antioxidant, anti-inflammatory, anticoagulant anti-atherosclerotic, anti-aging, and anti-tumor effects [10-16]. A study involving 80 liver cancer patients assessed liver function level, treatment effectiveness, and quality of life index and concluded that curcumin has obvious curative effects [17]. A study involving a rat model and in vitro assays found that curcumin has potential therapeutic effects against tumors [18].

BCLAF1 is a widely-expressed multifunctional, BCL2-related transcription factor that bears an arginine (R)-serine (S) functional domain and a DNA binding domain [19]. BCLAF1 is involved in various biological processes, including apoptosis, transcription regulation, and DNA damage repair [20-23]. In HCC, BCLAF1 is reported to induce autophagy, promote cell proliferation, and inhibit apoptosis under stress conditions.
We previously reported that ginsenoside CK induces autophagy in SMMC-7721 cells by mediating BCLAF1, regulating ERK1/2 pathway, and inducing mitochondrial apoptosis, and that suppression of BCLAF1 expression promotes HIF-1α ubiquitination, thereby inhibiting HIF-1α-mediated glycolysis and cell proliferation [25]. These findings suggest that BCLAF1 has multiple roles in tumorigenesis. In this study, we investigated the hypothesis that BCLAF1 mediates the anti-tumor effects of curcumin.

The mitochondrial apoptotic pathway modulates various cell death processes, including necrosis, apoptosis, pyrodeath and iron death. Thus, treatments targeting mitochondrial apoptosis may be effective in various diseases [26]. The mechanisms underlying mitochondrial apoptosis may improve the effectiveness of cancer treatment [27]. *In vitro* and *in vivo* studies showed that ginkgo biloba triggered S phase cell cycle arrest in HepG2 and SK-Hep-1 cells and promoted the release of Caspase-3 and Cyt-C, suggesting that in HCC, apoptosis is mediated by the mitochondrial pathway [28]. Treatment of BEL-7402 and HepG2 cells with Alder significantly induced apoptosis, reduced mitochondrial membrane potential. By activating mitochondrial DNA transcription, mitochondrial transcription factor B1 (TFB1M) inhibits apoptosis in HCC and promotes HCC growth, epithelial-mesenchymal transformation, and metastasis [29]. It is reported that isopropyl ester activated caspase signaling in Hep3B by increasing the expression levels of the active forms of Caspase-3, Caspase-8, Caspase-9, and Bax, while downregulating the anti-apoptotic factor, Bcl-2, and enhancing the levels of Cyt-C and ROS, thereby inducing apoptosis [30]. These findings indicate that the mitochondrial apoptosis pathway influences tumor cell apoptosis. Inhibiting PI3K/AKT signaling is reported to enhance curcumin-induced apoptosis in MCF7 cells [31-32].

Although curcumin is a widely studied pleiotropic, multi-active compound, its mechanism of action against liver cancer is unclear. In this study, we investigated the effect of BCLAF1 on the induction of mitochondrial apoptosis through curcumin-mediated PI3K/AKT/GSK3β signaling in HCC. This study sought to uncover mechanisms by which curcumin induced apoptosis in HCC and to elucidate potential therapeutic strategies for HCC treatment.

**Results**

**2.1 Curcumin inhibited the proliferation of HCC cells**

Fig. 1a shows the chemical structure of curcumin, a lipophilic polyphenol [33]. To assess the effect of curcumin on the proliferation of HCC cells, we treated HepG2 and SK-Hep-1 cells with curcumin at 10 µM, 20 µM, 40 µM, and 60 µM for 24, 48 and 72 h and then carried out CCK-8 analysis. The median inhibitory concentrations in HepG2 cells at 24, 48 and 72 h were 43.73 µM, 22.97 µM, and 21.17 µM, respectively. The median inhibitory concentrations of SK-Hep-1 cells at 24, 48 and 72 h were 40.11 µM, 27.76 µM, and 21.24 µM, respectively. HepG2 cells treated with curcumin at 10 µM, 20 µM, 40 µM, and 60 µM for 24 h had growth rates of 97.45%, 79.83%, 65.27%, and 30.26%, respectively. Under the same treatment conditions, SK-Hep-1 cells had growth rates of 99.91%, 94.02%, 71.73% and 40.21%, respectively (Fig. 1b). Cell cycle analysis using flow cytometry in HepG2 cells treated with vehicle and curcumin at 20 µM, 40 µM, and 60 µM, or 5-Fluorouracil (5FU, positive control) at 10 µM revealed the
proportions of cells in G0/G1 phase to be 46.46%, 53.30%, 56.60%, 62.63%, and 71.03%, the proportions of cells in S phase to be 24.59%, 25.21%, 22.18%, 22.80% and 16.77%, and the proportions of cells in G2/M phase to be 28.95%, 21.83%, 21.21%, 14.57% and 12.20%. Under the same treatment conditions, the proportions of SK-Hep-1 cells in G0/G1 phase were 49.60%, 52.58%, 61.28%, 65.26%, and 74.72%, the proportions of cells in S phase were 20.96%, 21.21%, 16.59%, 16.78%, and 12.96%, and the proportions of cells in G2/M phase were 29.44%, 26.21%, 22.13%, 17.97%, and 12.32%. DNA content in the G0/G1 phase increased significantly, while that in G2/M phase and S phase decreased relatively (Fig. 1c). These results indicated that cells were blocked in G0/G1 phase and could not enter S phase, resulting in a decrease in cell number in G2/M phase and decreased proliferation index.

2.2 Curcumin promoted apoptosis in HCC cells

Next, we treated HepG2 and SK-Hep-1 cells for 24 h using curcumin at 20 µM, 40 µM, and 60 µM, or 5FU at 10 µM and examined their effect on apoptosis. Apoptosis is characterized by chromatin shrinkage, which appears as dense structures upon Hoechst 33258 staining. Hoechst 33258 staining revealed that curcumin increased the number of apoptotic cells dose-dependently (Fig. 2a). Annexin V / PI double staining revealed the apoptosis rate to be 5.00%, 13.92%, 16.16%, 26.87% and 39.59%, respectively, in HepG2 cells and 9.30%, 18.24%, 26.43%, 30.58%, and 32.14%, respectively, in SK-Hep-1 cells. Compared with the control group, apoptosis was significantly elevated in cells treated with curcumin at 40 µM or with 5FU (Fig. 2b). Western blot analysis revealed that when compared with the control group, treatment with curcumin increased the levels of the apoptosis-related proteins, Cleaved caspase-3 and Cleaved caspase-9, dose-dependently (Fig. 2c) and also enhanced the expression of the pro-apoptotic factors, Bax and Cyt-C, while suppressing the levels of the anti-apoptotic factor, Bcl-2 (Fig. 2d). To determine if curcumin induced mitochondrial apoptosis pathways, we evaluated changes in mitochondrial membrane potential using a fluorescence probe and observed increased green fluorescence and decreased red fluorescence in curcumin-treated HCC cells, indicating reduced mitochondrial membrane potential (Fig. 2e). These results suggest that curcumin promotes apoptosis in HCC cells via the mitochondrial apoptosis pathway.

2.3 Curcumin inhibits PI3K/AKT/GSK-3β signaling

To evaluate if curcumin triggers apoptosis in HCC cells through the PI3K/AKT/GSK-3β pathway, we treated HepG2 and SK-Hep-1 cells for 24 h with curcumin at 20 µM, 40 µM, and 60 µM, or 5FU at 10 µM. Subsequent western blot analysis revealed that curcumin significantly reduced the levels of PI3K, p-PI3K, AKT, p-AKT, GSK-3β, and p-GSK-3β (Fig. 3a). To further test the effect of curcumin on the PI3K/AKT/GSK-3β pathway, we pretreated cells with LY294002, a PI3K inhibitor, and observed that treatment with curcumin (40 µM) enhanced the inhibition of the PI3K/AKT/GSK-3β pathway, upregulated Bax levels, and downregulated Bcl-2 levels (Fig. 3b).
2.4 BCLAF1 knockout enhanced curcumin-induced apoptosis in HCC cells

Western blot analysis revealed that treatment with curcumin decreased BCLAF1 levels in a concentration-dependent manner (Fig. 4a). To test if the effects of curcumin on the PI3K/AKT/GSK-3β pathway induced apoptosis in the HCC cell lines by modulating BCLAF1, we knocked out BCLAF1 in HepG2 and SK-Hep-1 cells using CRISPR/Cas9 technology and treated them with curcumin at 40 µM for 24 h. Cell cycle analysis showed that compared with the control group and curcumin group, the amount of DNA in the G0/G1 phase was markedly higher in the curcumin+sgRNA group, while the proportion in the S and G2/M phase was significantly lower (Fig. 4b). Annexin V/PI double staining showed that compared with the control group and curcumin-treated group, apoptosis was significantly higher in the curcumin+sgRNA group (Fig. 4c). Western blot analysis revealed that compared with the curcumin-treated group, the levels of Bax and Cyt-c were increased in the BCLAF1 knock out cells while the level of Bcl-2 decreased (Fig. 4d). Moreover, curcumin further inhibited the expression of PI3K/AKT/GSK-3β pathway-related proteins. The protein expression levels of p-PI3K, p-AKT, and p-GSK-3β in the curcumin+sgRNA group were dramatically decreased when compared with the curcumin alone group (Fig. 4e). Analysis of mitochondrial membrane potential showed that compared with the control group and the curcumin-treated group, the curcumin+sgRNA group had higher levels of green fluorescence and lower levels of red fluorescence, indicating decreased mitochondrial membrane potential (Fig. 4f).

2.5 Curcumin inhibits tumor growth in a nude mouse xenograft model of HCC and regulates mitochondrial apoptosis in the PI3K/AKT/GSK-3β pathway mediated by BCLAF1

Next, given that in vitro analyses suggested that curcumin inhibits the proliferation of HCC cell lines, we sought to evaluate the antitumor effects of curcumin in vivo. To this end, we established a nude mouse xenograft model of HCC. We treated the mice with curcumin at 20 µM, 40 µM, and 60 µM, or with 5FU at 10 µM and observed that curcumin inhibited tumor growth in a dose-dependent manner, as indicated by the tumor volumes of 1832.49±1001.37, 1285.93±547.63, 1117.17±752.54, 713.36±262.69 and 271.69±154.69 mm³, respectively (Fig. 5a). Analysis of tumor volume and growth inhibition curves revealed that when compared with the negative control group, the volume of the xenograft tumors decreased with increasing concentration of curcumin (Fig. 5b–c). Next, we performed pathological analysis of the tumors using H&E staining. This analysis found that when compared with the negative control group, curcumin altered the cell structure of the xenograft tumor cells, reduced their cell volume and nucleo-plasma ratio, and caused apoptosis (Fig. 5d). Immunohistochemical analysis showed that Bcl-2 and Bax localized to the cytoplasm, that curcumin enhanced Bax expression and inhibited BCLAF1 expression, and that BCLAF1 was expressed in the cytoplasm and nucleus (Fig. 5e). Western blot
analysis of apoptosis-related proteins showed that expression of the PI3K/AKT/GSK-3β pathway-related proteins, PI3K, p-PI3K, AKT, p-AKT, GSK-3β and p-GSK-3β was decreased, with a more apparent decrease in the levels of the phosphorylated proteins (Fig. 5f).

Materials And Methods

4.1 Cell culture

Human hepatoma cell lines (HepG2 and SK-Hep-1) were purchased from BeNa Culture CollectionKey (Beijing, China), the number of cell lines was 1×10^6/cell culture flask. HepG2 and SK-Hep-1 cells were thawed and cultured in Dulbecco's modified Eagle's medium (DMEM; Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco; Grand Island, NY, USA) and 100 U/mL penicillin-streptomycin (Gibco; Grand Island, NY, USA) in a humidity incubator (37 °C, 5%CO₂).

4.2 CCK-8 proliferation assay

The proliferation of HepG2 and SK-Hep-1 cells treated with curcumin (MCE; New Jersey, USA) was detected by CCK-8 assay (APExBIO; Texas, HOU, USA). The cells were inoculated in a 96-well plate with curcumin (10, 20, 40, 60 μM) for 24, 48 and 72 h, added 100 μL culture medium and 10 μL CCK-8 (5.0 mg/ml), incubated in humidity incubator (37 °C, 5%CO₂) for 2 h, the optical density was measured at 490 nm (OD) of the microplate reader (Bio-Tek; San Jose, CA, USA).

4.3 Tumor implantation

Thirty 6-week-old female BALB/ C-NU mice (number: 110324210103555631) were purchased from Sibafu Biotechnology Co., LTD (Beijing, China). Laboratory animal use permit: SYXK (Ji) 2021-006. Laboratory animal production license: SCXY (Jing) 2019-0010. HepG2 (1×10^6/mL) cells were subcutaneously inoculated in the axilla and randomly grouped (Curcumin: 20, 40, 60 μM, 5FU: 10 μM and vehicle: normal saline). When the tumor grew to 50-100 mm³. The experiment was divided into 5 groups, with 6 mice in each group, once a day for 15 consecutive days. After anesthesia, all nude mice were sacrificed to get tumor blocks, tumor blocks were frozen or fixed with 10% formalin and made into wax blocks for preservation. The length and width of tumor blocks were measured. The tumor volume and growth inhibition rate were calculated.

4.4 Analysis of cell morphology

After treating with curcumin and 5FU, morphological changes of HepG2, SK-Hep-1 cells and tumor tissues were observed by hematoxylin and eosin (H&E; Beyotime; Shanghai, China) and Hoechst 33342 staining (Beyotime; Shanghai, China). For H&E staining, after the wax block slices were baked, dewaxed by xylene, treated with high concentration to low concentration alcohol, washed with distilled water. Cells were
exposed with hematoxylin for 5 min and eosin for 2 min, alcohol gradient dehydration, xylene was treated with 20 min, dried, neutral gum was sealed and observed under light microscope (Olympus, Japan) at 100 × magnification. For Hoechst 33258 staining, fixed with 4% paraformaldehyde for 20 min and washed with PBS. 1mL Hoechst 33258 staining solution was added to each well and incubated at room temperature for 20 min. After washing with PBS, fluorescence microscope (Olympus, Japan) was used to observe and take photos (200 × magnification).

4.5 Cell cycle analysis

Cell cycling was analyzed by a cell cycle analysis kit (Beyotime). HepG2 and SK-Hep-1 cells were inoculated in six-well plates, treated with curcumin and 5FU for 24 h. According to the instructions, centrifuged at 1000 g for 5 min, washed with precolled PBS, centrifuged at 1000 g for 5 min to obtain cell precipitation, and fixed with precolled 70% anhydrous ethanol. Propidium iodide staining solution was prepared to stain, and cell precipitation was suspended. The cells were incubated for 30 min at 37 °C without light and analyzed by flow cytometry (Beckman Coulter, Inc., CA, USA). The same is true of tumor tissue.

4.6 Analysis of mitochondrial membrane potential

Mitochondrial membrane potential changes were analyzed by mitochondrial membrane potential detection kit (Beyotime). According to the instructions, the logarithmic growth stage cells of HepG2 and SK-Hep-1 cells were inoculated into 6-well plates. After 24 h, CCCP (10 μM) was added to treat cells for 30 min, 1 mL culture medium and 1 mL JC-1 working solution were mixed to each well, then incubated in 37 °C incubator for 40 min, washed twice by JC-1 dyeing buffer (1X), 2 mL culture solution was added to each well, and fluorescence microscope was used to observe and take photos at 400 × magnification.

4.7 Apoptosis analysis

HepG2 and SK-Hep-1 cells were inoculated in six-well plates. After 24 h of treatment with different concentrations of curcumin, the cells were digested with trypsin without EDTA, digestion was terminated by precooling PBS, followed by centrifugation. The cells were cleaned and centrifuged again. Cell suspension was prepared by V-FITC binding solution, added Annexin V-FITC, and incubated at room temperature with dark for 10 min. Then added PI at room temperature with dark for 5 min. Cell apoptosis rate was detected by flow cytometry (Beckman Coulter, Inc., CA, USA).

4.8 CRISPR/Cas9-mediated BCLAF1 knockout

HepG2 and SK-Hep-1 cells were collected and centrifuged, the supernatant was discarded and inoculated in a six-well plate. Each cell was divided into blank control group and sgRNA group. On the next day,
replaced with 1 mL culture medium (without serum and antibiotics), and 20 μL lentivirus vector was added to sgRNA group at the same time. After 12 h, replaced with conventional culture medium and culture continued. 48 h later, 6 μg/mL penicillomycin was added to each well for screening, and stable transfected cell lines with BCLAF1 gene knockout were obtained.

4.9 Immunohistochemistry staining

In the experimental group, the xenografts of nude mice were made into wax blocks, sliced and baked, dewaxed with xylene solution, dewaxed with ethanol gradient, washed by PBS and put into citrate. Endogenous peroxide blocker was added and incubated at room temperature for 30 min. Added goat serum protein. Primary antibody: BCLAF1, Bcl-2 and Bax was added for sealing for 20 min. The slices were placed in hematoxylin staining solution. After being removed and dried, the slices were sealed with neutral gum and randomly selected fields were placed under a microscope for observation and photography (400 × magnification).

4.10 Western blotting

HepG2 and SK-Hep-1 cells in logarithmic growth phase were treated with different concentrations of drugs. Extracted cells and tumor tissues protein. The protein concentration was determined by BCA method, and the loading quantity was calculated according to the sample concentration. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (100 V, 120 min), transferred to polyvinylidene fluoride (PVDF) membranes (100 V, 30-90 min), then the membranes was blocked with skim milk (5%), the primary antibodies were incubated overnight at 4 °C. The next day, incubated with anti-rabbit secondary antibody (1:5 000) at room temperature for 1 h, ECL luminescent solution was dropped. Target proteins were detected by Bio-RAD imaging system (Bio-RAD, Hercules, CA, USA)

4.11 Statistical analysis

All experiments were repeated three times. Data are expressed as mean ± standard deviation (SD), and differences between groups were analyzed by one-way analysis of variance and Student’s t-test. p < 0.05 was considered to be statistically significant. SPSS version 19.0 and GraphPad Prism 5.0 software were used to analyze the results.

Discussion

In China, primary liver cancer is associated with high morbidity and mortality [34]. Currently, liver cancer is mainly treated using chemotherapy, molecular targeted therapy, and immunotherapy [35]. Although curcumin has anticancer potential, the mechanisms through which it triggers apoptosis in hepatocellular carcinoma (HCC) are unclear.
Curcumin is known to regulate cell cycle progression and cause cancer cell death [36]. Using CCK-8 analysis, we show that curcumin inhibited the proliferation of human HCC cells in a concentration-dependent and time-dependent manner and caused cell cycle arrest at the G0/G1 phase. Apoptosis is a key process of regulating cell numbers and eliminating unwanted, potentially dangerous cells. Mitochondrial damage plays a central role in determining cell fate, especially in programmed cell death pathways [37]. Curcumin has been reported to induce mitochondria-dependent apoptosis in HepG2 cells [38]. Here, we hypothesized that curcumin might induce mitochondrial apoptosis in HCC cells. Hoechst staining analysis on human liver cancer cells showed that curcumin increased the number of apoptotic cells in a concentration-dependent manner and enhanced the expression levels of apoptosis proteins. Analysis of mitochondrial membrane potential showed that compared with the control group, cells treated with curcumin and 5FU had significantly lower mitochondrial membrane potential. Western blot analysis showed that the levels of the apoptosis-associated protein Cyt-C increased while the levels of Bcl-2/Bax decreased. These findings are consistent with past findings [39], suggesting that curcumin promotes mitochondrial apoptosis in HCC cells.

The PI3K/AKT signaling pathway is involved in the occurrence and development of various tumors through its effects on cell proliferation and apoptosis. GSK-3β, a key serine/threonine family kinase, is an AKT substrate [40] involved in the regulation of energy metabolism, cell growth, and apoptosis. Guo [41] proposed that GSK-3β is involved in dex-induced pancreatic β cell apoptosis. Curcumin ameliorates oxidative stress-induced apoptosis in osteoblasts by preserving mitochondrial function and activating AKT/GSK-3β signaling. Downregulating the levels of p-PI3K and p-AKT in osteoblasts inhibited the PI3K/AKT pathway [42]. GSK3β knockout reduced Dex-induced apoptosis in osteoblasts. However, the association between the PI3K/AKT/GSK-3β pathway and mitochondrial apoptosis is unclear. Our investigation of the relationship between apoptosis in hepatoma cells and the PI3K/AKT/GSK-3β pathway shows that curcumin downregulates the expression of PI3K, p-PI3K, AKT, p-AKT, GSK-3β, and p-GSK-3β. Inhibiting the PI3K/AKT/GSK-3β pathway in HCC cells using LY294002 and treating them with curcumin revealed that curcumin enhanced the inhibition of these pathway proteins, and gradually decreased Bcl-2/Bax levels. These results indicate that Bax promoted the expression of proteins that are associated with mitochondrial apoptosis, increased mitochondrial membrane permeability, released pro-apoptotic proteins, and induced apoptosis. These results suggest that curcumin may induce mitochondrial apoptosis in HCC cells by inhibiting the PI3K/AKT/GSK-3β pathway.

BCLAF1, a core protein gene of hepatitis C virus, is highly expressed in Huh-7 cells, and its expression is induced by apoptosis signaling [43]. Studies show that BCLAF1 influences the proliferation and invasion of tumor cells [44], induces senescence [21], and participates in multiple physiological and pathological processes [45]. Downregulation of BCLAF1 expression inhibits proliferation and affects cell cycle progression in human adrenal cortical small cell carcinoma cells [46]. BCLAF1 plays a key role in LMK-235-mediated apoptosis and may be a potential target for the treatment of diffuse large B-cell lymphoma [47]. Here, analysis of the association between curcumin-induced apoptosis and BCLAF1 found that curcumin inhibits BCLAF1 expression. Analysis of whether BCLAF1 mediates the effects of curcumin on mitochondrial apoptosis in HCC cells revealed that treating BCLAF1 knockout HepG2 and SK-Hep-1 cells
with curcumin (40 µM) significantly increased apoptosis, increased cell cycle arrest in the G0/G1 phase, and decreased the mitochondrial membrane potential. Moreover, it increased the level of Cyt-c and decreased the Bcl-2/Bax ratio, suggesting that curcumin-induced mitochondrial apoptosis in HCC cells was mediated by BCLAF1. The expression levels of pathway-related proteins in the sgRNA+curcumin group and curcumin (40 µM) group were markedly reduced. These results indicate that curcumin induced mitochondrial apoptosis in HepG2 and SK-Hep-1 \textit{in vitro} through BCLAF1-mediated inhibition of the PI3K/AKT/GSK-3β pathway.

To determine the effect of curcumin on HCC apoptosis \textit{in vivo}, we established a nude mouse xenograft model bearing HepG2 cells. These analyses revealed that treating the tumor xenograft model with curcumin reduced tumor volume in a concentration-dependent manner. Moreover, curcumin disrupted the structure of tumor tissue. \textit{In vivo} analysis of the effects of curcumin on the mitochondrial apoptosis pathway induced by BCLAF1 and the PI3K/AKT/GSK-3β pathway showed that curcumin treatment reduced the expression of BCLAF1 and Bcl-2, while increasing Bax levels, indicating that curcumin induced mitochondrial apoptosis in the xenograft tumor model and inhibited BCLAF1 expression. Treatment of the xenograft tumor mice with curcumin reduced the expression of pathway-related proteins, suggesting that curcumin inhibited the PI3K/AKT/GSK-3β pathway in the nude mouse xenograft model of HCC. These results indicate that curcumin induces mitochondrial apoptosis \textit{in vivo} by regulating BCLAF1 and inhibiting the PI3K/AKT/GSK-3β pathway. Taken together, these \textit{in vitro} and \textit{in vivo} findings show that curcumin induces mitochondrial apoptosis in HCC through BCLAF1-mediated suppression of the PI3K/AKT/GSK-3β pathway. Our findings highlight potential therapeutic strategies for the treatment of liver cancer.

\textbf{Conclusion}

Our data show that curcumin inhibited the proliferation of the human HCC cell lines HepG2 and SK-Hep-1 by inhibiting the expression of BCLAF1, inhibiting the activation of the PI3K/AKT/GSK-3β pathway, and promoting BCLAF1-regulated mitochondrial apoptosis. These findings are consistent with \textit{in vivo} observations on a xenograft nude mouse model of human HCC, which showed that curcumin inhibited tumor growth, downregulated BCLAF1 expression and pathway, and enhanced mitochondrial apoptosis. In conclusion, this study indicates that in HCC, curcumin may regulate the BCLAF1-mediated induction of mitochondrial apoptosis via the PI3K/AKT/GSK-3β pathway, thereby suppressing tumor growth.

\textbf{Declarations}

\begin{itemize}
  \item \textbf{Funding}
  
  This work was supported by the National Natural Science Foundation of China (81760728).

  \item \textbf{Competing interests}

  The authors have no relevant financial or non-financial interests to disclose.
\end{itemize}
• Ethics approval

The experiment procedures were approved by the Institutional Animal Care and Use Committee of Yanbian University.

• Consent to participate

Not applicable.

• Consent for publication

Not applicable.

• Availability of data and materials

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

• Code availability

Not applicable.

• Authors’ contributions

Xuewu Zhang and Chunhua Bai proposed the original design of this study; Jiaqi Zhao and Jielin Su conducted the experimental research and wrote the first draft of the paper. The remaining authors contributed to the improvement of the study, including literature retrieval, data analysis and the final draft of the paper.

References


**Figures**

**Figure 1**

Curcumin inhibited the proliferation of HCC cell lines. (a) Chemical structure of curcumin. (b) HepG2 and SK-Hep-1 cells were treated with curcumin at 10 µM, 20 µM, 40 µM, and 60 µM, and the rate of cell growth was examined using CCK-8 analysis and compared with that of the control group. Cell growth in cells treated with curcumin at 40 µM and 60 µM was reduced significantly. (c) HepG2 and SK-Hep-1 cells were treated with curcumin at 20 µM, 40 µM, and 60 µM or with 5FU at 10 µM. Cell cycle analysis using flow cytometry after propidium iodide staining revealed that, compared with the control group, the amount of DNA increased significantly with increasing concentrations of curcumin. * and ** indicate $P<0.05$ and $P<0.01$, respectively.
Figure 2

Curcumin promoted apoptosis in HCC cells. HepG2 and SK-Hep-1 cells were treated for 24 h with curcumin at 20 µM, 40 µM, and 60 µM, or with 5FU at 10 µM. (a) Hoechst 33258 staining and analysis of apoptotic cells under a fluorescent microscope (magnification: 200X). (b) Apoptosis analysis using flow cytometry after Annexin V and propidium iodide staining revealed that, compared with the control group, apoptosis increased with increasing curcumin concentration. (c) Western blot analysis of Cleaved...
caspase-3 and Cleaved caspase-9 levels. (d) Western blot analysis of the levels of mitochondrial apoptosis-related proteins, Bax, Bcl-2, and Cyt-c. (e) Analysis of mitochondrial membrane potential under a fluorescence microscope after staining with the fluorescent probe, JC-1 (magnification: 400X). * and ** indicate $P<0.05$ and $P<0.01$, respectively.

Figure 3

Curcumin inhibited the PI3K/AKT/GSK-3β pathway. HepG2 and SK-Hep-1 cells were treated with curcumin at 20 µM, 40 µM, and 60 µM, or with 5FU at 10 µM for 24 h. (a) Western blot analysis of the levels of the PI3K/AKT/GSK-3β pathway proteins PI3K, p-PI3K, AKT, p-AKT, GSK-3β, and p-GSK-3β. (b) Western blot analysis of the levels of the apoptotic proteins, Bax and Bcl-2, and the pathway proteins p-PI3K, p-AKT, and p-GSK-3β, after treatment with LY294002.
Figure 4

Curcumin enhanced apoptosis in HCC cells after BCLAF1 knockout. HepG2 and SK-Hep-1 BCLAF1 knockout cells were treated with 40 µM curcumin for 24 h. (a) Western blot analysis of BCLAF1 expression levels compared with the control group. (b) Western blot analysis of expression and the PI3K/AKT/GSK-3β pathway proteins p-PI3K, p-AKT, and p-GSK-3β. (c) Western blot analysis of levels of the apoptotic proteins Bax, Bcl-2, and Cyt-c compared with the control group and the curcumin (40 µM)
group. (d) Cell cycle analysis using flow cytometry revealed that, compared with the control group, cell cycle arrest in the G0/G1 phase was significantly higher in the curcumin (40 μM) group. (e) Apoptosis analysis using flow cytometry showed that, compared with the control group, apoptosis was significantly increased in the curcumin (40 μM) group. (f) Analysis of changes in membrane potential under a fluorescence microscope (magnification: 400X). *, **, †, and ‡‡, indicate $P<0.05$, $P<0.01$, $P<0.05$, and $P<0.01$, respectively.
Figure 5

Curcumin inhibited BCLAF1-regulated tumor growth in a nude mouse xenograft model of HCC and mediated mitochondrial apoptosis via the PI3K/AKT/GSK-3β pathway. (a) The nude mice were treated with curcumin at 20 µM, 40 µM and 60 µM, or with 5FU at 10 µM for 15 days. Mice were sacrificed under anesthesia and the tumors were collected, examined, and imaged. (b) Comparison of tumor volume (mm$^3$) in treated mice and control mice. c) Tumor suppression rate in treated mice (%) compared with the control group. (d) H&E analysis (magnification: 100X). (e) Subcellular localization and expression level of Bax, Bcl-2, and BCLAF1 were determined using immunohistochemical staining and imaged under a microscope (magnification: 400X). (f) Western blot analysis of the expression levels of the PI3K/AKT/GSK-3β pathway proteins, PI3K, p-PI3K, AKT, p-AKT, GSK-3β, and p-GSK-3β in treated mice compared with the control group. * and ** indicate $P<0.05$ and $P<0.01$, respectively.