

## Anti-tumor Effect and Its Mechanisms of Ursolic Acid on Human Esophageal Carcinoma Cell Eca-109 *in Vivo*

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### ABSTRACT

**Objective:** To investigate the anti-tumor effect and possible mechanisms of ursolic acid on human esophageal carcinoma *in vivo*. **Methods:** A transplanted tumor model by injecting Eca-109 cells into subcutaneous tissue of BALB/c nude mice was established. 40 nude mice bearing tumors were randomly divided into 4 groups and 0.2 ml saline or 0.2 ml ursolic acid (25–100 mg·kg<sup>-1</sup>·d<sup>-1</sup>) was injected into abdominal cavity respectively once everyday and lasted for fourteen days. The changes of tumor volume were measured continuously and tumor inhibition rate was calculated. The morphological changes of apoptosis were observed by electron microscope. The expressions of COX-2, bcl-2 and Bax protein in transplanted tumors were detected by immunohistochemistry. At last the PGE2 level of transplanted tumors was detected by radioimmunoassay. **Results:** Treatment of nude mice with 25, 50, or 100 mg·kg<sup>-1</sup>·d<sup>-1</sup> of ursolic acid significantly inhibited the growth of the human esophageal carcinoma tumor in nude mice and induced Eca-109 cells apoptosis as demonstrated by electron microscopy analyses. The expressions of COX-2 and bcl-2 in the transplanted tumors were decreased in ursolic acid groups, while the Bax increased. The PGE2 level of transplanted tumors was decreased in ursolic acid groups with a dose-related manner. **Conclusion:** Ursolic acid has anti-tumor effects against human esophageal carcinoma cells *in vivo*, which are likely mediated via induction of tumor cell apoptosis and inhibition of COX-2 and PGE2.

**Key words:** Ursolic acid; Esophageal carcinoma; Apoptosis; COX-2; PGE2

Ursolic acid (UA), a pentacyclic triterpene acid, is widely distributed in medical herbs and edible plants, such as *ligustrum lucidum*, *hedyotis diffusa*, *sambucus chinensis*, *forsythia*, hawthorn, apples and other fruits. UA has been shown to possess hypoglycemic<sup>[1]</sup>, anti-inflammatory<sup>[2]</sup>, anti-viral<sup>[3]</sup>, anti-oxidation<sup>[4]</sup> and immunomodulatory activity<sup>[5]</sup>. Now, attention has been focused on its antitumor activity. Some studies have shown that UA exhibits growth inhibition properties against many human cancer cell lines *in vitro*, including HepG2 Hepatoma cells<sup>[6]</sup>, MCF-7 breast cancer cells, Caco-2 colon cancer cells<sup>[7]</sup> and SNG-II endometrial cancer cells<sup>[8]</sup>. And these could be

explained through inhibition of DNA replication, induction of Ca<sup>2+</sup> release, inhibition of protein tyrosine kinases, and activation of caspases to induce cancer cell apoptosis. Another UA-induced apoptotic mechanism involves the down-regulation of the cellular inhibitor of apoptosis gene and inhibition of NF- $\kappa$ B activity. However, the effect of UA on cancer cells *in vivo* has rarely been reported. In this study, we investigated the effects of UA on the inhibition of proliferation, induction of apoptosis and expression of apoptosis-related proteins in transplanted human esophageal carcinoma in nude mice at the cell and molecular level, and further clarified its anti-tumor mechanisms.

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### MATERIALS AND METHODS

#### Materials

Human esophageal carcinoma cell line ECa-109

was supplied by the department of pathophysiology of Chongqing University of Medical Sciences. Ursolic acid was supplied by Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) and RPMI-1640 culture medium were obtained from GIBCO BRL (USA). The mouse monoclonal antibodies against human Cox-2, Bax, and bcl were purchased from Santa Cruz Biotechnology, Inc (USA). Sp-9000 Immunohistochemistry kits and horseradish peroxidase (HPR)-rabbit anti-mouse antibody (the second Ab) were purchased from Beijing Zhongshan Biotechnology Co (China). PGE2 Radioimmunoassay kits was obtained from Institute of Hematology of Suzhou University (China).

### Cell Culture and Preparation of UA

Eca-109 cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 mg/ml) at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. UA was dissolved in saline.

### Animal Experiment

A total of 50 mature male normal BALB/c nude mice, aged 6–8 w and weighing 15–20 g, were obtained from Shanghai Laboratory Animal Center of Chinese Academy of Sciences. Animals were housed in micro-isolator cages in a barrier facility under pathogen-free conditions at a monitored ambient temperature of 22°C. To establish tumors in the BALB/c nude mice, Eca-109 cells were subcutaneously injected into the forelimb region of 40 nude mice at a cell density of  $1 \times 10^7$  cells suspended in 100  $\mu$ l of PBS. Tumors were allowed to grow until the tumor volume was increased to approximately 0.2 cm<sup>3</sup>. They were then randomly divided into four groups (10/group), and given daily peritoneal injections (200  $\mu$ l) of following treatments for fourteen days: the first group was kept as control and treated with saline, the other groups were treated with UA (25, 50, 100 mg·kg<sup>-1</sup>·d<sup>-1</sup>), respectively. The nude mice were killed 12 h after last injection and tumor inhibition rate of UA was calculated. Tumor inhibition rate (IR)=(average weight of control group–average weight of treated-group)/average weight of control group $\times$ 100%. IR<30 was considered ineffective, while IR $\geq$ 30 was considered effective<sup>[9]</sup>.

### Electron Microscopy

Tumor tissues (1 mm<sup>3</sup>) were pre-fixed with 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer at 4°C for 2 h, and then rinsed thoroughly in phosphate buffer and postfixed in 1% OsO<sub>4</sub> at 4°C for 30 min. After being fixed, tissues were dehydrated through a graded ethanol series, and embedded in Epon (Epoxy resin, Spi supplies, West chester, USA). The ultrastructure of cells was analyzed in ultrathin sections (70 nm) in a TEM (Hitachi H-800, Tokyo, Japan) after the sections were stained with uranyl acetate and lead citrate.

### Immunohistochemistry

The tumor tissues were fixed with 4% paraformaldehyde at room temperature for 24 h. The paraffin-embedded specimens were cut into sections with a thickness of 5  $\mu$ m. Immunohistochemical staining for COX-2, bcl-2 and Bax was performed according to the standard streptavidin-peroxidase method described in the procedure program of streptavidin-peroxidase reagents kit. A previously known positive tumor tissue was used as a positive control. The immunohistochemical results were quantitatively analyzed by a biological image analysis system which consists of Nikon800u biology microscopy, Spot2 digital color camera and Metomorph biological image analysis software.

### Radioimmunoassay

The frozen tumor tissues (about 50 mg) were homogenized in 1 ml 0.9% sodium chloride solution and incubated in a shaking bath (37°C) for 15 min, then centrifuged at 7500 g for 15 min. Subsequently, the level of PGE2 in tissues' supernatant was detected using PGE2 (I125) RIA kit.

### Statistical Analysis

All data were expressed as  $\bar{x} \pm s$ . Statistical analysis were carried out by using the statistic software SAS 9.0 and t-test.  $P < 0.05$  was considered to be statistically significant.

## RESULTS

### Effect of UA on Tumor Growth in the Esophageal Cancer Model in Vivo

The body weight of nude mice (control group) was lower but there was no significant difference

compared to the normal nude mice (normal group). Treatment with various doses of UA slightly reduced body weight of nude mice (UA groups) compared to the other 2 groups. And treatment

significantly decreased the tumor growth with inhibition rates from 26.27% to 63.14% (Tab. 1). No death of nude mice caused by UA injection was observed.

Tab. 1. The inhibitory effect of UA on Eca-109 cells transplanted into nude mice ( $\bar{x} \pm s$ )

Groups	Change of mean weight of nude mice (g)		Mean weight of tumor (g)	Inhibition (%)
	Before treatment	After treatment		
Normal	16.06±2.42	18.26±2.26	0	0
Control	16.75±2.69	18.06±2.51	2.55±0.42	0
UA (25 mg)	17.13±3.08	17.44±2.47	1.88±0.64*	26.27
UA (50 mg)	16.50±3.05	16.09±2.38	1.30±0.26**	49.02
UA (100 mg)	17.09±2.85	15.80±2.39	0.94±0.25**	63.14

Each group consists of 10 nude mice. Compared with control groups: \* $P < 0.05$ , \*\* $P < 0.01$

### Ultrastructural Changes Tumor Tissues of Induced by UA

Tumor cell apoptosis in UA-treated group was confirmed by electron microscopy. Fig. 1 shows

representative picture of apoptotic tumor cells in UA-treated groups, which revealed cell shrinkage, chromatin condensation, irregularly shaped crescents at the nuclear edges, and fragmentation of cells into membrane-wrapped apoptotic bodies.

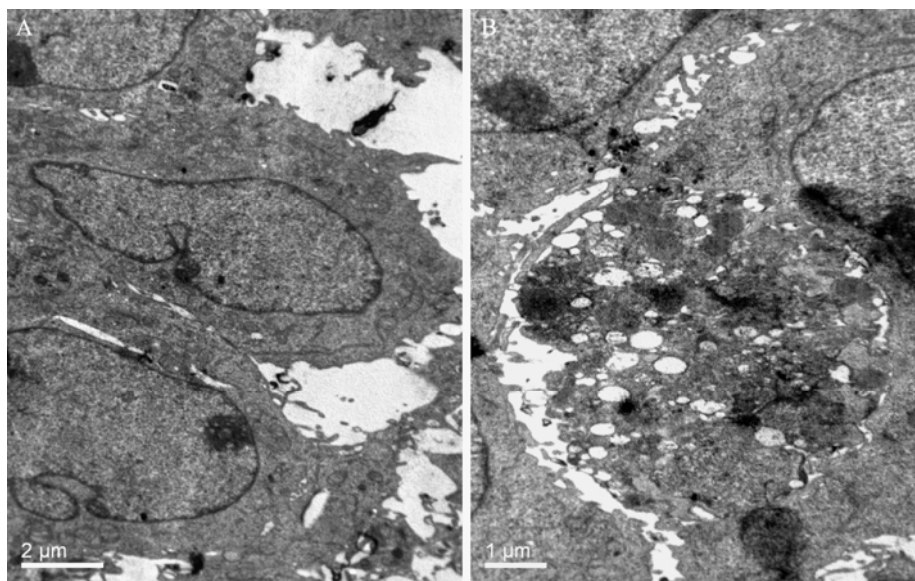


Fig. 1. Ultrastructural changes of Eca-109 cells induced by UA *in vivo*  
A: control Eca-109 cells (TEM  $\times 8000$ ) B: Eca-109 cells treated with UA (TEM  $\times 12000$ )

### Effects of UA on Expressions of Cox-2, bcl-2 and Bax

Treatment of UA significantly reduced the number of COX-2 and bcl-2 positive cells, with

weak to moderate staining in a dose-related manner. The number of Bax positive cells was increased in the UA-treated group in a dose-related manner (Fig. 2). Correspondingly, the ratio of bcl-2/Bax decreased significantly in the UA-treated group

compared with the control group (Tab. 2).

### Effects of UA on PGE2 Level

The concentration of PGE2 (pg/ml) in the

UA-treated group ( $425.76 \pm 48.00$ ,  $300.72 \pm 34.04$ ,  $167.36 \pm 50.40$ ) was lower than that in control group ( $573.45 \pm 55.22$ ). Compared with the control group, PEG2 level of UA-treated group has significant difference ( $P < 0.01$ ).

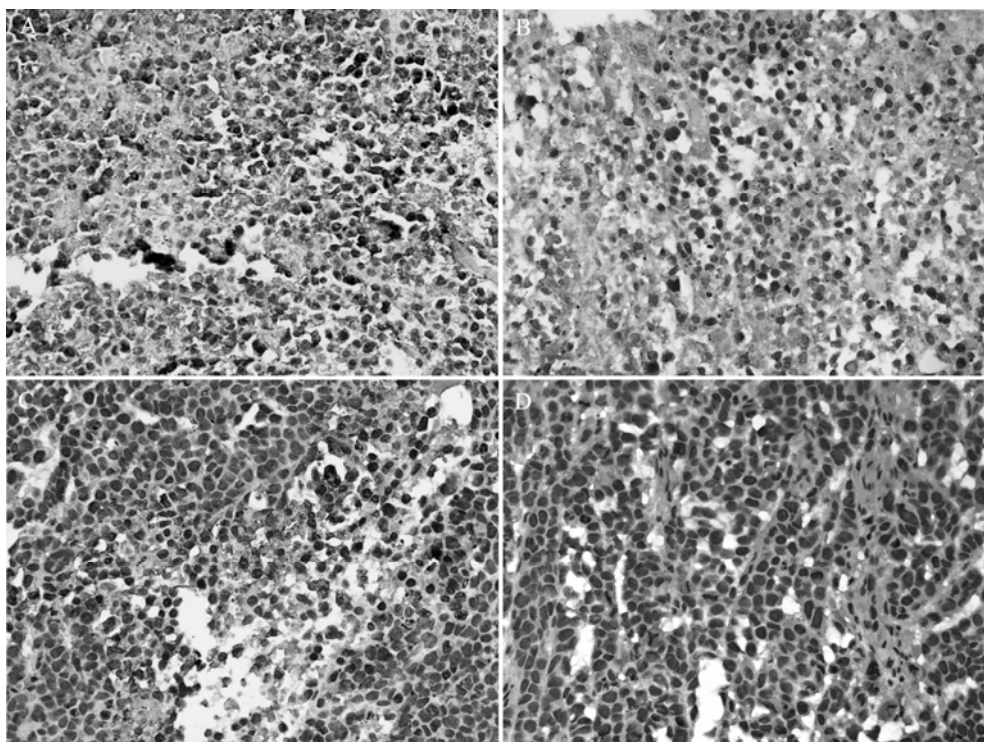


Fig. 2. Effects of UA on the expression of COX-2 in transplanted tumor DAB $\times 400$

A: control group B: UA-treated group ( $25 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ )

C: UA-treated group ( $50 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ ) D: UA-treated group ( $100 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ )

Tab. 2. Effect of UA on the expression of COX-2, bcl-2 and Bax in tumor tissue

Group	COX-2 (A)	bcl-2 (A)	Bax (A)	bcl-2/Bax
Normal	$0.00 \pm 0.00$	$0.00 \pm 0.00$	$0.00 \pm 0.00$	0
Control	$0.29 \pm 0.04$	$0.32 \pm 0.03$	$0.24 \pm 0.03$	1.34
UA (25 mg)	$0.22 \pm 0.03^*$	$0.28 \pm 0.03$	$0.27 \pm 0.04$	1.04
UA (50 mg)	$0.19 \pm 0.02^{**}$	$0.26 \pm 0.01^{**}$	$0.30 \pm 0.02^{**}$	0.87
UA (100 mg)	$0.16 \pm 0.03^{**}$	$0.23 \pm 0.02^{**}$	$0.32 \pm 0.03^{**}$	0.72

Compared with control group:  $*P < 0.05$ ,  $**P < 0.01$  A: Absorbance value.

## DISCUSSION

In terms of cancer treatment, chemotherapy has serious limitations, namely the lack of selectivity

of active ingredients and resistance of cancer cells to these chemicals<sup>[10]</sup>. Thus, it is urgent to find new compounds that can kill both parental cancer cells and drug resistant cells and differentiate between



normal and cancer cells in order to selectively kill cancerous cells with reduced toxicity. The search for anticancer agents with high efficiency and low toxicity from the natural vegetation has kindled great interest in recent years. UA is one such compound, which has been shown to inhibit the growth of a wide variety of tumor cells<sup>[6-8]</sup>. In this study, we have demonstrated that UA also has obvious antitumor effects on human esophageal carcinoma *in vivo*.

Apoptosis, a physiological process for killing cells, is critical for the normal development and function of multicellular organisms. Abnormalities in the control of cell death can contribute to a variety of diseases, including cancer. Apoptosis is a programmed cell death that can be induced by a variety of different stimuli such as growth factor deprivation, ionizing radiation, chemotherapeutic drugs and activation of cell death receptors<sup>[11]</sup>. Induced apoptosis of tumor cells is one of the effective treatment for cancer. Bax is a promoter for apoptosis and bcl-2 is an inhibitor. The ratio of bcl-2:Bax dictates the ultimate sensitivity or resistance of tumor cells to apoptosis. In this study, the morphological changes of apoptosis were observed under electron microscope. Immunohistochemistry showed that UA can increase the expression of Bax and reduce the expression of bcl-2 *in vivo*. Bax and bcl-2 are the apoptosis-related proteins of mitochondrial pathway. Our findings suggest that UA can induce apoptosis of esophageal carcinoma cells by mitochondrial pathway *in vivo*.

Among various prostaglandins, PGE<sub>2</sub> plays an important role in carcinogenic process. COX-2 is a crucial rate-limiting enzyme in PGE<sub>2</sub> biosynthesis, which converts arachidonic acid into prostaglandin G<sub>2</sub> (PGG<sub>2</sub>) and then prostaglandin H<sub>2</sub> (PGH<sub>2</sub>). COX-2 is induced in inflammation and in several epithelial cancers including esophageal cancer. Multiple lines of evidence suggest that COX-2 is important in carcinogenesis and overexpressed in cancer cells<sup>[12]</sup>. Reduced the expression of COX-2 and PEG2 may be effective ways to inhibit growth of tumor. In this study, Immunohistochemistry showed that UA can reduce the expression of COX-2 *in vivo*. Radioimmunoassay showed that UA also reduced the expression of PEG<sub>2</sub> with a dose-dependent characteristics. These suggest that UA can inhibit the growth of Eca-109 cells *in vivo* by reducing the expression of COX-2 and PEG<sub>2</sub>, which may be one of the anti-tumor mechanisms of UA.

In conclusion, UA is a potential anti-tumor agent for esophageal carcinoma with reduced

toxicity *in vivo*. However, poor water solubility of UA has confined its use. Although its derivatives have good water solubility, these derivatives possess no anti-neoplasm activity<sup>[13]</sup>. More work should be done to solve this problem.

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