p53 interferes with microtubule-stabilizing agent-induced apoptosis in prostate and colorectal cancer cells

JI YOUNG KIM[1,4], JIN-YONG CHUNG[1,4], SEUNG GEE LEE[1,3], YOON-JAE KIM[1,3], JI-EUN PARK[1,3], JEANHO YUN[2-4], YOUNG CHUL PARK[5], BYEONG GEE KIM[6], YOUNG HYUN YOO[1,3,4] and JONG-MIN KIM[1,3,4]

Departments of [1]Anatomy and Cell Biology, [2]Biochemistry, Dong-A University, Busan 602-714; [3]Mitochondria Hub Regulation Center, [4]Medical Research Science Center, Dong-A University, Busan 602-714; [5]Department of Microbiology and Immunology, Pusan National University School of Medicine, Yangsan, Gyeongnam 626-870; [6]Department of Biology, College of Natural Sciences, Pusan National University, Busan 609-735, Republic of Korea

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Abstract. Taxanes are microtubule-stabilizing agents that have anticancer activity against several types of human solid tumors. Although the primary mechanism of action of these drugs is well understood, the signaling pathways that confer resistance to these agents in certain types of cancer remain poorly understood. In particular, the association of p53 with the mechanism(s) of taxane-mediated cell death is still controversial. In this study, we showed that p53 has a profound inhibitory effect on docetaxel (Doc)-induced apoptosis in prostate and colorectal cancer cells and that caspases play a critical role in this process. Doc induced prostate cancer cell apoptosis at high levels in p53-null PC3 cells, at intermediate levels in p53-mutant DU145 cells and at low levels in p53 wild-type LNCaP cells. While transient overexpression of p53 in PC3 cells suppressed Doc-induced apoptosis, knockdown of p53 in LNCaP cells increased apoptosis. This finding was further confirmed using an isogenic pair of colorectal cancer cell lines, HCT-116 $p53^{-/-}$ and $p53^{+/+}$, indicating that p53 inhibits induction of apoptosis by Doc. To our knowledge, this is the first report describing that chemical or genetic knockout of p53 enhances the susceptibility of both prostate and colorectal cancer cells to Doc-induced apoptosis. These results may suggest an approach to stratify patients for regimens involving Doc.

Correspondence to: Dr Jong-Min Kim, Department of Anatomy and Cell Biology, Dong-A University, Dongdaeshin-dong 3-1, Seo-gu, Busan 602-714, Republic of Korea
E-mail: jmkim7@dau.ac.kr

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Introduction

Taxanes are microtubule-stabilizing agents (MSAs) that exhibit anticancer activity in several human solid tumors (1). Among these taxanes, docetaxel (Doc) has an increased affinity for tubulin (2) and has higher antitumor activity than paclitaxel (3). Doc has been approved for use in the clinical treatment of lung, breast and prostate cancer (4). Although the primary action mechanism of this drug is well understood, the signaling pathways that confer resistance to these agents in certain types of cancer remain poorly understood.

Several signal transduction pathways are involved in apoptosis evoked by taxanes (5). The upregulation of p53 and the cyclin-dependent kinase inhibitor (p21/Waf1) (6), phosphorylation of Bcl-2 (7) and activation of MAP kinases (8) have been implicated as mediators of Taxol-induced apoptosis. Mitotic catastrophe resulting from micronucleation or aberrant mitosis also appears to be a critical step during Doc-induced apoptosis (9). Recently, we demonstrated that binding of Smac/Diablo to survivin in the nucleus plays an important role in Doc-induced apoptosis of prostate cancer cells (10).

Although p53 is implicated in the modulation of taxane-induced cancer cell death, it remains controversial whether it plays a key role in MSA-mediated apoptosis. Several studies have suggested that Taxol-induced apoptosis is independent of the p53 status (11-16). In contrast, increased sensitivity to Taxol was observed in non-transformed cells in the absence of functional p53 (17). Similar results were observed in ovarian carcinoma cells (18). However, no further study has evaluated the hypothesis that deficiency or mutation of p53 may render cancer cells more susceptible to taxane-induced apoptosis.

In this study, we report that chemical or genetic knockout of p53 enhances susceptibility to Doc-induced apoptosis in both prostate and colorectal cancer cells. Our data suggest that p53 plays a pivotal role in suppressing Doc-induced apoptosis in prostate and colorectal cancer cells and that the p53 status could be used to stratify patients for Doc-containing treatment regimens.

Materials and methods

Reagents. Docetaxel (Taxotere) was obtained from Aventis Pharmaceuticals (Bridgewater, NJ, USA). Hoechst 33258, RNase A and propidium iodide (PI) were from Sigma Chemical Co. (St. Louis, MO, USA). 3,3'-Dihexyloxacarbocyanine iodide [$DiOC_6(3)$] was from Invitrogen Molecular Probes (Carlsbad, CA, USA).

Antibodies. The antibodies used included rabbit anti-p53 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); rabbit anti-cleaved caspase-3, -7, -8 and -9 (Cell Signaling Technology, Inc., Beverly, MA, USA); rabbit anti-tBID (p15) cleavage site-specific antibody (BioSource International, Camarillo, CA, USA); mouse anti-PARP and rabbit anti-phosphorylated p53 (Calbiochem, San Diego, CA, USA); mouse anti-β-actin (Sigma); mouse anti-XIAP (BD Pharmingen, San Diego, CA, USA). Horseradish peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and FITC-conjugated anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) were also used.

Cell culture and drug treatment. LNCaP, DU145 and PC3 (human prostate carcinoma cell lines; American Type Culture Collection, Rockville, MD, USA) were maintained in Dulbecco's modified Eagle's medium/ Nutrient Mixture F-12 HAM (Sigma) containing 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen) and 1.2 g/l sodium bicarbonate supplemented with 10 μg/ml penicillin-streptomycin (Invitrogen). HCT-116 $p53^{+/+}$ and $p53^{-/-}$ cells were provided by Dr Bert Vogelstein (Howard Hughes Medical Institute, Baltimore, MD, USA) and maintained in RPMI-1640 (Invitrogen) containing 10% heat-inactivated FBS and 2 g/l sodium bicarbonate supplemented with 10 μg/ml penicillin-streptomycin. Cells were incubated in a humidified incubator at 37˚C with 5% CO_2. Doc (10 mg) was dissolved in 7.8 ml of 1X PBS (pH 7.4), 7.8 ml of DMSO and 15.6 ml of absolute ethanol as 375 μM stock, and it was added to cells at 50% confluency.

Wild-type p53 transfection. For transfection, PC3 cells were seeded in 6-well plates and grown until they attained 50% confluency, and then 1.5 μg of p53 plasmid was introduced into the cells using Transfectin (Bio-Rad, Richmond, CA, USA) according to the manufacturer's instructions. After 24 h, the cells were treated with 5 nM Doc and then incubated at 37˚C for an additional 24 h.

Knockdown of p53 with small interfering RNA (siRNA). For transfection, cells were seeded in 6-well plates and grown until they attained 50% confluency, after which 6 μl of 10 μM stock siRNA (SignalSilence® p53 siRNA; Cell Signaling Technology, Inc.) was introduced into the cells using RNAiFect (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions.

Sub-G_1 analysis. Cells were harvested for cell cycle analysis, fixed with 95% ethanol (with 0.5% Tween-20) for 24 h, incubated with 0.05 mg/ml PI and 1 μg/ml RNase A at 37˚C for 30 min, and analyzed by flow cytometry using an Epics XL flow cytometer with analysis software (EXPO32™; Beckman Coulter, Miami, FL, USA). The cells in the sub-G_1 population were considered apoptotic.

Annexin V cell death assay. Cells were stained with Annexin V-FITC and PI using the Annexin V-FITC Apoptosis Detection kit (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's protocol. Briefly, $1x10^5$ cells were pelleted and resuspended in 100 μl of binding buffer. Subsequently, 5 μl of Annexin V-FITC and PI (0.05 mg/ml) were added to the cells, and cells were incubated for 15 min at room temperature (RT). After incubation, 400 μl of the binding buffer was added to the stained cells, and the cells were analyzed by flow cytometry.

Measurement of mitochondrial membrane potential. Cells ($5x10^5$) were incubated with 100 nM $DiOC_6$ at 37˚C for 30 min and then washed and resuspended with PBS; thereafter, the fluorescence was measured by flow cytometry.

Western blot analysis. For western blot analysis, whole cell lysates were prepared by incubating cell pellets in lysis buffer [30 mM NaCl, 0.5% Triton X-100, 50 mM Tris-HCl (pH 7.4), 1 mM Na_3VO_4, 25 mM NaF, 10 mM $Na_4P_2O_7$] for 30 min on ice. After insoluble fractions were removed by centrifugation at 14,000 rpm at 4˚C for 40 min, the supernatants were collected, and the protein concentration was determined using a BCA protein assay kit (Pierce Biotechnology, Inc., Woburn, MA, USA). Cell lysates (50 μg) were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane (Amersham Pharmacia Biotech). Membranes were incubated for 1 h at RT with a primary antibody in Tris-buffered saline containing 0.05% Tween-20 (TBS-T; pH 7.4) in the presence of 5% nonfat dry milk. After the membranes were washed in TBS-T, secondary antibody reactions were performed with an appropriate source of antibody conjugated with horseradish peroxidase. The signals were detected with an Enhanced-Chemiluminescence Detection kit (Amersham Pharmacia Biotech) in the LAS-3000 detector (Fuji, Japan). Immunoblotting for β-actin was performed in every experiment as an internal control.

Immunocytochemistry. Collected cells were attached on slide glass by cytospin centrifugation using a Cellspin™ (Hanil, Korea). Cells were fixed with 4% paraformaldehyde at RT for 30 min, washed with PBS for 10 min, and incubated with 0.2% Triton X-100 for 10 min. Then, cells were washed, incubated with the appropriate primary antibody in 1% bovine serum albumin at RT for 2 h. For the secondary antibody reaction, cells were incubated with the appropriate fluorescein (FITC)-conjugated secondary antibody at RT for 1 h. For counterstaining of the nucleus (when required), cells were incubated with PI (50 μg/ml) at RT for 15 min. Finally, cells were washed, mounted, and observed using a confocal microscope (LSM510; Carl Zeiss, Germany).

Statistics. Data are expressed as the mean ± SD of 3 or 4 separate experiments and analyzed by the Student's t-test. Mean values were considered statistically significant at $P<0.05$.

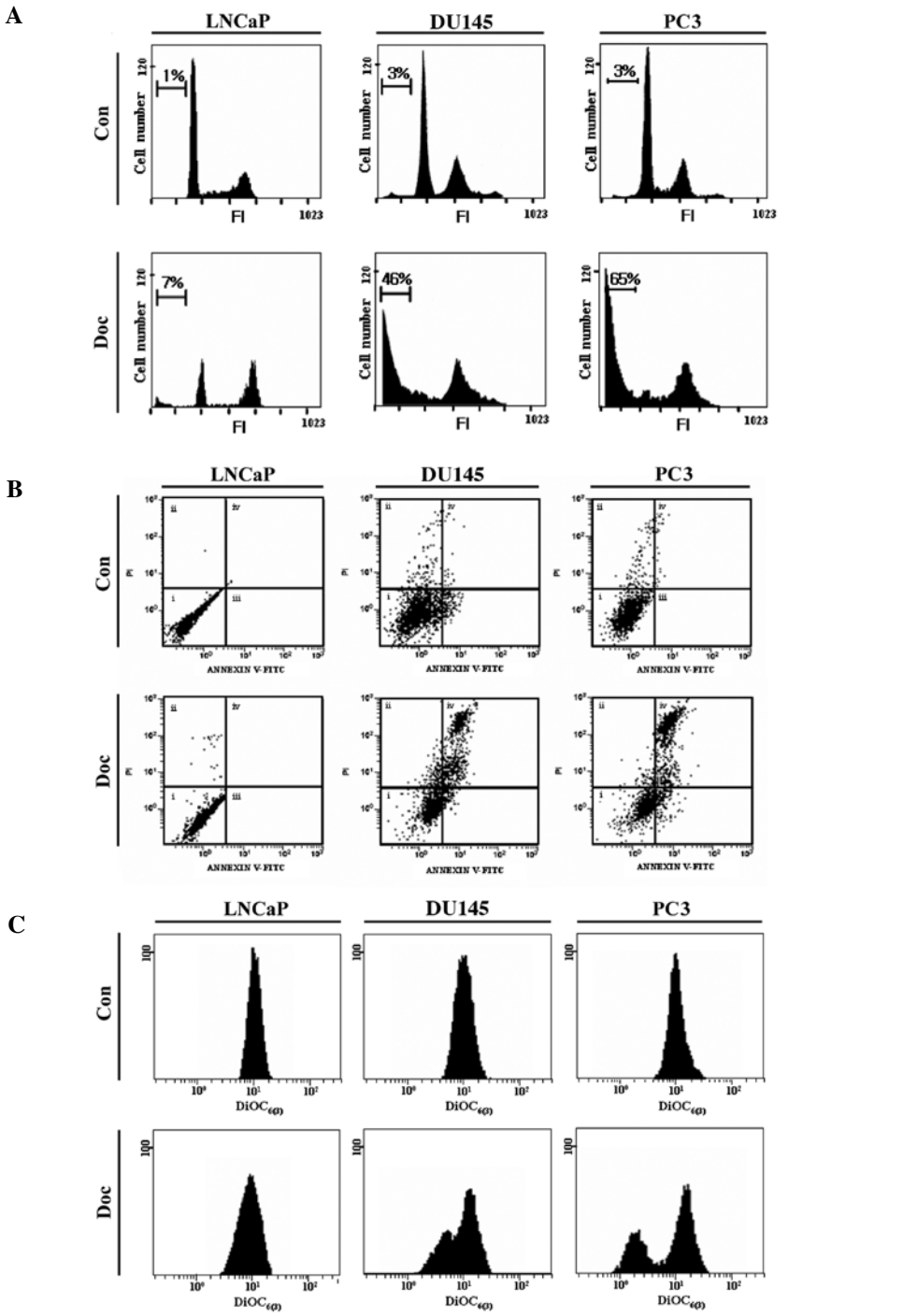

Figure 1. Induction of apoptosis in LNCaP, DU145 and PC3 prostate cancer cells after exposure to docetaxel. (A) Identification of apoptosis by sub-G_1 analysis with a flow cytogram. (B) Annexin V cell death assay. i, ii, iii and iv denote viable (live), necrotic, early apoptotic and late apoptotic regions, respectively. (C) Measurement of mitochondrial membrane potential after Doc (5 nM) treatment for 48 h. Con and Doc indicate control and docetaxel, respectively.

Results

Doc does not effectively induce apoptosis in p53 wild-type prostate cancer cells. To monitor the role of p53 in Doc-induced cancer cell death, three types of prostate cancer cells [LNCaP (p53 wild-type), DU145 (p53 mutant) and PC3 (p53 null)] with a different p53 status were primarily employed in this study. According to our previous study (10), 5 nM Doc (for 48 h) was considered as the optimal condition for the induction of apoptosis in these prostate cancer cell lines. Flow cytometric sub-G_1 analysis showed that significant cell death occurred in DU145 and PC3 cells, but not in LNCaP cells (Fig. 1A). The type of cell death induced by Doc was mostly apoptotic, as evaluated by Annexin V binding assay (Fig. 1B). Moreover, Doc-induced apoptosis was accompanied by depolarization of mitochondrial membrane potential (MMP; $\Delta\Psi m$) (Fig. 1C). These results indicate that Doc effectively induces apoptosis in prostate cancer cells that harbor defective p53.

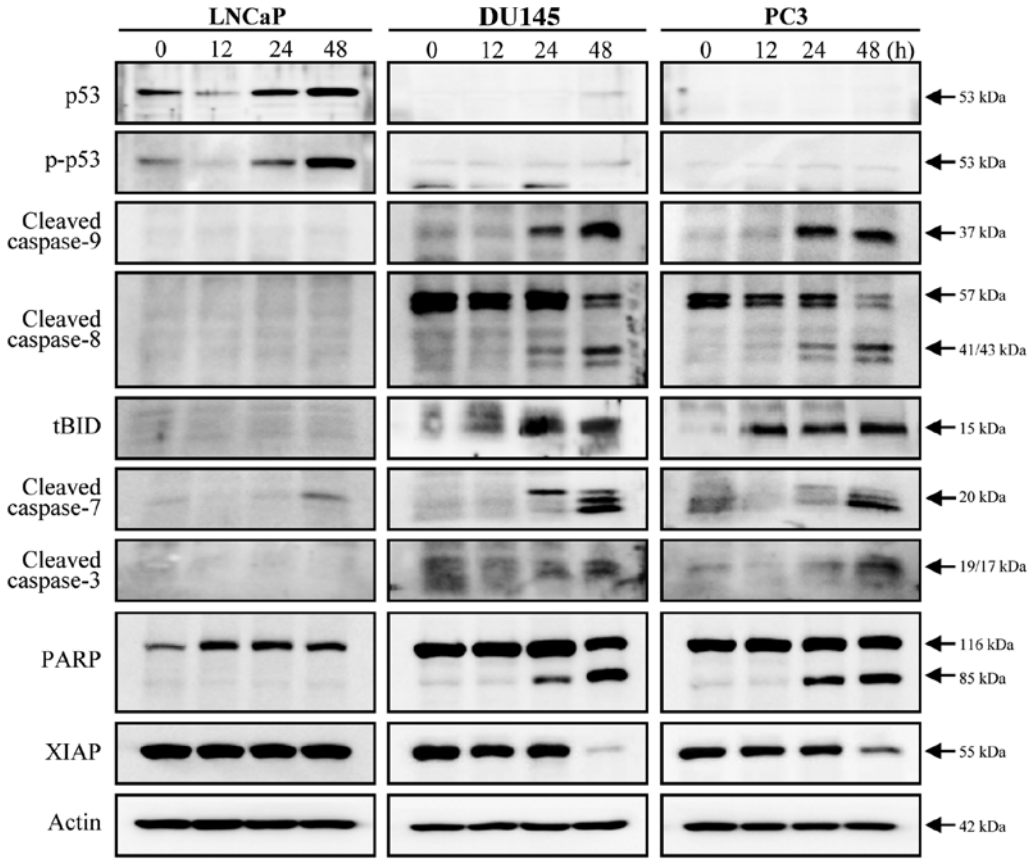

Figure 2. Changes in p53 and apoptosis-related proteins in LNCaP, DU145 and PC3 prostate cancer cells after exposure to docetaxel (Doc). Occurrence of caspase-dependent apoptosis after Doc treatment as determined by western blot analyses of p53, phospho-p53 (serine 15), caspase-9, -8, -3 and -7, tBID, XIAP and PARP. Actin was used as an internal (loading) control in the western blot analysis.

Doc-induced apoptosis in prostate cancer cells is accompanied by activation of multiple caspases. Changes in total p53 levels and phosphorylated p53 (at serine 15) after exposure to Doc were detected only in LNCaP cells (Fig. 2). While significant activation of caspase-9, -8, -7 and -3 was not observed in LNCaP cells, all caspases were cleaved in both DU145 and PC3 cells (Fig. 2). Appearance of tBID supports the evidence that the caspase-8 pathway was also associated with Doc-induced apoptosis, in conjunction with the caspase-9 pathway (Fig. 2). Cleavage of PARP and decreased levels of XIAP protein indicate that Doc-induced apoptosis in prostate cancer cells was caspase-3 dependent (Fig. 2).

Knockdown of p53 sensitizes LNCaP cells to Doc-induced apoptosis. In order to examine whether the ablation of p53 in LNCaP cells increases their susceptibility to Doc-induced apoptosis, we used siRNA-mediated knockdown of p53. As shown in Fig. 3A and B, the expression of endogenous p53 was dramatically reduced by siRNA transfection. p53 knockdown synergistically increased LNCaP cell death induced by Doc (Fig. 3C), which was associated with a caspase-dependent pathway (Fig. 3D). These results indicate that p53 plays a crucial role in the suppression of Doc-dependent apoptosis in prostate cancer cells.

Ectopic expression of p53 attenuates Doc-induced apoptosis in PC3 cells. We next tested whether transient transfection of p53 into PC3 cells suppresses Doc-induced apoptosis. An increase in total and phospho-p53 was detected in PC3 cells following transfection (Fig. 4A and C) and was associated with a significant repression of Doc-induced apoptosis (Fig. 4B). The inhibitory role of p53 in Doc-induced apoptosis was confirmed by the inhibition of the cleavage of caspase-3 and -7, and PARP that are provoked by Doc (Fig. 4C). These results imply that p53 interferes with Doc-induced apoptosis in prostate cancer cells.

p53-deficient colorectal cancer cells are more susceptible to Doc-induced apoptosis than those with wild-type p53. To determine whether p53 plays an inhibitory role in Doc-induced apoptosis in cells other than prostate cancer cells, an isogenic pair of colorectal carcinoma (HCT-116) cell lines differing only in their p53 status was investigated. Doc induced cell death in a higher number HCT-116 $p53^{-/-}$ cells when compared to the number in the HCT-116 $p53^{+/+}$ cells (Fig. 5A). Apoptosis occurred in a caspase-dependent manner, as revealed by cleavage of caspase-3 and -7, and PARP (Fig. 5B). Furthermore, the functional knockdown of p53 in HCT-116 $p53^{+/+}$ cells by either p53 siRNA or pifithrin-α (PFT-α), a p53 inhibitor, sensitized cells to Doc-induced apoptosis, which was concomitant with caspase-3 and -7, and PARP cleavage (Fig. 5C). Taken together, these results indicate that p53 also inhibits colorectal cancer cell apoptosis induced by Doc.

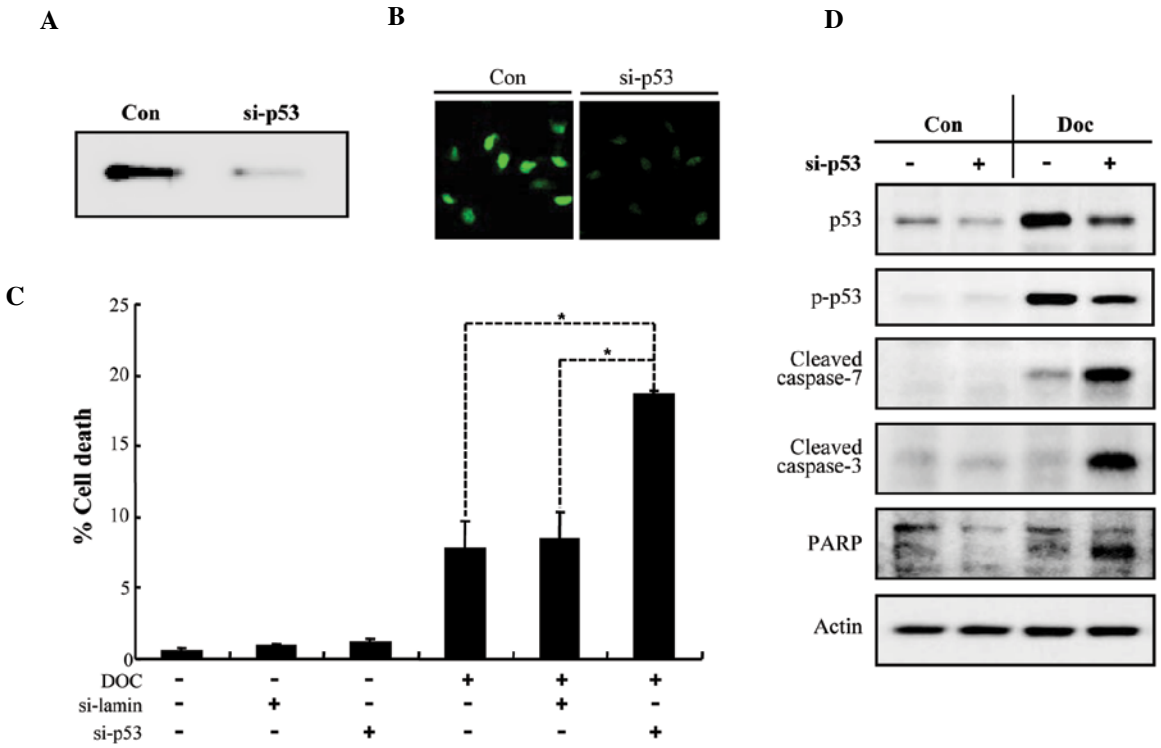

Figure 3. Loss of p53 function by p53 siRNA enhances docetaxel (Doc)-induced apoptosis in LNCaP cells. For the knockdown of the p53 gene in LNCaP cells, plasmid-based siRNA for p53 (si-p53) or control vector (si-lamin) were transfected for 24 h before Doc treatment. Downregulation of p53 proteins at 48 h after transfection as confirmed by (A) western blot analysis and (B) immunocytochemistry for p53. (C) Increase in the sub-G_1 population (apoptotic cells) in cells exposed to Doc by transfection of p53 si-RNA. After transfection, cells were exposed to Doc for 48 h and collected for sub-G_1 analysis by flow cytometry. *Significantly different from Doc or Doc + si-lamin ($P<0.05$). (D) Effect of p53 siRNA on apoptosis-related proteins in the absence or presence of Doc for 48 h. The same amount of proteins (~50 μg) was applied to SDS-PAGE followed by western blot analyses for p53, phospho-p53, cleaved caspase-3 and -7 and PARP. Actin was used as an internal (loading) control. Con and Doc indicate control and docetaxel, respectively.

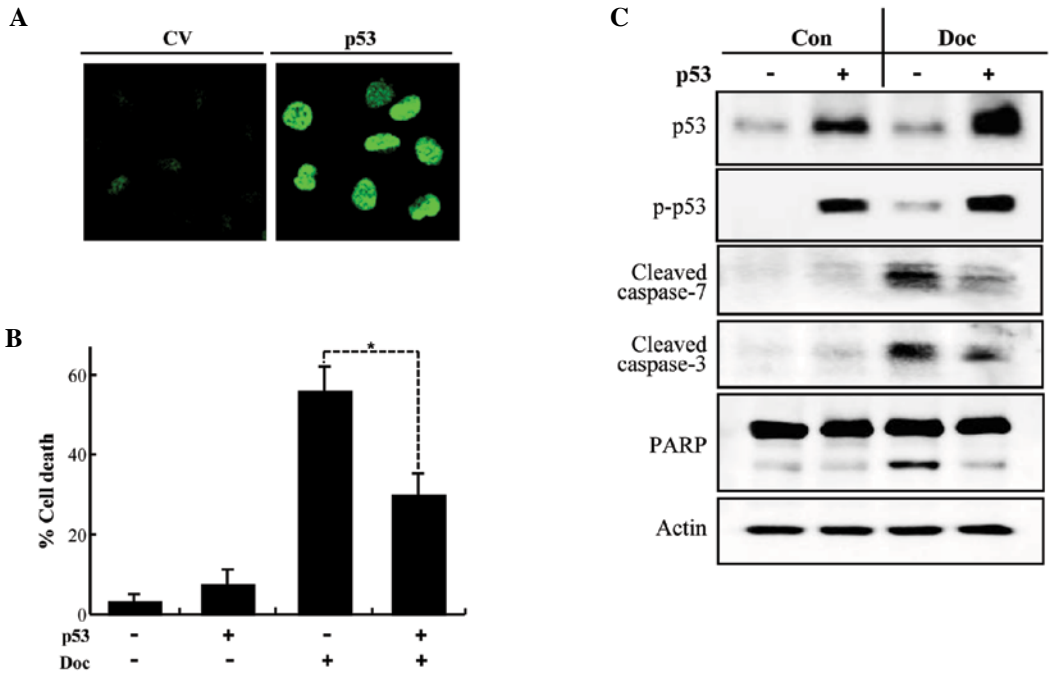

Figure 4. Ectopic expression of p53 inhibits docetaxel (Doc)-induced apoptosis in PC3 cells. For the transfection of the p53 gene, p53 plasmid or control vector (CV) was transfected in PC3 cells for 24 h before Doc treatment. (A) Overexpression of p53 proteins at 48 h after transfection, as confirmed by immunocytochemistry for p53. Microphotographs were captured with a confocal microscope. Original magnification, x800. (B) Reduction in the sub-G_1 population (apoptotic cells) in Doc-treated cells by transfection of p53. After transfection, cells were exposed to Doc for 48 h and collected for sub-G_1 analysis by flow cytometry. *Significantly different from the Doc-treated group ($P<0.05$). (C) Effect of exogenous p53 on apoptosis-related proteins in the absence or presence of Doc for 48 h. The same amount of protein (~50 μg) was applied to SDS-PAGE followed by western blot analyses for p53, phospho-p53, cleaved caspase-3 and -7 and PARP. Actin was used as an internal (loading) control. Con and Doc indicate control and docetaxel, respectively.

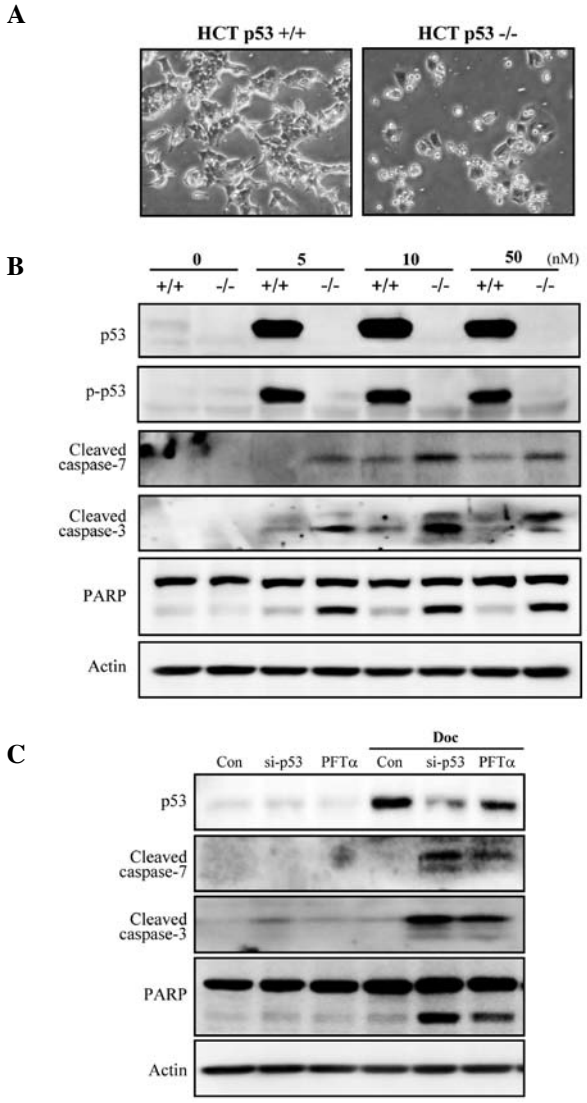

Figure 5. Role of p53 in docetaxel (Doc)-induced apoptosis in an isogenic pair of colorectal cancer cells. (A) Phase-contrast cellular morphology in HCT-116 $p53^{+/+}$ and HCT-116 $p53^{-/-}$ cells after exposure to Doc (10 nM) for 24 h. Original magnification, x100. (B) Effect of p53 on caspase-dependent apoptosis in the absence or presence of Doc for 24 h. HCT-116 $p53^{+/+}$ and HCT-116 $p53^{-/-}$ cells were treated with Doc at 0, 5, 10 and 10 nM for 24 h. The same amount of protein (~50 μg) was applied to SDS-PAGE followed by western blot analyses for p53, phospho-p53, cleaved caspase-3 and -7 and PARP. Actin was used as an internal (loading) control. (C) Effect of p53 knockdown either by siRNA or pifithrin-α (PFT-α) on caspase-dependent cell death in HCT-116 $p53^{+/+}$ cells. Western blot analysis was performed as described above (B). Con and Doc indicate control and docetaxel, respectively.

Discussion

The p53 gene is frequently mutated in human cancers (19), and p53 regulates cell cycle arrest, apoptosis, and DNA repair in a variety of cells (20). The role of p53 in the ultimate sensitivity to various anticancer drugs remains controversial. While several studies have demonstrated that cells lacking functional p53 are resistant to anticancer agents (21-23), others have reported contrasting findings (24-26). In this context, we examined whether the p53 status modulates the sensitivity to apoptosis in prostate and colorectal cancer cells after exposure to Doc.

In the present study, we demonstrated that p53 strongly inhibits Doc-induced apoptosis in prostate and colorectal cancer cells. Caspases are central mediators of this process. Doc treatment induced a graded apoptotic response in prostate cancer cell apoptosis; specifically, apoptosis was lowest in cells with wild-type p53 and highest in functionally null p53 PC3 cells. While transient overexpression of p53 in PC3 cells blocked Doc-induced apoptosis, knockdown of p53 in LNCaP cells enhanced apoptosis after Doc exposure. This finding was strongly supported by the results obtained with an isogenic pair of colorectal cancer cell lines, HCT-116 $p53^{-/-}$ and $p53^{+/+}$ cells. Taken together, these results clearly indicate that p53 exhibits an inhibitory role in the induction of apoptosis by Doc.

A co-relationship between defects in p53 and increased sensitivity to Taxol has not been reported in ovarian (12), colorectal (27), renal (16) and gastric (28) cancer cells. In contrast, loss of normal p53 function did confer sensitization to Taxol by increasing G_2/M arrest and apoptosis (17), and similar results were found in IGROV-1 ovarian carcinoma cells (18). This inconsistency may be attributed to the different origin or cellular constituents of each cancer cell type. Survivin is repressed by p53 (20) and is also implicated in the assembly process of microtubule polymerization during Doc-induced apoptosis in p53-mutated prostate cancer cells (10). Therefore, it is tempting to speculate that p53-mediated repression of survivin may block the MSA-induced cell death machinery in cancer cells. Further studies of the potential role of p53 in regulating microtubule dynamics are required in order to understand the relationship between p53 and taxane resistance in various cancer cells.

The findings of this study suggest that p53 is a potent inhibitor of Doc-induced apoptosis in both prostate and colorectal cancer cells. Thus, the genetic and functional status of p53 may be an important factor (biomarker) in guiding therapeutic strategies for prostate and colorectal cancer patients. A combinational treatment of Doc and p53-specific antagonists (i.e., PFT-α) could be considered for the development of a potential novel chemotherapy for taxane-resistant cancers in the near future.

Acknowledgements

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