Digestive and Liver Disease 51 (2019) 120–126

Contents lists available at ScienceDirect

Digestive and Liver Disease

journal homepage: www.elsevier.com/locate/dld

Liver, Pancreas and Biliary Tract

Both glypican-3/Wnt/β-catenin signaling pathway and autophagy contributed to the inhibitory effect of curcumin on hepatocellular carcinoma

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ARTICLE INFO

Article history:
Received 8 February 2018
Accepted 11 June 2018
Available online 21 June 2018

Keywords:
Apoptosis
Curcumin
Glypican-3
Hepatocellular carcinoma
HepG2
Wnt/β-catenin

ABSTRACT

Aim: The aim of this study is to investigate the role of glypican-3(GPC3)/wnt/β-catenin signaling pathway and autophagy in the regulation of hepatocellular carcinoma (HCC) growth mediated by curcumin.
Methods: HepG2 cells were treated with various concentrations of curcumin and/or GPC3-targeting siRNA in the presence or absence of 3-MA. Cell proliferation and apoptosis were determined by MTT and TUNEL assay, respectively. Expression of GPC3, β-catenin, c-myc, LC3, and Beclin1 was determined by western blotting. In addition, curcumin was tested in tumor xenografts mice model, Caliper IVIS Lumina II was used to monitor the tumor growth, and GPC3/wnt/β-catenin signaling proteins were determined by western blotting.
Results: Curcumin treatment led to proliferation inhibition and apoptosis induction in HepG2 cells in a concentration-dependent manner, and suppressed HCC tumor growth in vivo. Further analysis showed that curcumin treatment inactivated Wnt/β-catenin signaling and decreased GPC3 expression, silencing of GPC3 expression promoted the effects of curcumin on Wnt/β-catenin signaling. In addition, inhibiting autophagy by 3-MA relieved curcumin-dependent down-regulation of GPC3.
Conclusion: Curcumin suppressed HCC tumor growth through down-regulating GPC3/wnt/β-catenin signaling pathway, which was partially mediated by activation of autophagy.

1. Introduction

Hepatocellular carcinoma (HCC) is the most common liver malignancy, ranking as the fifth common cancer worldwide and the third common cause of cancer-related death [1]. It is estimated that there were 782,500 new cases of liver cancer in 2012 worldwide, and death rates of HCC have increased by 2.3% in men and 1.4% in women from 2006 to 2010, of which 466,100 new liver cases and 422,100 liver-related deaths occurred in China [2]. Even with the guidance of a treatment algorithm, treatment of patients with HCC is complicated. Surgical resection is the primary choice for treating HCC, but this treatment is often futile because patients with HCC are generally diagnosed at a late stage. Therefore, pharmaceutical drugs are indispensable for HCC treatment, especially for advanced HCC patients. Unfortunately, there are few drugs that are effective for clinical use, and thus, more efficient therapeutic agents remain to be developed.

Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is the primary active constituent extracted from the plant *Curcuma longa*. It is well documented that curcumin is endowed with anti-inflammatory, antioxidant and anti-tumor effects with low cytotoxicity to normal cells [3]; hence, curcumin is widely studied for treating various diseases, including HCC. It has been demonstrated that curcumin inhibits HCC growth by inhibiting cell proliferation and inducing apoptosis in vitro and in vivo, and an array of signaling pathways and molecular targets have been identified to be involved in its anti-tumor effects [4–9],

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https://doi.org/10.1016/j.dld.2018.06.012
1590-8658/

such as PI3K/Akt, Wnt/β-catenin, IGF, and VEGF. In addition, curcumin has also been shown to inhibit the epithelial–mesenchymal transition, migration and invasion of HCC cell lines [10–12]. However, the mechanism by which curcumin exerts its effects on HCC was still not clearly elucidated.

Glypican-3 (GPC3) is newly regarded as a potential biomarker for HCC diagnosis since it is highly expressed in HCC tissues but silenced in adult tissues. Recent studies have shown that GPC3 plays an important role in regulating proliferation, apoptosis, invasion and metastasis in HCC cells [13–16] and that silencing GPC3 leads to inhibition of HCC cell proliferation and induction of apoptosis. Further investigation revealed that the Wnt signaling pathway is critical for GPC3-mediated cell growth [14,17,18]. However, whether GPC3 is involved in the regulation of HCC tumor growth induced by curcumin remains unknown. In the present study, the effects of curcumin on GPC3/wnt/β-catenin signaling and the potential mechanism were investigated in vitro and in vivo.

2. Materials and methods

2.1. Chemicals and reagents

Curcumin, rapamycin and 3-MA were purchased from Sigma–Aldrich (St. Louis, MO, USA). Primers and siRNAs for GAPDH and GPC3 were synthesized by Sangon Biotech (Shanghai, China). FastStart Universal SYBR Green Master and protease inhibitor were purchased from Roche (Mannheim, Germany). Mouse anti-human polyclonal antibody against GPC3 was purchased from R&D Systems (Minneapolis, USA). Mouse anti-human monoclonal antibody against c-myc was purchased from Santa Cruz Biotechnology (CA, USA). Rabbit anti-human monoclonal antibodies against from LC3, Beclin-1, β-catenin, and cyclin D1 were obtained from Cell Signaling Technology (Boston, USA). Rabbit anti-human β-actin monoclonal antibody and RIPA lysis buffer were purchased from Google (USA). D-Luciferin was purchased from Cayman Chemical (USA).

2.2. Cell culture

HepG2 cells were purchased from ATCC (Manassas, USA) and maintained in DMEM with 10% FBS (Gibco, America), 1% penicillin-streptomycin (10,000 U/ml penicillin, 10 mg/ml streptomycin) at 37 °C in a humidified atmosphere containing 50 ml/l CO_2, and 0.25% trypsin was used for cell passage.

2.3. MTT assay

HepG2 cell viability was measured by MTT assay using a Cytotoxicity Detection kit (Beyotime, China). Briefly, HepG2 cells were plated in 96-well plates at a density of 2×10^4 cells/well and were allowed to adhere and grow for 24 h. The medium was then replaced with 100 μl/well of fresh medium containing various concentrations (0, 5, 10, 20 μmol/l) of curcumin for 24–72 h. Then, 20 μl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) was added and incubated at 37 °C for 4 h. Next, the medium was removed, and 150 μl of DMSO was added to each well. After the crystals were fully dissolved, absorbance was measured at 490 nm with Microplate Reader.

2.4. TUNEL apoptosis assay

Various concentrations of curcumin and siRNA-induced apoptosis of HepG2 cells was determined using a One Step TUNEL Apoptosis Assay Kit (Beyotime, China) according to the manufacturer's instructions. Briefly, curcumin-treated cells were washed twice with ice-cold PBS and fixed with Immunol Staining Fix Solution (Beyotime, China), and after permeabilizing with 0.5% Triton X-100 for 5 min, the cells were incubated with TUNEL detection reagent at 37 °C for 1 h, cellular fluorescence was observed under a fluorescence microscope.

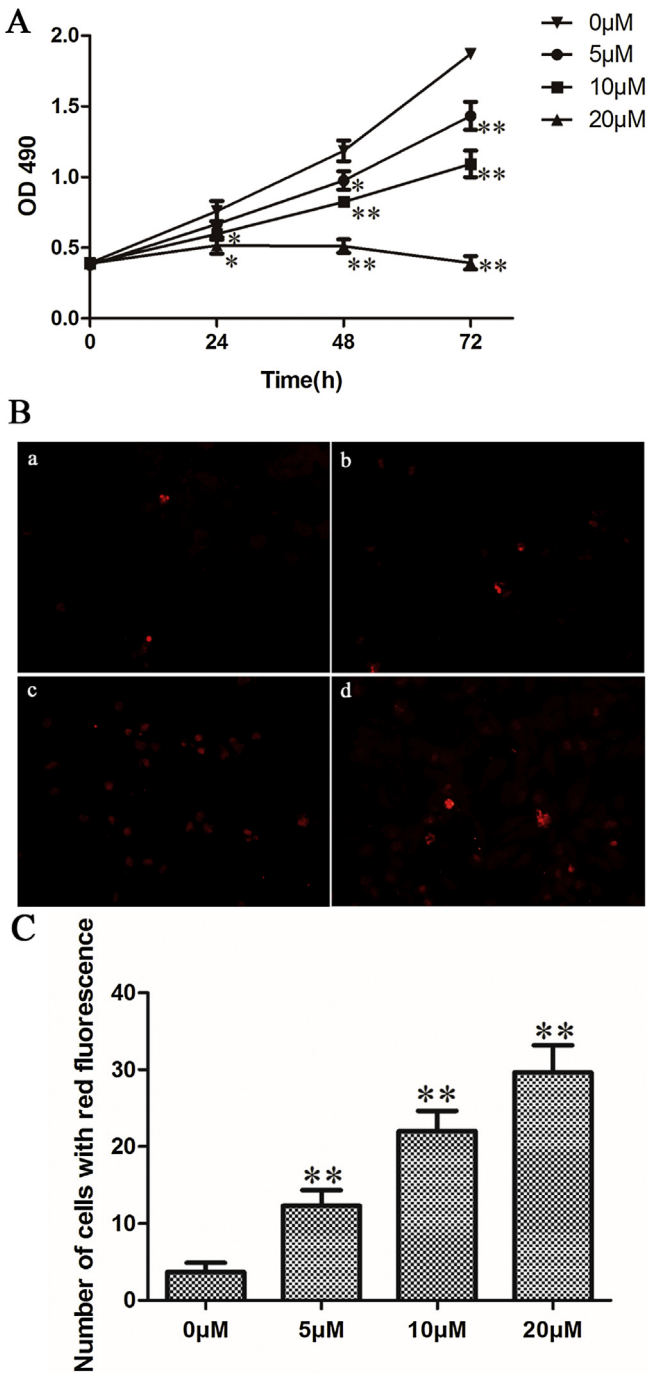

Fig. 1. Curcumin inhibited HepG2 cell proliferation and induced cell apoptosis. A, HepG2 cells were treated with various concentration of curcumin for 24–72 h, cell proliferation was determined by MTT assay (n = 3). B and C, HepG2 cells were treated with various concentration of curcumin for 24 h, cell apoptosis was determined by TUNEL assay (n = 3). a, 0 μM; b, 5 μM; c, 10 μM; d, 20 μM. *p < 0.05 vs. 0 μM; **p < 0.01 vs. 0 μM.

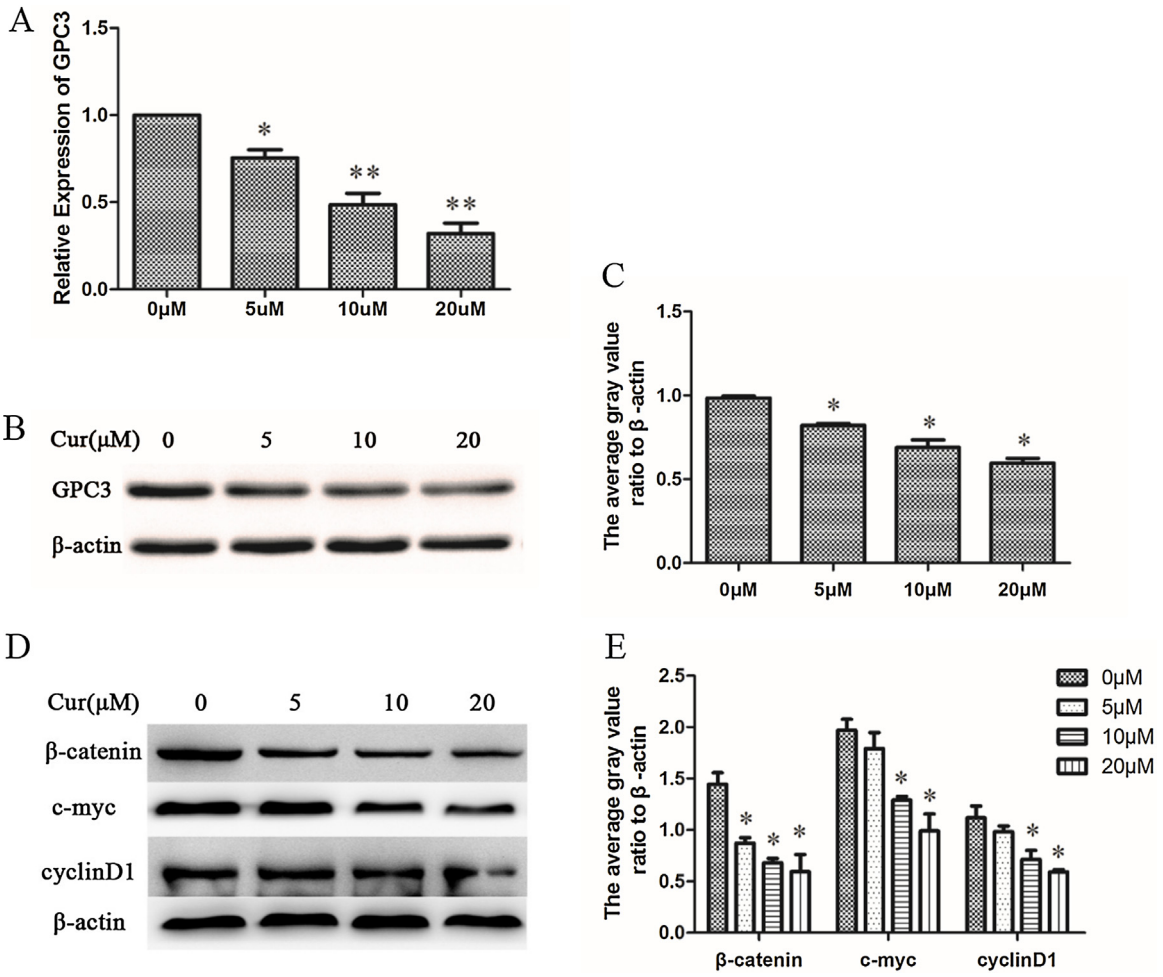

Fig. 2. Curcumin inhibited GPC3 expression and Wnt/β-catenin signaling in HepG2 cells. A, HepG2 cells were treated with curcumin at concentrations of 0, 5, 10, and 20 μM. GPC3 mRNA levels were determined by real-time RT-PCR (n = 3). B and C, Protein levels of GPC3 induced by curcumin were determined by western blotting. D and E, Expression of β-catenin, c-myc, and cyclin D1 were determined by Western blotting. *p < 0.05 vs. 0 μM; **p < 0.01 vs. 0 μM.

2.5. Real-time PCR

Cells were washed twice with ice-cold PBS, and 1 ml TRIzol was added into each 6-well plate. Total RNA was extracted using TRIzol reagent according to the manufacturer's instructions. RNA purity and concentration were measured using a UV spectrophotometer. Approximately 1 μg of total RNA from each sample was used to synthesize cDNA using a High-Capacity cDNA Reverse Transcription Kit (Waltham, MA, USA). Then, real-time PCR was performed by using a FastStart Universal SYBR Green Master kit on a StepOne Plus instrument (ABI, USA) using the following program: 30 s at 95 °C, 60 s at 60 °C, for 40 cycles. Primers used for PCR were as follows: GAPDH-F: 5′-CTGGGCTACACTGAGCACC-3′; GAPDH-R: 5′-AAGTGGTCGTTGAGGGCAATG-3′; GPC3-F: 5′-ATTGGCAAGTTATGTGCCCAT-3′; GPC3-R: 5′-TTCGGCTGGATAAGGTTTCTTC-3′, and the sizes of the products were 130 bp and 101 bp, respectively.

2.6. Western blot analysis

After treatment with curcumin, HepG2 cells were washed twice with ice-cold PBS, harvested in RIPA lysis buffer containing protease inhibitors and then lysed for 30 min on ice. Lysates were centrifuged at 12,000 rpm for 15 min at 4 °C, and the supernatant was collected. Protein concentration was determined by BCA (bicinchoninic acid, Beyotime, China) analysis. Equal amounts of protein extracts were separated by SDS-PAGE and then transferred onto a nitrocellulose membrane. The membranes were incubated with 5% non-fat milk for 1 h and then incubated with primary antibodies overnight at 4 °C; all antibodies were diluted 1:1000 with 3% non-fat milk. After washing with tris-buffered saline with Tween-20, the membranes were incubated with HRP-conjugated secondary antibodies at room temperature (RT) for 2 h. Immunoreactive proteins were visualized using an ECL kit (Millipore, Billerica, USA).

2.7. siRNA transfection

siRNA for GPC3 (sense: 5′-CCAGUGGUCAGUCAAAUUATT-3′ and antisense: 5′-UAAUUUGACUGACCACUGGTT-3′) and negative control siRNA (si-NC) were purchased from Shanghai Sangon Biotech (Shanghai, China). HepG2 cells were seeded into six-well plates and were cultured overnight. The cells at 70% confluence were then transfected with 20 nmol/L siRNA using Lipofectamine 2000 according to the manufacturer's protocol.

2.8. Tumor xenografts

Six 4-week-old BALB/c nude female mice were purchased from SJA Laboratory Animal Center (Hunan, China) and housed in SPF Animal Center in Hubei University of Medicine. Sterilized food and water were accessible ad libitum. All 6-week-old nude mice were randomly divided into one of two groups: a control group and cur-

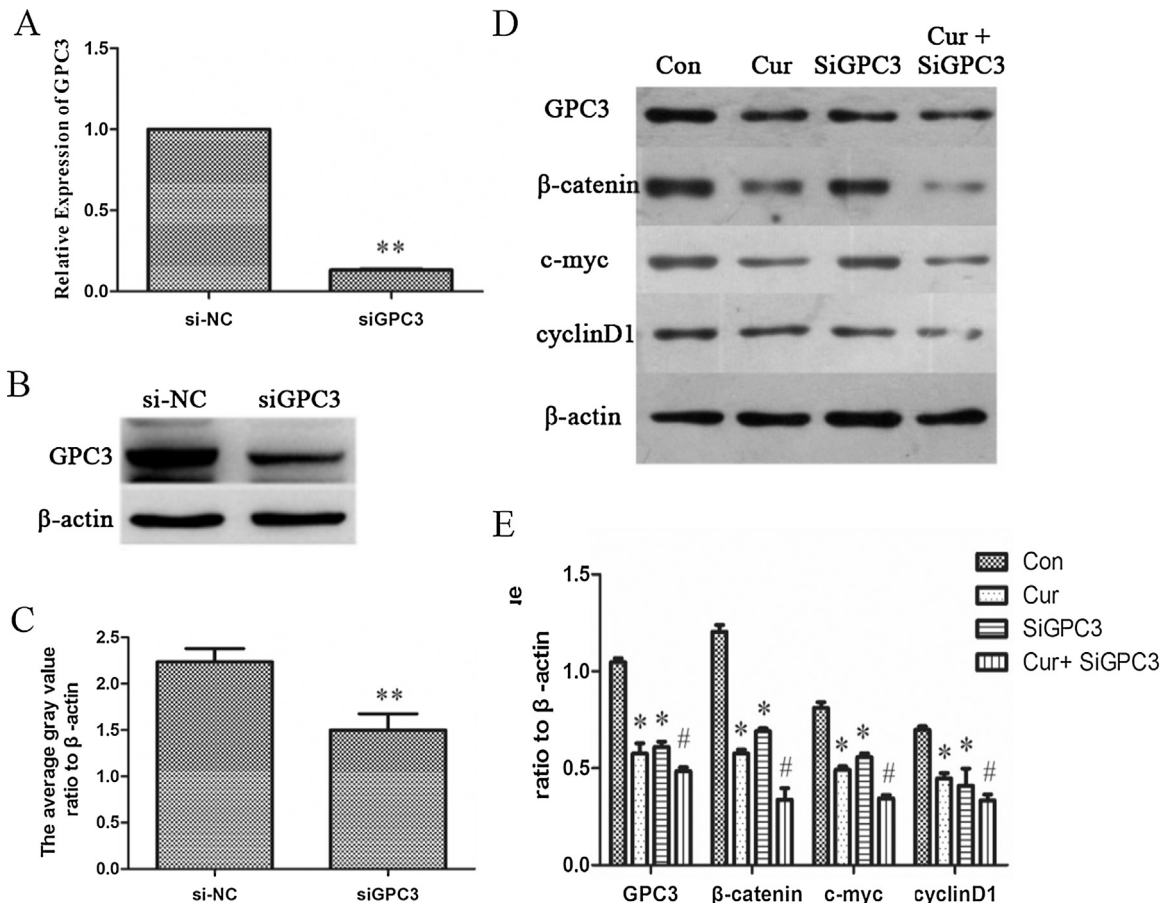

Fig. 3. Silencing of GPC3 inhibited wnt/β-catenin signaling in HepG2 cells. A, GPC3 mRNA level in HepG2 cells transfected with 20 nM siRNA for 12 h was determined by Real-time PCR (n = 3). **p < 0.01 vs. si-NC. B and C, GPC3 protein level in HepG2 cells transfected with 20 nM siRNA for 24 h was determined by Western blot (n = 3). **p < 0.01 vs. si-NC. D and E, Expression of GPC3, β-catenin, c-myc and cyclinD1 in HepG2 cells transfected with 20 nM siRNA in the presence or absence of 20 μM curcumin (n = 3). Con: control. Cur: curcumin. *p < 0.05 vs. Con; #p < 0.05 vs. Cur.

cumin group. A HepG2 cell line with stable luciferase expression (HepG2-luc) was generated, cultured, and then resuspended in PBS at 10^7 cells/ml. Next, 10^6 HepG2-luc cells in 0.1 ml of PBS were subcutaneously injected into both lower limbs of mice one day prior to curcumin treatment. Mice in the curcumin group were treated with 200 mg/kg curcumin per day by intraperitoneal injection for one month, while mice in the control group were injected with an equal volume of the solvent. Tumor species were obtained from the mice after they were anethetized with persistent isoflurane. All experiments were approved by the Institutional Animal Care and Use Committee of Hubei University of Medicine, and all animals received humane care and that study protocols comply with the institution's guidelines and animal research laws.

2.9. Caliper IVIS Lumina

Tumors were imaged by IVIS 200. Before imaging, 450 μl of 10 mg/ml D-luciferin dissolved in PBS was intraperitoneally injected into each mouse, and 15 min later, the mice were anesthetized with isoflurane and imaged by the IVIS 200 imaging system.

2.10. Statistics

Statistical analysis was conducted using SPSS, Version 22.0 (SPSS, Inc., Chicago, IL, USA). Each experiment was performed at least 3 times. Data are presented as the mean values ± standard deviation and were analyzed using Student's t-test. $P < 0.05$ was considered a statistically significant difference.

3. Results

3.1. Curcumin inhibited HepG2 cell proliferation and induced apoptosis by down-regulating GPC3 expression and wnt/β-catenin signaling

To investigate the role of curcumin in HCC cells, we initially examined the proliferation and apoptosis induced by various concentrations of curcumin (0, 5, 10, and 20 μmol/l) in HepG2 cells for 24–72 h by MTT and TUNEL assay, respectively. OD values represent cell viability or cell numbers. As shown in Fig. 1A, cell viability was significantly inhibited by curcumin in a dose- and time-dependent manner, and a considerable decrease of cell number was observed in the HepG2 cells treated with 20 μmol/l curcumin. This finding suggests a pro-apoptotic effect of curcumin in HepG2 cells, which was confirmed with increased cellular fluorescence by TUNEL assay (Fig. 1B, C).

To identify the effect of curcumin on the expression of GPC3, HepG2 cells were cultured in the presence of various concentrations of curcumin (0, 5, 10, and 20 μmol/l) for 24 h. As showed in Fig. 2A–C, curcumin treatment significantly decreased the expression of GPC3 at both the mRNA and protein levels in a dose-dependent manner. Furthermore, the expression levels of β-catenin, c-myc and cyclin D1 were also significantly reduced upon curcumin treatment (Fig. 2D, E).

3.2. GPC3 silencing enhanced curcumin-induced HepG2 cell apoptosis

To further explore the role of GPC3 in the anti-tumor effect induced by curcumin, GPC3-targeting siRNA was transfected into HepG2 cells in the presence or absence of curcumin. Using 20 nmol/l siRNA, GPC3 expression was decreased at both the mRNA and protein levels (Fig. 3A–C). It has been revealed that the cell surface attachment of GPC3 is required for Wnt signaling and to stabilize β-catenin in HCC cells [14]. In the present study, GPC3 silencing was shown to significantly reduce the expression levels of β-catenin, c-myc and cyclin D1 (Fig. 3D, E). Since curcumin inhibited GPC3 expression and the Wnt signaling pathway, we further examined whether the inhibitory effect of curcumin on Wnt signaling is GPC3-dependent. As a result, combined treatment with siGPC3 and curcumin led to a more profound inhibition of the expression levels of GPC3, β-catenin, c-myc and cyclin D1 than siGPC3 or curcumin treatment alone (Fig. 3D, E). In addition, a more potent inhibition of HepG2 cell proliferation (Fig. 4A) and enhanced apoptosis were documented in the case of combined treatment with siGPC3 and curcumin (Fig. 4B, C).

3.3. Curcumin suppressed HCC tumor growth by inhibiting GPC3/Wnt/β-catenin signaling

To verify the role of curcumin in HCC tumor growth in vivo, tumor xenografts were established. After curcumin administration for one month, the area of region of interest (ROI) and tumor sizes of the mice were significantly reduced than those in the control group (Fig. 5), suggesting a potent anti-HCC effect of curcumin in vivo. The western blotting analysis also demonstrated an inhibited expression of GPC3, β-catenin, c-myc and cyclin D1 (Fig. 5E, F), confirming that curcumin suppressed HCC tumor growth by inhibiting GPC3/Wnt/β-catenin signaling.

3.4. Autophagy mediated curcumin-induced inhibition of GPC3 protein expression

Our previous study showed that activation of autophagy could reduce GPC3 protein levels and down-regulate GPC3/Wnt/β-catenin signaling in HepG2 cells [19]. Given that autophagy plays an important role in tumor growth, we speculated that curcumin-dependent decreased expression of GPC3 may be partially mediated by activation of autophagy. To examine curcumin-induced autophagy, the two autophagy biomarkers LC3 and Beclin1 were analyzed by Western blot. Under the stimulation of curcumin for 12 h, the expression levels of both LC3 and Beclin1 were significantly up-regulated in HepG2 cells (Fig. 6A, B), indicating an inducible role of curcumin in autophagy. Next, 3-MA, the most common autophagy inhibitor, was used to disrupt autophagy. As expected, 3-MA pretreatment could maintain GPC3 protein levels in curcumin-treated HepG2 cells, suggesting that blocking autophagy counteracts curcumin-induced GPC3 down-regulation (Fig. 6C, D).

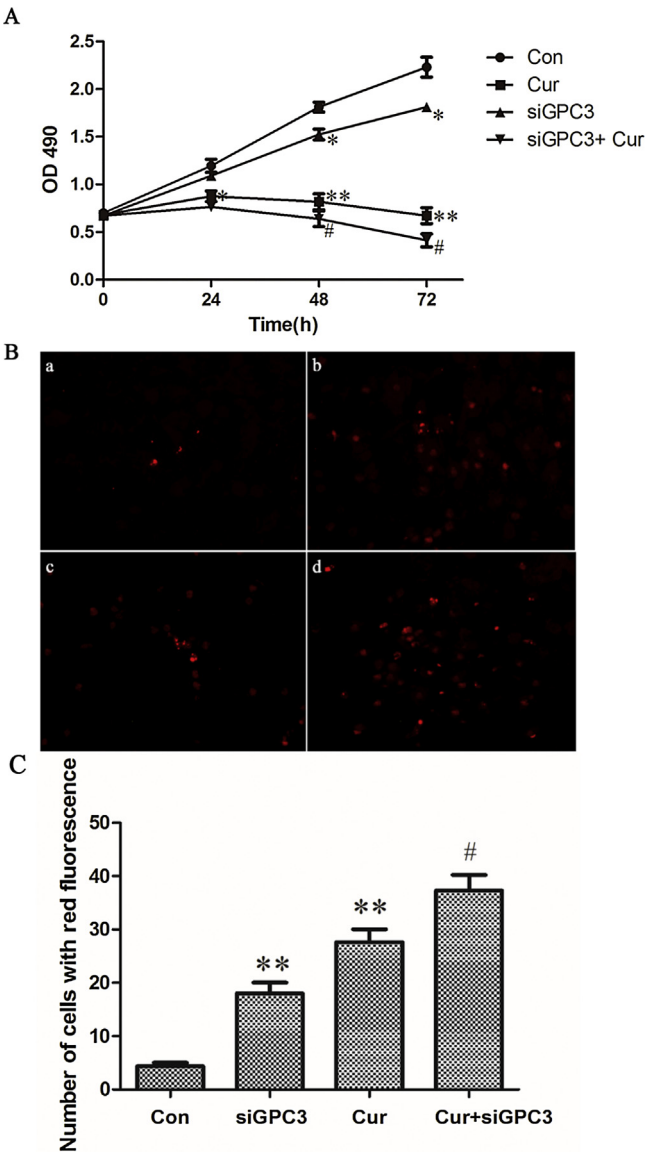

Fig. 4. Silencing of GPC3 promoted apoptosis induced by curcumin. A, After transfected with siRNA for 12 h, HepG2 cells were incubated for another 24 h in the presence or absence of 20 μM curcumin, cell proliferation was assessed by MTT (n = 3). B and C, Apoptosis of HepG2 cells transfected with siRNA in the presence or absence of 20 μM curcumin was assessed by TUNEL (n = 3). a, control; b, curcumin; c, siGPC3; d, curcumin + siGPC3. Con: control; Cur: curcumin. *p < 0.05 vs Con; **p < 0.01 vs siGPC3.

4. Discussion

Curcumin is one of the most widely used spice ingredients and is the primary active polyphenolic compound in *C. longa*. For centuries, curcumin has been extensively used in Ayurvedic medicine in India and South Asia because it is nontoxic to normal cells and exhibits several beneficial properties, such as anti-inflammatory, antioxidant and antiseptic activities [20]. It has been reported that administration of curcumin at 8000 mg per day for 3 months is tolerable for human beings, and thus, curcumin is widely used to treat a range of disease such as rheumatism, cataracts [21], skin diseases [3], and cancers, including HCC [22,23]. Several studies have shown that curcumin has a positive effect on the treatment of HCC, and potential molecular mechanisms for this effect have also been extensively reported. It has demonstrated that curcumin suppresses proliferation and induces apoptosis in HCC cells through several pathways [24] and was also shown to have a synergistic effect on the treatment of HCC when used in combination with other drugs, such as bevacizumab [25].

GPC3 is a member of heparan sulfate proteoglycan family and attaches to the cell membrane by its interaction with glycosyl phosphatidylinositol. A previous study showed that *GPC3* is one of the first transcripts to be expressed during malignant hepatocyte transformation, and GPC3 protein could be detected in approximately 50% of high-grade dysplastic macronodules in cirrhotic liver [26]. A panel of studies showed that GPC3 is highly expressed in most HCC tissues using genomics or histopathological detection methods [27–29], and the level of GPC3 in HCC was significantly associated

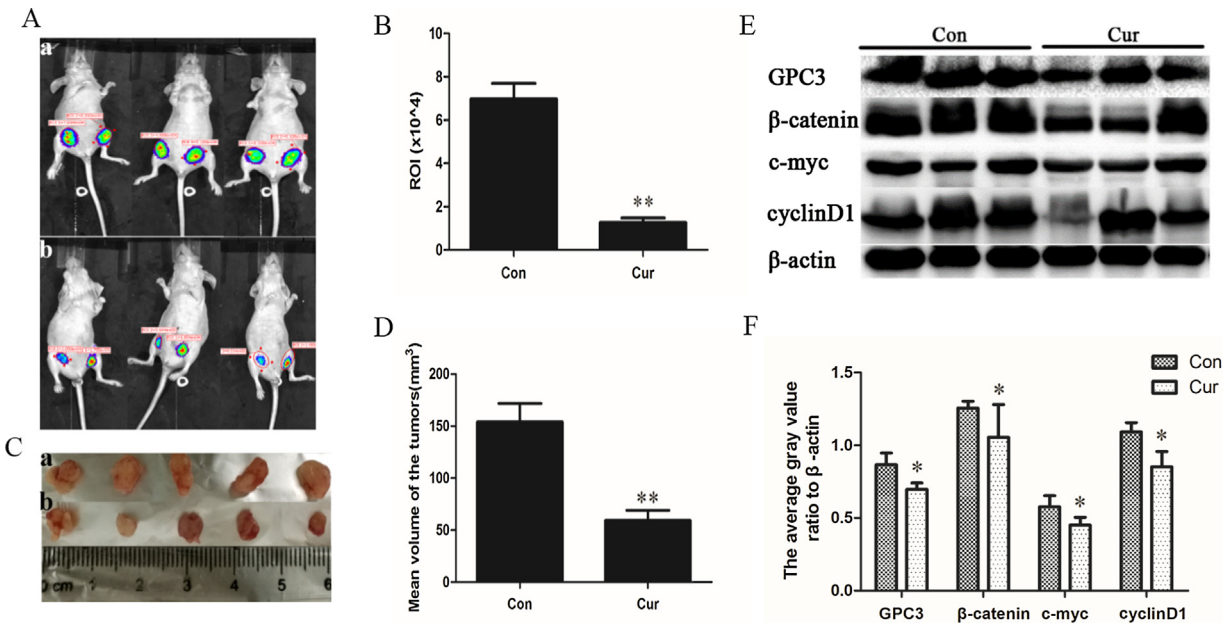

Fig. 5. Curcumin suppressed HCC tumor growth by inhibiting GPC3/Wnt/β-catenin signaling. A and B, Tumor-transplanted nude mice were treated with PBS as control (a, control group) or curcumin (b, curcumin treatment group) for one month, region of interest (ROI) in each mouse was monitored by IVIS. C and D, Tumors were imaged after resection from each mouse and tumor sizes were measured with rulers. D and E, Total protein lysates were prepared from tumor tissues resected from nude mice with xenografts; the levels of GPC3, β-catenin, c-myc, and cyclin D1 were determined by Western blot analysis. Con, control; Cur, curcumin. *p < 0.05; **p < 0.01.

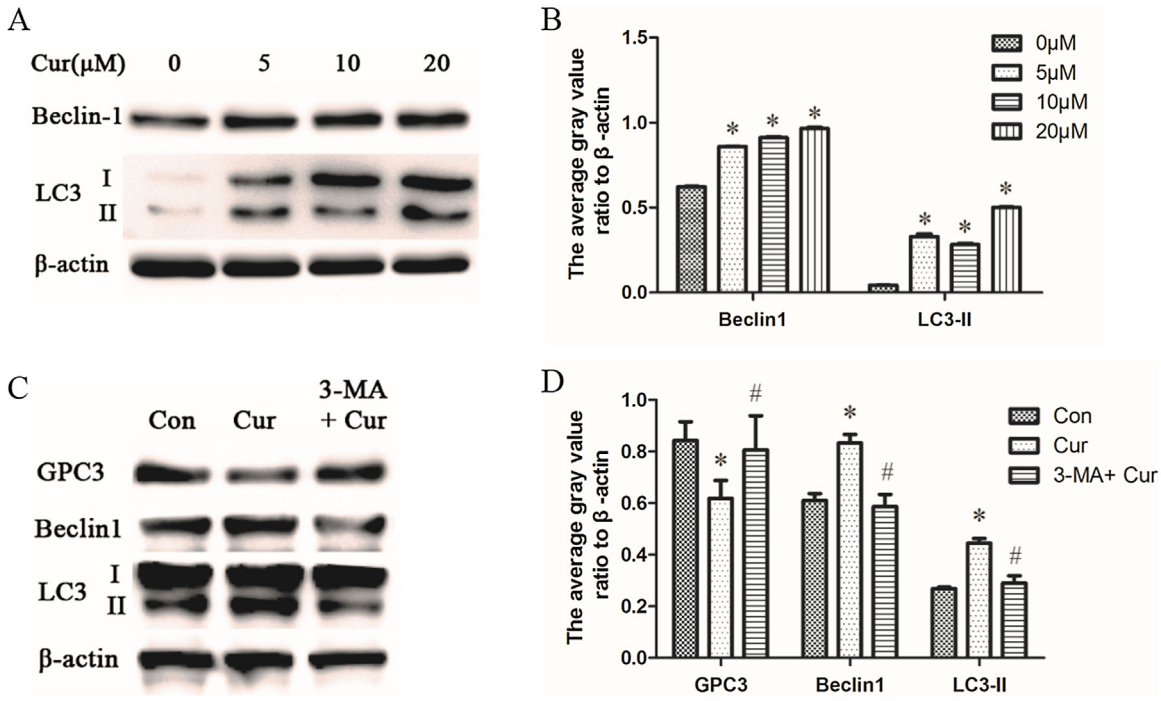

Fig. 6. Curcumin reduced GPC3 expression partially by autophagy activation. A and B, HepG2 cells were treated with curcumin at concentrations of 0, 5, 10, and 20 μM, and expression levels of Beclin1 and LC3 were determined by Western blot analysis. *p < 0.05 vs. 0 μM. C and D, HepG2 cells were stimulated with 20 μM curcumin in the presence or absence of 5 mM 3-MA, the expression levels of GPC3, Beclin1 and LC3 were determined by Western blot analysis. *p < 0.05 vs. Con; #p < 0.05 vs. Cur. Con, control; Cur, curcumin.

with tumor size, histopathological differentiation, and intrahepatic metastasis [30]. Recent studies have reported that GPC3 plays an important role in HCC tumor growth, invasion and metastasis. GPC3-silenced cells exhibited reduced proliferation in vitro and attenuated capacities in developing HCC tumors in nude mice [31].

In the present study, we demonstrated that GPC3 and wnt/β-catenin signaling was involved in the inhibitory effects induced by curcumin. In addition, knockdown of GPC3 inhibited Wnt/β-catenin pathway in HepG2 cells. Further analysis showed that GPC3 silencing enhanced curcumin-directed apoptosis and proliferation inhibition and led to further inhibition of the expression of β-catenin, c-myc and cyclin D1, suggesting that GPC3 was a potential therapeutic target for HCC treatment.

Autophagy is an important mechanism for maintaining cellular homeostasis in mammals by degrading unnecessary or abnormal organelles and proteins. Increasing studies have confirmed that autophagy plays a critical role in hepatocarcinogenesis and HCC development. It has been reported that GPC3 expression in HCC tissues was positively correlated with p62, which functions as an autophagy adapter for substrate degradation, suggesting a significant association between GPC3 expression and autophagy deficiency [32]. Our previous study revealed that GPC3 is negatively regulated by autophagy and that activation of autophagy inhibited the proliferation of HepG2 cells by inhibiting GPC3/Wnt/β-catenin signaling [19]. In the present study, both mRNA and protein levels of GPC3 were decreased upon curcumin treatment, while blockage of autophagy by 3-MA counteracted curcumin-induced GPC3 down-regulation, suggesting that curcumin-directed GPC3 down-regulation may probably, at least partially, occur through activation of autophagy; however, other mechanisms remain to be elucidated.

In conclusion, the present study demonstrated that curcumin represses HCC tumor growth in vitro and in vivo by inhibiting GPC3/Wnt/β-catenin signaling pathway and that this effect was partially mediated by autophagy. Therefore, curcumin may be clinically applied as a treatment or as an adjuvant in HCC treatment, and GPC3 might be an alternative therapeutic target for HCC treatment.

Conflict of interest

None declared.

Funding

This study was supported by National Science Foundation of China [81541140; 81641028]; Foundation for Innovative Research Team of Hubei University of Medicine [2014CXG05]; Foundation for Innovative Research Groups of Natural Science Foundation of Hubei Province of China [2018CFA031], and Key Program for Precision Medicine of Taihe Hospital [2016JZ05].

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