

The synergistic effect of organic silicone quaternary ammonium salt and 5-fluorouracil on hepatocellular carcinoma *in vitro* and *in vivo*

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Hepatocellular carcinoma (HCC) is the third most common type of cancer worldwide, causing over 370 000 deaths per year, with approximately half of them in China.

Chemotherapy is the optimal treatment for patients with advanced HCC, although chemoresistance has become a significant obstacle to successful liver cancer surgery. In this paper, we have assessed the characteristics of drugs to explore the effects of individual and combined action of organic silicone quaternary ammonium salt (Jieyoushen) and 5-fluorouracil (5-FU). The results of MTT assays showed that single and combined action of Jieyoushen and 5-FU can inhibit the proliferation of liver carcinoma cell lines in a dose-dependent and time-dependent manner, respectively. Electron microscopy and Hoechst 33342 staining showed characteristic apoptotic bodies in apoptotic cells treated with Jieyoushen and 5-FU. Flow cytometry results indicated that the percentage of cells at G0/G1 phase gradually increased, whereas it gradually decreased during the S phase after treatment.

Introduction

Hepatocellular carcinoma (HCC) is the main form of liver cancer. The incidence of HCC has substantially increased around the world (Chen *et al.*, 2009; Tanizaki *et al.*, 2010; Jemal *et al.*, 2011; Jiao *et al.*, 2012). In China alone, more than 401 000 new patients were diagnosed with HCC and more than 371 000 patients died of this disease in 2008 (Ferlay *et al.*, 2010).

Surgical treatment is considered to be the best choice for HCC (Takayama *et al.*, 2010); however, only a small proportion of patients are candidates for radical resection at the time of diagnosis, owing to the fact that HCC rarely presents with characteristic symptoms in the early stage and over 80% of patients lose their chance for curative hepatectomy when the diagnosis of HCC is confirmed (Jia *et al.*, 2012; Yang *et al.*, 2012). For the management of advanced HCC, systemic chemotherapy with classical cytotoxic agents offers a marginal survival benefit (Thomas *et al.*, 2008; Wang *et al.*, 2012). Compared with local treatments, such as radiation and surgery, chemotherapy is a form of systemic treatment that may reach tumor cells wherever they have spread (Ramalingam and Belani, 2008). At present, more than 100 drugs are being used for chemotherapy, either alone or in combination. However, HCCs are resistant to anticancer drugs

Taken together, these results suggest that the combination of Jieyoushen with 5-FU exerts a synergistic anticancer effect on HCC growth and that targeted therapeutic strategies may improve HCC sensitivity to chemotherapy. *European Journal of Cancer Prevention* 23:372–384 © 2014 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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(Asghar and Meyer, 2012; Jia *et al.*, 2012). Traditional systemic chemotherapy has a low curative rate and results in many toxic side effects in liver cancer; hence, it is not widely accepted by clinical practitioners. Recently, the application of new chemotherapeutic drugs and the new combination regimens have been proved to be effective against advanced-stage HCC (Wan *et al.*, 2009; Ca *et al.*, 2012; Xie *et al.*, 2012). Moreover, it is desirable to explore the alternative modalities of combination chemotherapy for advanced HCC.

5-Fluorouracil (5-FU) is one of the optimal drugs for the treatment of HCC. The thymidylate synthase inhibition and the misincorporation of fluoronucleotide into RNA and DNA is generally thought to be the main effective mechanism against cancer (Llovet Josep, 2002; Lopez *et al.*, 2006; Mukai *et al.*, 2006; Xu *et al.*, 2007; Bu *et al.*, 2008). However, the rapid development of acquired resistance to 5-FU has limited its clinical usage (Yoo *et al.*, 2009), and its underlying molecular mechanism remains undefined. To enhance the clinical use of 5-FU, a number of drug combinations have been investigated (Shang *et al.*, 2007; Yang *et al.*, 2008; Morabito *et al.*, 2009). The results of such experimental and clinical studies showed that some drugs can not only be effective against various types of cancer but can also improve the effectiveness of 5-FU

significantly and reduce toxicity greatly. The combinative mechanism is needed to further identify which one plays the dominant role in the anticancer process.

Jieyoushen is a type of polymeric surfactant, and its main component is a quaternary ammonium salt. Nowadays it is mostly used as a postoperative physical antimicrobial material (Liu *et al.*, 2010). When water-soluble liquid Jieyoushen is sprayed on skin surfaces or mucosal areas, it immediately solidifies and forms an invisible antimicrobial layer with a dual overlapping structure: the bonded film and the positive-charge film. The bonded film comprises macromolecular agents, securely bonded to the body surface by means of a chemical, which has a long-acting effect on preventing microbial growth. The positive-charge film comprises cationic activators that form a reticulate film with the positive charge of the skin surface or mucosal area. The positive-charge film strongly absorbs pathogenic microorganisms that have negative charge, such as bacteria, fungi, and viruses. If the respiratory enzyme of the pathogenic microorganisms on which they rely for existence is out of action, they will die due to lack of oxygen supply (Li *et al.*, 2011). However, it has not been published in the literature that Jieyoushen is an antitumor agent. The mechanism of tumor metastasis prevention is indeterminate. In our previous research, the anticancer activity of Jieyoushen *in vivo* was tested in a rat intra-abdominal tumor model, and the result showed that Jieyoushen can prevent the spread of intra-abdominal tumors in rats. Moreover, the incidence rate differed significantly between the treatment and control groups ($P < 0.05$). Hence, it was concluded that Jieyoushen can be used to prevent tumor implant metastasis when it is used alone or in combination with other chemotherapeutic drugs to decrease the required dose of chemotherapy and minimize toxicity in patients.

In this study, the synergistic effect and mechanisms of action of the combination of Jieyoushen with 5-FU have been investigated in human HCC SMMC7721 cell lines and R15 cell lines of the rat. The synergy, additivity, or antagonism of these agents from growth inhibition, cell cycle distribution, apoptosis, and expression of caspase-3 and caspase-8, Bax, Bcl-2, and survivin proteins were carefully evaluated to understand the combinative action of the two drugs and find the possible mechanism of action. We hope that these results will bring out new therapeutic means for preventing the metastasis of HCC in surgery.

Materials and methods

Materials

Jieyoushen was provided by the Nanjing Shenqi Technology Development Co. Ltd (Jiangsu, China). 5-FU (0.25 g/10 ml) was purchased from Tianjin Jinyao Amino Acid Co. Ltd (Tianjin, China). The primary anti-Bax, anti-Bcl-2, anti-caspase-8, anti-caspase-3, anti-survivin, and anti- β -actin

antibodies were obtained from Bioworld Technology Inc. (Louis Park, Minnesota, USA). The annexin V-FITC/propidium iodide (PI) apoptosis detection kit was purchased from Invitrogen Technology Inc. (Carlsbad, California, USA). Six- to 8-week-old male Wistar rats (Lanzhou University Experimental Animal Center, Lanzhou, China) were used in the in-vivo experiments. A cage, surgical instruments, 10% chloral hydrate (0.35 ml/100 g weight), DMEM high-sugar medium, and fetal calf serum were used.

Cell culture

Human liver carcinoma cell lines (SMMC7721), hepatocyte lines (LO2), and rat liver carcinoma cell lines (R15) were obtained from American Type Culture Collection (ATCC, Manassas, Virginia, USA). They were cultured in RPMI 1640 medium (Gibco, Grand Island, New York, USA), supplemented with 10% heat-inactivated fetal bovine serum, 50 U/ml of penicillin, and 50 μ g/ml of streptomycin, 15 mmol/l (pH 7.2) HEPES, 2 mmol/l L-glutamine, and incubated at 37°C in a humidified incubator containing 5% CO₂. The treatment groups were divided into different concentrations from 6 to 48 h.

Growth inhibition assay

Liver carcinoma cells were seeded in 96-well plates at a density of 5×10^3 cells/well in quintuplicate, and 200 μ l Jieyoushen (1/1000, V%), 10 μ mol 5-FU, and the combination (1:1, V/V) solution were added to each well at a final concentration. Cells were incubated for 0, 6, 12, 24, and 48 h. The serum-free medium group was set as the bottom control, and a positive control group at a density of 5×10^3 cells/well was also seeded. Meanwhile, hepatocyte LO2 cells served as the negative control group. After the treatment, 50 μ g/10 μ l of MTT (Sigma, Santa Clara, California, USA) was mixed into these wells and the cells were further incubated for another 4 h; thereafter, 150 μ l of DMSO (Sigma) was added to each well after removing the supernatant. After shaking the plate for 15 min, cell viability was measured by reading the absorbance at a wavelength of 490 nm using a Model 680 Elisa Reader (Bio-Rad, Hercules, California, USA). Survival rates were calculated according to the percentage of positive controls. The experiment was repeated three times.

Transmission electron microscopy observation

Transmission electron microscopy was performed to detect morphological changes. Briefly, human liver carcinoma cell lines SMMC7721 and rat liver carcinoma cell lines R15 were treated with Jieyoushen (1/1000, V%), 5-FU (10 μ mol), and the combination (1:1, V/V) for 24 h. After intervention with different concentrations of drugs, cells were digested with 0.25% pancreatin and centrifuged at 1000 rpm for 5 min, followed by repeated washing with PBS, supernatant removal, and fixing with 2.5% precooled glutaraldehyde overnight at 4°C. Ultrathin sections of copper were prepared, and cells were rinsed once with PBS and then fixed with 1% osmic acid

for 1 h. Samples were then dehydrated by acetone and embedded in epoxide resin. After staining with uranyl acetate and lead citrate, the sections were observed under a transmission electron microscope.

Hoechst staining

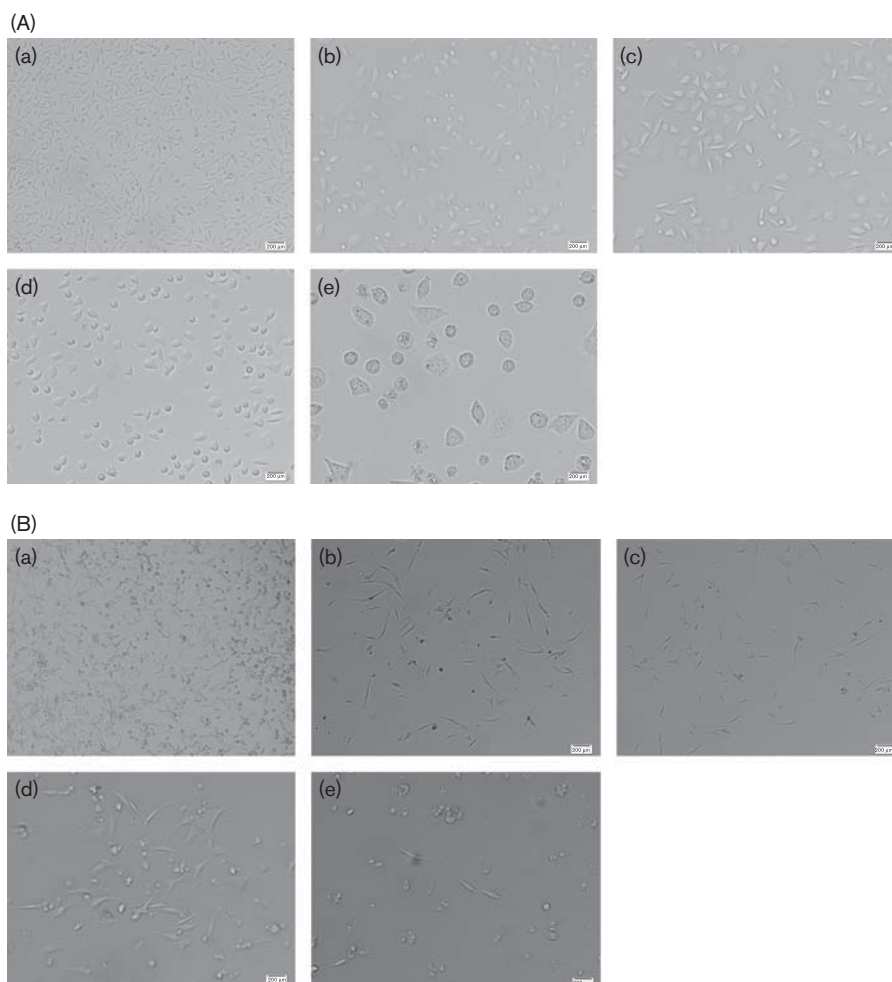
Liver carcinoma cells (4×10^4 /well) were cultured in 24-well plates in RPMI 1640, which was supplemented with 10% fetal bovine serum. At the same time, the duplicate culture plates were prepared. Then, human liver carcinoma cell line SMMC7721 and rat liver carcinoma cell line R15 were treated with Jieyoushen (1/1000, V%), 5-FU (10 μ mol), and the combination (1 : 1, V/V) for 24 h. After intervention, cells were washed twice with cold PBS (pH 7.2). Then they were fixed with 4% precooled neutral paraformaldehyde overnight at 4°C. Thereafter, the cells were washed with PBS and stained with 0.5 ml Hoechst 33342 (Sigma) for 8 min at room temperature, followed

by another wash with PBS. An excitation wavelength of 350 nm and an emission wavelength of 460 nm were used to observe morphological changes under a fluorescence microscope (Olympus X71; Olympus, Tokyo, Japan).

Flow cytometric analysis of cellular cycle and apoptosis

Cells were seeded in 75-cm² culture flasks. The culture medium was replaced with fresh medium when cells had reached 80% confluency and then cells were exposed to Jieyoushen (1/1000, V%), 5-FU (10 μ mol), and the combination (1 : 1, V/V) for 24 h. They were then washed with PBS, fixed in ice-cold 70% ethanol, and stored at -20°C for further use. Before analysis, cells were washed and suspended at a concentration of 5×10^6 cells/ml in PBS and incubated with 0.1 mg/ml RNaseA and 40 μ g/ml PI at 37°C for 20 min. Samples were then analyzed using a FACS Calibur Cell Sorting System (BD FacsCalibur; BD Bioscience, Franklin, New Jersey, USA).

Fig. 1



The morphological change when SMMC7721 (A) and R15 (B) cells were treated with the mixtures of 1/1000 Jieyoushen and 10 μ mol 5-fluorouracil for 6, 12, 24, and 48 h. (a) Control; (b) 6 h; (c) 12 h; (d) 24 h; (e) 48 h.

Western blot analysis of the expression of Bax, Bcl-2, caspase-8, caspase-3, and survivin proteins

Cells were inoculated and fused to 80% confluency and treated with the same drugs as used in the MTT experiment. The medium was collected by centrifugation and the cell monolayer was washed with PBS. Protein extraction was carried out using RIPA (Beyotime Biotechnology, Haimen, Jiangsu, China). Cell extracts were collected and centrifuged at 14000*g* in a microcentrifuge for 20 min at 4°C. The supernatants were collected, and protein concentration was determined using Bradford's method. Cell extracts were either used immediately or stored at -80°C. They were boiled at 100°C in denaturation buffer for 7 min after taking steps to prevent the degradation of proteins. For SDS-PAGE, 60 µg of protein from each extract was loaded into each well of the gel. A condensation protein electrophoresis gel was run at 80 and 100V, and proteins were transferred onto polyvinylidene fluoride membranes at 200 mA in an ice bath for 1–2 h. Protein bands were determined using a prestained molecular weight marker (SM0671; Fermentas, Burlington, Ontario, Canada) for reference. Membranes were blocked with TBST buffer (50 mmol/l Tris-HCl, pH 7.4, 150 mmol/l NaCl, 0.1% Tween 20) containing 5% (w/v) fat-free dried milk for 2 h at room temperature and incubated with primary antibody overnight at 4°C. Membranes were then washed with TBST and incubated with species-specific HRP-conjugated secondary antibodies for 2 h at room temperature. The membrane underwent additional washing, and the immunoreactive protein was visualized using the chemiluminescent reagent ECL (Minipore, Darmstadt, Germany) according to the manufacturer's protocol. Differences in protein expression were examined by densitometry analysis using Image-pro plus 6.0 software (Media Cybernetics Inc, Bethesda, Maryland, USA). The experiments were repeated at least twice to confirm the results.

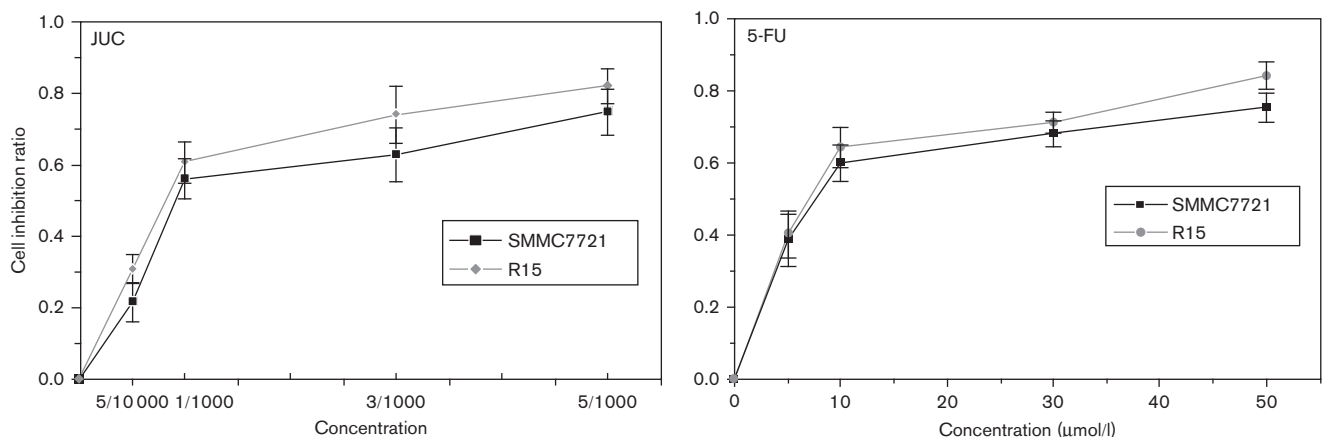
Animal experiment

Forty male Wistar rats, weighing 150 g, were randomly divided into the control group and the experimental group by a random number table method. R15 cells were collected when they reached 80% or more confluency, and cell vitality was tested using trypan blue staining to ensure that cell vitality was higher than 95%. The cell concentration was diluted to 6×10^6 /ml with physiological saline. The rats were anesthetized with 10% chloral hydrate, and then fixed on the operating table. We made a 3 cm incision in the middle of the abdomen between the xiphoid process and the pubic symphysis, and then separated the peritoneum using an abdominal tractor. In the treatment group, Jieyoushen (2 ml) was sprayed twice into the abdominal cavity mucous membrane, and then 1 ml cell suspension was absorbed and injected into the enterocoelia. In the control group, 1 ml cell suspension was absorbed and injected into the enterocoelia directly. The abdominal cavity was closed after treatment. The mice were killed with a lethal dose of anesthetic after 5 weeks. We opened the abdominal cavity again beside the old wound. The formation of an implanted metastatic tumor was observed, and the number of rats forming a metastatic tumor was recorded. The tumor growth situation was also recorded by measuring the weight of the tumor. Two tumor tissues were stained with hematoxylin and eosin to observe the pathological features.

Statistical analyses

The results were analyzed using SPSS 15.0 software (SPSS Inc., Chicago, Illinois, USA) and were expressed as mean \pm SD. One-way analysis of variance and Tukey's post-hoc tests were used for determining the differences between groups, and a *P* value of less than 0.05 was considered statistically significant.

Fig. 2



The inhibitory effect of Jieyoushen (JUC) and 5-fluorouracil (5-FU) on SMMC7721 and R15 cells. SMMC7721 and R15 cells were treated with various concentrations of Jieyoushen and 5-FU for 24 h. Dose-dependent inhibition of liver carcinoma cell growth could be observed ($P < 0.05$, Tukey's post-hoc analysis). After cells were incubated, cell proliferation was determined by MTT assay.

Results

Morphological observation under light microscopy

It can be seen from Fig. 1 that the cells had changed from the original epithelioid or polygonal shape to round shape, the cell membrane had shrunk, and many nuclei had become pyknotic; the refractive index of the cells had decreased or cytoplasm containing partly multivesicular bodies and dense granules had appeared (Fig. 1).

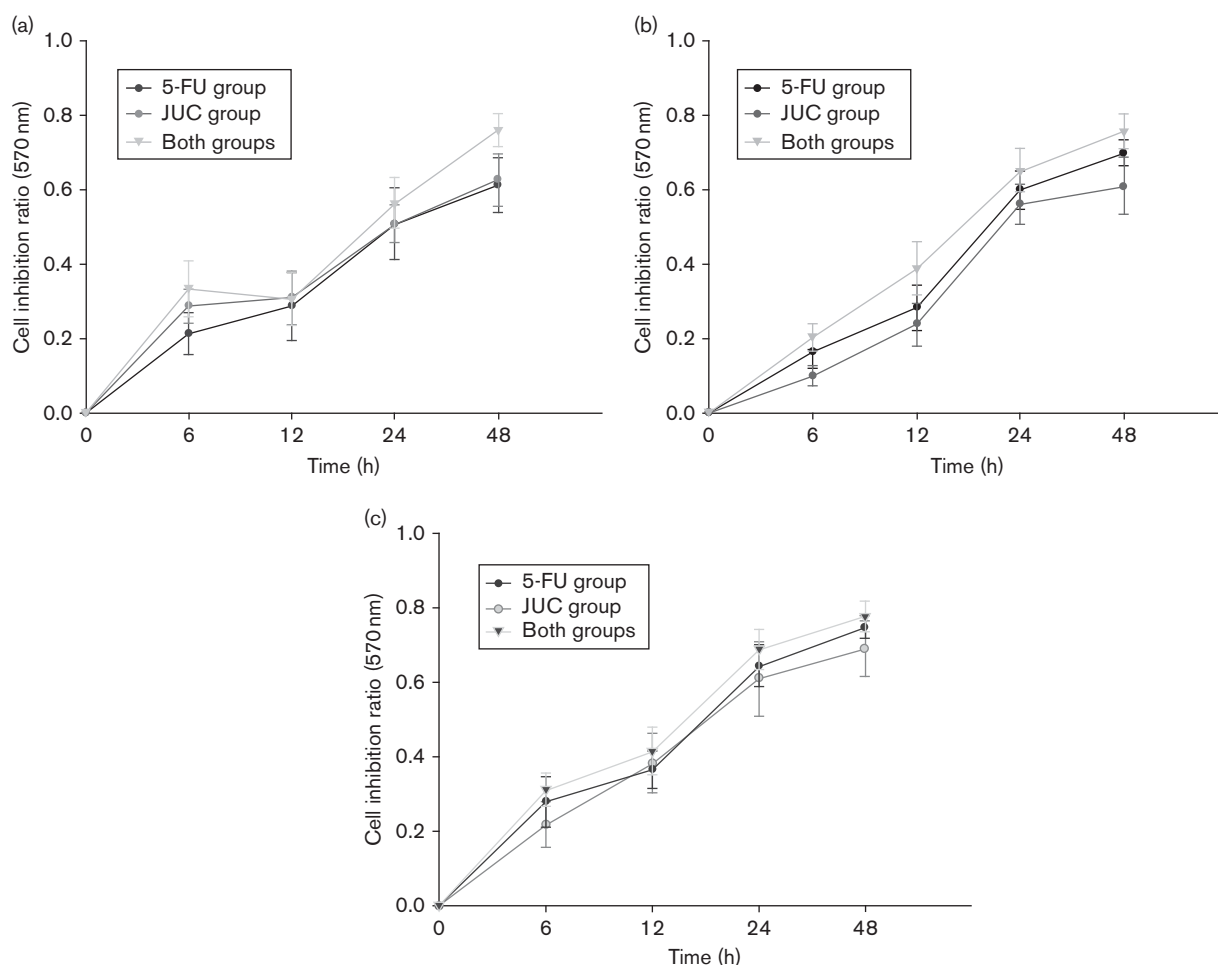
Cell survival assay

The results of the MTT assay showed that Jieyoushen and 5-FU could inhibit the proliferation of SMMC7721 and R15 cells after 24h exposure (Fig. 2). Moreover, the observed inhibitory effect was dose dependent. After SMMC7721 and R15 cells had been treated with 5/10 000, 1/1000, 3/1000, and 5/1000 Jieyoushen (dilution rate) for 24h, the cell inhibition ratios of SMMC7721 were 21.68 ± 5.77 , 56.03 ± 5.43 , 62.83 ± 7.64 , and $74.73 \pm$

6.21% , respectively. Similarly, the cell inhibition ratios of R15 were 30.95 ± 4.21 , 60.81 ± 5.90 , 74.01 ± 7.92 , and $82.14 \pm 4.91\%$, respectively. After SMMC7721 and R15 cells had been treated with 5, 10, 30, and 50 μmol 5-FU for 24h, the cell inhibition ratios of SMMC7721 were 38.48 ± 7.14 , 59.92 ± 5.17 , 68.13 ± 3.53 , and $75.27 \pm 4.26\%$, respectively. Similarly, the cell inhibition ratios of R15 were 40.18 ± 6.34 , 64.33 ± 5.47 , 71.24 ± 2.93 , and $84.12 \pm 3.91\%$, respectively. Considering the comprehensive contrast effect, 1/1000 Jieyoushen and 10 μmol 5-FU were chosen to investigate the combinative effect of Jieyoushen and 5-FU in inhibiting the growth of HCC cell lines.

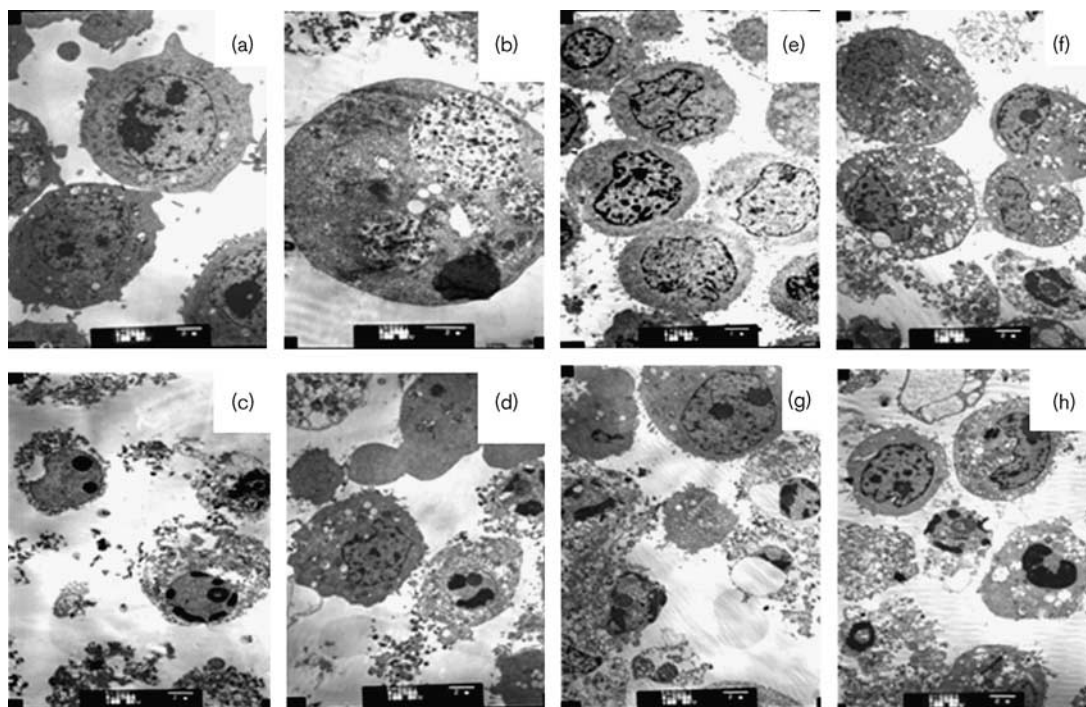
As shown in Fig. 3, compared with the results of 5-FU or Jieyoushen alone, markedly decreased cell viability was observed in the combination groups when they were treated with 5-FU and Jieyoushen. The cell inhibition ratios for SMMC7721 and R15 cells treated with 5-FU

Fig. 3



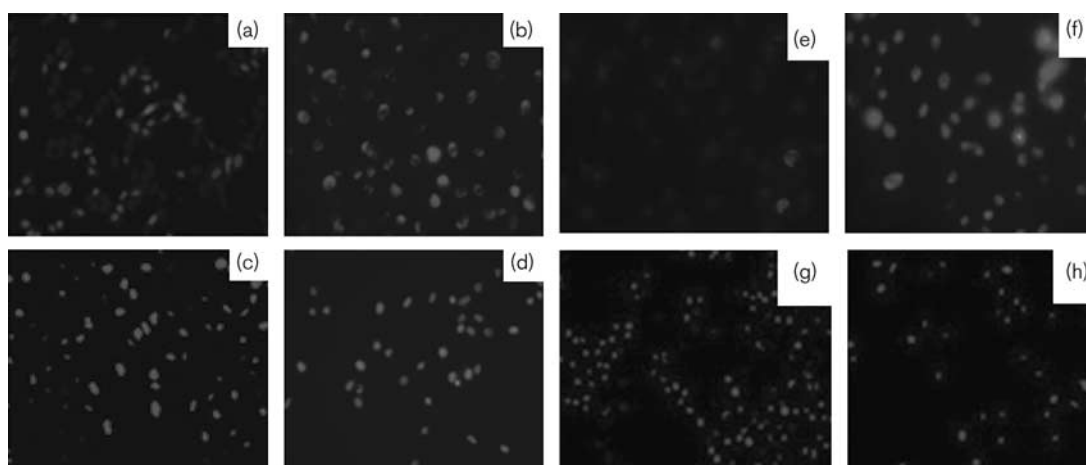
Cell proliferation inhibiting effects of drugs on SMMC7721 (b) and R15 (c) liver carcinoma cells and hepatocyte lines LO2 (a). The cells were treated with 1/1000 Jieyoushen (JUC), 10 μmol 5-fluorouracil (5-FU) and the combination for 6, 12, 24, and 48 h. Time-dependent inhibition of liver carcinoma cells growth could be observed ($P < 0.05$, Tukey's post-hoc analysis). After cells were incubated, cell proliferation was determined by MTT assay.

Fig. 4



Effects of drugs on ultrastructure of SMMC7721 and R15 cells by transmission electron microscope, including blank control group (a, e), 10 μmol 5-fluorouracil (5-FU) (b, f), 1/1000 Jieyoushen (c, g), and the combination group (d, h) for 24 h; the karyopyknosis and chromatic agglutination ($\times 2500$), apoptotic cells ($\times 2500$) are shown in the right picture.

Fig. 5

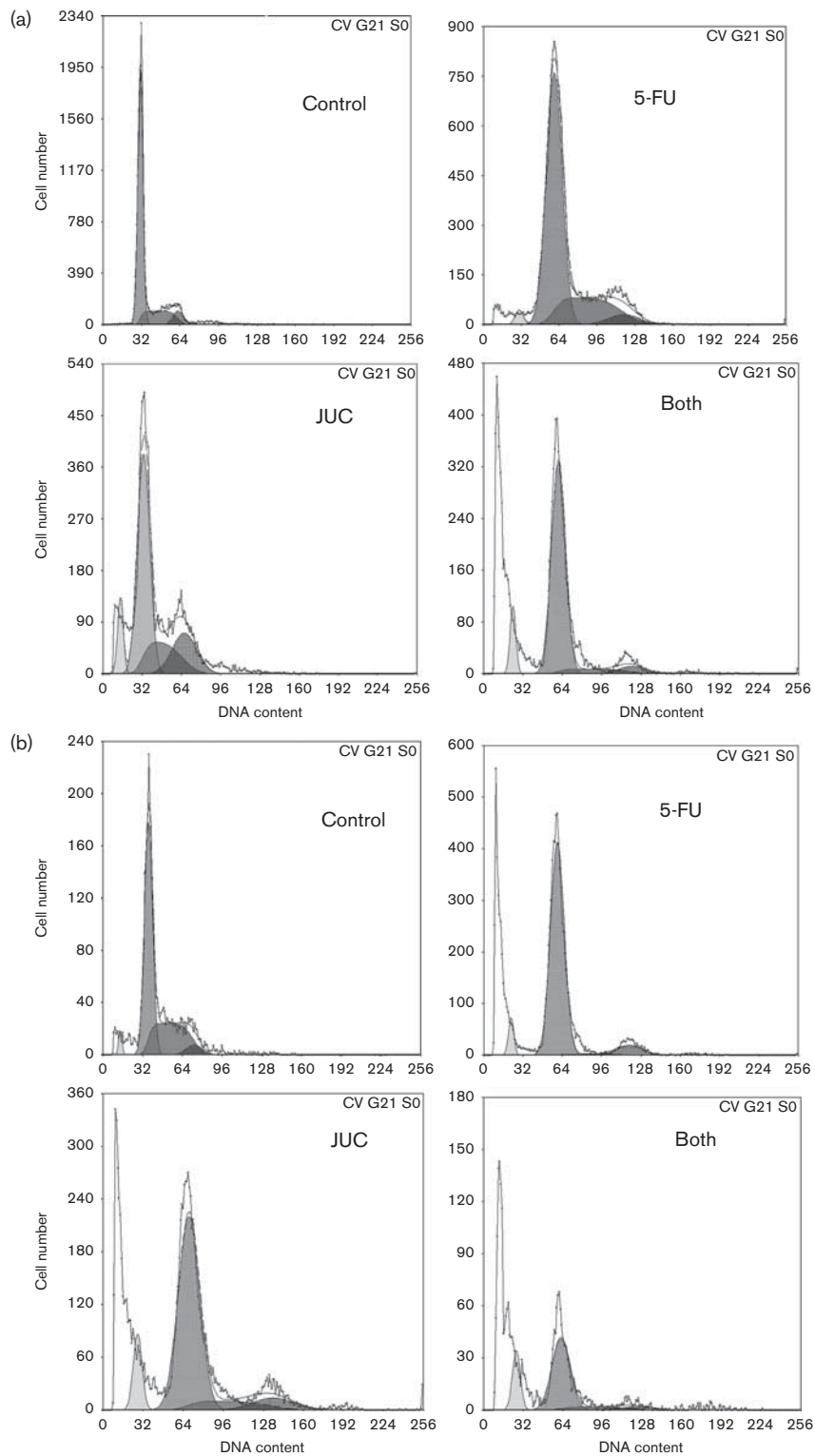


Morphological change and the number of apoptotic nuclei formed after SMMC7721 and R15 cells were treated with these drugs for 24 h, stained with Hoechst 33258 and analyzed under fluorescence microscope ($\times 20$). (a) SMMC7721 control; (b) SMMC7721 10 μmol 5-fluorouracil (5-FU); (c) SMMC7721 1/1000 Jieyoushen; (d) SMMC7721 both groups; (e) R15 control; (f) R15 10 μmol 5-FU; (g) R15 1/1000 Jieyoushen; (h) R15 both groups.

(10 $\mu\text{mol/l}$) alone were 59.92 ± 5.17 and $64.33 \pm 5.47\%$ at 24 h, respectively. The cell inhibition ratios for SMMC7721 and R15 cells treated with 1/1000 of pristine Jieyoushen alone were 56.03 ± 5.43 and $60.81 \pm 5.90\%$ at 24 h, respectively. Compared with Jieyoushen, 5-FU

showed a more potent effect in reducing cell growth. After we treated 5-FU with 1/1000 of pristine Jieyoushen for 24 h, the antitumor activities of 5-FU was seen to be enhanced significantly in a time-dependent manner in both cell lines. It is noteworthy that the cell inhibition

Fig. 6



FACS analyses of the cell cycle of SMMC7721 (a) and R15 (b) cells treated with 1/1000 Jieyoushen (JUC), 10 μ mol 5-fluorouracil (5-FU), or their combination for 24 h.

ratios for SMMC7721 and R15 cells treated with 1/1000 of pristine Jieyoushen plus 10 $\mu\text{mol/l}$ 5-FU increased to 65.05 ± 5.87 and $68.67 \pm 5.29\%$, respectively. These data indicate that the combination of Jieyoushen and 5-FU synergistically inhibits cell growth in HCC cell lines.

Transmission electron microscopy for ultrastructural changes and analysis of apoptosis

It can be seen from Fig. 4 that SMMC7721 and R15 cells showed increased heterochromatin within the nucleus, as well as chromatin margination, chromatin clump shrinkage within the cytoplasm, incomplete cell membranes, and increasing numbers of apoptotic cells, necrotic cells, or autophagic bubbles within the cytoplasm. Besides the above ultrastructural changes, R15 cells showed a large number of autophagic vesicles and apoptotic cells, and more cells dissolved and were necrotic. This evidence suggests that the joint action of 5-FU and Jieyoushen could inhibit the proliferation of SMMC7721 and R15 cells.

Hoechst staining

Hoechst 33342 staining was used to observe the morphology of apoptotic cells. The results showed that the control group exhibited regular nuclear morphology, uniform staining, and light blue color. After the joint action for 24 h, compared with the control group, cell morphology reduced and nuclear condensation or fragmentation block formed characteristic apoptotic bodies (Fig. 5).

Flow cytometric detection of cell cycle and apoptosis

Cell cycle analysis

The cell cycle results showed that the two cell lines underwent the same changes in cell cycle, which are described below.

The cell cycle analysis revealed that when SMMC7721 cells were treated with 10 μmol 5-FU for 24 h, compared with the control phase, the percentage of cells in the G0/G1 phase was significantly increased ($P < 0.05$). However, when treated with 1/1000 Jieyoushen alone for 24 h, the cell cycle distribution was not significantly changed. When a mixture of these two agents was used, the most remarkable change occurred. Compared with cells treated with either 5-FU or Jieyoushen alone ($P < 0.05$) (Fig. 6a), cells mostly accumulated in the G0/G1 phase and there was a significant decrease in the S phase population. Similar regulation can be found when R15 cells were treated with 1/1000 Jieyoushen, 10 μmol 5-FU, or their combination for 24 h (Fig. 6b). Therefore, it can be concluded that the mixture of Jieyoushen and 5-FU had a synergistic effect on the treatment of SMMC7721 and R15 cells (Tables 1 and 2).

The detection of the occurrence of apoptosis in two kinds of cells

Jieyoushen and 5-FU in the combination group could not only induce human hepatocarcinoma cell apoptosis but could make it senescent. In the flow cytometry, we used PI and annexin V-FITC. On treating the two kinds of cells with 5-FU (10 μmol) or both 5-FU and 1/1000 Jieyoushen for 24 h, it was found that the population of PI-negative and annexin V-positive cells increased as well as that of PI-positive and annexin V-positive cells (from $26.9 \pm 0.6\%$ for 5-FU treatment to $35.9 \pm 0.7\%$ for Jieyoushen plus 5-FU treatment). The experiments were repeated three times ($P < 0.05$, one-way analysis of variance). The number of viable apoptotic cells and nonviable apoptotic cells in the sample was ascertained (Pietra *et al.*, 2001). The results show that Jieyoushen and 5-FU in combination could induce a synergistic effect, which can increase the percentage of apoptosis in human hepatocarcinoma cells (Fig. 7).

Table 1 Cell cycle distribution of SMMC7721 cells treated with 1/1000 Jieyoushen, 10 μmol 5-fluorouracil or their combination for 24 h

Groups	Dose	G0/G1 (%)	S (%)	G2/M (%)
Control	–	54.88 \pm 6.34	26.159 \pm 5.63	18.961 \pm 7.70
5-FU	10 μmol	69.016 \pm 9.25 \blacktriangle	25.609 \pm 8.17	5.375 \pm 12.38 \blacktriangle
Jieyoushen	1/1000	57.301 \pm 10.73	21.784 \pm 3.41	20.914 \pm 3.54
Combination	10 μmol + 1/1000	85.774 \pm 4.85*	8.080 \pm 4.39*	6.145 \pm 1.26*

5-FU, 5-fluorouracil.

*Represent that differences between the combination group and control group have statistical significance ($P < 0.05$).

\blacktriangle Represent that differences between 5-FU group and control group have statistical significance ($P < 0.05$).

Table 2 Cell cycle distribution of R15 cells treated with 1/1000 Jieyoushen, 10 μmol 5-fluorouracil, or their combination for 24 h

Groups	Dose	G0/G1 (%)	S (%)	G2/M (%)
Control	–	57.614 \pm 5.73	30.928 \pm 8.71	11.457 \pm 6.32
5-FU	10 μmol	90.023 \pm 11.24 \blacktriangle	7.784 \pm 13.44 \blacktriangle	2.193 \pm 1.31 \blacktriangle
Jieyoushen	1/1000	78.679 \pm 9.82 \blacklozenge	11.512 \pm 5.45 \blacklozenge	9.809 \pm 3.03
Combination	10 μmol + 1/1000	80.523 \pm 6.29*	7.480 \pm 4.13*	11.997 \pm 2.89

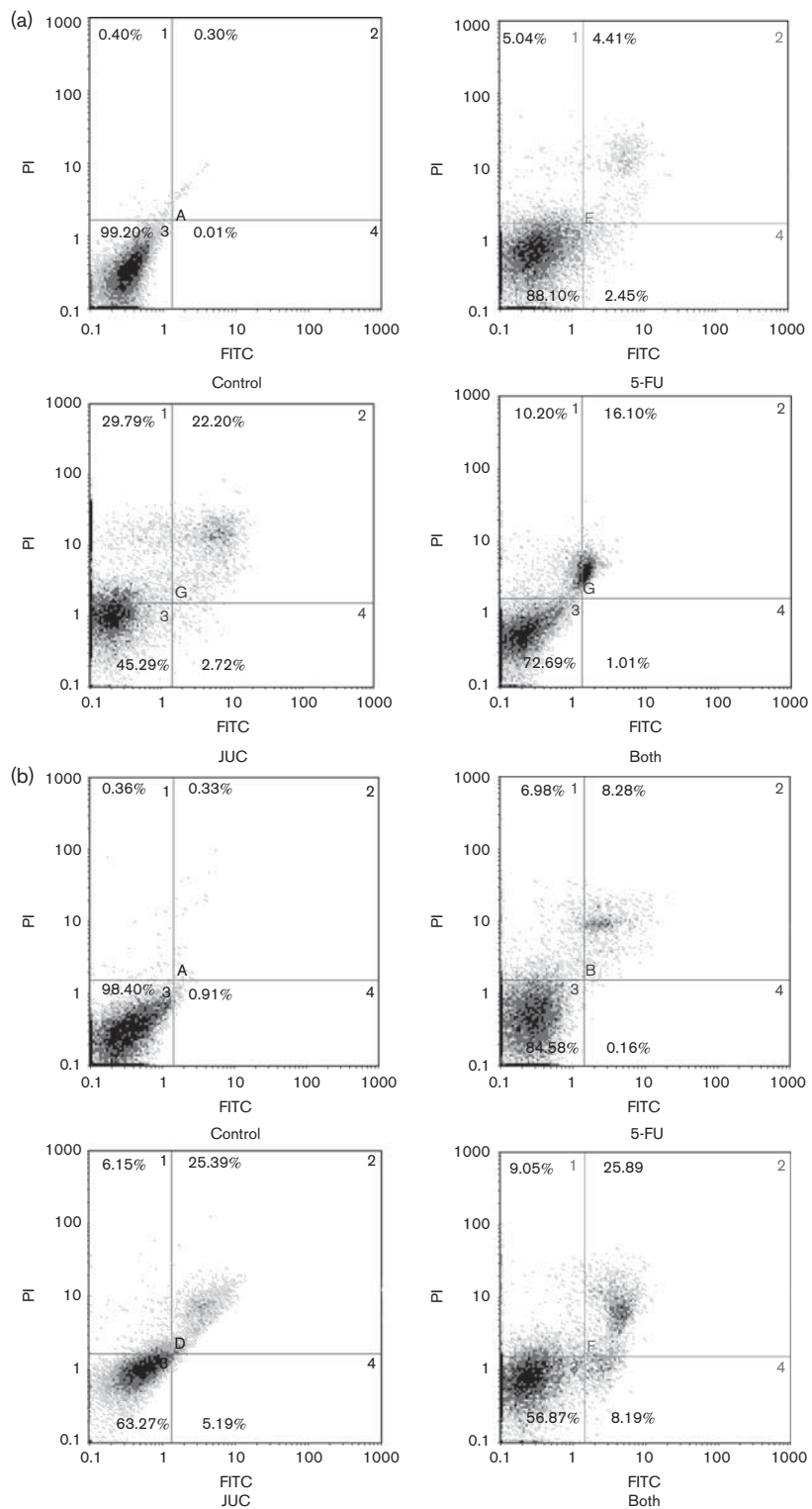
5-FU, 5-fluorouracil.

*Represent that differences between the combination group and control group have statistical significance ($P < 0.05$).

\blacktriangle Represent that differences between 5-FU group and control group have statistical significance ($P < 0.05$).

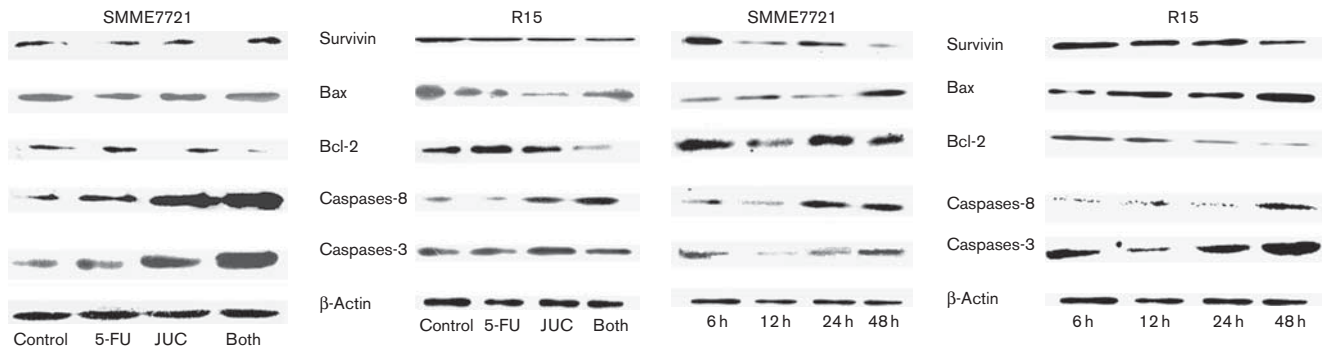
\blacklozenge Represent that differences between Jieyoushen group and control group have statistical significance ($P < 0.05$).

Fig. 7



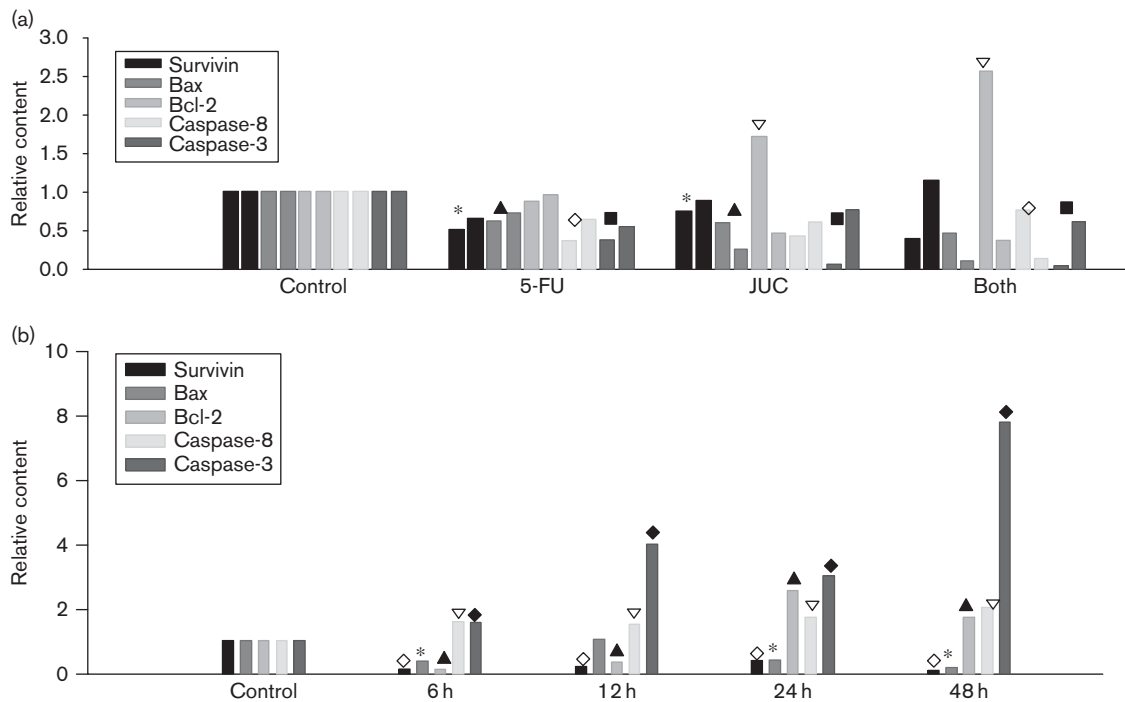
Apoptosis of SMMC7721 (a) and R15 (b) cells treated with 1/1000 Jieyoushen (JUC), 10 μmol 5-fluorouracil (5-FU), and the combination for 24 h. The percentage of apoptotic cells was determined by flow cytometry. The annexin V-positive cells were defined as apoptotic cells, including viable apoptotic cells (the annexin V-positive, PI-negative) and nonviable apoptotic cells (the annexin V/PI-positive).

Fig. 8



Expression of caspase-3 and caspase-8, Bax, Bcl-2, and survivin protein as detected by western blotting when SMMC7721 and R15 cells were treated with 1/1000 Jieyoushen (JUC), 10 μ mol 5-fluorouracil (5-FU), and the combination for 24 h, and the mixtures of 1/1000 Jieyoushen and 10 μ mol 5-FU for 6, 12, 24, and 48 h.

Fig. 9



The comparison of the relative content of proteins, the relative to the change in quantity of protein expression in two kinds of cell. *Bax protein; \blacktriangle Bcl-2 protein; ∇ caspase-8 protein; \diamond caspase-3 protein; \blacksquare survivin protein, compared with the control group, treated groups have obvious statistical significance (a) ($P < 0.05$). There is time-dependent relationship in the protein expression (b) ($P < 0.05$).

Analysis of the change of related protein levels in the mitochondrial pathway

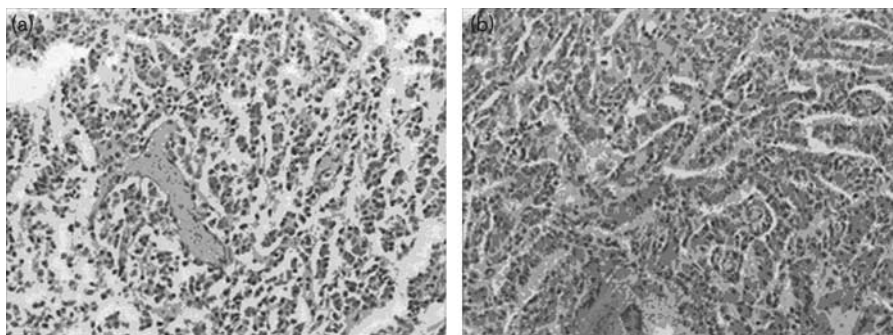
Compared with the control group, the combination treatment group could significantly increase Bax, caspase-8, and caspase-3 protein levels in SMMC7721 and R15 cells at 24h, and significantly decrease Bcl-2 protein expression. However, compared with the control group, the content of survivin protein was significantly decreased. Also, a time-dependent relationship in the protein expression was found

(Figs 8 and 9). The related transduction mechanism is shown in Fig. 11.

The influence of Jieyoushen in the formation of implanted metastatic tumors in rats

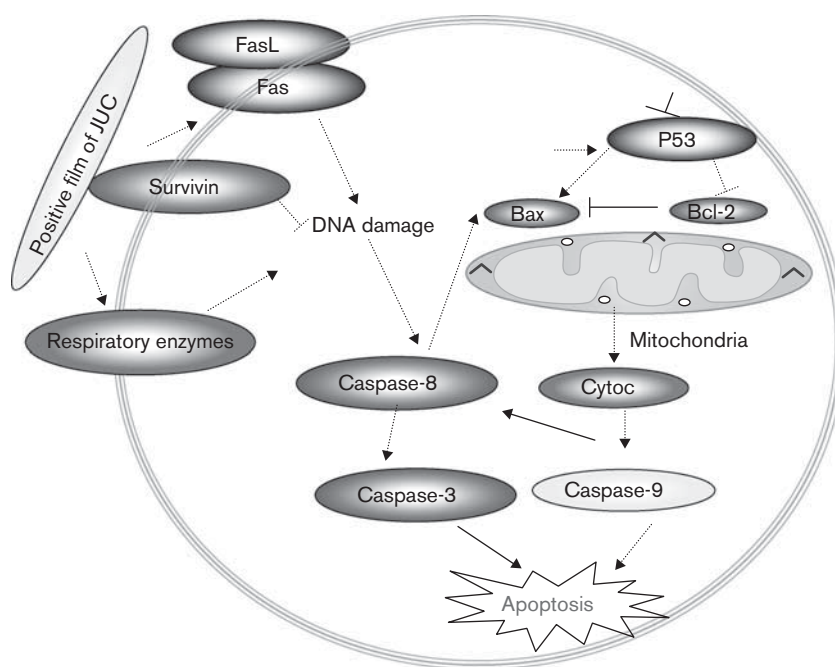
In in-vivo experiments, the tumor metastasis model demonstrated that Jieyoushen can inhibit cell proliferation and induce cell apoptosis significantly. Owing to surgery-related injury and postoperative infection, there

Fig. 10



Intraperitoneal tumor formation in the control group (a) and experimental group (b). Hematoxylin and eosin staining of sections of tumors of both the groups show no pathological morphology difference (microscopic observation, 10 × 10).

Fig. 11



The mitochondrial apoptosis pathway. JUC, Jieyoushen.

Table 3 The influence of Jieyoushen in implanting metastatic tumor formation model of rat

	Control group	Jieyoushen treatment group
Number of rats	18	17
Tumor burdened rats	12 (66.7)	5 (29.4)

were three dead rats in the treatment group and two in the control group, which accounts for about 12.5% of the total number of rats. It can be seen in Table 3 that tumor was formed in 12 rats in the control group, and the incidence rate was about 66.7%. In contrast, tumor was detected in five rats in the Jieyoushen treatment group

and the incidence rate was about 29.4%. Moreover, the incidence rate differed significantly between the treatment and control groups ($P < 0.05$). It can also be seen from Fig. 10 that there was no obvious difference in the tumor pathological morphology between the experimental group and the control group.

Discussion

HCC is generally diagnosed when patients have already reached an advanced stage of the disease and it is not eligible for curative therapies. Potentially curative treatment options include surgical resection or liver

transplantation, which can offer patients adequate liver function and tumor treatment (Muller, 2006). Implantation metastasis is difficult to avoid during operation, which leads to the relapse of liver cancer. In addition, in many cases, implantation metastases are a predominant cause of morbidity and mortality. Because of the high incidence of implantation metastases, regional strategies directed toward the liver have been developed in an attempt to improve patient survival. The individuals not eligible for curative therapy are generally treated with local ablative methods such as radiofrequency ablation or alcohol ablation, and systemic chemotherapy can be offered either alone or in combination to selected groups of patients. However, HCC is not sensitive to chemotherapy, and the development of side effects and drug resistance is the biggest challenge for chemotherapy. Multiple factors are implicated in increasing resistance to chemotherapeutic agents, including reduction of intracellular drug accumulation, DNA damage repair by the modulation of proliferative or antiapoptotic proteins, etc. (Siddik, 2003). These causes eventually lead to an unsatisfactory clinical therapeutic effect. Therefore, more and more research is being carried out to solve this difficult problem. According to the characteristics of each drug, the researchers have adopted the synergistic effect of several drugs to treat diseases. A combination of drugs is recommended to optimize the treatment schedules, in order to achieve the best form of therapy and/or minimize side effects (Eichhorn *et al.*, 2004; Johnston *et al.*, 2007).

In animal models, Jieyoushen has been proved to be very successful in inhibiting tumor development. Therefore, Jieyoushen can be a useful adjunct for improving the effectiveness of chemotherapeutic agents in the treatment of liver cancer. 5-FU and its derivatives, as a classic chemotherapy drug, can cause cell injury by inhibiting thymidylate synthesis. It has become a mainstay of treatment for advanced-stage HCC. However, the evolution of HCC chemotherapy was delayed by the resistance to 5-FU and side effects (Yau *et al.*, 2008; Yang *et al.*, 2012). The required dose must be decreased and the host toxicity must be minimized to improve the effects of 5-FU. In our present study, compared with either agent alone, the combination of Jieyoushen with 5-FU showed a better inhibitory effect. The possible mechanism is that Jieyoushen had been sprayed on the surface of tissues, which formed an invisible protective film, which can prevent the adhesion of the tumor cell to the treated tissue surface. The mechanism is different from that of other general antitumor drugs. Jieyoushen not only has a chemical–drug-directed antitumor effect, but can also form a layer of protective film on the tissue surface, which can prevent cancer cells from growing in organization.

In principle, survival signals are ideal targets for anti-cancer therapeutic strategies because blocking these signals leads to the death of cells that are dependent on them. Our experiment showed that the combination treatment of Jieyoushen with 5-FU significantly inhibited

HCC growth. The apoptosis-related protein in the mitochondrial pathway that may cause the cascade effect of additional functional proteins may be involved in this inhibition. In this study, we observed that the induction of apoptosis by the combination of Jieyoushen and 5-FU seems to be mediated through the modulation of protein levels of the *Bcl-2* family, such as *Bcl-2* and *Bax*. Moreover, *p53* plays important roles in Jieyoushen and 5-FU-induced apoptosis in HCC cells.

It is well known that the interplay between members of the *Bcl-2* protein family can regulate the mitochondrial (or intrinsic) apoptosis pathway (Lei *et al.*, 2012). Following apoptotic stimuli, cytochrome *c* is released into the cytosol from the mitochondria as a result of mitochondrial outer membrane permeabilization, which is followed by the formation of apoptotic bodies and caspase activation (Danial and Korsmeyer, 2004). The key, essentially irreversible, step in the commitment to apoptotic death is the permeabilization of the mitochondrial outer membrane. *Bcl-2* is an upstream effector molecule in the apoptotic pathway and has been identified as a potent suppressor of apoptosis (Hockenbery *et al.*, 1993).

We found that the combination of Jieyoushen and 5-FU significantly downregulated *Bcl-2* protein and upregulated levels of *Bax* protein in SMMC7721 and R15 cells. These suggest an involvement of an intrinsic apoptotic pathway by which alantolactone induces apoptosis in SMMC7721 and R15 cells. However, western blot analysis revealed that Jieyoushen, in combination with 5-FU, led to enhanced processing of initiator *caspase-8* in SMMC7721 and R15 cells, and these data suggest a possibility of a cross-talk between the two pathways, as further activated *caspase-8* can activate *caspase-3*. Therefore, effective inhibition of *caspase-3* and *caspase-9* could be critical in providing a targeted pathway for cancer prevention and treatment. Finally, the *caspase-3* protein executed the instruction of apoptosis, leading to cell death. We observed that Jieyoushen, in combination with 5-FU, led to a dose-dependent decrease in the protein level in SMMC7721 and R15 cells, which may downregulate the expression of antiapoptotic target genes such as *Bcl-2*, from which we can conclude that these may favor the apoptosis of SMMC7721 and R15 cells. *p53* is a transcription factor that regulates cell cycle progression and DNA repair, and we infer that this may favor apoptosis of SMMC7721 and R15 cells. This multitasking is important for the suppression of tumor formation, as well as for mediating the cellular responses to many standard DNA damage-inducing cancer therapies (Vazquez *et al.*, 2008). When the combination of Jieyoushen and 5-FU was used, the DNA damage and cell cycle change were first caused by an internal mechanism.

Our result indicates that the combination of Jieyoushen and 5-FU significantly increased the expression of *p53* in SMMC7721 and R15 cells, which may lead to cell cycle arrest and upregulation of *Bax* expression (Rocha and Perkins, 2005). These show that *p53* is an independent factor in the

combination of Jiyoushen with 5-FU that induced apoptosis. Meanwhile, the nano-positively charged layer damaged the cell membrane. Further, the drugs damaged the cell directly, and then caspase-8 protein was activated and influenced the proteins of the mitochondrial membrane, such as Bax, Bcl-2, and so on. These led to the apoptosis of the classic mitochondrial pathway. This mechanism is shown in Fig. 11.

In summary, the combination of Jiyoushen with 5-FU could inhibit the proliferation of liver carcinoma cell lines and prompt cell apoptosis, which originated from the shielding effect of the positive film. This physical film can render cell respiration enzymes anaerobic and induce cell death (He *et al.*, 2012). Finally, the cell membrane signal ended. The ability of the combination of Jiyoushen with 5-FU to inhibit cell proliferation and induce apoptosis is caused by a multipronged cascade of events. In future studies, the complex coupling reaction that occurred between the positive membrane and the membrane surface will be further discussed. As both agents have been widely used in clinical practice, intraoperative cases of metastasis are necessary to identify the curative effect of combined treatment. We will develop this combination as a potential new strategy and use it as an adjunct to infusion chemotherapy in the future.

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Conflicts of interest

There are no conflicts of interest.

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有机聚硅氧烷季铵盐和5-氟尿嘧啶对体外和体内 肝细胞癌的协同作用

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肝细胞癌 (HCC) 是全世界第三大最常见的癌症类型, 造成每年超过37万人死亡, 其中约有半数是中国。化疗是治疗晚期肝细胞癌患者的最佳治疗方法, 虽然化疗耐药已成为肝癌手术成功的显著障碍。在本文中, 我们已经评估药物的特点, 以探索有机硅季铵盐(洁悠神)和5-氟尿嘧啶 (5-FU) 的单一作用和联合作用的影响。MTT测定的结果表明, 洁悠神和5-FU的单一作用和联合作用能分别以剂量依赖性和时间依赖性的方式抑制肝癌细胞系的增殖。电子显微镜和Hoechst 33342染色显示凋亡细胞中有特点的凋亡小体用洁悠神和5-FU治疗。流式细胞仪检测结果表明, 细胞在G0/G1期的比例逐渐增加, 而治疗后在S期逐渐下降。综合考虑, 这些结果表明, 洁悠神与5-FU联合应用对肝细胞癌生长具有协同抗癌作用, 且目标治疗方法可以提高肝细胞癌对化疗的敏感性。

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引言

肝细胞癌 (HCC) 是肝癌的主要形式。HCC的发病率在全世界已经显著增加 (Chen等人, 2010年; Jemal等人, 2011年; Jiao等人, 2012年; Chen等人, 2009年)。仅在中国, 超过40.1万例新患者被诊断有肝癌, 2008年有超过37.1万例患者死于这种疾病 (Ferlay等人, 2010年)。手术治疗被认为是肝癌最好的选择 (Takayama等人, 2010年); 但是, 仅一小部分患者在诊断时适合接受根治性切除术, 因为HCC很少在早期阶段出现特征性症状, 且超过80%的患者在确诊有肝癌的时候已经失去了肝切除术治疗的机会 (Jia等人, 2012年; Yang等人, 2012年)。对于晚期肝癌的管理, 全身化疗与传统的细胞毒性药物为患者带来了很小的生存益处 (Thomas等人, 2008年; Wang等人, 2012年)。与放疗和手术等局部治疗相比, 化疗是全身性治疗的一种形式, 它可能达到肿瘤细胞扩散的地方 (Ramalingam和Belani, 2008年)。目前, 100多种药物正在用于化疗, 或单独使用或联合使用。然而, HCC对抗癌药物有耐药性 (Asghar和Meyer, 2012年; Jia等人, 2012年)。传统全身化疗的治愈率较低且会导致许多肝癌毒副作用, 因此, 没有被临床操作者广泛接受。近日, 新型化疗药物和新型联合治疗方案的应用已被证明能有效阻止晚期肝细胞癌 (Wan等人, 2009年; Ca等人, 2011年; Xie等人, 2012年)。此外, 理想的治疗方法是探索晚期HCC联合化疗的替代方法。

5-氟尿嘧啶 (5-FU) 是治疗 HCC 的最佳药物之一。胸苷酸合成酶抑制和含氟核苷错误掺入 RNA 和 DNA 一般被认为是对抗癌症的主要有效机制 (Llovet Josep, 2002年; Lopez 等人, 2006年; Mukai 等人, 2006年; Xu 等人, 2007年; Bu 等人, 2008年)。然而, 对 5-FU 获得性耐药的快速发展限制了它的临床应用 (Yoo 等人, 2009年), 其潜在分子机制仍然

不确定。为了提高 5-FU 的临床使用, 已经研究大量联合用药方式 (Shang 等人, 2007 年; Yang 等人, 2008 年; Morabito 等人, 2009 年)。上述实验和临床研究的结果表明, 有些药物不仅能有效对抗各种癌症, 但还可以提高 5-FU 的显著疗效, 和大幅度地降低毒性。需要在联合用药机制中进一步确定哪一种药物在抗癌过程中起到主导作用。

洁悠神是一种聚合物表面活性剂, 其主要成分是季铵盐。现在它主要用作术后物理抗菌材料 (Liu 等人, 2010 年)。当水溶性液体洁悠神喷洒在皮肤表面或粘膜区域时, 它立即固化, 形成一个具有双层重叠结构的透明抗菌层: 胶联膜和正电荷膜。胶联膜由大分子试剂构成, 它通过化学方式牢固地连接体表, 对防止微生物生长起到长效作用。正电荷膜由使用皮肤表面或粘膜区域正电荷形成网状膜的阳离子活化剂构成。正电荷膜强烈吸附具有负电荷的病原微生物, 如细菌, 真菌和病毒。如果病原微生物赖以生存的呼吸酶失去作用, 他们将死于缺氧 (Li 等人, 2011 年)。然而, 文献中并没有发表洁悠神属于抗肿瘤剂的内容。肿瘤转移预防的机制尚未确定。在我们以前的研究中, 洁悠神的体内抗癌活性在大鼠腹腔内肿瘤模型中测试, 其结果表明, 洁悠神能防止大鼠腹腔内肿瘤扩散。此外, 治疗组和对照组之间的发病率有显著差异 ($P < 0.05$)。因此, 可以得出结论, 洁悠神可以在单独用药或与其它化疗药物联合用药时用于防止植入肿瘤转移, 为患者减少化疗所需的剂量并最大限度地减少毒性。

在这项研究中, 探讨洁悠神与 5-FU 联合用药对人 HCC SMMC7721 细胞株和大鼠 R15 细胞系的协同作用和机制。对这些药物在胱氨酰酶-3、胱氨酰酶-8、Bax、Bcl-2 和存活蛋白的生长抑制、细胞周期分布、细胞凋亡和表达中的协同、相加、或拮抗作用进行仔细评估, 以了解两种药物的联合作用并发现可能的作用机制。我们希望这些结果将为预防肝细胞癌手术转移带来新的治疗手段。

材料和方法

材料

洁悠神由南京神奇科技开发有限公司 (江苏

省, 中国) 提供。5-FU (0.25g/10ml) 购自于天津金耀氨基酸有限公司 (天津, 中国)。抗 Bax、抗 Bcl-2、抗胱氨酰酶-8、抗胱氨酰酶-3、抗存活素和抗 β -肌动蛋白等一抗购自于 Bioworld 科技公司 (Louis Park, 明尼苏达州, 美国)。膜联蛋白 V-FITC/碘化丙啶 (PI) 的细胞凋亡检测试剂盒购自于 Invitrogen 科技公司 (卡尔斯巴德, 加利福尼亚州, 美国)。在体内实验中使用 6-8 周龄雄性 Wistar 大鼠 (兰州大学实验动物中心, 兰州, 中国)。还使用一只笼子、几个手术器具、10% 水合氯醛 (0.35ml/100g 重量)、DMEM 高糖培养基、和胎牛血清。

细胞培养

人肝癌细胞系 (SMMC7721)、肝细胞系 (LO2) 和大鼠肝细胞系 (R15) 购自于美国模式培养物集存库 (ATCC, 马纳萨斯, 弗吉尼亚州, 美国)。将其在 RPMI 1640 培养基 (Gibco, Grand Island, 纽约州, 美国), 辅以 10% 热灭活胎牛血清、50U/ml 青霉素、和 50 μ g/ml 链霉素、15mmol/l (pH 7.2) HEPES、和 2 mmol/l 的 L-谷氨酰胺中 37 $^{\circ}$ C 下含 5% CO₂ 的潮湿培养箱中培养。各治疗组按照 6-48 小时的不同浓度进行分组。

生长抑制试验

肝癌细胞以 5×10^3 细胞/孔一式五份的密度接种于 96 孔板, 并将 200 μ l 洁悠神 (1/1000, V%), 10 μ mol 的 5-FU 和两者合并使用 (1:1, V/V) 的溶液以最终浓度加入每个孔。细胞培养 0、6、12、24、和 48 小时。在无血清培养基组被设定为底部对照组, 同时以 5×10^3 细胞/孔的密度为阳性对照组接种。同时, 肝细胞 LO2 细胞作为阴性对照组。治疗后, 将 50 μ g/10ml 的 MTT (Sigma 公司, 圣克拉拉, 加利福尼亚, 美国) 混合到这些孔中, 并将细胞再培养 4 小时, 此后, 先清除上清液, 再加入 150ml 的 DMSO (Sigma 公司) 至每个孔。摇晃平板 15 分钟后, 通过使用 680 型酶标仪 (Bio-Rad 公司, Hercules, 加利福尼亚州, 美国) 在 490nm 波长下读取吸光度, 测定细胞存活率。根据阳性对照的百分比计算生存率。实验重复 3 次。

透射电子显微镜法观察

采用透射电子显微镜法检测形态学变化。简言之, 采用洁悠神 (1/1000, V%)、5-FU (10 μ mol)、和联合用药 (1: 1, V/V) 处理人肝癌细胞系 SMMC7721 和大鼠肝癌细胞株 R15 共 24 小时。用不同浓度的药物介入后, 细胞用 0.25% 胰酶消化并以 1000rpm 离心 5 分钟, 随后用 PBS 反复清洗, 除去上清液, 并用 2.5% 预冷的戊二醛在 4 $^{\circ}$ C 下整夜固定。制备超薄铜切片和用 PBS 冲洗细胞一次, 然后用 1% 锇酸固定 1 小时。再将样品用丙酮脱水, 并包埋在环氧树脂中。用乙酸双氧铀和柠檬酸铅染色后, 在透射电子显微镜下观察切片。

Hoechst 染色

肝癌细胞 (4 \times 10⁴/孔) 在 RPMI 1640 的 24 孔板中培养, 辅以 10% 胎牛血清。在同一时间, 制备两份培养板。采用洁悠神 (1/1000, V%)、5-FU (10 μ mol)、和联合用药 (1: 1, V/V) 处理人肝癌细胞系 SMMC7721 和大鼠肝癌细胞株 R15 共 24 小时。介入后, 将细胞用冷冻 PBS (pH7.2) 中洗涤两次。然后将其用 4% 预冷的中性多聚甲醛在 4 $^{\circ}$ C 下整夜固定。此后, 将细胞用 PBS 洗涤并在室温下用 0.5ml 的 Hoechst 33342 (Sigma 公司) 染色 8 分钟, 随后再用 PBS 洗涤。以 350nm 的激发波长和 460nm 的发光波长在荧光显微镜下分别观察形态变化 (奥林巴斯 X71 型; 奥林巴斯公司, 日本, 东京)。

细胞周期和细胞凋亡的流式细胞术分析

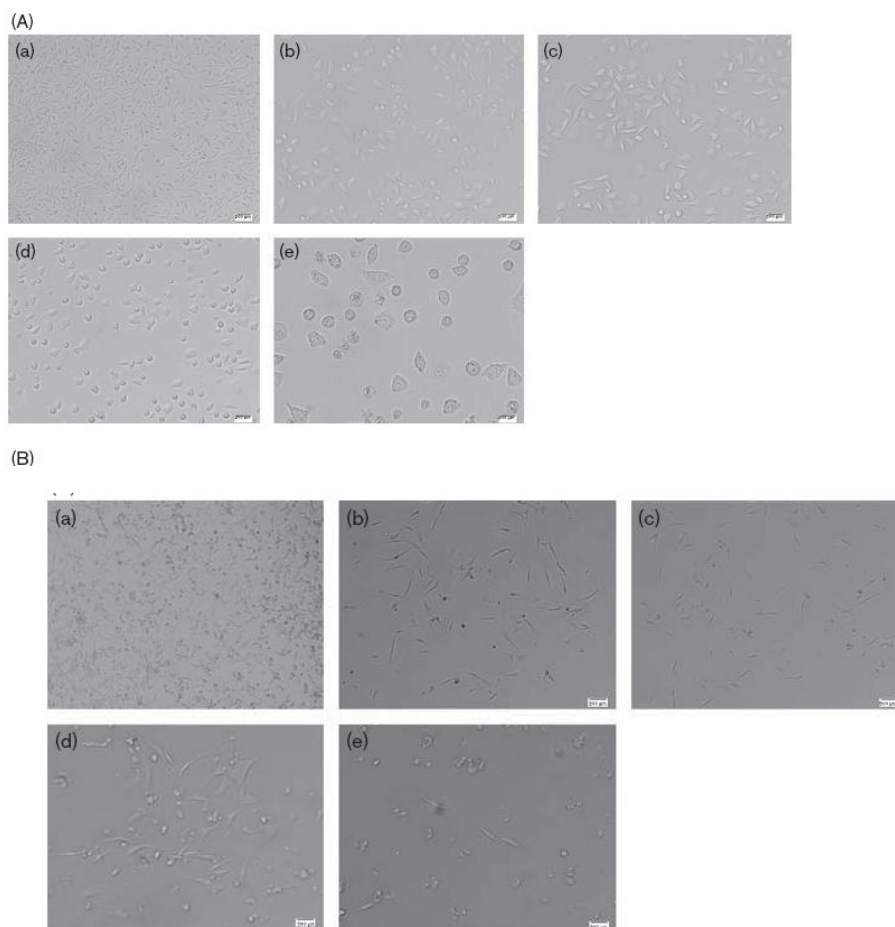
细胞在 75-cm² 培养瓶中接种。当细胞达到 80% 融合度的时候, 将原有培养基换成新鲜培养基, 然后将细胞暴露于洁悠神 (1/1000, V%)、5-FU (10 μ mol)、和联合用药 (1: 1, V/V) 共 24 小时。再将其用 PBS 洗涤, 固定

于冰冻的 70% 乙醇中, 并在 -20 $^{\circ}$ C 下保存备用。分析前, 将细胞洗涤并悬浮于 5 \times 10⁶ 细胞/ml 浓度的 PBS 中和用 0.1 mg/ml 的 RNA 酶 A 和 40 mg/ml 的 PI 在 37 $^{\circ}$ C 下培养 20 分钟。然后使用 FACS Calibur 细胞分选系统 (BD FACSCalibur; BD Bioscience 公司, 富兰克林, 新泽西州, 美国) 分析样品。

Bax、Bcl-2、胱氨肽酶-8、胱氨肽酶-3、和存活蛋白表达的 Western blot 分析

将细胞接种和融合至 80% 融合度, 并使用与 MTT 实验相同药物处理。培养基用离心法收集, 细胞单层用 PBS 洗涤。蛋白浸提使用 RIPA (碧云天生物技术研究所, 海门市, 江苏省, 中国)。收集细胞浸提液并在 41 $^{\circ}$ C 下 14000 g 微量离心 20 分钟。收集上清液, 并采用 Bradford 方法测定蛋白质浓度。细胞浸提液被立即使用或保存在 -80 $^{\circ}$ C 下。采取措施防止蛋白质降解后, 将其在变性缓冲液中 100 $^{\circ}$ C 下煮沸 7 分钟。进行十二烷基硫酸钠聚丙烯酰胺凝胶电泳 (SDS-PAGE), 将各浸提液中的 60 mg 蛋白质上样到各孔的凝胶中。在 80 和 100V 下进行凝胶电泳用于蛋白质浓缩, 蛋白质在冰浴中 200mA 下转移到聚偏氟乙烯膜上共 1-2 小时。使用预染分子量标记物 (SM0671; Fermentas 公司, 伯灵顿, 安大略省, 加拿大) 测定蛋白质条带。膜用含 5% (w/v) 无脂奶粉的 TBST 缓冲液 (50mmol/l 的 Tris-HCl, pH 值 7.4, 150mmol/l 的 NaCl, 0.1% 吐温 20) 在室温下阻断 2 小时, 用一抗在 4 $^{\circ}$ C 下整夜培养。然后将膜用 TBST 洗涤, 并用具有种特异性的 HRP-缀合的二抗在室温下培养 2 小时。再次洗涤膜, 并根据制造商的方案使用化学发光试剂 ECL (Minipore 公司, 达姆施塔特, 德国) 观察免疫反应性蛋白。使用 Image-Pro plus 6.0 软件 (Media Cybernetics 公司, 贝塞斯达, 马里兰州, 美国) 进行密度分析, 检测蛋白表达的差异。该实验至少重复两次, 以确认结果。

Fig. 1



当用1/1000的洁悠神和10 mmol 5-氟尿嘧啶共同处理SMC7721 (A)和d R15 (B)细胞时，6, 12, 24,和48小时的形态变化。(a) 对照; (b) 6小时; (c)12小时; (d) 24小时; (e) 48小时。

动物实验

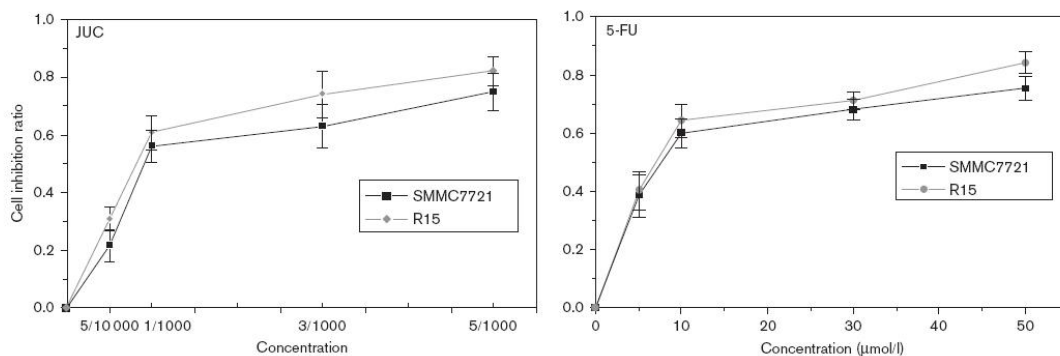
40只雄性Wistar大鼠，体重150 g，由随机数表法随机分为对照组和实验组。R15细胞达到80%或更高的融合时进行收集，并用台盼蓝染色对细胞活力进行了测试，以确保细胞活力为95%以上。用生理盐水将细胞浓度稀释到 $6 \times 10^6 / \text{ml}$ 。用10%水合氯醛将大鼠麻醉，然后固定在手术台上。我们在腹部中间剑突和耻骨联合之间的部分做一个3厘米的切口，然后用腹腔牵引机分离腹膜。在治疗组中，喷洒两次洁悠神（2毫升）到腹腔粘膜中，然后1毫升细胞悬浮液被吸收和注入腹腔。在对照组中，1毫升细胞悬浮液被直接吸收和注入腹腔。治

疗后关闭腹腔。5周后用致死剂量的麻醉剂将小鼠杀死。我们在旧的伤口旁边再次打开腹腔。观察到形成了植入的转移性肿瘤，记录形成转移性肿瘤大鼠的数量。通过测量肿瘤的重量，同时记录肿瘤的生长情况。两个肿瘤组织用苏木精和曙红染色，观察病理特征。

统计分析

采用SPSS 15.0软件（SPSS公司，美国伊利诺伊州芝加哥）对结果进行了分析，并以均数±标准差进行表达。用单向方差和Tukey的事后检验分析来测定两组之间的差异，如P值小于0.05，则被认为有显著统计学意义。

Fig. 2



洁悠神 (JUC) 和5-氟尿嘧啶 (5-FU) 对SMMC7721和R15细胞的抑制作用。用不同浓度的洁悠神和5-FU处理SMMC7721和R15细胞24小时。可观察到随剂量增加而增强的癌细胞生长的抑制作用 ($P < 0.05$, Tukey事后分析)。细胞培养后, 通过MTT检验确定细胞增殖。

结果

光镜下形态学观察

从图1中可以看出。该细胞从原始上皮样或多边形的形状转变为圆形, 细胞膜已经萎缩, 许多晶核浓缩, 细胞的折射率有所减少或已经出现了含有部分多泡体和致密颗粒的胞质 (图1)。

细胞存活实验

MTT assay的结果表明, 洁悠神和5-FU可在24小时作用后 (图2) 抑制SMMC7721和R15细胞的增殖。此外, 所观察到的抑制作用是随着剂量的增加而增强的。在用5/10 000, 1/1000, 3/ 1000, 5/1000的洁悠神 (稀释率) 处理

SMMC7721和R15细胞24小时后, SMMC7721的细胞抑制率分别为 21.68 ± 5.77 , 56.03 ± 5.43 , 62.83 ± 7.64 和 $74.73 \pm 6.21\%$ 。类似地, R15的细胞抑制率分别为 30.95 ± 4.21 , 60.81 ± 5.90 , 74.01 ± 7.92 和 $82.14 \pm 4.91\%$ 。在用5, 10, 30和50mmol的5-FU处理SMMC7721和R15细胞24小时后, SMMC7721的细胞抑制率分别为 38.48 ± 7.14 , 59.92 ± 5.17 , 68.13 ± 3.53 和 $75.27 \pm 4.26\%$ 。同样, R15的细胞抑制率分别为 40.18 ± 6.34 , 64.33 ± 5.47 , 71.24 ± 2.93 和 $84.12 \pm 3.91\%$ 。考虑到全面的对比效果, 选择1/1000洁悠神和10mmol 5-FU来调查在抑制HCC细胞株的生长方面洁悠神和5-FU联合使用的效果。

Fig. 3

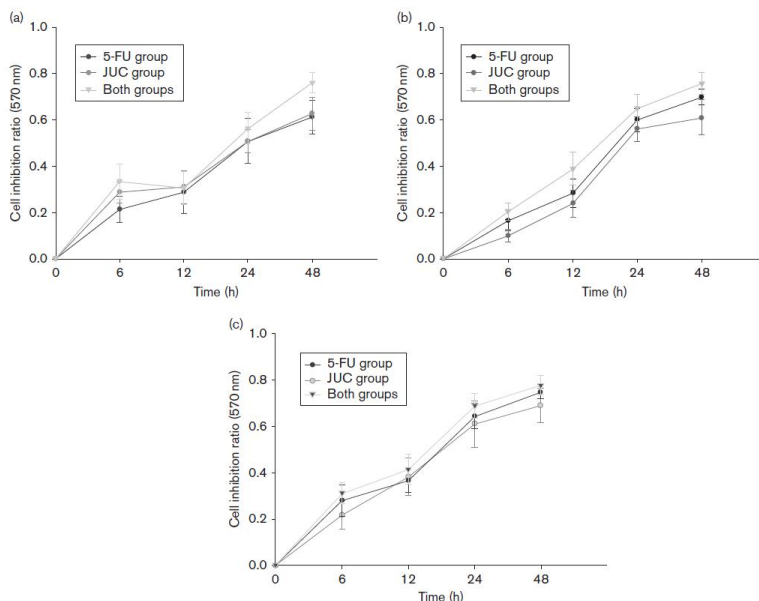


图3 药物对SMMC7721(b)和R15(c)肝癌细胞以及干细胞系LO2(a)的细胞增殖抑制作用。细胞经1/1000洁悠神(JUC)、10 μ mol5-氟尿嘧啶(5-FU)以及联合处理6、12、24和48小时。可以观察到对肝癌细胞生长的时间依赖性抑制(P<0.05, Tukey事后分析)。细胞培养后, 用MTT分析检测细胞增殖。

如图3所示, 与单独使用5-FU或洁悠神的结果比较, 当使用5-FU和洁悠神处理时, 在联合组观察到显著降低的细胞活力。仅用5-FU (10mmol/l) 处理的SMMC7721和R15细胞在24小时后细胞抑制率分别为59.92 \pm 5.17和64.33 \pm 5.47%。仅用1/1000的洁悠神原液处理的SMMC7721和R15细胞在24小时后细胞抑制率分别为56.03 \pm 5.43和60.81 \pm 5.90%。与洁悠神相比, 5-FU在减少细胞生长方面更为有效。我们用1/1000的洁悠神原液处理5-FU 24

小时后, 在两种细胞株中, 5-FU的抗肿瘤活性显著地随着时间的增加而增强。值得注意的是, 用1/1000的洁悠神原液加上10 mmol / L的5-FU处理的SMMC7721和R15细胞的细胞抑制率分别增加至65.05 \pm 5.87和68.67 \pm 5.29%。这些数据表明, 洁悠神和5-FU的联合共同抑制了HCC细胞株的细胞生长。

关于超微结构变化及细胞凋亡分析的透射电子显微镜检查

Fig. 4

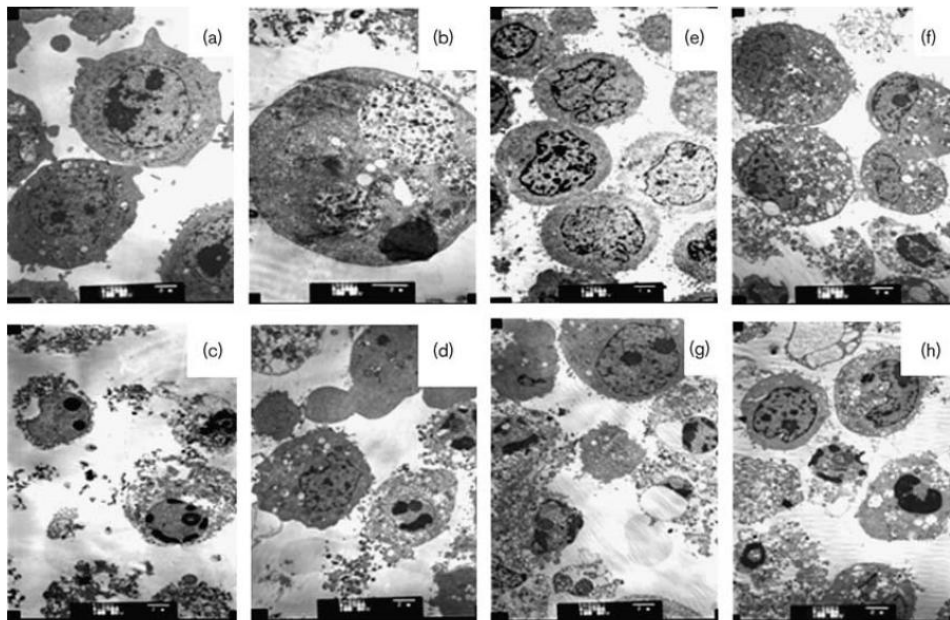


图4 经透射电子显微镜观察药物24小时后对SMMC7721和R15细胞超微结构的作用, 包括空白对照组(a,e)、10 μ mol5-氟尿嘧啶(5-FU)(b, f)、1/1000 洁悠神(c, g)以及联合组(d, h); 右图显示核固缩和染色质凝集(\times 2500)、细胞凋亡(\times 2500)。

从图4中可以看出, SMMC7721和R15细胞的细胞核内异染色质增加, 细胞质内染色质边集、染色质丛收缩、细胞膜不完整, 凋亡细胞、坏死细胞、或细胞质内自噬泡的数量不断增加。除了上述超微结构方面的变化, R15细胞显示大量的自噬泡和凋亡细胞, 更多的溶解和坏死细胞。这一证据表明, 5-FU和洁悠神的共同作用可抑制SMMC7721和R15细胞的增殖。

Hoechst染色

用Hoechst33342染色法观察凋亡细胞的形态。结果表明对照组显示出常规细胞核形态、均匀染色和淡蓝色。在共同作用24小时后, 与对照组比较, 细胞形态减退, 核凝缩或碎裂块形成特色凋亡体(图5)。

Fig. 5

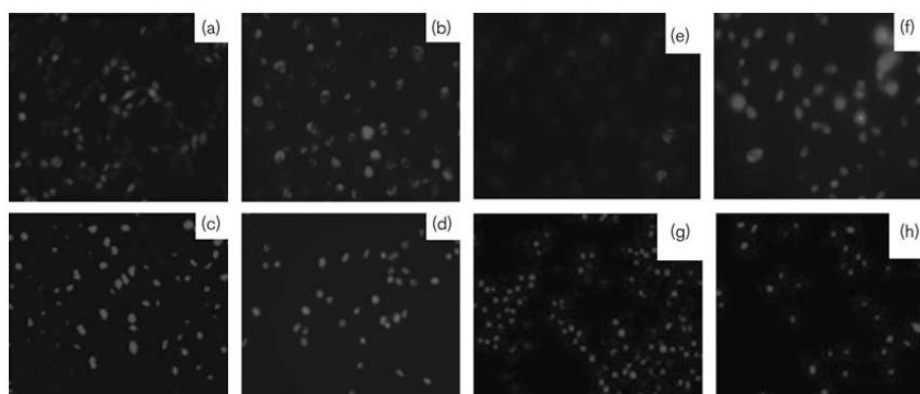


图 5 SMMC7721 和 R15 细胞由药物处理 24 小时,用 Hoechst 33258 染色,然后用荧光显微镜($\times 20$)分析形态学变化和凋亡细胞核形成的数量。(a) SMMC7721 对照; (b) SMMC7721 10 μmol 5-氟尿嘧啶(5-FU); (c) SMMC7721 1/1000 洁悠神; (d) SMMC7721 1/1000 洁悠神 + 10 μmol 5-FU; (e) R15 对照; (f) R15 10 μmol 5-FU; (g) R15 1/1000 洁悠神; (h) R15 1/1000 洁悠神 + 10 μmol 5-FU。

流式细胞术检测细胞周期和凋亡

细胞的周期分析

细胞周期的结果表明,如下所述,在两种细胞株在细胞周期中经历了相同的变化。

细胞周期分析显示,当SMMC7721细胞用10 mmol 5-FU处理24小时后,与对照组相比较,细胞在G0/G1阶段的百分比显著增加 ($P < 0.05$)。然而,当用1/1000洁悠神单独

处理24小时后,细胞周期分布没有显著变化。当联合使用这两种制剂时,发生了最显著的变化。与单独使用5-FU或洁悠神 ($P < 0.05$) 处理的细胞相比(图6a),细胞大多聚集在G0/G1阶段,数量在S阶段显著减少。当用1/1000洁悠神、10 mmol 5-FU,或共同使用24小时(图6b)处理R15细胞时,可以发现类似的调节作用。因此,可以得出结论:洁悠神和5-FU的结合对处理SMMC7721和R15细胞具有协同作用(表1和2)。

Fig. 6

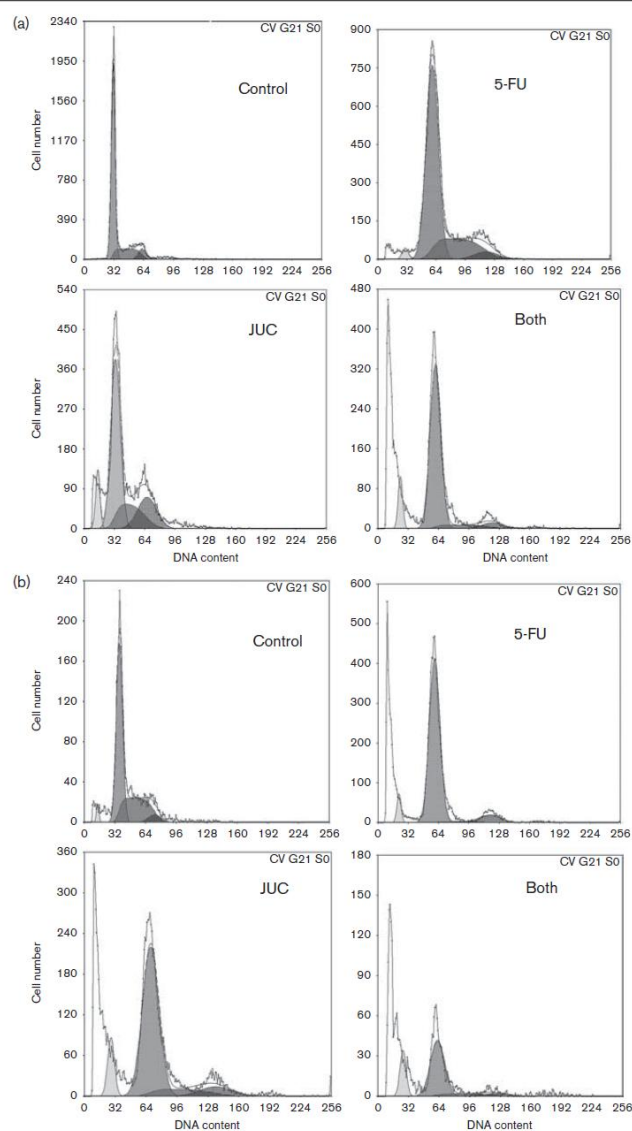


图6 流式细胞仪分析 SMMC7721(a)和 R15(b)细胞经 1/1000 洁悠神(JUC)、10 μ mol5-氟尿嘧啶(5-FU) 以及联合处理 24 小时后的细胞周期。

表 1 用 1/1000 洁悠神、10 μ mol5-氟尿嘧啶或联合处理 SMMC7721 细胞 24 小时后的细胞周期分布

组别	剂量	G0/G1 (%)	S (%)	G2/M (%)
对照组	—	54.88 \pm 6.34	26.159 \pm 5.63	18.961 \pm 7.70
5-FU 组	10 μ mol	69.016 \pm 9.25▲	25.609 \pm 8.17	5.375 \pm 12.38▲
洁悠神组	1/1000	57.301 \pm 10.73	21.784 \pm 3.41	20.914 \pm 3.54
联合治疗组	10 μ mol+1/1000	85.774 \pm 4.85*	8.080 \pm 4.39*	6.145 \pm 1.26*

5-FU, 5-氟尿嘧啶

*表示联合治疗组和对照组的差异有统计学意义(P<0.05).

▲表示 5-FU 组和对照组的差异有统计学意义(P<0.05)

表 2 用 1/1000 洁悠神、10 μ mol5-氟尿嘧啶或联合处理 R15 细胞 24 小时后的细胞周期分布

组别	剂量	G0/G1 (%)	S (%)	G2/M (%)
对照组	—	57.614 \pm 5.73	30.928 \pm 8.71	11.457 \pm 6.32
5-FU 组	10 μ mol	90.023 \pm 11.24▲	7.784 \pm 13.44▲	2.193 \pm 1.31▲
洁悠神组	1/1000	78.679 \pm 9.82◆	11.512 \pm 5.45◆	9.809 \pm 3.03
联合治疗组	10 μ mol+1/1000	80.523 \pm 6.29*	7.480 \pm 4.13*	11.997 \pm 2.89

5-FU, 5-氟尿嘧啶

*表示联合治疗组和对照组的差异有统计学意义(P<0.05).

▲表示 5-FU 组和对照组的差异有统计学意义(P<0.05)

◆表示洁悠神组和对照组的差异有统计学意义(P<0.05)

在两种细胞中检测到细胞凋亡的发生

联合用药组中的洁悠神与5-FU不仅能引起人肝癌细胞凋亡,还可以使其衰老。在流式细胞仪中,我们使用PI和膜联蛋白V-FITC。在用5-FU(10 mmol)或同时使用5-FU和1/1000洁悠神处理两种细胞24小时后,人们发现,PI-阴性和膜联蛋白V-阳性细胞的数量增加,且PI-阳性和膜联蛋白V-阳性细胞的数量也增加

(从5-FU治疗的26.9 \pm 0.6%到洁悠神联合5-FU治疗的35.9 \pm 0.7%)。本实验重复3次(P<0.05,单因素方差分析)。确定样本中凋亡活细胞和凋亡死细胞的数量(彼得拉等人,2001)。结果表明,洁悠神和5-FU的联合治疗可引发协同作用,从而可以提高凋亡细胞在人肝癌细胞中的百分比(图7)。

Fig. 7

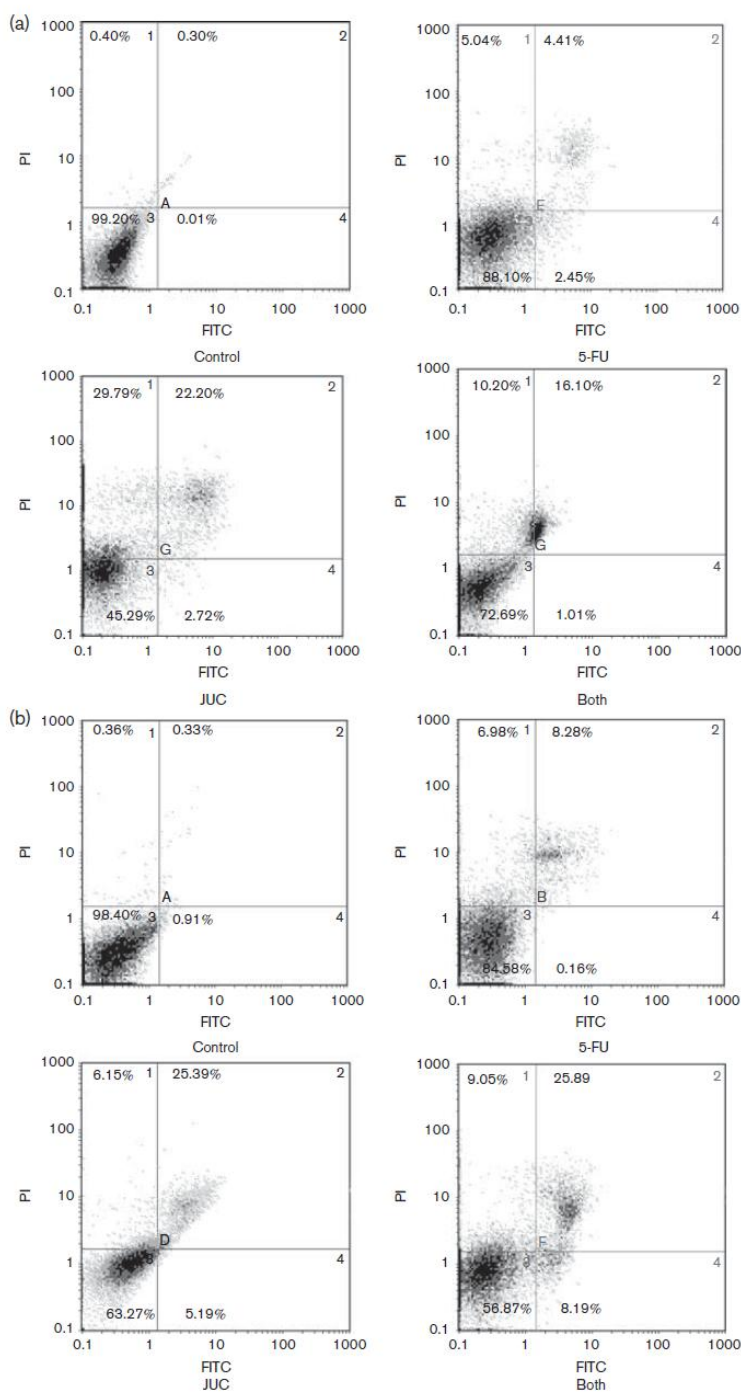


图 7 SMMC7721(a)和 R15(b)细胞经 1/1000 洁悠神(JUC)、10 μ mol5-氟尿嘧啶(5-FU)以及联合处理 24 小时后的细胞凋亡。用流式细胞仪测定凋亡细胞百分比。annexin V 阳性细胞被定义为凋亡细胞，包括存活的凋亡细胞（annexin V 阳性、PI 阴性）和不能存活的凋亡细胞（annexin V/PI-阳性）。

相关蛋白水平的线粒体途径改变的分析

与对照组相比，联合治疗组在第24小时可显著

增加SMMC7721和R15细胞的Bax、caspase-8和caspase-3的蛋白水平，并显著降低Bcl-2蛋

白的表达。然而，与对照组相比，存活蛋白的含量显著下降。另外，蛋白质表达随着时间的

变化而变化的关系被发现（图8和图9）。相关的转导机制见图11。

Fig. 8

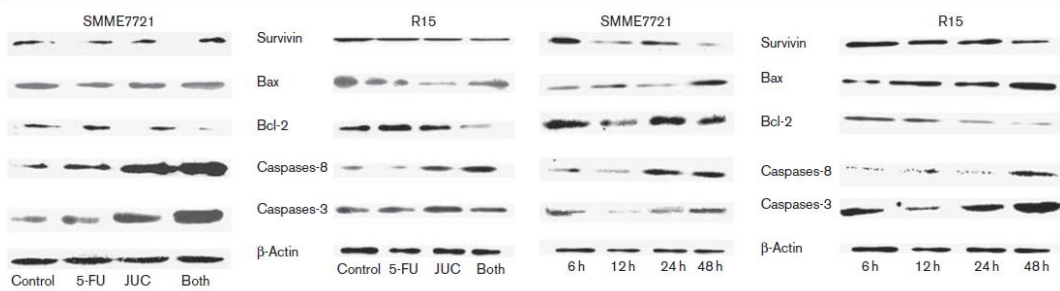


图 8 SMME7721(a)和 R15(b)细胞经 1/1000 洁悠神(JUC)、10 μ mol5-氟尿嘧啶(5-FU)以及联合处理 24 小时、以及 1/1000 洁悠神和 10 mmol 5-FU 混合物处理 6、12、24、48 小时后，用免疫印迹法检测胱天蛋白酶-3 和胱天蛋白酶-8、Bax, Bcl-2 以及 survivin 蛋白的表达。

Fig. 9

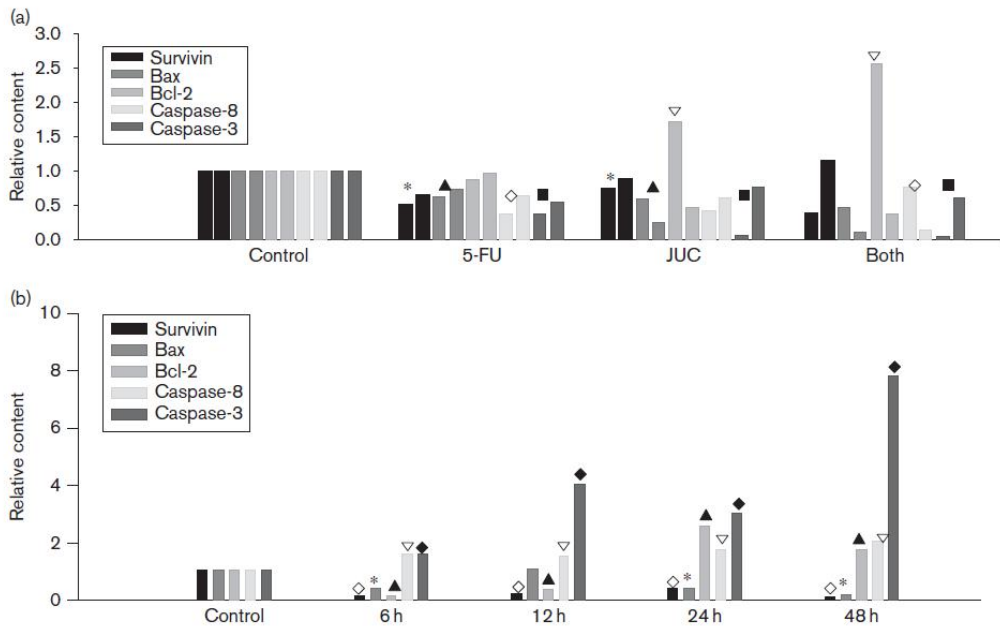


图9 比较两种细胞的蛋白质相对含量，相对于蛋白表达量的变化。

*Bax 蛋白 ▲Bcl-2 蛋白；▽胱天蛋白酶-8 蛋白；◇胱天蛋白酶-3 蛋白；■survivin 蛋白，与对照组相比，治疗组有明显的统计学意义(a) (P<0.05)。蛋白表达有时间依赖性关系(a) (P<0.05)。

Fig. 10

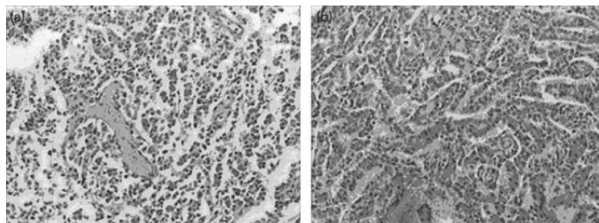


图 10 对照组(a)和实验组(b)中腹腔内肿瘤的形成。两组肿瘤切片用苏木精和曙红染色，显示无病理形态学差异（显微镜观察，10×10）。

Fig. 11

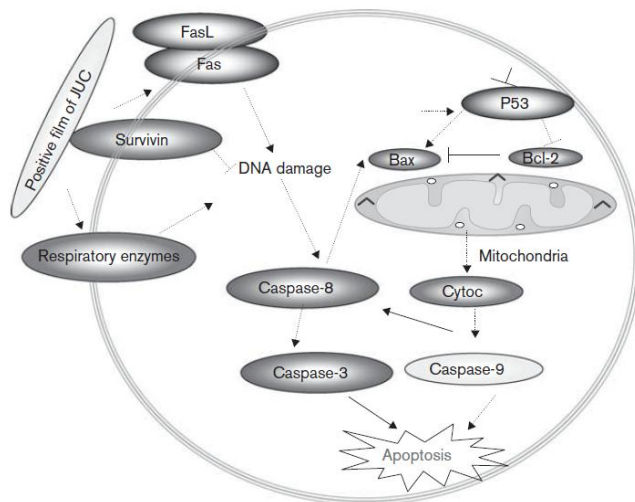


图11线粒体凋亡途径，JUC，洁悠神

表 3 洁悠神对小鼠植入转移性肿瘤形成模型的影响

	对照组	洁悠神治疗组
小鼠数量	18	17
负载肿瘤的小鼠	12(66.7)	5 (29.4)

洁悠神对大鼠体内形成植入转移性肿瘤的影响

在体内实验中，肿瘤转移模型表明，洁悠神能抑制细胞增殖，引起细胞凋亡的作用显著。由于手术相关的损伤和术后感染，治疗组有三只老鼠死去，对照组有两只老鼠死去，约占大鼠总数的12.5%。表3中可以看出，对照组中12只大鼠形成肿瘤，发病率约为66.7%。相比而言，洁悠神治疗组中5只大鼠检出肿瘤，发病率约为29.4%。此外，发病率在治疗组和对照组之间有显著差异（P<0.05）。从图10中还可以看出，实验组和对照组大鼠之间的肿瘤病理形态无明显差异。

讨论

肝癌一般确诊时，患者已经到了疾病的晚期，无法根治。可能的治疗方法包括外科手术切除或肝移植，可以为患者提供足够的肝功能和肿瘤治疗（穆勒，2006）。在手术期间，很难避

免移植转移，导致肝癌的复发。另外，在许多情况下，移植转移是导致发病率和死亡率的主要原因。由于移植转移的高发病率，为了提高患者生存率，制定了针对肝脏区域的战略。不能进行根治性治疗的个人一般采用局部烧蚀方法治疗，例如射频消融或酒精消融。全身化疗可以单独或结合其他疗法给选择的患者组。然而，肝癌对化疗不敏感，副作用和耐药性是化疗的最大挑战。越来越严重的化疗剂耐药问题牵涉到多种因素，包括细胞内药物聚集的减少，通过调整增殖或抗凋亡细胞进行的蛋白DNA损伤修复等（Siddik，2003）。这些原因最终导致临床治疗效果的不理想。因此，越来越多的研究正在开展，以解决这一难题。根据各药物的特性，研究人员已经采用了几种药物的协同作用来治疗疾病。建议联合用药以优化治疗方案，从而达到最佳治疗并/或尽量减小副作用（Eichhorn等人，2004；Johnston等人，2007）。

在动物模型中, 洁悠神已被证明能非常成功地抑制肿瘤生长。因此, 洁悠神可以作为一种有用的辅助方法, 来提高肝癌治疗中化疗药物的有效性。5-FU 及其衍生物, 作为经典的化疗药物, 能通过抑制胸苷酸合成来造成细胞损伤, 已成为治疗晚期肝癌的中流砥柱。然而, 对 5-FU 的耐药及副作用阻碍了肝癌化疗的进展。为了提高 5-FU 的效果, 必须减少所需的剂量, 宿主毒性必须最小化。在我们目前的实验中, 与单一的制剂相比, 洁悠神联合 5-FU 显示出更好的抑制作用。其可能的机制是: 洁悠神喷于组织表面后, 形成隐形保护膜, 能预防肿瘤细胞附着到已处理的组织表面。这种机理与其他一般抗肿瘤药物不一样。洁悠神不仅具有化学药物引导的抗肿瘤作用, 而且能在组织表面形成一层保护膜, 预防肿瘤细胞在组织中的生长。

原则上, 存活信号是抗癌治疗策略的理想目标, 因为阻断这些信号能导致依赖于信号的细胞死亡。我们的实验显示洁悠神和 5-FU 的联合治疗显著抑制了 HCC 生长。线粒体途径中凋亡相关的蛋白能引起附加功能蛋白的级联效应, 也可能参与了这种抑制。该研究中, 我们观察到洁悠神联合 5-FU 诱导的凋亡似乎是通过调节 Bcl-2 家族的蛋白水平介导的, 如 Bcl-2 和 Bax。另外, p53 在洁悠神和 5-FU 诱导的 HCC 细胞凋亡中起着重要的作用。

众所周知, Bcl-2 蛋白家族成员之间的相互作用可以调节线粒体 (或内在) 凋亡途径。在凋亡刺激后, 细胞色素 C 从线粒体释放到胞质中, 作为线粒体外膜通透化的结果, 之后凋亡小体形成, 蛋白酶激活。导致凋亡的关键步骤是线粒体外膜的透化, 这基本上是不可逆的。Bcl-2 是凋亡途径中的一种上游效应因子, 已被确定为细胞凋亡的强效抑制物。

我们发现洁悠神联合 5-FU 能显著地下调 SMMC7721 和 R15 细胞中的 Bcl-2 蛋白, 上调 Bax 蛋白水平。这说明一种内在凋亡途径的参与, 通过该途径土木香内酯诱导 SMMC7721 和 R15 细胞凋亡。然而, 蛋白印迹分析显示洁悠神联合 5-FU 能加强 SMMC7721 和 R15 细胞中引发剂胱天蛋白酶-8 的进程, 这些资料说明两种途径之间串扰的可能性, 因为进一步活化的胱天蛋白酶-8 能活化胱天蛋白酶-3。因

此, 有效抑制胱天蛋白酶-3 和胱天蛋白酶-9 在提供肿瘤的预防和治疗的目标途径中是很关键的。最终, 胱天蛋白酶-3 蛋白完成了凋亡的指令, 导致细胞死亡。我们观察到洁悠神联合 5-FU 能减少 SMMC7721 和 R15 细胞中蛋白质水平的细胞依赖性, 这能抑制抗凋亡靶细胞的表达, 如 Bcl-2, 由此我们可总结出这可能有利于 SMMC7721 和 R15 细胞的凋亡。p53 是一种转录因子, 调节细胞周期进程和 DNA 修复, 我们推断这可能有利于 SMMC7721 和 R15 细胞的凋亡。这种多重任务处理对于抑制肿瘤形成是很重要的, 对于调节许多标准 DNA 损伤诱导的癌症疗法的细胞反应也很重要。当联合使用洁悠神和 5-FU 时, 内部机制首先引起 DNA 损伤和细胞周期变化。

我们的结果显示联合使用洁悠神和 5-FU 能显著增加 SMMC7721 和 R15 细胞中 p53 的表达, 这能导致细胞周期阻断, 并上调 Bax 表达。这说明 p53 在洁悠神和 5-FU 的联合使用中是一种独立的诱导凋亡的因子。同时, 纳米正电荷层破坏细胞膜。药物进一步直接损坏细胞, 然后激活胱天蛋白酶-8 蛋白, 影响线粒体膜的蛋白质, 如 Bax, Bcl-2 等。这些导致了经典的线粒体途径的凋亡。图 11 中显示了这种机制。总之, 洁悠神联合 5-FU 能抑制肝癌细胞系的增殖, 提示细胞凋亡, 这源于正电荷膜的屏蔽作用。这种物理膜能使细胞呼吸酶厌氧, 诱导细胞死亡。最终, 细胞膜信号结束。洁悠神联合 5-FU 抑制细胞增殖和诱导凋亡的能力是由各种事件的多元级联引起的。今后的研究将进一步讨论正电荷膜和膜表面之间发生的复杂偶联反应。因为两种制剂都广泛用于临床实践, 转移术中情况是必要的, 以确定联合治疗的疗效。我们将发展这种联合, 以作为潜在的新策略, 并在将来用于辅助灌注化疗。

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利益冲突

不存在利益冲突。