

Curcumin attenuates resistance to irinotecan via induction of apoptosis of cancer stem cells in chemoresistant colon cancer cells

PENGFEI SU^{1*}, YONG YANG^{2*}, GUOXIN WANG¹, XIAOWU CHEN¹ and YONGLE JU¹

¹Department of Gastrointestinal Surgery, Shunde Hospital, Southern Medical University, Shunde, Guangdong 528300; ²Department of General Surgery, Heping Hospital Affiliated with Changzhi Medical College, Changzhi, Shanxi 046000, P.R. China

Received February 6, 2018; Accepted May 30, 2018

DOI: 10.3892/ijo.2018.4461

Abstract. Resistance to conventional chemotherapeutic agents, including irinotecan (CPT-11), 5-fluorouracil and capecitabine is a major cause for therapeutic failure in patients with colorectal cancer (CRC). Increasing evidence has demonstrated that cancer cells exhibiting stem cell-like characteristics are associated with the development of resistance to chemotherapeutic agents. As a plant polyphenol, curcumin has been demonstrated to have the ability to ameliorate resistance of CRC to chemotherapeutic agents, but the associations among curcumin, cancer stem cells (CSCs) and chemoresistance of CRC remain unclear. The present study established a CPT-11-resistant colon cancer cell line, LoVo/CPT-11 cells, and detected the expression levels of CSC identification markers [cluster of differentiation (CD)44, CD133, epithelial cell adhesion molecule (EpCAM) and CD24] in parental cells and CPT-11-resistant cells. It was revealed that the expression levels of the colon CSC markers in LoVo/CPT-11 cells were significantly higher compared those in parental cells at the mRNA and protein level. The effect of curcumin on the chemoresistance to CPT-11 and the expression levels of CSC identification markers in LoVo/CPT-11 cells separately treated

with curcumin and CPT-11 were further investigated. The results revealed that curcumin significantly attenuated chemoresistance to CPT-11, and treatment with curcumin resulted in a significant reduction of the expression levels of CSC identification markers. Furthermore, a tumor sphere formation assay was used to enrich colon CSCs from LoVo/CPT-11 cells, and demonstrated that curcumin efficiently diminished the traits of colon CSCs, as evidenced by the inability to form tumor spheres, the reduction in the expression of CSC identification markers, and apoptosis-induced effects on sphere-forming cells treated with curcumin alone or in combination with CPT-11. Altogether, the present data demonstrated that curcumin attenuated resistance to chemotherapeutic drugs through induction of apoptosis of CSCs among colon cancer cells. These findings may provide novel evidence for the therapeutic application of curcumin in CRC intervention.

Introduction

In the last decade, the incidence and mortality rates of colorectal cancer (CRC) have decreased to a certain extent owing to improved screening, diagnostic and therapeutic technical expertise for cancer treatment. Despite these positive developments, CRC remains one of the most common cancer types and the primary cause for cancer-associated mortality worldwide (1). For the majority of patients with recurrent or metastatic CRC, chemotherapy is a palliative but relatively effective treatment (2). At present, irinotecan (CPT-11) in the presence or absence of other chemotherapeutic agents, including 5-fluorouracil and capecitabine, is used to reduce the symptoms in patients with advanced CRC (3). However, the 5-year survival rate of patients with metastatic or recurrent CRC is <10% (4). Chemoresistance is one of the major obstacles to treating this malignancy (5); thus, the identification of agents that are able to attenuate chemoresistance in cancer cells is required.

Curcumin, a dietary polyphenolic compound extracted from *Curcuma longa*, has been well-researched, and possesses anti-inflammatory, anticancer, apoptotic and anti-metastatic activities (6-10). It regulates several processes, including cell proliferation, genes regulating apoptosis and growth

Correspondence to: Professor Xiaowu Chen or Professor Yongle Ju, Department of Gastrointestinal Surgery, Shunde Hospital, Southern Medical University, 1 Jiazi Road, Shunde, Guangdong 528300, P.R. China

E-mail: 1428616947@qq.com

E-mail: richardju66@163.com

*Contributed equally

Abbreviations: CRC, colorectal cancer; CSC, cancer stem cell; CD133, cluster of differentiation 133; CD44, cluster of differentiation 44; CD24, cluster of differentiation 24; EpCAM, epithelial cell adhesion molecule; CPT-11, irinotecan; Cur, curcumin

Key words: colorectal cancer, chemoresistance, irinotecan, curcumin, cancer stem cell, apoptosis

factors (11). Its low toxicity, low cost and highly efficient anti-cancer functions further demonstrate the potential of curcumin in preventing or treating cancer (12). Accumulating evidence has suggested that curcumin targets cancer stem cells (CSCs) in numerous types of human cancer. For example, Zhu *et al.* (13) reported that lung CSC is suppressed by curcumin through inhibition of the wnt/ β -catenin and sonic hedgehog signaling pathways. Additionally, Mukherjee *et al.* (14) demonstrated that curcumin inhibits breast CSC migration by amplifying the E-cadherin/ β -catenin negative feedback loop. CSCs, which comprise a minor subset of cells within the tumor, are the primary contributor to the development of resistance to chemotherapeutic agents, and subsequent cancer metastasis and recurrence (15,16). It has been reported that the majority of chemotherapeutic agents presently in use lack the capacity to eliminate CSCs (17). However, the associations between curcumin, and colon CSCs and chemoresistance in CRC remain unclear. Furthermore, it has been reported that the resistance of CSCs to chemotherapeutic agents is a result of active DNA repair mechanisms, high expression of ABC transporters and resistance to apoptosis (18). In addition to the aforementioned mechanisms, autophagy is activated in CSCs in response to various anticancer therapies as a protective mechanism, thus providing chemoresistance (19). Visibly, there are a variety of mechanisms associated with the chemoresistance of CSCs. Therefore, the mechanisms of curcumin targeting CSCs in CRC chemoresistance require further investigation.

Based on the aforementioned observations, CPT-11-resistant cells and sphere-forming cells were developed in the present study to explore the effects of curcumin on chemoresistance. Following the induction of resistance to CPT-11 in LoVo cells, the changes in the expression levels of drug-resistant-associated proteins and CSC identification markers were detected. Next, the extent of chemoresistance and the expression levels of CSC markers in CPT-11-resistant cells were evaluated following treatment with curcumin. Furthermore, the effect of curcumin on tumor sphere formation in colon CSCs, as well as the expression levels of CSC identification markers in sphere-forming cells were investigated. Finally, whether curcumin targets CSCs via induction of apoptosis was explored.

Materials and methods

Cell lines and cell cultures. Human colon cancer LoVo cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) (both from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1% penicillin/streptomycin (Beijing Solarbio Science & Technology, Co., Ltd., Beijing, China) in a humidified 37°C incubator with 5% CO₂. CPT-11 (Jiangsu Hengrui Medicine Co., Ltd., Lianyungang, China)-resistant cells, referred to as LoVo/CPT-11 cells, were established by repetitive treatment of the LoVo cells with increasing concentrations of CPT-11 over a 10-12-month period, based on the methods described in our previous studies (20).

Antibodies and reagents. Primary rabbit polyclonal antibodies against cluster of differentiation (CD)133 (cat. no. 18470-1-AP), CD44 (cat. no. 15675-1-AP), epithelial cell adhesion molecule

(EpCAM; cat. no. 21050-1-AP), CD24 (cat. no. 18330-1-AP) and β -actin (cat. no. 20536-1-AP) were purchased from ProteinTech Group, Inc. (Chicago, IL, USA). Rabbit polyclonal antibodies against ATP binding cassette subfamily B member 1 (ABCB1; cat. no. ab155421), cleaved caspase-3 (cat. no. ab13847), cleaved caspase-9 (cat. no. ab2324), cleaved caspase-8 (cat. no. ab25901), BCL2 associated X apoptosis regulator (Bax; cat. no. ab53154), apoptosis regulator Bcl-2 (Bcl-2; cat. no. ab196495), and the horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibodies (cat. no. ab7090), were purchased from Abcam (Cambridge, UK). Curcumin was purchased from Merck KGaA (Sigma-Aldrich, Darmstadt, Germany).

Growth inhibition assay. A Cell Counting kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used to assess the inhibition of cell growth in response to CPT-11 (0, 5, 10, 20, 40, 80, 120, 160 and 200 μ M) and curcumin (0, 2.5, 5, 10, 15, 20, 25, 30, 35 and 40 μ M). Briefly, LoVo and LoVo/CPT-11 cells (1.0×10^4 cells/well) were seeded onto 96-well plates in 100 μ l culture medium (RPMI-1640, 10% FBS and 1% penicillin/streptomycin)/well. Drugs diluted with the culture medium were added to each well after 24 h of plating and then co-incubated for 24 h. At the end of the treatment, the culture medium was replaced with fresh culture medium containing 10% CCK-8 reagent and the reaction was allowed to proceed for 2 h. Absorbance was measured at the wavelength of 450 nm. All assays were performed as three replicates and the results were indicated as a percentage compared with the corresponding control group.

Tumor sphere formation assay. LoVo/CPT-11 cells were cultured in serum-free Dulbecco's modified Eagle's medium/F12 (Gibco; Thermo Fisher Scientific, Inc.) with 20 ng/ml epidermal growth factor, 10 ng/ml basic fibroblast growth factor (all from PeproTech, Inc., Rocky Hill, NJ, USA), 2% B27 supplement (Thermo Fisher Scientific, Inc.), and 5 μ g/ml insulin (Sigma-Aldrich; Merck KGaA). Cells were then plated into ultralow-attachment 6-well plates (Corning Incorporated, Corning, NY, USA) at a density of 2.0×10^4 cells/well, and fresh medium was added every two days. Tumor sphere formation was observed, and images were captured using a light microscope (original magnification, $\times 100$; Olympus BX51; Olympus Corporation, Tokyo, Japan).

Detection of CD133-positive cells by flow cytometry. The percentages of CD133-positive cells in LoVo, LoVo/CPT-11 and sphere-forming cells were measured using a flow cytometry assay. Briefly, cells were separately centrifuged (4°C, 5 min, 100 \times g) and washed with ice-cold PBS. Subsequently, 1×10^6 cells were incubated with 5 μ l of phycoerythrin-conjugated mouse anti-human monoclonal antibody CD133 (1:50; cat. no. 12-1339-41; Affymetrix; Thermo Fisher Scientific, Inc.) in the dark at 4°C for 1 h. Next, the cells were washed with ice-cold PBS and each sample was measured using a flow cytometer. The results were analyzed with FlowJo 7.6.1 software (FlowJo LLC, Ashland, OR, USA).

Effects of curcumin on cells. To analyze the effect of curcumin on the chemoresistance of LoVo/CPT-11 cells, different

concentrations of curcumin (0, 2.5 and 5 μ M) were used to treat LoVo/CPT-11 cells in combination with CPT-11, then the results were analyzed via a growth inhibition assay. The half maximal inhibitory concentration (IC_{50}) of CPT-11 in cells treated with curcumin was calculated. Furthermore, cells were treated with different concentrations of curcumin (0, 2.5 and 5 μ M) and CPT-11 (0, 10, 20, 40 and 100 μ M) individually for 24 h, and the expression levels of CSC identification markers were further explored using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis. For colon cancer CSCs, sphere-forming cells were treated with various concentrations of curcumin (0, 2.5, 5, 10 and 20 μ M) and CPT-11 (0, 10, 20, 40 and 100 μ M) separately or in combination. For the combined experiment, sphere-forming cells were separated into four groups: Control group, curcumin (5 μ M) group, CPT-11 (100 μ M) group and combined group (5 μ M curcumin and 100 μ M CPT-11). After 3 days of treatment, the tumor spheres with a sphere diameter $>50 \mu$ m were imaged and counted the sphere-forming efficiency (SFE) was normalized to the control group and calculated as a percentage. After 3 days of various treatments, the expression of CSC identification markers and apoptosis-associated proteins were detected.

Apoptosis assessment by flow cytometry. Apoptosis distribution was detected using a fluorescein isothiocyanate Annexin V Apoptosis Detection kit (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's protocol. In brief, sphere-forming cells were treated with curcumin and CPT-11 individually and in combination for 24 h, then cells were collected and washed with cold PBS twice. Subsequently, the cells were resuspended in 100 μ l binding buffer, and incubated with Annexin V (5 μ l) and PI (1 μ l) at 4°C in the dark for 15 min. Finally, the samples were detected using a flow cytometer within 1 h. Results were analyzed using WinMDI v2.9 software (The Scripps Research Institute, San Diego, CA, USA).

RT-qPCR assay. Total cellular mRNA from LoVo, LoVo/CPT-11 and sphere-forming cells were extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Subsequently, 500 ng of purified mRNA was reverse-transcribed into cDNA following the manufacturer's protocol (Beijing Transgen Biotech Co., Ltd., Beijing, China). The qPCR amplification was performed in triplicate using a Light Cycler[®] 480 II (Roche Applied Science, Penzberg, Germany). The reaction system (20 μ l) contained the cDNA template, forward and reverse primers, ddH₂O, and 2X SYBR Green qPCR Super Mix (Beijing Transgen Biotech Co., Ltd.). The PCR steps included incubations for 10 min at 95°C, followed by 40 cycles with each cycle consisting of 15 sec at 95°C and 1 min at 60°C. Expression levels of each target gene were standardized using β -actin as an internal control, and the result was calculated using the $2^{-\Delta\Delta C_q}$ method (21). The primers of CD133, CD44, EpCAM, CD24, ABCB1 and β -actin (Sangon Biotech Co., Ltd., Shanghai, China) were used as follows: CD133 forward, 5'-TTCTTGACCGACTGAGACCCA-3' and reverse, 5'-TCATGTTCTCCACGCCTCTT-3'; CD44 forward, 5'-CTGCCGCTTTCAGGTGTA-3' and reverse, 5'-CATTGTGGGCAAGGTGCTATT-3'; EpCAM forward, 5'-AATCGTCAATGCCAGTGTACTT-3'

and reverse, 5'-TCTCATCGCAGTCAGGATCATAA-3'; CD24 forward, 5'-CTCCTACCCACGCAGATTTATTC-3' and reverse, 5'-AGAGTGAGACCACGAAGAGAC-3'; ABCB1 forward, 5'-TTGCTGCTTACATTCAGGTTTCA-3' and reverse, 5'-AGCCTATCTCCTGTCGCATTA-3'; β -actin forward, 5'-CATGTACGTTGCTATCCAGGC-3' and reverse, 5'-CTCC TTAATGTACGCACGAT-3'.

Western blot assay. Cells were collected using a plastic scraper, washed with cold PBS and then solubilized in ice-cold protein extract solution RIPA containing protease inhibitors (Beyotime Institute of Biotechnology, Shanghai, China). The supernatant was used for western blot analysis following clarification. Protein concentration was determined using a BCA kit (Beyotime Institute of Biotechnology) and standardized between the samples. Equal amounts (30 μ g) of protein were then separated by SDS-PAGE using 8-12% polyacrylamide gel and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with Tris-buffered saline Tween-20 (TBST) containing 5% skim milk for 1 h at room temperature. The membranes were then incubated in primary antibodies including anti-CD44, anti-CD133, anti-EpCAM, anti-CD24 (all 1:500), anti-ABCB1, anti- β -actin (both 1:3,000), anti-cleaved caspase-3, anti-cleaved caspase-9, anti-cleaved caspase-8, anti-Bax, anti-Bcl-2 (all 1:1,000) at 4°C overnight. Following washing with TBST, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (1:5,000) for 1 h at room temperature. The bands were visualized using a Western Bright ECL HRP substrate kit (EMD Millipore) in a Kodak Image station (Kodak, Rochester, NY, USA). The expression levels of the proteins were analyzed using ImagingJ 1.48 software (National Institutes of Health, Bethesda, MD, USA). Specific β -actin protein was used as the loading control to normalize the sample amounts.

Statistical analysis. All data are expressed as the mean \pm standard deviation of three independent experiments. Statistical comparisons were determined using unpaired t-tests or one-way analysis of variance with Tukey's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference. All statistical calculations were performed using SPSS software (version 20.0; IBM Corp., Armonk, NY, USA), Graphs were prepared using GraphPad Prism software (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA).

Results

CPT-11-resistant cells express higher levels of ABCB1 and CSC markers compared with parental cells. Via long-term culturing of original CPT-11-sensitive LoVo cells by gradual adaptation to increasing CPT-11 concentrations, CPT-11-resistant LoVo cells were successfully established. The resistance to CPT-11 in LoVo/CPT-11 cells was examined by treating these cells with different concentrations of CPT-11. The growth inhibitory rates were compared with those of their parental cells using a CCK-8 assay. The results demonstrated that the growth inhibitory rates of LoVo/CPT-11 cells treated with CPT-11 were significantly decreased when compared with the parental cells from concentration $\geq 5 \mu$ M (Fig. 1A). The IC_{50} of CPT-11 in LoVo/CPT-11

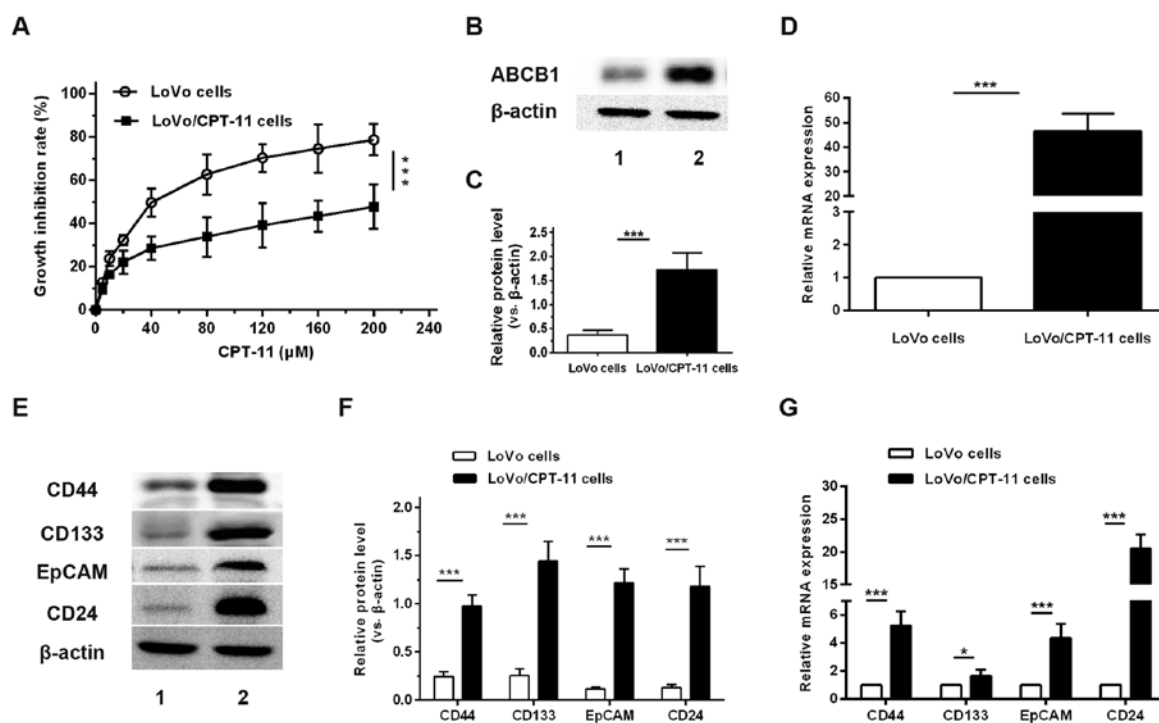


Figure 1. Growth inhibitory rates, and the expression of ABCB1 and CSC identification markers in LoVo and LoVo/CPT-11 cells. (A) The growth inhibitory rates of LoVo and LoVo/CTP-11 cells treated with various concentrations of CPT-11 for 24 h, as determined using a cell counting kit-8 assay. (B) Representative western blotting image, (C) quantification of protein expression and (D) mRNA expression of ABCB1 in LoVo and LoVo/CPT-11 cells. (E) Representative western blotting image, (F) quantification of protein expression and (G) mRNA expression of CSC identification markers in LoVo and LoVo/CPT-11 cells. Relative protein expression was measured using ImageJ. β -actin was used as a loading control. Each bar represents the mean \pm standard deviation of three independent experiments. * $P < 0.05$ and *** $P < 0.001$ vs. LoVo cells. 1, LoVo cells; 2, LoVo/CPT-11 cells. ABCB1, ATP-binding cassette Subfamily B Member 1; CSC, cancer stem cell; CPT-11, irinotecan.

cells were $268.72 \pm 2.43 \mu\text{mol/l}$, remarkably higher compared with that of parental cells ($43.27 \pm 1.64 \mu\text{mol/l}$). The expression of ABCB1 in mRNA and protein levels between LoVo and LoVo/CPT-11 cells was compared. The results revealed that the mRNA and protein expression of ABCB1 was significantly increased in LoVo/CPT-11 cells compared with parental cells (Fig. 1B-D). Based on these data, LoVo/CPT-11 cells were effectively established. To further determine whether the chemoresistance of CRC cells is associated with CSC, the expression of CSC identification markers in LoVo/CPT-11 cells were examined. As expected, the mRNA and protein expression levels of CD44, CD133, EpCAM and CD24 were significantly increased in LoVo/CPT-11 cells compared with the parental cells (Fig. 1E-G).

Curcumin reduces chemoresistance to CPT-11 and down-regulates the expression levels of CSC markers. Curcumin inhibited the growth of LoVo/CPT-11 cells in a concentration-dependent manner. At 2.5 and 5 μM curcumin concentrations, the growth inhibitory rate was 2.67 ± 0.03 and $4.35 \pm 0.24\%$, respectively (Fig. 2A). To determine whether curcumin reduces LoVo/CPT-11 cell chemoresistance, the cells were treated with CPT-11 in the presence or absence of low concentrations of curcumin. The growth inhibitory rates were significantly increased in the presence of 2.5 and 5 μM curcumin (Fig. 2B), with the IC_{50} of CPT-11 reduced from 268.72 ± 2.43 to 150.63 ± 6.51 and $144.48 \pm 3.22 \mu\text{M}$, respectively.

To further explore the possibility that curcumin attenuated chemoresistance via reducing CSC-like characteristics,

LoVo/CPT-11 cells were treated with different concentrations of curcumin and CPT-11 individually for 24 h. The results revealed that, with the exception of CD133 mRNA expression, the protein expression of CD133, and the mRNA and protein expression levels of CD44, EpCAM and CD24 were inhibited by curcumin in a concentration-dependent manner (Fig. 2C-E), whereas CPT-11 had no significant effect on the expression of these markers (Fig. 2F-H).

Tumor sphere formation from CPT-11-resistant cells in serum-free medium (SFM) culture. LoVo/CPT-11 cells were plated in SFM containing several appropriate growth factors in order to form stable cell spheroids. In order to verify the CSC-like characteristics of sphere-forming cells, the percentages of CD133-positive cells were detected. As demonstrated in Fig. 3, the percentage of CD133-positive cells was significantly increased in LoVo/CPT-11 cells and sphere-forming cells, particularly in sphere-forming cells compared with parental cells. These results confirmed the characteristics of CSC in sphere-forming cells and further verified that LoVo/CPT-11 cells acquired CSC-like characteristics.

Curcumin diminishes the activity of colon CSCs and the expression levels of CSC identification markers. To detect the effect of curcumin and CPT-11 on colon CSCs, sphere-forming cells were treated with curcumin and CPT-11 separately or in combination for 3 days. As shown in Fig. 4, curcumin produced a concentration-dependent reduction in the SFE with almost no sphere formation following 10 μM curcumin treatment. In

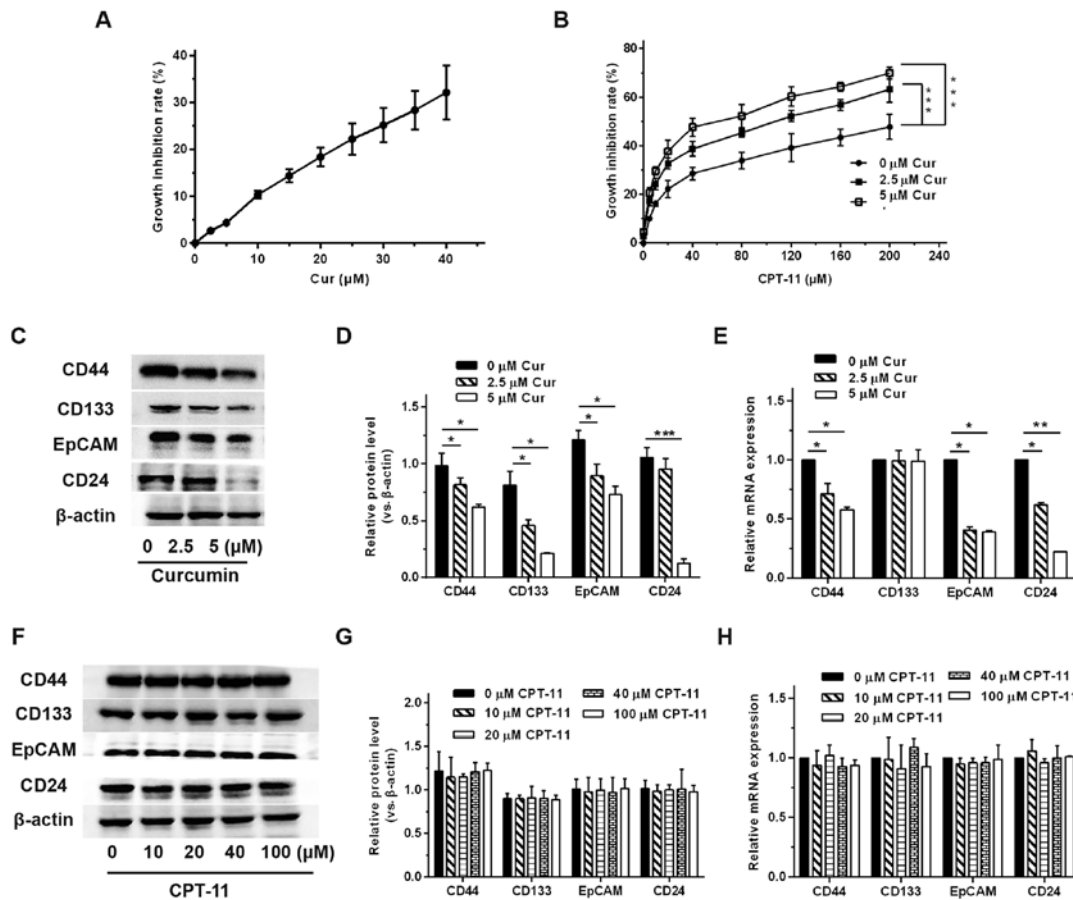


Figure 2. Growth inhibitory rates and the changes in CSC markers in LoVo/CPT-11 cells following various treatments. The growth inhibitory rates of LoVo/CPT-11 cells treated with (A) curcumin alone or (B) a combination of CPT-11 and curcumin for 24 h was determined using a cell counting kit-8 assay. (C) Representative western blotting image, (D) quantification of protein expression and (E) mRNA expression of CSC identification markers in LoVo/CPT-11 cells treated with curcumin. (F) Representative western blotting image, (G) quantification of protein expression and (H) mRNA expression of CSC identification markers in LoVo/CPT-11 cells treated with CPT-11. β -actin was used as a loading control. Each bar represents the mean \pm standard deviation of three independent experiments. * P <0.05, ** P <0.01 and *** P <0.001 vs. untreated control cells. CSC, cancer stem cell; CPT-11, irinotecan.

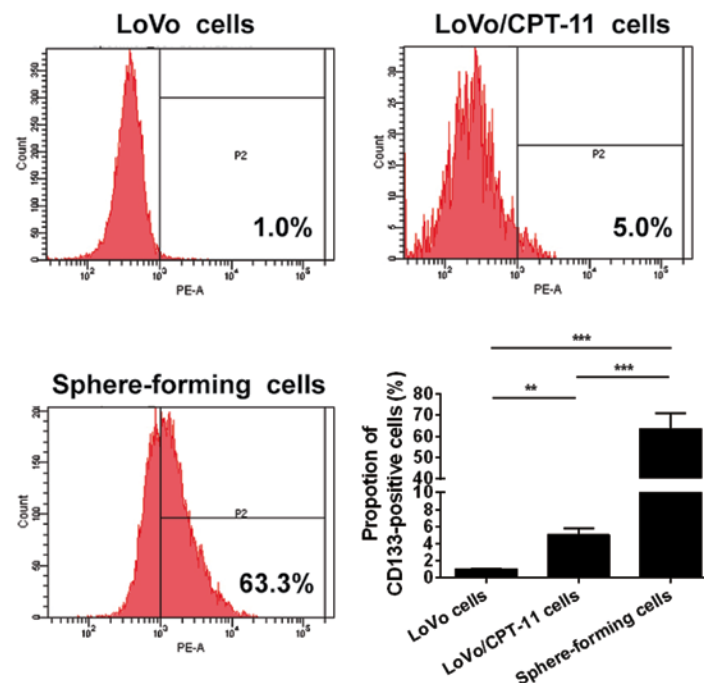


Figure 3. Percentages of CD133-positive cells in LoVo, LoVo/CPT-11 and sphere-forming cells. Each bar represents the mean \pm standard deviation of three independent experiments. ** P <0.01 and *** P <0.001. PE-A, PE-conjugated anti-CD133 antibody. CPT-11, irinotecan; PE, P-phycoerythrin; CD133, cluster of differentiation 133.

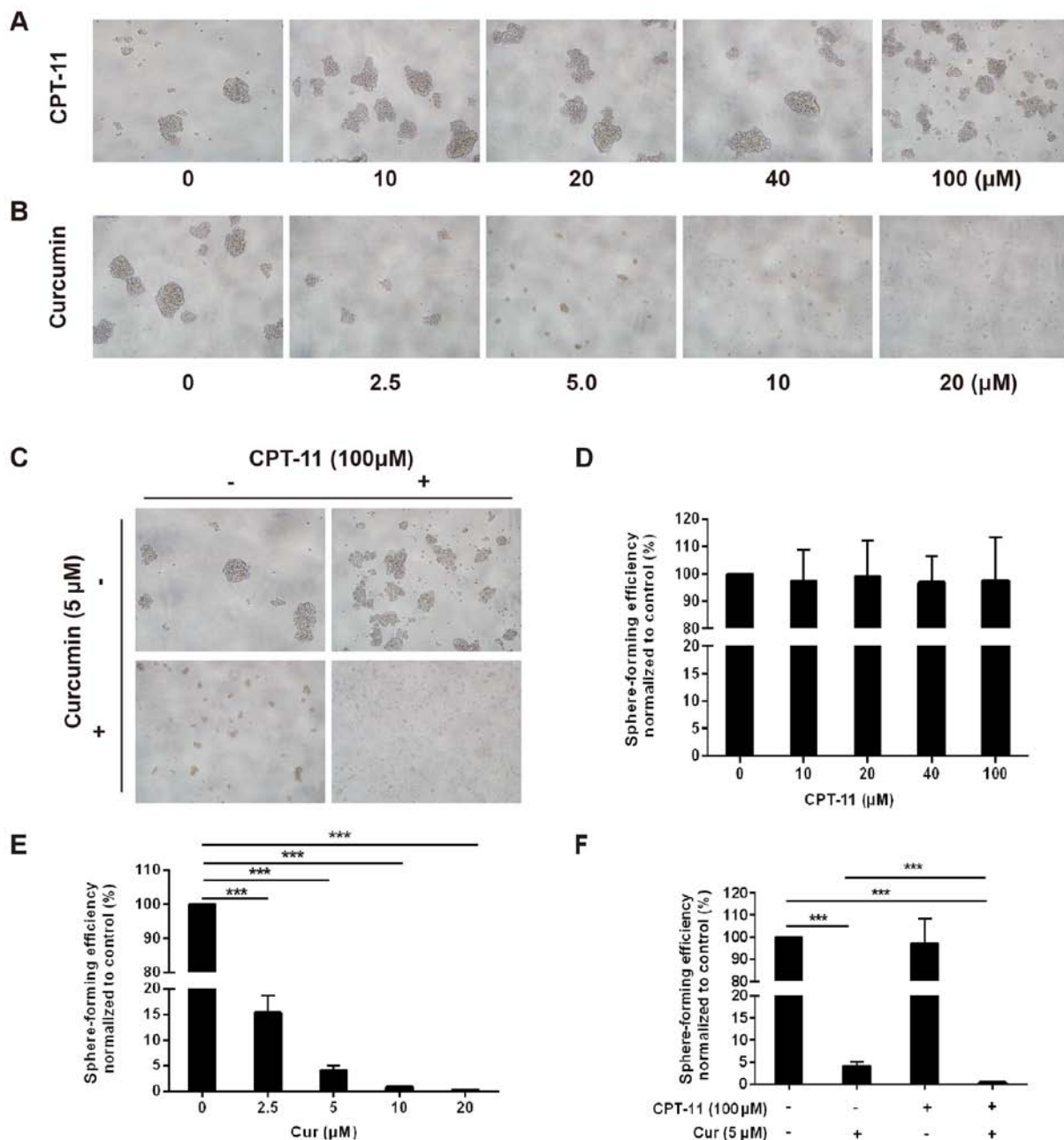


Figure 4. Changes in morphology and the number of tumor spheres in sphere-forming cells. LoVo/CPT-11 cells were plated as single cells in ultra-low attachment 6-well plates at 2.0×10^4 cells/well. Then, the cells were treated separately on the third day with various concentrations of CPT-11 and curcumin. The tumor sphere changes were observed under a light microscope 3 days later. (Original magnification, $\times 100$). The morphology changes in sphere-forming cells (A) CPT-11, (B) curcumin and (C) combined treatments. The normalized sphere-formation efficiency following (D) CPT-11, (E) curcumin and (F) combined treatments. Each bar represents the mean \pm standard deviation of the three independent experiments. *** $P < 0.001$ vs. control cells. CPT-11, irinotecan.

contrast, no significant differences in the SFE were observed following CPT-11 treatment alone. Furthermore, Fig. 4C and F demonstrate that the inhibitory effect in the combined curcumin and CPT-11 group, was significantly increased compared with that in the group treated with $5 \mu\text{M}$ curcumin alone. Additionally, the mRNA and protein expression levels of CSC identification markers (CD44, CD133, EpCAM and CD24) were significantly inhibited following treatment with curcumin alone or in combination with CPT-11 compared with untreated cells (Fig. 5).

Curcumin induces apoptosis of colon CSCs. To further to understand the effect of curcumin on colon CSCs, the effects

of curcumin on apoptosis in sphere-forming cells were investigated. The results of flow cytometry analysis indicated that cell apoptosis was induced in the presence of curcumin in sphere-forming cells, and the apoptosis-inducing effect of combined curcumin and CPT-11 was significantly increased compared with that of curcumin alone (Fig. 6A and B). Additionally, western blot analysis was used to measure the expression levels of proteins associated with apoptosis. Fig. 6C and D demonstrate that the expression level of anti-apoptotic protein Bcl-2 was significantly decreased, and the levels of pro-apoptotic proteins cleaved caspase-8, cleaved caspase-9, cleaved caspase-3 and Bax were significantly increased in the presence of curcumin compared with untreated control group.

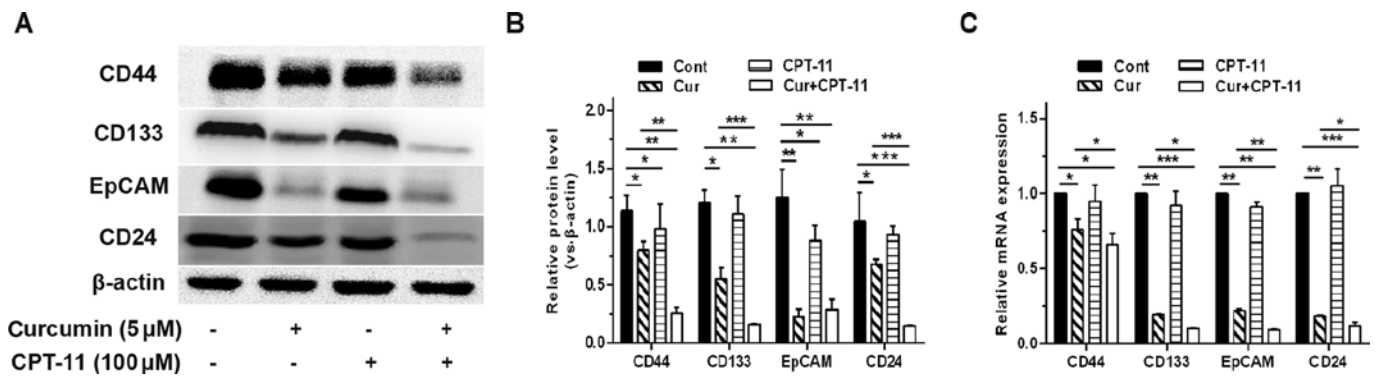


Figure 5. Changes in CSC identification markers in sphere-forming cells following various treatments. The protein expression (A) and the relative protein expression (B) and the mRNA expression (C) of CSC markers in sphere-forming cells in different groups. β-actin was used as a loading control. Each bar represents the mean ± standard deviation of the three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 vs. control cells. CSC, cancer stem cell.

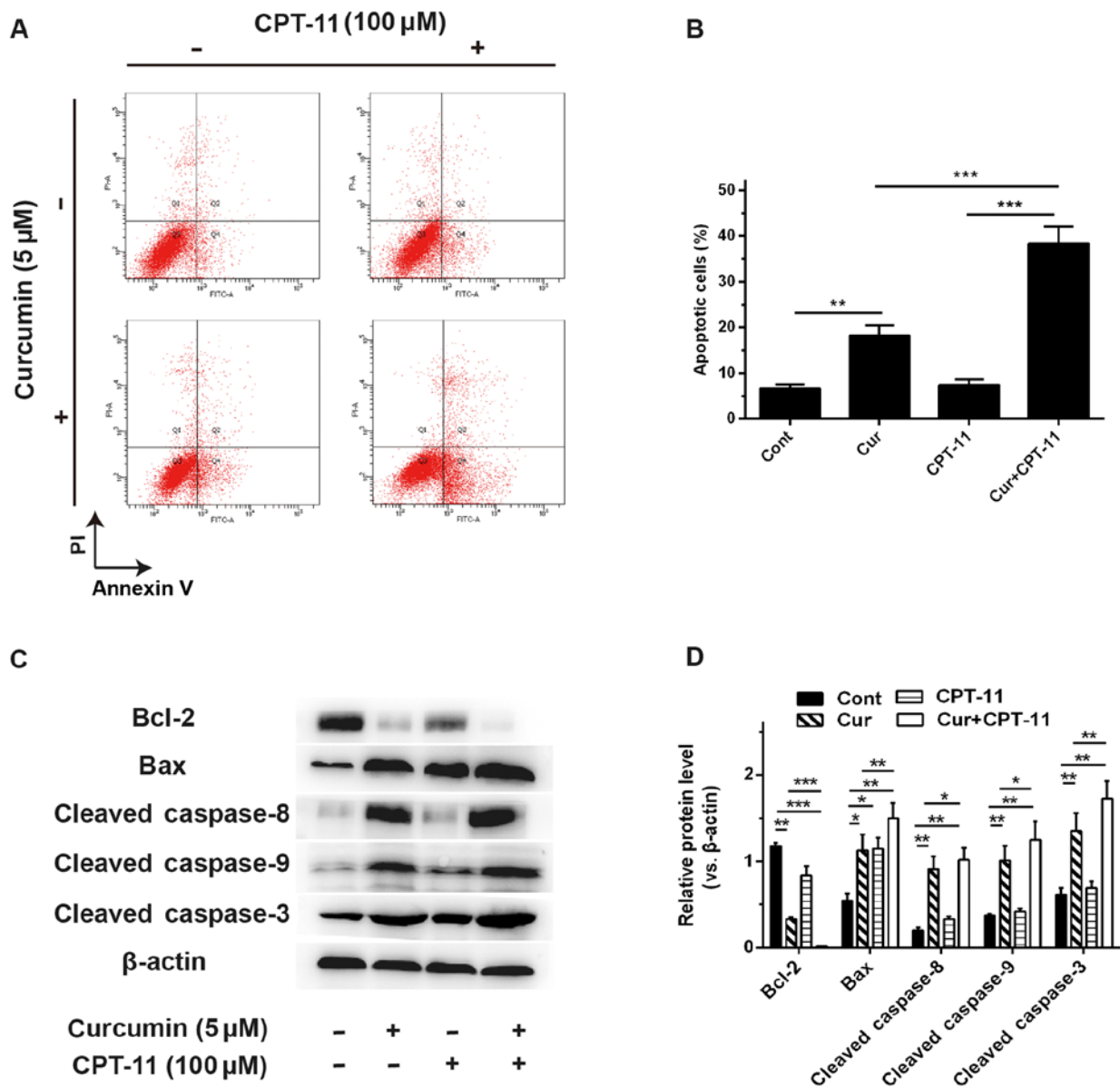


Figure 6. Rates of apoptosis and the expression of apoptosis-associated proteins in sphere-forming cells. (A) The flow cytometry profiles and (B) quantitative analysis of the apoptosis rate of spheroid-forming cells following treatment with curcumin and CPT-11 separately and in combination. (C) Representative blot of apoptosis-associated protein expression and (D) quantification of protein expression in spheroid-forming cells following treatment with curcumin and CPT-11 separately and in combination. β-actin was used as a loading control. Each bar represents the mean ± standard deviation of the three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 vs. control cells. CPT-11, irinotecan.

Discussion

CRC is the fourth leading cause of cancer-associated mortalities worldwide, and is associated with an unsatisfactory prognosis and <10% rate of 5-year survival rate (4). Development of chemoresistance is considered to be an important obstacle to the achievement of satisfactory therapeutic effects for CPT-11 treatment. There is increasing experimental evidence indicating that CSCs drive tumor initiation, invasion and metastasis, and contributes to chemoresistance, consequently promoting unrestricted tumor progression (22-24). Understanding the mechanisms of chemoresistance and identifying the agents that may reverse it are of great importance for achieving effective therapeutic strategies.

In the present study, a CPT-11-resistant cell subline was derived from the human colon cancer LoVo cell line, which was confirmed to exhibit increased cell viability compared with of the parental cell line in the presence of different concentrations of CPT-11. A previous study demonstrated that ABC transporters contribute to the chemoresistance by pumping anticancer agents out of the cells (25). When compared with parental cells, the expression level of ABCB1 (also known as P-glycoprotein) was significantly increased in LoVo/CPT-11 cells. In addition, it has been reported that the high expression of ABC transporters accounts for the chemoresistance property of CSCs (26). Based on this observation, the expression of specific cell surface or cytoplasmic biomarkers of CSCs was further investigated. Since widely accepted biomarkers of colon CSCs have not yet been defined, four potential and frequently-used biomarkers, including CD44, CD133, CD24 and EpCAM (also known as ESA) were chosen to identify colon CSCs (27-30). As a transmembrane glycoprotein, CD44 possesses a particular cell adhesion function, and consequently assists the matrix adhesion and migration of CSCs (31). In the prominin family of pentaspan transmembrane glycoproteins, CD133, also called Prominin-1 or AC133, was the first member to be verified, and now is widely used to identify or isolate CSCs from various types of tumor, including that of the pancreas, colon and liver (32). Zhang *et al* (33) demonstrated that CD133-positive colon cancer cells exhibited increased abilities to form tumor spheres and initiate tumor development compared with CD133-negative cells. Similarly, CD24 and EpCAM have been reported to be candidates for identifying or sorting CSCs from colon cancer cells (34). Even though CD44, CD133, EpCAM and CD24 are commonly used to identify CSCs in CRC, the specificity of each marker for the identification of CSCs has not yet been defined. Thus, the use of a single biomarker to identify CSCs may not be reliable, and a group of biomarkers is preferable to identify CSCs, as was used in the current study. In the present study, the data indicated that the expression of CD44, CD133, CD24 and EpCAM at the mRNA and protein levels were significantly higher in LoVo/CPT-11 cells compared with that of parental cells. In addition, the proportion of CD133-positive cells was significantly increased in CPT-11-resistant cells. Consequently, LoVo/CPT-11 cells acquired CSC-like characteristics, and the proportion of CSCs derived from LoVo/CPT-11 cells was increased compared with that in LoVo cells. This observation necessitates the exploration and development of anticancer agents possessing CSC-targeting ability.

CPT-11 combined with 5-fluorouracil is one of the standard chemotherapeutic options for metastatic CRC. Nevertheless, the effectiveness of CPT-11 and other agents is restricted as they ineffectively target CSCs, which contributes to the development of chemoresistance (17). In the current investigation, it was demonstrated that curcumin inhibited the growth of LoVo/CPT-11 cells in a concentration-dependent manner. Considering how the cytotoxic effect of curcumin may interfere with the results, low concentrations of curcumin were chosen to study its effect on the chemoresistance of LoVo/CPT-11 cells to CPT-11. The results revealed that curcumin increased the effectiveness of CPT-11 in inhibiting the growth of LoVo/CPT-11 cells. The IC₅₀ of CPT-11 in drug-resistant cells was significantly reduced by treatment with low concentrations of curcumin. Furthermore, the data indicated that curcumin significantly downregulated the protein expression of the CSC-associated biomarkers CD44, CD133, CD24 and EpCAM, with similar results observed with mRNA expression. These results demonstrated that curcumin may reduce chemoresistance to CPT-11 through the targeting of CSCs. Thus, further investigations directly involving CSCs were performed.

Several methods have been reported to isolate CSCs, including the side population (SP) cell sorting method, magnetic activated cells sorting (MACS), fluorescence activated cells sorting (FACS) and SFM culture (35). Hoechst staining is toxic to cells, and CSCs isolated via the SP cell sorting method may exhibit decreased self-renewal, tumorigenicity and other CSC properties; thus, the use of this technique is limited (36). FACS and MACS require knowledge of specific surface markers of CSCs, and questions have been raised regarding the specificity and reliability of markers for the identification of CSCs as widely accepted specific surface markers of CSCs have not yet been defined (37). Binding of an antibody to its receptor during these two methods may result in cell activation, influencing the biology of cells (38). In addition, cells are treated with enzymes prior to collection for isolation, so the majority of surface antigens may be damaged and perhaps this is one of the major disadvantages of the use of surface markers to isolate cancer stem cells. Certain factors, including the low viability of isolated cells, high cost and the difficulty of using complex equipment are other limitations of MACS or FACS use (36). Accumulating studies have demonstrated that the formation of floating tumor spheres in SFM is one of the major characteristics of CSCs, and only CSCs are able to form tumor spheres in SFM (39,40). The tumor sphere formation assay has been reported for culturing mammospheres (41), neurospheres (42) and colonospheres (15), and is considered to be a convenient method for investigating CSCs (43). Considering the aforementioned reasons, SFM was used to generate and culture tumor spheres from LoVo/CPT-11 cells in anchorage-independent conditions. In sphere-forming cells, the proportion of CD133-positive cells, indicating the characteristics of CSCs (33,44), increased over 63- and 12-fold when compared with parental cells and CPT-11-resistant cells, respectively. Further investigation revealed that curcumin significantly inhibited tumor sphere formation, and decreased the expression of CD44, CD133, EpCAM and CD24 at the mRNA and protein levels in the absence or presence of CPT-11, while CPT-11 alone had almost no inhibitory effect. Furthermore, the inhibitory effect of the combination of

curcumin and CPT-11 on sphere formation and expression of CSC markers was significantly compared with that of curcumin alone; we hypothesized that this may be the result of a synergistic effect. These results were in accordance with the results in LoVo/CPT-11 cells and taken together suggests that CSCs contribute to acquisition of chemoresistance. Furthermore, these results suggest that curcumin ameliorates chemoresistance via targeting and eliminating CSCs.

The association between the inhibitory effect of curcumin and apoptosis was further explored. The results of the flow cytometry assay suggested that curcumin significantly induced apoptosis of CSCs, while CPT-11 had no significant effect on induction of apoptosis. Furthermore, curcumin in combination with CPT-11 demonstrated a stronger effect compared with curcumin alone. Curcumin may not only be an 'effector' itself, but also serve as a 'catalyzer' for CPT-11 in inducing apoptosis, resulting in a synergistic effect between curcumin and CPT-11. This may be investigated in future studies. In order to achieve higher credibility, western blot assays were used to verify the effect of curcumin on the induction of apoptosis. As a family of cysteine aspartic acid-specific proteases, caspases are essential components of apoptotic pathways, which include the mitochondrial and death receptor pathways (45). The activation of distinct caspase cascades is known to be involved in activating and cleaving certain proteins that are essential to the physiological process of apoptosis (46). Caspase-8, the initiator of the death receptor pathway, is activated by tumor necrosis factor, which then activates the downstream effector protease. In addition, caspase-8 participates in the mitochondrial apoptosis pathway, and the release of cytochrome *c* activates caspase-9 and other effector proteases. The two apoptosis pathways lead to the activation and cleavage of downstream protease caspase-3, provoking further activation events (47). The Bcl-2 family, including pro-apoptosis members such as Bax and anti-apoptosis members such as Bcl-2, has been identified as upstream regulators of the caspase cascade (48). In the present study, the results of flow cytometry assay and western blot assay indicated that the caspase apoptotic pathway is involved in curcumin-induced apoptosis in LoVo/CPT-11 CSCs. Notably, the flow cytometry assay revealed that CPT-11 did not induce apoptosis, but western blot analysis revealed reduced expression levels of Bcl-2, and increased levels of Bax and cleaved-caspase-3 following CPT-11 treatment. The possible reasons may be as follows: Firstly, apoptosis is characterized by a variety of functional and morphological changes in the plasma membrane and nucleus, these changes result in a change in the fluorescence intensity in cells. Flow cytometry detects apoptosis via analyzing the difference of fluorescence intensity among normal cells, apoptotic cells and dead cells, it reflects apoptosis at a cellular level, while the expression of apoptosis-associated proteins detected by western blot assay reflects apoptosis at a molecular level (45,49). Secondly, 7-ethyl-10-hydroxy-camptothecin (SN-38), the metabolic product of CPT-11, induces DNA damage, and is the primary mechanism for the pharmacological effect of CPT-11 (50). Studies have reported that CPT-11 induces apoptosis of colon cancer cells through the p53 signaling pathway, and CPT-11 alone or in combination with other drugs may induce apoptosis via altering the expression of Bcl-2 family, caspase family and survival-associated genes in cancer cells (50,51). CPT-11 or its metabolic product

SN-38 possibly intervenes in apoptosis-associated pathways, influencing the expression of Bax, Bcl-2 and cleaved-caspase-3 in LoVo/CPT-11 CSCs. However, on account of the active DNA repair mechanisms, high expression of ABC transporters, and resistance to apoptosis in CSCs, the function and morphology of plasma membrane and nucleus may not change (18,52).

Several other cellular signaling pathways are known to regulate cell apoptosis and proliferation, and we hypothesize that the phosphatidylinositol 3-kinase (PI3K)/AKT serine/threonine kinase (AKT), mitogen-activated protein kinases (MAPK)/extracellular signal-regulated kinase (ERK) and signal transducer and activator of transcription (STAT) signaling pathways are associated with the effects of curcumin observed in the present study. The PI3K/AKT signaling pathway is dysregulated in numerous types of human cancer, and regulates the apoptotic response via interaction with the key mediators of the apoptotic process (53). Activation of this pathway results in the phosphorylation of transcription factors, which increases the expression of anti-apoptotic members and decreases the levels of pro-apoptotic proteins in the Bcl-2 family, subsequently inhibiting the release of cytochrome *c* from the mitochondria, and activating the apoptotic caspase cascade (47). The MAPK/ERK signaling pathway is associated with the cell proliferation and apoptosis, and cross-talk between the PI3K/AKT and MAPK/ERK pathways exists in numerous cancer cells (54-56). PI3K/AKT signaling pathway abrogation may lead to a compensatory activation of the MAPK/ERK signaling pathway (57). The STAT pathway has a role in relaying extracellular signals from the cytoplasm to the nucleus, regulating the expression of several genes involved in cell cycle progression and apoptosis, and curcumin analogues may reduce the expression of the STAT downstream target gene and induce apoptosis in colon cancer stem cells (58). Thus, the natural compound curcumin possibly induces apoptosis and suppresses proliferation in CSCs via attenuation of apoptosis-associated signaling pathways, including the PI3K/AKT, MAPK/ERK and STAT pathways, and it may be worth investigating in future studies.

Taken together, these findings suggest that CSCs serve an important role in the development of chemoresistance in CRC cells. The results of the present study indicated that the natural compound curcumin effectively attenuated chemoresistance of CRC cells via targeting and inducing apoptosis in CSCs, hence eliminating CSCs. The current study provides evidence for the clinical therapeutic potential of curcumin as a supplement to conventional chemotherapy in patients with CRC experiencing resistance to conventional anticancer drugs.

Acknowledgements

The authors would like to thank the public experimental platform (Research Center of Clinical Medicine of Nanfang Hospital Affiliated to Southern Medical University, Guangzhou, Guangdong, China) for providing experimental facilities.

Funding

The present study was supported by the China Postdoctoral Science Foundation (grant no. 2015M582358, received by

GW) and the Project of Traditional Chinese Medicine Bureau of Guangdong Province (grant no. 20141307, received by XC).

Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

XC and PS conceived and designed the experiments. PS and YY performed the experiments and acquired data. YJ and GW provided the methodology, analyzed and interpreted the data. PS and YY drafted the manuscript. YJ directed the writing of the manuscript and revised the manuscript. YJ and PS critically revised the manuscript. All authors have given final approval of the version to be published.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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