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Original Article

Oleanolic acid suppresses the proliferation of human bladder cancer by Akt/mTOR/S6K and ERK1/2 signaling

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Abstract: Oleanolic acid has significant pharmacological activities, such as anti-tumor, regulating blood sugar level and liver protection, which are more effective compared with free aglyconeoleanolic acid. However, it is still unknown if oleanolic acid affects the proliferation of human bladder cancer. We utilized T24 cells to study the effect of oleanolic acid on the proliferation and apoptosis of human bladder cancer. In this study, we found that the anticancer effect of oleanolic acid significantly suppressed cell proliferation and increased apoptosis and caspase-3 activity of T24 cells. Furthermore, Akt, mTOR and S6K protein expression was greatly inhibited in T24 cells under oleanolic acid treatment. Meanwhile, ERK1/2 of phosphorylation protein expression was significantly promoted by oleanolic acid treatment. Taken together, we provided evidences that oleanolic acid was Akt/mTOR/S6K and ERK1/2 signaling-targeting anti-tumor agent. These findings represent new evidences that oleanolic acid suppresses the proliferation of human bladder cancer by Akt/mTOR/S6K and ERK1/2 signaling, and oleanolic acid may be used to prevent human bladder cancer.

Keywords: Oleanolic acid, bladder cancer, Akt, mTOR, S6K, ERK1/2

Introduction

Bladder cancer is one of the most common tumors and its morbidity is ascending year by year [1]. Statistics from WHO demonstrates that morbidity of bladder cancer ranks sixth among male general tumors [2]. Furthermore, its morbidity ranks second among urologic neoplasms, which is behind that of prostatic cancer [2]. However, high re-currency and death rates of bladder cancer cannot be regarded as being in the same category with that of prostatic cancer and other urologic neoplasms.

As far as ischemia reperfusion injury is concerned, two death forms of nerve cells are cell necrosis and cell apoptosis [3]. Among these forms, apoptosis is also called programmed cell death. Activation of related genes, encoding and synthesis of related proteins are urgently required by this active programmed cell death process [4].

As a kind of serine/threonine protein kinase, mTOR is closely associated with activation of various signal transduction pathways [5]. It participates in essential biological process as cell growth and apoptosis, such as transcription, interpretation, metabolism, angiogenesis and regulation of cell cycles. Thus, it plays a fundamental role in cell growth and survival [6]. As a signaling molecule, phosphatidylinositol-3-kinase (P13K) exists in cells. When P13K on the surface of cytomembrane feels some special signal stimulus, it activates Akt [7]. Then, activated Akt could further phosphorylate a series of apoptosis proteins to promote the survival of nerve cells. Studies also found that the activation of P13K could increase size of central chord and quantities of synapsis. It was predicted that it was related with activation of Akt/S6K as mTOR was a substrate of Akt [8].

As a drug with natural source, oleanolic acid can be widely found in food, medicinal herb and other plants, which exists in free form or being

combined into glycosides [9]. In recent years, researches on antineoplastic mechanisms of oleanolic acid are not rare. It was suggested that oleanolic acid could inhibit the proliferation of colon cancer cells and lung carcinoma cells through various mechanisms [10, 11]. In this study, we intended to examine that oleanolic acid suppresses the proliferation of human bladder cancer. Our results showed that oleanolic acid inhibited human bladder cancer cells via Akt/mTOR/S6K and ERK1/2 signaling.

Materials and methods

Cell line culture

Human T24 cells were obtain from and cultured using Dulbecco's Modified Eagle Medium (DMEM, Gibco-BRL, Rockville, IN, USA) supplemented with 10% of fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO, USA), 4 mM glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin (Thermo Fisher Scientifi, Inc., Waltham, MA,USA) in a 5% CO_2 and humidified atmosphere at 37°C.

MTT assay

2×10^3 T24 cells per well were seeded in 96-well plates and cultured with 10, 20 and 50 μM of oleanolic acid for 1 and 2 days. 50 μL 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, 1 μg/mL, Sigma-Aldrich, St. Louis, MO, USA) was added to cell media and cultured in a 5% CO_2 and humidified atmosphere at 37°C for 4 h. Then medium was discarded 150 μL dimethylsulfoxide (DMSO, Invitrogen Company, Australia) were loaded in each well. Cell proliferation was measured at 570 nm with a reference wavelength of 655 nm with Microplate Reader Model 550 (BioRad Laboratories, Japan).

Apoptosis detection

4×10^5 T24 cells per well were seeded in 6-well plates and cultured with 10, 20 and 50 μM of oleanolic acid for and 2 days. T24 cells per well were stained with 50 μL Annexin V and 50 PI Apoptosis Detection Kit (Biovision, CA, USA) at darkness. The percentage of apoptotic cells measured using Flow cytometry (EPICS® ALTRA™; Olympus).

Active caspase-3 level

4×10^5 T24 cells per well were seeded in 6-well plates and cultured with 10, 20 and 50 μM of oleanolic acid for and 2 days. T24 cells per well were lysed in a lysis buffer containing 50 mM Tris-HCl. Miscible liquids was were centrifuged at 12,000 rpm for 20 min at 4°C and then supernatants were collected. Protein concentration was measured using BCA (Beyotime, Jiangsu, China). Equal quantities of protein were used to analyze using PathScan® Cleaved Caspase-3 (Asp175) Sandwich ELISA kit (Signaling Technology, Inc, Danvers, MA, USA). Active caspase-3 level was quantified using Microplate Reader Model 550 (BioRad Laboratories, Japan) at a wavelength of 450 nm.

Protein extraction and western blot analysis

4×10^5 T24 cells per well were seeded in 6-well plates and cultured with 10, 20 and 50 μM of oleanolic acid for and 2 days. T24 cells per well were lysed in a lysis buffer containing 50 mM Tris-HCl. Miscible liquids was were centrifuged at 12,000 rpm for 20 min at 4°C and then supernatants were collected. Protein concentration was measured using BCA (Beyotime, Jiangsu, China). Equal quantities of protein were separated to electrophoresis on 12% SDS-PAGE, and then transferred to a polyvinylidene difloride membrane (Thermo Fisher Scientific, Inc.). The membrane was blocking with 5% non-fat milk in Tris-buffered saline (TBS) for 1 h and incubated with rabbit anti-Akt (1:3000, Santa Cruz, USA), anti-phosphorylation-Akt (1:3000, Santa Cruz, USA), anti-mTOR (1:4000, Santa Cruz, USA), anti-S6K (1:4000, Santa Cruz, USA), anti-phosphorylation-ERK1/2 (1:2000, Santa Cruz, USA) and anti-β-actin (1:5000, Tiangen, Beijing, China) overnight at 4°C. The membrane was washed with Tris-buffered saline with 0.1% Tween 20 (TBST-T) followed by incubation with goat anti-rabbit horseradish peroxidase (HRP) conjugated secondary antibody (1:5000, Thermo Fisher Scientifi, Inc. USA) for 1 h at room temperature.

Statistical analysis

All values were reported as means ± SD. Student's t-test and 1-way repeated-measures ANOVA were used to compare groups. Data were considered to be statistically significant when and $P<0.01$ (**).

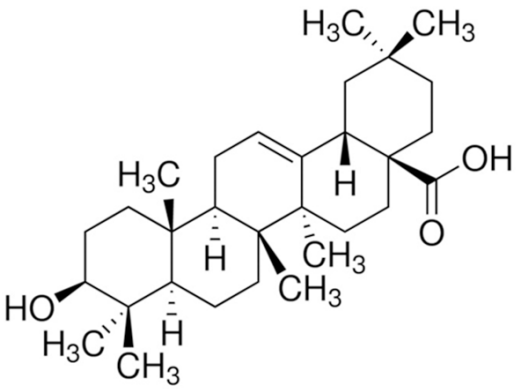

Figure 1. The chemical structure of oleanolic acid.

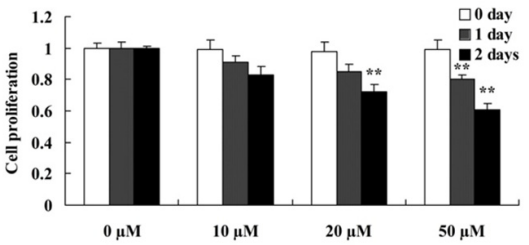

Figure 2. Oleanolic acid suppresses the cell proliferation of human bladder cancer. **P<0.01 compared with 0 μM oleanolic acid group.

Results

Oleanolic acid suppresses the cell proliferation of human bladder cancer

The chemical structure of oleanolic acid was expressed at **Figure 1**. We examined the effect of oleanolic acid on the cell proliferation of human bladder cancer. Oleanolic acid did indeed decrease the cell proliferation of T24 cells in a dose- and time-dependent manner, as shown in **Figure 2**. We observed that 20-50 μM oleanolic acid significantly reduce the cell proliferation of T24 cells with an incubation period of 3 days (**Figure 2**). Meanwhile, 50 μM oleanolic acid oleanolic acid significantly reduce the cell proliferation of T24 cells with an incubation period of 2 days (**Figure 2**).

Oleanolic acid suppresses the apoptosis of human bladder cancer

We used Annexin V/PI assay to detect the anticancer effect of oleanolic acid on suppresses the apoptosis of human bladder cancer. As shown in **Figure 3**, 50 μM oleanolic acid could

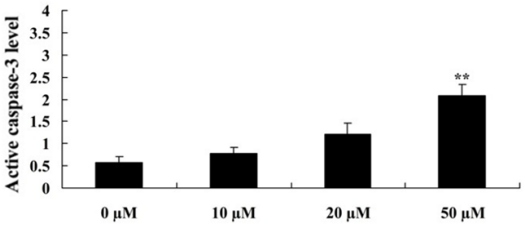

Figure 3. Oleanolic acid suppresses the apoptosis of human bladder cancer. **P<0.01 compared with 0 μM oleanolic acid group.

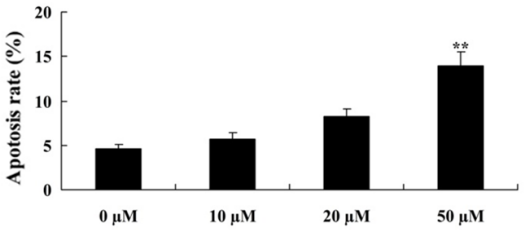

Figure 4. Oleanolic acid suppresses the capase-3 of human bladder cancer. **P<0.01 compared with 0 μM oleanolic acid group.

also induce apoptosis, which has statistical significance.

Oleanolic acid suppresses the capase-3 of human bladder cancer

Furthermore, we tested the anti-cancer effect of oleanolic acid on the capase-3 activity of human bladder cancer. The capase-3 activity of T24 cells exposed with 50 μM oleanolic acid was significantly increased, as shown in **Figure 4**.

Oleanolic acid suppresses the proliferation of human bladder cancer through Akt signaling

We explored molecular mechanism of oleanolic acid on human bladder cancer, we measured Akt and phosphorylation-Akt (p-Akt) protein expressions of T24 cells. Interestingly, 50 μM oleanolic acid also significantly inhibited p-Akt/Akt rate in T24 cells, compared with untreated group (**Figure 5**).

Oleanolic acid suppresses the proliferation of human bladder cancer through mTOR signaling

Next, we employed mTOR signaling to investigate molecular mechanism of oleanolic acid on

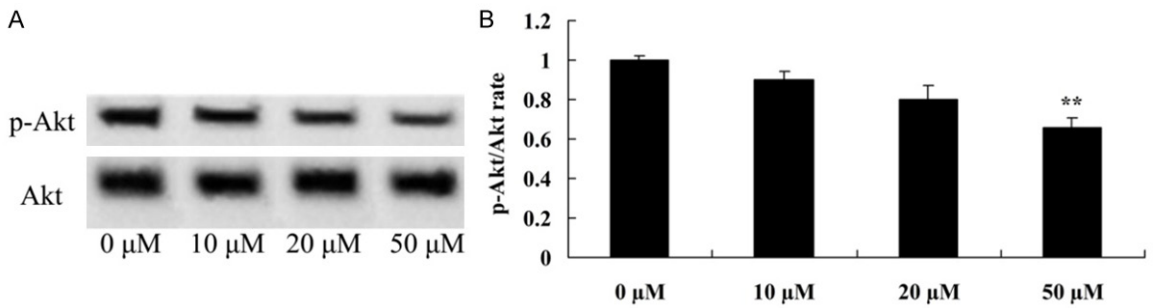

Figure 5. Oleanolic acid suppresses the proliferation of human bladder cancer through Akt signaling. Akt and p-Akt protein expression using Western blot analysis (A) and statistical analysis of Akt and p-Akt protein (B) in T24 cells. **P<0.01 compared with 0 μM oleanolic acid group.

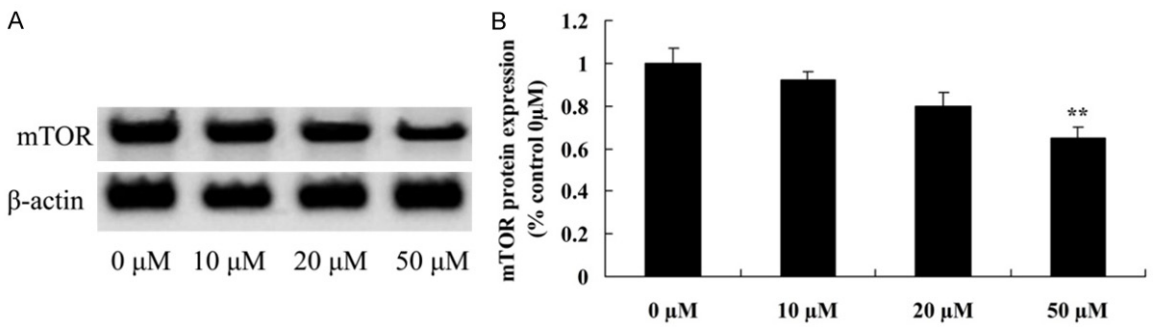

Figure 6. Oleanolic acid suppresses the proliferation of human bladder cancer through mTOR signaling. mTOR protein expression using Western blot analysis (A) and statistical analysis of mTOR protein (B) in T24 cells. **P<0.01 compared with 0 μM oleanolic acid group.

human bladder cancer. Significantly, T24 cells exposed with 50 μM oleanolic acid were suppressed mTOR protein expression, compared with untreated group (**Figure 6**).

Oleanolic acid suppresses the proliferation of human bladder cancer through S6K signaling

Also, we investigated if oleanolic acid showed a similar effect on the proliferation of human bladder cancer through S6K signaling. We found that S6K protein expression was significantly inhibited when T24 cells exposed with 50 μM oleanolic acid (**Figure 7**).

Oleanolic acid suppresses the proliferation of human bladder cancer through ERK1/2 signaling.

Furthermore, we probed the anti-cancer effect of oleanolic acid on ERK1/2 signaling, phosphorylation-ERK1/2 (p-ERK1/2) was analyzed using western blot analysis. As shown in **Figure 8**, 50 μM oleanolic acid was able to suppress p-ERK1/2 protein expression of T24 cells (**Figure 8**).

Discussion

Akt could inhibit cell apoptosis by regulating families related with cell apoptosis. It can also hand down survival signals by phosphorylation of mTOR and its downstream molecules as P70S6K and 4E-BP1 [12]. Thus, apoptosis of cells independent on p53 is inhibited and cell survival is promoted. Activated PI3K/Akt may further activate mTOR through TSC1/2. PI3K/Akt/mTOR signal transduction pathways play an important role in occurrence and progression of tumor and drug resistance by inducing survival, differentiation and vascularization [13]. In consequence, it becomes a new target for intervention treatment of tumors. In various malignant cancers as bladder cancer and non-small cell lung cancer, the activation of PI3K/Akt pathway could resist cell apoptosis triggered by chemotherapy and radiotherapy [14]. Selective inhibition of PI3K or Akt activity would

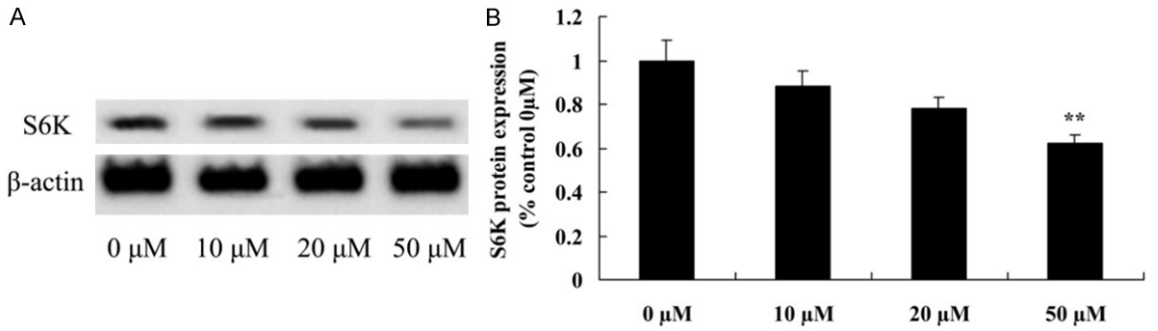

Figure 7. Oleanolic acid suppresses the proliferation of human bladder cancer through S6K signaling. S6K protein expression using Western blot analysis (A) and statistical analysis of S6K protein (B) in T24 cells. **P<0.01 compared with 0 μM oleanolic acid group.

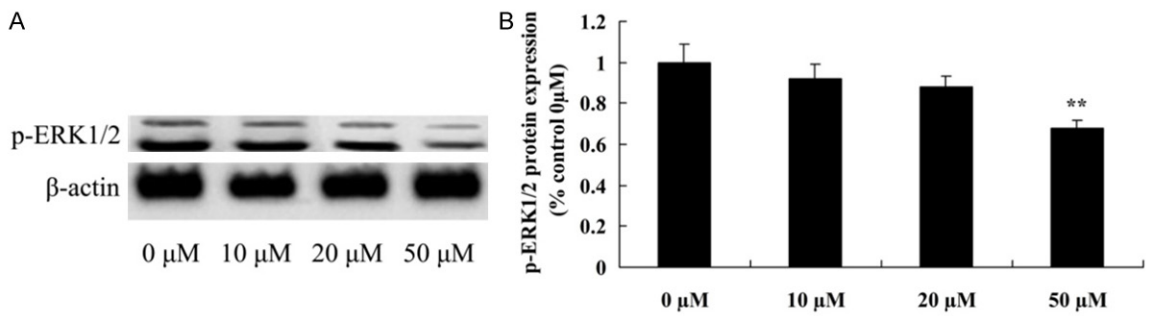

Figure 8. Oleanolic acid suppresses the proliferation of human bladder cancer through ERK1/2 signaling. p-ERK1/2 protein expression using Western blot analysis (A) and statistical analysis of p-ERK1/2 protein (B) in T24 cells. **P<0.01 compared with 0 μM oleanolic acid group.

decrease phosphorylation levels of Akt and increase sensitivity of apoptosis resulting from chemotherapy and radiotherapy [15]. Therefore, we found that oleanolic acid could reduce the cell proliferation, induced apoptosis and increased caspase-3 activity of T24 cells. Some literature reports that oleanolic acid suppress cell growth of gallbladder cancer, Liver cancer (Hep-G2), lung cancer (A549), gastric cancer (BGC-823), breast cancer (MCF-7) and prostatic cancer (PC-3) tumor cell lines [16, 17].

P70S6K is one downstream target of PI3K/Akt/mTOR signal pathways, whose functions are direct phosphorylation of RPS15a [18]. Therefore, it can facilitate interpretation of mRNA and related expressions of cell growth and differentiation. It plays an important role in cell growth and proliferation [15]. mTOR/P70S6K signal pathway plays an essential role in cell growth, differentiation, proliferation, migration and survival [12]. In this study, we originally provided evidences that oleanolic acid also significantly inhibited p-Akt/Akt rate in T24 cells. Lu et al. reported that OA induces apoptosis of MKN28 cells through AKT and JNK signaling pathways [19].

mTOR signal pathway occupies core status in cell growth. Activation of mTOR signal pathway could inhibit cell apoptosis induced by various stimulus; promote cycle progress, cell survival and proliferation [20]. Mtor signal pathway plays important role in proliferation of normal cells. Furthermore, it is closely associated with transition of carcinoma cells from normal cells, growth and proliferation of carcinoma cells. It also participates in vascularization, invasion and metastasis of tumor cells [21]. This data demonstrated that, treatment with oleanolic acid significantly suppressed mTOR protein expression in T24 cells. Zhou et al. reported that oleanolic acid is anti-tumor effects on osteosarcoma cells through mTOR and caspases-3. New evidences also indicated that oleanolic acid induces protective autophagy

through JNK and mTOR pathways in some tumor cell lines [9].

As ERK1/2/MAPK pathway takes part in cell proliferation, differentiation, apoptosis and metastasis, it is closely associated with tumorigenesis [22]. Countless studies have demonstrated that about 30% of tumors are involved with this pathway. Immunohistochemical method has been employed to test up-regulation of phosphorylation of ERK1/2 in salivary gland tumors such as adenoid cystic carcinoma and mucoepidermoid carcinoma [23]. Our data showed that oleanolic acid significantly inhibited S6K protein expression and suppressed p-ERK1/2 protein expression of T24 cells. Liu et al. suggest that oleanolic acid induces human hepatoma cell apoptosis through the activation of caspase-3 and ERK1/2 pathway [24]. Moreover, Liu et al also found that oleanolic acid inhibits proliferation and invasiveness of Kras-transformed cells via inhibiting the activation of Akt/mTOR/S6K signaling [25]. The oleanolic acid should be investigated for their anti-cancer activity in further studies.

Taken together, oleanolic acid suppresses the proliferation and induced apoptosi of T24 cells through inhibition of Akt/mTOR/S6K and ERK1/2 signaling. Our data indicated that oleanolic acid may be a promising agent for human bladder cancer therapy and may be applied for clinical treatment.

Disclosure of conflict of interest

None.

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