Puerarin inhibits bladder cancer cell proliferation through the mTOR/p70S6K signaling pathway

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Abstract. Puerarin, as a novel oncotherapeutic agent, may exert anticancer effects and inhibit the proliferation of cancer cells. To explore the effects of puerarin on human bladder cancer cells, and to elucidate the potential mechanism underlying these effects, a Cell Counting Kit-8 assay was used to examine the proliferation of T24 and EJ cells following puerarin treatment. The effects of puerarin treatment on the cell cycle were detected by flow cytometry (FCM), while puerarin-induced cell apoptosis was detected by terminal deoxynucleotidyl transferase dUTP nick end labeling and FCM, and the cellular ultrastructural morphological changes were observed by transmission electron microscopy. Cell invasion was examined using a Transwell assay with Matrigel. The expression levels of mechanistic target of rapamycin (mTOR), phosphorylated (p)-mTOR, p70-S6 kinase (p70S6K) and p-p70S6K proteins in the mTOR signaling pathway were then assessed by western blotting. The results demonstrated that puerarin may inhibit bladder cancer cell viability, block the cell cycle in the G0/G1 phase and induce apoptosis in bladder cancer cells. The expression levels of p-mTOR and p-p70S6K proteins were downregulated, while no change was observed in the expression levels of mTOR and p70S6K proteins when T-24 and EJ cells were treated by puerarin. In the present study, puerarin was demonstrated to inhibit the viability of human bladder cancer cells. These effects may be due to the puerarin-induced downregulation of proteins in the mTOR/p70S6K signaling pathway, and the present study may provide the experimental basis for puerarin to be considered as a promising novel anti-tumor drug for the treatment of bladder cancer.

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Introduction

Bladder cancer is increasingly common globally and its morbidity and mortality rates are the fourth and seventh highest in men as estimated by the American Cancer Society in 2015, respectively (1). Therefore, bladder cancer is a major burden to public health. In total, ~75% of newly diagnosed bladder cancer cases are non-muscle-invasive bladder cancer (NMIBC) (2,3). The recommended treatment for patients with NMIBC is transurethral resection of bladder tumor. However, bladder cancers have a high recurrence rate, and ~25% of patients with NMIBC develop into muscle-invasive bladder cancer following treatment (4,5). Although numerous chemotherapeutic drugs have been demonstrated to inhibit tumor recurrence and progression, their toxic side effects and chemosensitivity reduce the overall therapeutic effect for patients with NMIBC (6-9). Therefore, the identification of novel adjuvants or alternative agents for patients with NMIBC is urgently required. Previous studies have revealed that certain plants and microorganisms have anticancer effects, often characterized by low toxicity and few side-effects (10,11).

Puerarin is the main isoflavone glycoside isolated from the traditional Chinese herb *Radix pueraria lobate* (12). Puerarin has been widely used as an antidiuretic, antipyretic and diaphoretic due to its various medicinal properties (12). Previous studies have demonstrated that puerarin may be used to treat neurodegenerative disorders (13,14) and cardio-cerebrovascular disease (15,16). In addition, puerarin may inhibit the apoptosis of human osteoblasts through the extracellular signal-regulated kinase signaling pathway (17). Puerarin may also exert anticancer effects and inhibit the growth of esophageal cancer cells, and this effect is associated with the mitochondrial pathway (18). It also inhibits proliferation and induces apoptosis in glioblastoma (19), gastric cancer (20) and colon cancer (21) cell lines. However, the effect of puerarin on human bladder cancer are unclear, and the underlying mechanisms remain elusive. Therefore, the present study investigated

the anticancer effects and potential mechanisms underlying the effect of puerarin on human bladder cancer.

Materials and methods

Cell culture and reagents. Human bladder cancer T24 cell line and its derivative, the EJ cell line, were purchased from the China Center for Type Culture Collection (Wuhan University, Wuhan, China) (22). The cells were maintained in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Puerarin was purchased from Shandong Fangming Pharmaceutical Group Co., Ltd. (Heze, China; injection grade; Chinese FDA approval no. H20033292). Dimethyl sulfoxide was purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Fetal bovine serum (FBS) was obtained from Gibco; Thermo Fisher Scientific, Inc. The bladder cancer T24 and EJ cell lines were cultured in RPMI-1640 medium with 10% FBS and maintained at 37˚C in a humidified atmosphere of 5% CO_2. The medium was changed every 2-3 days, and cells were subcultured until they reached 90% confluency prior to being harvested using trypsin.

Cell viability assay with Cell Counting Kit-8 (CCK-8). CCK-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was utilized to quantify T24 and EJ cell viability. Cells were seeded onto 96-well plates at a density of $1x10^5$ cells/well for 24 h, and then incubated with RPMI-1640 medium containing various dilutions of puerarin (0.01, 0.1, 1, 10 and 100 μmol/l) and negative control (completed untreated) at 37˚C in a 5% CO_2 humidified atmosphere for 24, 48 and 72 h. Following incubation for the indicated times, 10 μl CCK-8 solution was added to each well and incubated for 2 h at 37˚C to examine the effect of puerarin on bladder cancer cell proliferation. Colorimetric analysis was performed at a wavelength of 490 nm. Three independent experiments were performed in triplicate.

Transwell cell invasion assays. T24 and EJ cells were seeded in 12-well culture plate at a density of $4x10^5$ cells/well and incubated with puerarin (100 μmol/l) at 37˚C in a 5% CO_2 humidified atmosphere for 24, 48 and 72 h, with completely untreated cells used as the negative control group. The cells were then suspended in serum free RPMI-1640 medium and plated at a density of $2x10^5$ cells/well in the upper chamber of Transwell plates with polycarbonate membranes (pore size, 8 μm) and diluted Matrigel coating (BD Biosciences, Franklin Lakes, NJ, USA). Complete medium (10% FBS RMPI-1640; 600 μl) was added to the lower chamber. Following incubation for 18 h at 37˚C in a 5% CO_2 humidified atmosphere, the cells that passed through the filters into the bottom wells were fixed in 100% methanol for 30 min at 4˚C and stained with 0.5% crystal violet for 15 min at 37˚C. The number of cells in 10 randomly selected fields (magnification, x100) from each well was counted under an optical microscope (CX21; Olympus Corporation, Tokyo, Japan). The invasion assays were repeated at least three times.

Transmission electron microscopy. To observe the morphological changes of bladder cancer cell lines induced by puerarin with different time and concentration, T24 and EJ cells were pretreated with puerarin (100 μmol/l) for 0, 24, 48 and 72 h at 37˚C, or were completely untreated in the negative control group. Additionally, T24 cells were treated with different concentrations of puerarin (0, 1, 10 and 100 μmol/l) for 72 h at 37˚C. The cells were then collected with 447.2 x g centrifugation for 5 min at room temperature and fixed with 2.5% glutaraldehyde for 2 h at 4˚C. Then the sample was treated with 1% osmium tetroxide for 30 min at 4˚C and dehydrated in increasing concentrations of acetone (50, 70, 90 and 100%; cat no. PYG0013; Boster Biological Technology, Pleasanton, CA, USA) at room temperature. The sample was embedded in embedding resin (cat no. 18109; Epon 812 embedding kit; Ted Pella, Inc., CA, USA) for 24 h at 60˚C, and a 50 mm ultrathin section was prepared with a microtome. The ultrastructure of cells was detected by transmission electron microscopy (Tecnai G2, FEI; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at x100 magnification.

Cell cycle and apoptosis assay by flow cytometry (FCM). T24 cells were seeded in 4- wells plates at a density of $4x10^5$ cells/well and incubated with puerarin (100 μmol/l) at 37˚C in a 5% CO_2 humidified atmosphere for 0, 24, 48 and 72 h, and control cells being completely untreated. The cells were then collected by trypsin and centrifugation. A total of 500 μl binding buffer (including precooled 70% ethanol and 0.5 mmol/l EDTA) was added to each tube and incubated overnight in a 4˚C refrigerator. Cells were then resuspended, centrifuged at a speed of 447.2 x g for 5 min at room temperature and washed twice with PBS. PBS containing 0.1% Triton X-100 and 50 μg/ml RNAse was applied to the resuspended cells. Subsequently, cells were incubated in 90 μl propidium iodide (PI) buffer (Sigma-Aldrich; Merck KGaA) for 30 min at room temperature in the dark, and cell cycle analyses were performed by FCM within 1 h. Cells were collected and subjected to Annexin V and PI staining using an Annexin V-fluorescein isothiocyanate apoptosis detection kit (Vazyme, Piscataway, NJ, USA), following the manufacturer's protocol. Apoptotic cells were then detected by FCM (BD FACSCalibur; BD Biosciences).

Cell apoptosis detection by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. T24 and EJ cells with $4x10^5$/well cell density were pretreated with puerarin (0 and 100 μmol/l) and incubated at 37˚C in a 5% CO_2 humidified atmosphere for 24, 48 and 72 h, with negative control cells being completely untreated. Cell apoptosis was analyzed by TUNEL assay, according to the manufacturer's protocol (TUNEL apoptosis assay kit; Roche Diagnostics GmbH, Mannheim, Germany). Apoptosis of cells was analyzed by counting the positive cells, as well as the total number of cells, at 10 randomly selected fields at x400 magnification in a blinded manner using a fluorescence microscope (IX71; Olympus Corporation).

Western blot analysis. To observe the effect of puerarin on the expression level of protein in bladder cancer lines by different time and concentration, T24 cells, following treatment with puerarin (0 and 100 μmol/l), were incubated for 0, 24, 48 and 72 h, respectively. Additionally, T24 and EJ cells were treated with different concentrations of puerarin (0, 1 and 100 μmol/l) for 72 h, respectively. Cells were then collected using trypsin

and were centrifuged at a speed of 12,745.2 x g for 15 min at 4˚C, and lysed for 30 min at 4˚C by lysis buffer containing 50 mM Tris (pH 7.4), 10% glycerol, 50 mM NaCl, 1 mM EDTA and 1% Triton X-100. All protein extraction processes were performed according to the product specification. A bicinchoninic protein assay kit (Beyotime Institute of Biotechnology, Haimen, China) was used to measure the protein concentration. Equal amounts of protein (50 μg) were then separated by electrophoresis on 12% SDS-PAGE and proteins were transferred to polyvinylidene fluoride membranes. The membranes were blocked in 5% non-fat milk solution for 2-4 h at 4˚C in a freezer and washed twice with TBST solution (0.1% Tween-20). Membranes were incubated overnight at 4˚C with the following antibodies: Polyclonal rabbit mammalian target of rapamycin (mTOR; cat no. A00003; 1:1,000, Boster Biological Technology, Pleasanton, CA, USA); polyclonal rabbit phosphorylated (p-) mTOR (cat no. 20301782-1; 1:2,000; Bioworld Technology, Inc., St. Louis Park, MN, USA); polyclonal rabbit P70-S6 kinase 1 (p70S6K; cat no. 20313303-1; 1:2,000; Bioworld Technology, Inc.); and polyclonal rabbit p-p70S6K (cat no. 20314827-1; 1:2,000; Bioworld Technology, Inc.); β-actin (cat no. BM0626; 1:1,000; Boster Biological Technology). Membranes were then washed three times with TBST solution, incubated with horseradish peroxidase-conjugated secondary antibodies (goat anti-mouse immunoglobulin G; cat no. BA1051 and cat no. BA1055; 1:800; Boster Biological Technology) for 2 h at 4˚C freezer, and the expression of proteins was then detected with an enhanced chemiluminescence kit (super-sensitive ECL ready-to-use substance kit; cat no. AR1173; Boster Biological Technology) with GeneSys imaging software (Gene Gnome, version: 1.5.9.0; Syngene, Frederick, MD, USA).

Statistical analysis. SPSS software (version 16; SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. All data were calculated as the mean ± standard deviation. One-way ANOVA and the Student-Neuman-Keuls method were utilized to analyze the results between treated and control groups, and a Student's t test was used to compare two groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Puerarin inhibits the viability of bladder cancer cells. Human bladder T24 and EJ cells were pretreated with puerarin (0, 0.01, 0.1, 1, 10 and 100 μmol/l) for 24, 48 and 72 h. Subsequently, cell viability was measured using the CCK-8 assay, and cell viability was revealed to be inhibited in a concentration- and time-dependent manner by puerarin treatment. As presented in Fig. 1, the relative viability of T24 and EJ cells following 100 μmol/l puerarin treatment for 72 h was 47 and 50%, respectively, compared with the untreated control cells. With increased incubation time, the relative cell viability of T24 and EJ cells treated with puerarin decreased compared with the untreated control cells. Therefore, a dosage of 100 μmol/l puerarin and incubation periods of 24, 48 and 72 h were used for subsequent experiments.

Puerarin inhibits the invasion of bladder cancer cells. Following pretreatment of human bladder cancer T24 and EJ cells with 0 or 100 μmol/l puerarin for 24, 48 and 72 h, cell invasion was measured by Transwell assay. As presented in Fig. 2, puerarin treatment significantly inhibited the invasion of bladder cancer T24 and EJ cells, compared with the control group ($P<0.05$).

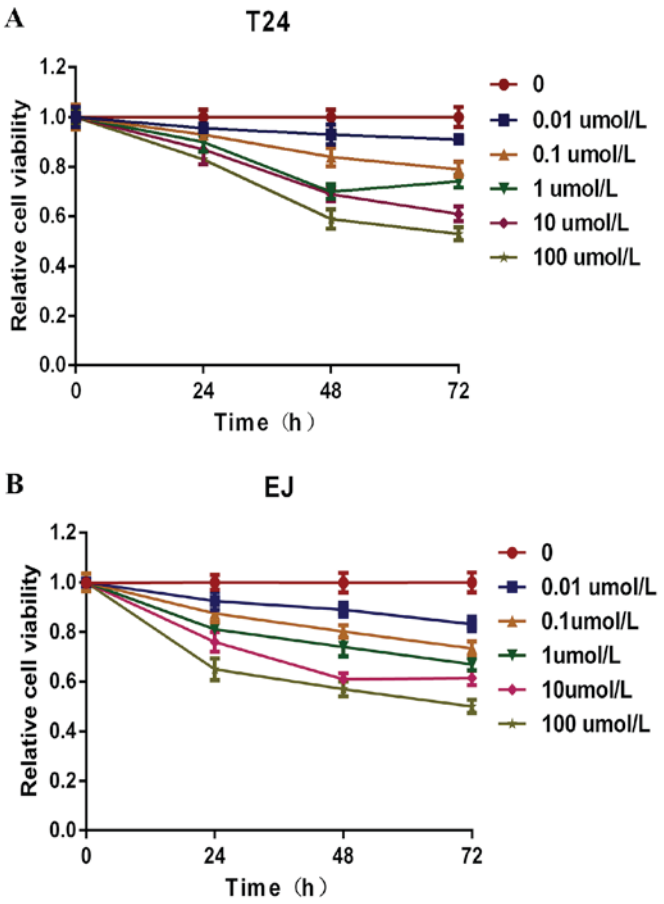

Figure 1. Puerarin inhibited bladder cancer T24 and EJ cell viability. Effects of puerarin on the viability of (A) T24 and (B) EJ cells. Values are given as a percentage of untreated control cells. The data are presented as the average of triplicate results from a representative experiment. Bars represent the standard deviation.

Morphological changes in bladder cancer cells are induced by puerarin treatment. Transmission electron microscopy was used to examine the morphological changes induced by puerarin. As presented in Fig. 3, the untreated control groups contained cells with intact nuclear membranes, large and circular nuclei, increased chromatin, abundant mitochondria and endoplasmic reticulum with normal morphology. However, for T24 and EJ cells treated with puerarin for 24 h, lumped chromatin accumulation was observed inside the nuclear membrane, the mitochondria were impaired due to pyknosis, resulting in membrane ruptures and fewer mitochondria overall in the puerarin-treated group, and a large number of autophagocytic vacuoles were formed. Compared with puerarin treatment for 24 h, when the T24 and EJ cells were incubated with puerarin for 48 and 72 h, the cells became smaller, an increased number of organelles were lost, the nuclear membranes were partially disrupted and the nuclei were broken up (Fig. 3). The T24 cells treated with different concentrations of puerarin (1, 10 and 100 μmol/l) for 72 h exhibited the same inhibitory effect (Fig. 3).

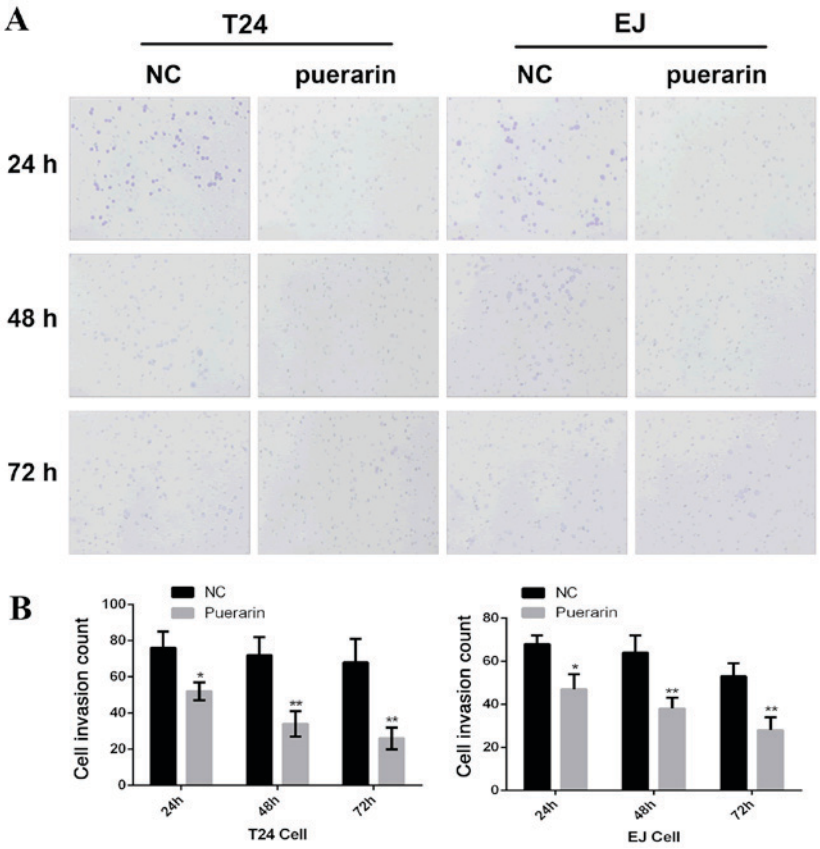

Figure 2. Puerarin inhibited the invasion of bladder cancer cells. (A) T24 and EJ cells treated with puerarin (100 μmol/l) for 24, 48 and 72 h, and then harvested. Invasion was analyzed by Transwell assay with Matrigel; magnification, x100. (B) The quantified number of invaded cells. The data are presented as the average of triplicate results from a representative experiment. Bars, standard deviation. *P<0.05, **P<0.01 vs. negative control.

Puerarin affects the cell cycle and apoptosis of bladder cancer cells. FCM was applied to detect alterations in the cell cycle distribution induced by puerarin treatment in the T24 cell line. As presented in Fig. 4, compared with the 0 h (completed untreated) control group, cultivating T24 cells with 100 μmol/l puerarin for 24, 48 and 72 h resulted in 10.5, 12.8 and 17.5% increases in the percentage of cells in the G0/G1 phase, respectively. This was accompanied by a decrease in the percentage of cells in the G2/M phase, but no significant difference was observed in the percentage of cells in the S phrase, which indicated that puerarin induces cell cycle arrest at the G0/G1 phase in bladder cancer T24 cells. The results also revealed that the apoptotic rate of T24 cells increased following treatment with puerarin for 24, 48 and 72 h (Fig. 4).

Apoptotic effect of puerarin in bladder cancer cells. A TUNEL assay was performed to detect the apoptosis rate of bladder cancer T24 and EJ cells treated with puerarin. Compared with the control group, the results demonstrated the apoptosis of T24 and EJ cells was increased following treatment with puerarin for 24, 48 and 72 h (Fig. 5).

Antitumor effects of puerarin are mediated by the mTOR signaling pathway. The mTOR signaling pathway is involved in the tumorigenesis, development and prognosis of bladder urothelium carcinoma, and is essential for the regulation of autophagy (23,24). Therefore, to investigate the mechanisms underlying puerarin- induced cell apoptosis, western blotting

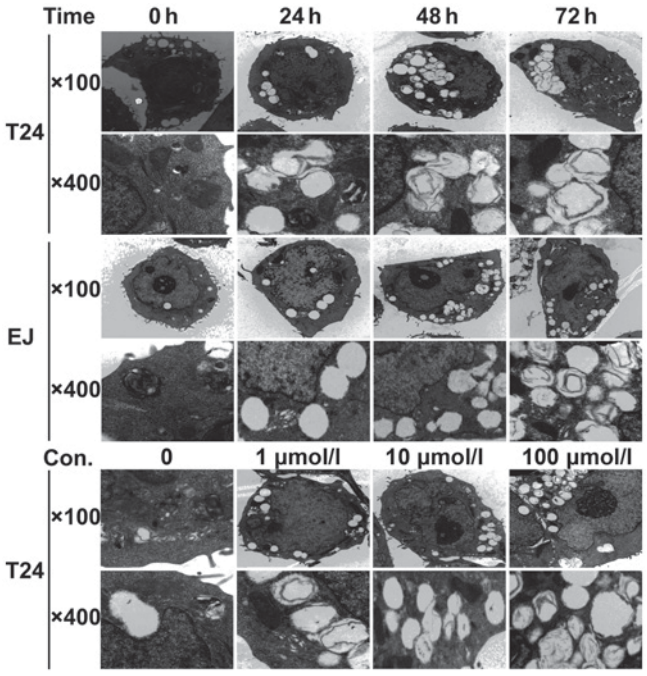

Figure 3. The ultrastructural morphological changes of T24 and EJ cells treated with puerarin, under a transmission electron microscope. Original magnification, x100.

was performed to confirm the expression levels of associated proteins. As is presented in Fig. 6, compared with the

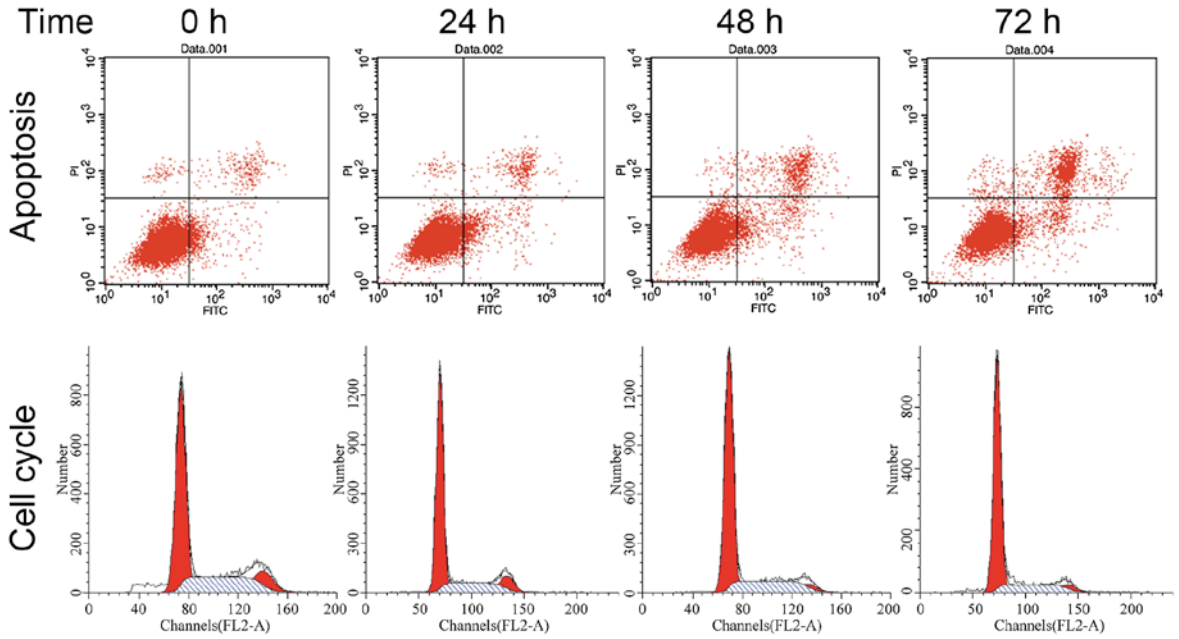

Figure 4. Puerarin affected the cell cycle and apoptosis of T24 cells. T24 cells were treated with puerarin (100 μmol/l) for 24, 48 and 72 h, and then harvested. The cell cycle was analyzed by flow cytometry, and the apoptotic fraction of cells was detected by Annexin V staining following puerarin treatments. FITC, fluorescein isothiocyanate; PI, propidium iodide.

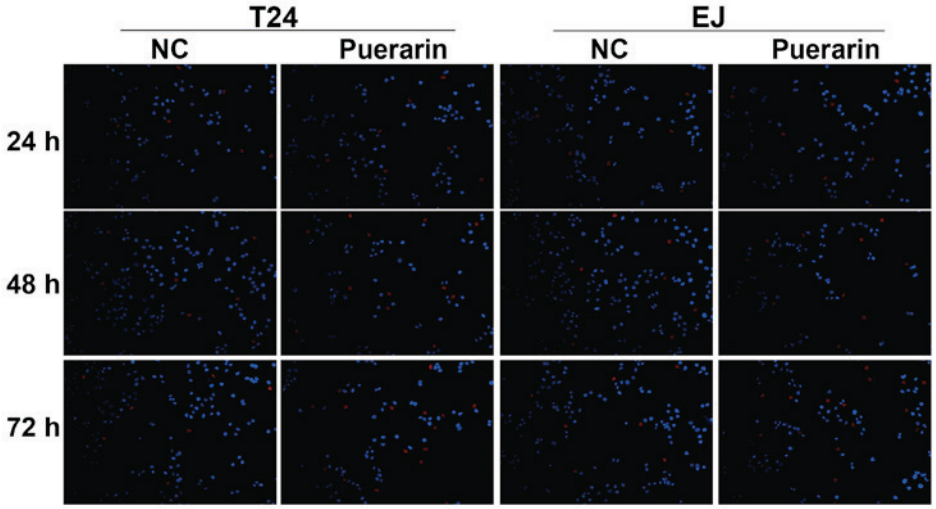

Figure 5. Puerarin induced apoptosis in bladder cancer T24 and EJ cells. T24 and EJ cells were treated with puerarin (100 μmol/l) for 24, 48 and 72 h, and then harvested. Apoptotic properties were detected by TUNEL assay. Magnification: x100. NC, negative control.

control group, the protein expression level of p-mTOR and p-p70S6K decreased in a dose-dependent manner following puerarin-treatment in T24 and EJ cells, while no changes were observed in the expression level of mTOR and p70S6K in puerarin-treated T24 and EJ cells. In addition, the level of p-mTOR and p-p70S6K decreased in a time-dependent manner following treatment of T24 cells with puerarin, while no changes were observed in the expression level of mTOR and p70S6K in puerarin-treated T24 cells (Fig. 6).

Discussion

The search for alternative anticancer agents has led to renewed interest in traditional medicine (25,26). Puerarin, as a traditional Chinese medicine, has protective effects on the nervous and cardiovascular system and may additionally prevent osteoporosis, inflammation and liver injury (14,15,17). Multiple studies have revealed that puerarin induces cell apoptosis, suppresses cell proliferation and increases the chemosensitivity of cancer cells (19-21). Thus, in the present study, a detailed investigation was conducted on the effects of puerarin on bladder cancer cells. As revealed by CCK-8 assay, the viability of T24 and EJ cells was inhibited by puerarin in a dose- and time-dependent manner. The morphological changes induced by puerarin indicated that cells were undergoing apoptosis. With the TUNEL assay, puerarin treatment was revealed to significantly promote the proportion of apoptotic cells. Puerarin may induce bladder cancer cell autophagy and induce cell cycle arrest at the G0/G1

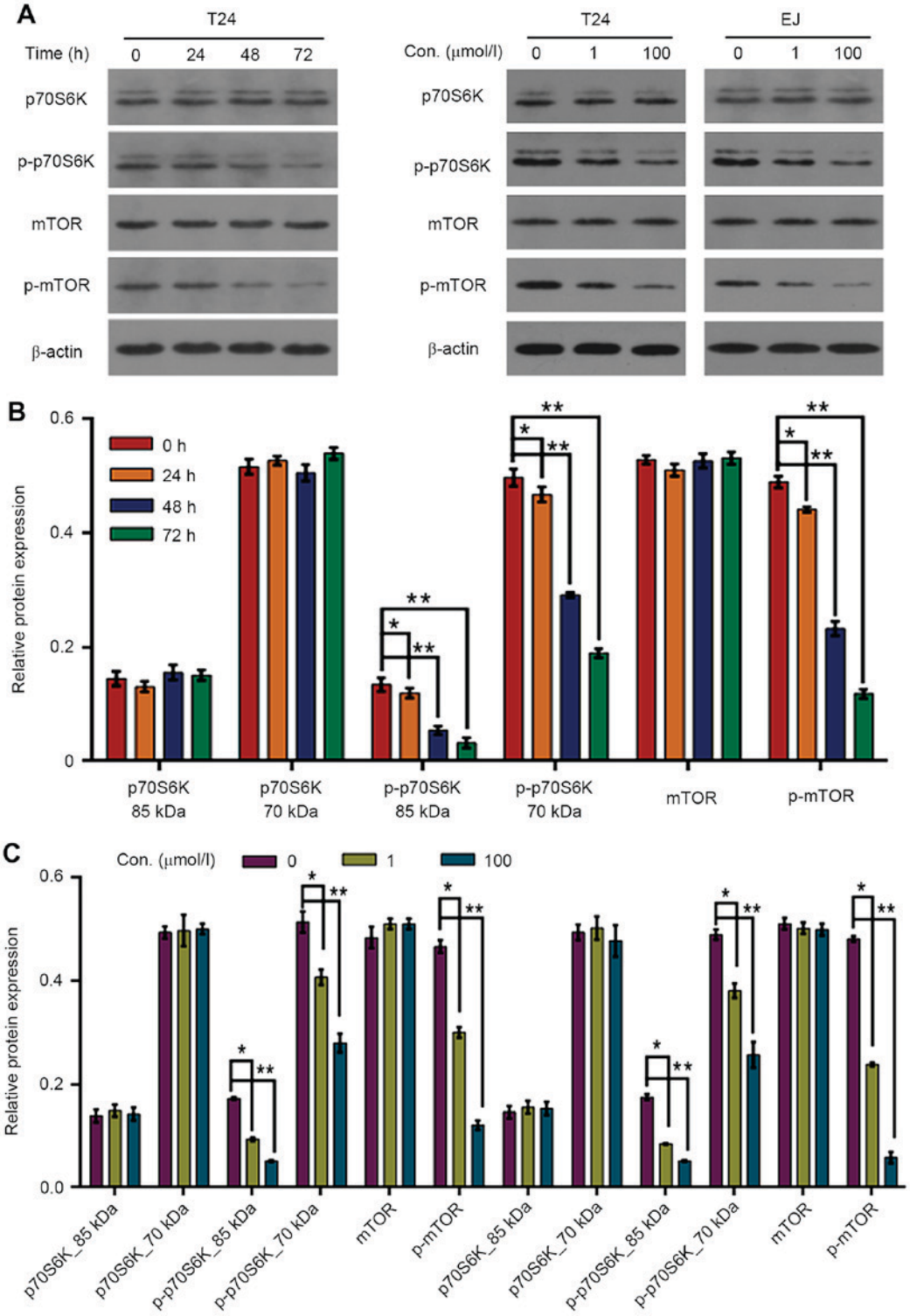

Figure 6. Effect of puerarin on the expression levels of mTOR/p70S6K signaling pathway proteins in T24 and EJ cell lines. (A) Protein levels of mTOR, p70S6K, p-mTOR and p-p70S6K in T24 and EJ cells following puerarin treatment were detected by western blot analysis; (B) Relative protein expression following puerarin treatment in T24 cells for 0, 24, 48 and 72 h. *P<0.05, **P<0.01 vs. 0 h. (C) Relative protein expression following puerarin treatment in T24 and EJ cells at 0, 1 and 100 μmol/l. *P<0.05, **P<0.01 vs. 0 μmol/l. mTOR, mechanistic target of rapamycin signaling; p-mTOR, phospho-mTOR; p70S6K, p70-S6 kinase; p-p70S6K, phosphor-P70S6K.

phase. In addition, Transwell assays revealed that puerarin may inhibit cell invasion. These results confirmed that puerarin treatment resulted in an inhibitory effect on bladder cancer cells.

The mTOR signaling pathway is involved in cancer pathogenesis and progression. p70S6K, which is downstream of the mTOR signaling pathway, is associated with tumor formation (23,27). Loss of p70S6K promotes cell cycle progression and cell proliferation (27). p70S6K also mediates the effects of oncogenic protein kinase B (Akt) signaling on mRNA translation, cell growth and tumor progression (28). The activation of the mTOR/S6K axis stimulates protein

synthesis and cell growth (29). Notably, the mTOR signaling pathway is associated with regulation of energy balance and autophagy as a survival pathway (24). Autophagy is a major degradation pathway in eukaryotic cells, which is essential for removing damaged organelles and macromolecules from the cytoplasm and recycling amino acids during periods of starvation (30,31). Therefore, the present study investigated whether inhibition of the mTOR signaling pathway by puerarin leads to the induction of autophagy. The results indicated that the protein expression levels of p-mTOR and p-p70S6K decreased in a time-dependent manner following treatment of bladder cancer cells with puerarin, and that no changes were observed in the protein expression level of mTOR and p70S6K following puerarin treatment in bladder cancer cells.

Yu and Li (21) reported that puerarin induces apoptosis in colon cancer HT-29 cells and suppressing cell proliferation, and puerarin treatment increases the expression level of BCL2-associated X protein (Bax) and decreases the expression level of c-myc and B cell lymphoma-2 (Bcl-2) (21). Yang *et al* (19) also reported that puerarin exerts antitumor effects through suppressing the expression of p-Akt and Bcl-2, and promotes the expression of Bax in glioblastoma cells. Puerarin inhibited proliferation and induced apoptosis in SMMC-7721 hepatocellular carcinoma cells via the mitochondria-dependent pathway (32). In addition, combined with or without 5-fluorouracil, puerarin significantly suppressed proliferation and markedly increased apoptosis in esophageal cancer cells *in vitro* and *in vivo* (18). These previous studies demonstrated similar results to the present study, and thus strengthen them.

A number of previous studies have demonstrated that cell cycle arrest is associated with the inhibition of cancer cell proliferation. For example, Lin *et al* (33) demonstrated that puerarin inhibits the growth of breast cancer cells through inducing apoptosis and cell cycle arrest in the G2/M phase. Although the results of the present study may differ from those of Lin *et al* (33), Gan and Yin (34) reported that puerarin treatment leads to cell proliferation inhibition via inducing mantle cell lymphoma cell cycle arrest in the G1 phase, and this mechanism may involve the phosphoinositide-3 kinase/Akt and nuclear factor-κB signaling pathway. In accordance with this previous study, the results of the present study revealed that puerarin induces bladder cancer cell cycle arrest at the G0/G1 phase. Due to the aforementioned results, it may be concluded that puerarin affects bladder cancer cell viability and induces apoptosis, which is mediated by the mTOR/p70S6K signaling pathway.

Although puerarin has already been widely applied in experimental research and clinical trials in China with high efficiency (14,15,17,35,36), limitations exist. The low aqueous solubility and intestinal permeability values may lead to a lower blood concentration following oral administration of puerarin (37). In order to acquire improved therapeutic effects of puerarin, investigators are attempting to design nanoparticles or other puerarin encapsulations and delivery systems to improve the effect of treatment (38).

In conclusion, the present study demonstrated that puerarin-induced apoptosis in human bladder cancer cells was mediated by activation of the mTOR/p70S6K signaling pathway. Puerarin may serve as a novel therapeutic strategy in the inhibition of carcinogenesis and progression of bladder cancer. However, additional studies are required to affirm the effect of puerarin on bladder cancer *in vivo* and to verify whether puerarin may be used as part of an intravesical treatment.

Acknowledgements

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