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Ursolic acid inhibits proliferation and induces apoptosis of HT-29 colon cancer cells by inhibiting the EGFR/MAPK pathway*

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Abstract: Objective: To investigate the effects of ursolic acid on the proliferation and apoptosis of human HT-29 colon cancer cells. Methods: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and flow cytometry assays were performed to evaluate the effects of ursolic acid on the growth and apoptosis of HT-29 cells. Western blot analysis was applied to investigate the inhibitory effects of ursolic acid on the phosphorylation of the epidermal growth factor receptor (EGFR), extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (p38 MAPK), and the activity of B cell leukemia-2 (Bcl-2), B cell leukemia-xL (Bcl-xL), caspase-3, and caspase-9. Results: Ursolic acid inhibited the growth of HT-29 cells in dose- and time-dependent manners. The median inhibition concentration (IC_{50}) values for 24, 48, and 72 h treatment were 26, 20, and 18 μmol/L, respectively. The apoptotic rates of 10, 20, and 40 μmol/L ursolic acid treatments for 24 h were 5.74%, 14.49%, and 33.05%, and for 48 h were 9%, 21.39%, and 40.49%, respectively. Ursolic acid suppressed the phosphorylation of EGFR, ERK1/2, p38 MAPK, and JNK, which is well correlated with its growth inhibitory effect. 10, 20, and 40 μmol/L ursolic acid significantly inhibited the proliferation of EGF-stimulated HT-29 cells ($P<0.05$). Cell proliferation was most significantly inhibited when treated with 10 and 20 μmol/L ursolic acid combined with 200 nmol/L AG 1478 or 10 μmol/L U0126 ($P<0.01$). Besides, it also down-regulated the expression of Bcl-2 and Bcl-xL and activated caspase-3 and caspase-9. Conclusion: Ursolic acid induces apoptosis in HT-29 cells by suppressing the EGFR/MAPK pathway, suggesting that it may be a potent agent for the treatment of colorectal cancer.

Key words: Colon cancer, Ursolic acid, Epidermal growth factor receptor (EGFR), Mitogen-activated protein kinase (MAPK), Apoptosis

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INTRODUCTION

Colorectal carcinoma (CRC), one of the most commonly seen cancers globally (Jemal *et al*., 2008), is the fifth highest cancer in incidence and mortality in the Chinese population and is still increasing (Cao *et al*., 2008; Sung *et al*., 2005). A number of genes and proteins, such as the epidermal growth factor receptor (EGFR), have now been found to be abnormally expressed in cancers (Dougherty *et al*., 2008). The mitogen-activated protein kinase (MAPK) pathway is one of the main downstream effectors of the EGFR, including the extracellular signal-regulated kinase (ERK), the c-Jun N-terminal kinase (JNK)/ stress-activated protein kinase (SAPK), and the p38 MAPK. A number of studies demonstrated that the EGFR/MAPK pathway was aberrantly activated in CRC, which is believed to be responsible for the proliferation, survival, and metastasis of cancer cells, suggesting a potential therapeutic value to identify approaches or drugs to inhibit this pathway (Roberts and Der, 2007; Schwartsmann *et al*., 2005).

A defect in the apoptosis pathway is another common event in many types of cancers including colorectal cancer. The overexpression of the B cell

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leukemia-2 (Bcl-2) family proteins, namely, Bcl-2 and Bcl-xL, has been shown as two critical factors blocking apoptosis (Kroemer, 1997). Besides, a series of cysteine proteases known as caspases are key initiators and executors of apoptosis in response to various intrinsic or extrinsic death signals. Therefore, modulation of these molecules may help to reestablish the apoptotic machinery and promote cell death.

Over the past decades, the treatment options for colorectal cancer have undergone tremendous changes; however, the clinical results are far from satisfaction (Saltz, 2008). Many traditional Chinese medicinal herbs, such as *Hedyotic diffusa* and *Radix actinidiae*, are commonly used in colorectal cancer treatment in China, but the mechanism is not yet understood. Ursolic acid (Fig.1a) is an active compound present in these herbs. Previous studies reported that ursolic acid exhibits a broad range of pharmacological properties such as anti-inflammatory, antiviral, antioxidant, hepatoprotective, cytotoxic, anti-tumor, anti-angiogenesis, anti-metastasis activities (Ikeda *et al*., 2008). Recent studies showed that ursolic acid inhibited the proliferation and induced apoptosis of HCT-15 and CO115 colon carcinoma cells (Xavier *et al*., 2009). Moreover, ursolic acid activated the intestinal alkaline sphingomyelinase in HT-29 human colon carcinoma cells in vitro and in vivo (Tan *et al*., 2006; Andersson *et al*., 2003; 2008). Because of the critical role of EGFR/MAPK signal pathway in CRC, we hypothesize that ursolic acid may inhibit the proliferation and induce the apoptosis of HT-29 cells through EGFR/MAPK signal pathway.

MATERIALS AND METHODS

Materials

Ursolic acid was purchased from Sigma (MO, USA), dissolved in dimethyl sulfoxide (Sigma, MO, USA), and stored at −20 °C. Human recombinant epidermal growth factor (EGF), U0126, and AG 1478 were purchased from Cell Signal Technology (MA, USA). All the primary antibodies used in the study including phospho-EGFR, EGFR, phospho-ERK1/2, ERK1/2, phospho-JNK, and phospho-p38 MAPK, caspase-3, caspase-9, Bcl-2, and Bcl-xL were purchased from Cell Signal Technology. The secondary antibodies, horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin G (IgG) or anti-mouse IgG, were purchased from Santa Cruz Biotechnology (CA, USA).

Cell line and culture conditions

Human HT-29 colon cancer cells were purchased from American Type Culture Collection (VA, USA) and were maintained in McCoy's 5A medium (Invitrogen, NY, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, and 1% (v/v) penicillin and streptomycin (Invitrogen, NY, USA). Cells were grown at 37 °C in a humidified atmosphere consisting of 5% CO_2.

Cell proliferation assay

Cell proliferation was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining as described by Denizot and Lang (1986) with minor modification. Cells (3×10^3~6×10^3) were incubated in 96-well plates in the presence (10, 20, 40 μmol/L) or absence of ursolic acid for 48 h in a final volume of 200 μl. At the end of the treatment, 20 μl MTT (5 mg/ml in phosphate buffered saline (PBS)) was added to each well, and the samples were incubated for an additional 4 h at 37 °C. The purple-blue MTT formazan precipitate was dissolved in 100 μl dimethyl sulfoxide (DMSO). The activity of the mitochondria reflecting cellular growth and viability was evaluated by an ELX800 Micro Plate Reader (Bio-Tek Instruments Inc., Highland Park, USA) at 570 nm.

Apoptosis assay

Apoptotic rates were determined by flow cytometry analysis using a fluorescence-activated cell sorting (FACS) caliber (Becton Dickinson, CA, USA) and annexin V-fluorescein isothiocyanate (FITC)/ propidium iodide (PI) kit (Sigma, MO, USA). Staining was performed according to the manufacturer's instructions. The percentage of the early apoptosis was calculated by annexin V-positivity and PI-negativity, while the percentage of the late apoptosis plus necrosis was calculated by annexin V-positivity and PI-positivity.

Western blot analysis

Western blot analysis was performed according to the Cell Signaling Technology Company's

instruction with a minor adjustment. Briefly, cells (5×10^5 well^{-1}) were incubated in the presence or absence of drugs for various time intervals and lysed in a sample buffer (Pierce, Rockford, IL, USA). Protein (60 μg) was applied to a 5% to 10% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a nitrocellulose membrane, and then detected using the proper primary and secondary antibodies using chemiluminescence kit (Pierce, Rockford, IL, USA).

Statistical analysis

The Student's *t*-test was used for comparison between groups and $P<0.05$ was considered significant.

RESULTS

Ursolic acid inhibited the proliferation and induced the apoptosis of HT-29 cells

Previous studies showed that ursolic acid had strong inhibitory effects against a panel of cancer cell lines (Subbaramaiah *et al*., 2000; Shishodia *et al*., 2003). In this study, we examined whether ursolic acid could inhibit the proliferation of HT-29 cells. As shown in Fig.1b, ursolic acid inhibited the growth of HT-29 cells in dose- and time-dependent manners. The median inhibition concentration (IC_{50}) values for 24, 48, and 72 h were 26, 20 and 18 μmol/L, respectively.

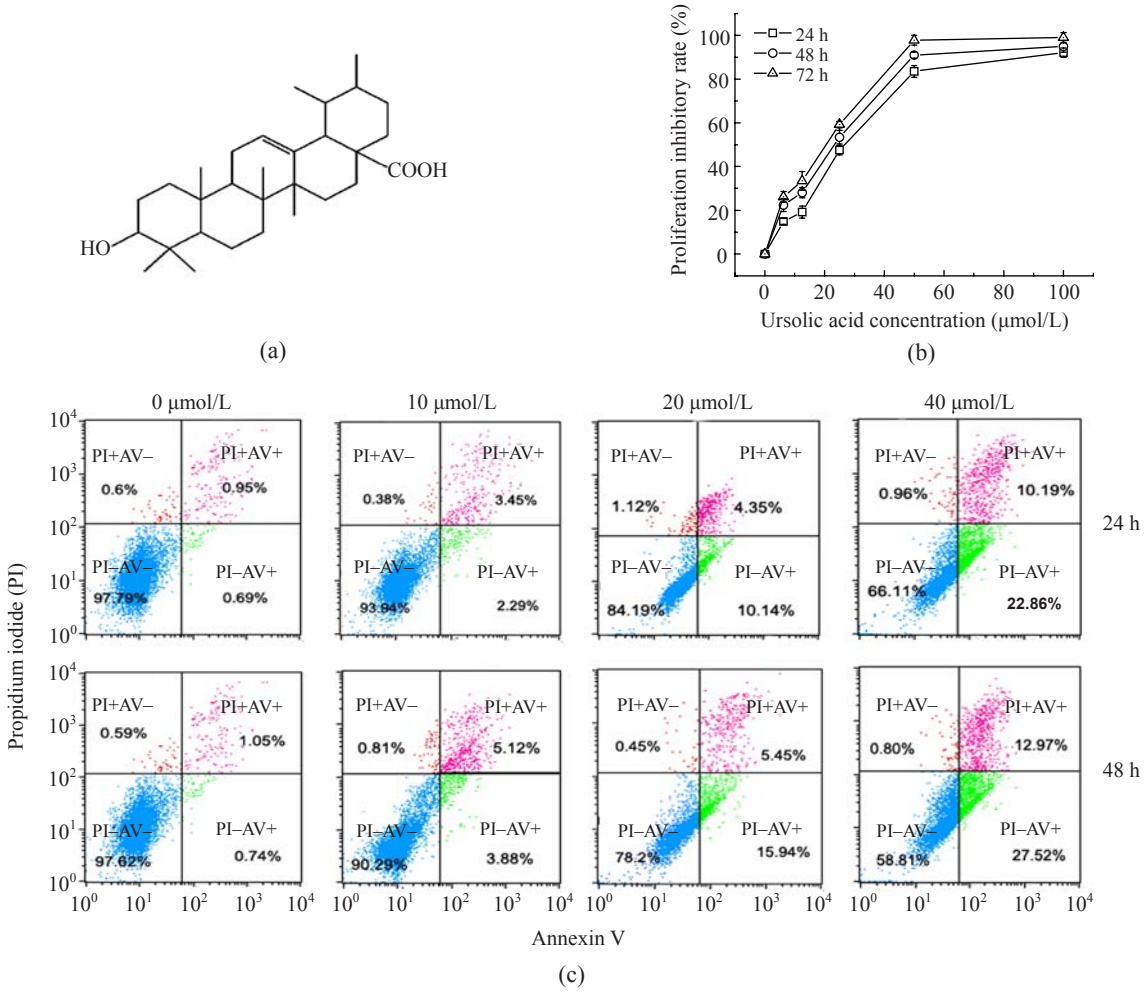

Fig.1 Effects of ursolic acid on the proliferation and the apoptosis of HT-29 cells

(a) The chemical structure of ursolic acid; (b) Ursolic acid inhibited the proliferation of HT-29 cells in dose- and time-dependent manners. Data are presented as mean±*SD* from three independent experiments; (c) Ursolic acid caused strong apoptotic death in HT-29 cells after 24 or 48 h treatment. Cells were collected after 0, 10, 20, and 40 μmol/L ursolic acid treatments, stained with annexin V/PI, and evaluated by FACS analysis

We next examined the effect of ursolic acid on apoptotic death of HT-29 cells by FACS analysis by annexin V/PI staining. Fig.1c shows the percentages of viable, early apoptotic, late apoptotic, and dead cells treated with 0, 10, 20, and 40 μmol/L ursolic acid for 24 and 48 h, respectively. Annexin V/PI staining of the control cells (no ursolic acid treatment) was observed in a large population of viable cells. However, cells treated with 40 μmol/L ursolic acid for 24 h resulted in a clear shift from live cell population to early and late apoptotic cell population, and stronger apoptotic effect was observed at 48 h. As shown in the bottom panel of Fig.1c, ursolic acid induced a significant increase in the early and late apoptotic cells at 10, 20, and 40 μmol/L, and the total percentages of apoptotic cells (including the early and late apoptotic cells) were 9%, 21% and 41%. This quantitative analysis of cell death clearly demonstrated that ursolic acid induced the apoptosis of HT-29 cells in both dose- and time-dependent manners.

Ursolic acid inhibited the proliferation of HT-29 cells by suppressing the phosphorylation of EGFR

Previous reports have demonstrated that EGFR was overexpressed in a variety of human epithelial malignancies including colon carcinoma (Grandis and Sok, 2004). Here, we examined whether ursolic acid could inhibit the EGFR signaling pathway. As shown in Fig.2a, cells stimulated with 100 ng/ml EGF in the presence of serum for 10 min significantly increased phospho-EGFR in HT-29 cells, compared with the control cells. As expected, AG 1478, a specific pharmacological inhibitor of EGFR, at 200 nmol/L decreased the EGF-induced phosphorylation of EGFR in HT-29 cells. On the other hand, cells treated with 10, 20, and 40 μmol/L ursolic acid for 24 h decreased the phosphorylation of EGFR in a clear dose-dependent manner. The suppression effect of ursolic acid at 40 μmol/L was comparable to that of AG 1478. Ursolic acid also decreased the phosphorylation of EGFR in a time-dependent manner (Fig.2b). In order to investigate whether the anti-proliferative effect of ursolic acid was correlated with the suppression of EGFR phosphorylation, we further tested the proliferative rate of HT-29 cells treated with or without ursolic acid after EGF stimulation for 48 h. As shown in Fig.2c, the proliferation rate of EGF-stimulated HT-29 cells increased by 39% compared with the control cells. This increase is correlated with the increase of EGFR phosphorylation by Western blotting analysis. Cells treated with AG 1478 showed 68.5% decrease in proliferation rate compared with the EGF-stimulated cells. Meanwhile, cells treated with 10, 20, and 40 μmol/L ursolic acid showed significant dose-dependent inhibition on cell proliferation. The rates of proliferation decreased by 38.5%, 65.9%, and 78.4%, respectively, compared with EGF-stimulated cells. Cells treated with both

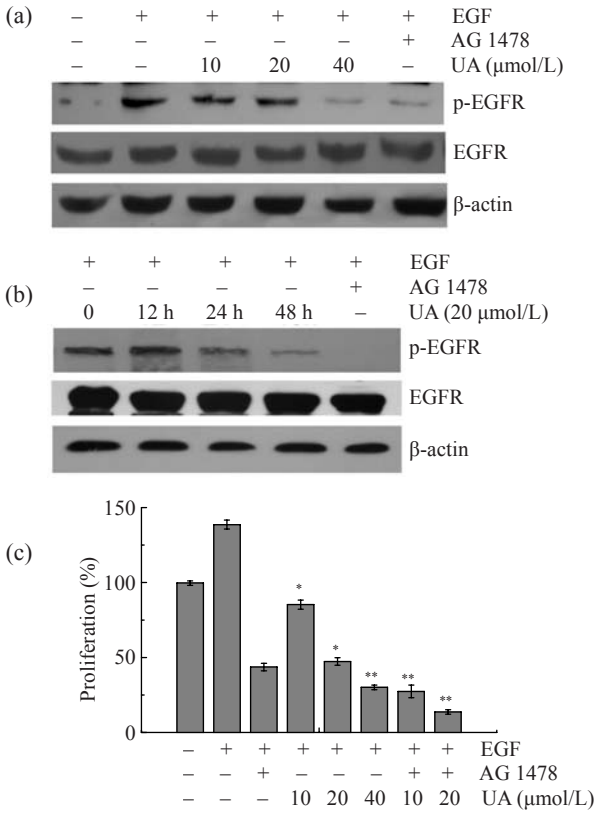

Fig.2 Ursolic acid (UA) inhibited EGFR phosphorylation of HT-29 cells

(a) HT-29 cells were stimulated by 100 ng/ml EGF in the presence of serum for 10 min before being treated with or without ursolic acid at various concentrations or AG 1478 at 200 nmol/L for 24 h; (b) HT-29 cells were stimulated by 100 ng/ml EGF for 10 min before being treated with 20 μmol/L ursolic acid for 12, 24, and 48 h or 200 nmol/L AG 1478 for 48 h; (c) Ursolic acid diminished EGF-induced cell proliferation in a dose-dependent manner. Ursolic acid, combined with AG 1478, had the strongest inhibitory effect against the EGF-stimulated proliferation of HT-29 cells for 48 h. Values (percentage of control) given were derived from the average of at least three independent experiments (±*SD*); * and ** denote statistical significance at $P<0.05$ and $P<0.01$, respectively, compared with EGF-stimulated cell proliferation

ursolic acid and AG 1478 showed a stronger inhibition than treated by either one of the two, suggesting that ursolic acid and AG 1478 synergetically inhibited HT-29 cells. These findings demonstrate that ursolic acid inhibits the proliferation of HT-29 cells by suppressing EGFR phosphorylation.

Ursolic acid inhibited the phosphorylation of ERK1/2, p38 MAPK, and JNK in HT-29 cells

Since several lines of evidence have indicated that overexpression and activation of the MAPK pathway play an important part in the progression of CRC (Fang and Richardson, 2005), we examined the possible involvement of the MAPK pathway in HT-29 cells treated with ursolic acid. As shown in Fig.3a, ursolic acid significantly inhibited the activation of ERK1/2 in a dose-dependent manner, and ursolic acid (40 μmol/L for 48 h) near completely inhibited the phosphorylation of ERK1/2. This is comparable with the inhibitory effect of U0126, a specific pharmacological inhibitor of MEK1/2, which is the upstream molecule of ERK1/2. Moreover, ursolic acid decreased the phosphorylation of ERK1/2 in a time-dependent manner (Fig.3b). Cells treated with 20 μmol/L ursolic acid for 48 h resulted in a nearly complete inhibition of ERK1/2 phosphorylation. This is comparable with the inhibitory effect of U0126. We further tried to delineate whether the growth inhibition of HT-29 cells under ursolic acid treatment was correlated with the inhibition of the EGFR-ERK1/2 signaling pathway. As shown in Fig.3c, the proliferation of HT-29 cells treated with EGF increased by 39% compared with the control cells. The proliferation of cells treated with 10 μmol/L U0126 for 48 h decreased significantly, suggesting a close relationship between the inhibition of ERK1/2 and the decreased cell proliferation. The treatments of ursolic acid at 10, 20, and 40 μmol/L for 48 h inhibited cell growth in a clear dose-dependent manner. 40 μmol/L ursolic acid showed a similar inhibitory effect to U0126, which is well correlated with the decrease of ERK1/2 phosphorylation by Western blotting analysis. The strongest inhibition effect was also observed when cells were treated with 20 μmol/L ursolic acid and 10 μmol/L U0126 for 48 h. We further examined whether ursolic acid had any effect on the phosphorylation of p38 MAPK and JNK, two other terminal serine/threonine kinases in the MAPK pathway. As shown in Fig.3d, ursolic acid dose-dependently decreased the phosphorylation of p38 MAPK and JNK and reached an almost complete inhibition at 40 μmol/L. The results demonstrate that

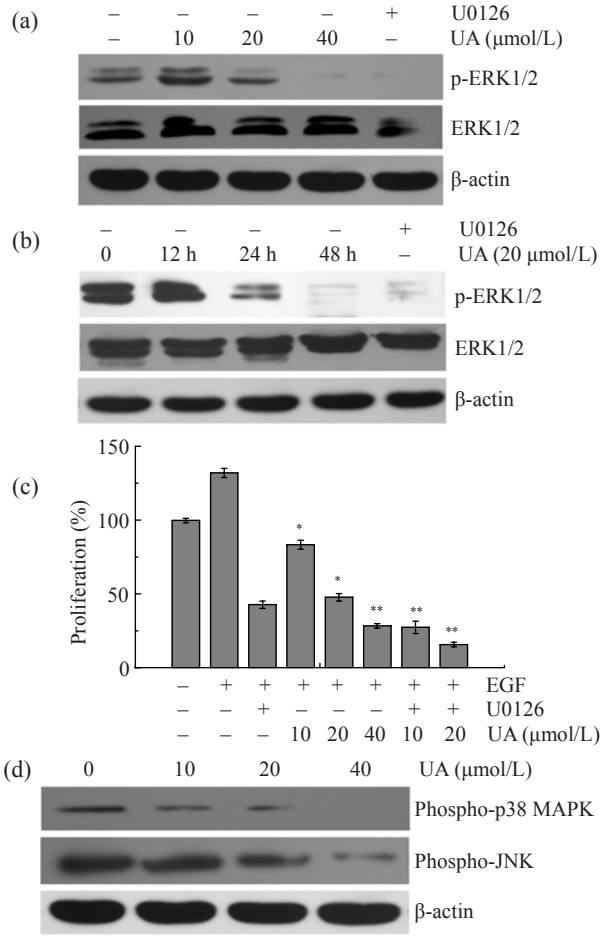

Fig.3 Ursolic acid (UA) inhibited ERK1/2, p38 MAPK, and JNK phosphorylation in HT-29 cells

(a) Cells were treated with varying concentrations of ursolic acid (10, 20, and 40 μmol/L) or U0126 at 10 μmol/L for 48 h. Meanwhile, p-ERK1/2 and total ERK1/2 were detected by Western blot analysis; (b) Cells were treated with 20 μmol/L ursolic acid for 12, 24, and 48 h or 10 μmol/L U0126 for 48 h. Meanwhile, p-ERK1/2 and total ERK1/2 were detected by Western blot analysis; (c) HT-29 cells were stimulated by 100 ng/ml EGF in the presence of serum for 10 min and were then treated with varying concentrations of either ursolic acid, U0126, or a combination of both for 48 h. Cell proliferation rate was determined by MTT assay. Values (percentage of control) are shown as mean±standard deviation of triplicate samples. * and ** denote statistical significance of $P<0.05$ and $P<0.01$, respectively, compared with EGF-stimulated cell proliferation; (d) Ursolic acid dose-dependently inhibited the phosphorylation of p38 MAPK and JNK. Cells were treated with 0, 10, 20, and 40 μmol/L ursolic acid for 48 h

ursolic acid inhibited the phosphorylation of ERK1/2, p38 MAPK, and JNK in both dose- and time-dependent manners, in agreement with ursolic acid's anti-proliferation effect in HT-29 cells.

Ursolic acid induced apoptosis of HT-29 cells via down-regulation of Bcl-2 and Bcl-xL and activation of caspase-3 and caspase-9

Since ursolic acid induced apoptosis of HT-29 cells (Fig.1c), we further assessed the effect of ursolic acid on the Bcl-2 and caspase family proteins, two most relevant classes of apoptotic-regulatory proteins. As shown in Fig.4a, ursolic acid down-regulated the expression of Bcl-2 and Bcl-xL in a dose-dependent manner. Ursolic acid also activated caspase-3 and caspase-9, and the clear cleaved caspase-3 and caspase-9 were seen at the treatment of ursolic acid at 40 μmol/L for 48 h. The results suggest that ursolic acid induced apoptosis in HT-29 cells via the down-regulation of Bcl-2 and Bcl-xL and the activation of caspase-3 and caspase-9 (Fig.4b).

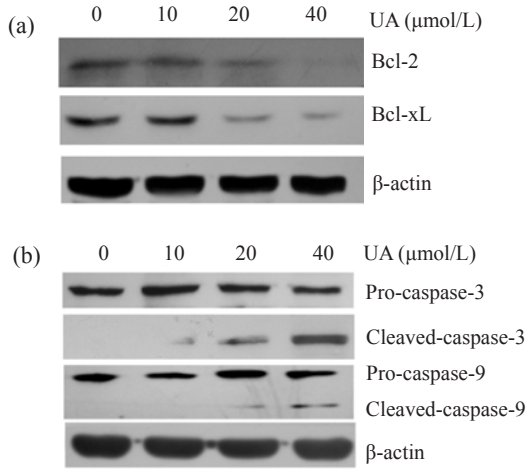

Fig.4 Ursolic acid (UA) down-regulated the expression of Bcl-2 and Bcl-xL and activated caspase-3 and caspase-9

(a) Cells were treated with 0, 10, 20, and 40 μmol/L ursolic acid for 48 h and the expression levels of Bcl-2 and Bcl-xL were detected by Western blot analysis; (b) Cells were treated with 0, 10, 20, and 40 μmol/L ursolic acid for 48 h and the activation of caspase-3 and caspase-9 was detected by Western blot analysis

DISCUSSION

Understanding the mechanism of CRC carcinogenesis has been increasingly important (Li and Lai, 2009). Many studies indicated that the EGFR/MAPK pathway played a critical role in colon cancer carcinogenesis, which has interested many oncologists in developing novel non-toxic natural alternative therapeutic reagents interfering with this signal pathway. Previous studies reported that ursolic acid could inhibit the proliferation and induce the apoptosis in a number of cancer cells including the CRC cells (Huang *et al*., 1994; Hsu *et al*., 2004; Manu and Kuttan, 2008; Pathak *et al*., 2007). The possible mechanisms include inhibiting nuclear factor kappaB (NF-κB) or phospho-protein kinase B (Akt) activation, down-regulating cyclooxygenase-2 (COX2), matrix metalloproteinase-9 (MMP-9) and Cyclin D1, increasing the DNA repair ability, activating the Bcl-2 and caspase family proteins, increasing the alkaline sphingomyelinase (Alk-SMase) activity, etc. (Huang *et al*., 1994; Cha *et al*., 1996; Xavier *et al*., 2009; Shishodia *et al*., 2003). Our study demonstrates that ursolic acid inhibits the proliferation of HT-29 cells by suppressing the EGFR/MAPK signaling pathway. We found that ursolic acid inhibited the phosphorylation of EGFR, ERK1/2, p38 MAPK and JNK in HT-29 cells in dose- and time-dependent manners. The inhibition effect on cell proliferation resulted from the suppression of the EGFR/MAPK pathway, which is in accordance with a previous report that ursolic acid could inhibit phorbol 12-myristate 13-acetate (PMA)-mediated activation of protein kinase C, ERK1/2, JNK, and p38 MAPK in 184B5/HER human mammary epithelial cells (Subbaramaiah *et al*., 2000). However, in BRAF mutated CO115 colon cells, it has been reported that ursolic acid had no effect on phospho-ERK but could decrease Akt phosphorylation (Xavier *et al*., 2009). This result indicates that ursolic acid might activate multiple signal pathways depending on the type of cancer cells. The detailed mechanisms may need further investigation. In this study, we showed that ursolic acid induced strong apoptosis in HT-29 cells. The apoptotic effect may be achieved by activation of caspase-3 and caspase-9 and inhibition of Bcl-2 and Bcl-xL expression, which well agrees with others' reports (Manu and Kuttan, 2008; Huang *et al*., 1994).

In conclusion, this study shows that ursolic acid is a multi-target compound that strongly inhibits the EGFR/MARK pathway, down-regulates the expression of Bcl-2 and Bcl-xL, and activates caspase-3 and

caspase-9 in HT-29 cells. Th results suggest that ursolic acid, an active compound present in traditional Chinese medicinal herbs *Hedyotic diffusa* and *Radix actinidiae*, may be a potent agent for the treatment of colorectal cancer.

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