Establishment and characterization of a docetaxel-resistant human prostate cancer cell line

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Abstract. The aim of the present study was to establish a novel docetaxel-resistant prostate cancer cell line and investigate its biological characteristics. The human prostate cell line, PC-3, was exposed to docetaxel, the concentrations of which were increased in a stepwise manner in the medium to select the drug-resistant cell line, PC-3/DTX. The morphological features were observed using inverted microscopy. The growth curves of PC-3 and PC-3/DTX cells were drawn to calculate the doubling time. Flow cytometry was performed to determine cell-cycle distribution. A 3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2H tetrazolium bromide assay was performed to test the drug resistance of PC-3 and PC-3/DTX cells. Western blot analysis was conducted to determine the protein expression levels of the mammalian target of rapamycin (mTOR) signaling pathway, which may serve a role in regulating drug resistance in the two cell lines. PC-3/DTX cells exhibited changes in morphology, proliferation rate, doubling time and cell-cycle distributions, compared with PC-3 cells. PC-3/DTX cells were 10.9-fold resistant to docetaxel in comparison with PC-3 cells. The results showed that PC-3/DTX cells overexpressed Rictor and p-AKT(S473) proteins, which are specific subunits or downstream substrates of mTORC2. The new findings suggested that the mTORC2 signaling pathway may serve an important role in the regulation of docetaxel drug resistance of PC-3 cells. In conclusion, PC-3/DTX cells may be applied to study the resistance of anticancer drugs and to identify methods to overcome resistance.

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Abbreviations: PC-3/DTX, docetaxel-resistant PC-3 cells; mTOR, mammalian target of rapamycin; Akt, protein kinase B; p-AKT, phosphorylated Akt; ADT, androgen deprivation therapy; CRPC, castration-resistant prostate cancer; mCRPC, metastatic castration-resistant prostate cancer; CCK8, Cell Counting kit-8; PI, propidium iodide; OD, optical density; IC_{50}, 50% inhibitory concentration; Td, growth curves and doubling time; PTEN, phosphatase and tensin homolog; mTORC1, mTOR complex 1; mTORC2, mTOR complex 2; PI3K, phosphoinositide 3-kinase; p70 S6K1, p70 S6 kinase 1; 4E-BP1, eukaryotic initiation factor 4E binding protein 1

Key words: prostate cancer, docetaxel, drug resistance, mTOR, PC-3/DTX

Introduction

The estimated number of new prostate cancer cases was ~1.3 million with 359,000 associated deaths worldwide in 2018, with prostate cancer ranking as the second most common cancer and the fifth leading cause of cancer-associated mortality in males (1). Although it is highly curable if the tumor is locally confined, patients with advanced late-stage disease have a much lower 5-year survival rate (~30%) (2). Androgen deprivation therapy (ADT) is the standard-of-care for patients with advanced prostate cancer. Although highly effective initially, tumors eventually progress and transform themselves into castration-resistant prostate cancer (CRPC) (3). Metastatic CRPC (mCRPC) remains the main clinical challenge and a major cause of mortality (4). In patients with mCRPC, docetaxel currently serves as the standard first-line chemotherapy. However, in addition to its long-term toxicity, the benefits of docetaxel are generally restricted, and drug resistance occurs inevitably in nearly all patients. Clinical data have demonstrated that, in addition to the ~50% of patients who fail to respond to docetaxel inherently, in most patients

who initially respond to the drug, disease progression will recur within 1 year from the start of treatment, indicating the generation of acquired chemoresistance (5).

In this regard, docetaxel resistance has become a major clinical issue to overcome. Although extensive research has been performed into docetaxel resistance, there is no mechanism to explain in detail the clinical response to docetaxel therapy. Mammalian target of rapamycin (mTOR) is an oncoprotein that is generally dysregulated in human cancer (6). In prostate cancer, the mTOR pathway is frequently hyperactivated (7). The pivotal role of mTOR in prostate cancer makes it a potential target for therapeutic interventions.

To completely understand drug resistance, cultured cell lines resistant to anticancer drugs need to be primarily established. In the present study, a docetaxel-resistant prostate cancer cell line was established and the characteristics of docetaxel resistance in this cell line were examined. Furthermore, the mTOR signaling components were compared between the parental cells and cells that developed resistance to docetaxel to analyze the impact of mTOR signaling on the acquired docetaxel-resistant phenotype in preclinical CRPC models.

Materials and methods

Anticancer agents. Docetaxel (Sanofi S.A.) was dissolved in 95% ethanol to the stock concentration of 100 nm and stored at 4°C.

Drug-resistant cancer cell lines. Docetaxel-resistant clones of PC-3 cells were developed by exposing cells to docetaxel at an intermittently increasing concentration. In brief, PC-3 cells were seeded onto a 96-well plate and exposed to 0.1 nM docetaxel for 24 h. Next, the medium was changed to normal culture medium (F-12 medium with 10% fetal bovine serum), and 100 U/ml penicillin and streptomycin (all purchased from (Thermo Fisher Scientific, Inc.). Following the PC-3 cells being cultured three times, the cells were continuously incubated with a higher concentration of 0.2 nM docetaxel for 24 h, prior to the medium being changed to normal cultured medium. This step was repeated with 0.50, 0.75, 1.00, 1.50, 2.00, 5.00, 7.50, 10.00, 15.00, 20.00 and 30.00 nM docetaxel. PC-3 cells that were resistant to 30 nM docetaxel were named PC-3/DTX cells and stored for further investigation.

Cell culture. The human prostate cancer PC-3 cell line was purchased from American Type Culture Collection, Manassas, VA, USA (CRL-1435). In brief, PC-3 cells and docetaxel-resistance PC-3 cells (PC-3/DTX) were cultured in F-12 medium (Thermo Fisher Scientific, Inc.), supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.), penicillin (100 U/ml; Thermo Fisher Scientific, Inc.) and streptomycin (100 U/ml; Thermo Fisher Scientific, Inc.). The cultures were maintained at 37°C in a 95% humidified atmosphere with 5% CO_2.

Growth curves of PC-3 and PC-3/DTX cells detected by CCK8 assay. Cell proliferation were measured by Cell Counting Kit-8 (CCK-8; cat. no. C0037, Beyotime Institute of Biotechnology). To compare the viability of PC-3 and PC-3/DTX cells, cells were seeded onto 96-wells plates at a density of $2.0x10^3$ cells/well. After 24 h incubation at 37°C in a 95% humidified atmosphere with 5% CO_2, 10 μl CCK8 solution was added into each well, and incubated at 37°C for 1 h. Next, the absorption at 450 nm was measured using a microplate spectrophotometer (MD I3X). This measurement was repeated each day until day 5, and growth curves were drawn. Each experiment was repeated three times, and the average values were taken. The cell doubling cycle was calculated using the following equation: $T=txlg2/lg (OD_t-OD_0)$, where T is the population doubling time, t is the time of continuous culture, OD_t is the final absorption of cells, and OD_0 is the initial absorption of cells.

Evaluation of cell inhibition by MTT assay. The MTT cell viability/cytotoxicity assay kit (cat. no. C0009, Beyotime Institute of Biotechnology) was used for this assay. PC-3 and PC-3/DTX cells were seeded onto 96-well plates at a density of $1x10^4$ cells/well at 37°C in a 95% humidified atmosphere with 5% CO_2 for 24 h. Following washing with PBS, the following concentrations of docetaxel: 0.781, 1.562, 3.125, 6.250, 12.500, 25.000, 50.000 and 100.000 nM, were added to the cells, which were then incubated for 72 h. After introducing 10 μl MTT solution for 4 h, formazan was dissolved using formazan solvent in the kit and 100 μl formazan was added to each well. The absorption at 570 nm was measured using a microplate spectrophotometer (MD I3X). Each experiment was repeated three times, and the average values were used for analyses. Resistance indices (RIs) were calculated as the ratio of the 50% inhibitory concentration (IC_{50}) values of PC-3/DTX to that of PC-3 cells.

Cell-cycle detection by flow cytometric analysis. For cell cycle analysis, the cells were processed using the cell cycle and apoptosis analysis kit (cat. no. C1052, Beyotime Institute of Biotechnology). PC-3 and PC-3/DTX cells were harvested and resuspended at a density $2x10^5$ cells/ml in 500 μl binding buffer. According to the instruction of the kit, 5 μl propidium iodide (PI) and RNase were added to the samples and incubated at 4°C for 30 min. Next, the cells were resuspended in 500 μl PBS and analyzed using a flow cytometer (BD-FACS-Calibur; BD Biosciences). The fluorescence of PI was monitored at 488 nm. Fluorescence intensity was quantified by flow cytometry analysis. Each plot represented 20,000 viable cells, and non-viable cells were excluded from flow cytometric analysis by appropriate gating. All data analyses were performed using FCS express V3.0 (BD Biosciences).

Western blot analysis. Cells were collected in RIPA buffer (containing 0.2% Triton X-100, 5 mmol/l EDTA, 1 mmol/l PMSF, 10 mg/ml leupeptin, 10 mg/ml aprotinin, added with 100 mmol/l NaF, and 2 mmol/ Na_3VO_4) and lysed for 30 min on ice. Protein concentration was assayed using the Bio-Rad protein kit (Beyotime Institute of Biotechnology, Haimen, China), and equal amounts of 10 μl sample were loaded per well on a sodium dodecyl sulfate-polyacrylamide gel. Subsequently, proteins were transferred onto 0.45 μm pore sized positively-charged nylon polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA) and blocked with blotto (5% dry milk in PBS with 0.1% Tween-20) at room temperature for 1 h. The cells were incubated with the following primary
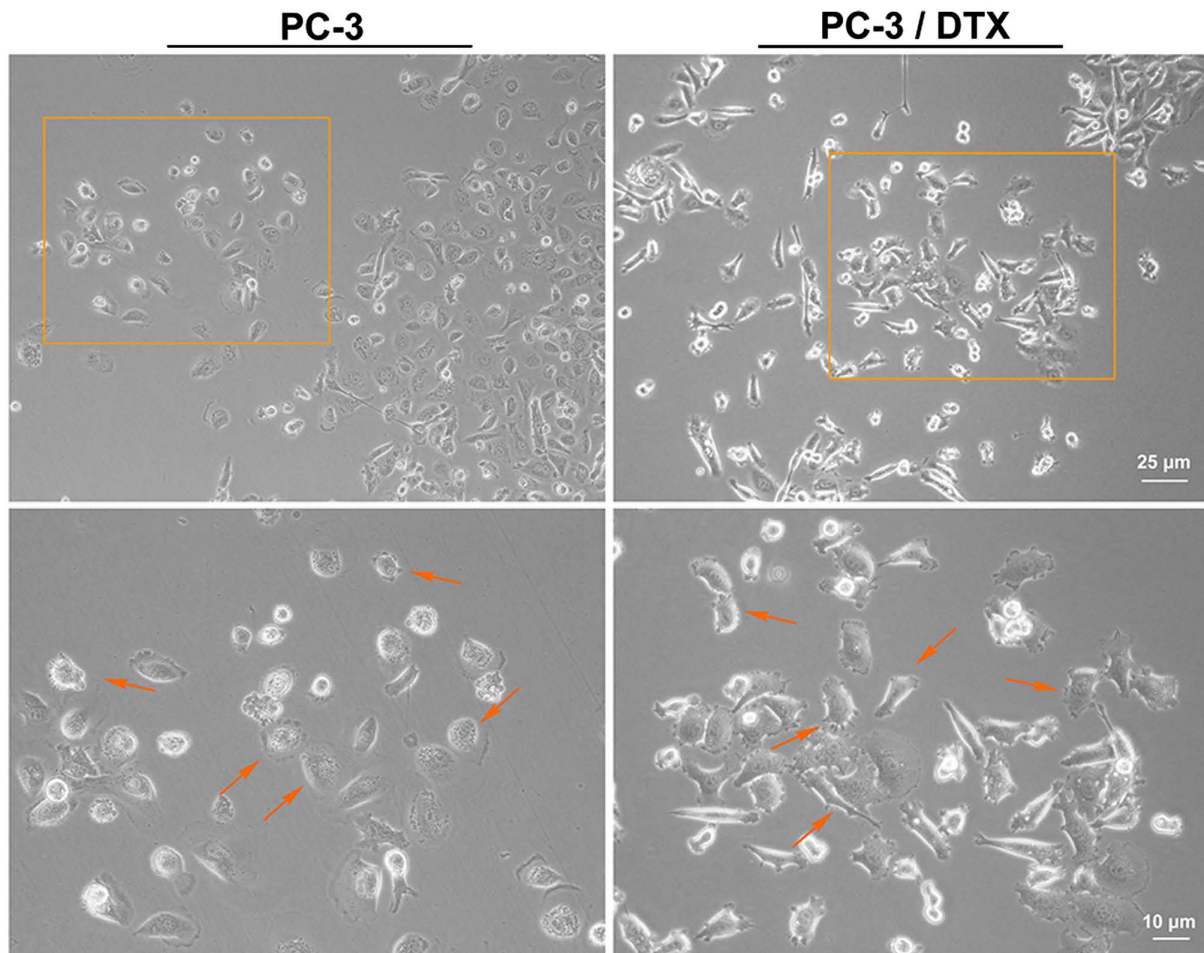
Figure 1. Establishment of the docetaxel-resistant PC-3/DTX cell line. The phenotype of PC-3 and PC-3/DTX cells were captured using an inverted microscope. The bottom panel is a magnification of indicated section in the upper panel. The arrow indicates the typical morphology of PC-3 and PC-3/DTX cells. All images represent three independent experiments (n=3). Scale bar=10 µm. PC-3/DTX, docetaxel-resistance PC-3 cells.

antibodies: Anti-Rictor [1:1,000; Cell Signaling Technology (CST), Inc.; cat. no. 2114], anti-Raptor (1:1,000; CST; cat. no. 2280T), anti-AKT (1:1,000; ProteinTech Group, Inc.; cat. no. 10176-2-AP), anti-pAKT (S473; 1:1,000; CST; cat. no. 9271T), anti-p70 S6 kinase 1 (1:1,000; CST; cat. no. 2708), anti-p70S6k (T389; 1:1,000; CST; cat. no. 9234T), anti-4EBP1 (1:1,000; CST; cat. no. 9644T), anti-4EBP1-S65 (1:1,000; CST; cat. no. 9451T) and anti-GAPDH (1:1,000, CST; cat. no. 5174T) overnight at 4˚C, followed by washing three times with PBST (0.1% Tween-20). They were then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG(H+L) secondary antibodies (Beyotime Institute of Biotechnology; 1:1,000; cat. no. A0208) in TBS-T for 1 h at room temperature. The blots were detected using an enhanced chemiluminescent substrate (Beyotime Institute of Biotechnology).

Statistical analysis. All data are presented as the mean ± standard deviation for the indicated number of separate experiments. Three independent experiments were performed for each study. Comparisons of differences in the quantitative data among groups were performed using an unpaired t-test. P<0.05 was considered to indicate a statistically significant difference. SPSS 19 software (IBM Corp.) was used for statistical analyses.

Results

Establishment of the docetaxel-resistant PC-3/DTX cell line. PC-3 cells were exposed to increasing concentrations of docetaxel intermittently for 12 months to establish a stable docetaxel-resistant cell line, PC-3/DTX. As shown in Fig. 1, the cell phenotype was captured using an inverted microscope (magnifications, x10 and x20). Compared to the PC-3 cells, the gap junction was increased and more irregular cell margins were observed in PC-3/DTX cells. The cell inhibition analysis demonstrated that the IC_{50} values for PC-3 and PC-3/DTX cells were 4.75±0.05 and 52.00±0.04 nM, respectively (Fig. 2). The resistance index of PC-3/DTX cells was 10.9 times that of PC-3 cells, suggesting that PC-3/DTX cells exhibited a high resistance to docetaxel.

Growth curves and doubling time (T_d) of PC-3 and PC-3/DTX cells. The cell growth curves are presented in Fig. 3. No significant differences were observed between the growth curves of PC-3 and PC-3/DTX cells in 2 days. After 2 days, PC-3/DTX cells grew more slowly than PC-3 cells, as can be observed by the decrease in the growth curve. The T_d of PC-3 and PC-3/DTX cells was 25.34±0.02 and 28.87±0.75 h, respectively.
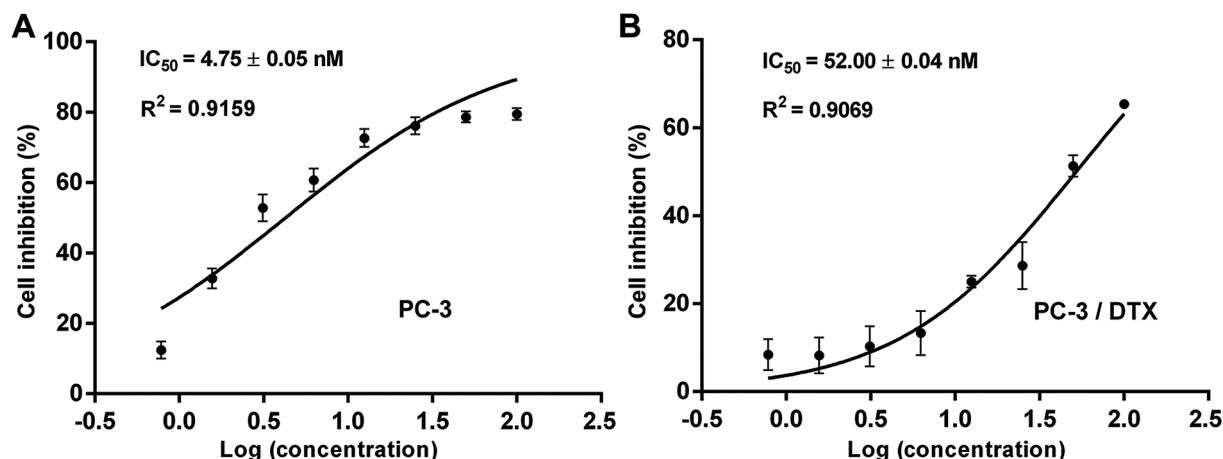
Figure 2. The evaluation of cell inhibition in PC-3 and PC-3/DTX cells treated with docetaxel using MTT assays. (A) PC-3 cells were treated with increasing concentrations of docetaxel over 72 h. (B) Sensitivity of PC-3/DTX with the paralleled treatment. The absorption at 570 nm was measured by SpectraMax i3x (MD). PC-3/DTX, docetaxel-resistance PC-3 cells; IC_{50}, 50% maximum inhibitory concentration.
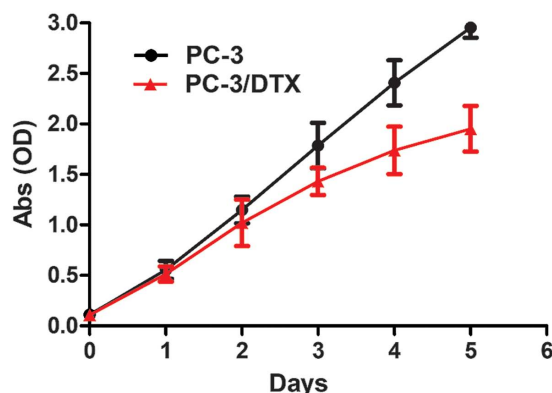

Figure 3. The growth curves of PC-3 and PC-3/DTX cells. The cell growth curves were plotted with culture time on the x-axis and the average Abs per day on the y-axis. Data are expressed as the mean ± standard deviation. n=3. PC-3/DTX, docetaxel-resistance PC-3 cells, Abs, absorbance.

Cell-cycle analysis of PC-3 and PC-3/DTX cells assessed by flow cytometry. The cell cycle of PC-3 and PC-3/DTX cells was assessed by flow cytometry. The results showed significant changes in the G_0/G_1 phase and S phase between PC-3 and PC-3/DTX cells (Fig. 4). The distributions of PC-3 and PC-3/DTX cells in the G_0/G_1 phase were 48.09±2.20 and 60.48±2.10% and those in S phase were 35.61±1.80 and 20.99±0.38%, respectively. Compared with PC-3 cells, the number of PC-3/DTX cells in the G_0/G_1 phase was 12.39% higher ($P<0.05$) and 14.62% lower in the S phase ($P<0.01$). There were no significant differences between the two cell lines with respect to the G_2/M phase.

Protein changes in the mTORC2 signal pathway in PC-3/DTX cells. The expression of Rictor and p-AKT(S473), which are specific subunits or downstream substrates of mTORC2, were significantly upregulated in PC-3/DTX cells (Fig. 5A and B). By contrast, the expression of Raptor, p70S6K (T389) and 4EBP1-S65, which are specific subunits or downstream substrates of mTORC1, exhibited no notable changes (Fig. 5C and D).

Discussion

Chemotherapy is the main method of treatment for cancer. However, the occurrence of drug resistance is one of the main reasons for the poor effect of chemotherapy in patients with cancer. In patients with mCRPC, docetaxel serves as the standard first-line chemotherapy following failure of ADT. Docetaxel is a microtubule stabilizer that may inhibit microtubule disassembly, thereby leading to G2/M cell-cycle arrest and several forms of cell death, including apoptosis and mitotic catastrophe (5). However, various mechanisms have been studied to interpret the existence of drug resistance that may be attributable for the unsatisfactory clinical outcomes in patients, for which the activation of compensatory pro-survival signaling pathways independent of the AR is one potential mechanism (5). Several preclinical studies have demonstrated that docetaxel may increase Akt phosphorylation (p-Akt) at S473, a direct downstream target of mTORC2, in prostate cancer cells (8). In line with this, compared with drug-naïve cells, prostate cancer cells that have gained resistance to docetaxel after an extremely long-term drug exposure exhibit greater expression of p-Akt (S473) (8). These findings indicated the possibility that the mTORC2 pathway, as indicated by increased p-Akt, may cause CRPC cells to become refractory to docetaxel, a putative mechanism that has not previously been investigated.

Few studies have focused on prostate cancer drug-resistant cell lines, which has impeded research on the mechanism of drug resistance in prostate cancer. The establishment of drug-resistant cell lines is important to study the biological characteristics, drug-resistant mechanism, and methods to overcome drug resistance.

In the present study, a docetaxel-resistant prostate cancer cell line was established *in vitro* as a model to investigate chemotherapy resistance by intermittently exposing prostate cancer parental cells to a high concentration of docetaxel with time-stepwise increments. The drug concentrations were increased to select cancer cell with drug resistance; this method may accurately simulate the biological changes of tumor resistance. Compared with the parental PC-3 cells, PC-3/DTX cells
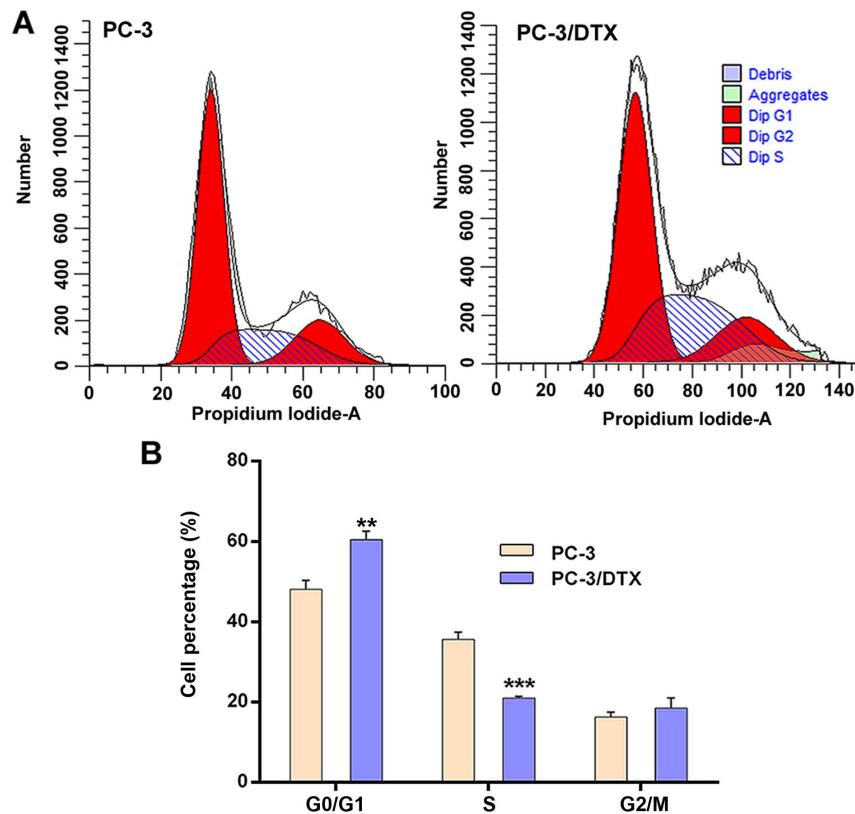
Figure 4. The cell cycle analysis of PC-3 and PC-3/DTX cells assessed by flow cytometry. (A) The cell cycle of PC-3 and PC-3/DTX cells. The fluorescence of PI was monitored at 488 nm. (B) Quantification of fluorescence intensity by flow cytometry analysis. Each plot represented 20,000 viable cells (non-viable cells were excluded from flow cytometry analysis by appropriate gating). Data are expressed as the mean ± standard deviation. n=3. **P<0.01, ***P<0.001 vs. control. PC-3/DTX, docetaxel-resistance PC-3 cells.
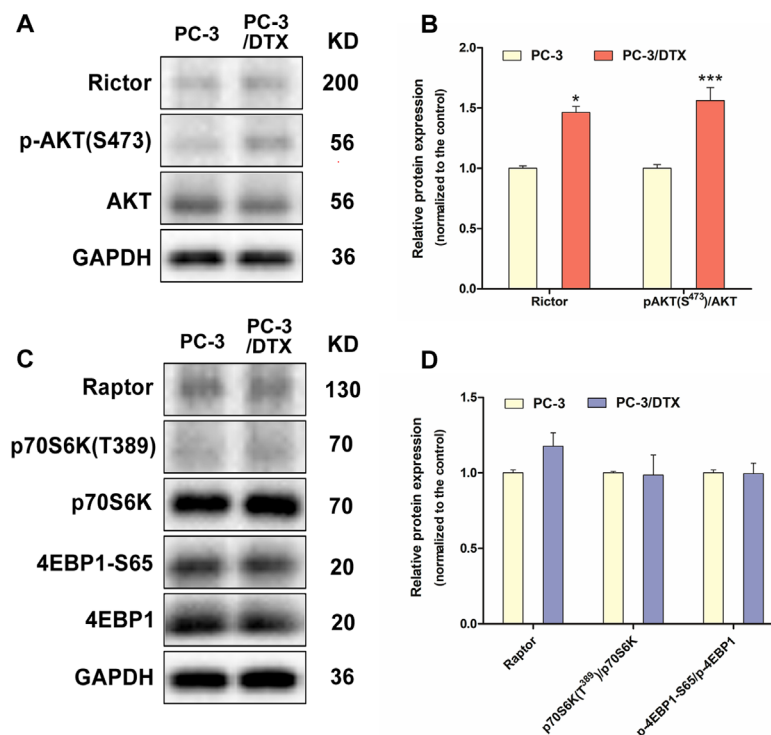

Figure 5. Western blot analysis of protein expression associated with the mTORC2 and mTORC1 signal pathway in the docetaxel-resistant PC-3 cells. (A) The protein expression of Rictor, p-Akt (S473) and Akt. (B) Quantification of protein expression levels of Rictor, p-Akt (S473) and Akt. (C) The protein expression of Raptor, p70 S6K1 (T389), p70 S6K1, 4E-BP1-S65 and 4E-BP1. (D) Quantification of the protein expression levels of Raptor, p70 S6K1 (T389), p70 S6K1, 4E-BP1-S65 and 4E-BP1. Data are presented as the mean ± standard deviation of independent experiments performed in triplicate. *P<0.05, ***P<0.001 vs. control. PC-3/DTX, docetaxel-resistance PC-3 cells; mTOR, mammalian target of rapamycin; Akt, protein kinase B; p-Akt, phosphorylated Akt; 4E-BP1, eukaryotic initiation factor 4E binding protein 1; p70 S6K1, p70 S6 kinase 1.

showed 10.9-times more resistance to docetaxel, indicating that PC-3/DTX cells exhibited significant drug resistance to docetaxel. In the present study, the growth of resistant cells and parental cells was found to be similar, and the T_d of PC-3/DTX and PC-3 cells did not exhibit a significant difference (28.87 vs. 25.34 h). The difference in the cell proliferation rate was marked with 3-5 days, meaning that PC-3/DTX cells showed a delay in the initiation of the logarithmic growth. More PC-3/DTX cells were in the G_0/G_1 phase and fewer cells were in the S phase. A comparison of the biological characteristics of the PC-3 and PC-3/DTX cells revealed that the growth of the drug-resistant cells was relatively slower, and the cell cycle of PC-3/DTX cells was altered.

mTOR is an oncoprotein that is generally dysregulated in human cancer. In prostate cancer, the mTOR pathway is frequently hyperactivated primarily due to the loss of function of the upstream tumor suppressor, phosphatase and tensin homolog (PTEN), which occurs in ~40% of primary tumors and ~70% of metastatic lesions (6,7). Consequently, aberrant mTOR expression has been revealed to be associated with disease progression and poor clinical outcomes in prostate cancer (9). Notably, PTEN loss is also found in up to 80% of mCRPC patients (10), suggesting a high prevalence. This clinical observation is supported by the preclinical findings demonstrating that loss of PTEN is a driving force for developing castration resistance in mice (11). In line with this, cumulating evidence strongly indicates that Akt/mTOR signaling, which is PTEN downstream, is activated in advanced prostate cancer, particularly in CRPC (9).

It has been reported that the mTOR pathway serves a crucial role in cancer; it regulates cell growth and cell survival. Two distinct protein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), are involved in the regulation of cell function. Therefore, the present study evaluated the protein expression of the mTOR signaling pathway in docetaxel-resistant PC-3/DTX cells. Functionally, mTOR serves a pleiotropic role in the regulation of malignant phenotypes, including proliferation, metabolism, angiogenesis and drug resistance. At the molecular level, mTOR functions as two distinct complexes, mTORC1 and 2, downstream of PI3K signaling. Raptor and Rictor are two key proteins composing mTORC1 and 2, respectively. mTORC1 phosphorylates two downstream targets, p70 S6K1 and eukaryotic initiation factor 4E binding protein 1 (4E-BP1), regulating protein translation, cell size, cell proliferation, and survival. By contrast, mTORC2 may directly phosphorylate AGC family proteins, including Akt and PKCα, and regulates cell survival, cytoskeleton rearrangement, and cell migration (6,12). Preclinical studies have demonstrated that mTORC1 promotes tumor growth, invasion, and angiogenesis in prostate cancer models (13-16). Notably, mTORC2 is critical for the prostate cancer tumorigenesis driven by PTEN loss, but is dispensable for normal prostatic epithelial functions (17), supporting the selective role of mTORC2 in prostate cancer. The results of the present study demonstrated that PC-3/DTX cells exhibited stronger mTORC2 activity and downstream signaling pathways. This new finding suggested that the mTORC2 signaling pathway may serve an important role in the regulation of the docetaxel resistance of PC-3 cells. In future studies, the impact of mTORC2 signaling on the acquired docetaxel resistance of CRPC cells and the potential of agents targeting mTORC2 in reversing docetaxel resistance *in vitro* should be investigated.

In conclusion, a stable docetaxel-resistant PC-3 cell line was established. This cell line may serve as a useful cell model to further study the molecular mechanisms of prostate cancer drug resistance and may lead to the establishment of novel therapeutic strategies for prostate cancer.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

JL was the principal investigator in the study and was responsible for the literature search, study design, data interpretation, and manuscript writing and revision. YH and DL were the main executors of the study, contributed toward carrying out the study, data analysis, and manuscript writing. YZ and XL performed cell culture. YD participated in the design of the present study and manuscript revision. DZ, LW and QZ participated in the design of the present study and data interpretation. All authors read and approved the final manuscript, and agreed to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the study were appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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