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Cucurbitacin E induces apoptosis of human prostate cancer cells via cofilin-1 and mTORC1

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Abstract. Cucurbitacin E is an important member of the cucurbitacin family and exhibits inhibitory effects in various types of cancer. Cucurbitacin is a potential antineoplastic drug; however, its anticancer effect in human prostate cancer (PC) remains unknown. The aim of the present study was to determine whether the effect of cucurbitacin E on the cell viability and apoptosis of the human PC cell line, LNCaP, was mediated by cofilin-1- and mammalian target of rapamycin (mTOR). The results of the present study demonstrated that cucurbitacin E significantly exhibited cytotoxicity, suppressed cell viability (P<0.0001) and induced apoptosis (P=0.0082) in LNCaP cells. In addition, it was demonstrated that treatment with cucurbitacin E significantly induced cofilin-1 (P=0.0031), p-mTOR (P=0.0022), AMP-activated protein kinase (AMPK; P=0.0048), cellular tumor antigen p53 (p53; P=0.0018) and caspase-9 (P=0.0026) protein expression in LNCaP cells, suggesting that cucurbitacin E exerts its effects on LNCaP cells through cofilin-1, mTOR, AMPK, p53 and caspase-9 signaling. These results suggested that cucurbitacin E maybe used as a therapeutic agent in the treatment of human PC.

Introduction

Prostate cancer (PC) is a type of malignant cancer that affects male prostate tissue, and is a result of abnormal and disordered acinar cell growth (1). Currently, PC is the second most frequently observed cancer worldwide and its morbidity rate is increasing annually (2). According to the World Health Organization (WHO), there were 899,000 new PC cases in 2008, which accounted for ~14% of new cancer cases in men worldwide (3). According to a prediction by the WHO, the number of patients with PC will have increased to 1.7 million by 2030 (4). Epidemiological studies have reported that the morbidity rate of PC is dependent on geography and ethnicity, and is more prevalent in developed countries (5,6).

Cofilin-1 is an actin-associated protein with a low molecular weight that is present in eukaryotic cells (7). The genes encoding cofilin-1 are located on chromosome 11q13 and are expressed in various types of non-muscle tissue, including the liver and brain (8). The remodeling of the actin cytoskeleton serves an important role in the processes of invasion and metastasis of cancer cells (9). The regulation of actin and actin-associated proteins, includingcofilin-1, is important in the formation of pseudopodium (8). Cofilin-1 is an essential regulatory factor for cancer cell metastasis and invasion, and its overexpression leads to increased cancer cell migration (10). Therefore, cofilin-1 inhibition may significantly reduce the invasiveness of cancer cells (11). In melanoma cells, exogenous cofilin-1 signaling may upregulate the expression of matrix metalloproteinases 2 and 14 (11). Cofilin-1 is therefore able to promote matrix degradation, facilitating pseudopodium-mediated invasion.

Mammalian target of rapamycin (mTOR) is an important signal transduction molecule, which regulates transcription and translation through the integration of extracellular signals (12). mTOR complex 1 (mTORC1) is the primary form of mTOR and has an important role in cell growth, proliferation, differentiation and apoptosis (12-14). mTORC1 consists of mTOR, TORC subunit LST8 and regulatory-associated protein of mTOR (RPTOR) (14). A previous study demonstrated that the dysregulation of elements of the mTOR signaling pathway was associated with the occurrence, development and metastasis of various types of cancer (15).

Cucurbitacin E is a member of the cucurbitacin family, which are a group of tetracyclic triterpenoids extracted from cucurbitaceous plants (16). Studies have demonstrated that cucurbitacin E possesses a number of pharmacological functions, including anticancer, anti-viral and anti-inflammatory effects (17,18). Cucurbitacin E is able to inhibit the activation of signal transducer and activator of transcription 3 (STAT3)-associated transcription factors, and disrupt the mitogen-activated protein kinase signaling pathway (19). Cucurbitacin E induces G2/M cell cycle arrest and apoptosis in cancer cells, and subsequent cytoskeletal changes (17). Therefore, the present study aimed to investigate whether cucurbitacin E induces the apoptosis of human PC through cofilin-1 and mTORC1 signaling.

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Materials and methods

Materials. Cucurbitacin E was obtained from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany) and its formula is shown in Fig. 1. Dimethyl sulfoxide (DMSO), SDS, MTT and lactate dehydrogenase (LDH) were also obtained from Sigma-Aldrich (Merck Millipore). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were obtained from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). The Cell Death Detection ELISA kit was obtained from Roche Diagnostics (Indianapolis, IN, USA). Polyvinylidene difluoride (PVDF) membranes were obtained from GE Healthcare Life Sciences (Chalfont, UK).

Cell culture. The human PC cell line, LNCaP, was obtained from the Experiment Center of Yan'an University (Yan'an, China), and cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were incubated at 37˚C in a humidified atmosphere containing 5% CO_2.

Cell viability assay. Cell viability was determined using the MTT assay, as previously described (15). Briefly, LNCaP cells were seeded in 96-well plates at a density of 3,300 cells/well and incubated overnight at 37˚C, prior to treatment with 0, 0.01, 0.1, 1, 10 and 100 μM cucurbitacin E. At 24 and 48 h following treatment with cucurbitacin E, 20 μl MTT solution (0.1 mg/ml) was added to the cells and the plates were subsequently incubated at 37˚C for 4 h. Following incubation, 150 μl DMSO was added for 20 min to dissolve the formazan crystals. The absorbance at 570 nm was measured using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA) and the cell viability was subsequently calculated using the following formula: $(A570_{sample} - A570_{blank})/(A570_{control} - A570_{blank}) \times 100$.

Cytotoxicity assay. Cytotoxicity was determined using the LDH assay, as previously described (15). Briefly, LNCaP cells were seeded in 96-well plates at a density of 3,300 cells/well and incubated overnight at 37˚C prior to treatment with 0, 0.1, 1 and 10 μM cucurbitacin E. After 24 h, 100 μl LDH solution was added to the cells and the plates were incubated at 37˚C for 30 min. The absorbance at 490 nm was measured using a microplate reader.

Detection of apoptosis. LNCaP cells were seeded into 6-well plates at a density of $1x10^5$ cells/well, in triplicate, prior to treatment with 0, 0.1, 1 and 10 μM cucurbitacin E for 24 h. Apoptotic nucleosomes were measured using the Cell Death Detection kit (KeyGen, Shanghai, China) with 10 μg Annexin V-fluorescein isothiocyanate and 5 μg propidium iodide for 15 min in darkness according to the manufacturer's protocol. Apoptosis rate was analyzed by flow cytometry (Epics XL Flow Cytometer; Beckman Coulter, Inc., Brea, CA, USA). The data were analyzed using the ModFit LT program (Verity Software House, Topsham, ME, USA).

Western blot analysis. LNCaP cells were seeded into 6-well plates at a density of $1x10^5$ cells/well, in triplicate, prior to treatment with 0, 0.1, 1 and 10 μM cucurbitacin E for 24 h. Cells were lysed on ice in radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) and homogenized. Total protein concentration was subsequently assessed using a Bradford Assay kit (Beyotime Institute of Biotechnology). Equal amounts of protein (50 μg) were resolved on a 12% w/v gel using SDS-PAGE and transferred onto a PVDF membrane. The membrane was blocked using 5% milk in TBS containing Tween-20 at 37˚C for 1 h and incubated with the following primary antibodies overnight at 4˚C: Mouse anti-cofilin-1 (1:500, sc-53934); mouse phosphorylated (p)-anti-mTOR (1:500, sc-293132); anti-AMP-activated protein kinase (AMPK) (1:400, sc-25792); mouseanti-P53 (1:2,000; sc-6243), mouse anti-caspase-9 (1:400, sc-47698; all Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and mouse anti-β-actin (AF0003, 1:500; Beyotime Institute of Biotechnology). The membrane was then incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibodies (40,000, A0208 or A02581; Beyotime Institute of Biotechnology) for 2 h at room temperature. β-actin was used as the loading control. Protein bands were visualized using New-SUPER ECL (cat. no. KGP1127; Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) and the band intensity was quantified using ImageJ software (version 3.0; National Institutes of Health, Bethesda, MA, USA).

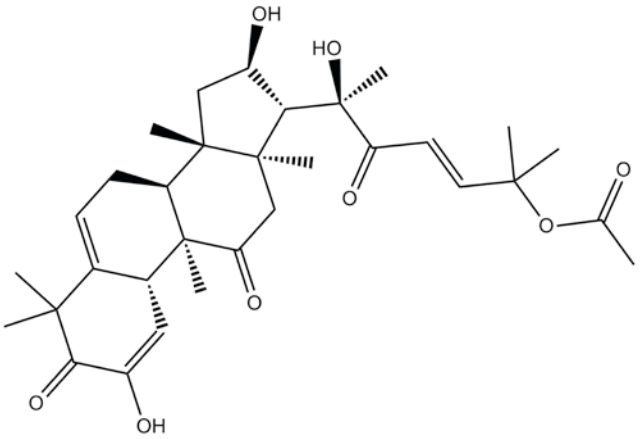

Figure 1. Chemical structure of cucurbitacin E.

Statistical analysis. Values are presented as the mean ± standard error of the mean. Statistical analysis was performed using GraphPad Prism 4.0 (GraphPad Software Inc., La Jolla, CA, USA), and the results were analyzed using analysis of variance and the Bonferroni post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Treatment with cucurbitacin E suppresses LNCaP cell viability. The MTT assay was performed to demonstrate that treatment with cucurbitacin E reduces the viability of human PC cells. Treatment with cucurbitacin E was shown to suppress LNCa cell viability in a dose- and time-dependent manner (Fig. 2). Treatment with 10 and 100 μM cucurbitacin E for 24 h significantly decreased LNCaP cell viability compared with the untreated cells ($P=0.0092$ and 0.0081, respectively; Fig. 2A). Treatment with 0.1-100 μM cucurbitacin E for 48 h significantly decreased LNCaP cell viability compared with

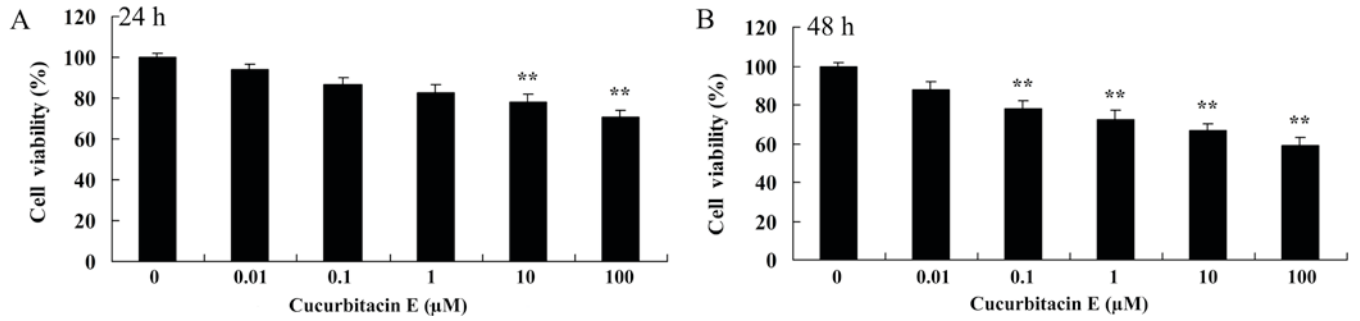

Figure 2. Treatment with cucurbitacin E suppresses LNCaP cell viability. Cell viability was evaluated using the MTT assay at (A) 24 and (B) 48 h following treatment with cucurbitacin E. **P<0.01 vs. untreated cells.

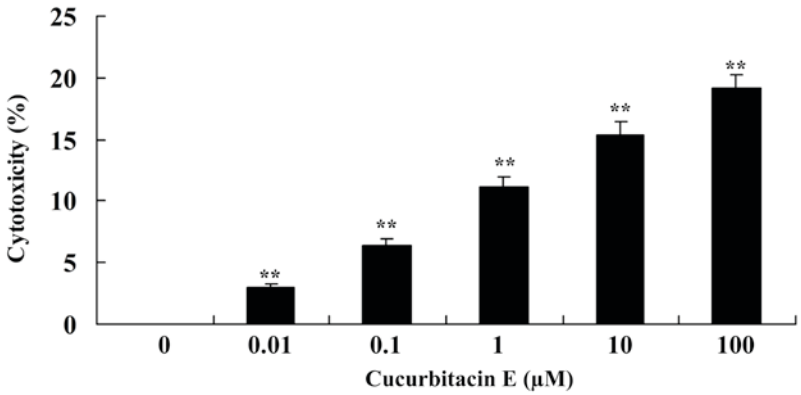

Figure 3. Cucurbitacin E exhibits cytotoxicity in LNCaP cells. **P<0.01 vs. untreated cells.

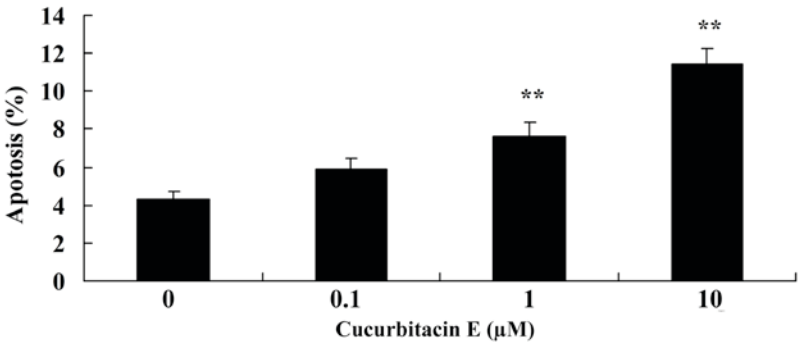

Figure 4. Treatment with cucurbitacin E induces apoptosis in LNCaP cells. **P<0.01 vs. untreated cells.

the untreated cells (P=0.0079, 0.0063, 0.0055 and 0.0041 for 0.1, 1, 10 and 100 μM, respectively; Fig. 2B).

Cucurbitacin E exhibits cytotoxicity in LNCaP cells. The LDH assay was performed to analyze the cytotoxicity of cucurbitacin E on human PC cells. Cucurbitacin E significantly exhibited marked cytotoxicity on LNCaP cells in a dose-dependent manner (P=0.00092, 0.0079, 0.0065, 0.0050 and 0.00021 for 0.01, 0.1, 1, 10 and 100 μM, respectively; Fig. 3).

Treatment with cucurbitacin E induces apoptosis of LNCaP cells. The Cell Death Detection ELISA kit was used to measure the effect of cucurbitacin E on the apoptosis of human PC cells. Treatment with 1 and 10 μM cucurbitacin E for 24 h led to significantly increased apoptosis in LNCaP cells, as compared with the untreated cells (P=0.0076 and 0.0045, respectively; Fig. 4).

Treatment with cucurbitacin E induces cofilin-1 protein expression. The effect of treatment with 0, 0.1, 1 and 10 μM cucurbitacin E for 24 h on cofilin-1 protein expression in LNCaP cells was evaluated. Cofilin-1 protein expression was significantly increased following treatment with 1 and 10 μM cucurbitacin E compared with the untreated cells (P=0.0076 and 0.0045, respectively; Fig. 5A and B).

Treatment with cucurbitacin E induces mTOR protein expression. To determine whether the induction of cofilin-1 expression impacted on the expression of other proteins, mTOR protein expression was evaluated using western blotting. mTOR protein

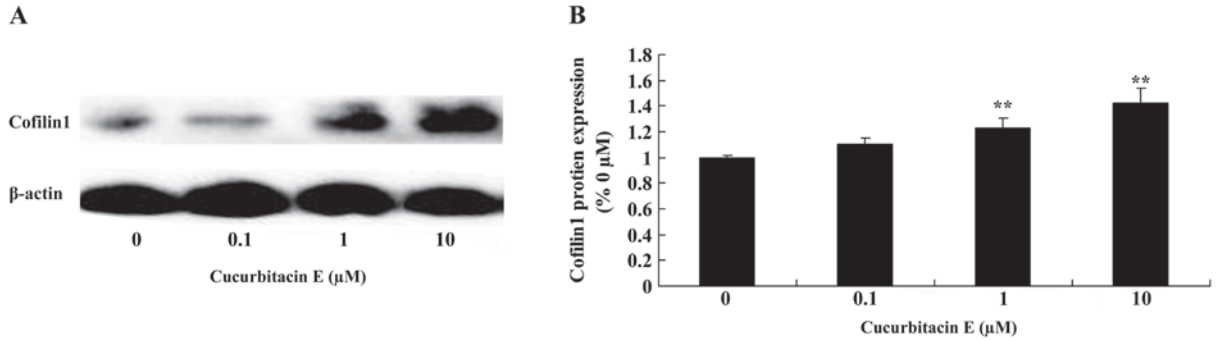

Figure 5. Treatment with cucurbitacin E induces cofilin-1 protein expression. (A) Western blot analysis demonstrated increased cofilin-1 protein expression following treatment with cucurbitacin E. (B) This was confirmed by protein band quantification relative to cofilin-1 expression in the untreated cells. **P<0.01 vs. untreated cells.

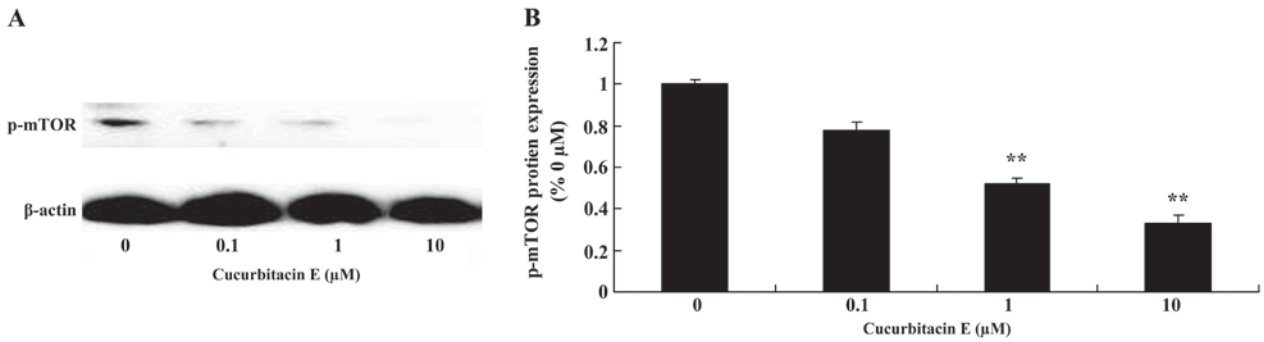

Figure 6. Treatment with cucurbitacin E induces mTOR protein expression. (A) Western blot analysis demonstrated increased mTOR protein expression following treatment with cucurbitacin E. (B) This was confirmed by protein band quantification relative to mTOR expression in the untreated cells. **P<0.01 vs. untreated cells. mTOR, mammalian target of rapamycin.

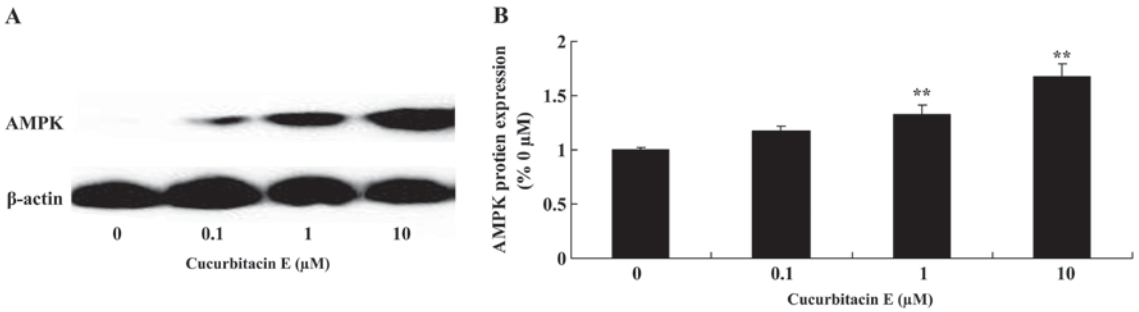

Figure 7. Treatment with cucurbitacin E induces AMPK protein expression. (A) Western blot analysis demonstrated increased AMPK protein expression following treatment with cucurbitacin E. (B) This was confirmed by protein band quantification relative to AMPK expression in the untreated cells. **P<0.01 vs. untreated cells. AMPK, AMP-activated protein kinase.

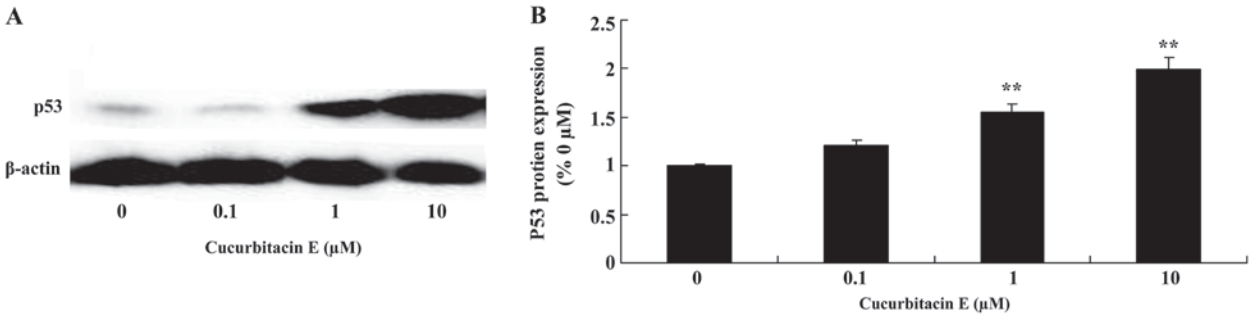

Figure 8. Treatment with cucurbitacin E induces p53 protein expression. (A) Western blot analysis demonstrated increased p53 protein expression following treatment with cucurbitacin E. (B) This was confirmed by protein band quantification relative to p53 expression in the untreated cells. **P<0.01 vs. untreated cells. p53, cellular tumor antigen p53.

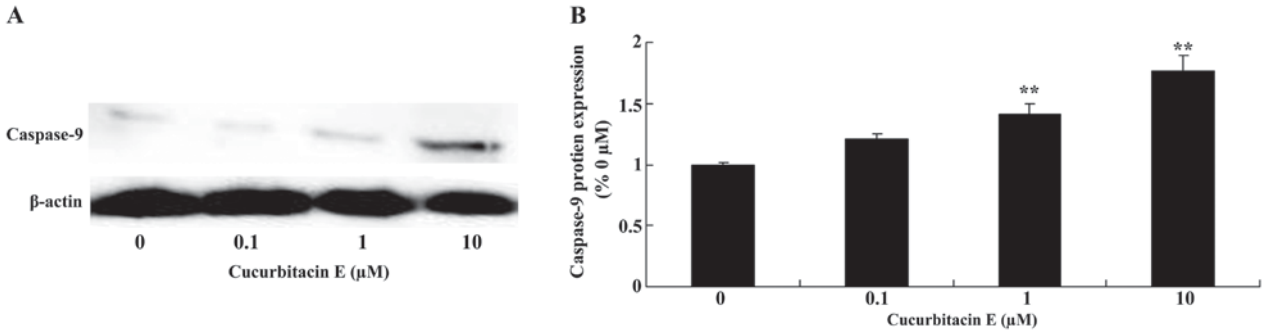

Figure 9. Treatment with cucurbitacin E induces caspase-9 protein expression. (A) Western blot analysis demonstrated increased caspase-9 protein expression following treatment with cucurbitacin E. (B) This was confirmed by protein band quantification relative to caspase-9 expression in the untreated cells. **P<0.01 vs. untreated cells.

expression was significantly increased following treatment with 1 and 10 μM cucurbitacin E compared with the untreated cells (P=0.0030 and 0.0009, respectively; Fig. 6A and B).

Treatment with cucurbitacin E induces AMPK protein expression. To determine changes in the expression of apoptotic proteins in LNCaP cells following treatment with cucurbitacin E, AMPK protein expression was evaluated using western blotting. AMPK protein expression was significantly increased following treatment with 1 and 10 μM cucurbitacin E compared with the untreated cells (P=0.0011 and P<0.0001, respectively; Fig. 7A and B).

Treatment with cucurbitacin E induces p53 protein expression. The effect of treatment with 0, 0.1, 1 and 10 μM cucurbitacin E for 24 h on p53 protein expression in LNCaP cells was evaluated. p53 protein expression was significantly increased following treatment with 1 and 10 μM cucurbitacin E compared with the untreated cells (P=0.0021 and P<0.0001, respectively; Fig. 8A and B).

Treatment with cucurbitacin E inducescaspase-9 protein expression. The effect of treatment with 0, 0.1, 1 and 10 μM cucurbitacin E for 24 h on caspase-9 protein expression in LNCaP cells was evaluated. Caspase-9 protein expression was significantly increased following treatment with 1 and 10 μM cucurbitacin E compared with the untreated cells (P=0.0050 and 0.0023, respectively; Fig. 9A and B).

Discussion

PC is the most frequently occurring cancer in Western countries and a primary cause of cancer-associated mortality (4). Although castration is effective in patients with early stage PC, other therapeutic regimes are limited by the development of hormonal-refractory or hormone-resistant PC (20). The present study demonstrated that cucurbitacin E exerted cytotoxic effects on LNCaP cells, decreasing the cell viability and inducing apoptosis. Previous studies demonstrated that treatment with cucurbitacin E induced apoptosis in triple-negative breast cancer, ovarian cancer and PC cells (21-23).

Cofilin-1 is a low molecular weight polypeptide expressed universally in eukaryotes (9). Cofilin-1 expression has been shown to be dysregulated in various types of cancer tissue, including bladder, breast and esophageal cancer, and affects the occurrence, progression, diagnosis, prognosis and treatment of cancer (8). The results of the present study demonstrated that treatment with cucurbitacin E induced cofilin-1 protein expression in LNCaP cells. Nakashima *et al* (24) suggested that cucurbitacin E inhibits cofilin-1 phosphorylation in human leukemia cells.

mTORC1 is an important regulatory factor of cell growth, proliferation and cell cycle progression by regulating the phosphorylation of its downstream proteins (25). RPTOR interacts with mTOR and mediates trophic signal transduction to regulate cell size and mTOR-associated protein expression (26). The mTOR signaling pathway is an important signaling pathway; its overactivation is able to promote the growth and proliferation of cancer cells, inhibit apoptosis and autophagy, and promote cell migration, invasion, metastasis and angiogenesis (27). mTOR is closely associated with the genesis and development of cancer. Previous studies have demonstrated that mTOR protein expression is significantly upregulated in various types of cancer tissue, including PC, liver cancer, cervical cancer, colorectal cancer, lung adenocarcinoma, esophageal squamous carcinoma, non-small cell lung cancer and extra hepatic bile duct carcinoma (25,28). The results of the present study demonstrated that treatment with cucurbitacin E induced mTOR protein expression inhuman PC cells. Zha *et al* (29) suggested that cucurbitacin E induces autophagy through AMPK and mTORC1 signaling.

AMPK regulates the formation of the apoptosome through activation of the p53 signaling pathway and caspase-9 precursors (26). AMPK activates a number of proteases and endonucleases, and inhibits DNA repairase, which leads to the damage of cytoskeletal proteins and nucleoproteins, and subsequent apoptosis (30). The results of the present study demonstrated that treatment with cucurbitacin E induced AMPK, p53 and caspase-9 protein expression in LNCaP cells. Sun *et al* (31) reported that cucurbitacin E treatment may be effective against pancreatic cancer by activating p53 and STAT3 signaling. Huang *et al* (18) demonstrated that treatment with cucurbitacin E induced G2/M cell cycle arrest through the activation of caspase-8 and -9. The present study demonstrated that treatment with cucurbitacin E induced apoptosis in human PC cells via cofilin-1 and mTORC1 signaling. These results suggested that cucurbitacin E may be a potential therapeutic candidate for the treatment of PC.

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