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Functional p53 Determines Docetaxel Sensitivity in Prostate Cancer Cells

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Abstract

BACKGROUND—Docetaxel is the first line treatment for castration resistant prostate cancer (CRPC). However, docetaxel resistance rapidly develops. Identifying the critical mechanisms giving rise to docetaxel resistance is the major challenge in advanced prostate cancer.

METHODS—The effects of docetaxel on human DU145, PC3, LNCaP, and C4-2 prostate cancer cells were examined in cell culture, and p53 expression were analyzed by Western blot analysis. The potential role of p53 in docetaxel sensitivity in prostate cancer cells was tested by either p53 silencing using shRNA or p53 overexpression by introducing wild-type p53.

RESULTS—We found that DU145 (mutant p53) and PC3 (p53 null) cells were less sensitive than LNCaP and C4-2 cells expressing functional p53 in response to docetaxel. Docetaxel treatment induces considerably higher apoptosis in LNCaP and C4-2 cells than in DU145 and PC3 cells in a dose dependent manner. Docetaxel increases the levels of ser15 phosphorylation of p53 in a dose dependent manner in both LNCaP and C4-2 cells, while has no effect on the levels of ser15 phosphorylation of p53 in DU145 cells. These results suggest that p53 phosphorylation is associated with docetaxel sensitivity in prostate cancer cells. To further confirm whether p53 activation can induce cell sensitivity to docetaxel treatment, we used p53 shRNA to knock down p53 expression in C4-2 cells and determined the cells response to docetaxel treatment. Knockdown of p53 significantly down regulated p53 phosphorylation and blocked docetaxel induced apoptotic cell death compared to the vector control. To further confirm this observation, we established a stable knock out p53 in C4-2 cells. Down regulation of p53 in the stable p53 knock out C4-2 cells significantly inhibited docetaxel induced apoptotic cell death. We also used wild-type (WT) p53 to over express p53 in DU145 cells, and found that expression of WT-p53 in DU145 cells increased their sensitivity to docetaxel.

CONCLUSIONS—These results demonstrate that docetaxel induces p53 phosphorylation and that p53 status is a crucial determinant of docetaxel sensitivity in prostate cancer cells.

Keywords

prostate cancer; docetaxel; p53

INTRODUCTION

Although androgen deprivation therapy (ADT) is initially effective for treating localized and androgen responsive prostate cancer, most patients will relapse due to development of castration-resistant prostate cancer (CRPC) after 2–3 years [1]. Docetaxel is the first line treatment for CRPC. While the use of docetaxel has proved to be beneficial, only about 48% of patients respond to docetaxel and prednisone combination treatment with a median survival of 2.5 months over the control group [2]. Docetaxel is a cytotoxic antimicrotubule agent that binds to the β -tubulin subunit of microtubulin, resulting in stabilizing microtubules and preventing depolymerization, which leads to the inhibition of microtubule dynamics and cell cycle arrest and eventually apoptotic cell death [3–5].

Wild type p53 is known as a tumor suppressor gene involved in cellular response to DNA damage results in induction of a series of target genes such as p21 and Bax, leading to growth arrest or apoptosis [6]. p53 is one of the most commonly mutated genes in human cancer and loss of wild-type function through mutation or deletion can have profound effects on how cells respond to cell stress, such as ionizing radiation, free radicals, hypoxia, and chemotherapy. p53 expression usually is low in the normal condition, and binds with Mdm2 in the cytoplasm. The exposure of cell stresses such as ionizing radiation, free radicals, hypoxia, and chemical reagents disrupts the p53-Mdm2 complex and subsequently leads to the phosphorylation of p53. The activated p53 rapidly translocates into the nucleus and binds to the DNA consensus to activate the downstream target genes. Phosphorylation of p53 has been reported in cells exposed to different conditions, such as ionizing radiation, UV irradiation, topoisomerase I inhibitor camptothecin, and selenium [7–11]. The phosphorylation of p53 might stabilize p53 protein through stabilizing the p53–p300 complex and disassociating the p53-Mdm2 complex [12,13]. Mutant p53 or the lack of p53 in the cell usually causes drug resistance [14]. p53 protein is physically associated with tubulin in vivo and in vitro, and localizes to cellular microtubules [15]. Given the importance of microtubulin in mediating the effects of docetaxel on cellular response and the association of p53 to tubulin, it is possible that p53 may play a role in how cells respond to docetaxel treatment.

In this study, we investigated the potential roles of p53 in the development of docetaxel resistance in prostate cancer cells. We found that docetaxel is more sensitive in C4-2 and LNCaP cells that express wild-type p53 than DU145 cells that express mutant p53. We demonstrate that docetaxel induces p53 phosphorylation in LNCaP and C4-2 cells but not in DU145 cells, and that p53 status is a crucial determinant of docetaxel sensitivity in prostate cancer cells.

MATERIALS AND METHODS

Reagents and Cell Culture

C4-2, LNCaP, PC3, and DU145 cells were maintained in RPMI1640 supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 0.1 mg/ml streptomycin. The cells were maintained at 37°C in a humidified incubator with 5% carbon dioxide.

Plasmids and Cell Transfection

p53 shRNA plasmid was cloned in pBabe-U6 vector, the target sequence is GACTCCAGTGGTAATC-TAC [16]. Wild type (WT)-p53 plasmid was cloned in pcDNA3.1 vector. C4-2 and DU145 cells were transiently transfected by expressing plasmids using Attractene (QIAGEN). C4-2 p53 shRNA and C4-2 pBabe-U6 vector stable clones were selected in RPMI1640 medium supplemented with 1.0 µg/ml puromycin for 2 weeks after being transfected with p53 shRNA and control vector and then maintained in RPMI1640 medium containing 1.0 µg/ml puromycin.

Preparation of Nuclear and Cytosolic Extracts

C4-2 cells and C4-2 shRNA p53 stable clone cells were treated with DMSO or different doses of docetaxel overnight. Cells were harvested, washed with PBS twice, and resuspended in a low salt buffer (10 mmol/L HEPES-KOH [pH 7.9], 1.5 mmol/L MgCl₂, 10 mmol/L KCl, and 0.1% NP40) and incubated on ice for 30 min. Nuclei were precipitated by 3,000g centrifugation at 4°C for 10 min. The supernatant was collected as the cytosolic fraction. After washing once with the low salt buffer, the nuclei were lysed in a high salt lysis buffer (50 mmol/L Tris-HCl [pH 8], 150 mmol/L NaCl, 1% Triton X-100) and were vigorously shaken at 4°C for 30 min. The nuclear lysate was precleared by 10,000 rpm centrifugation at 4°C for 15 min. Protein concentration was determined using the Coomassie Plus protein assay kit (Pierce, Rockford, IL).

Immunofluorescence Assay

2×10^4 C4-2 control cells and C4-2 shRNA p53 stable clone cells were plated in 8-well chamber slides and treated with 2.5 nM docetaxel overnight. Cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and incubated with 1% BSA to block nonspecific binding. Cells were incubated with anti-ser15 phosphorylation of p53 (Cell Signal) and anti-tubulin antibodies (Santa Cruz Biotechnology) overnight. Intracellular ser15 phosphorylation of p53 was visualized with FITC-conjugated secondary antibodies, tubulin was visualized with Texas red conjugated secondary antibodies and nuclei were visualized with DAPI.

Western Blot Analysis

Whole cell protein extracts were resolved on SDS-PAGE. Proteins were then transferred to nitrocellulose membrane. After blocking for 1 hr at room temperature in 5% milk in PBS/0.1% Tween-20, membranes were incubated overnight at 4°C with the indicated primary antibodies. Following secondary antibody incubation, immunoreactive proteins were visualized with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Cell Growth Assay

C4-2, LNCaP, and DU145 cells were seeded in 12-well plates at a density of 1×10^5 cells/well in RPMI1640 media containing 10% FBS. The cells were treated as indicated and total cell numbers were counted.

Cell Death ELISA

Cells were seeded in 12-well plates (1×10^5 cells/well) and treated with vehicle alone or with different doses of docetaxel for 24 hr. Mono- and oligonucleosomes in the cytoplasmic fraction were measured by the Cell Death Detection ELISA kit (Roche) according to the manufacturer's instructions. Briefly, floating and attached cells were collected and homogenized in 400 µl of incubation buffer. Five microliters of the supernatant diluted in 95

μ l of incubation buffer were used in the ELISA. The wells were coated with anti-histone antibodies and then incubated with the lysates, horseradish peroxidase-conjugated anti-DNA antibodies, and subsequently the substrate. Absorbance was determined at 405 nm.

Clonogenic Assay

C4-2 cells and C4-2 shRNA p53 stable clone cells were treated with DMSO or different doses of docetaxel for 5 hr in FBS conditions. Cells were plated at equal density (1,000 cells/dish) in 100 mm dishes for 14 days. The cells were rinsed with PBS before staining with 0.5% crystal violet/4% formaldehyde for 30 min and the number of colonies was counted.

Statistical Analysis

All data are presented as means $\pm$ standard deviation of the mean (SD). Statistical analyses were performed with Microsoft Excel analysis tools. Differences between individual groups were analyzed by one-way analysis of variance (ANOVA) followed by the Scheffé procedure for comparison of means. $P < 0.05$ was considered statistically significant.

RESULTS

Differential Sensitivity to Docetaxel in Human Prostate Cancer Cells

LNCaP, C4-2, PC3, and DU145 cells were treated with increasing doses of docetaxel, and cell numbers were counted after 48 hr treatment. As shown in Figure 1A, DU145 and PC3 cells were less sensitive than LNCaP and C4-2 cells in response to docetaxel, consistent with previous reports showing that both DU145 and PC3 cells are more resistant to docetaxel treatment compared to LNCaP cells. One of the mechanisms of docetaxel-mediated cytotoxicity is inducing apoptotic cell death. We therefore measured apoptosis induced by docetaxel in these cells. As shown in Figure 1B, docetaxel treatment induces considerably higher apoptosis in LNCaP and C4-2 cells than in DU145 and PC3 cells in a dose dependent manner. Collectively, these results demonstrate that LNCaP and C4-2 cells are more sensitive to docetaxel treatment compared to DU145 and PC3 cells.

Docetaxel Induces p53 ser15 Phosphorylation in p53 Wild-Type LNCaP and C4-2 Cells but not in p53-Mutant DU145 Cells

LNCaP and C4-2 cells express functional p53 while DU145 cells express mutant p53 [17]. To determine whether functional p53 is associated with the differential sensitivity observed in prostate cancer cells in response to docetaxel treatment, LNCaP, C4-2, and DU145 cells were treated with different doses of docetaxel and cell lysates were collected for Western blot analysis. Since PC3 cells express null-p53, we chose DU145 cells which express mutant p53 for this study. As shown in Figure 2A,B, docetaxel increases the levels of ser15 phosphorylation of p53 in a dose and time dependent manner in C4-2 cells, while having no effect on the levels of ser15 phosphorylation of p53 in DU145 cells. The ser15 phosphorylation of p53 induced by docetaxel is also observed in LNCaP cells (Fig. 2A). p53 is usually translocated into the nucleus after activation [14,18–20]. We demonstrated that docetaxel treatment induced ser15 phosphorylation of p53 expression in C4-2 cells with the majority located in the nucleus but not in the cytoplasm (Fig. 3A,B). These results suggest that p53 phosphorylation is associated with docetaxel sensitivity in prostate cancer cells.

p53 Status Is a Crucial Determinant of Docetaxel Sensitivity

Our data showed that LNCaP and C4-2 cells are more sensitive than DU145 and PC3 cells in response to docetaxel treatment, and that the p53 status in these cells is associated with the differential docetaxel sensitivity. To test whether p53 activation affects cell sensitivity to

docetaxel treatment, we knocked down p53 expression using p53 shRNA in C4-2 cells and determined the cells response to docetaxel treatment. As shown in Figure 4A, docetaxel treatment induced the levels of ser15 phosphorylation of p53 in C4-2 cells expressing shRNA vector control, while p53 shRNA significantly decreased the levels of the ser15 phosphorylation of p53 induced by docetaxel. Knockdown p53 expression by p53 shRNA significantly down regulated ser15 phosphorylation of p53 and blocked docetaxel induced apoptotic cell death compared to the shRNA vector control as indicated by decreased levels of cleaved PARP protein (Fig. 4A) and lower levels of apoptosis (Fig. 4B). To further confirm this observation, we transiently transfected wild-type (WT)-p53 into DU145 cells and treated the cells with docetaxel, the results showed that overexpression of WT-p53 into DU145 cells increased their sensitivity to docetaxel (Fig. 4C). Docetaxel could induce WT-p53 phosphorylation at Ser15 site and increase the p53 target gene p21 expression in DU145 cells (Fig. 4D). We further confirmed these observations in C4-2 cells by stably knocking down of p53 expression in cells (Fig. 5A). Docetaxel treatment increased the levels of phosphorylation of p53 in vector control C4-2 cells, while had minimum increase in the levels of phosphorylation of p53 in the stable p53 knock down C4-2 cells (Fig. 5B). Consistent with the previous observation, down regulation of p53 expression significantly inhibited docetaxel effects, including inhibition of cell growth and induction of apoptotic cell death (Fig. 5C–E). Clonogenic ability of C4-2 vector control cells was inhibited by docetaxel treatment in a dose dependent manner; knock down of p53 expression in the C4-2 stable cells was able to reduce the inhibitory effect of docetaxel (Fig. 6A,B). Collectively, these results demonstrate that p53 status is a crucial determinant of docetaxel sensitivity in prostate cancer cells.

DISCUSSION

Docetaxel is a taxane that has been widely used against different cancers such as ovarian cancer, lung cancer, breast cancer and is the first line treatment for castration-resistant prostate cancer [21–26]. While the use of docetaxel has proven to be beneficial, resistance rapidly develops and only about 48% of patients respond to docetaxel and prednisone combination treatment with a median survival of 2.5 months over the control group [27]. Identifying the critical mechanisms giving rise to treatment resistance is the major challenge in advanced prostate cancer. Recent studies linked docetaxel sensitivity to androgen receptor in prostate cancer cells [28,29]. In this study, we investigated the potential mechanisms of docetaxel resistance that occurred after docetaxel treatment in prostate cancer cells. We found that DU145 and PC3 cells that express mutant or null p53 were less sensitive than LNCaP and C4-2 cells that express wild-type p53 in response to docetaxel. In addition, we found functional p53 is associated with the differential sensitivity observed in prostate cancer cells in response to docetaxel treatment.

We utilized in vitro prostate cancer cell models to determine cells response to docetaxel treatment. We observed that LNCaP and C4-2 cells are more sensitive to docetaxel treatment than DU145 and PC3 cells. Our results of docetaxel sensitivity in the prostate cancer cell lines are consistent with several reports showed that LNCaP cells are more sensitive than DU145 and PC3 cells [27,30–34]. However a few reports showed that LNCaP cells are more resistant to docetaxel treatment than DU145 or PC3 cells [35,36]. The reason for this discrepancy between the results of different studies is unclear, possibly due to the different passages or variants of LNCaP cells used by different laboratories. It is well known that docetaxel blocks microtubule activity during cell division leading to apoptosis [26]. It binds to the β -subunit of the tubulin heterodimer, which is the key constituent protein of cellular microtubules. The binding of docetaxel accelerates the polymerization of tubulin, and stabilizes the microtubules network, thereby inhibiting their depolymerization. This inhibition of microtubule depolymerization results in the arrest of the cell division cycle at

G2/M phase, leading to apoptosis of the cancer cells eventually [3,37]. Our results also demonstrate that docetaxel induces apoptotic cell death in prostate cancer cells and that docetaxel treatment resulted in significant higher apoptosis in LNCaP and C4-2 cells than DU145 and PC3 cells. These results suggest that docetaxel induced cell death is partly due to induction of apoptosis in prostate cancer cells.

LNCaP and C4-2 cells express wild-type p53, while DU145 and PC3 cells express mutant and null p53 [38]. To understand the potential role of p53 in prostate cancer cells toward docetaxel sensitivity, we analyzed p53 activity in response to docetaxel treatment in prostate cancer cells. Docetaxel treatment increases the levels of ser15 phosphorylation of p53 in a dose dependent manner in both LNCaP and C4-2 cells, while having little effect on the levels of ser15 phosphorylation of p53 in DU145 cells. The reason for the failure of docetaxel in inducing the phosphorylation of mutant p53 in DU145 cells is not clear. Carroll et al. [17] reported that two mutations in the p53 gene, a mutation at codon 274 (pro > leu) and a second mutation at codon 223 (val > phe), were found in DU-145 cells. Whether these mutations are involved in preventing docetaxel-mediated p53 phosphorylation in DU145 cells needs further investigation. To further confirm whether p53 activation can induce cell sensitivity to docetaxel treatment, we used p53 shRNA to knock down p53 expression in C4-2 cells and determined the cells response to docetaxel treatment. Knockdown of p53 expression by p53 shRNA significantly blocked docetaxel induced apoptotic cell death compared to the vector control, while overexpression of wild-type p53 in DU145 cells increased their sensitivity to docetaxel. The ability to resensitize DU145 cells (express endogenous mutant phosphorylated p53) to docetaxel by introduction of the wild-type p53 suggests that there may be a major functional difference between phosphorylated wild-type p53 and phosphorylated mutant p53.

p53 status has an important relationship with cancers, restoring endogenous p53 expression leads to regression of autochthonous lymphomas and sarcomas in mice without affecting normal tissues [39]. Caraglia et al. [3] found wild type status of p53 correlates with the sensitivity to the growth-inhibiting effects induced by docetaxel in human epithelial cells. p53 mutations increase cell proliferation and survival, and in some settings promote genomic instability and resistance to certain chemotherapies, brief reactivation of endogenous p53 in p53-deficient tumors can produce complete tumor regressions [40]. Although mutations of p53 occur at relatively low frequency (4–20%) in early stage of prostate cancer, the frequency of p53 mutations is increased (50–79%) in advanced stages of prostate cancer, as well as in recurrent and metastatic disease [41]. Currently, there is no report about the p53 status from recurrent docetaxel treated prostate cancer patients. A study from breast cancer patients harboring p53 mutation showed less responsive (44%) to docetaxel treatment compared to patients with wild-type p53 (62%) [42]. Although the difference was not statistically significant and needed further validation in a larger patient population, it suggested that p53 mutations might play a certain role in the development of docetaxel resistance in breast cancer patients. Taken together, loss of p53 function is responsible for docetaxel resistance and therapeutic strategies aimed at activation of p53 may be effective for resensitizing cancer cells from docetaxel resistance to response.

In summary, we found that p53 status is a crucial determinant of docetaxel sensitivity in prostate cancer cells. An investigation of p53 status in patients before and after docetaxel treatment is under way to determine the clinical usefulness of p53 status for patient selection and prognosis in response to docetaxel treatment.

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Fig. 1. LNCaP and C4-2 cells are more sensitive to docetaxel treatment than DU145 and PC3 cells. **A:** Docetaxel inhibits the growth of LNCaP, C4-2, DU145, and PC3 prostate cancer cells. Cells were plated in 12-well plates and treated with different concentrations of docetaxel. The cell number was counted after 24 hr. **B:** Effect on apoptosis. Mono- and oligo-nucleosomes in the LNCaP, C4-2, DU145, and PC3 cell cytoplasmic fraction were analyzed by cell death detection ELISA as described in the Materials and Methods Section. Apoptosis is expressed as enrichment factor, which was calculated by using the absorbance of each treated sample divided by its corresponding control.

Fig. 2. Docetaxel activated the p53 pathway in C4-2 and LNCaP cells but not in DU145 cells. **A:** C4-2, LNCaP and DU145 cells were treated with different concentrations of docetaxel for 24 hr in FBS condition; whole cell lysates were collected and subjected to Western blot analysis using the antibodies as indicated. **B:** C4-2 cells were treated with 5 nM docetaxel, the whole cell lysates were collected at different time points and subjected to Western blot.

Fig. 3.

Docetaxel activated p53 in the nucleus. **A:** C4-2 cells were treated with different doses of docetaxel in FBS conditions overnight. Cytoplasmic and nuclear proteins were immunoblotted with phosphor-p53 (ser15), p53, HSP90, and POLII antibodies. POLII and HSP90 were used as loading controls for nuclear and cytoplasmic proteins, respectively. **B:** C4-2 cells were treated with control and 2.5 nM docetaxel in FBS conditions overnight. Cells were processed for immunofluorescent staining of phosphor-p53 (ser15)-FITC, Tubulin-Texas red and Nuclei were stained with DAPI.

Fig. 4.

Down regulation of p53 protein decreases docetaxel sensitivity. **A:** C4-2 cells were transiently transfected with p53 shRNA or vector for 24 hr, followed by docetaxel treatment for another 24 hr. Whole cell lysates were collected and subjected to Western blot analysis using the antibodies as indicated. **B:** Down regulation of p53 protein reduces docetaxel-mediated apoptosis. C4-2 cells were transiently transfected with p53 shRNA or vector for 24 hr, followed by docetaxel treatment for 24 hr. Mono- and oligo-nucleosomes of cytoplasmic fraction were analyzed by cell death detection ELISA as described in the Materials and Methods Section. **C:** Transiently transfected WT-p53 into DU145 cells could sensitize docetaxel cells, DU145 cells were transiently transfected with WT-p53 or pcDNA for 24 hr, followed by docetaxel treatment for another 24 hr, counted cell number and calculated the cell survival rate. **D:** DU145 was transiently transfected with WT-p53 or pcDNA for 24 hr, followed by docetaxel treatment for another 24 hr. Whole cell lysates were collected and subjected to Western blot analysis using the antibodies as indicated. *, significant differences between the groups ($P < 0.05$).

Fig. 5.

Stable knockdown of p53 protein in C4-2 cells increases docetaxel resistance. **A:** C4-2 p53 shRNA stable clone (C4-2 sip53 No. 2) express lower levels of p53 and phosphor-p53 (ser15) proteins compared to C4-2 cell control cells. **B:** C4-2 control cells and C4-2 p53 shRNA stable clone cells were treated with different doses of docetaxel for 24 hr; whole cell lysates were collected and subjected to Western blot analysis using the antibodies as indicated. **C:** Effect on cell survival. C4-2 control cells and C4-2 p53 shRNA stable clone cells were treated with docetaxel for 24 hr, cell numbers were counted and expressed as % of cell survival. **D:** Effect on apoptosis. C4-2 control cells and C4-2 p53 shRNA stable clone cells were treated with different doses of docetaxel for 24 hr; mono- and oligo-nucleosomes of cytoplasmic fraction were analyzed by cell death detection ELISA. **E:** Photograph of C4-2 control cells and C4-2p53 shRNA stable clone cells treated with different doses of docetaxel. *, significant differences between the groups ($P < 0.05$).

Fig. 6. Knock down of p53 expression reverses docetaxel-mediated inhibition of clonogenic ability. C4-2 control cells and C4-2 p53 shRNA stable clone cells were treated with different doses of docetaxel for 5 hr. Cells (1,000 cells/dish) were plated on 10 cm dishes, and the colonies were stained with 0.5% crystal violet after 3 weeks (A). The scanned images of the colonies were quantified with imaging software(B).*, significant differences between the groups ($P < 0.05$).