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Qingjie Fuzheng Granule and 5-fluorouracil Inhibit Colorectal Cancer Growth in a Synergistic Manner via the Phosphatidylinositol 3-Kinase/Protein Kinase B Pathway

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Abstract: Our goal is to learn how Qingjie Fuzheng Granule (QFG) and 5-fluorouracil (5-FU) work together to treat colorectal cancer. Research Tools and Procedures: Human colorectal cells and xenograft mice were utilized to study the synergistic anti-cancer capacity of QFG and 5-FU, which targets the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signaling pathway. Chou-Talalay was employed to generate this index. Findings: The growth of HCT-8 cells was suppressed by a synergistic effect of QFG and 5-FU. The combination of QFG with 5-FU in HCT-8 cells and mice resulted in apoptosis induced by regulation of associated proteins, inhibition of proliferation, migration, and invasion. In addition, enzymes involved in 5-FU metabolism, including thymidylate synthase and dihydropyrimidine dehydrogenase, were down-regulated by QFG and 5-FU. Furthermore, by inhibiting the PI3K/AKT signaling pathway, it eased cancer-related behaviors such as migration, invasion, and proliferation. Findings: By inhibiting the PI3K/AKT signaling pathway, the anti-cancer actions of QFG and 5-FU are enhanced.

Keywords: Phosphatidylinositol 3-kinase/protein kinase B signaling pathway, Qingjie Fuzheng granule, 5-fluorouracil, colorectal cancer, synergistic impact

INTRODUCTION

A malignant tumor of the gastrointestinal tract, colorectal cancer (CRC) has a high incidence rate and ranks second or third in terms of fatality. [1] Although surgery is now the gold standard for treating CRC, individuals who have postoperative recurrence, missed surgical opportunities, or metastatic disease still primarily get chemotherapy. (2, 3). One of the most frequent chemotherapy medications used to treat colorectal cancer is 5-fluorouracil (5-FU). As a chemotherapeutic treatment that depends on the cell cycle, 5-FU prevents tumor cell growth by interfering with DNA synthesis, which in turn stops cell cycles. [5] Although 5-FU is very effective against cancer, it has been used with caution due to its adverse effects, which may harm healthy organisms and lower patients' quality of life when taken in large quantities over an extended length of time. In addition, therapeutic efficacy is further diminished as a result of primary and secondary chemotherapy resistance. [6] Therefore, there is an immediate need to develop better therapies to increase the effectiveness of chemotherapy and enhance the quality of life for patients with colorectal cancer.

The growth and chemoresistance of colorectal cancer cells are regulated by the phosphatidylinositol-3-kinase/protein kinase B (PI3K/AKT) signaling system. [7] In a typical scenario, extracellular signals trigger PI3K activity, which in turn activates AKT. Finally, CRC cell survival is enhanced by blocking apoptosis via the negative regulation of B-cell lymphoma-2-associated X protein (Bax) by activated AKT. [8] At the same time, AKT activation triggers EMT, which improves CRC cell migration and invasion and makes tumor spread easier. [9] Therefore, reducing PI3K/AKT activity is helpful for many reasons, including making CRC cells more sensitive to chemotherapy, reducing their ability to proliferate and metastasize, and triggering cell death. [10]

Utilizing two or more medications in conjunction with one another or in a certain order to accomplish therapeutic objectives is known as combination therapy, and it is a commonly used method in the treatment of many disorders. the eleventh This approach not only improves results by making the treatments more effective, but it also lessens their side effects and slows the development of drug resistance. A synergistic effect occurs when the effects of several medications work together. One way to classify a synergistic impact is by its strength; there are two main types: additive and reinforcing. The combined impact of the two medications is what is meant by an additive effect. To have an augmentation effect, all of the parts working together have a stronger impact than each one working alone. Drug combinations may be quantitatively defined by Chou-Talalay's combination index (CI), which measures the additive (CI = 1), synergistic (CI < 1), and antagonistic (CI > 1) effects. [12] Finding effective chemoprotectors and potentiators, decreasing chemo toxicities, and increasing chemotherapy sensitivity are all critical issues that must be addressed immediately.

The Chinese herbal remedy Qingjie Fuzheng Granules (QFG) contains roasted malt, Astragalus membranaceus, Hedyotis diffusa, and Scutellaria barbata. Some of the qualities possessed by these herbs include the ability to remove heat, eliminate toxins, boost vitality, and eliminate food. Inducing apoptosis and reducing proliferation via the PI3K/AKT signaling pathway, prior research has shown that QFG has an anti-cancer effect on CRC cells. In the treatment of colorectal cancer, clinical findings have shown that the chemotherapeutic effectiveness is enhanced when 5-FU-based chemotherapy regimens are coupled with QFG. References [13,14]: Nevertheless, the mechanism of 5-FU coupled with QFG in CRC is not yet the subject of any investigation. This study's overarching goal is to learn more about the possible mechanism of action of 5-FU with QFG in CRC and their synergistic anti-tumor impact.

Materials and Methods

Roswell Park Memorial Institute (RPMI) 1640 medium (c11875500bt), fetal bovine serum (FBS) (10099141), penicillin and streptomycin (15140122) were obtained from Life Technologies; crystalline violet (g1062, Solarbio); Cell Counting Kit 8 (CCK8) kit (KGA3111018), Annexin-V FITC/PI kit (KGA108) and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) kit (KGA704) were required from KeyGene Biotech Co., Ltd. (Nanjing, China); Transwell chamber (3422, Corning Inc.); Antibodies to Bax (50599-2), Bcl-2 (12789-1), CDK4 (11026-1-AP), cyclin D1 (60186), p21 (10355-1-AP), Vimentin (10366-1-AP), PI3K (60225-1), and GAPDH (60004) were purchased from Proteintech (Rosemount, IL, USA). N-cadherin (14215s), E-cadherin (14472s), cleaved caspase-3 (9664), AKT (4685S), p-AKT (4060S) were purchased from Cell Signaling Technology, Inc., (Danvers, MA, USA). The enhanced chemiluminescent substrate for horseradish peroxidase (HRP) activity was acquired from US Everbright (S6009S, U.S. Everbright, China). Other reagents, except for explicitly mentioned, were obtained from Nest (Wuxi, China).

Cell culture

HCT-8 and HCT-116 cells were cultured in RPMI 1640 complete medium which contained 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. When the cell confluence reached 80%~90% and the cells were subcultured.

Animals

A total of 40 SPF-grade male BALB/c nude mice, acquired from Slike Co. Ltd., (Shanghai, China), weighting 18–20 g, were kept independently ventilated animal cage. The mice were allowed to eat and drink *ad libitum*. All experimental procedures were performed in accordance with the Guidelines for Animal Experimentation of Fujian University of Traditional Chinese Medicine and were approved by the Fujian Institute of Traditional Chinese Medicine Animal Ethics Committee (FJTCM IACUC 2021018).

Cell viability assay

HCT-8 and HCT-116 cells were inoculated in 96-well plates (100 μL/well) at a density of 1.0×10^5 cells/mL, and there are 6 replicates in each group. The cells were cultured overnight at 37°C, 5% CO_2, and saturated humidity. Afterward, treatment with different drugs for 24 h or 48 h, respectively. The culture supernatant was then removed, and a 10% CCK8 solution was prepared by mixing 1 mL CCK8 stock solution with 9 mL phosphate-buffered saline (PBS). Then 100 μL of the CCK8 solution was added to each well and incubated for 2 h at 37°C. Finally, the absorbance at 450 nm was measured using an enzyme marker. Cell viability (%) was calculated as follows: Cell viability (%) = A value of experimental group/A value of control group × 100%

Drug interaction analysis

According to the unified theory of Chou-Talalay, the drug interaction between QFG and 5-FU was calculated with CompuSyn software (ComboSyn, Inc., New York, NY, USA).[15] Different ratio of QFG and 5-FU [Table 1] were used to quantify the CI, which provides a quantitative representation of pharmacological interactions to assess drug synergy combination treatments. CI <1 indicates the synergistic effect of QFG and 5-FU.

In vivo nude mouse transplantation tumor study

A total of 4×10^6 HCT-8 cells, mixed 1:1 (V:V) with Matrigel™, were subcutaneously injected in the right flank of the mice to initiate tumor growth. When the tumors reached 3 mm in diameter, the mice were randomly divided into four groups (n = 10) according to the size of the tumors: Control group, QFG group (1 g/kg), 5-FU group (25 mg/kg), and QFG + 5-FU group (1 g/kg + 25 mg/mL). The drugs were administered immediately according to the group assignment. The QFG group was given QFG by gavage at a dose of

Table 1: The ratio of Qingjie Fuzheng Granule and 5-fluorouracil

Drug combination fixed ratio	
Ratio (QFG: 5-FU)	**Drug concentration (mg/mL)**
160:1	$0.125:7.81\times10^{-4}$
	$0.25:1.56\times10^{-3}$
80:1	$0.125:1.56\times10^{-3}$
	$0.25:3.13\times10^{-3}$
40:1	$0.125:3.13\times10^{-3}$
	$0.25:6.25\times10^{-3}$
20:1	$0.125:6.25\times10^{-3}$
	$0.25:1.25\times10^{-2}$

QFG: Qingjie Fuzheng Granule, FU: Fluorouracil

1 g/kg. The 5-FU group was given 5-FU intraperitoneally at 25 mg/kg. QFG + 5-FU group was given QFG at 1 g/kg orally, meanwhile, given 5-FU intraperitoneally at 25 mg/kg. The control group was given the same volume of saline. QFG was administered once a day for 6 consecutive days per week; 5-FU was injected once every other day. The length (L) and width (W) of the tumors were recorded every other day, and calculated the tumor volume as the following formula: Tumor volume = $\pi/6 \times L \times W^2$. During the experiment, the weight of each nude mouse was recorded. At the end of the experiment, the animals were anesthetized, the tumors were excised and weighed.

Colony formation

Treatment with QFG (0.25 mg/mL), 5-FU (3.13 μg/mL) and the combination for 24 h. Control cells (untreated) were also processed in parallel. Then 500 cells/well were collected and seeded into 12-well plates and incubated for 8 days, fixed with 4% paraformaldehyde (1 mL/well) for 20 min and stained with 0.1% crystal violet for another 20 min. Finally, the number of clones was counted.

Flow cytometry

Treatment with different drugs for 24 h, 1×10^6 cells were collected, and re-suspended in 500 μL of 1× binding buffer followed by washed twice with cold PBS. Annexin V-FITC and PI (both 5 μL) were added and incubated for an additional 15 min in the dark. For each sample, 10,000 events were collected and analyzed.

4′,6-Diamidino-2-phenylindole staining assay

Twenty-four hours after treated with different drugs, 4′,6-Diamidino-2-phenylindole (DAPI) staining was performed. In brief, the cells were fixed with 4% paraformaldehyde for 15 min, followed by three washes with PBS. Next, 10% DAPI staining solution was added and incuated for 15 min in the dark, then imaged using a fluorescence microscope (×200).

Migration and invasion assay

Twenty-four hours after treated with different drugs, 50,000 HCT-8 cells were seeded in the upper chamber of transwell plates. Matrigel coating was applied for the invasion assay, while no Matrigel was used for the migration assay. The upper chamber contained RPMI-1640 basic midium, and the lower chamber contained 700 μL of RPMI-1640 with FBS. The plates were incubated at 37°C for 14 h. Then, the both the upper and lower chambers were fixed with 4% paraformaldehyde for 20 min, and stained with crystal violet for 15 min. Finally, cells in the lower layer of chamber was photographed using an inverted microscope (×200).

Western blot

The proteins were extracted using radioimmunoassay buffer containing inhibitor cocktail (Thermo Fisher Scientific, USA). The samples were then centrifuged and obtain final supernatants. The concentration of total protein was detected with BCA (bovine serum albumin) protein assay kit (Thermo Fisher Scientific, USA). The proteins (30 μg) that were denatured at 100°C in a metal bath were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electro-transferred to polyvinylidene difluoride membranes, sealed with 5% milk and incubated with primary antibodies (1:1000) at 4°C overnight. After the membranes were incubated with the corresponding secondary antibodies (HRP-conjugated 1:5000) for 1 h at 25°C, the Image Lab (Bio-Rad Laboratories, Inc., Berkley, California, USA) was used to detect proteins.

Immunohistochemistry

After inactivation of endogenous peroxidase with 3% hydrogen peroxide and blocked with goat serum (G9023, Sigma), the slides were incubated with primary antibodies against Ki-67 (1:200) at 4°C overnight. On the following day, the slides were incubated with biotinylated secondary antibodies (KIT-9710, MXB Biotechnologies, China) and streptavidin peroxidase (KIT-9710, MXB Biotechnologies China) at 37°C, respectively. After for a 30 min incubation, the slides were washed 3 times with PBS before each reagent change. Finally, the sections were stained using a DAB kit (DAB-0031, Solarbio, China) and hematoxylin was used for restaining for 1 min (G1140, Solarbio, China). Five random fields from each slice were imaged at ×400 magnification. The positive expression in tumor tissues was quantified using a true-color multifunctional cellular image analysis system (Image-Pro Plus, Media Cybernetics).

TUNEL staining

The tumor tissues, fixed with 4% paraformaldehyde, were treated with Triton X-100, and then sealed with goat serum. They were subsequently incubated with TUNEL immunofluorescence reagent. After stained with DAPI, the tissues were photografted using a confocal laser scanning microscope (ZEISS, Germany).

Optimal time for phosphatidylinositol 3-kinase/protein kinase B pathway activation

After 10 h' culture, HCT-8 cells were changed into RPMI 1640 blank medium for 24 h. Subsequently, 10% FBS was added at 0, 5, 15, 30, 60 and 120 min, respectively, then p-AKT was analyzed by Western blot.

The combined effect of Qingjie Fuzheng Granule and 5-fluorouracil on survival, apoptosis, migration, and invasion of HCT-8 cells after phosphatidylinositol 3-kinase/protein kinase B pathway activation

HCT-8 cells were pretreated for 24 h, followed by a 30-min culture to activate PI3K/AKT pathway. Subsequently, morphological observation, colony formation, DAPI staining, migration, and invasion assays were performed.

Statistical analysis

The data are presented as mean ± standard deviation, and statistical analyses were performed using Graphpad Prism (version 8.2.1 Windows version, GraphPad Software, San Diego). The unpaired Student's *t*-test was applied to compare two groups, and one-way analysis of variance, followed by *post hoc* Fisher's least significant difference testing was applied to compare multiple groups. $P < 0.05$ was considered statistically significant.

RESULTS

Synergistic effects of Qingjie Fuzheng Granule and 5-fluorouracil in colorectal cancer cells

Treatment with QFG and 5-FU for 24 h or 48 h, Chou-Talalay was used to calculate the CI. As shown in Figure 1, the CI values were all <1, that indicated a synergistic anti-cancer effect of QFG and 5-FU. The synergistic effect was more significant in HCT-8 cells. The synergistic effect of QFG/5-FU at a ratio of 80:1 was stronger in CRC HCT-8 cells. Therefore, the best concentrations of QFG and 5-FU were 0.25 mg/mL and 3.13×10^{-3} mg/mL respectively, in HCT-8 cells.

Qingjie Fuzheng Granule combined with 5-fluorouracil synergistically inhibited intestinal cancer growth

The synergistic activity of QFG combined with 5-FU on HCT-8 cells was assesses *in vitro* and *in vivo*. As shown in Figure 2a, treatment with QFG and 5-FU significantly reduced the viability of HCT-8 cells in a time-dependent manner ($P < 0.05$). Furthermore, the combination of QFG and 5-FU induced to a decrease in the number of attached and detached HCT-8 cells after 24 h [Figure 2b]. Moreover, we explored the synergistic effect of QFG and 5-FU using xenograft mice, as shown in Figure 2c and d, compared with treatment with QFG or 5-FU alone, the combination significantly inhibited the tumor volume ($P < 0.05$). In addition, the combination of QFG and 5-FU resulted in a reduction in tumor weight as shown in Figure 2e ($P < 0.05$). Taken together, these data suggest that QFG combined with 5-FU effectively synergizes to inhibit the growth of intestinal cancer both *in vitro* and *in vivo.*

Qingjie Fuzheng Granule and 5-fluorouracil synergisticly inhibited the proliferation of HCT-8 cells

To investigate the effects of QFG combined with 5-FU on the proliferation of HCT-8 cells *in vitro* and *in vivo*, colony formation assays and immunohistochemical staining were performed. As shown in [Figures 3a and b], QFG combined with 5 FU inhibited the ability of clonal goblet formation compared with single drug intervention ($P < 0.05$). Similarly, QFG combined with 5-FU inhibited Ki-67 expression in tumor tissues [Figure 3e and f]. Moreover, as shown in Figure 3c, d, g and h, QFG combined with 5-FU decreased CDK4 and Cyclin

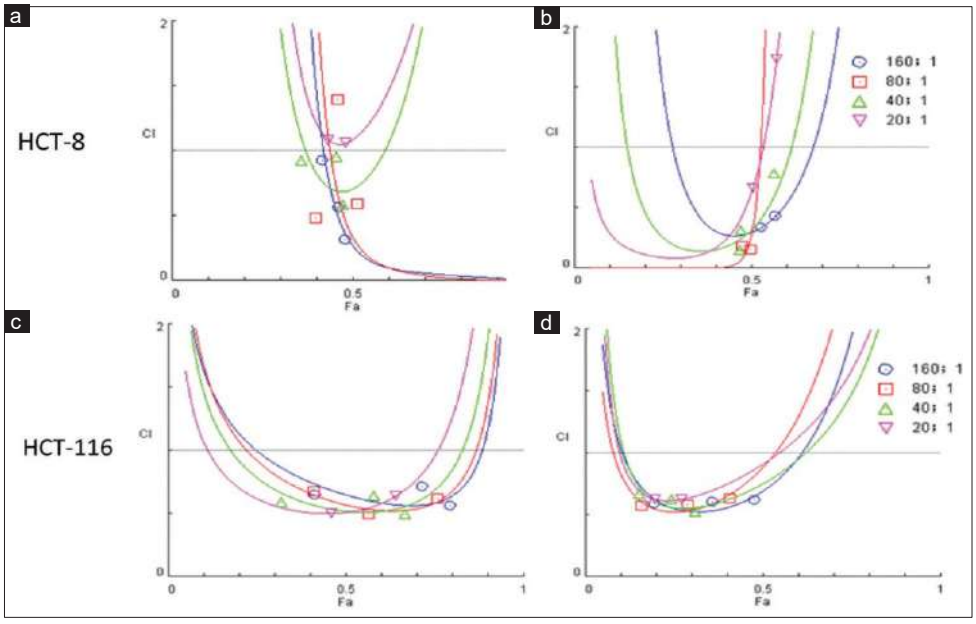

Figure 1: Synergistic effects of Qingjie Fuzheng Granule (QFG) and 5-fluorouracil (5-FU) on colorectal cancer. (a) 24-h combination index (CI) values of different ratios of QFG combined with 5-FU intervention in HCT-8 cells. (b) 48-h CI values of different ratios of QFG combined with 5-FU intervention in HCT-8 cells. (c) 24-h CI values of different ratios of QFG and 5-FU intervention in HCT-116 cells. (d) 48-h CI values of different ratios of QFG combined with 5-FU intervention in HCT-116 cells

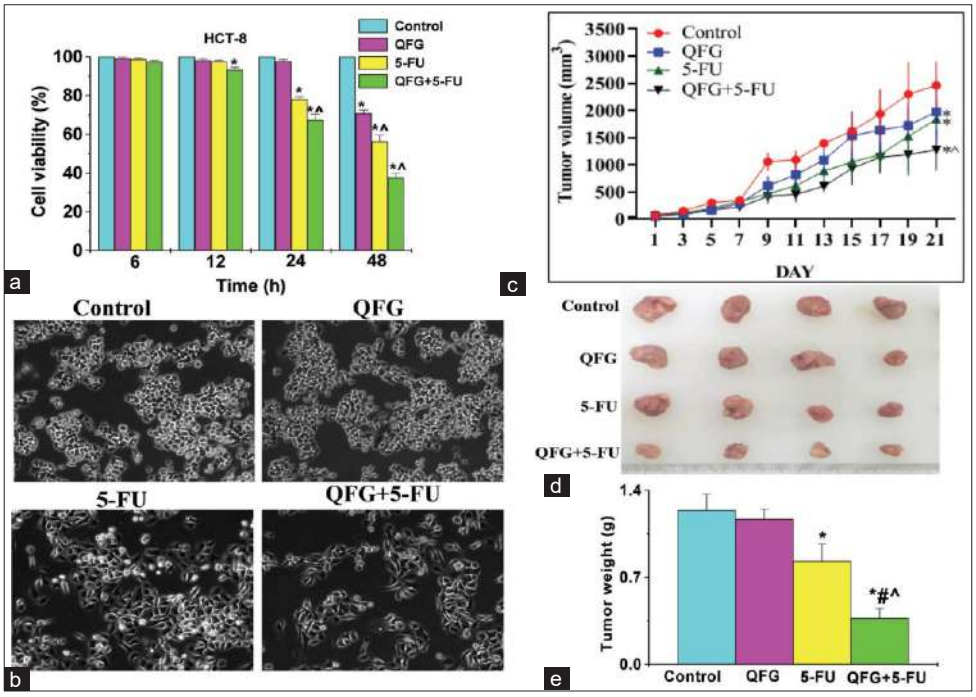

Figure 2: Synergistic inhibition of intestinal cancer growth by QingjieFuzheng Granule (QFG) and 5-fluorouracil (5-FU) *in vitro* and *in vivo*. (a) HCT-8 cells viability. (b) Morphology of HCT-8 cells (×200, scale bar = 100 μm). (c) Tumor volume in xenograft mice. (d) Representative photographs of tumors in xenograft mice. (e) Tumor weight. *$P < 0.05$ compared with the control group; ^ $P < 0.05$ compared with the QFG group; #$P < 0.05$ compared with the 5-FU group. QFG: QingjieFuzheng Granule, 5-FU: 5-Fluorouracil

D1 protein expression while increased p53 and p21 levels. Taken together, these results suggest that QFG combined with 5-FU synergistically inhibits the proliferation of HCT-8 cells both *in vitro* and *in vivo*.

Qingjie Fuzheng Granule and 5-fluorouracil synergisticly promoted apoptosis of HCT-8 cells

As shown in Figure 4a-d, QFG combined with 5-FU promoted apoptosis in HCT-8 cells compared to the single drug groups, as demonstrated by Annexin V/PI staining ($P < 0.05$) and DAPI staining assay ($P < 0.05$). Furthermore, in xenograft mice, treatment with QFG and 5-FU increased the expression of TUNEL positive cells ($P < 0.05$) as shown by immunofluorescence staining [Figure 5a and b]. In addition, apoptosis-related proteins expression was determined. As shown in Figures 4e and f and 5c and d, QFG combined with 5-FU increased Bax and cleaved-caspase-3 protein expression while decreased of Bcl-2 protein expression, both *in vitro* and *in vivo* ($P < 0.05$). These results suggest that QFG and 5-FU synergistically promotes apoptosis *in vitro* and *in vivo*.

Qingjie Fuzheng Granule combined with and 5-fluorouracil synergistically inhibited migration and invasion of HCT-8 cells

As shown in Figure 6a-d, compared to the individual treatment, QFG and 5-FU inhibited migration and invasion of HCT-8 cells (both $P < 0.05$). Meanwhile, we detected EMT-related proteins expression. As shown in Figure 6e-h, QFG combined with 5-FU increased E-cadherin protein expression and decreased N-cadherin and Vimentin protein expression in both HCT-8 cells and tumor tissues. These results further support the synergistic effect of QFG and 5-FU on the metastasis of intestinal cancer cells.

Qingjie Fuzheng Granule combined with 5-fluorouracil inhibited of dihydropyrimidine dehydrogenase and thymidylate synthase expression of HCT-8 cells

To investigate the effect of 5-FU in CRC cells, we examined the expression of 5-FU metabolism-related proteins. As shown in Figure 7, we found that QFG and 5-FU inhibited dihydropyrimidine dehydrogenase (DPD) and thymidylate synthase (TS) protein expression of HCT-8 *in vitro* and *in vivo* (both $P < 0.05$) [Figure 7].

Qingjie Fuzheng Granule combined with 5-fluorouracil synergistically inhibited phosphatidylinositol 3-kinase/protein kinase B signaling pathway of HCT-8 cells

To explore the effect of QFG combined with 5-FU on the PI3K/AKT pathway, we examined it by western blot analysis and found that QFG and 5-FU decreased p-PI3K and p-AKT protein expression both in HCT-8 cells and tumor tissues [Figure 8]. These results suggest that QFG combined with 5-FU synergistically inhibited intestinal cancer growth by suppressing the PI3K/AKT signaling pathway.

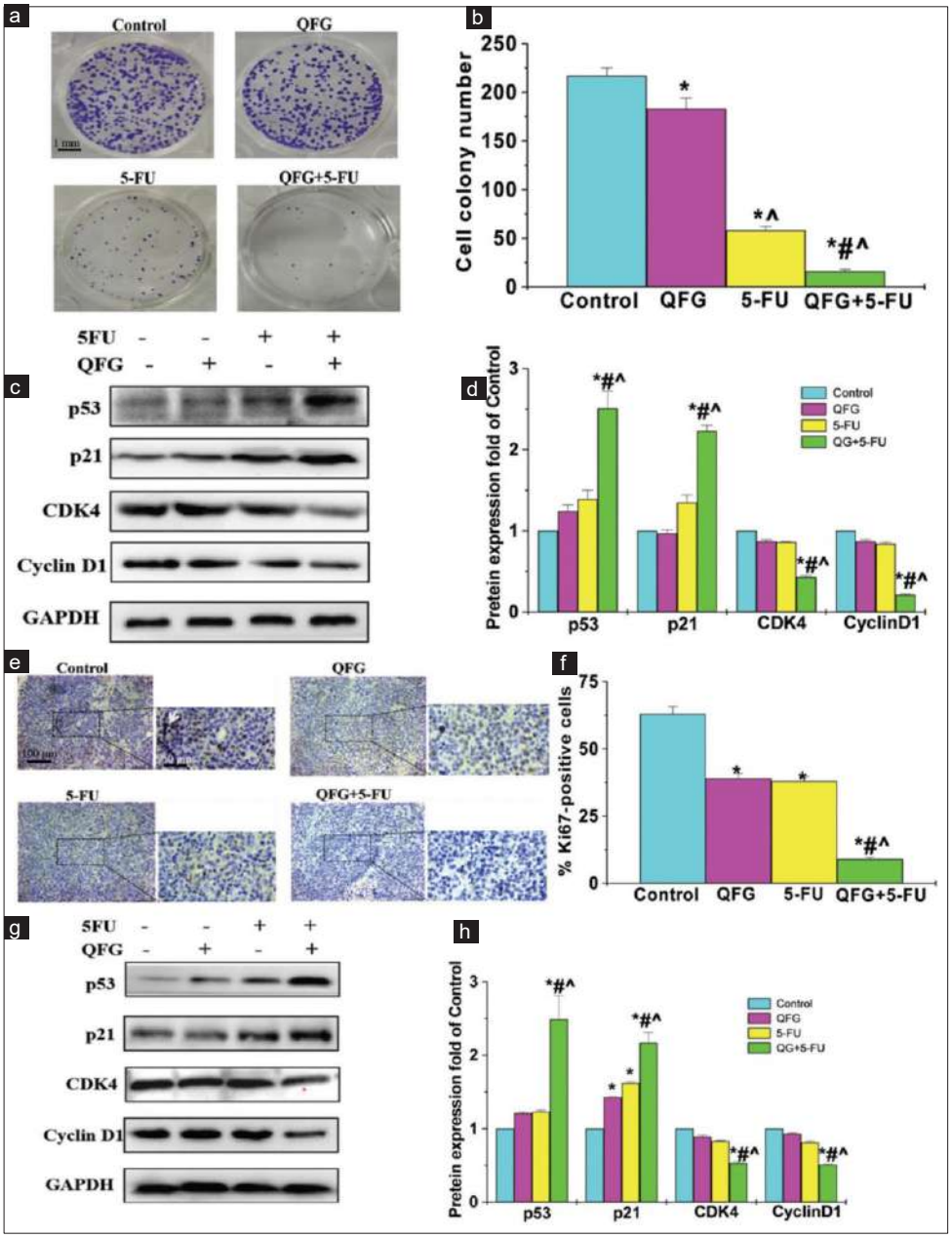

Figure 3: Qingjie Fuzheng Granule (QFG) and 5-fluorouracil (5-FU) synergistically inhibits the proliferation of HCT-8 cells *in vitro* and *in vivo*. (a and b) Colony formation assay. (c and d) Proliferation-related proteins expression in HCT-8 cells. (e and f) Ki67 expression in xenograft mice (×400, scale bar = 50 μm). (g and h) Expression of proliferation-related proteins in xenograft mice. The arrow refers to Ki-67 positive cell. *$P < 0.05$ compared with the control group; ^ $P < 0.05$ compared with the QFG group; #$P < 0.05$ compared with the 5-FU group. QFG: Qingjie Fuzheng Granule, 5-FU: 5-Fluorouracil

Qingjie Fuzheng Granule combined with 5-fluorouracil regulated proliferation, apoptosis, migration and invasion of HCT-8 cells through blocking phosphatidylinositol 3-kinase/protein kinase B pathway

FBS, as an important exogenous environmental factor containing lots of growth factors, plays an important role in cell signaling by binding to growth factor receptors on the cell membrane and inducing changes in the signaling pathway-related proteins. To explore the effect of FBS on the PI3K/AKT pathway, we subjected HCT-8 cells to 10% FBS intervention under starvation condition for different durations. The results are shown in Figure 9a and b demonstrate that 10% FBS intervention for 30 min significantly upregulated p-AKT protein expression ($P < 0.05$), indicating the activation of the PI3K/AKT signaling pathway in HCT-8 cells by 10% FBS intervention.

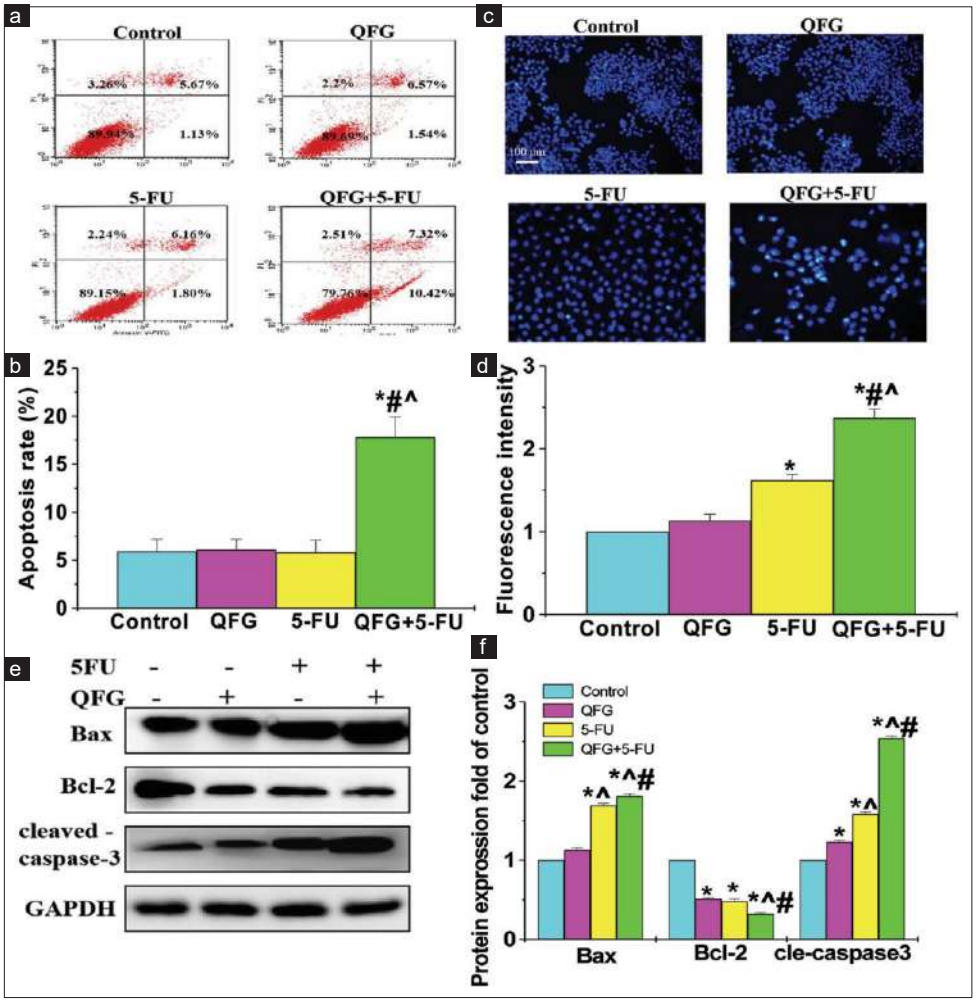

Figure 4: Qingjie Fuzheng Granule (QFG) combined with 5-fluorouracil (5-FU) synergisticly promoted apoptosis in HCT-8 cells. (a and b) Annexin V/PI staining analyzed by flow cytometry. (c and d) DAPI staining (×200, bar = 100 μm). (e and f) Expression of apoptosis-related protein in HCT-8 cells. *P < 0.05 compared to the control group; ^ P < 0.05 compared to the QFG group; #P < 0.05 compared to the 5-FU group. QFG: Qingjie Fuzheng Granule, 5-FU: 5-Fluorouracil

Next, we investigated whether QFG combined with 5-FU synergistically regulated the proliferation, apoptosis, migration, and invasion of HCT-8 cells via PI3K/AKT pathway. Moreover, QFG combined with 5-FU blocked the activstion of AKT induced by FBS [Figure 9c and d]. As shown in Figure 10a, QFG and 5-FU significantly inhibited cell growth in HCT-8 cells. Similarly, the results in Figure 10b and c demonstrated that QFG and 5-FU reduced the number of colonies which was induced by PI3K/AKT pathway activation (P < 0.05). Furthermore, QFG and 5-FU promoted apoptosis significantly (P < 0.05) as shown in Figure 10d and e. Moreover, QFG and 5 FU suppressed migration and invasion (both P < 0.05) which were promoted by PI3K/AKT pathway activation as shown in Figure 10f and g and Figure 10h and i. In conclusion, these results suggest that QFG and 5-FU synergistically regulated the growth process of HCT-8 cells by regulating the PI3K/AKT pathway.

Discussion

CRC ranks as the third most common malignant tumor in the world.[1] Current treatment choices for CRC primarily include surgery, radiotherapy and chemotherapy, with chemotherapy being the main approach for patients with inoperable or postoperative metastatic tumors.[2] 5-FU, the first-line chemotherapy regimen, is widely used in CRC. However, the side effects which can easily damage the normal organism, induce drug resistance and reduce patients' quality of life limited the use of 5-FU. Consequently, it is urgent to discover novel treatment strategies to overcome the limitations associated with chemotherapy. Combination therapy involving the simultaneous administration of multiple drugs has emerged as a promising approach. Traditional Chinese Medicine has better advantages in reducing toxicity and increasing efficacy, Combination therapy of TCM and 5-FU not only increase the therapeutic effects, but alleviate the side effects at lower dose. This

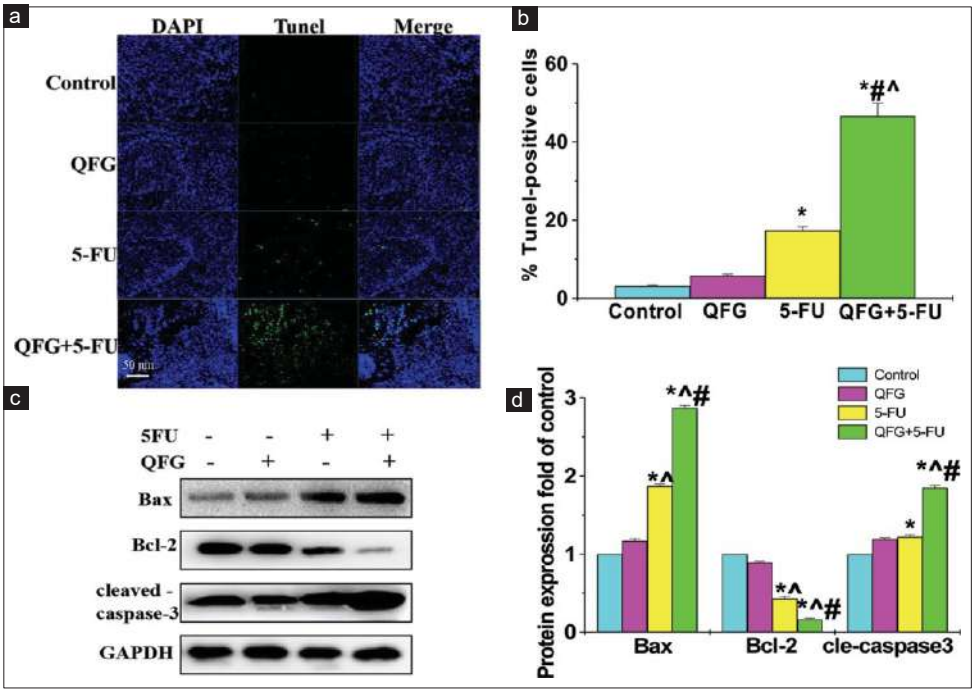

Figure 5: Qingjie Fuzheng Granule combined with 5-fluorouracil synergisticly promoted apoptosis in xenograft mice. (a and b) TUNEL staining assay (×400, bar = 50 μm). (c and d) The expression of apoptosis-related proteins in xenograft tissues. QFG: Qingjie Fuzheng Granule, 5-FU: 5-Fluorouracil

approach aims to achieve synergistic efficacy while reducing toxicity.[12]

Patients' quality of life and chemotherapy effectiveness are both improved greatly by QFG, a clinical anti-tumor compound that is often utilized. To add to that, QFG may shield the gut mucosal from 5-FU. References [13,14]: It was proposed that there could be a synergistic impact when QFG and 5-FU are combined. Prior research has shown that QFG may control CRC development by inhibiting the PI3K/AKT pathway. [13] Multiple processes, including tumor growth, apoptosis, metastasis, and resistance to 5-FU treatment, are influenced by the tumor-related genes that are downstream targets of the PI3K/AKT signaling pathway. These genes include p21, Bcl-2, EMT, DPD, TS, and others. On the basis of these results, the purpose of this research is to investigate the combined effects of QFG and 5-FU on colorectal cancer. Furthermore, we endeavored to investigate how QFG and 5-FU impacted the PI3K/AKT pathway and its subsequent processes, such as CRC cell proliferation, apoptosis, metastasis, and chemoresistance. Understanding how QFG and 5-FU work together to combat CRC is the objective. The unending advancement of tumors is mostly due to the unchecked growth of malignant cells. [16] As a crucial tumor suppressor and one of the major regulators of cell division, p53 is an important player in the field. One gene that suppresses cell cycle-dependent protein kinases is P21, which is a downstream gene of p53 [17]. Cyclin D1-CDK4 is unable to phosphorylate Rb (G1 phase cycle) because a complex formed by P21 and Cyclin D1-CDK4 suppresses protein kinase activity. protein that acts as an inhibitor. The G1 phase block and inhibition of cell proliferation are caused by Rb's inactive state binding to E2F, which inactivates E2F. pp. 18–21 The colony formation test was used to determine that a mixture of QFG and 5-FU may decrease cell growth in this investigation. We found that the combination of QFG and 5-FU decreased Ki67 positivity in tumor tissues by immunohistochemical assay. We also found that the combination of QFG and 5-FU increased p53 and p21 protein expression while inhibiting Cyclin D1 and CDK4 protein expression by Western bolt assay, compared to single drug. The results of the experiment show that it is possible to suppress the growth of colorectal cancer cells by combining QFG with 5-FU. The process of programmed cell death, or apoptosis, is controlled by genes and results in the inevitable cessation of life. [8] In apoptosis, the Bcl-2 family is involved, and Bax dimers help open and increase the permeability of the mitochondrial membrane. A reduction in mitochondrial membrane permeability follows an increase in Bcl-2 expression, and Bcl-2 may form heterodimers with Bax. As a result, cystathionase (Caspases) is activated when cellular mitochondrial membrane disruption triggers the release of cytochrome-c. Cell death is orchestrated by these cleaved caspases, one of which being Caspase 3. pages 22–24 This research used a battery of tests, including western blotting, flow cytometry, DAPI staining, and TUNEL immunofluorescence, to confirm elevated apoptosis.

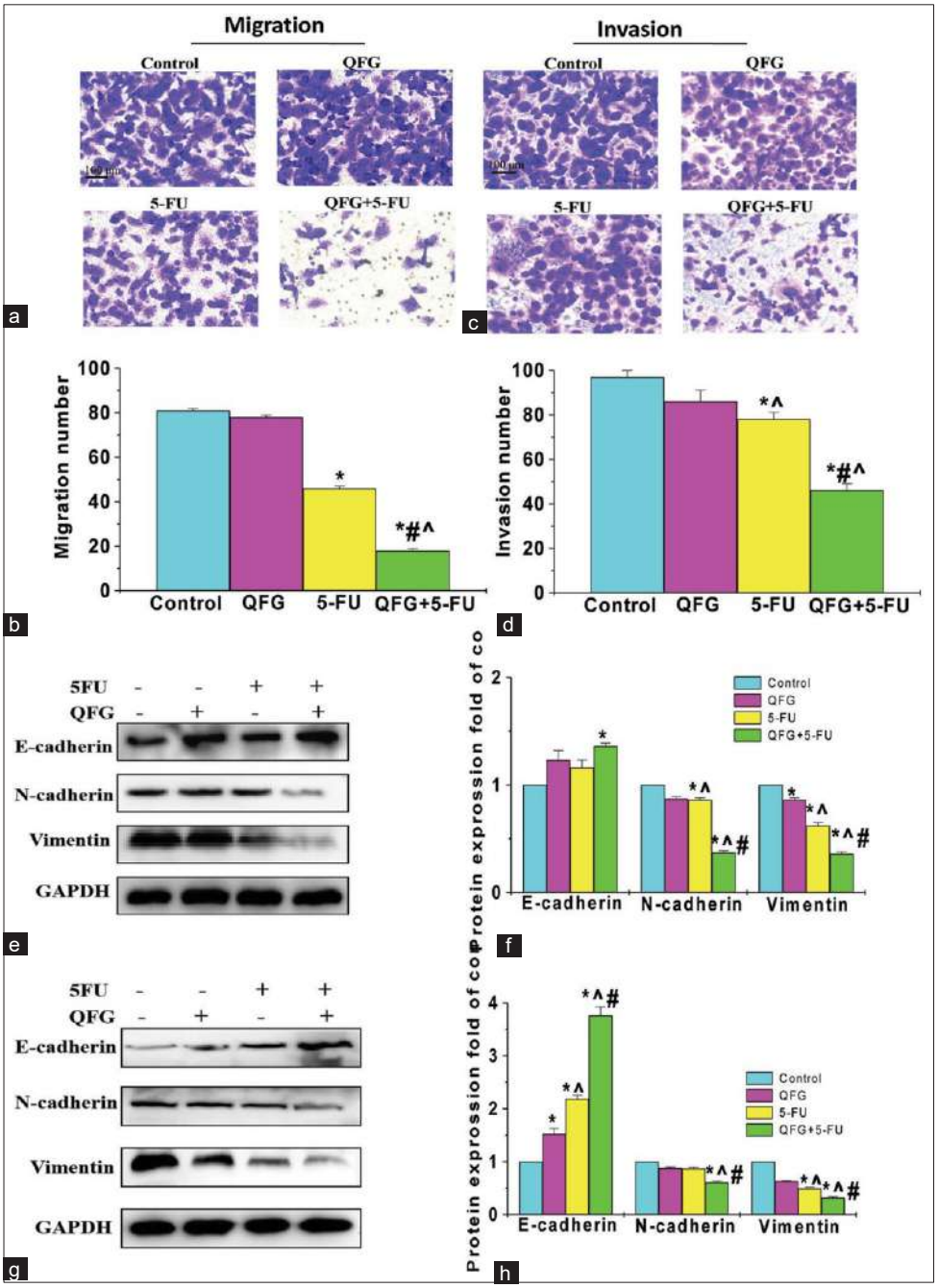

Figure 6: Qingjie Fuzheng Granule (QFG) and 5-fluorouracil (5-FU) inhibited migration and invasion. (a and b) Migration assay and statistic analysis in HCT-8 cells (×200, bar = 100 μm). (c and d) Invasion assay and statistic analysis in HCT-8 cells (×200, bar = 100 μm). (e and f) EMT-related proteins expression in HCT-8 cells. (g and h) EMT-related proteins expression in xenograft mice. *P < 0.05 compared to the control group; ^ P < 0.05 compared to the QFG group; #P < 0.05 compared to the 5-FU group. QFG: Qingjie Fuzheng Granule, 5-FU: 5-Fluorouracil

EMT is a process in which epithelial cell phenotype transformed to mesenchymal cell phenotype under specific physiological or pathological conditions, which promotes the loss of intercellular adhesions, increases the metastatic capacity of cancer cells, inhibits apoptosis, and is an important factor affecting tumor recurrence and metastasis.[25-28] Cell-triggered EMT, which results in loss of epithelial cell integrity, and transcriptional repression of genes encoding epithelial cell-specific proteins such as E-Cadherin by transcription factors, contribute to the degradation of adherens junctions, and their adherens junctions are replaced by proteins capable of greater junctional flexibility (e.g., N-Cadherin), leading to cell separation and enhanced cell motility.[29-34] Epithelial genes

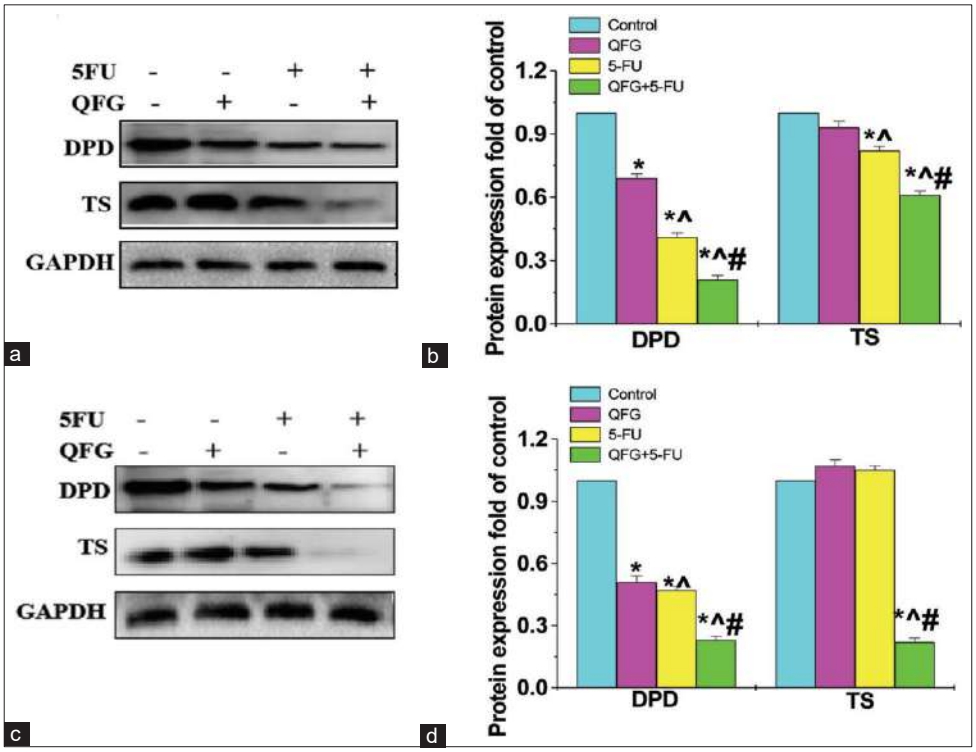

Figure 7: Qingjie Fuzheng Granule (QFG) and 5-fluorouracil (5-FU) inhibited DPD and TS expression. (a and b) DPD and TS expression in HCT-8 cells. (c and d) DPD and TS in tumor tissues. *$P < 0.05$ compared with the control group; ^ $P < 0.05$ compared with the QFG group; #$P < 0.05$ compared with the 5-FU group. QFG: Qingjie Fuzheng Granule, 5-FU: 5-Fluorouracil

are repressed by transcription factors while mesenchymal genes are activated, upregulate Vimentin (a waveform protein) expression, increase extracellular deposition of fibronectin, thereby facilitating metastasis.[35-37] In this study, migration and invasion assays revealed that QFG combined with 5-FU significantly reduced the migrated and invased ability of CRC cells. In-depth study, treatment with QFG and 5-FU affected the expression of EMT-related factor, inhibited EMT occurrence, and synergistically suppressed tumor cell metastasis.

5-FU blocks the production of DNA in cells, which is how it fights tumors. Because it facilitates the conversion of dUMP to dTMP, the only thymidine nucleotide required for DNA synthesis and repair, TS is also recognized as the 5-FU target enzyme. Downregulation of DPD promotes 5-FU chemosensitivity, and DPD is a rate-limiting enzyme in 5-FU degradation. Sections 38–40 This research found that the combination of QFG and 5-FU substantially reduced DPD and TS expression compared to the single-drug therapy. This suggests that the combination of the two drugs may enhance the effectiveness of 5-FU by inhibiting 5-FU-related negative regulatory enzymes.

Tumor growth may be accelerated by the PI3K/AKT signaling pathway. In order to partially activate AKT and then modulate its activity, PI3K activation may produce PIP3, which acts as a second messenger to bring PDK1 and AKT proteins to the plasma membrane. route that goes downstream. Tables 41–43 AKT produces several outcomes: 1) The phosphorylation of AKT hinders cell proliferation by affecting factors like p21; 2) The phosphorylation of AKT affects apoptosis by preventing the activity of BAD (Bcl-2-associated cell death agonist); 3) The phosphorylation of AKT positively controls the NF-κB pathway, which regulates EMT and impacts cell metastasis. positions 11, 44, and 45 Treatment with QFG and 5-FU, as revealed by the results of the western blot experiment, disrupted the PI3K/AKT signaling pathway, according to this research. In this work, the PI3K/AKT signaling pathway was stimulated by 10% FBS, and the effects of the two medicines coupled with QFG were investigated. 10% FBS triggered the AKT signaling pathway. The combined treatment of QFG and 5-FU controlled the proliferation, apoptosis, and metastasis of HCT-8 cells, which were followed by the activation of PI3K and AKT, according to functional tests. The findings show that the PI3K/AKT signaling system's downstream pathway may be regulated by combining QFG and 5-FU therapy, which in turn controls the proliferation, apoptosis, metastasis, and 5-FU chemoresistance of CRC.

CONCLUSIONS

In summary, we demonstrated for the first time that combined treatment QFG with 5-FU has a synergistic ability against CRC, which inhibits cell proliferation, metastasis, induces apoptosis. The downstream of PI3K/

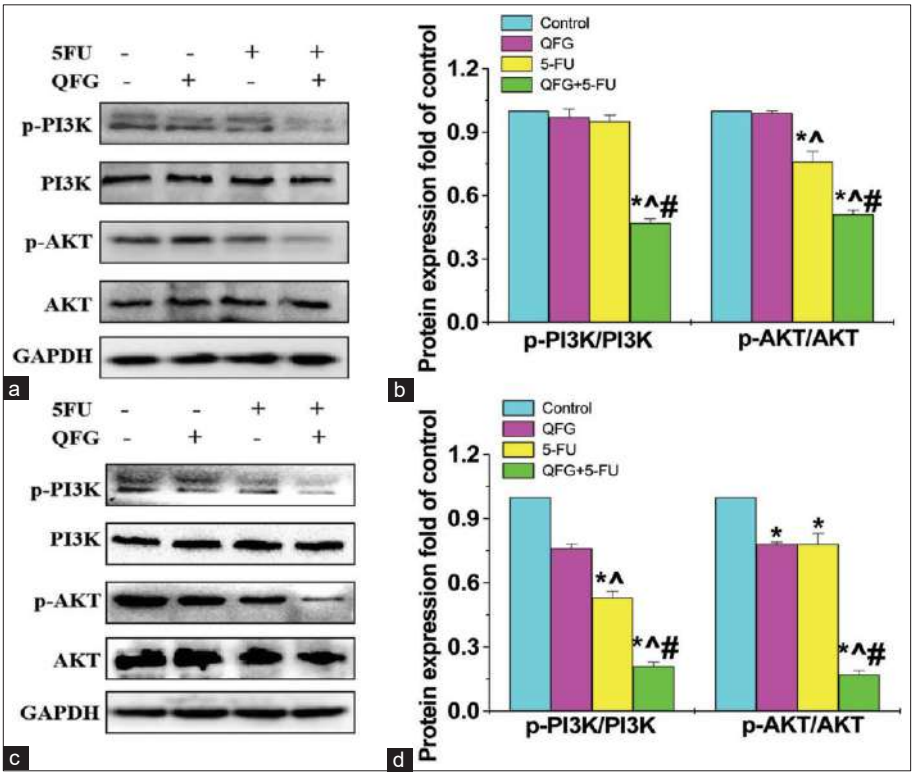

Figure 8: Qingjie Fuzheng Granule (QFG) combined with 5-fluorouracil (5-FU) synergisticly inhibited the PI3K/AKT signaling pathway. (a and b) QFG and 5-FU blocked phosphorylation of PI3K and AKT in HCT-8 cells. (c and d) QFG and 5-FU blocked phosphorylation of PI3K and AKT in tumor tissues. *P < 0.05 compared to the control group; ^ P < 0.05 compared to the QFG group; #P < 0.05 compared to the 5-FU group. QFG: Qingjie Fuzheng Granule, 5-FU: 5-Fluorouracil

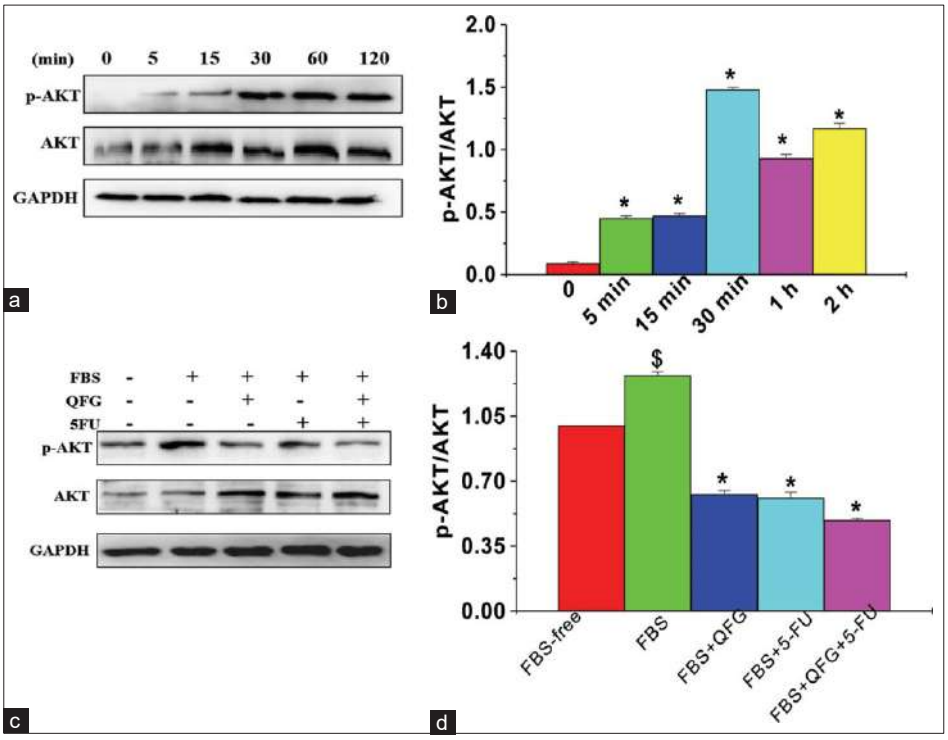

Figure 9: Effect of Qingjie Fuzheng Granule (QFG) and 5-fluorouracil (5-FU) on the activation of the PI3K/AKT pathway by 10% FBS. (a and b) FBS induced AKT phosphonation in different time. (c and d) QFG and 5-FU inhibited the AKT phosphonation. $$P$ < 0.05 compared with the FBS Free group; *P < 0.05 compared with the FBS group. QFG: Qingjie Fuzheng Granule, 5-FU: 5-Fluorouracil

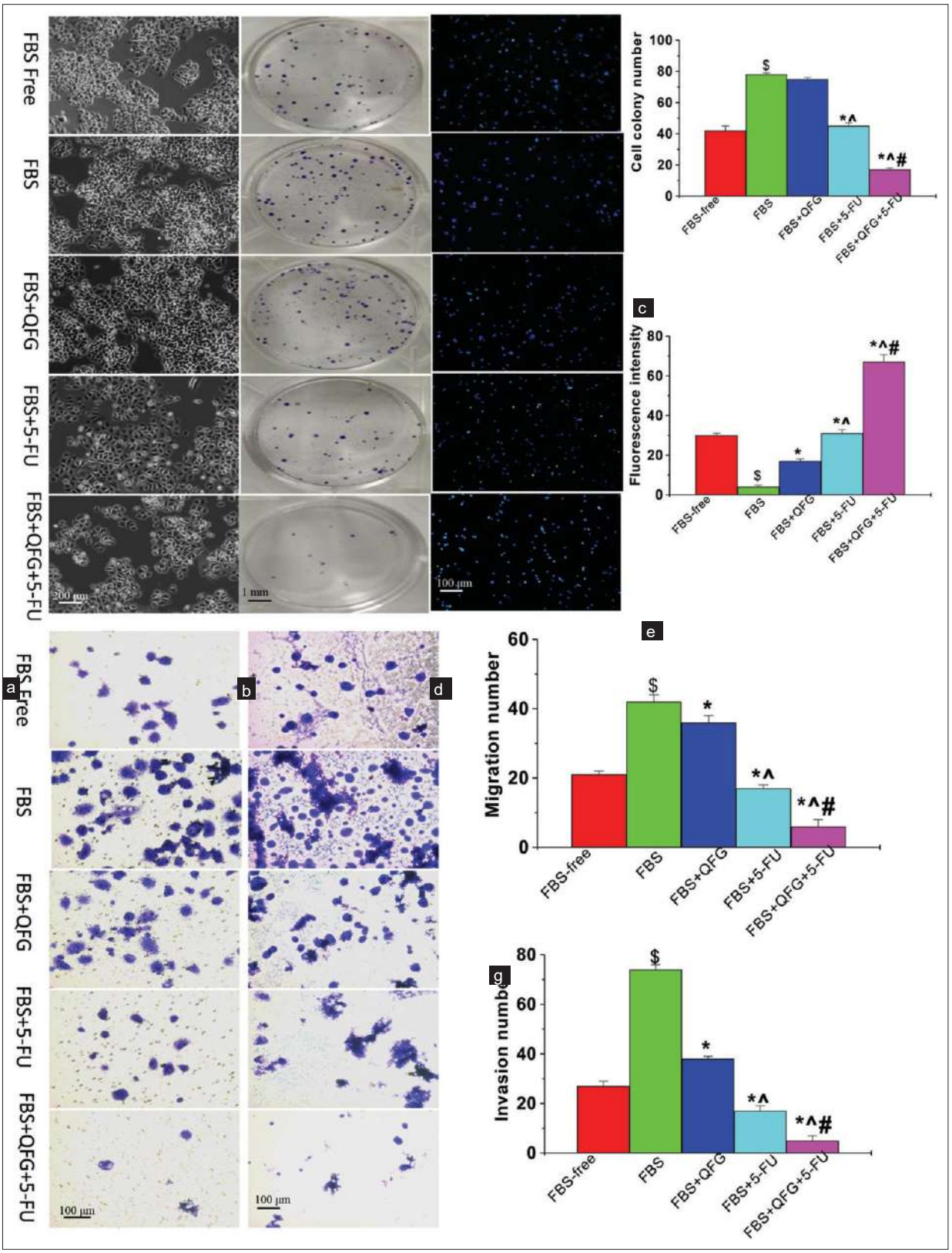

Figure 10: Qingjie Fuzheng Granule (QFG) combined with 5-fluorouracil (5-FU) regulated proliferation, apoptosis, migration and invasion via inhibiting PI3K/AKT pathway. (a) Morphology. (b and c) Colony formation and statistical analysis; (d and e) DAPI staining and statistical analysis (×200, ba *r* = 100 μm). (f and g) Migration and statistical analysis (×200, bar = 100 μm); (h and i) Invasion and statistical analysis (×200, bar = 100 μm). $*P* < 0.05 compared with the FBS-Free group; **P* < 0.05 compared with the FBS group; ^ *P* < 0.05 compared with the FBS + QFG group; #*P* < 0.05 compared with the FBS + 5-FU group. QFG: Qingjie Fuzheng Granule, 5-FU: 5-Fluorouracil

AKT signaling pathway involves tumor proliferation,

apoptosis, metastasis and other related processes. Our results demonstrate that the combined treatment of QFG with and 5-FU exerts its inhibitory capability on CRC cells though regulating PI3K/AKT signaling pathway. This regulation leads to the modulation of gene expressions such as p53 and p21, resulting in a synergistic inhibition of CRC proliferation. Moreover, the combination therapy modulates the genes expression, such as Bax and Bcl-2, leading to a synergistic induction of CRC apoptosis. In addition, it regulates the EMT process, synergistically inhibiting CRC metastasis. Furthermore, the combination therapy inhibits the expression of DPD and TS, which enhances the efficacy of 5-FU and further contributes to the inhibition of CRC progression.

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