Anticancer effect of docetaxel induces apoptosis of prostate cancer via the cofilin-1 and paxillin signaling pathway

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Abstract. Prostate cancer is a common multiple malignant tumor occurring in males. Prostate cancer mortality is the 2nd most common of all tumor types in Western countries and the mortality of morbidity is 13% in the USA. The present study aimed to investigate the anticancer effect of docetaxel on inducing the apoptosis of prostate cancer via the cofilin-1 and paxillin signaling pathway. Treatment with docetaxel (1-50 nM) disposed the human LNCaP prostate cancer cells for 24 h. Cell growth and cytotoxicity were subsequently measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and lactate dehydrogenase assay, respectively. Docetaxel-induced cell death was analyzed using flow cytometric and caspase-3 assays. Reverse transcription-quantitative polymerase chain reaction analysis was used to detect the gene expression of cofilin-1 and western blots were used to determine the protein expression of paxillin. Treatment with docetaxel inhibited cell growth, promoted cytotoxicity, activated apoptosis and increased caspase-3 activity in the LNCaP cells. Notably, administration of docetaxel reduced the gene expression of cofilin-1 and the protein expression of paxillin in the LNCaP cells. Additionally, knockdown of cofilin-1 advanced the anticancer effect of docetaxel against LNCaP cells through suppression of the paxillin pathway. The present findings demonstrated that the anticancer effect of docetaxel induces the apoptosis of prostate cancer via the suppression of the cofilin-1 and paxillin signaling pathways, which will assist in setting a stage for the clinical treatment of prostate cancer.

Introduction

Prostate cancer is a common cancer of the male genitourinary system, with the 3rd highest morbidity worldwide (1).

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According to GLOBOCAN 2008, a project that offered the estimated cancer incidence, mortality and prevalence worldwide in 2008 by the World Health Organization, the morbidity of prostate cancer in 2008 was 14%, ranking 2nd among male cancer morbidities worldwide, just behind lung cancer (2). A significant difference of prostate cancer morbidity emerged in different parts of the world, with a differential of 25:1 (2). The age standardized morbidity of prostate cancer in developing countries is 0.012%, the cumulative incidence of 0-74-year-old male is 1.4%, which is 6th highest worldwide (2). By contrast, the age standardized morbidity in developed countries is 0.062% and the cumulative incidence of 0-74-year-old male is 7.8%, which is the highest worldwide (2).

Cofilin-1 is a major member of cofilin/actin depolymerizing factors, the monomer of which combines with actin monomers and depolymerizes them from actin bottoms in two ways; One of which is increasing the depolymerization speed of actin monomer from actin bottoms, the other is cutting the actin microfilament into fragments (3). Reconstruction of actin skeleton serves a decisive role in tumor cell invasion and metastasis (4). Actin regulatory systems, including cofilin-1, are crucial in regulating the formation of cancer cell pseudopods (5). Changes in the expression and activity of cofilin-1 were revealed *in vivo*, in tissues of oral squamous cell carcinoma, renal cell carcinoma and ovarian cancer, as well as in the carcinoma cell lines cultured *in vitro* (5,6). A previous study demonstrated that the activation of cofilin-1 led to the lamellipodia formation of breast cancer cells (7). When the expression of cofilin-1 in the mammary gland MTLn3 cell line was restrained, the invasive pseudopods of breast cancer cells failed to completely mature, which explained the important role of cofilin-1 in the formation of cancer cell pseudopods (7,8).

As one of the downstream regulatory proteins of focal adhesion kinase (FAK), paxillin is able to control cell metastasis and migration (9). Paxillin is closely associated with the mitogen activated protein kinase (MAPK)/FAK signaling pathway, since paxillin and FAK are required for the MAPK/FAK signaling pathway to regulate the adhesion process of fibroblasts. Proepithelin is involved in the migration and invasion of cancer cells by activating extracellular-regulated kinase 1/2 and forming the paxillin-FAK complex (10). However, the mechanism by which the paxillin-FAK complex effects the processes that prostaglandin 2 influences the

attachment, migration and invasion of cancer cells remains to be elucidated (10,11).

As a type of docetaxel drugs, docetaxel is the first cytotoxic drug with a definite effect on hormone refractory prostate cancer (12). Experiments *in vitro* revealed that the growth of the prostate cancer cell line was effectively inhibited by docetaxel, a drug whose anticancer activity is higher than taxol because of higher intracellular drug level and longer retention time (13). Besides, docetaxel is free of serious adverse reactions, particularly to the heart (14). With the effects of inhibiting prostate specific antigen increments, relieving pain and reducing adverse reactions from chemotherapy, docetaxel can delay tumor progression, reduce the symptoms and significantly prolong patient lives (15). The present study hypothesized that the anticancer effect of docetaxel induces the apoptosis of prostate cancer via the cofilin-1 and paxillin signaling pathways.

Materials and methods

Reagents. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were acquired from Hyclone (St. Louis, MO, USA) and Thermo Fisher Scientific, Inc. (Waltham, MA, USA), respectively. Streptomycin, penicillin, 3.3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) assays were acquired from Sigma-Aldrich (St. Louis, MO, USA). The EnzChek Caspase-3 assay kit was acquired from Molecular Probes (Eugene, OR, USA).

Cell culture and assays of cell growth. The human androgen-sensitive human LNCaP prostate cancer cells were obtained from the central laboratory of The Second Hospital of Shandong University (Shandong, China) and were grown in DMEM, supplemented with 10% FBS and antibiotics (100 mg/ml streptomycin and 100 IU/ml penicillin) in a 5% CO_2-humidified incubator at 37°C.

Assays of cell growth and cytotoxicity. LNCaP cells ($5x10^3$ cells/well) were seeded into 96-well plates and were incubated with docetaxel (1-50 nM) for 24 h. Following treatment, 20 μl MTT was added into each well and incubated in a 5% CO_2-humidified incubator at 37°C. Subsequently, 150 μl dimethyl sulfoxide was added to each well and the cells were agitated for 20 min. Cell growth was measured using a Synergy H1 plate reader (Bio-Tek, Seattle, WA, USA) at 540 nm. Meanwhile, LNCaP cells ($5x10^3$ cells/well) were seeded into 96-well plates and incubated with docetaxel (1-50 nM) for 24 h. Following treatment, the cells were lysed using 0.1% (w/v) Triton-X-100 in (0.9%) NaCl. LDH was subsequently added to each well and the cytotoxicity was determined spectrophotometrically at 490 nm on a Synergy H1 plate reader, according to the manufacturer's protocol.

Assays of cell apoptosis. LNCaP cells ($1x10^6$ cells/well) were seeded into 6-well plates and incubated with docetaxel (5, 7.5 or 10 nM) for 24 h. Following treatment, cell apoptosis was measured on a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA) using Annexin V-fluorescent isothiocyanate

(FITC)/propidium iodide (PI) staining (BestBio, Shanghai, China), according to the manufacturer's protocol. LNCaP cells were washed twice with cold phosphate-buffered saline (PBS) and resuspended using 500 μl binding buffer (BestBio). Annexin V-FITC and PI (5 μl) were added and incubated for 30 min at 4°C in the dark. The samples were analyzed by flow cytometry and data was analyzed with FACSDiva™ software, version 7.0 (BD Biosciences).

Assays of caspase-3 activity. LNCaP cells ($1x10^6$ cells/well) were seeded into 6-well plates and incubated with docetaxel (5, 7.5 and 10 nM) for 24 h. Following treatment, caspase-3 activity was measured at 460 nm spectrophotometrically using EnzChek Caspase-3 assay kit, according to the manufacturer's protocol on a Synergy H1 plate reader.

Western blotting. LNCaP cells ($1x10^6$ cells/well) were seeded into 6-well plates and incubated with docetaxel (5, 7.5 and 10 nM) for 24 h. Following treatment, LNCaP cells were incubated with lysis buffer (PBS containing 1% Triton-X-100 and protease inhibitors) for 20 min at 4°C. The supernatants were harvested by centrifugation at 13,800 x g for 10 min at 4°C. A bicinchoninic acid Protein Assay kit (Thermo Fisher Scientific, Inc.) was used to measure the protein concentrations. Equal quantities (500 μg/ml) were separated using 10-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane by standard procedures (Bio-Rad Laboratories, Inc., Munich, Germany). The PVDF membrane was blocked with 5% non-fat dry milk in PBS containing 0.1% Tween 20 (pH 7.6; PBST) for 2 h. The membrane was subsequently incubated with the appropriate antibodies: mouse monoclonal anti-Phosphorylated (p-) cofilin-1 (cat. no. sc-53934) or mouse monoclonal p-paxillin (1:2,000; Santa Cruz Biotechnologies, Inc., Santa Cruz, CA, USA; cat. no. sc-365379), diluted in PBST overnight at 4°C. The membranes were washed three times with PBST and incubated with a goat anti-mouse secondary antibody (1:1,000; Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China) diluted in PBST for 2 h at room temperature. The membranes were incubated with an enhanced chemiluminescence kit (Amersham Biosciences, Freiburg, Germany) and the protein levels were quantitatively analyzed using ImageJ software (imagej.nih.gov/ij/).

Small interfering (si)RNA complex formation. Cofilin-1 siRNA and control siRNA were purchased from Sangon Biotech (Shanghai, China). LNCaP cells ($1x10^6$ cells/well) were seeded into 6-well plates and were allowed to grow to 70-80% confluence prior to transfection. The medium was replaced with fresh medium and the LNCaP cells were transfected with a mixture of 100 nmol/l siRNA and Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol.

Statistical analysis. All statistical analyses were performed using SPSS 18.0 software (IBS SPSS, Chicago, IL, USA) and are expressed as the mean ± standard deviation from at least three experiments. Comparisons between two groups were
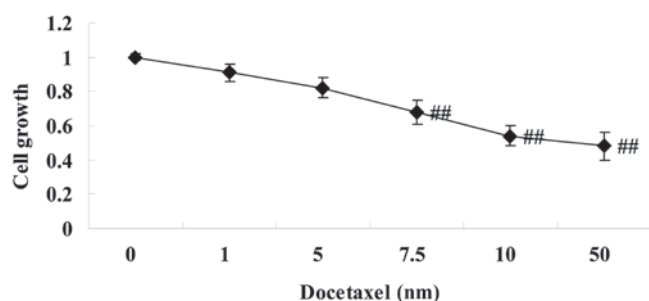
Figure 1. Anticancer effect of docetaxel on cell growth in LNCaP cells (##P<0.01 vs. 0 nm docetaxel group).
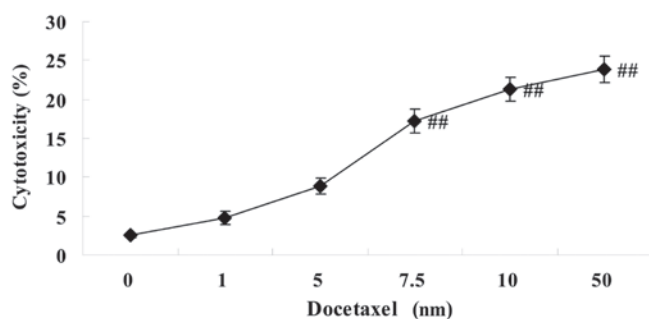

Figure 3. Anticancer effect of docetaxel on cell apoptosis in LNCaP cells (##P<0.01 vs. 0 nm docetaxel group).

Figure 2. Anticancer effect of docetaxel on cytotoxicity in LNCaP cells (##P<0.01 vs. 0 nm docetaxel group).

Figure 4. Anticancer effect of docetaxel on the activity of caspase-3 in LNCaP cells (##P<0.01 vs. 0 nm docetaxel group).

performed using Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Anticancer effect of docetaxel on cell growth in LNCaP cells. To address the hypothesis that docetaxel exerts anticancer effects on human prostate cancer, the present study initially investigated the effect of pretreatment with docetaxel (1-50 nM) on LNCaP cells for 24 h. The effects of increasing drug concentrations were first measured using MTT assays. At 24 h post-exposure, cell growth of LNCaP cells was suppressed in a dose-dependent manner, as measured by MTT assays (Fig. 1). Notably, 7.5-50 nm docetaxel significantly suppressed cell growth of LNCaP cells, compared with the 0 nm docetaxel group (Fig. 1).

Anticancer effect of docetaxel on cytotoxicity in LNCaP cells. As shown in Fig. 2, these docetaxel-mediated anticancer effects on cytotoxicity were enhanced, as demonstrated using an LDH assay. Notably, 7.5-50 nm docetaxel significantly increased the cytotoxicity of LNCaP cells, compared with the 0 nm docetaxel group (Fig. 2).

Anticancer effect of docetaxel on cell apoptosis in LNCaP cells. To determine whether docetaxel has anticancer effects on human prostate cancer, the effect of treatment with docetaxel (5, 7.5 and 10 nM) was assessed in LNCaP cells for 24 h. Compared with the 0 nm docetaxel group, treatment with docetaxel (7.5 and 10 nM) significantly promoted cell apoptosis in LNCaP cells (Fig. 3).
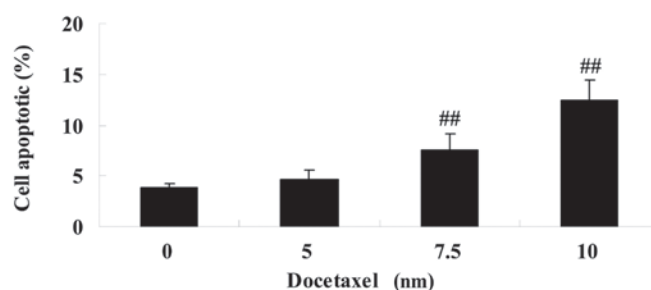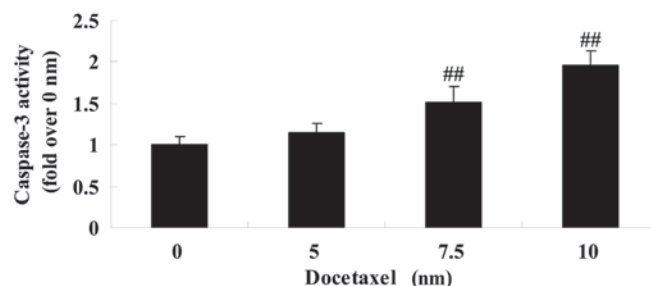
Anticancer effect of docetaxel on the activity of caspase-3 in LNCaP cells. To investigate the molecular mechanism of docetaxel on human prostate cancer, the effect of docetaxel on the activity of caspase-3 was assessed in LNCaP cells following 24 h exposure. As shown in Fig. 4, treatment with docetaxel (7.5 and 10 nM) significantly increased the activity of caspase-3 in LNCaP cells compared with the 0 nm docetaxel group (Fig. 4).

Anticancer effect of docetaxel on cofilin-1 in LNCaP cells. To analyze the molecular mechanism of docetaxel on human prostate cancer, the effect of docetaxel on the protein expression of p-cofilin-1 was assessed in LNCaP cells at 24 h post-exposure. As shown in Fig. 5, treatment with docetaxel (7.5 and 10 nM) significantly suppressed the protein expression of p-cofilin-1 in LNCaP cells.

Anticancer effect of docetaxel on paxillin in LNCaP cells. To research the molecular mechanism of docetaxel on human prostate cancer, the effect of docetaxel on the protein expression of p-paxillin was assessed in LNCaP cells after 24 h docetaxel treatment. A significant inhibition of the protein expression of p-paxillin was observed in the LNCaP cells following treatment with 10 nM docetaxel compared with the 0 nm docetaxel group (Fig. 6).

Knockdown of cofilin-1 enhances cell death and causes apoptosis. To further investigate the molecular mechanism of docetaxel on human prostate cancer, the effect of knockdown of cofilin-1 on the anticancer effect of docetaxel was assessed in LNCaP cells. Following treatment with docetaxel (7.5 nM) and also cofilin-1 siRNA, a significant decrease in the protein expression of p-cofilin-1 was observed in the LNCaP cells
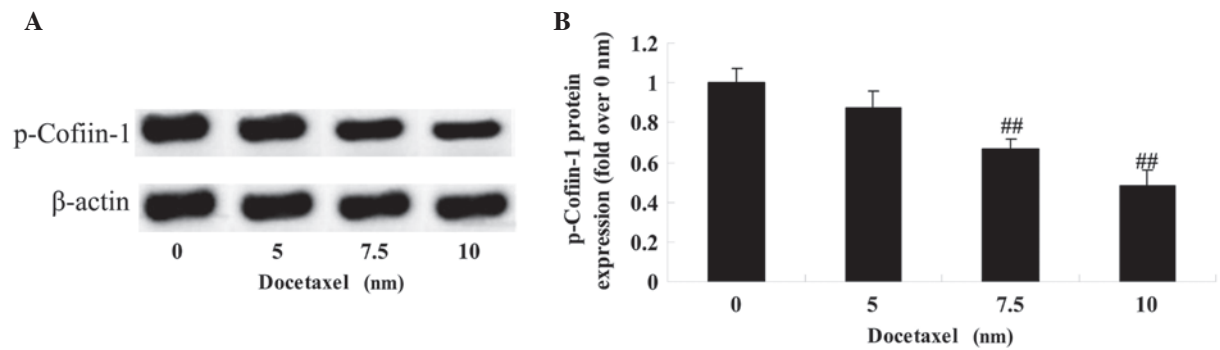
Figure 5. Anticancer effect of docetaxel on cofiin-1 in LNCaP cells. (A) The anticancer effect of docetaxel on the protein expression of p-cofiin-1 was assessed by western blotting in LNCaP cells. (B) The protein expression was quantified as the fold-change compared with the untreated cells ($^{\#\#}P<0.01$ compared with 0 nm docetaxel group). p-, phosphorylated.
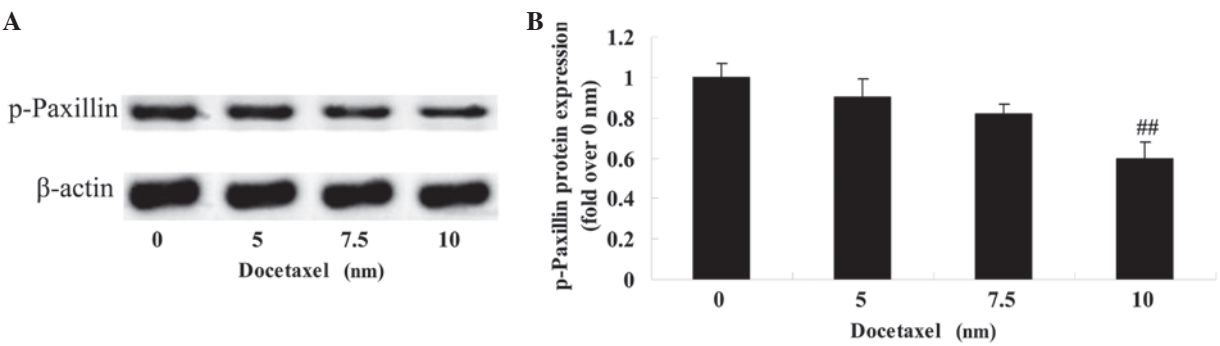

Figure 6. Anticancer effect of docetaxel on the expression of p-paxillin in LNCaP cells. (A) The anticancer effect of docetaxel on the protein expression of p-paxillin was assessed by western blotting assays. (B) The protein expression levels of p-paxillin was determined as the fold-change against the levels in the untreated cells ($^{\#\#}P<0.01$ compared with 0 nm docetaxel group).
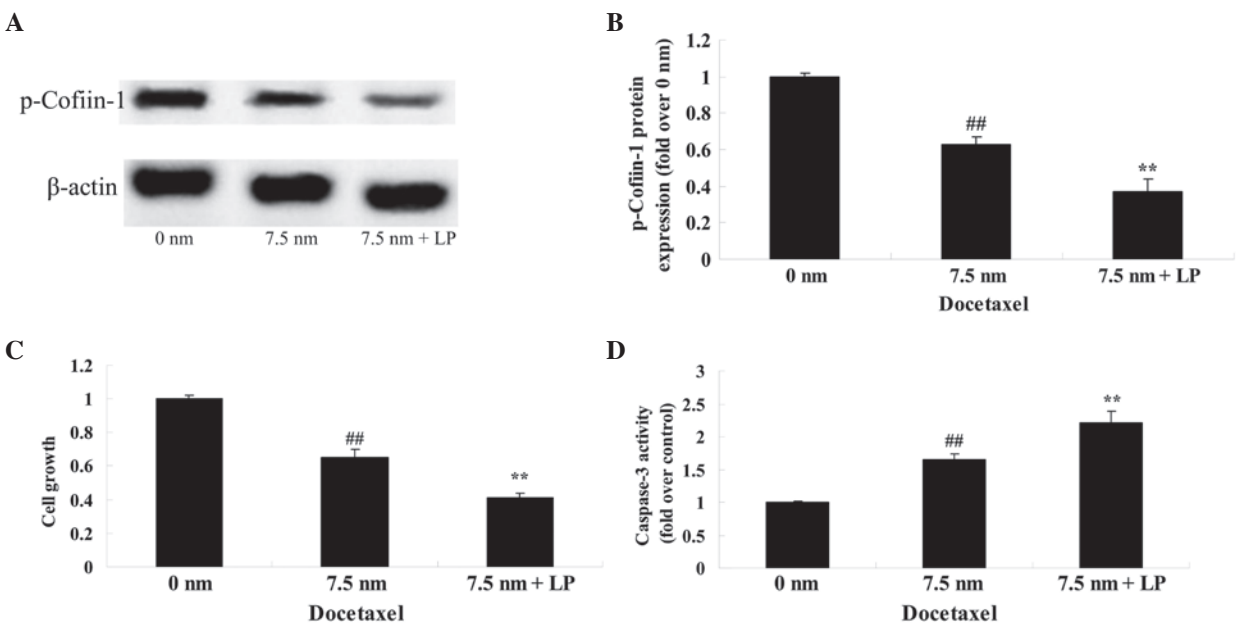

Figure 7. Knockdown of cofilin-1 enhances cell death and causes apoptosis. (A) The anticancer effect of docetaxel on the protein expression levels of p-cofiin-1 was assessed by western blotting and (B) the protein expression levels were quantified as the fold-change compared with the untreated cells. The anticancer effect of docetaxel on cell growth was determined in LNCaP cells treated with (C) 7.5 nm or (D) 7.5 nm docetaxel + LP. $^{\#\#}P<0.01$ compared with the 0 nm docetaxel group; $^{**}P<0.01$ compared with 7.5 nm docetacel group. LP, cofiin-1 small interfering RNA.

(Fig. 7). However, the effect of docetaxel on cell growth and cell apoptosis were significantly reduced, however, were increased by the combined exposure, compared with the 0 nm docetaxel group (Fig. 7).

Discussion

As a common male malignancy in Occident, prostate cancer has the 2nd highest mortality among malignant tumor types (1). The latest data shows that in America, 217,730 newly diagnosed cases of prostate cancer occurred in 2010, accounting for 28% of all tumor types. Of this, 32,050 patients succumbed to prostate cancer in 2010, accounting for 11% of cancer mortality (16). The prostate cancer morbidity in China is markedly less compared with in Western countries, however, its trend has escalated perpendicularly in the past 10 years due to the westernized lifestyle, aging population and the popularization of prostate specific antigen screening (17). As a result of the significant rises in morbidity and mortality at the end of 20th century, prostate cancer has become a major disease influencing Chinese males, and requires increased attention (18). The present *in vitro* investigation clearly revealed that pretreatment with docetaxel significantly suppressed cell growth, increased the cytotoxicity, increased the apoptosis, and induced the activity of caspase-3 in the LNCaP cells.

Cofilin-1 is a type of eukaryon low molecular weight protein, combining with actin. Cofilin-1 is important in cell migration since it produces lamellipodia by highly localized activities and determines the direction of cell movement (5). It was shown previously that cofilin-1 is a type of regulatory factor for cancer cell metastasis and invasion, whose overexpression in protein raises the tumor migration rate (5). Therefore, inhibiting its expression can significantly reduce cancer cell invasion (3). Cofilin-1 was found by researches to be overexpressed in several types of tumor (8). The present findings supported an earlier study showing that treatment with docetaxel significantly reduced the protein expression of p-cofilin-1 in LNCaP cells. Following knockdown of cofilin-1 expression, docetaxel significantly inhibited cell growth and promoted the apoptosis of LNCaP cells. Pérez-Martínez *et al* (19) suggested that docetaxel enhances cytotoxicity through knockdown of cofilin-1 in human prostate cancer cells (19). Additionally, it was also shown that docetaxel induced apoptosis via the targeting of cofilin-1 pathways in prostate cancer cells.

Paxillin is expressed in human muscular tissue and other tissues, with the exception of nervous tissue and blood platelets. Cytoskeletal proteins combined with paxillin, including actin, tubulin, vinculin and actopaxin, are essential for embryonic development, damage repair and tumor associated cell migration (20). A previous study has shown that paxillin has regulatory functions for adhesion plaque, cell migration and cell dissemination (21). There exists a certain association between paxillin, and the invasion and metastasis of tumor cells since the invasion and metastasis of tumor cells are directly associated with changes of adhesive force and locomotive ability. The biological function of paxillin and the specific binding proteins requires further investigation, however paxillin is likely to become a novel target of tumor treatments (22). Therefore, the present findings suggested that docetaxel significantly inhibited the protein expression of p-paxillin in LNCaP cells. Lu *et al* (23) indicated that docetaxel inhibits vascular endothelial growth factor through suppression of the phosphorylation of paxillin in human endothelial cell migration (23).

In conclusion, the present studies revealed that the anticancer effect of docetaxel suppressed cell growth, increased cytotoxicity, induced apoptosis and activated caspase-3 activity in human LNCaP prostate cancer cells, therefore leading to protein expression levels of cofilin-1 and paxillin. The present study suggested that therapies using novel specific signaling molecules may prove useful for the anticancer effect of docetaxel on prostate cancer or other cancer types.

Acknowledgements

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