Ursolic acid activates the apoptosis of prostate cancer via ROCK/PTEN mediated mitochondrial translocation of coflin-1

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Abstract. Ursolic acid has various pharmacological activities, and can reduce blood fat as well as having antitumor, anti-inflammatory and antiviral properties. However, the pro-apoptotic mechanism by which ursolic acid influences human prostate cancer requires additional study. The aim of the present study was to assess whether ursolic acid activates the apoptosis of prostate cancer and to investigate the mechanism by which the Rho-associated protein kinase 1 (ROCK1)/phosphatase and tensin homolog (PTEN) signaling pathway performs a role in ursolic acid-mediated coflin-1 to induce apoptosis in human prostate cancer. Firstly, the present study determined the pro-apoptotic mechanism by which ursolic acid influences the cell proliferation and apoptosis of human prostate LNCaP cancer cells. Caspase-3/9 activities and ROCK1, PTEN, Cofilin-1 and cytochrome c protein expression levels were also analyzed. In the present study, it is reported that the pro-apoptotic mechanism of ursolic acid potently suppressed the cell proliferation of human prostate LNCaP cancer cells. The present study revealed that the mediation of ROCK1/PTEN-cofilin-1/cytochrome c protein expression activates caspase-3/9 activities which subsequently induced the apoptosis of human prostate cancer cells. In conclusion, these findings demonstrated that ursolic acid activates the apoptosis of prostate cancer via ROCK/PTEN mediated coflin-1/cytochrome c which mediated caspase-3/9 activities.

Introduction

In western countries, prostate cancer is the most common type of non-malignant skin tumor and is the leading cause of cancer-associated mortality (1). Castration remains the standard method for the treatment of prostate cancer, particularly metastatic prostate cancer, however it cannot successfully treat this disease (2). It is predicted that ~80% of patients with prostate cancer experience a recurrence of clinical symptoms or a change to the volume of prostate cancer following the removal of the androgen (3). However, following a median treatment period of 18-24 months, almost all patients enter the hormone independent phase of prostate cancer (4).

Cofilin is a type of actin-binding protein that exists in eukaryotes, and has a low molecular weight (5). The Cofilin-1 gene is located on hormone 11q13 and is expressed in various non-muscular tissues, particularly in the liver and brain (6). The progression of tumor cells occurs in a complexed micro-environment through migration (7), by invasion into pseudopods. During the invasion and metastasis of tumor cells, Cofilin-1 performs an essential role in the remodeling of the actin skeleton (8). A previous study suggested that expression levels of Cofilin-1 and changes to cellular activities have been identified in tissues of oral squamous cell carcinoma, renal cell carcinoma and ovarian cancer as well as in in vitro cultured carcinoma cell lines (9).

Ursolic acid (UA) is a pentacyclic triterpenoid, with the chemical name, molecular formula and molecular weight of (3β)-3-Hydroxy-urs-12-en-28-oic acid, C30H48O3 and 456, respectively (10). UA has a wide distribution, and exists in the form of dissociation or glycoside in Sambucus chinensis, Foulum eriobotryae, bearberry, glossy privet fruits, plantain herbs, hawthorn, selfheal and Oldenlandia diffusa (11). UA has low toxicity and few side effects, and has various pharmacological activities including anti-hepatitic, anti-tumor, anti-inflammatory, anti-viral and reducing blood lipids (12-14). Additional investigation into the effect of UA on the apoptosis of prostate cancer and its possible signal transduction pathway may provide a potential drug target for the clinical treatment of patients with prostate cancer.

Materials and methods

Cell culture. Human prostate cancer LNCaP cells were cultured in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.),
100 g/ml streptomycin and 100 U/ml penicillin and maintained at a high humidity, at 37°C under 5% CO₂.

**Cell viability analysis.** Cell viability was measured using an MTT assay (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). LNCaP cells were seeded in 96-well plates at 1x10⁴ cells/well and incubated with varying concentrations of ursolic acid (0-80 µM) and incubated for 48 h at 37°C. The LNCaP cells were prepared using a Proteo JET cytoplasmic protein extraction kit (Fermentas; Thermo Fisher Scientific, Inc.). Protein concentration was measured using bicinchoninic acid (BCA; Beyotime Institute of Biotechnology, Haimen, China). Proteins (50 µg per lane) were loaded onto 10-12% SDS-PAGE for separation by electrophoresis, transferred onto polyvinylidene difluoride (PVDF) membranes, and blocked using TBST (and 0.1% Tween-20) containing 5% non-fat milk. The PVDF membranes were then incubated overnight at 4°C with the following primary antibodies: Anti-ROCK (dilution, 1:1,000; cat. no. sc-33779); anti-phosphorylation-PTEN (dilution, 1:1,000; cat. no. sc-101789); anti-Cofilin-1 (dilution, 1:1,000; cat. no. sc-33779); anti-Cytochrome c (dilution, 1:3,000; cat. no. sc-7159); and β-actin (dilution, 1:1,000; cat. no. sc-7210) (all from Santa Cruz Biotechnology, Inc. Dallas, TX, USA). PVDF membranes were subsequently incubated with secondary antibody (1:2,000; cat. no. sc-2004; Santa Cruz Biotechnology, Inc. Dallas, TX, USA) for 1 h at 37°C and were visualized using BeyoECL Plusenhanced chemiluminescence (P0018, Beyotime Institute of Biotechnology) and analyzed using Image Lab_3.0 (Bio-Rad Laboratories, Inc.).

**Flow cytometric analysis of apoptosis.** An Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) kit (BD Biosciences, Franklin Lakes, NJ, USA) was used to measure apoptosis. LNCaP cells were seeded in 6-well plates at a density of 1x10⁶ cells/well (n=3), incubated with varying concentrations of ursolic acid (0-80 µM) and incubated for 48 h at 37°C. The LNCaP cells were prepared using a Proteo JET cytoplasmic protein extraction kit (Fermentas; Thermo Fisher Scientific, Inc.). Protein concentration was measured using bicinchoninic acid (BCA; Beyotime Institute of Biotechnology, Haimen, China). Proteins (50 µg per lane) were loaded onto 10-12% SDS-PAGE for separation by electrophoresis, transferred onto polyvinylidene difluoride (PVDF) membranes, and blocked using TBST (and 0.1% Tween-20) containing 5% non-fat milk. The PVDF membranes were then incubated overnight at 4°C with the following primary antibodies: Anti-ROCK (dilution, 1:1,000; cat. no. sc-33779); anti-phosphorylation-PTEN (dilution, 1:1,000; cat. no. sc-101789); anti-Cofilin-1 (dilution, 1:1,000; cat. no. sc-33779); anti-Cytochrome c (dilution, 1:3,000; cat. no. sc-7159); and β-actin (dilution, 1:1,000; cat. no. sc-7210) (all from Santa Cruz Biotechnology, Inc. Dallas, TX, USA). PVDF membranes were subsequently incubated with secondary antibody (1:2,000; cat. no. sc-2004; Santa Cruz Biotechnology, Inc. Dallas, TX, USA) for 1 h at 37°C and were visualized using BeyoECL Plusenhanced chemiluminescence (P0018, Beyotime Institute of Biotechnology) and analyzed using Image Lab_3.0 (Bio-Rad Laboratories, Inc.).

**Analysis of caspase-3 and caspase-9 protease activity.** Caspase-3 and caspase-9 protease activity was measured using Caspase 3 Activity Assay Kit and Caspase 9 Activity Assay Kit (Promega Corporation, Madison, WI, USA). LNCaP cells were seeded in 96-well plates at 1x10⁴ cells/well and incubated with varying concentrations of UA (0-80 µM) for 48 h at 37°C. Subsequently, 100 µl of Caspase-Glo 3 or Caspase-Glo 9 reagent was added to each well and incubated at room temperature for 2 h. Caspase-3 and caspase-9 protease activity was measured using a TD 20/20 luminometer (Promega Corporation).

**Statistical analysis.** Data are presented as the mean ± standard deviation and were analyzed using the statistical software SPSS version 11.0 (SPSS, Inc., Chicago, IL, USA). Statistical analysis was performed using a one way analysis of variance. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Ursolic acid suppresses cell proliferation of prostate cancer.** The chemical structure of ursolic acid is shown in Fig. 1. The present study evaluated whether UA suppresses the cell proliferation of LNCaP prostate cancer using an MTT assay. When compared with 0 µM, ursolic acid caused a decrease in cell proliferation of LNCaP cells in a time- and dose-dependent manner (Fig. 2). The decrease in cell proliferation following treatments with: 50 µM of ursolic acid for 1 day; 30-50 µM ursolic acid for 2 days; and 20-50 µM of ursolic acid for 3 days were statistically significant (Fig. 2).

**Ursolic acid activates ROCK of prostate cancer.** To additionally investigate the effect of UA on the ROCK pathway of prostate cancer, the present study examined the protein expression of ROCK and cleaved ROCK in LNCaP cells. In comparison with the control group (0 µM ursolic acid), ROCK protein expression in LNCaP cells was significantly reduced and cleaved ROCK protein expression was significantly elevated in the 20 and 40 µM ursolic acid treatment groups (Fig. 3).

**Ursolic acid activates PTEN of prostate cancer.** To additionally investigate whether ursolic acid affects the PTEN pathway of prostate cancer, the present study detected the protein expression of phosphorylated PTEN (p-PTEN) in all the experimental groups. In comparison with the control group (0 µM ursolic acid), the p-PTEK protein expression of LNCaP cells was significantly promoted by 20 or 40 µM of ursolic acid (Fig. 3).

**Ursolic acid activates cofilin-1 of prostate cancer.** To improve the understanding of how ursolic acid affects cofilin-1 of prostate cancer, the present study detected cofilin-1 in LNCaP cells. Cofilin-1 protein expression in cytoplasmic LNCaP cells was observed to be significantly enhanced by treatment with 20 or 40 µM ursolic acid, compared with the control group (Fig. 4).

**Ursolic acid activates cytochrome c of prostate cancer.** The present study also examined the expression of cytochrome c in LNCaP cells in order to understand how ursolic acid affects cytochrome c in prostate cancer. As demonstrated in Fig. 4, there was a significant increase in cytochrome c protein.
Ursolic acid activates apoptosis of prostate cancer. The apoptosis of LNCaP cells was also investigated in order to investigate the effect of ursolic acid on prostate cancer cells. Fig. 5 demonstrates that treatments with 20 and 40 µM ursolic acid activate apoptosis of prostate cancer LNCaP cells.
ursolic acid significantly induced apoptosis of LNCaP cells in comparison with the 0 µM ursolic acid group (control).

**Ursolic acid activates caspase-3 and caspase-9 activities of prostate cancer.** The present study also investigated the mechanism of apoptosis, by examining caspase-3 and caspase-9 activities of LNCaP cells. Compared with the 0 µM ursolic acid group, caspase-3 and caspase-9 activities of LNCaP cells were significantly increased by the 20 and 40 µM ursolic acid treatment groups (Fig. 6).

**Discussion**

In western countries, prostate cancer is the most common type of cancer for males and also the leading cause of cancer associated mortality (3). This is also now observed in China. Castration is an effective treatment option for early prostate cancer patients (15). In the present study, it was observed that ursolic acid suppressed cell proliferation and induced the apoptosis of LNCaP cells. Park et al (16) suggested that ursolic acid induced apoptosis in prostate cancer PC-3 cells via caspase-9 and -3. Zhang et al (17) suggested that ursolic acid inhibits the proliferation and promotes apoptosis in human ovarian cancer. By inducing actin to connect with proteins and myosin, ROCK regulates protein phosphorylation through the contraction of actomyosin (18). The contraction of actomyosin is important for cell movement, and the inhibition of ROCK activity may negatively affect the contraction of actomyosin (19). Microtubules perform an essential role in maintaining cell polarity and extra-cellular transportation. The interaction between ROCK and Diaphanous-related formin (Dia) contribute to the regulation of cell polarity and canaliculus (20). The present study identified that ursolic acid significantly inhibited ROCK protein expression and elevated cleaved ROCK protein expression in LNCaP cells. Li et al (21) suggested that ursolic acid promotes the apoptosis of gastric cancer cells via the ROCK/PTEN pathway.

The importance of PTEN can be demonstrated by its frequent destruction to cancer cells (21). PTEN is the first known phosphatase which can inhibit tumor activity. In tumor cells, mechanisms which regulate the expression and functional changes of PTEN include the regulation of PTEN transcription, post-transcriptional regulation of encoding RNA, modification following interpretation and protein interactions (22). Slight changes to the expression levels of PTEN may influence the occurrence and progression of tumors (23). The present study demonstrated that ursolic acid significantly promoted p-PTEK protein expression in prostate cancer LNCaP cells. Wu et al (24) also reported that ursolic acid induced apoptosis in K562 cells by upregulating PTEN gene expression and cytochrome c. Li et al (21) suggested that ursolic acid promotes the apoptosis of gastric cancer cells through the ROCK/PTEN pathway.

Cofilin-1 is a fundamental regulatory factor in the invasion and metastasis of cancer cells (25). The overexpression of cofilin-1 increases the speed of tumor migration, and the inhibition of its expression can therefore significantly reduce the invasion of tumor cells (6,9). The present study revealed that ursolic acid significantly enhanced cofilin-1 protein expression in LNCaP cells. Li et al (21) reported that ursolic acid promotes the apoptosis of gastric cancer cells via ROCK/PTEN mediated cofilin-1 expression in the SGC-7901 cell line.

Cytochrome c is the control center for cell movement, and is not only the center of cellular respiratory chains and oxidative phosphorylation, but is also the regulatory center of cell apoptosis (26). The release of cytochrome c is a key step in cellular apoptosis. Under the conditions of deoxyadenosine triphosphate (dATP), cytochrome can combine with apoptotic protease activating factor 1 (APaf-1), which in turn promotes the formation of polymers and also enhances the formation of apoptosisosome (27). Activated caspase-9 results in the activation of other caspases including caspase-3. Caspase-3 triggers cascade reactions of Caspases, which subsequently results in apoptosis (28). A new study demonstrated that ursolic acid significantly increased the protein expression of cytochrome...
c and augmented the activities of caspase-3 and caspase-9 in LNCaP cells (28). In addition, Shyu et al (12) suggested that ursoic acid can induce the apoptosis of human hepatocellular carcinoma cells via the activation of caspase-9 and caspase-3. Similarly, Park et al (16) suggested that ursoic acid induced apoptosis in prostate cancer PC-3 cells through caspase-9 and caspase-3. Wu et al (24) also reported that ursoic acid induced apoptosis following the upregulation of PTEN and cytochrome c in K562 cells.

In conclusion, the present study demonstrates that ursoic acid activates the apoptosis of prostate cancer, at least in part by directly targeting ROCK/PTEN mediated mitochondrial translocation of cofilin-1. In future studies, the authors aim to focus on the drug development of ursoic acid for the treatment of human prostate cancer.

References


