Original Article

Combined Antitumor Effect of Ursolic Acid And 5-Fluorouracil on Human Esophageal Carcinoma Cell Eca-109 *In Vitro*

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ABSTRACT

Objective: To study the combined antitumor effect and possible mechanisms of ursolic acid with 5-fluorouracil (5-FU) on human esophageal carcinoma cell Eca-109 *in vitro*.

Methods: Eca-109 cells were treated with ursolic acid (10−50 μmol/L) and/or 5-fluorouracil (48.0−768.8 μmol/L) for 48 h *in vitro*. And then cell proliferation was determined by MTT assay. Cell cycle and apoptosis rate were analyzed by flow cytometry (FCM). The morphological changes of apoptosis were observed by fluorescent microscopy. At last the expression of $P27^{kip1}$, *bcl*-2 and *bax* were detected by western blot.

Results: Results: In comparison with single agent treatment, the combination of ursolic acid and 5-fluorouracil produced greater efficacy in growth inhibition, cell cycle arrest at G0/G1 phase, and apoptosis induction ($P<0.05$). Western blot analysis showed that the combination use of ursolic acid and 5-fluorouracil suppressed the expression of *bcl*-2 and increased the expressions of *bax* and $P27^{kip1}$.

Conclusion: Ursolic acid combined with 5-fluorouracil showed adjuvant antiproliferative effects on human esophageal carcinoma cell Eca-109 in vitro, which mainly due to the induction of cell cycle arrest as well as apoptosis.

Key words: Ursolic acid; 5-fluorouracil; Eca-109 cells; Apoptosis; Cell cycle; $P27^{kip1}$

INTRODUCTION

Recently, many antitumor compounds with new structural features and mechanism of action have been isolated from herbal medicines. Ursolic acid (UA) is a triterpenoid compound that exists widely in food, medicinal herbs, and other plants. Previous studies show that UA has numerous pharmacological activities including antioxidant activity, as well as anti-inflammatory, anti-cancer, and hepatoprotective effects[1−6]. Our previous studies show that ursolic acid inhibit the proliferation of human esophageal carcinoma cell Eca-109 *in vivo*[7]. To date, however, no study has been conducted in the evaluation of the combination effects of ursolic acid and chemotherapy drugs against human esophageal carcinoma cells. Therefore, the present study was designed to investigate the antitumor effects of combining ursolic acid with 5-fluorouracil (5-FU) on human esophageal carcinoma cell Eca-109 *in vitro*, with special emphasis on the changes in certain key protein expressions relevant to the regulation of cell cycle and apoptotic pathway.

MATERIALS AND METHODS

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Materials

Human esophageal carcinoma cell line Eca-109 was supplied by the department of pathophysiology of Chongqing Medical University. Ursolic acid, Hoechst 33258, Propidium iodide (PI) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) were supplied by Sigma Chemical Co. (St. Louis, MO, USA). 5-fluorouracil was obtained from Pharmaceutical factory of Nantong (China). Fetal bovine serum (FBS) and RPMI-1640 culture medium were obtained from GIBCO BRL (USA). The monoclonal mouse anti-human $P27^{kip1}$, *bax*, and *bcl*-2 antibodies were purchased from Santa Cruz Biotechnology, Inc. (USA). Horseradish peroxidase (HPR)-rabit anti-mouse antibody (the second Ab) was purchased from Beijing Zhong Shan Biotechnology Co (China).

MTT Assay

The effect of ursolic acid, 5-fluorouracil and their combination on the proliferation of Eca-109 cells was measured by MTT assay. Briefly 5×10^3 cells/well were grown in 96-well plates overnight. Cells were incubated with 10, 20, 30, 40, and 50 µmol/L ursolic acid; 48.0, 96.1, 192.2, 384.4, and 768.8 µmol/L 5-fluorouracil or their combination (The ratio of the two drugs combined was 1:1) for 48 h, then 10 µl of MTT (1 mg/ml) reagent was added to each well, and the cells were further incubated at 37°C for 4 h. The supernatant was removed and 200 µl DMSO in isopropanol was added to each well to solubilize the formazan product. The absorbance at wavelength of 570 nm was measured by a micro ELISA reader. The negative control well contained medium only. The ratios of the absorbance of treated cells relative to those of the control wells were calculated and expressed as percentage of growth inhibition.

Analysis of Cell Cycle Distribution and Cell Apoptosis

Cell cycle and cell apoptosis analyses were carried out using flow cytometry. Eca-109 cells (1×10^6/well) were seeded in six-well plates and allowed to attach overnight. Growing cells were treated with 10–50 µmol/L ursolic acid, 48.0–768.8 µmol/L 5-fluorouracil, or their combination for 48 h. The cells were then washed twice with PBS, centrifuged at 500× g for 5 min, fixed in 70% ethanol at 4°C. Before DNA analyses, the cells were washed again with PBS, treated with 50 µg/ml RNase, and stained with 100 µg/ml PI in the dark. Flow cytometry analysis was performed within 1 h according to the manufacturer's instructions.

Fluorescent Microscopy

Eca-109 cells (1×10^6) were grown on coverslips placed into six-well plates. After drug treatment as described above, cells were fixed with cold methanol and acetic acid at 4°C overnight, stained with Hoechst 33258 for 30 min in the dark, washed again in PBS and finally mounted in mounting medium. Processed cells were observed with a fluorescence microscope.

Western Blot

Eca-109 cells (1×10^6/well) were seeded in 6-well plates. Cells were treated as described above. The cells were scraped and washed twice by centrifugation at 500× g for 5 min at 4°C. The pellet was resuspended in lysis buffer supplemented with proteases and phosphotase inhibitors and incubated for 1 h at 4°C. The lysate was collected by centrifugation at 14,000×g for 40 min at 4°C, and the supernatant was stored at -20°C. 50 µg of denatured protein was separated on a sodium dodecyl sulfate-polyacrylamide gel (12% acrylamide) and transferred to PVDF membrane, which was then blocked overnight in 5% skim milk in TBST. For immunoblotting, the membrane was incubated for 1 hour with the antibody against *bax*, *bcl*-2, and $P27^{kip1}$. Afterward, it was rinsed by TBST and incubated with the second antibody (1:1000) for 1 h. The final blots were assessed with an ECL Western blotting detection system. The protein bands were scanned by computer and the relative expressions of protein were calculated by image anlysis software (Quantity One).

Statistical Analysis

All data were expressed as $\bar{x}\pm s$. Statistical analysis was carried out by using the statistic software SAS 9.0. Statistical analysis was performed using *t*-test, two-tailed distribution, assuming two-sample unequal variance. $P<0.05$ was considered to be statistically significant.

RESULTS

Combined Effects of UA and 5-FU on the Viability of Eca-109 Cells

Cell proliferation was inhibited strongly by

ursolic acid in a dose dependent manner. Treating Eca-109 cells with 5-fluorouracil also inhibited cellular proliferation in a dose dependent manner. Compared with single treatment, combination treatment inhibited cell proliferation and viability more significantly (*P*<0.05) (Table 1).

Combined Effect of UA and 5-FU on Cell Cycle and Cell Apoptosis

Eca-109 cells treated with ursolic acid were arrested in the G0/G1 phase in a typical dose dependent manner, and this effect was more evident when combined with 5-fluorouracil (Table 2). The percentage of apoptotic cells was increased to 14.41%−72.05% when treated with ursolic acid and 5-fluorouracil for 48 h, more than that with single treatment of ursolic acid (2.69%−56.12%) or 5-fluorouracil (12.63%−62.38%) (*P*<0.05).

Table 1. The inhibitory effects of UA, 5-FU, and their combination on Eca-109 cells for 48 ($\bar{x}\pm s$)

UA	5-fluorouracil (μmol/L)					
(μmol/L)	0	48.0	96.1	192.2	384.4	768.8
0	0	23.12±1.36[a]	34.32±1.97[a]	47.84±2.33[a]	59.16±1.61[a]	71.26±1.43[a]
10	6.26±1.29	32.48±1.50[a]	—	—	—	—
20	17.38±1.07[a]	—	41.19±2.01[a]	—	—	—
30	33.82±1.71[a]	—	—	56.38±1.42[ab]	—	—
40	54.39±1.58[a]	—	—	—	70.75±2.61[ab]	—
50	67.04±1.22[a]	—	—	—	—	82.27±1.86[ab]

Compared with control groups: [a]*P*<0.05; Combination effect analysis: [b]*P*<0.05.

Table 2. The effect of UA and 5-FU on the cycle distribution and cell apoptosis of Eca-109 cells ($\bar{x}\pm s$)

UA (μmol/L)	5-FU (μmol/L)	G_0/G_1 phase	S-phase	G_2/M phase	Apoptosis	Proliferation index
0	0	53.43±1.41	25.72±1.33	20.85±1.45	1.09±0.82	46.57±3.81
10	0	61.30±0.41[a]	24.19±0.63	14.15±2.01	2.69±0.77	38.34±2.28[a]
20	0	67.39±1.20[a]	19.35±1.19[a]	13.26±0.91	10.06±1.27[a]	32.61±1.62[a]
30	0	74.36±2.40[a]	14.21±0.89[a]	11.43±0.73	24.31±1.08[a]	25.64±1.97[a]
40	0	76.26±1.39[a]	12.52±0.75[a]	11.22±0.55	41.36±2.19[a]	23.74±1.10[a]
50	0	82.19±0.37[a]	10.32±0.82[a]	7.49±1.28	56.12±0.41[a]	17.81±2.07[a]
0	48.0	50.51±2.12	21.33±1.04	28.10±3.13	12.63±1.69	49.49±3.12
0	96.1	56.20±1.56[a]	24.13±2.26	19.80±2.10	21.89±2.56[a]	44.00±1.01
0	192.2	61.82±2.61[a]	27.48±1.49[a]	10.00±0.94	33.91±2.37[a]	37.55±2.03[a]
0	384.4	69.36±2.97[a]	27.13±1.17[a]	3.50±0.49	47.59±1.40[a]	30.64±1.18[a]
0	768.8	75.19±2.81[a]	22.34±2.50	2.40±0.84	62.38±1.67[a]	24.81±1.27[a]
10	48.0	67.83±2.52[a]	19.78±1.54	12.30±1.04	14.41±2.10	32.17±2.66
20	96.1	73.91±1.36[ab]	21.39±1.67	4.70±0.79	27.20±1.51[ab]	26.09±2.54[ab]
30	192.2	78.43±2.64[ab]	19.61±1.43	1.90±0.23	40.05±2.67[ab]	21.57±1.33[ab]
40	384.4	82.83±1.56[ab]	15.44±1.01[ab]	1.70±0.41	58.46±2.44[ab]	17.17±1.21[ab]
50	768.8	87.69±2.37[ab]	10.08±1.19[ab]	1.50±0.34	72.05±2.87[ab]	11.59±0.92[ab]

Compared with control groups: [a]*P*<0.05; Combination effect analysis: [b]*P*<0.05.

Hoechst 33258 Staining

After treatment of Eca-109 cells with UA, 5-FU, and their combination for 48 h, marked morphological changes of cell apoptosis such as condensation of chromatin, nuclear fragmentations and apoptotic bodies were found clearly using Hoechst 33258 staining (Figure 1). Compared with

single treatment, apoptotic cells significantly increased in combination treatment.

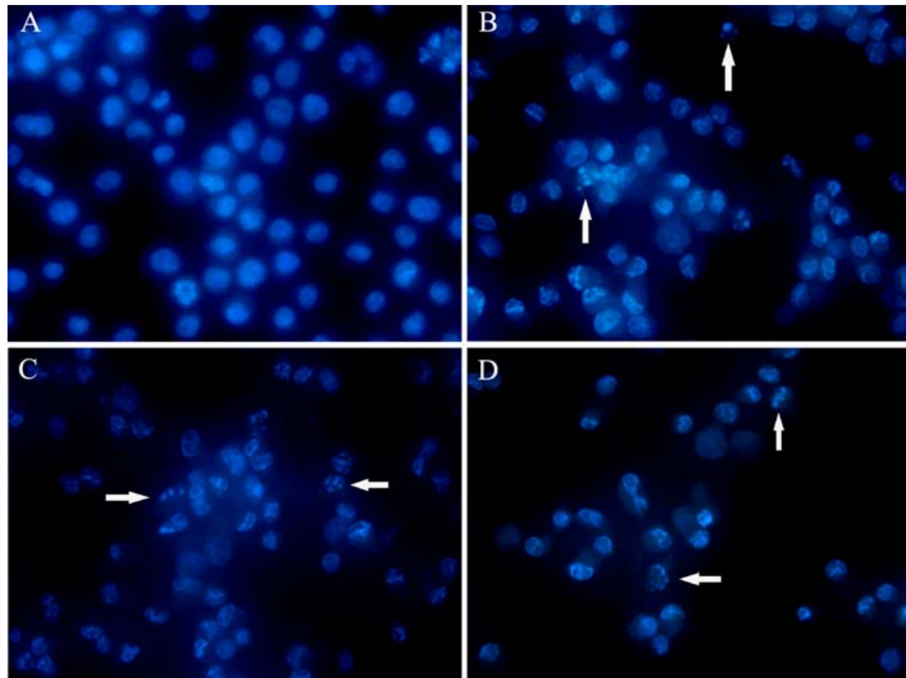

Figure 1. Apoptosis of Eca-109 cells observed by Hoechst 33258 staining. A: Control group; B: UA-treated group; C: 5-FU-treated group; D: Combination treatment group.

Combined Effect of UA and 5-FU on Expression of *bcl*-2, *bax*, $P27^{kip1}$

Using Western blotting analysis, we assessed the effects of UA, 5-FU, and their combination treatment on the protein expressions of *bcl*-2, *bax* and $P27^{kip1}$. Treatment of Eca-109 cells with UA (10−50 μmol/L) for 48 h resulted in decrease of the protein expression of *bcl*-2 and increase of *bax* and $P27^{kip1}$. As shown in Figure 2 and Figure 3, compared with single treatment, combination treatment with UA and 5-FU reduced the level of *bcl*-2 expression more significantly. The level of *bax* and $P27^{kip1}$ proteins in combination treatment cells were higher than those in single-treated cells.

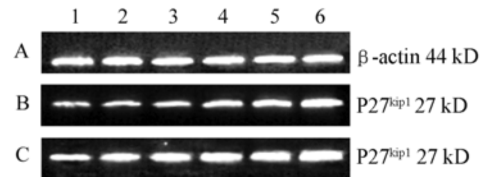

Figure 2. Western blot analysis of $P27^{kip1}$. A: β-action; B: UA-treated group; C: Combination treatment group. Lane 1: Control group; Lanes 2−6: UA (10−50 μmol/L or combined with 5-FU (48.0−768.8 μmol/L).

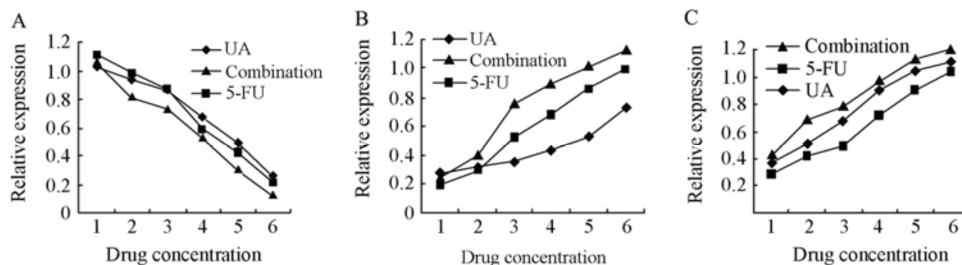

Figure 3. The relative expressions of *bcl*-2, *bax* and $P27^{kip1}$ Eca-109 cells treated with UA, 5-Fu, and their combination for 48 h. A: *bcl*-2/β-action; B: *bax*-2/β-action; C: $P27^{kip1}$/β-action; Drug concentration 1: Control group; Drug concentration 2−6: UA (10−50 μmol/L), 5-FU (48.0−768.8 μmol/L) or their combination.

DISCUSSION

The treatments for human esophageal carcinoma with chemotherapy rarely uses a single drug but usually combines two or more drugs with different mechanisms of action. An optimal treatment of combination chemotherapy may increase the therapeutic efficacy, decrease the toxicity toward the host or non-target tissues, and minimize or delay the development of drug resistance[8, 9]. In our study, we investigated the viability of human esophageal carcinoma cell Eca-109 under the influence of ursolic acid and 5-fluorouracil and in combination. Our study demonstrated that ursolic acid, when combined with anti-tumor agent 5-fluorouracil, showed adjuvant antiproliferative effects on Eca-109 cells *in vitro*.

Apoptosis is essential for the development and maintenance of tissue homeostasis and the elimination of unwanted or damaged cells from multicellular organisms. The aberrant regulation of apoptosis has been observed in many forms of tumors[10]. *bax* and *bcl*-2 are the apoptosis-related proteins of mitochondrial pathway. Studies have reported that *bcl*-2 inhibits apoptosis by preventing cytochrome c release from the mitochondria and inhibiting caspase activation[11], and *bax* is a promoter for apoptosis. The ratio of *bcl*-2: *bax* dictates the ultimate sensitivity or resistance of tumor cells to apoptosis. To probe the possible mechanism by which ursolic acid enhances the cytotoxic effects of 5-fluorouracil, we performed some experiments on cell apoptosis. Flow cytometry analysis showed that ursolic acid exhibited strong effects of inducing apoptosis on Eca-109 cells *in vitro*. And fluorescent microscopy showed ursolic acid treatments led to the occurrence of typical morphological changes of apoptosis. Western blotting analysis showed that treating Eca-109 cells with ursolic acid significantly inhibited the *bcl*-2 expression and increased *bax* expression, indicating that ursolic acid induced apoptosis through mitochondrial pathway in vitro, which was consistent with our previous report[7]. Compared with single treatment, combination treatment with ursolic acid and 5-fluorouracil reduced the level of *bcl*-2 expression and increased the level of *bax* expression more significantly, indicating that ursolic acid combined with 5-fluorouracil showed adjuvant effects of inducing apoptosis on Eca-109 cells *in vitro*.

$P27^{kip1}$ protein, members of the KIP family, inhibits the activities of cyclin D-, E-, and A-dependent kinases and induce cell-cycle arrest to allow cells to stop and repair DNA lesions before DNA replication occurs in S phase. Low levels of $P27^{kip1}$ are reported to be causally related to tumorigenesis. The level of $P27^{kip1}$ expression decreases during human esophageal carcinoma development and progression[12]. In this study, we found that the administration of ursolic acid, 5-fluorouracil or their combination resulted in accumulation of cells at G0/G1 phase. Affected cells are unable to successfully enter cell cycle. Ultimately, the prolonged mitotic arrest in most of these cells may lead to apoptosis. In order to get a better understanding of the mechanism by which ursolic acid, 5-fluorouracil and their combination leads to greater G0/G1 arrest, we further investigated the status of a key protein, $P27^{kip1}$ known to regulate G0/G1 transition. We clearly demonstrated that the level of $P27^{kip1}$ protein was increased in Eca-109 cells treated with either ursolic acid or 5-fluorouracil alone. These changes in combination treatment group were significantly greater than that in single treating group, indicating that treating Eca-109 cells with ursolic acid or 5-fluorouracil or their combination resulted in increased expression of $P27^{kip1}$, leading to G0/G1 arrest.

In summary, we have found that ursolic acid, when combined with 5-fluorouracil, has adjuvant antiproliferative effects, which mainly due to the induction of cell cycle arrest as well as apoptosis. The possible mechanisms underlying the action might be attributed to the changes in certain key proteins relevant to the regulation of cell cycle and/or apoptosis. This investigation suggests an important potential clinical application of the combination regimen for the treatment of esophageal carcinoma patients.

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