Antitumor effects of curcumin in human bladder cancer *in vitro*

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Abstract. Bladder cancer is one of the major causes of cancer-associated mortality, with a high incidence. Curcumin, a polyphenol compound extracted from turmeric, has been identified to regulate tumor progression. However, the therapeutic effect of curcumin in human bladder cancer has not yet been determined. In the present study, the effects of curcumin on cell growth, apoptosis and migration of bladder cancer cell lines were evaluated using an MTT assay, a Transwell assay and flow cytometry, and the associated mechanisms were investigated using western blot analysis. Curcumin was identified to decrease the growth of T24 and 5637 cells in a dose- and time-dependent manner. The present study confirmed that curcumin is able to inhibit cell migration and promote apoptosis of bladder cancer through suppression of matrix metalloproteinase signaling pathways *in vitro*. The anticancer effects of curcumin on bladder cancer cells may benefit clinical practice in the future.

Introduction

Bladder cancer is one of the major causes of cancer-associated mortality, with a high incidence (1). The majority of patients with bladder cancer initially present with non-muscle-invasive disease; however, disease recurrence is observed in between 30 and 56% of patients undergoing surgery (2). Radical cystectomy with urinary diversion is currently the standard treatment for those patients with refractory non-muscle-invasive and muscle-invasive bladder cancer (3). Although there has been recent progress in the development of treatments, the prognosis for patients with advanced bladder cancer following cystectomy is poor (4,5). Therefore, identification of effective molecular therapeutic targets to treat advanced bladder cancer is urgently required.

Curcumin is a dietary antioxidant derived from turmeric, which may possess anti-inflammatory, anti-proliferative and

pro-apoptotic properties (6,7). Curcumin induces the activation of pro-apoptotic and anti-tumorigenic signaling pathways (8). However, the therapeutic potential of curcumin against various cancer cells, its effects, and the molecular pathways involved in suppression of bladder cancer growth and metastasis remain unclear. In the present study, the anticancer effects of curcumin on bladder cancer *in vitro* were examined. It was investigated whether curcumin induces bladder cancer cell apoptosis and inhibits bladder cancer cell survival and invasion.

Materials and methods

Reagents and cell culture. Curcumin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was prepared by dissolving it in dimethylsulfoxide (DMSO) at a stock concentration of 5,000 mM and stored at -20°C. Serial dilutions were prepared in culture medium. Human urinary bladder transitional cell carcinoma (T24 and 5637) cells were obtained from the Chinese Academy of Science (Shanghai, China). T24 and 5637 cell lines were maintained in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Biological Industries, Cromwell, CT, USA). All media contained 100 units/ml penicillin and 100 μg/ml streptomycin. All cell lines were maintained at 37°C in a humidified incubator containing 5% CO_2. The cells that entered the exponential growth period were selected for experiments.

Proliferation assay (MTT assay). T24 and 5637 cells in the exponential growth phase were inoculated on 96-well plates at a density of $4x10^3$/well, and sterile PBS was added to the edge well as a control. After 24 h at 37°C, curcumin was added at the concentrations of 5, 10, 20, 30 and 40 μmol/l. The experimental control group (0.1% DMSO) and zero group were set up, and each group contained 3 wells. Following treatment, 10 μl MTT solution was added to each well for 2 h at 37°C, prior to dissolving the formazan product using DMSO, and cell viability was determined by measuring the absorbance at 540 nm using a microplate spectrophotometer (Molecular Devices, LCC, Sunnyvale, CA, USA). The mean percentage of viable cells ± standard deviation generated from three independent experiments are reported.

Determination of cell apoptosis by flow cytometry. Following treatment, apoptosis was detected using the Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit (BD Biosciences, Franklin Lakes, NJ, USA). T24 and 5637 cells
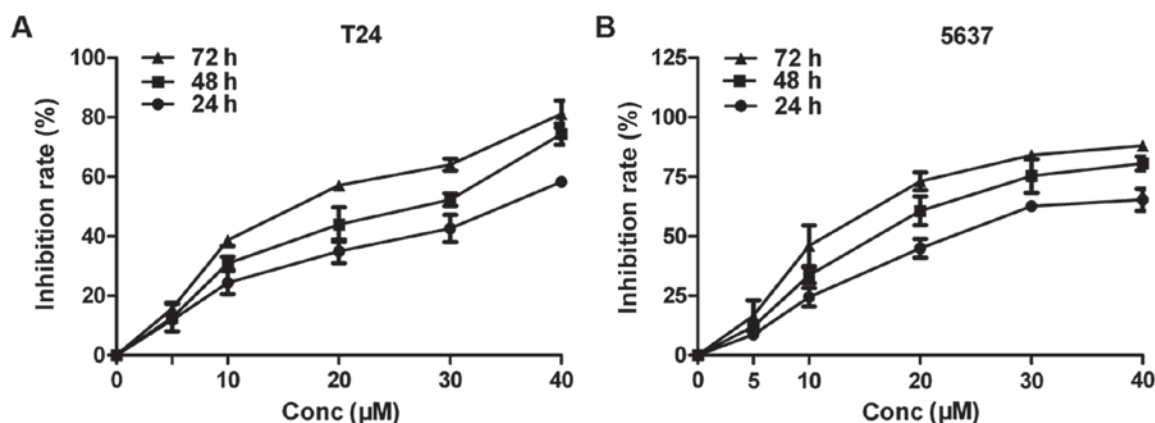
Figure 1. Inhibitory effect of curcumin on T24 and 5637 cells after 24, 48 and 72 h, measured using an MTT assay. (A) The survival rate of T24 cells with various concentrations of curcumin. (B) The survival rate of 5637 cells with various concentrations of curcumin. Conc, concentration.

were detached by trypsinization and washed three times in PBS, centrifuged at 1,000 x g for 5 min at 4˚C and resuspended in 195 µl Annexin V-FITC binding buffer. A 5 µl volume of Annexin V-FITC was added and mixed. The cells were then stained with Annexin V-FITC binding buffer in the dark for 10 min at room temperature. Subsequently, cells were centrifuged at 1,000 x g for 5 min at 4˚C and resuspended in 190 µl Annexin V-FITC binding buffer. Finally, 10 µl propidium iodide (PI) staining solution was added and mixed. The cells were kept on ice for 30 min in the dark and immediately subjected to flow cytometry analysis. The data were analyzed using Cell Quest BD FACStation™ v6.0x (BD Biosciences, Franklin Lakes, NJ, USA). The experiment was repeated three times.

Caspase 3/7 enzyme activity assay. Caspase 3/7 enzyme activity was determined using the Caspase-Glo 3/7 assay (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol. Cells (10^4 cells/well) were plated in a 96-well plate in 100 µl culture medium in the absence or presence of 5, 10 and 20 µmol/l curcumin. Caspase-Glo 3/7 reagent (100 µl) was added to each well and the plates were incubated at room temperature for an additional 1 h. Finally, the luminescence of each sample was measured by a luminometer (Beckman Coulter, Inc., Brea, CA, USA; DTX 880 Multimode Reader).

Cell migration assay. Transwell inserts with a pore size of 8 mm from Corning Incorporated (Corning, NY, USA) were used to determine tumor cell migration capacity. Following culture for 24 h at 37˚C, T24 and 5637 cells were starved in medium without FBS for 24 h, and $5x10^4$ cells were then resuspended in the FBS-free medium and placed in the upper chambers in triplicate. The cells remaining on the upper membrane were removed with cotton wool, whereas the cells that had migrated to the bottom of the membrane were fixed with 95% ethanol and stained with 0.1% crystal violet. Five visual fields of each insert were randomly selected and images were captured under a light microscope at x200 magnification. All experiments were performed in triplicate.

Western blot analysis. Cells were inoculated into the culture flask at $1x10^4$ cells/cm². Following adherence, cells were treated with 10 µmol/l curcumin or 0.1% DMSO (negative control group). Following incubation for 24 h at 37˚C, cells were lysed using the mammalian protein extraction reagent, radioimmunoprecipitation buffer (Beyotime Institute of Biotechnology, Haimen, China), supplemented with a protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland) and PMSF (Roche Diagnostics). Protein (30 µg) were separated by SDS-PAGE (10% gels), transferred to a polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). and blocked with 5% skimmed milk at room temperature for 1 h. Cells were washed with TBS-Tween-20 (TBST) three times for 5 min. The primary antibodies against matrix metalloproteinase (MMP)-2 (rabbit mAb; cat no., 87809; dilution, 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), MMP-9 (rabbit mAb; cat. no., 13667; dilution, 1:1,000; Cell Signaling Technology, Inc.) and tissue inhibitor of metalloproteinase-2 (TIMP-2; rabbit mAb; cat. no., 5738; dilution, 1:1,000; Cell Signaling Technology, Inc.) were added to the membrane prior to incubation at 4˚C overnight. The membrane was washed with TBST three times for 5 min. The secondary antibody (1:10,000; anti-rabbit IgG, horseradish peroxidase-linked antibody; cat. no., 7074; Cell Signaling Technology, Inc.) was added to the membrane and agitated for 1 h at room temperature prior to washing three times with TBST for 5 min. The blot was imaged using an enhanced chemiluminescent imaging system (Pierce; Thermo Fisher Scientific, Inc.).

Statistical analysis. All values are presented as the mean + standard error of the mean. For the MTT assay, the results are presented as the mean ± standard deviation. Statistical significance was determined using Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Curcumin inhibits the proliferation of bladder cancer cells. To determine the inhibitory effect of curcumin on bladder cancer cells, an MTT assay was performed. The concentration of curcumin used was 5, 10, 20, 30 and 40 µmol/l. When the concentration of curcumin was >10 µmol/l, the action time was >24 h, the growth of cells was inhibited in a time- and dose-dependent manner and the difference was statistically significant (P<0.001; Fig. 1A and B). These results indicated
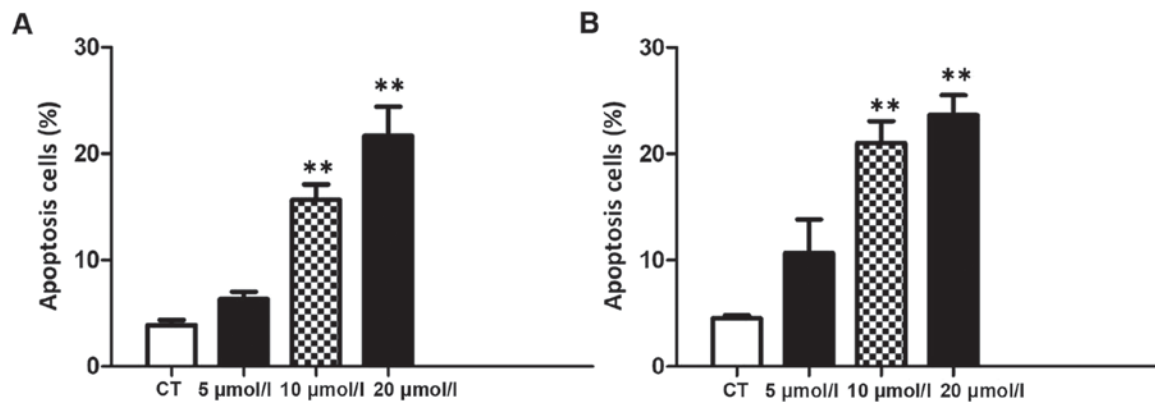
Figure 2. (A) Apoptosis in T24 cells treated with various concentrations of curcumin after 24 h. (B) Apoptosis in 5637 cells treated with various concentrations of curcumin after 24 h. **P<0.01 vs. control.
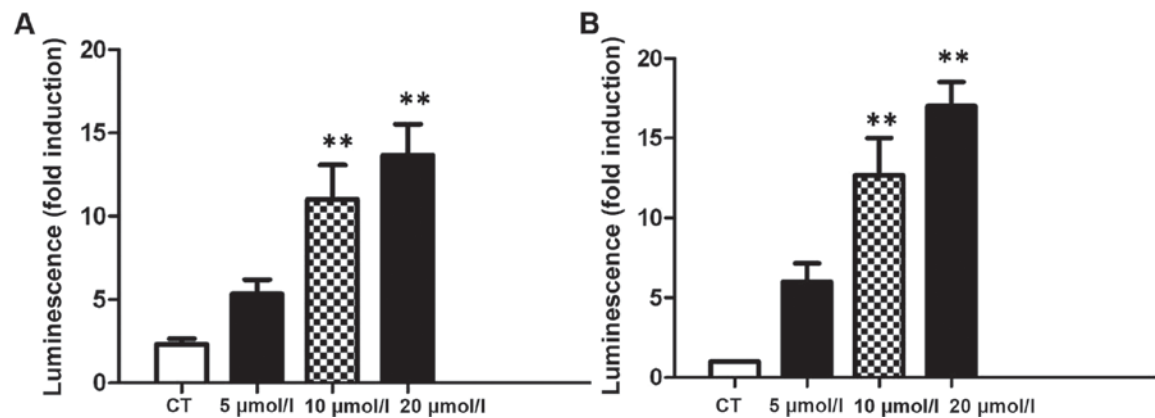

Figure 3. (A) Increasing concentrations of curcumin-stimulated caspase activity in T24 cells. (B) Increasing concentrations of curcumin stimulated caspase activity in 5637 cells. **P<0.01 vs. control.

that curcumin exhibited a strong inhibitory effect on the survival rates of T24 and 5637 cells. In the subsequent tests, 5, 10 and 20 μmol/l curcumin were selected to investigate the effects of curcumin on bladder cancer cells and explore the underlying molecular mechanisms.

Effect of curcumin on apoptosis of bladder cancer cells. To determine whether curcumin induces apoptosis of T24 and 5637 cells, Annexin V FITC/PI staining was performed. Annexin V and PI staining results indicated that, with an increase in curcumin concentration after 24 h, the number of apoptotic cells was also markedly increased (P<0.01; Fig. 2A and B). These results indicated that curcumin induces increased apoptosis in T24 and 5637 cells.

Caspase 3/7 enzyme activity in response to curcumin in bladder cancer cells. In order to evaluate whether caspases serve roles in curcumin-induced apoptosis of bladder cancer cells, the levels of caspase 3/7 were measured following treatment with various concentrations of curcumin for 24 h. The results revealed that there was a dose-dependent increase in caspase 3/7 enzyme activity in curcumin in T24 and 5637 cells (Fig. 3A and B). These results demonstrated that there was a dose-dependent increase in caspase 3/7 enzyme activation in curcumin-treated T24 and 5637 cells which induced significantly increased apoptosis compared with the control.

Effect of curcumin on the migratory ability of bladder cancer cells. The Transwell invasion chamber experiments demonstrated that the number of T24 and 5637 cells passing through the polycarbonate membrane in the high concentration group was significantly less compared with that in the control group and the low concentration group (P<0.01; Fig. 4A and B). Compared with the negative control group, the number of transmembrane cells gradually decreased as the drug concentration of curcumin increased, and the differences between each group were statistically significant (P<0.01).

Effect of curcumin on expression of MMP-2, MMP-9 and TIMP-2 in T24 cells. The expression of MMP-2, MMP-9 and TIMP-2 was examined using western blotting in T24 cells (Fig. 5). The results indicated that the expression of MMP-2 and MMP-9 proteins was significantly decreased (P<0.01), and that of the TIMP-2 protein was significantly increased, in the 10 μM curcumin group compared with the control group. These results confirmed that curcumin inhibits cell metastatic potential via MMP pathways.

Discussion

Bladder cancer is one of the most frequent urological malignancies, which is characterized by increasing incidence and mortality (9). It has been proposed that chemotherapy represents
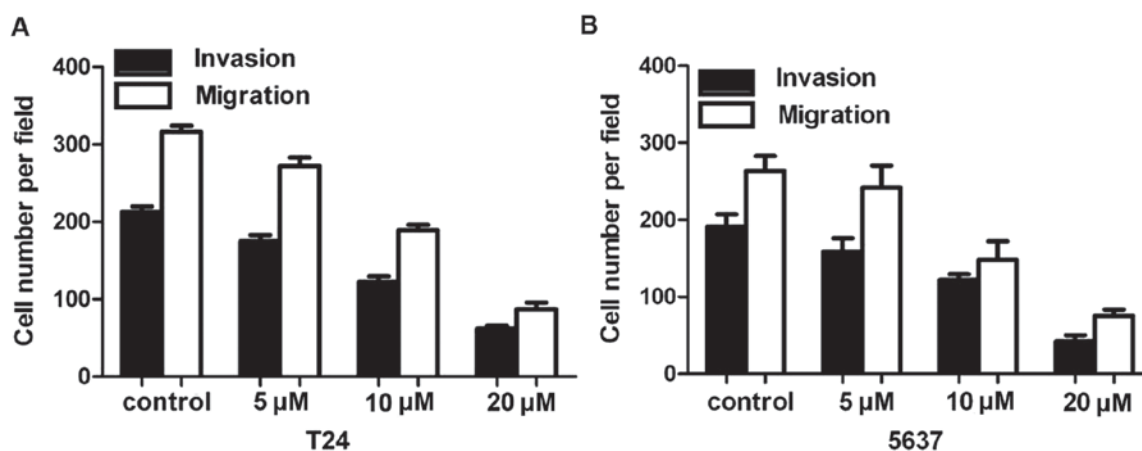
Figure 4. Migration and invasion of (A) T24 and (B) 5637 cells treated with various concentrations of curcumin after 24 h.
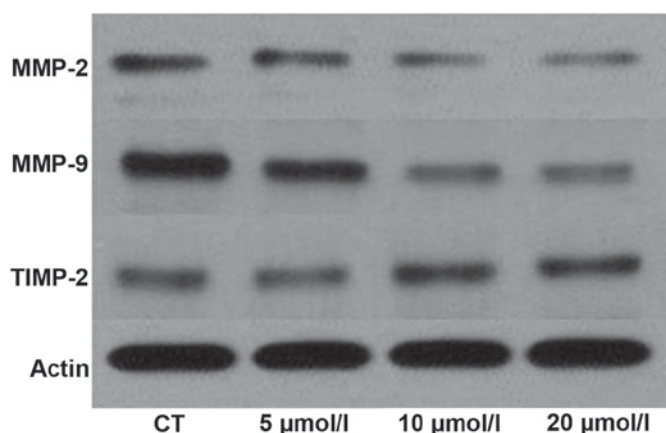

Figure 5. Western blot analysis of the expression of proteins after T24 cells were treated with curcumin. The levels of MMP-2, MMP-9 and TIMP-2 were analyzed by western blot analysis. β-actin was used as a loading control. MMP, matrix metalloproteinase; TIMP-2, tissue inhibitor of metalloproteinase-2; CT, control.

important modality for patients with bladder cancer (10). However, the prognosis of bladder cancer remains poor (11). Early diagnosis and effective treatment are essential in order to increase the life expectancy of patients with bladder cancer.

Curcumin is a polyphenol compound extracted from turmeric that has been identified to regulate tumor progression (12,13). Previous studies have focused on curcumin due to its anticancer properties (14,15). However, there have been few studies on the effect of curcumin on the survival and invasion of bladder cancer cells. In the present study, the underlying molecular mechanism of curcumin in bladder cancer was investigated by studying the effect of curcumin on proliferation and apoptosis of the human bladder cancer cell lines T24 and 5637.

Several effects of curcumin have been identified. One of them is the effect of curcumin on coronary heart disease (15,16). In addition, curcumin has been safely demonstrated to function as an antitumor drug (17,18). The efficacy of curcumin on the proliferation of bladder cancer *in vitro* was examined, and the results demonstrated that curcumin inhibits cell proliferation in a time- and dose-dependent manner. The results indicated that curcumin is a potential anticancer drug for the treatment of bladder cancer. The results of the present study identified that curcumin may cause significant growth inhibition in bladder cancer cell lines, which was in accordance with the aforementioned previous studies (19,20).

Curcumin exhibited the ability to effectively modulate the apoptotic effect of cancer cells (20,21). The results of the present study identified that the number of apoptotic cells increased markedly with an increase in curcumin concentration, indicating that curcumin induces apoptosis in two human urinary bladder transitional cell carcinoma cells. In addition, caspase 3/7 activity was examined, and the results indicated that curcumin stimulated a marked increase in caspase activity beginning at concentrations of 10 and 20 μmol/l.

A previous study has demonstrated that curcumin may inhibit the metastasis of melanoma cells *in vitro* (22). The results of the present study are in agreement with previous studies (23,24); curcumin inhibited the migration of T24 and 5637 cells. To date, few studies have investigated the molecular mechanisms through which curcumin inhibits metastasis (25). Curcumin may impede cell metastasis via downregulation of Src and focal adhesion kinase activity, two important factors in integrin signal transduction (26). In the present study, curcumin inhibited the migration of bladder cancer cells by inhibiting MMP signaling pathways. MMPs belong to a family of Zn^{2+}-dependent endogenous proteolytic enzymes. MMPs degrade almost all components of the extracellular matrix, with the exception of polysaccharides, and are involved in invasion and metastasis of tumors. The results of western blot analysis indicated that the expression of MMP-2 and MMP-9 proteins was significantly decreased in the 10 μM curcumin group compared with the control group, which confirmed that curcumin inhibits cell metastatic potential via MMP signaling pathways.

In conclusion, the results of the present study identified that the anticancer effects of curcumin inhibited cell proliferation and migration, and promoted apoptosis of bladder cancer cell through the suppression of MMP signaling pathways. Additional studies are required to determine the *in vivo* anticancer effects of curcumin in animal models, in order to evaluate the therapeutic potential of curcumin on bladder cancer cells and the potential benefits of curcumin for clinical practice in the future.

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