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Basic Study

Qingjie Fuzheng granules inhibit colorectal cancer cell growth by the PI3K/AKT and ERK pathways

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Abstract**BACKGROUND**

Qingjie Fuzheng granules (QFGs) are part of a traditional Chinese medicine formula, which has been widely used and found to be clinically effective with few side effects in various cancer treatments, including colorectal cancer (CRC). However, the precise mechanisms and molecular signaling pathways involved in the activity of QFGs' anticancer effect have not been reported in the literature. In this study, we hypothesized that QFGs can inhibit the growth of colorectal cancer cells, and that its mechanism is closely related to one or more intracellular signal transduction pathways.

AIM

To better evaluate the mechanism underlying the anti-cancer effect of QFGs on the CRC cell lines HCT-116 and HCT-8.

METHOD

First, we measured cell viability and cytotoxicity by performing MTT and lactate dehydrogenase (LDH) assays. We evaluated the role of QFGs in cell proliferation and apoptosis by assessing colony formation and analyzing Hoechst 33258 staining. Second, cell cycle and apoptosis rates were measured by fluorescence activated cell sorting, and the expression levels of survivin, cyclin D1, CDK4, p21, Bax, Bcl-2, Fas, FasL, and cleaved-caspase-3/-8/-9 were measured by performing western blots and caspase activity assays. Furthermore, inhibitors of caspase-3/-8/-9 were used to elucidate the specific apoptosis pathway induced by QFGs in cancer cells. Finally, activation of the PI3K/AKT and ERK signaling pathways

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was examined using the western blot assay to investigate the possible mechanism.

RESULTS

MTT and LDH assays revealed that after 0.5-2.0 mg/mL of QFGs treatment, cell viability was reduced by (6.90% ± 1.03%)–(59.70% ± 1.51%) (HCT-116; $P < 0.05$) and (5.56% ± 4.52%)–(49.44% ± 2.47%) (HCT-8; $P < 0.05$), and cytotoxicity was increased from 0.52 ± 0.023 to 0.77 ± 0.002 (HCT-116; $P < 0.01$) and from 0.56 ± 0.054 to 0.81 ± 0.044 (HCT-8; $P < 0.01$) compared with the non-QFGs treatment groups. Additionally, colony formation and Hoechst 33258 staining assays showed that QFGs inhibited proliferation and induced apoptosis in CRC cells. QFGs also increased the expression levels of Bax, Fas and FasL, decreased the level of Bcl-2, and stimulated the activation of caspase-3/-8/-9, which were revealed by western blot and caspase activity assays. In contrast, when adding the three caspase inhibitors, the suppression effect of QFGs on cell viability and apoptosis were markedly inhibited. Moreover, QFGs suppressed the phosphorylation levels of PI3K, AKT and ERK.

CONCLUSION

These results demonstrated that QFGs can inhibit CRC cell proliferation and induce apoptosis by suppressing the PI3K/AKT and ERK signaling pathways.

Key words: Qingjie Fuzheng granules; Colorectal cancer; Proliferation; Apoptosis; PI3K/AKT; ERK

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Core tip: To study the effect and mechanism of Qingjie Fuzheng granules (QFGs) on colorectal cancer (CRC) cells, we measured cell viability and cytotoxicity by performing MTT and LDH assays. We also evaluated the role of QFGs in cell proliferation by conducting colony formation, cell cycle and western blot assays. We evaluated the role of QFGs in cell apoptosis by assessing both Hoechst 33258 and Annexin-V/PI staining and performing western blot assays. Furthermore, the activation of PI3K/AKT and ERK signaling pathways was examined using the western blot assay to investigate the possible mechanism. All results demonstrated that QFGs inhibited CRC cell growth by suppressing the PI3K/AKT and ERK signaling pathways.

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INTRODUCTION

As a result of changing lifestyles and aging populations, the prevalence of colorectal cancer (CRC) remains high and accounts for approximately 25% of the world's cancer-related deaths^[1-3]. Although surgical resection and chemoradiotherapy are the most commonly used clinical options, surgery is not an option for all patients, and long-term chemoradiotherapy can cause adverse side effects, such as drug resistance, recurrence and metastasis^[4-7]. Therefore, the search for novel therapies has attracted worldwide attention.

Natural products, which often have fewer side effects than synthetic drugs, are important in the treatment of many diseases and have a long history of use in China. Therefore, natural products have been studied by many researchers to find better antitumor drugs^[8-11]. Qingjie Fuzheng granules (QFGs) is a traditional Chinese medicine (TCM) formula (Table 1) that consists of a mixture of four herbs (*Scutellaria barbata* D. Don, malt, *Hedyotis diffusa* Willd, and Astragalus) that together confer properties of anti-inflammation, antioxidative, antibacterial, immunity enhancement and digestion promotion. QFGs have been widely used and found to be clinically effective in various cancer treatments, including CRC, and have few side effects.

However, the precise mechanisms and molecular signaling pathways involved in the activity of QFGs' anticancer effect have not been reported in the literature.

CRC develops because of a cell growth imbalance caused by excessive proliferation or lack of apoptosis. Eukaryotic cell proliferation is controlled by the cell cycle, which consists of the G₀, G₁, S, G₂ and M phases. In the detection of cell cycle progression, the G₁/S transition is one of the main checkpoints^[12]. The main regulatory factors in G₁/S progression are cyclin D1 and cyclin-dependent kinase 4 (CDK4), which can form complexes to regulate this progress^[13-15]. A CDK inhibitor, p21, can change the function of CDK-cyclin complexes by binding to them and then suppressing cell proliferation^[16]. Normal cell apoptosis can eliminate surplus, redundant, and aberrant cells in animals, so it is essential for normal tissue maintenance. Disorders in this process trigger many diseases, including CRC^[17-19]. The pathways involved in the apoptotic process are the mitochondria-dependent pathway, also called the intrinsic apoptosis pathway, and the death receptor-mediated apoptosis pathway^[20]. The former is modulated by the Bax (proapoptotic) and Bcl-2 (anti-apoptotic) family proteins^[21], which control the release of apoptotic correlation factors, such as cytochrome C (Cyt C)^[22]. When intracellular damage occurs, mitochondria-dependent apoptosis is triggered. Then, Cyt C, together with Apaf-1 and caspase-9, cleaves caspase-3^[23]. Receptor-mediated apoptosis originates from outside the cell, with the binding of the Fas ligand (termed FasL or CD95L) to the Fas receptor (termed CD95). Once the death receptor pathway is successfully activated, the Fas-associated death domain and caspase-8 will accumulate, and caspase-8 will be cleaved. Then, caspase-8 cleaves caspase-3, which generates the activated form of caspase-3 that serves as the ultimate activator of apoptosis^[24]. Therefore, one of the key approaches in the development of antitumor drugs is to promote apoptosis and inhibit tumor cell proliferation, two processes that typically promote cancer growth. There are multiple signaling pathways that regulate cancer growth, including the PI3K/AKT and ERK signaling pathways, and abnormal activation of these signaling pathways can lead to irregular expression of these factors.

The aim of this study is to better understand the mechanism underlying the potential anticancer effect of QFGs by investigating their biological function using the human CRC cell variants HCT-116 and HCT-8. Our results showed that QFGs inhibit proliferation and increase apoptosis in HCT-116 and HCT-8 cells by inactivating the PI3K/AKT and ERK pathways.

MATERIALS AND METHODS

Cell culture

The human colon carcinoma HCT-8 and HCT-116 cell lines were purchased from the American Type Culture Collection. The two cell lines were cultured in Roswell Park Memorial Institute-1640 medium (C11875500BT; Life Technologies Corp. Grand Island, United States) containing 10% fetal bovine serum, 1% penicillin, and 1% streptomycin, and were grown at 37 °C in 5% CO₂.

Preparation of QFGs and caspase inhibitors

QFGs were obtained and prepared as previously described^[11]. Briefly, QFGs powder was dissolved in 1 × PBS (store concentration of QFGs is 200 mg/mL) and stored at 20 °C. Inhibitors of caspase-3/-8/-9 (Z-DEVE-FMK, ab120488; Z-IETD-FMK, ab141382; Z-LEHD-FMK, ab142026, Abcam, CA, United States) were dissolved in DMSO to a concentration of 10 mM and stored at -20 °C.

MTT assays

HCT-8 and HCT-116 cells were placed into 96-well plates (1 × 10⁵ cell/well). After 12 h, the cells were treated with different doses of QFGs (0.5, 1 and 2 mg/mL) and grown for 24 h, or the cells were treated with a designated dose of QFGs (2 mg/mL) in combination with inhibitors of caspase-3 (Z-DEVE-FMK), caspase-8 (Z-IETD-FMK), and caspase-9 (Z-LEHD-FMK) at a concentration of 10 μmol/L each and then incubated for 24 h. Then, MTT (0.5 mg/mL) was added to each well (100 μL/well) and incubated for 4 h. Subsequently, all wells were treated with DMSO (100 μL/well). Absorbance at 570 nm in each well was measured by using an ELISA reader (Infinite M200 PRO; Tecan Austria GmbH, Austria).

Lactate dehydrogenase assays

Cells were seeded into 12-well plates (1 × 10⁵ cell/well), treated with different dose of QFGs (0.5, 1 and 2 mg/mL) and grown for 24 h. Then, a lactate dehydrogenase (LDH) release assay kit (Beyotime, Shanghai, China) was used to determine the LDH activity according to the kit's manual.

Table 1 Composition of Qingjie Fuzheng granules

Common name	Latin name	Part used	Daily adult dose, g
Spreading Hedyotis herb	<i>Hedyotis diffusa</i> Willd	Dried root	15
Malt	<i>Hordeum vulgare</i> L.	Dried seed	15
Astragalus	<i>Radix astragali</i>	Dried root	15
Scutellaria barbata	<i>Scutellaria barbata</i> D. Don	Dried body	15

Colony formation assays

After treatment with different concentrations of QFGs (0.5, 1, and 2 mg/mL) for 24 h, a colony formation assay was performed as described previously^[11].

Hoechst 33258 staining

QFGs and 10 µmol/L caspase inhibitors (Z-DEVE-FMK, Z-IETD-FMK, Z-LEHD-FMK) were added to the cells and grown for 24 h. Subsequently, 4% paraformaldehyde was used to fix the cells for 15 min. Then, 4% paraformaldehyde was removed and 1× PBS was used to rinse the cells three times. Then, Hoechst 33258 (c0003; Beyotime, Shanghai, China) (100 µL/well) was added to all wells in the dark for 15 min. The Hoechst 33258 solution was then removed, and 1× PBS was used to rinse the stained cells three times, followed by 1× addition of fresh PBS. An inverted fluorescence microscope (Leica DMI4000B; Leica Camera AG, Solms, Germany) was used to observe and photograph the cells.

Cell cycle assays

Cell cycle was measured in the HCT-8 and HCT-116 cells after treatment with the indicated concentrations of QFGs (0.5, 1 and 2 mg/mL). Cell cycle progression was estimated by using a propidium iodide (PI) kit (KGA512; KeyGen Biotech, Nanjing, China) and fluorescence activated cell sorting according to the manufacturer's instructions.

Annexin V-FITC/PI staining flow cytometry assays

Cells were seeded into 6-well plates (1.5×10^5 cell/well), treated with different doses of QFGs (0.5, 1 and 2 mg/mL) and grown for 24 h. Then, cells were stained by using an Annexin V/PI kit (KGA108; KeyGen BioTech, Nanjing, China) according to the kit's manual. Annexin V-positivity and PI-negativity (lower-right quadrant) represented early apoptotic cells, whereas Annexin V-positivity and PI-positivity (upper-right quadrant) represented late apoptotic cells.

Caspase activity assays

A caspase activity assay kit (caspase-3, KGA204; caspase-8, KGA304; caspase-9, KGA404; KeyGen BioTech, Nanjing, China) was used to detect the activity of caspases according to the manufacturer's instructions. In brief, cell lysates were prepared after the addition of the indicated reaction buffer (provided in the kit) at 37 °C for 4 h in the dark. Absorbance at 405 nm was measured by using an ELISA reader.

Western blot analysis

When CRC cells were treated with different doses of QFGs (0.5, 1 and 2 mg/mL) for 24 h, a cell lysis buffer containing a cocktail to lyse the cells was added. The bicinchoninic acid assay was used to detect the total protein concentrations, and 50 µg of total protein was used for electroblotting. Five percent skim milk was used to block the NC membranes, and then primary antibodies against Fas, FasL, p-PI3K and p-AKT (ab-110021, ab-15285, ab182651, ab38449; 1:1000, Abcam, CA, United States), p-ERK (sc-16982; 1:1000, Santa Cruz Biotechnology, CA, United States), cleaved-caspase-3, cleaved-caspase-8, cleaved-caspase-9, β-actin, Bcl-2 and Bax (#9662, #4790, #9508, #4967, #4223, #5023; 1:1000, Cell Signaling, Beverly, MA, United States), PI3K, AKT and ERK (13329-1-AP, 10176-2-AP, 16443-1-AP; 1:2000, Proteintech, United States) were added at 4 °C overnight. On the second day, the appropriate HRP-conjugated secondary antibodies (goat anti-mouse IgG secondary antibody, #L3032; goat anti-rabbit IgG secondary, #L3012; 1:5000, Signalway Antibody, PA, United States) were added and the SuperSignal West Pico Chemiluminescent Substrate was used to detect the signal.

Statistical analysis

One-way ANOVA and SPSS software (version 18.0) were used to analyze all of the data in this study. The data are expressed as the mean ± standard deviation. $P < 0.05$

indicated statistical significance.

RESULTS

QFGs decreased cell viability and increased cytotoxicity in HCT-116 and HCT-8 cells

The MTT assays and LDH activity assays were used to evaluate the effect of QFGs on the growth of the two cell types. QFGs inhibited cell viability in a dose-dependent manner (Figure 1A, B), showing cytotoxicity at 0.5–2.0 mg/mL (Figure 1C, D). In Figure 1A and B, cell viability after treatment with QFGs (0.5–2.0 mg/mL, 24 h) decreased by (6.90% ± 1.03%)–(59.70% ± 1.51%) (HCT-116) and (5.56% ± 4.52%)–(49.44% ± 2.47%) (HCT-8) relative to the viability in control cells ($P < 0.05$). In Figure 1C and D, treatment with QFGs (0.5–2.0 mg/mL, 24 h) increased the LDH activity rate of the cells from 0.52 ± 0.023 to 0.77 ± 0.002 (HCT-116) and from 0.56 ± 0.054 to 0.81 ± 0.044 (HCT-8) relative to that in control cells ($P < 0.01$). These results proved that QFGs treatment reduced cell viability and increased cytotoxicity in both cell types.

QFGs inhibited the proliferation of HCT-116 and HCT-8 cells by arresting the cell cycle

Cell colony formation assays were used to evaluate the changes in cell growth after treatment with QFGs. As shown in Figure 2A, QFGs dose-dependently inhibited colony formation in HCT-116 and HCT-8 cells. Subsequently, cell cycle assays were used to verify the proliferation-inhibiting effects of QFGs. As shown in Figure 2B–E, the percentages of S phase in HCT-116 cells after treatment with 0, 0.5, 1, and 2 mg/mL QFGs were 44.7% ± 2.77%, 33.45% ± 3.30%, 16.50% ± 2.12%, and 12.86% ± 2.51%, respectively ($P < 0.01$), and the percentages of S phase in HCT-8 cells after treatment with 0, 0.5, 1, and 2 mg/mL QFGs were 44.55% ± 3.32%, 26.71% ± 2.17%, 25.60% ± 2.19%, and 21.99% ± 3.30%, respectively ($P < 0.01$). These results suggested that QFGs can inhibit proliferation of both cell types by arresting the cell cycle.

QFGs induced apoptosis of HCT-116 and HCT-8 cells via the mitochondria-dependent and death receptor pathways

Hoechst 33258 staining assays were used to evaluate the changes in cell nuclear morphology after treatment with QFGs (Figure 3A). The degree of staining was low in untreated cells, but it gradually increased in the other three groups, which indicated a gradual increase in apoptosis. Subsequently, Annexin V-FITC/PI assays were used to verify the apoptosis-inducing effect of QFGs. As shown in Figure 3B, apoptosis percentages in HCT-116 cells after treatment with 0, 0.5, 1, and 2 mg/mL QFGs were 4.07% ± 0.48%, 11.87% ± 0.5%, 12.77% ± 0.67%, and 31.13% ± 0.73%, respectively ($P < 0.01$), and apoptosis percentages in HCT-8 cells after treatment with 0, 0.5, 1, and 2 mg/mL QFGs were 2.23% ± 0.50%, 9.34% ± 0.69%, 17.19% ± 0.55%, and 33.93% ± 0.93%, respectively ($P < 0.01$). We also found that QFGs upregulated the expression of cleaved-caspase-3/-8/-9 in both cell types (Figure 3C, D; $P < 0.01$). At the same time, the caspase activity was measured using a commercial caspase activity assay kit. Identical to the western blot results, the activities of caspase-3/-8/-9 were significantly enhanced by QFGs treatment in both cell types (Figure 3E, F; $P < 0.01$). These results indicated that QFGs induced apoptosis in the two cell types, and suggested that apoptosis occurred *via* both the mitochondria-dependent and death receptor-mediated pathways.

To further confirm these findings, various specific caspase inhibitors were used. As shown in Figure 4A, Z-DEVD-FMK, Z-IETD-FMK, and Z-LEHD-FMK markedly inhibited the inhibitory effect of QFGs on cell viability in both cell types ($P < 0.05$ and 0.01, respectively). In addition, we used the Hoechst 33258 staining assay to detect nuclear morphological changes, and all three caspase inhibitors clearly inhibited the apoptosis-induced effect of QFGs (2 mg/mL) in both cell types (Figure 4B). These findings proved that QFGs induced apoptosis *via* the mitochondria-dependent and death receptor-mediated pathways in both HCT-116 and HCT-8 cells.

QFGs regulated the expressions of survivin, cyclin D1, CDK4, p21, Bax, Bcl-2, Fas, and FasL in HCT-116 and HCT-8 cells

During cell cycle regulation, survivin is a key protein that indicates cell cycle progression, and the complex of cyclin D1 and CDK4 directly regulates cell cycle progression^[13]. In a previous study, overexpression of this complex has been found to induce cell proliferation, whereas p21 inhibited the effect of the cyclin D1/CDK4 complex^[14]. During the regulation of cell apoptosis, Bax and Bcl-2 regulate the mitochondria-dependent pathway^[21], while Fas and FasL activate the death receptor-

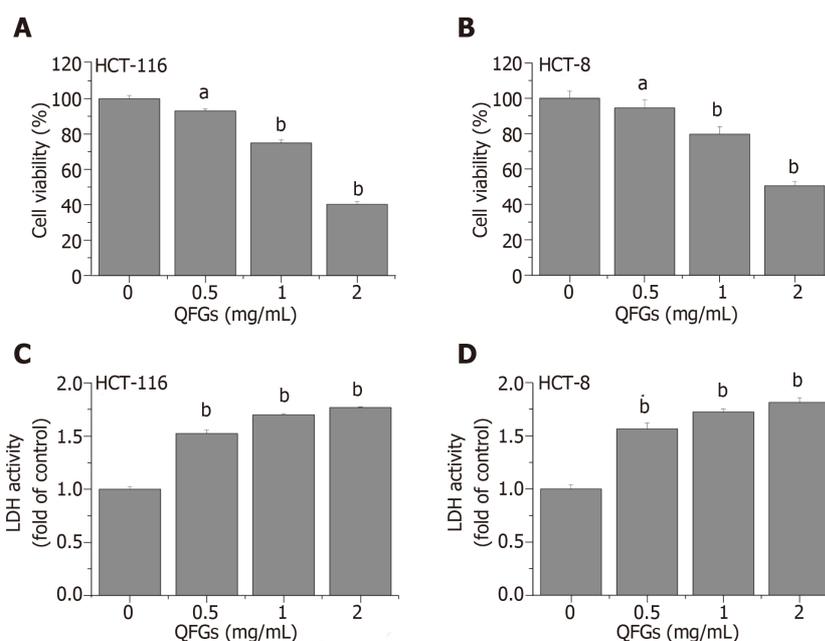


Figure 1 Effect of QFGs on the viability and cytotoxicity of HCT-116 and HCT-8 cells. A, B: Cell viability was measured by MTT assay; C, D: Cytotoxicity was measured by LDH assay. ^a $P < 0.05$, ^b $P < 0.01$ vs the control group. QFGs: Qingjie Fuzheng granules; LDH: Lactate dehydrogenase.

mediated pathway^[24]. We found that QFGs can inhibit proliferation *via* arrest of the cell cycle in HCT-116 and HCT-8 cells. We used western blotting to test the protein expression levels of survivin, cyclin D1, CDK4, and p21 after the cells were treated with QFGs. As shown in **Figure 5A** and **B**, we found that QFGs downregulated the expression of survivin, cyclin D1 and CDK4, but upregulated the level of p21 in both cell types ($P < 0.05$ and 0.01 , respectively). Since we found that QFGs induced apoptosis *via* the mitochondria-dependent and death receptor-mediated pathways in HCT-116 and HCT-8 cells, we used western blotting to test the protein expression levels of Bcl-2, Bax, Fas, and FasL after the cells were treated with QFGs. As shown in **Figure 5C** and **D**, we found that QFGs decreased the expression of Bcl-2 but promoted the expression of Bax, Fas, and FasL in both cell types ($P < 0.05$ and 0.01 , respectively). Briefly, these findings suggest that QFGs inhibit proliferation *via* cell cycle arrest and induce apoptosis *via* the mitochondria-dependent and death receptor-mediated pathways in HCT-116 and HCT-8 cells.

QFGs suppressed the PI3K/AKT and ERK signaling pathways in HCT-116 and HCT-8 cells

Cancer occurrence and progression are highly associated with the regulation of multiple signaling pathways, including PI3K/AKT and ERK^[25,26]. To further explore the potential mechanisms underlying the observed anticancer effects of QFGs, we examined the expression of major regulation factors involved in the PI3K/AKT and ERK signaling pathways. As shown in **Figure 6A** and **B**, after treatment with QFGs, the ratios of the phosphorylation expression level to the total expression level were significantly downregulated in PI3K, AKT and ERK in both cell types ($P < 0.05$ and 0.01 , respectively), which suggested that the anticancer effect of QFGs on CRC cells occurs *via* suppression of the PI3K/AKT and ERK signaling pathways.

DISCUSSION

CRC is a deadly disease, primarily due to its high rate of metastasis and recurrence in patients. Multidrug combination therapy and surgical treatment are the main therapeutic methods that can significantly improve patients survival. However, there are many patients with cancer that is drug resistant and recurrent after traditional clinical treatment^[27,28]. Therefore, there is a need to discover new kinds of anticancer drugs, such as herbal products. In recent years, TCM has attracted increasing attention in the field of oncology because of its relative security and long history of application^[29-32]. QFGs are a four-herb TCM formula that consists of *Scutellaria barbata* D. Don, malt, *Hedyotis diffusa* Willd, and Astragalus. In the past few years, some

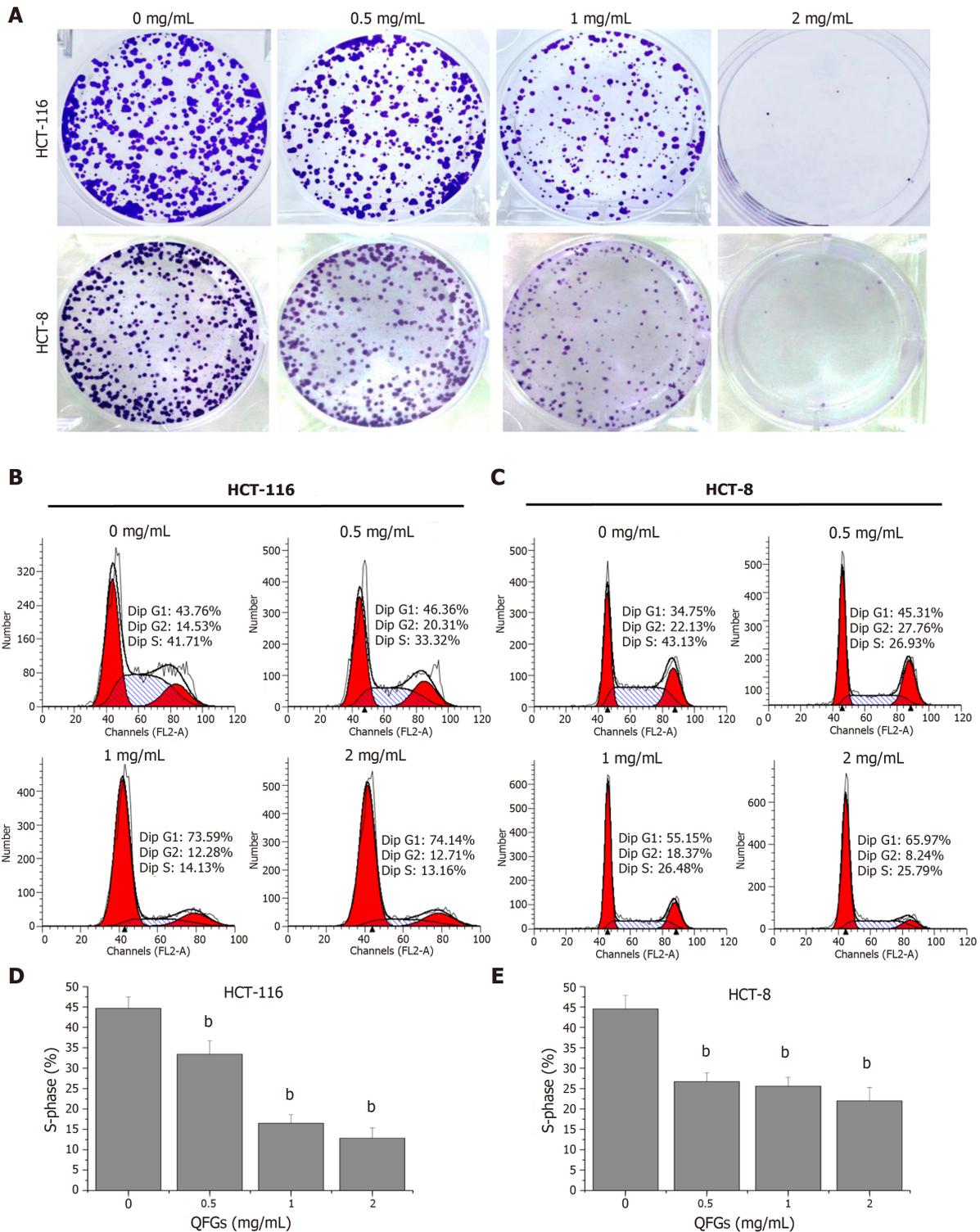
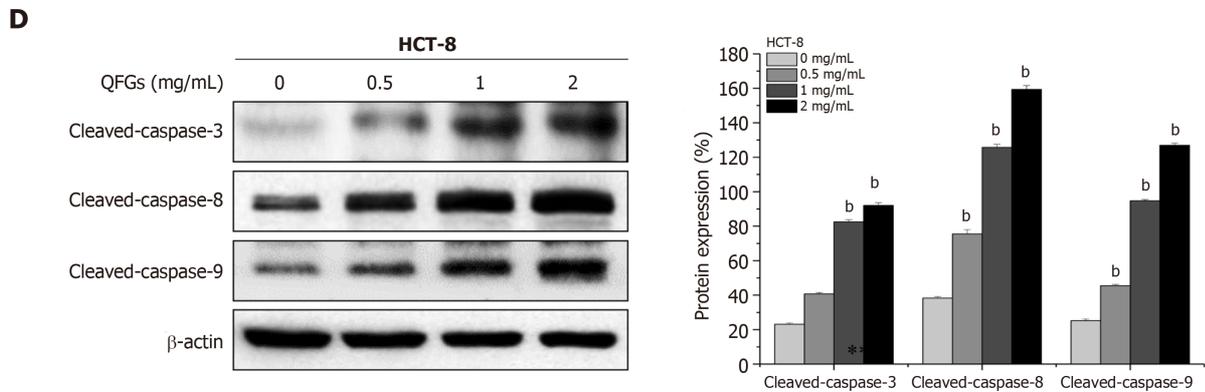
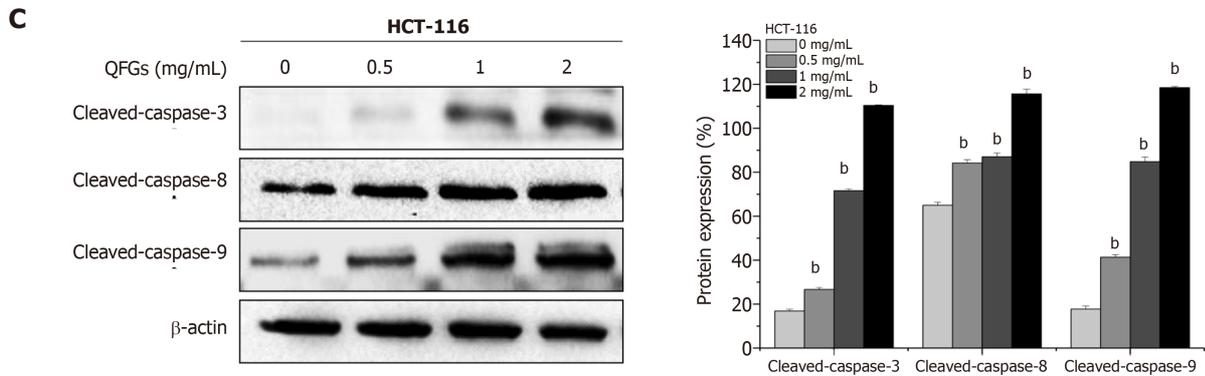
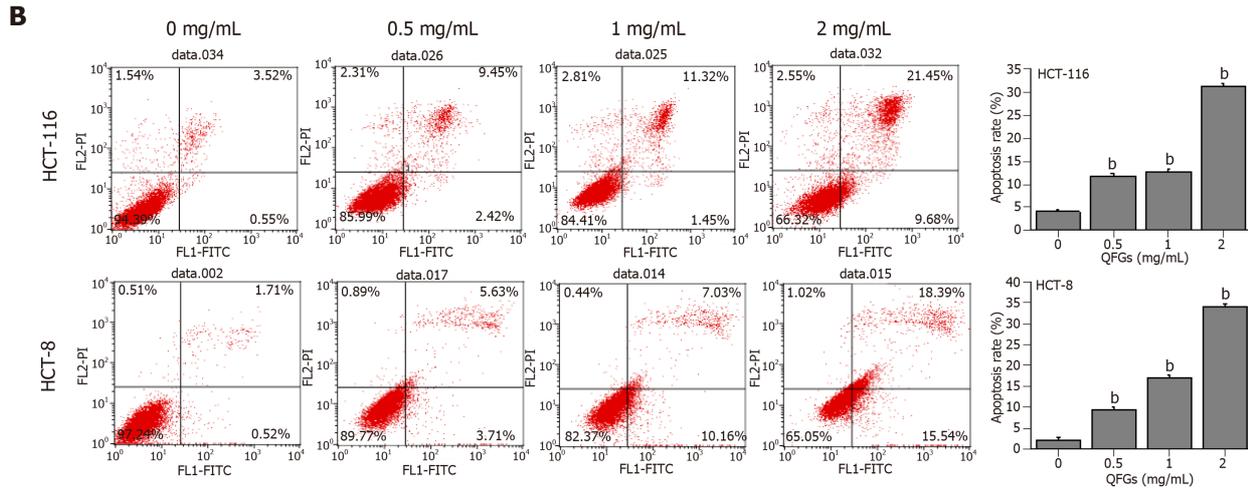
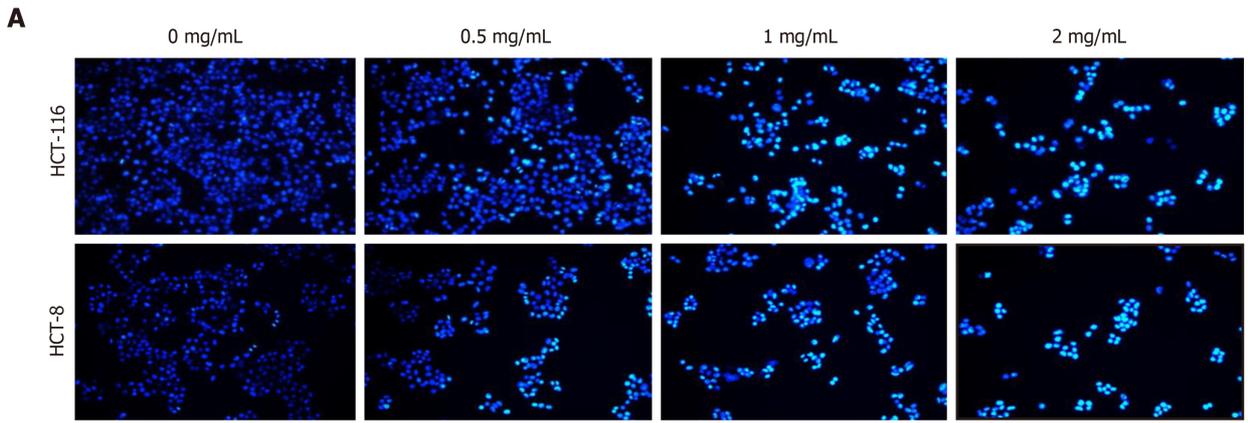


Figure 2 Effect of QFGs on the proliferation of HCT-116 and HCT-8 cells. A: Cell growth ability was measured by colony formation assays; B, C: Cell proliferation was detected by cell cycle assays with flow cytometry analysis; D, E: Quantification of FACS analysis. ^b*P* < 0.01 vs the control group. QFGs: Qingjie Fuzheng granules; FACS: Fluorescence activated cell sorting.

studies have proven that *Hedyotis diffusa* Willd and *Scutellaria barbata* D. Don are capable of promoting apoptosis and inhibiting growth and angiogenesis in many types of cancer cells, including CRC^[33,34]. Malt could boost the movement of Qi and improve food digestion, and Astragalus is a vital component in many TCM formulas that have been used in clinics to cure many cancer patients, reduce the incidence of complications, and improve the quality of life of cancer patients^[35]. However, there are no reports on the underlying signaling pathways and mechanisms involved in QFGs anticancer activity.



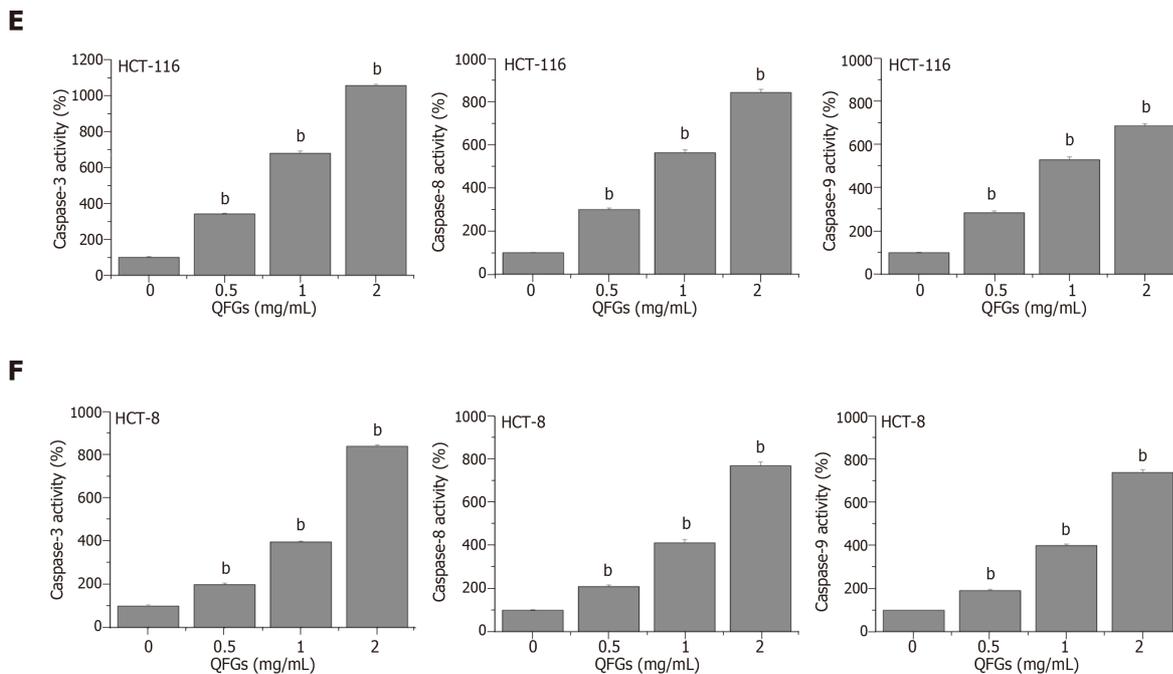


Figure 3 Effect of QFGs on HCT-116 and HCT-8 cells apoptosis. A: Cell apoptosis was detected by Hoechst 33258 staining and visualized under a fluorescent microscope (200 ×); B: Cell apoptosis was detected by Annexin V-FITC/PI staining with flow cytometry analysis, and the analysis results were quantified; C, D: The expression of cleaved caspase-3, caspase-8 and caspase-9 were determined by western blot. β -actin was used as an internal control. Each band is representative of triplicate experiments. The band densities of western blots were quantitatively analyzed; E, F: The activities of caspase-3, caspase-8 and caspase-9 were determined by using a caspase activity assay kit. ^b $P < 0.01$ vs the control group. QFGs: Qingjie Fuzheng granules; PI: Propidium iodide; FITC: Fluorescein isothiocyanate.

In this study, we found that QFGs exhibited significant anticancer effects on HCT-116 and HCT-8 cells. The anticancer effect of QFGs is mainly through the inhibition of proliferation and induction of apoptosis, which are mechanisms commonly exploited in tumor therapy. As demonstrated in the current study, QFGs reduced cell viability and increased cytotoxicity in HCT-116 and HCT-8 cells in a dose-dependent manner. Furthermore, we used cell colony formation, nuclear staining, and flow cytometry assays to demonstrate that these effects in HCT-116 and HCT-8 cells resulted from the inhibition of proliferation and induction of apoptosis by QFGs.

Cell proliferation is regulated by the cell cycle, which consists of the G0, G1, S, G2 and M phases. DNA synthesis is completed in S phase, which is responsible for the initiation and completion of DNA replication^[20]. Therefore, the G1/S transition is one of the two main checkpoints in the cell cycle. Using a cell cycle assay, we observed that the inhibitory effects of QFGs on HCT-116 and HCT-8 cell proliferation were associated with blocking the G1 to S phase transition. The G1/S process is highly regulated by cyclin D1, which forms complexes with CDK4^[21,22]. Overexpression of the cyclin D1/CDK4 complex can enhance cell proliferation, whereas p21 can bind to this complex and inhibit its activity^[24]. Therefore, the expression of CDK4, cyclin D1 and p21 indicate the proliferation state of HCT-116 and HCT-8 cells to some extent. This study proved that QFGs administration upregulates p21 protein expression while downregulating cyclin D1 and CDK4 protein expression. These results showed that QFGs inhibit HCT-116 and HCT-8 cell proliferation.

Cell apoptosis or programmed death, which is an essential process in a healthy organism, removes surplus and damaged cells^[36-38]. Failure to execute the apoptosis process may lead to various diseases, such as cancer^[39] and autoimmune diseases^[40], whereas too much apoptosis may lead to neurodegenerative diseases^[41]. There are two pathways involved in apoptosis: the mitochondria-dependent and death receptor-mediated pathways. The mitochondria-dependent pathway is initiated by caspase-9, and the death receptor pathway is initiated by caspase-8. Both pathways ultimately rely on the activation of caspase-3^[17-19].

In the mitochondria-dependent apoptosis pathway, mitochondrial dysfunction directly leads to the occurrence of apoptosis and is central to the apoptotic pathway^[42]. Mitochondrial outer membrane permeabilization (MOMP), an essential event in the mitochondria-mediated apoptosis pathway, causes the transfer of Cyt C and other apoptotic proteins from the mitochondria into the cytosol, which leads to caspase activation and apoptosis^[43]. Caspases are the key proteins in the regulation of cell apoptosis. During mitochondria-mediated apoptosis, caspase-3 is an important

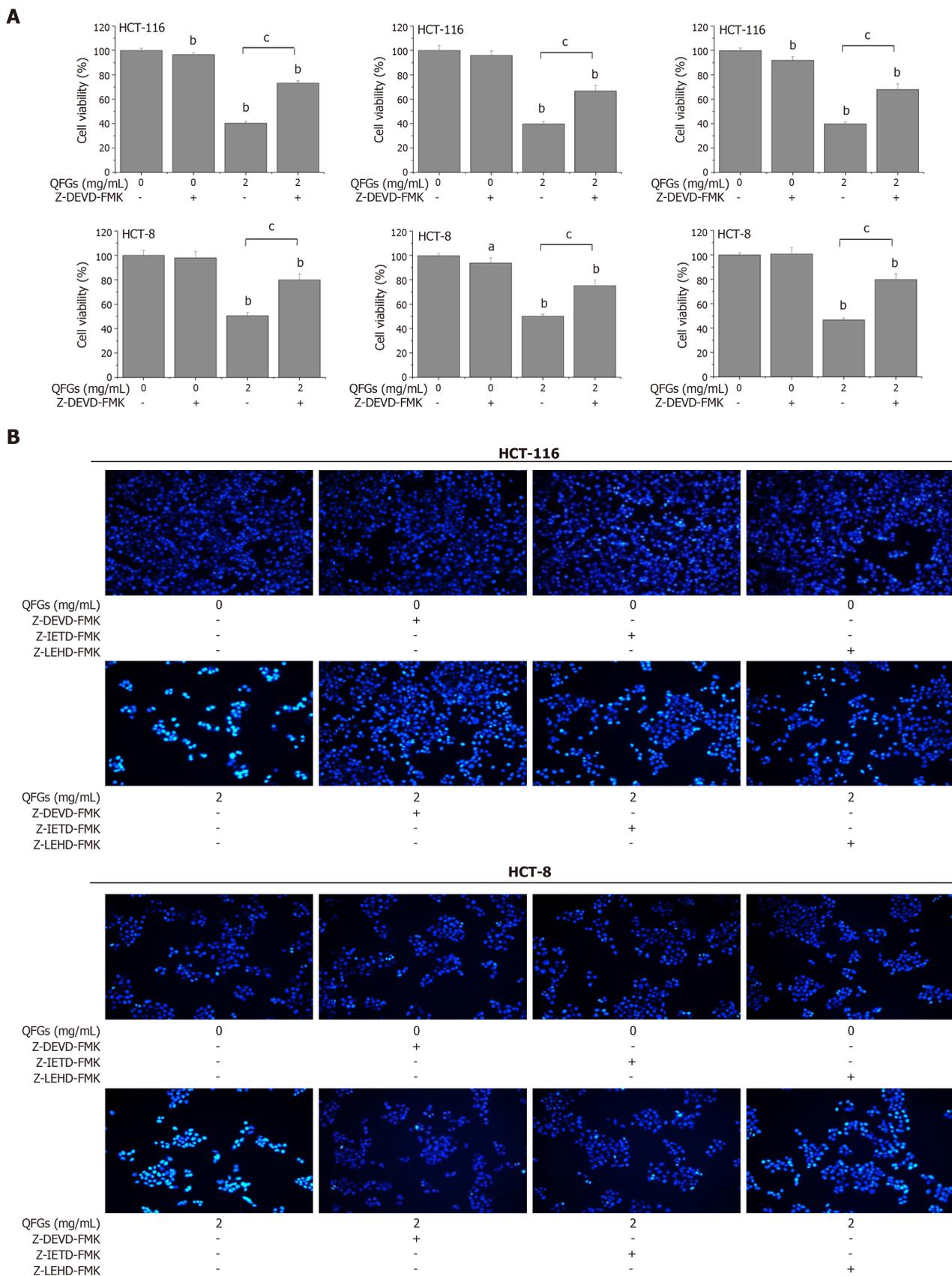


Figure 4 Effect of QFGs on the mitochondria-dependent and death receptor pathways in HCT-116 and HCT-8 cells. A: The cells were treated with or without QFGs (2 mg/mL) in combination with different caspase inhibitors (Z-DEVD-FMK, Z-IETD-FMK, Z-LEHD-FMK) for 24 h, and then cell viability was measured by MTT assay for each combination. ^a $P < 0.05$, ^b $P < 0.01$ vs the control group, ^c $P < 0.01$ vs the inhibitor group; B: Cells were treated with or without QFGs (2 mg/mL) in combination with different caspase inhibitors (Z-DEVD-FMK, Z-IETD-FMK, Z-LEHD-FMK) for 24 h, and then apoptosis in the HCT-116 and HCT-8 cells was detected by Hoechst 33258 staining and visualized under a fluorescent microscope (200 ×). QFGs: Qingjie Fuzheng granules; Z-DEVD-FMK: Inhibitor of caspase-3; Z-IETD-FMK: Inhibitor of caspase-8; Z-LEHD-FMK: Inhibitor of caspase-9.

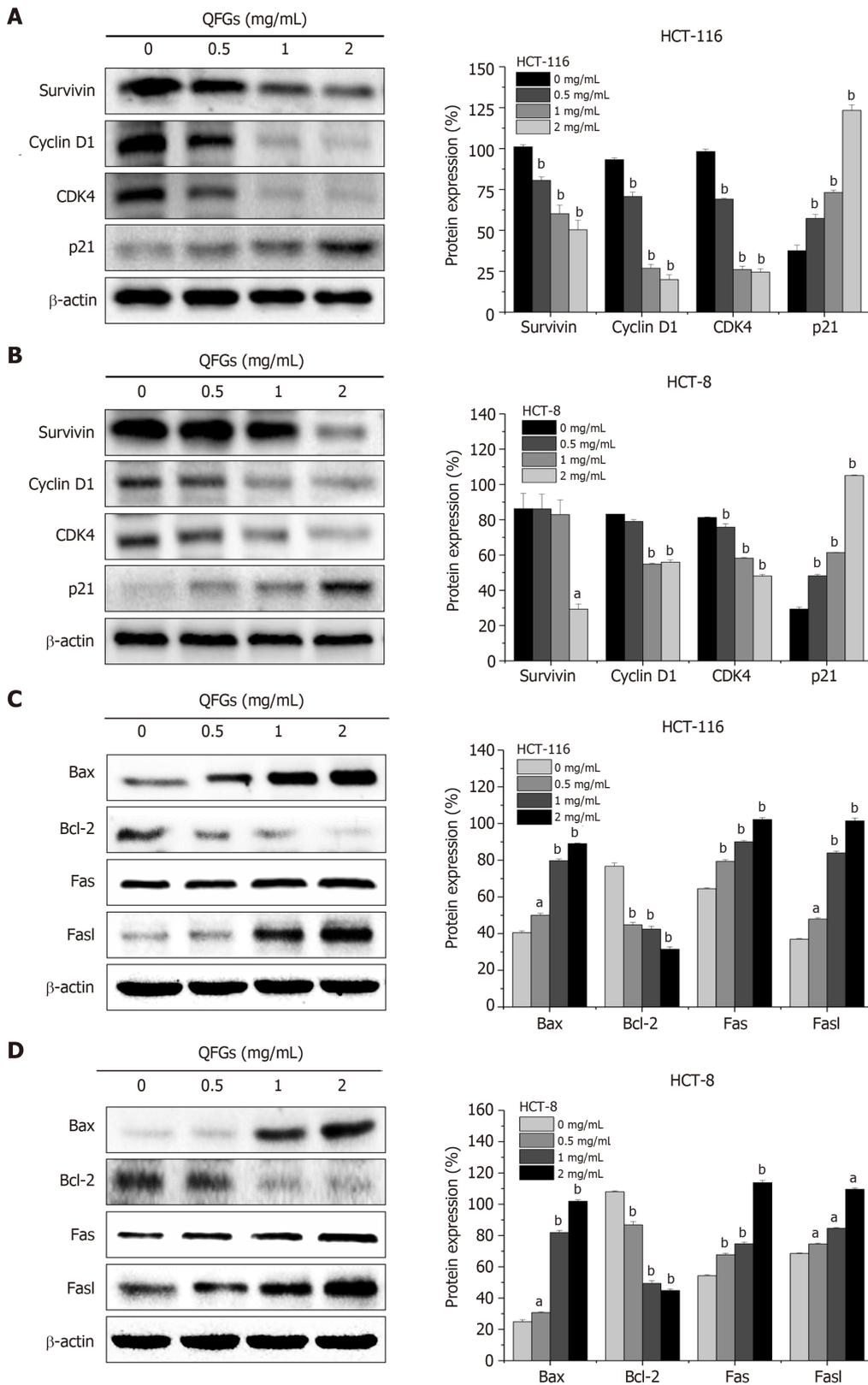


Figure 5 Effects of QFGs on the expression of survival, cyclin D1, CDK4, p21, Bax, Bcl-2, Fas, and FasL in HCT-116 and HCT-8 cells. A, B: The expression levels of survival, cyclin D1, CDK4, and p21 were determined by western blot. β -actin was used as an internal control. Each band is representative of triplicate experiments. The band densities of western blots were quantitatively analyzed; C, D: The expressions of Bax, Bcl-2, Fas, and FasL were determined by western blot. β -actin was used as an internal control. Each band is representative of triplicate experiments. The band densities of western blots were quantitatively analyzed. ^a $P < 0.05$, ^b $P < 0.01$ vs the control group. QFGs: Qingjie Fuzheng granules; CDK4: Cyclin-dependent kinase 4; Bcl-2: B cell leukemia/lymphoma 2; Bax: Bcl2 associated X; FasL: Fas ligand.

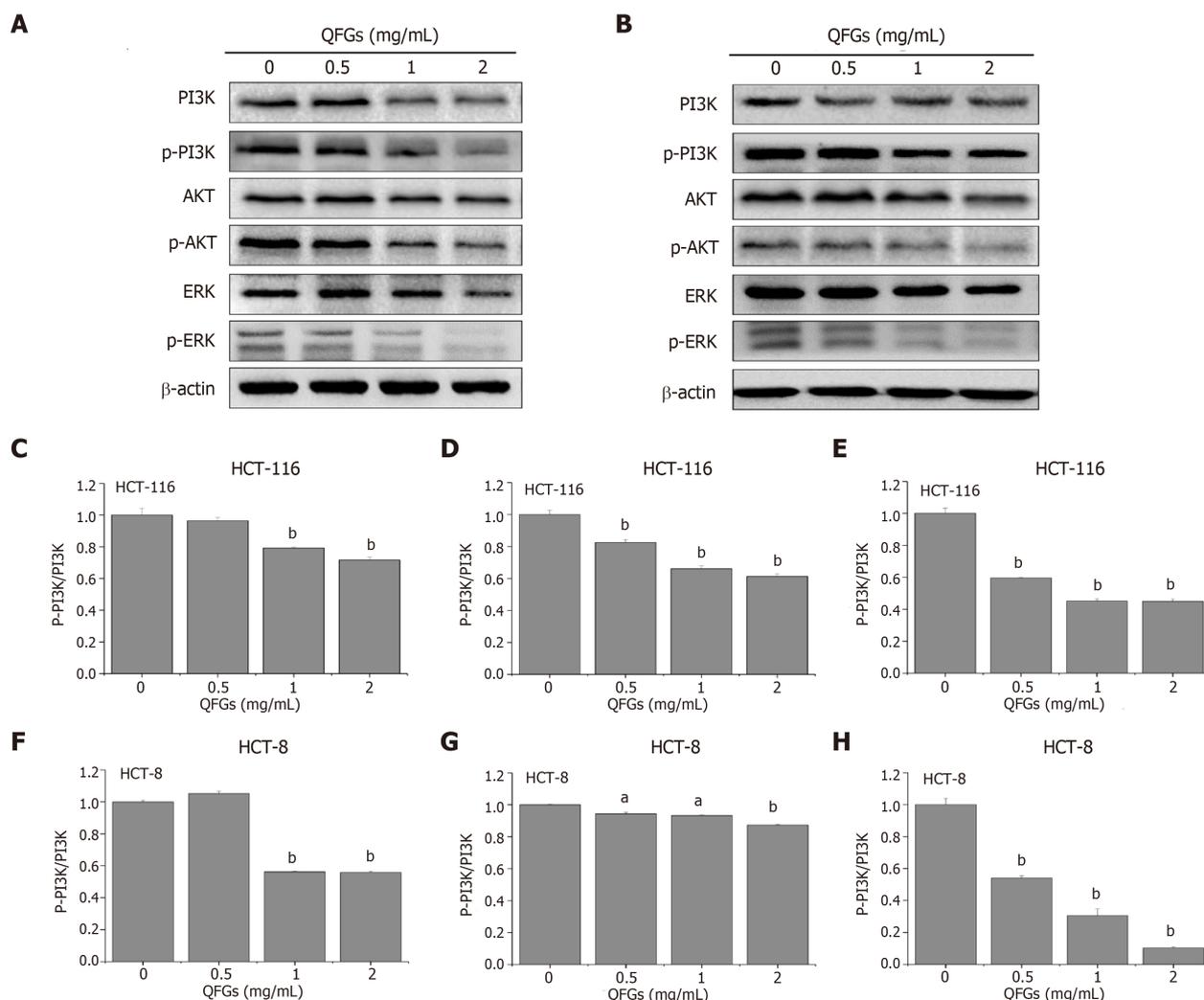


Figure 6 Effect of QFGs on the regulation of PI3K/AKT and ERK signaling pathways in HCT-116 and HCT-8 cells. A, B: The expression and phosphorylation levels of PI3K, AKT and ERK in cells were determined by western blot. β -actin was used as an internal control. Each band is representative of triplicate experiments; C-H: The ratio of phosphorylation expression level/total expression level was quantitatively analyzed. ^a $P < 0.05$, ^b $P < 0.01$ vs the control group. QFGs: Qingjie Fuzheng granules; PI3K: Phosphatidylinositol 3-kinase; pPI3K, PhosphorylatedPI3K; AKT: Protein kinase B; pAKT: PhosphorylatedAKT; ERK: Extracellular regulated protein kinases.

activator that can be cleaved by its upstream initiators, such as caspase-9. The present study showed that QFGs promoted the activation of both caspase-9 and caspase-3 in HCT-116 and HCT-8 cells. In addition, the process of HCT-116 and HCT-8 cell death induced by QFGs was followed by an increase in the cleavage of caspases-9 and caspase-3, which then promotes the molecular cascade leading to apoptosis. The Bcl-2 protein is the key regulatory protein in mitochondria-dependent apoptosis. Some studies have reported that MOMP occurs when proapoptotic Bax-like proteins form pores in the mitochondria; however, the effect of anti-apoptotic Bcl-2-like members on MOMP is opposite to that of proapoptotic Bax-like proteins on MOMP. Therefore, the ratio of Bax to Bcl-2 is the key to determining cell survival [44,45]. Our study proved that QFGs administration upregulated Bax protein expression and downregulated Bcl-2 protein expression. These results showed that QFGs induce HCT-116 and HCT-8 cell apoptosis *via* the mitochondria-dependent pathway.

During the death receptor apoptosis pathway, caspase-3 is also the ultimate activator of apoptosis. Caspase-3 is cleaved by its downstream initiators, such as caspase-8. In this study, we discovered that both caspase-8 and caspase-3 can be cleaved by QFGs in HCT-116 and HCT-8 cells. In addition, as noted earlier, HCT-116 and HCT-8 cell death induced by QFGs was followed by increased cleavage of caspase-8 and caspase-3, which accelerates apoptosis. In this pathway, death signals are transmitted *via* cell surface receptors that communicate with the FasL/Fas signaling pathway, which is part of the death receptor pathway. After binding to FasL, Fas trimerizes and interacts with Fas-associated protein with a death domain, which contributes to the cleavage of caspase-8 and caspase-10 and leads to activation

of downstream effector caspases, including caspase-3, caspase-6 and caspase-7, ultimately causing apoptosis^[24]. In this study, we demonstrated that QFGs treatment upregulated FasL and Fas protein expression. These results showed that QFGs induce HCT-116 and HCT-8 cell apoptosis through the extrinsic apoptosis pathway.

To determine if the two classic apoptosis pathways were both involved in this study, we added caspase-3/-8/-9 inhibitors and performed the MTT and Hoechst 33258 staining assays to test cell viability and cell apoptosis once again. We found that all three inhibitors markedly inhibited the inhibitory effect of QFGs on cell viability in CRC cells, and inhibited the apoptosis induced by QFGs. This result verified that QFGs induced apoptosis *via* the mitochondria-dependent and death receptor apoptosis pathways in HCT-116 and HCT-8 cells, which directly revealed the multi-target inhibitory effects of QFGs on CRC cells.

The pathogenic mechanisms underlying the development of cancer, including CRC, are heterogeneous and regulated by multiple signaling pathways, including PI3K/AKT and ERK^[25,26]. Previous studies have reported that the PI3K/AKT and ERK signaling pathways regulate cell growth, apoptosis and metastasis^[46]. As one of the important intracellular signal transduction pathways, PI3K/AKT signaling has been reported to play important roles in cell survival, apoptosis and metastasis^[47]. In previous studies, activated AKT existed in CRC tumors, which has been shown to be a poor prognostic factor for CRC patients^[48]. Overexpression of downstream factors of AKT may result in the activation of the PI3K signaling pathway^[46]. ERK signaling is also an important pathway that highly regulates cell proliferation and apoptosis. ERK is a mitogen-activated protein kinase (MAPK), which can be activated by MAPK kinase kinase (*e.g.*, Raf), MAPK kinase (*e.g.*, MEK), and MAPK (*e.g.*, ERK)^[49]. Activation of the ERK pathway regulates the expression of various genes and proteins that mediate cell proliferation and apoptosis. The present study demonstrated that QFGs suppressed the activations of PI3K, AKT and ERK, which showed that the anticancer effect of QFGs acts on CRC cells *via* the PI3K/AKT and ERK signaling pathways.

ARTICLE HIGHLIGHTS

Research background

Colorectal cancer (CRC) is a major public health problem, representing the third cause of cancer deaths worldwide. Surgery and adjuvant chemotherapy are the main treatment for CRC. However, 40-50% of patients still die due to recurrence, metastases and drug resistance. In addition, severe side effects caused by chemotherapy agents lead to the deterioration of patient quality-of-life and therapeutic application. Therefore, the search for novel therapies has attracted worldwide attention. Qingjie Fuzheng granules (QFGs) is a traditional Chinese medicine formula with properties of anti-inflammation, antioxidative, antibacterial, immunity enhancement, and digestion promotion. QFGs has been widely used and found to be clinically effective in various cancer treatments, including CRC, and has few side effects. However, the precise mechanisms and molecular signaling pathways involved in the activity of QFGs' anticancer effects have not been reported in the literature. In this study, we hypothesized that QFGs can inhibit the growth of CRC cells, and that its mechanism is closely related to one or more intracellular signal transduction pathways.

Research motivation

To better understand the mechanism underlying the potential anti-cancer effect of QFGs on the human CRC cell variants HCT-116 and HCT-8.

Research objectives

To elucidate the effect of QFGs on the biological function of CRC cells, and to investigate this biological function to explore the exact mechanism of QFGs effects on CRC cells.

Research methods

First, cell viability and cytotoxicity were measured by performing MTT and LDH assays. We evaluated the role of QFGs in cell proliferation and apoptosis by assessing colony formation using Hoechst 33258. Second, cell cycle and apoptosis levels were measured by fluorescence-activated cell sorting. The expression levels of survivin, cyclin D1, CDK4, p21, Bax, Bcl-2, Fas, FasL, and cleaved-caspase-3/-8/-9 were measured by performing western blotting and caspase activity assays. Furthermore, inhibitors of caspase-3/-8/-9 were also used to elucidate the exact apoptosis pathway induced by QFGs in cancer cells. Finally, activation of the PI3K/AKT and ERK signaling pathways was examined using the western blot assay to investigate the possible mechanism.

Research results

MTT and LDH assays revealed that after 0.5-2.0 mg/mL of QFGs treatment, cell viability was reduced by (6.90% ± 1.03%)–(59.70% ± 1.51%) (HCT-116; $P < 0.05$) and (5.56% ± 4.52%)–(49.44% ± 2.47%) (HCT-8; $P < 0.05$). Cytotoxicity was increased from 0.52 ± 0.023 to 0.77 ± 0.002 (HCT-116; P

< 0.01) and from 0.56 ± 0.054 to 0.81 ± 0.044 (HCT-8; $P < 0.01$) compared with non-QFGs treatment groups. Additionally, colony formation and Hoechst 33258 staining assays showed that QFGs inhibited proliferation and induced apoptosis in CRC cells. QFGs also increased the expression levels of Bax, Fas, and FasL, decreased the level of Bcl-2, and stimulated the activation of caspase-3/-8/-9, which were revealed by western blot and caspase activity assays. In contrast, upon adding the three caspase inhibitors, the suppression effect of QFGs on cell viability and apoptosis were markedly inhibited. Moreover, QFGs suppressed the phosphorylation levels of PI3K, AKT and ERK.

Research conclusions

These results demonstrated that QFGs inhibit CRC cell proliferation and induce apoptosis by suppressing the PI3K/AKT and ERK signaling pathways. This indicated that QFGs are a potential new therapeutic treatment for CRC and other cancers.

Research perspectives

Traditional Chinese Medicine (TCM) is important for the treatment of many cancers and has a long history of clinical use. If the effects and mechanisms of TCM are further elucidated, it may provide a more effective treatment for many cancer types.

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