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Original Article

Curcumin Reverses 5-Fluorouracil Resistance by Promoting Human Colon Cancer HCT-8/5-FU Cell Apoptosis and Down-regulating Heat Shock Protein 27 and P-Glycoprotein*

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ABSTRACT **Objective:** To investigate the potential mechanisms that curcumin reverses 5-fluorouracil (5-FU) multidrug resistance (MDR). **Methods:** Cell growth and the inhibitory rate of curcumin (2–25 μg/mL) and/or 5-FU (0.05–1000 μg/mL) on human colon cancer HCT-8 and HCT-8/5-FU (5-FU-resistant cell line) were determined using cell counting kit-8 (CCK-8) assay. Apoptosis and cell cycle after 5-FU and/or curcumin treatment were detected by flow cytometry (FCM) and transmission electron microscopy (TEM). The expression of the multidrug resistance related factors p-glycoprotein (P-gp) and heat shock protein 27 (HSP-27) genes and proteins were analyzed by reverse transcription polymerase chain reaction (RT-PCR) and Western blotting (WB), respectively. **Results:** The inhibitory rate of curcumin or 5-FU on HCT-8 and HCT-8/5-FU cells proliferation at exponential phase were in a dose-dependent manner, HCT-8 cell line was more sensitive to curcumin or 5-FU when compared the inhibitory rate of HCT-8/5-FU. The 50% inhibitory concentration (IC_{50}) of combination 5-FU and curcumin (4.0 μg/mL) in HCT-8/5-FU was calculated as 179.26 μg/mL, with reversal fold of 1.85. Another IC_{50} of combination 5-FU and curcumin (5.5 μg/mL) in HCT-8/5-FU was calculated as 89.25 μg/mL, with reversal fold of 3.71. Synergistic effect of 5-FU and curcumin on HCT-8 and HCT-8/5-FU cells were found. The cell cycle analysis performed by FCM showed that HCT-8 and HCT-8/5-FU cells mostly accumulated at G_0/G_1 phase, which suggested a synergistic effect of curcumin and 5-FU to induce apoptosis. FCM analysis found that the percentage of apoptosis of cells treated with curcumin, 5-FU and their combination were significantly increased compared to the control group ($P<0.05$), and the percentage of apoptosis of the combination groups were slightly higher than other groups ($P<0.05$). The mRNA levels of P-gp (0.28±0.02) and HSP-27 (0.28±0.09) in HCT-8/5-FU cells treated with combination drugs were lower than cells treated with 5-FU alone (P-gp, 0.48±0.07, $P=0.009$; HSP-27, 0.57±0.10, $P=0.007$). The protein levels of P-gp (0.25±0.06) and HSP-27 (0.09±0.02) in HCT-8/5-FU cells treated with combination drugs were decreased when compared to 5-FU alone (P-gp, 0.46±0.02, $P=0.005$; HSP-27, 0.43±0.01, $P=0.000$). **Conclusions:** Curcumin can inhibit the proliferation of human colon cancer cells. Curcumin has the ability of reversal effects on the multidrug resistance of human colon cancer cells lines HCT-8/5-FU. Down-regulation of P-gp and HSP-27 may be the mechanism of curcumin reversing the drug resistance of HCT-8/5-FU to 5-FU.

KEYWORDS curcumin, Chinese medicine, 5-fluorouracil, multidrug resistance, HCT-8, HCT-8/5-FU, colon cancer, P-gp, HSP-27

Colorectal cancer (CRC) is the third deadliest and widely diagnosed cancer in the world.[1] Almost 5,60,000 people are lost due to colon cancer worldwide every year.[2] Chemotherapy plays an important role in the comprehensive treatment of colon cancet. To date, 5-fluorouracil (5-FU) is the therapeutic mainstay for the treatment of colon cancer, but the emergence of multidrug resistance (MDR) usually leads to therapy failure.[3] It is necessary to find a strong agent with minimal side effects that can reverse the effects of MDR to improve the effect of chemotherapy on

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MDR tumors.[4] Several mechanisms have been suggested to explain MDR acquisition. Among them, drug efflux due to increased expression and activity of ATP-binding cassette (ABC) transporters including P-glycoprotein (P-gp), is the most frequently proposed one. P-gp is a transmembrane efflux pump which acts as a multidrug transporter that prevents the accumulation of chemotherapeutic drugs. In a variety of suggested factors, heat shock protein 27 (HSP-27) has been implicated in inducing functional ABC transporters in cancer cells.[5] Aberrant level and induced phosphorylation of HSP-27 have been suggested in MDR acquisition.

Curcumin (Cur) is a hydrophobic polyphenol derived from the rhizomes of *Curcuma longa*. The yellow-orange pigment curcumin has been used for centuries in cooking as well as in Chinese medicine (CM).[6] Combined with other therapeutic agents, curcumin exhibits anti-cancer effect on the breast cancer,[7,8] hepatoma cells,[9] cervical cancer,[10] and oral cancer.[11] Furthermore, curcumin combines with 5-FU can enhance chemosensitization to 5-FU-based chemotherapy, induce apoptosis and reverse the resistance to 5-FU in human CRC cells.[12-14] Nevertheless, the exact role of curcumin in the reversal of resistance to 5-FU has not been clearly understood. The present study aimed to determine the therapeutic effect of curcumin alone or in combination with 5-FU for the treatment of human colon cancer cells HCT-8/5-FU (5-FU resistant), and investigate the potential and mechanism of curcumin in the reversal of resistance to 5-FU in human colon cancer cells.

METHODS

Reagents and Antibodies

HCT-8 and HCT-8/5-FU cells were purchased from KeyGEN BioTECH Co., Ltd. (China). Curcumin and 5-FU were purchased from Sigma-Aldrich (USA); cell counting kit-8 (CCK-8) and Annexin V/PI apoptotic detection kit were purchased from Bestbio Co. (China). Fetal bovine serum (FBS) was obtained from Zhejiang Tianhang Biotechnology Co., Ltd. (China). RPMI 1640 medium was purchased from Life Technologies (USA). Penicillin and streptomycin were purchased from Thermo Fisher Hyclone (USA). BCA protein assay was purchased from Beyotime Institute of Biotechnology (China). Rabbit monoclonal antibodies against human HSP-27, P-gp and β-actin were purchased from Abcam (UK). Horseradish-peroxidase (HRP) conjugated anti-rabbit IgG was purchased from Beijing Zhong Shan Golden Bridge Biological Technology Co., Ltd. (China).

Cell Culture

According to the manufacturer's manual, the frozen cells of human colon cancer HCT-8 and HCT-8/5-FU was quickly thawed in a 37 ℃ water bath. Aseptically, the HCT-8 and HCT-8/5-FU suspension were transferred to a 15 mL tube with 10 mL of respective RPMI 1640 medium. All media contained 10% FBS, 100 μg/mL penicillin and 100 μg/mL streptomycin. Cells were seeded in 96-well plates at 5.0×10^3 cells/well in a final volume of 100 μL and cultured at 37 ℃ with 5% CO_2. After cancer cells were cultured overnight, the medium was changed to fresh RPMI 1640 medium for 1, 2, 3, 4, 5, 6, 7 d, then underwent CCK-8 for growth analysis. Cancer cells in exponential phase were chosen for the following experiments.

Cytotoxicity of Curcumin for HCT-8 and HCT-8/5-FU Cells

The cells were planted in the flat 96-well culture plates at a seeding density of 5×10^3 cells/well in quintuplicate. After the overnight incubation, 100 μL curcumin was added to wells in serial concentrations (0, 2, 4, 8, 10, 12, 16, 20, 25 μg/mL). After 48 h incubation, the culture medium was discarded and refreshed with RPMI-1640. Simultaneously, 10 μL CCK-8 was added into each well containing 100 μL of the culture medium, co-incubated for an additional 4 h at 37 ℃. Cells treated with 0.1% dimethyl sulfoxide (DMSO) served as control. All experiments were repeated six times. Results were analyzed by CCK-8 assay and the absorbance optical density (OD) of 450 nm was measured by Varioskan Flash Spectral Scanning Multimode Reader (USA). The percentage of cytotoxicity was calculated using the following formula: inhibitory rate (IR, %) = $[1 - OD450_{test}/OD450_{control}] \times 100\%$. 5%, 10%, 25% and 50% inhibitory concentration (IC_5, IC_{10}, IC_{25} and IC_{50}) were evaluated by probit analysis using Pharmacologic Calculation System. X_1 and X_2, the concentrations less than IC_{10} of curcumin in the resistant cell line, were calculated.

Cytotoxicity of 5-FU and Determination of Multi-drug Resistance

HCT-8 and HCT-8/5-FU cells were planted in the flat 96-well culture plates at a seeding density of 5×10^3 cells/well in quintuplicate. After the overnight incubation, 100 μL 5-FU was added to wells in serial concentrations (HCT-8: 0, 0.05, 0.1, 0.2, 0.4, 0.8, 3.2,

6.4, 12.8 μg/mL; HCT-8/5-FU: 0, 30, 60, 90, 150, 300, 600, 1,000 μg/mL). Cells treated with 0.1% DMSO served as control. All experiments were repeated 6 times. Results were analyzed by CCK-8 assay. IC_{50} were evaluated, from which reversal index was calculated. The degree of resistance was calculated by resistant index (RI) as the following formula: RI= IC_{50} (HCT-8/5-FU)/IC_{50} (HCT-8).

Reversal Effect Assay

The ability of curcumin to reverse MDR was evaluated in HCT-8/5-FU cells by CCK-8 assay. HCT-8/5-FU cells were planted in the flat 96-well culture plates at a seeding density of 5×10^3 cells/well in quintuplicate. After the overnight incubation, 100 μL combinations of 5-FU (0, 30, 60, 90, 150, 300, 600, 1,000 μg/mL) with curcumin (0, X_1, X_2 μg/mL) were added to the wells. Cells treated with 0.1% DMSO served as control. All experiments were repeated 6 times. Results were analyzed by CCK-8 assay. The IC_{50} of combinations of 5-FU and curcumin were calculated in HCT-8/5-FU cells. The reversal fold (RF) values, as potency of reversal, were obtained from fitting the data=IC_{50} (5-FU)/IC_{50} (5-FU+curcumin).

Analysis of Synergism

HCT-8 and HCT-8/5-FU cells were planted in the flat 96-well culture plates at a seeding density of 5×10^3 cells/well in quintuplicate. After the overnight incubation, 100 μL combinations of 5-FU with curcumin were added into wells of HCT-8 and HCT-8/5-FU. Cells treated with 0.1% DMSO served as control. All experiments were repeated 6 times. Results were analyzed by CCK-8 assay.

The synergistic effect of 5-FU and curcumin was analyzed by applying the modified Bürgi formula (i.e., Jin equation).[15] The formula is $q = EA + B/(EA + EB - EA \times EB)$, where EA + B, EA, and EB are the average IR of the combination treatment, curcumin alone, and 5-FU alone, respectively. The q value < 0.85 indicates antagonism, 0.85–1.15 indicates additive effects, and ⩾1.15 indicates synergism.

Cell Cycle Distribution

To investigate the effect of curcumin and 5-FU on colon cells, cell cycle distribution were performed. For monolayer cultures 1×10^6 cells/plate were seeded in 35-mm tissue culture discs. After 80%–90% confluency, cancer cells were divided into 8 groups and treated with different concentrations of curcumin and 5-FU combination for 48 h before cell cycle distribution analysis. After treatment, cells were washed once with phosphate buffer saline (PBS), collected by trypsinization and centrifugation, and washed twice with ice-cold PBS. Cells at 1×10^6 per sample were fixed in 3 mL of 70% cold ethanol at –20 ℃ overnight. After centrifugation (1,000 r/min for 5 min), the fixed cells were washed with PBS once and re-suspended with 500 μL of PBS containing 100 μg/mL RNase and 5 μg/mL propidium iodide and incubated at room temperature in the dark for 30 min. Flow cytometric analysis was performed using FACScan (BD FACSCalibur™, USA) for determining the percentage of cells in various phases of the cell cycle. At least 10,000 events were counted.

Cell Apoptosis Detection

The effect of curcumin and 5-FU on cell apoptosis was assessed by flow cytometry using Annexin V-FITC assay kit according to manufacturer's protocol. Cell's treatment and grouping information were same as cell cycle distribution. After being treated for 48 h, cancer cells were washed once with PBS, harvested with trypsin, collected by centrifugation (1,000 r/min for 5 min), and washed twice with cold PBS. Cells at 4×10^5 were then re-suspended in Annexin V binding buffer at a final concentration of 1×10^6 cells/mL. Then cells were stained with 5 μL of Annexin V-FITC for 15 min and 10 μL propidium iodide (to distinguish the necrotic cells) for 5 min at 2–8 ℃ in the dark. After incubation, the fraction of apoptotic cells was analyzed by flow cytometry (BD FACSCalibur™, USA) using 488 nm excitation and determined with FlowJo software (Tree Star, USA).

Transmission Electron Microscopy Assay

Transmission electron microscopy (TEM) was performed to assess the effects of curcumin and 5-FU combination on the ultrastructure of colon cells. Cells were fixed for 1 h in Karnovsky's fixative followed by post-fixation in 1% OsO_4 solution. After dehydration in an ascending alcohol series, cultures were embedded in Epon and cut ultrathin with a Reichert-Jung Ultracut E (Darmstadt, Germany). Sections were contrasted with a mixture of 2% uranyl acetate/lead citrate and examined with a TEM (Zeiss, Jena, Germany).

Quantitative Real-Time PCR Analysis

After HCT-8/5-FU cells were treated with

different concentrations of curcumin and were co-treated with 5-FU for 48 h, the mRNA expression of P-gp and HSP-27 were detected by quantitative polymerase chain reaction (qRT-PCR). All the primer sequences are listed in Table 1. Total RNA was extracted from colon cells by using TRIzol Reagent. The cDNA synthesis and qRT-PCR were performed according to the manufacturer's instructions. For all of the qRT-PCR, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The expression of P-gp and HSP-27 genes was calculated relative to that in untreated cells and normalized for GAPDH mRNA using the $2^{-\triangle\triangle Ct}$ method. All studies were repeated in triplicate.

Table 1. Information of Primers

Primer	Sequence (5' to 3')	Length of production (bp)
P-gp	F: CGTGGGGCAAGTCAGTTCAT	139
	R: TCCTTCCAATGTGTTCGGCA	
HSP-27	F: GGAGATCACCGGCAAGCAC	115
	R: GGAGGAGGAAACTTGGGTGG	
GAPDH	F: TGTTGCCATCAATGACCCCTT	202
	R: CTCCACGACGTACTCAGCG	

Notes: F, forward, R, reverse.

Western Blotting Assays

After being treated for 48 h, cancer cells were collected and lysed. Protein concentration was determined using BCA protein assay according to the manufacturer's instructions. For each sample, about 40 μg of protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred onto polyvinylidene difluoride membrane. The membrane was blocked in 5% skim milk in Tris-buffered saline with Tween 20 buffer for 1 h and then incubated with individual primary antibodies overnight at 4 ℃. The membrane was washed by TBST buffer for 3 times and then incubated with secondary antibody-conjugated horseradish peroxidase for 1 h at room temperature. The protein bands on the membrane could be visualized on X-ray film using the enhanced chemiluminescence (ECL) kit. The protein levels were normalized by β-actin. In Western blot assay, the P-gp and HSP-27 antibodies were used at 1:200. Immunoreactive were detected with Quantity One (Biorad, USA).

Statistical Analysis

The dates were analyzed by SPSS 19 software package (SPSS, Chicago, Illinois, USA). All data were displayed as mean ± standard deviation ($\bar{x} \pm s$). One-way analysis of variance (ANOVA) and Student's *t*-test were employed to determine the statistical significance between different groups. All reported confidence intervals were calculated at the 95% level and all report *P*-values are two tailed.

RESULTS

Exponential Phase of HCT-8 and HCT-8/5-FU

After a week of culture and observation, both HCT-8 and HCT-8/5-FU cells reached the exponential phase in 2 days and the stagnate phase on day 4 (Figure 1). All the following tests were carried out during the exponential phase of the cell growth.

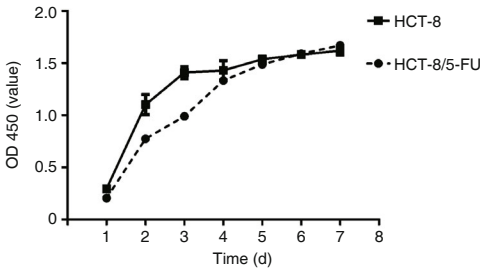

Figure 1. Cell Growth Curve

Inhibitory Effect of Curcumin on HCT-8 and HCT-8/5-FU Cells Proliferation

The CCK-8 assay demonstrated that the IR of curcumin on HCT-8 and HCT-8/5-FU cells proliferation at exponential phase was in a dose-dependent manner (Figure 2A). However, the sensitivity of two cell lines to curcumin was considerably different. HCT-8 cell line was more sensitive to curcumin, when compared the IR of HCT-8/5-FU. The IC_5, IC_{10}, IC_{25} and IC_{50} of curcumin were calculated and listed in Table 2. The IC values were adjusted to approximate concentrations (A-IC) which were listed in Table 2. The concentrations (X_1 and X_2) less than IC_{10} of curcumin were generally regarded as a safe concentration in the resistant cell line. X_1 was calculated as 4.0 μg/mL (A-IC_5) and X_2 was calculated as 5.5 μg/mL (A-IC_{10}), they were chosen to calculate the RF.

Inhibitory Effect of 5-FU on HCT-8 and HCT-8/5-FU Cells Proliferation

IR of 5-FU on HCT-8 (Figure 2B) and HCT-8/5-FU (Figure 2C) cells proliferation at exponential phase were in a dose-dependent manner. The HCT-8 cell line was more sensitive to 5-FU compared with the IR of HCT-8/5-FU. The IC_{50} of 5-FU on HCT-8 and HCT-8/5-FU

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cells were 4.47 μg/mL and 331.10 μg/mL respectively (Table 2). So the RI of HCT-8/5-FU was 74.12-fold.

Table 2. IC Values of Curcumin or 5-FU on Two Cell Lines (μg/mL)

Drug	Cell line	IC_5 (A-IC_5)	IC_{10} (A-IC_{10})	IC_{25}	IC_{50}
Curcumin	HCT-8	3.81	4.91	7.14	10.38
	HCT-8/5-FU	4.16 (4.0)	5.55 (5.5)	8.48	12.96
5-FU	HCT-8	–	–	0.47	4.47
	HCT-8/5-FU	–	–	96.40	331.10

Note: A-IC, appoximate inhibitory concentration (A-IC).

Inhibitory Effect of 5-FU Combined with Curcumin on HCT-8/5-FU Cells Proliferation

As shown in Figure 2C, 5-FU and curcumin had a synergistic effect on HCT-8/5-FU cells, the IR was higher in 5.5 μg/mL curcumin combined with 5-FU than 4.0 μg/mL curcumin with 5-FU. IC_{50} of 5-FU combined with 4.0 μg/mL curcumin was calculated as 179.26 μg/mL, for this RF was calculated as 1.85. IC_{50} of 5-FU combined with 5.5 μg/mL curcumin were calculated as 89.25 μg/mL, from which the RF were calculated as 3.71.

Synergistic Effect of 5-FU and Curcumin on HCT-8 and HCT-8/5-FU Cells

The IR and synergistic effect (*q*) of 3 concentrations of curcumin (IC_5, IC_{25}, IC_{50}) combined with 5-FU (IC_{25}) are shown in Table 3. The IR of curcumin and 5-FU on two cell lines was in a dose-dependent manner, which was higher when curcumin in a high concentration. However, the synergistic effect was on the opposite, the *q* was lower when curcumin in a high concentration. Regarding the concentrations, the IR of IC_{25} were higher than IC_5 of curcumin, the synergistic effect was higher than IC_{50}. Moreover, the concentration of curcumin less than IC_{50} was safer. As a result, IC_{25} of curcumin was chosen as the optimal reversing concentration.

Table 3. Synergistic Effect of Curcumin and 5-FU on Two Cell Lines

Cell line	Dose of 5-FU (μg/mL)	Dose of Cur (μg/mL)	IR (%)	*q* value
HCT-8	0.47	3.81	40.01	1.39
	0.47	7.14	58.79	1.34
	0.47	10.38	67.75	1.08
HCT-8/5-FU	96.40	4.16	47.49	1.65
	96.40	8.44	56.00	1.28
	96.40	12.96	69.94	1.12

Note: IR, inhibition rate

Effect of 5-FU and/or Curcumin on the Cell Cycle

DNA-FCM with propidium iodide identified a sub-population of cells. The cell cycle graphs demonstrated that the percentage of cells in the G_0/G_1 phase was significantly increased when treated with 5-FU and/or curcumin on HCT-8 (Appendix 1A), compared with the control ($P<0.05$). Meanwhile, the S phase of the cell cycle was obviously decreased following treatment of 5-FU and/or curcumin on HCT-8/5-FU (Appendix 1B), compared with the control ($P<0.05$). Cells in the G_2/M phase were significantly reduced with a combination treatments of 5-FU and curcumin (IC_{50}), compared to control ($P<0.05$). HCT-8 and HCT-8/5-FU cells mostly accumulated at G_0/G_1 phase. Cells were increased at G_0/G_1 phase and decreased at S and G_2/M phase when 5-FU combined with curcumin (Figure 3).

Cell Apoptosis Caused by 5-FU and/or Curcumin

The percentage of apoptotic cells was significantly increased after 5-FU and/or curcumin treatment (Figure 4, Appendix 2). The percentage of apoptotic cells was further increased following treatment with the mixtures of the two agents in comparison with either drug alone in HCT-8/5-FU, the percentage of apoptotic cells positively correlated with concentrations of curcumin.

Cell Apoptosis Examined by TEM

As shown in Figure 5, after exposed to 5-FU

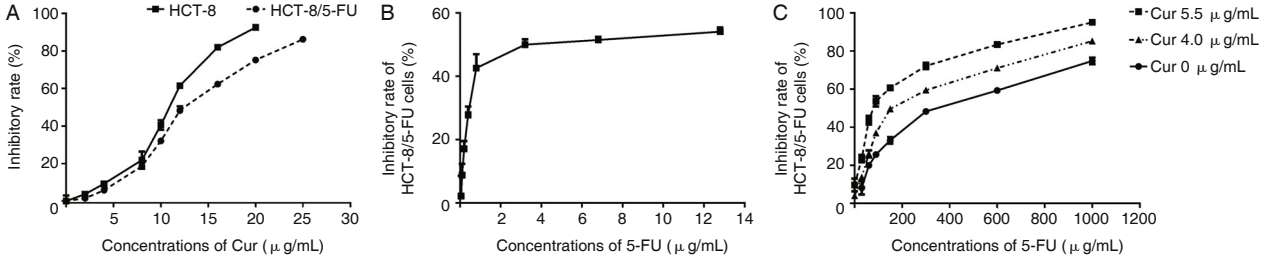

Figure 2. Inhibitory Rate of Curcumin and/or 5-FU on Two Cell Lines

Notes: A: inhibitory rate of curcumin on HCT-8/5-FU and HCT-8 cells; B: inhibitory rate of 5-FU on HCT-8 cells; C: inhibitory rate of 5-FU alone or combined with curcumin on HCT-8/5-FU cells.

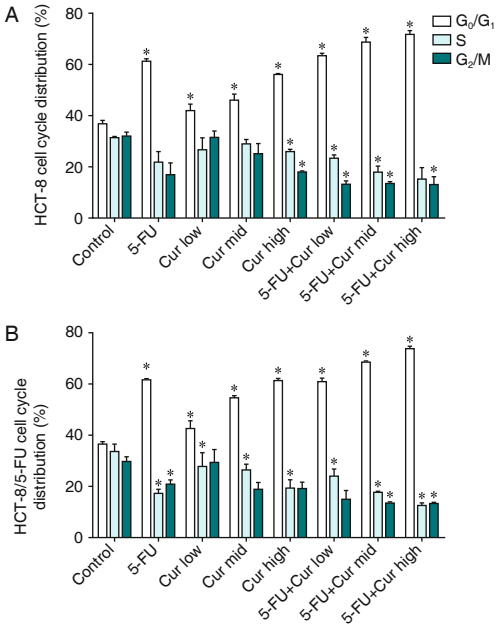

Figure 3. Analyses of Cell Cycle of Two Cell Lines Treated with 5-FU, Curcumin or Their Combination

Note: *P<0.05 *vs*. the same cell phase of control group

(IC_{25}) and/or curcumin (IC_5, IC_{25}, IC_{50}) for 48 h, the ultrastructural morphology of HCT-8 (Figure 5A) and HCT-8/5-FU (Figure 5B) cells were characterized by cell shrinkage and blebbing condensation of nuclear

Table 4. Grouping Information of Drug Intervention (μg/mL)

Group	HCT-8		HCT-8/5-FU	
	5-FU	Cur	5-FU	Cur
Control	0.00	0.00	0.00	0.00
5-FU (IC_{25})	0.47	0.00	96.40	0.00
Cur low (IC_5)	0.00	3.81	0.00	4.16
Cur mid (IC_{25})	0.00	7.14	0.00	8.44
Cur high (IC_{50})	0.00	10.38	0.00	12.96
5-FU (IC_{25})+Cur low (IC_5)	0.47	3.81	96.40	4.16
5-FU (IC_{25})+Cur mid (IC_{25})	0.47	7.14	96.40	8.44
5-FU (IC_{25})+Cur high (IC_{50})	0.47	10.38	96.40	12.96

Note: IC, inhibitory concentration.

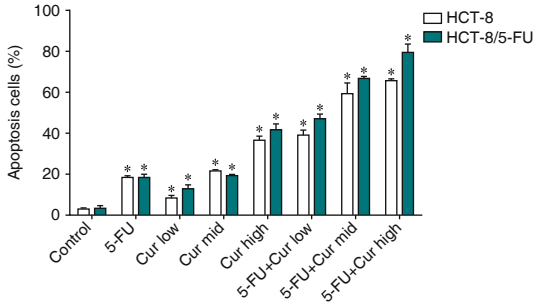

Figure 4. Effect of 5-FU and/or Curcumin on Apoptosis in HCT-8 and HCT-8/5-FU Cells

Note: *P<0.05 *vs*. the same cell line of control group.

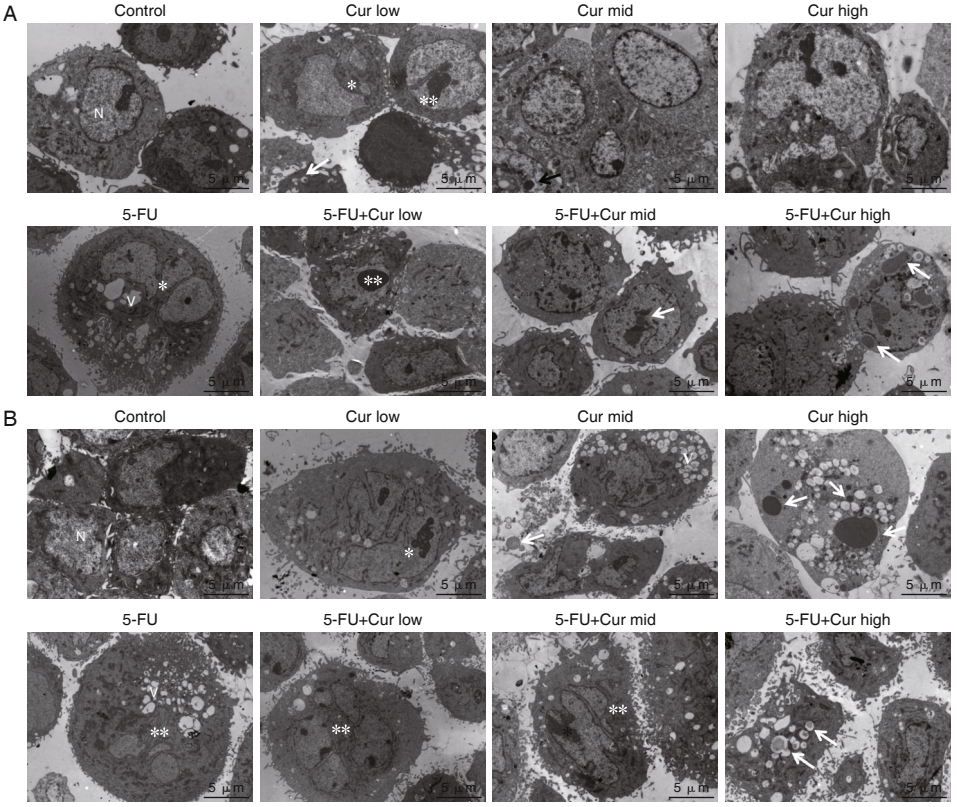

Figure 5. Ultrastructural Morphology of HCT-8 and HCT-8/5-FU Cells after Treated with Curcumin and/or 5-FU

Notes: A: the apoptosis examined by TEM of HCT-8 cells; B: the apoptosis examined by TEM of HCT-8/5-FU cells; *nuclear fragmentation; **nucleus with condensed chromatin; arrow indicates apoptotic body; N, nuclear; V: vacuole; scale bar = 5 μm.

chromatin and nuclear fragment. The apoptotic bodies were also visible in cells treated with combination of curcumin and 5-FU. Cells in control group retained their normal cellular morphological characteristics.

mRNA Levels of P-gp and HSP-27 in Two cell Lines Decreased after Treatment

As shown in Figure 6A, the expression of P-gp and HSP-27 mRNA were significantly decreased compared to the control group (P<0.05). To further analyze the data, we found the expression of P-gp (0.28±0.02) and HSP-27 (0.28±0.09) in HCT-8/5-FU were markedly decreased with the mixtures of the 2 agents in comparison with 5-FU alone (P-gp, 0.48±0.07, P=0.009; HSP-27, 0.57±0.10, P=0.007) in HCT-8/5-FU cell line.

Protein Levels of P-gp and HSP-27 Decreased in HCT-8/5-FU Cell Line after Treatment

As shown in Figure 6B, the protein levels of P-gp and HSP-27 were significantly decreased after treated with 5-FU or curcumin or their combination compared to control group (P<0.05). The protein levels of P-gp (0.25±0.06) and HSP-27 (0.09±0.02) were lower with the combination in comparison with 5-FU alone (P-gp, 0.46±0.02, P=0.005; HSP-27, 0.43±0.01, P=0.000) in HCT-8/5-FU cell line (Figure 6B).

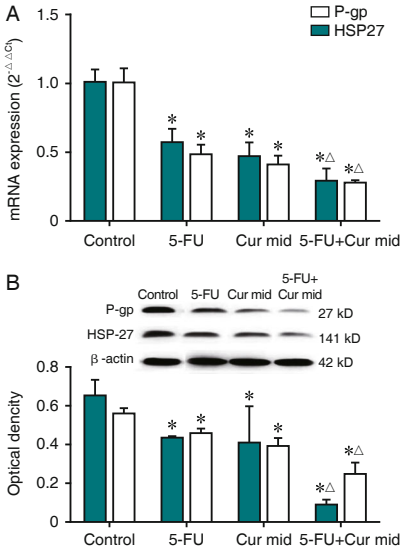

Figure 6. mRNA and Protein Expressions of HSP-27 and P-gp by 5-FU and /or Curcumin Treatment in HCT-8/5-FU

Note: A: the mRNA expression of HSP-27 and P-gp; B: the protein levels of HSP-27 and P-gp. *P<0.05 *vs*. control group; △P<0.05 *vs*. 5-FU group

DISCUSSION

Curcumin is a natural product with important therapeutic properties useful to treat human cancer. It has been previously used in herbal medicine and as a dietary compound with non-toxic effects.[16,17] In the present study, synergistic effect of 5-FU and curcumin on HCT-8 and HCT-8/5-FU cells were investigated. The results assumed that curcumin inhibited the proliferation of two colon cancer cell lines and enhanced the cytotoxicity of 5-FU on HCT-8/5-FU cells by inducing cell apoptosis, cell cycle arrest and down-regulating the expression of MDR acquisition related factors HSP-27 and P-gp.

A lot of studies elucidate that curcumin can reverse the MDR of chemotherapy in cancer cell by negative regulation of inflammatory cytokines, transcription factors, protein kinases, reactive oxygen species (ROS) and oncogenes, promote apoptosis, radiosensitization, chemosensitization of cancer cells, reduce angiogenesis and metastasis to inhibit tumor proliferation.[18-20] Noratto, et al[21] reported that curcuminoids could mediate the drug resistance suppression in colon cancer by reactive oxygen species-induced disruption of the microRNA-27a-ZBTB10-Sp axis. In colon cancer mice, absorbable curcumin and bevacizumab significantly inhibited the tumor growth and there was no observable side effect induced by absorbable curcumin.[22] Lu, et al[23] found that the sensitivity of cancer cells to vincristine, cisplatin, fluorouracil, and hydroxycamptothecin was enhanced, and the expression of the multidrug resistance gene and P-glycoprotein were significantly suppressed after vincristine-resistant cell line of human colon cancer treated with curcumin at concentrations greater than 25 μmol/L. Ye, et al[24] found that curcumin can reverse the drug resistant in lung cancer cells by inhibiting the expression of hypoxia inducible factor 1α and activating caspase-3. These findings suggest that curcumin may represent a reversal agent for the chemosensitization of cancer cells.

In our study, the treatment of HCT-8 and HCT-8/5-FU cells with different concentrations curcumin inhibited the growth of the cells in a concentration-dependent manner. Compared with control and 5-FU group, combined curcumin and 5-FU treatment resulted in an even profound inhibitory effect in cell proliferation. The IC_{50} of combination 5-FU and curcumin was significantly decreased, showing a synergistic effect on up-regulation of cell apoptosis and cell cycle arrest. Our results once again confirm that curcumin can enhance the sensitivity of cancer cell to 5-FU.

The mechanism of MDR formation is complex. Previous study showed that curcuminoids enhance the anticancer activity of the chemotherapeutic drug 5-FU due to the suppression of MDR1.[21] P-gp-mediated MDR is considered to be the classic mechanism of resistance.[25] P-gp is encoded by MDR1 genes, also called ABCB1 and works in a similar manner to a pump to extrude anticancer drugs out of cells. P-gps expressed in the plasma membrane are mediators of MDR, actively effluxing a wide range of amphiphilic drugs irrespective of concentration gradient, thereby reduce intracellular drug levels to less than therapeutic concentrations.[26] Overexpression of P-gp in various cancer cells has prompted numerous research groups to search effective inhibitors for this glycoprotein. Series of dates report has shown that down-regulating P-gp can reverse the drug resistance of MDR human cancer cells.[27] Recent research reveals that curcumin was a modulator of P-gp in cancer, which has been associated with inhibiting both P-gp function and expression.[28] In keeping with previous studies, our study found that the expression of P-gp significantly reduced in combination of curcumin and 5-FU group compared to control and 5-FU groups in HCT-8/5-FU cells. This result suggest that curcumin may reverse the drug resistance of HCT-8/5-FU cells to 5-FU by down-regulating the expression of P-gp.

HSPs are involved in tumour immunity, and are correlated with survival and drug resistance in numerous types of cancer.[29] HSP-27 belongs to HSPs family and has been reported associating with MDR.[30,31] Researches showed that HSP-27 has been implicated in drugs induce functional ABC transporters in cancer cells.[5,32] Forced expression of HSP-27 reverses P-gp-mediated drug efflux and MDR1 gene expression in Adriamycin-resistant human breast cancer cells.[32] In the present study, we found the level of HSP-27 was obviously decreased when HCT-8/5FU cells co-treated with curcumin and 5-FU compared to 5-FU alone. The trend of HSP-27 level was similar as P-gp, which suggested that there was an interaction between HSP-27 and P-gp. However, this hypothesis needs further research.

In conclusion, this study provides an important mechanism based knowledge with potential utility in overcoming drug resistance induced by 5-FU. Curcumin is a new reversal agent for the chemosensitization of cancer cells to 5-FU by promoting cell apoptosis, cycle arrest and inhibiting cell proliferation. The mechanism of reversing drug resistance in HCT-8/5-FU to 5-FU may be contributed to down-regulating the expression of P-gp and HSP-27 levels.

Conflict of Interest

The authors declared that they had no conflict of interests.

Author Contributions

He WT, Zhang T and Zhang HL conceived of the study, participated in its design and coordination, and drafted the manuscript. Zhu YH carried out the flow cytometry. Abulimiti P and Zeng FY were responsible for cell culture and CCK-8 assay. Zhang LP and Luo LJ performed the statistical analysis, transmission electron microscopy analysis and PCR. Xie XM performed Western blot assay.

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