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Analysis of resistance-associated gene expression in docetaxel-resistant prostate cancer cells

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Abstract. Docetaxel-based chemotherapy is the standard treatment for metastatic castration-resistant prostate cancer (CRPC). However, a number of patients with metastatic CRPC are refractory to docetaxel or develop docetaxel resistance. The underlying molecular mechanisms of docetaxel resistance remain unclear, which is a significant burden to the management of metastatic prostate cancer. In the present study, the differential gene expression between docetaxel-sensitive (PC3) and docetaxel-resistant (PC3DR2) prostate cancer cells was identified using DNA microarrays, western blot analysis and reverse transcription-quantitative polymerase chain reaction. Of the genes implicated in cancer-associated pathways, insulin-like growth factor 1 receptor, DBF4 homolog, sterile α motif and leucine zipper-containing kinase AZK, Patched 1, serpin peptidase inhibitor, clade E, member 1 and breast cancer 2 (BRCA2) were >3-fold upregulated in PC3DR2 cells compared with PC3 cells. BRCA2 knockdown with small interfering RNA decreased the docetaxel resistance of PC3DR2 cells. These results suggest that BRCA2 serves an important role in the docetaxel resistance of prostate cancer cells. In addition, BRCA2 modulation may be a strategy to partially reverse docetaxel resistance in prostate cancer.

Introduction

Prostate cancer, the most common non-cutaneous malignancy, is the second leading cause of cancer-associated mortality for males in the US, behind lung cancer (1). As localized prostate cancer can be cured by surgery or radiotherapy, the major public health burden comes from the metastatic stage of prostate cancer, for which there are currently limited curative options. A typical first-line treatment for metastatic prostate cancer is androgen deprivation therapy. However, the initial response to hormone therapy is not maintained for a long time; hormone therapy resistance, which leads to a disease state termed castrate-resistant prostate cancer (CRPC), emerges within a median time of 1.5 years (2). A previous study of patients with CRPC revealed that ~67% of patients responded to long-term androgen hormone blockade; the median survival time of non-responder patients was <1 year (2).

Docetaxel was approved by the US Food and Drug Administration in 2004 and has been evaluated in randomized Phase III trials for patients with metastatic CRPC to overcome the limitations of hormone therapy (3). Docetaxel chemotherapy prolonged median overall survival by ~3 months when compared with mitoxantrone and prednisone, and exhibited palliative benefits for a number of patients with metastatic CRPC (3). Docetaxel exerts its effect by targeting microtubules, which consist of filamentous polymers of α- and β-tubulin heterodimers and are critical for cell division (4). Docetaxel binds to β-tubulin and stabilizes microtubule structures, which inhibits the mitotic spindle apparatus. Thus, docetaxel-susceptible cells exhibit mitotic arrest, leading to apoptosis (5).

Although docetaxel was the first cytotoxic therapy demonstrated to exhibit a survival benefit in patients with CRPC, the median time to prostate-specific antigen progression is limited to 6-8 months, and additional chemotherapy options at progression are required (6). Furthermore, ~50% of patients with CRPC do not respond to docetaxel therapy, presenting a significant clinical problem (7). Furthermore, initial responders to docetaxel treatment ultimately develop docetaxel resistance. Proposed mechanisms for this resistance include the inhibition of drug accumulation into cancer cells, circumventing the cytotoxic effect via the upregulation of alternative growth pathways and the development of apoptosis resistance (5). However, the exact mechanism for docetaxel resistance has yet to be elucidated. *De novo* and acquired resistance to docetaxel chemotherapy are likely to be the main limitations to its efficacy.

A treatment regimen to overcome docetaxel resistance may be a viable alternative therapeutic strategy as there are currently a limited number of treatment options available to patients with CRPC. In our previous study, a cisplatin-resistant bladder cancer

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cell line was established to identify the genes associated with cisplatin resistance in bladder cancer (8). A docetaxel-resistant prostate cell line (PC3DR2) was subsequently established using the same method (9); resistance-associated genes in this cell line were examined in the present study through DNA microarray, western blot analysis and reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

In the present study, PC3DR2 cells were identified to exhibit a 3-fold increase in the expression of insulin-like growth factor 1 receptor (IGF1R), DBF4 homolog (DBF4), sterile α motif and leucine zipper-containing kinase AZK (ZAK), patched 1 (PTCH1), serpin peptidase inhibitor, clade E, member 1 (SERPINE1) and breast cancer 2 (BRCA2) from cancer-associated pathways compared with a docetaxel-sensitive cell line (PC3). BRCA2 small interfering (si)RNA knockdown restored docetaxel sensitivity in PC3DR2 cells, suggesting that BRCA2 may be associated with docetaxel resistance in human prostate cancer cells.

Materials and methods

Cell lines and chemicals. PC3 CRPC cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (MediaTech, Inc.; Corning Incorporated, Corning, NY, USA), 100 U/ml penicillin and 100 mg/l streptomycin (Gibco; Thermo Fisher Scientific, Inc.) with 5% CO_2 at 37˚C. A docetaxel-resistant CRPC cell line (designated PC3DR2) was generated by serial desensitization of PC3 cells as previously described (8,9). Docetaxel was obtained from Sanofi-Aventis Korea Co., Ltd. (Seoul, South Korea).

Cytotoxicity assay. The cytotoxic effect of docetaxel was determined using a Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc., Rockville, MD, USA). Approximately 4,000 cells were seeded in 96-well plates with 100 μl DMEM, and the cells were treated with increasing doses of docetaxel (0, 0.005, 0.01, 0.1, 0.5, 1, 2.5, 5, 10, 20 or 50 μM diluted with DMEM) for 72 h under the aforementioned conditions. Following incubation, 10 μl CCK-8 solution was added and the absorbance at 450 nm was determined 3 h after further incubation under the same conditions.

Apoptosis and survival-associated protein expression. Total protein was extracted from PC3DR2 cells with or without 2 μg/ml docetaxel treatment using radioimmunoprecipitation assay lysis buffer [containing 50 mM tris-HCl (pH 8.0), 150 mM sodium chloride, 1.0% NP-40, 0.5% sodium doxycholate, 0.1% sodium dodecyl sulfate and 1 mM phenylmethylsulfonyl fluoride]. Protein concentrations were determined using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). Equal amounts of protein (20 μg) were separated by 8-12% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA) blocked with Tris-buffered saline with Tween-20 containing 5% skimmed milk for 1 h at room temperature.

Membranes were incubated overnight at 4˚C with primary antibodies diluted to 1:1,000 against Akt (cat. no., #4060), phosphorylated (p)-Akt (cat. no., #4685), phosphoinositide 3-kinase (PI3K; cat. no. 4257), p-PI3K (cat. no. 4228), mechanistic target of rapamycin (mTOR, 2983), p-mTOR (cat. no. 2971), p70 ribosomal S6 kinase (p70S6; cat. no. 2708), p-p70S6 (cat. no. 9205), glycogen synthase kinase-β (GSK-β, cat. no. 9315), p-GSK-β (cat. no. 9323), p-eukaryotic translation initiation factor 4E-binding protein 1 (P-4E-BP1; cat. no. 2855), p-inhibitor of nuclear factor κB kinase α (p-IKKα; cat. no. 2694), cyclin A (cat. no. 4656), B1 (cat. no. 4138) and D1 (cat. no. 2978), cell division cycle 2C (CDC2C; cat. no. 9112), p-CDC2C (cat. no. 9111), retinoblastoma protein (pRb; cat. no. 9308), p21 (cat. no. 2946), caspases 3 (cat. no. 9664) and 8 (cat. no. 9496), poly (ADP-ribose) polymerase (PARP; cat. no. 9542) cellular inhibitor of apoptosis (cIAP) 1 (cat. no. 4952) and 2 (cat. no. 3130), β-actin (cat. no. 4970; all Cell Signaling Technology, Inc., Danvers, MA, USA), B-cell lymphoma 2 (Bcl-2; cat. no. sc-7382) and Bcl-2-associated agonist of cell death (Bad; cat. no. sc-8044) and X-apoptosis regulator (Bax; cat. no. sc-70405; all Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Following incubation with secondary antibodies (anti-mouse, cat. no. sc-2055; dilution, 1:1,000; anti-rabbit, cat. no. sc-2004, dilution, 1:5,000, Santa Cruz Biotechnology, Inc.) for 1 h at room temperature, protein expression was detected using an enhanced chemiluminescence western blot substrate kit (Pierce™ ECL Western Blotting Substrate kit; Thermo Fisher Scientific, Inc.).

Microarray analysis. Total RNA was extracted from PC3 and PC3DR2 cells using the RNeasy® Protect Mini kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's protocol. RNA samples with high RNA integrity numbers (RIN>9.0). Agilent 2100 Bioanalyzer System and RNA kits developed by Agilent Technologies (Santa Clara, CA, USA) with A260/280 ratios of 1.8-2.1 were used for cDNA synthesis. Amplification cycles of RNA to cDNA and cDNA to biotin-labeled RNA were performed with the GeneChip IVT Express kit (Affymetrix; Thermo Fisher Scientific, Inc.). RNA was hybridized to a GeneChip Human Genome HG-U133 Plus 2.0 array (Applied Biosystems; Thermo Fisher Scientific, Inc.); all microarray steps were performed according to the manufacturer's protocol. The MAS5 algorithm in GenPlex software ver. 3.0 (Istech Corp., Seoul, Korea) was used for analyzing the CEL file data. The Affymetrix Microarray Suite, MicroDB, and Data Mining Tool software v. 5.0 (Thermo Fisher Scientific, Inc.) were used to annotate 54,120 probe sets with 17,084 genes from the UniGene database. Following global scaling regression normalization, the data was log transformed to base 2. Gene expression levels in PC3 and PC3DR2 cells compared using the n-fold method. Differentially expressed gene clusters were analyzed using GeneCluster 1.0 (MIT, Cambridge, MA, USA). GenMAPP was used to analyze the functional pathways associated with differentially expressed genes. (http://www.genmapp.org).

RT-qPCR. Total RNA was extracted from PC3 and PC3DR2 cells using the RNeasy® Protect Mini kit as aforementioned. cDNA was produced from 1 μg total RNA using oligo(dT) primers and Omniscript reverse transcriptase enzyme (both Qiagen, Inc.) according to the manufacturer's protocol. qPCR was performed with the cDNA produced from 10 ng RNA with the FastStart Universal SYBR Green Master mix (Roche Diagnostics,

Table I. Primer sequences for reverse transcription-quantitative polymerase chain reaction.

Gene symbol	Gene name	Direction	Primer sequence (5'-3')
IGF1R	Insulin-like growth factor 1 receptor	Forward	GTCCTCCTTGATGGTGGAAT
		Reverse	GTTCAAAACCTTGCCCACAT
BRCA2	Breast cancer 2	Forward	ATGCAAATGCATACCCACAA
		Reverse	AGGTGGCCCTACCTCAAAAT
DBF4	DBF4 homolog	Forward	GGGTAACTGGAAGCCATGAA
		Reverse	CATGAGCCACAGGAGAGTCA
ZAK	Sterile α motif and leucine zipper-containing kinase AZK	Forward	GCTGCCTTCCTTTGAGATTG
		Reverse	CCGCTTCCCTGTAATGTTGT
PTCH1	Patched 1	Forward	AGGGATTCCAAGGTGGAAGT
		Reverse	TGGCCTCTTTGCTTCAGATT
SERPINE1	Serpin peptidase inhibitor, clade E, member 1	Forward	TATCCTTGCCCTTGAGTGCT
		Reverse	AGTGGCTGGACTTCCTGAGA
CDKN2C	Cyclin-dependent kinase inhibitor 2C	Forward	ACGTCAATGCACAAAATGGA
		Reverse	TCATGAATGACAGCCAAACC
CDC6	Cell division cycle 6 homolog	Forward	TCTGATTCCCAAGAGGGTTG
		Reverse	TTCTGCTGAAGAGGGAAGGA
CDC25C	Cell division cycle 25 homolog C	Forward	TGGGGAGATAACTGCCACTC
		Reverse	AAGCTGTGCTGGGCTACATT
CCNE2	Cyclin E2	Forward	CCGAAGAGCACTGAAAAACC
		Reverse	GAATTGGCTAGGGCAATCAA
WNT3	Wingless-type MMTV integration site family, member 3	Forward	CGCCTCGGAGATGGTAGTAG
		Reverse	AAAGTTGGGGGAGTTCTCGT
FLI1	Friend leukemia virus integration 1	Forward	TGCACTCAGCTGACCACTCT
		Reverse	TTTCCAAGTTCTGGGACCAC
CUL2	Cullin 2	Forward	GCATAGGACTGCATTCAGCA
		Reverse	GCGATGTCTGTGGAGTAGCA

Indianapolis, IN, USA) using a 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The sample was incubated at 95˚C for 15 min, followed by 40 cycles of 95˚C for 15 sec, annealing at 58˚C for 30 sec and extension at 72˚C for 30 sec. GAPDH was used as the reference gene. Fold change in gene expression was calculated following the $2^{-\Delta\Delta Cq}$ method (10). Primer sequences are presented in Table I.

siRNA preparation and transfection. si-BRCA2 specific for long-form BRCA2 was synthesized by Invitrogen; Thermo Fisher Scientific, Inc., according to published sequences (11,12). Scrambled siRNA (si-SCR) was obtained from Dharmacon (cat. no., #D-001210-01; GE Healthcare, Chicago, IL, USA). For transfection, 10 nM siRNA was mixed with DharmaFECT® 1 transfection reagent (Dharmacon; GE Healthcare) and used according to the manufacturer's protocol.

Statistical analysis. Unless indicated otherwise, datasets consist of >3 replicates. Data are presented as the mean ± standard deviation. Statistical significance between groups was determined using an unpaired Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Establishment of a docetaxel-resistant prostate cancer cell line (PC3DR2). A docetaxel-resistant prostate cancer cell line (PC3DR2) was generated by serial desensitization. To confirm the docetaxel resistance, PC3 and PC3DR2 cells were exposed to increasing doses of docetaxel (0, 0.005, 0.01, 0.1, 0.5, 1, 2.5, 5, 10, 20 or 50 μM) for 72 h. The extent of the cytotoxic effect of docetaxel was determined using a CCK-8 assay. As presented in Fig. 1, docetaxel decreased the viability of PC3

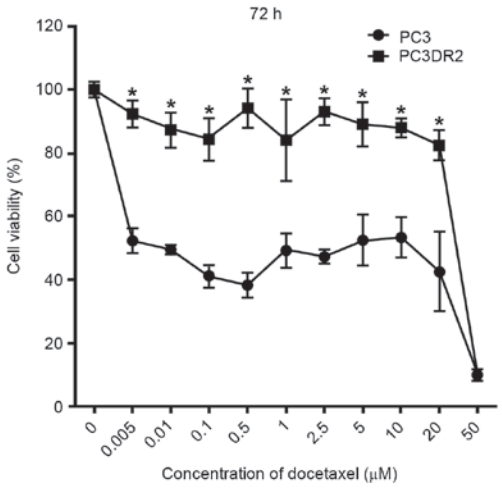

Figure 1. Cell Counting Kit-8 assays following 72 h of treatment with docetaxel in PC3 and PC3DR2 prostate cancer cells. Results represent the mean ± standard deviation (n=3). *P<0.05 compared with PC3 cells by t-test.

Table II. Number of upregulated and downregulated genes and signaling pathways in PC3DR2 cells compared with PC3 cells.

Fold change cut-off	Regulation	Genes	Significant pathways
2	Up	1,227	471
	Down	1,190	362
3	Up	392	162
	Down	243	67

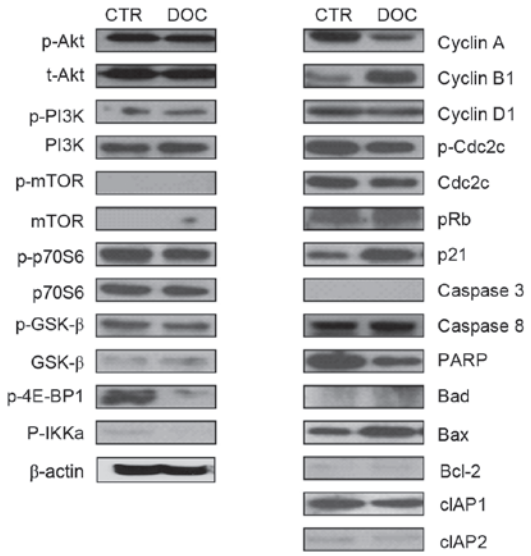

Figure 2. Protein expression analysis with western blotting in PC3DR2 prostate cancer cells. CTR, untreated PC3DR2 cells; DOC, docetaxel-treated PC3DR2 cells; p, phosphorylated; t-, total; PI3K, phosphoinositide 3-kinase; mTOR, mechanistic target of rapamycin; p70S6, p70 ribosomal S6 kinase; GSK-β, glycogen synthase kinase-β; 4E-BP1, eukaryotic translation initiation factor 4E-binding protein 1; IKKa, inhibitor of nuclear factor-κB kinase α; Cdc2C, cell division cycle 2C; pRb, retinoblastoma protein; PARP, poly (ADP-ribose) polymerase; Bcl-2, B-cell lymphoma 2; Bad, Bcl-2-associated agonist of cell death; Bax, Bcl-2-associated X-apoptosis regulator; cIAP, cellular inhibitor of apoptosis.

cells in dose-dependent manner at 72 h; a concentration of 0.005 μM docetaxel was sufficient to suppress proliferation of PC3 cells by 52% at 72 h. However, PC3DR2 cells were significantly more resistant to all concentrations of docetaxel ≤20 μM at 72 h ($P<0.05$); the 20 μM PC3DR2 group exhibited ~80% viability, whereas the 20 μM PC3 group exhibited ~50% viability. Thus, it was established that PC3DR2 cells exhibited significant resistance to docetaxel doses ≤20 μM.

Effect of docetaxel resistance in PC3DR2 cells on cell cycle, survival and apoptosis signaling pathways. To investigate the characteristics of signaling molecules and response of PC3DR2 cells to docetaxel treatment, alterations to proteins in cell cycle-, survival-, and apoptosis-associated signaling pathways in PC3DR2 cells were examined following docetaxel treatment. Levels of cell survival-(Akt, PI3K, p70S6, mTOR and GSKβ), cell cycle-(cyclin D1, p-CDC2C and pRb) and apoptosis-(caspases 3 and 8, Bad, Bcl-2, cIAP1 and 2) associated signaling molecules were not altered in PC3DR2 cells following docetaxel treatment. Notably, 4E-BP1, a repressor of mRNA translation, was inactivated by docetaxel treatment in PC3DR2. However, expression of some cell cycle-associated molecules (cyclin A, cyclin B1 and p21) and Bax was changed following docetaxel treatment (Fig. 2).

DNA microarray analysis of PC3DR2 cells. A total of 17,084 genes were analyzed; 1,227 genes were 2-fold upregulated, whereas 1,190 genes were 2-fold downregulated between PC3 and PC3DR2 cells. A total of 392 genes were 3-fold upregulated, whereas 243 genes were 3-fold downregulated (Table II). In addition, to identify docetaxel resistance-associated genes, 13 differentially expressed genes associated with biological processes possibly associated with docetaxel resistance were identified, including DBF4, cyclin-dependent kinase inhibitor 2C, cell division cycle 6 homolog, cell division cycle 25 homolog C, ZAK, SERPINE1, cyclin E2 (CCNE2), wingless-type MMTV integration site family, member 3, PTCH1, IGF1R, cullin 2, BRCA2 and Friend leukemia virus integration 1 (FLI1; Table III).v

Validation using RT-qPCR of candidate genes in docetaxel resistance. The results of the microarray analysis for the previously named genes was validated using RT-qPCR (Figs. 3-5, Table III). Of the 13 genes, those verified using RT-qPCR included DBF4, ZAK, SERPINE1, PTCH1, IGF1R and BRCA2. IGF1R, PTCH1 and BRCA2 are associated with pathways in cancer. DBF4 is associated with cell cycle. ZAK is associated with the mitogen-activated protein kinase (MAPK) signaling pathway, SERPINE1 and CCNE2 are associated with the p53 signaling pathway. We hypothesized that these genes may be associated with docetaxel resistance in PC3DR2 cells.

BRCA2 siRNA knockdown abolishes docetaxel resistance in PC3DR2 cells. Following the confirmation of the upregulation of DBF4, ZAK, SERPINE1, PTCH1, IGF1R and BRCA2 gene expression in PC3DR2 cells, the genes were investigated for their direct involvement in docetaxel resistance using an siRNA system. siRNA for each gene was transfected into PC3DR2 cells and the cell viability was determined using a CCK-8 assay following docetaxel treatment. si-BRCA2 was confirmed by RT-qPCR and western blotting to decrease the relative level of BRCA2 protein in PC3DR2 cells, whereas scrambled siRNA did not significantly affect the level of BRCA2 expression (Fig. 6A and B). The transfection of siRNA against DBF4, ZAK, SERPINE1, PTCH1 and IGF1R did not affect the docetaxel resistance of PC3DR2 cells (data not shown), whereas si-BRCA2 transfection significantly reduced docetaxel resistance at 48 and 72 h (Fig. 6C and D), suggesting that BRCA2 overexpression may be associated with docetaxel resistance in prostate cancer cells.

Discussion

Although docetaxel represents the most effective chemotherapeutic agent for patients with CRPC, once drug resistance develops, there are limited effective therapeutic strategy options for advanced CRPC. Thus, investigating the resistance mechanism of prostate cancer cells against docetaxel is of marked

Table III. Genes associated with cell cycle and cancer signaling pathways that were differentially expressed in PC3DR2 cells relative to PC3 cells, as determined by DNA microarray and RT-qPCR analyses.

		Fold-change in PC3DR2 cells	
Gene[a]	Pathway	Microarray	RT-qPCR[b]
DBF4	Cell cycle	24.9	5.2[c]
CDKN2C	Cell cycle	3.8	31.2
CDC6	Cell cycle	3.4	1.2
CDC25C	Cell cycle	3.2	0.7
ZAK	MAPK signaling pathway	4.6	6.2[c]
SERPINE1	p53 signaling pathway	3.7	3.4[c]
CCNE2	p53 signaling pathway	3.7	1.4[d]
WNT3	Pathways in cancer	3.3	1.4
PTCH1	Pathways in cancer	3.6	3.2[c]
IGF1R	Pathways in cancer	4.4	3.1[c]
CUL2	Pathways in cancer	4.6	1.5
BRCA2	Pathways in cancer	3.2	3[e]
FLI1	Transcriptional misregulation in cancer	9.0	1.4[e]

[a]Gene names are defined in Table I. [b]Relative to GADPH. [c]P<0.001, [d]P<0.01, [e]P<0.05 compared with PC3 cells, Student's t-test. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; MAPK, mitogen-activated protein kinase.

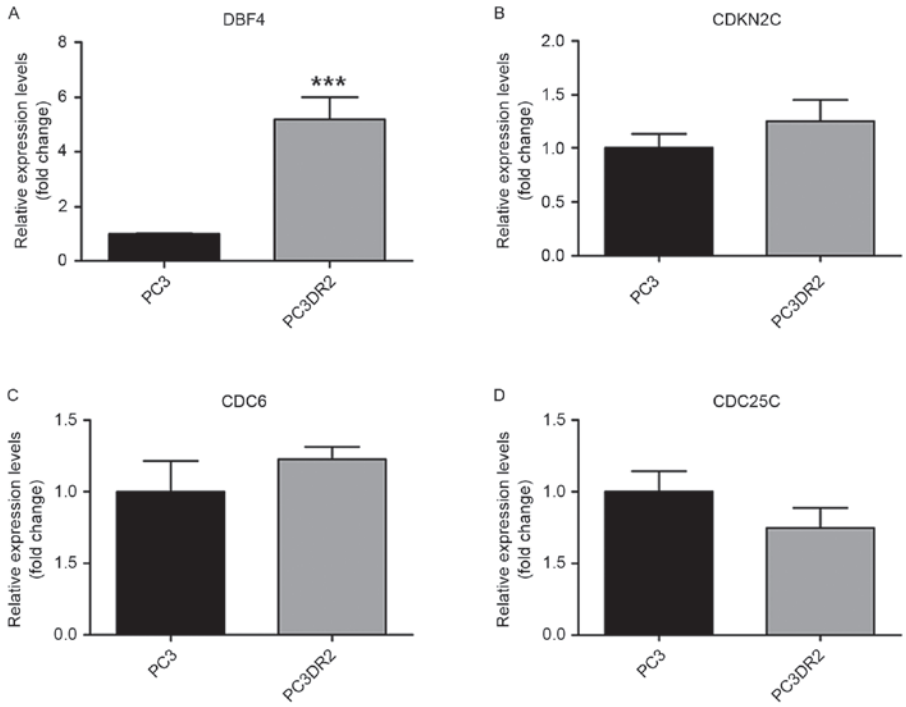

Figure 3. Cell cycle-associated gene expression analysis using qPCR in PC3 and PC3DR2 prostate cancer cells. (A) DBF4, (B) CDKN2C, (C) CDC6 and (D) CDC25C. The qPCR data are presented as relative values normalized to those of the internal control (GAPDH). Results represent the mean ± standard deviation (n=3). ***P<0.001 compared with PC3 cells by t-test.

urgency. In our previous study, human docetaxel-resistance prostate cancer cell line (PC3DR2) from docetaxel-sensitive prostate cancer cell line (PC3) were generated by serial desensitization (9); in the present study, differential gene expression between PC3 and PC3DR2 cells were compared with a DNA microarray. O'Neill *et al* (13) also manipulated docetaxel-resistant prostate cancer cell lines and suggested that multiple mechanisms contribute to docetaxel resistance in partial agreement with our results, although this study focused on the nuclear factor-κB pathway, indicating that multiple mechanisms may be involved in docetaxel resistance. In the present study, it was confirmed using western blotting analysis that the expression of a number of molecules, including those associated with the cell cycle, survival and apoptosis, were

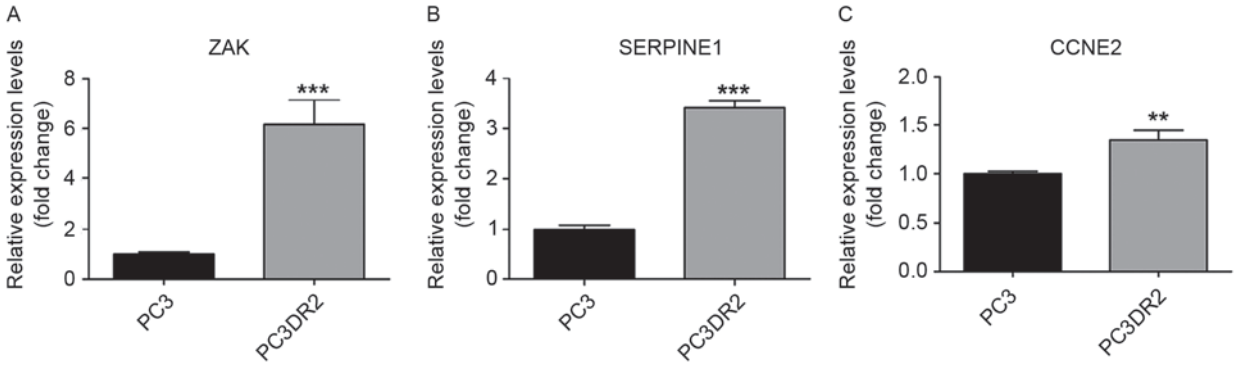

Figure 4. Signaling pathway-associated gene expression analysis using qPCR in PC3 and PC3DR2 prostate cancer cells. (A) ZAK, (B) SERPINE1 and (C) CCNE2. The qPCR data are presented as relative values normalized to those of the internal control (GAPDH). Results represent the mean ± standard deviation (n=3). **P<0.005 and ***P<0.001 compared with PC3 cells by t-test.

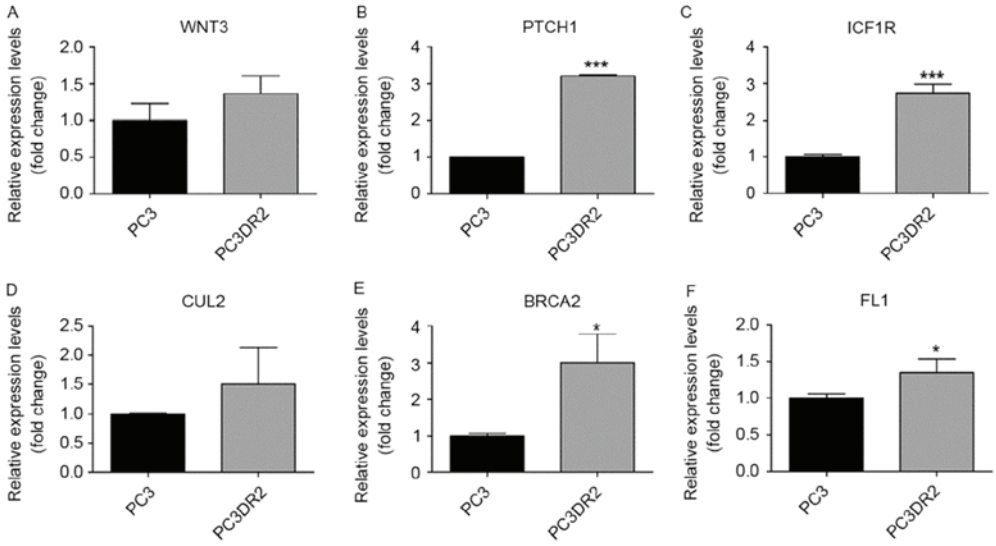

Figure 5. Cancer-associated pathway gene expression analysis by qPCR in PC3 and PC3DR2 prostate cancer cells. (A) WNT3, (B) PTCH1, (C) IGF1R, (D) CUL2, (E) BRCA2 and (F) FLI1. qPCR data are presented as relative values normalized to those of the internal control (GAPDH). Results represent the mean ± standard deviation (n=3). *P<0.05 and ***P<0.001 compared with PC3 cells by t-test.

unchanged in PC3DR2 cells subsequent to docetaxel treatment. Using microarray analysis confirmed by RT-qPCR, six overexpressed genes (IGF1R, DBF4, ZAK, PTCH1, SERPINE and BRCA2) associated with cancer signaling pathways were identified in PC3DR2 cells, exhibiting a >3-fold increase compared with PC3 cells in the RT-qPCR data. To confirm the association between the overexpression of these genes and docetaxel resistance, an siRNA against each gene was transfected into PC3DR2 cells. BRCA2 knockdown abolished the docetaxel resistance in PC3DR2 cells. These results suggest the novel hypothesis that BRCA2 overexpression may be involved in docetaxel resistance.

Docetaxel stabilizes tubulin subunits in microtubules, leading to apoptosis (5). In addition, it has been demonstrated that docetaxel leads to an antitumor effect by inducing the phosphorylation of Bcl-2 (14). Suggested docetaxel resistance mechanisms include: i) Overexpression of the p-glycoprotein drug efflux pump; ii) mutation of the drug-binding site; iii) Expression of another tubulin isoform; iv) Activation of a growth factor-associated pathway; and v) Use of an alternative metabolic pathway (5,15). In the present study, BRCA2 overexpression was identified as an additional possible mechanism for docetaxel resistance in PC3DR2 cells.

BRCA1 and 2 are well-known breast cancer susceptibility genes considered to be classical tumor-suppressor genes, since the loss of both alleles is required to promote carcinogenesis (11,12,16,17). A recent study demonstrated that the 12-year prostate cancer-specific survival rate was 94.3% for patients without and 61.8% for patients with a BRCA2 mutation, suggesting that the survival time for patients with a BRCA2 mutation is markedly below the average for prostate cancer (18). Mutations of BRCA genes increase the risk of prostate cancer and are associated with disease characteristics and therapeutic outcomes (19). It has been demonstrated that the functional loss of BRCA2 affects the focal development of prostate cancer (20) and the potential for the disease to spread through upregulation of matrix metalloproteinase-9 (21). Conversely, the decreased expression of BRCA2 mRNA predicts a favorable response to docetaxel in breast cancer (22). The results of the present study revealed that BRCA2 knockdown abolished

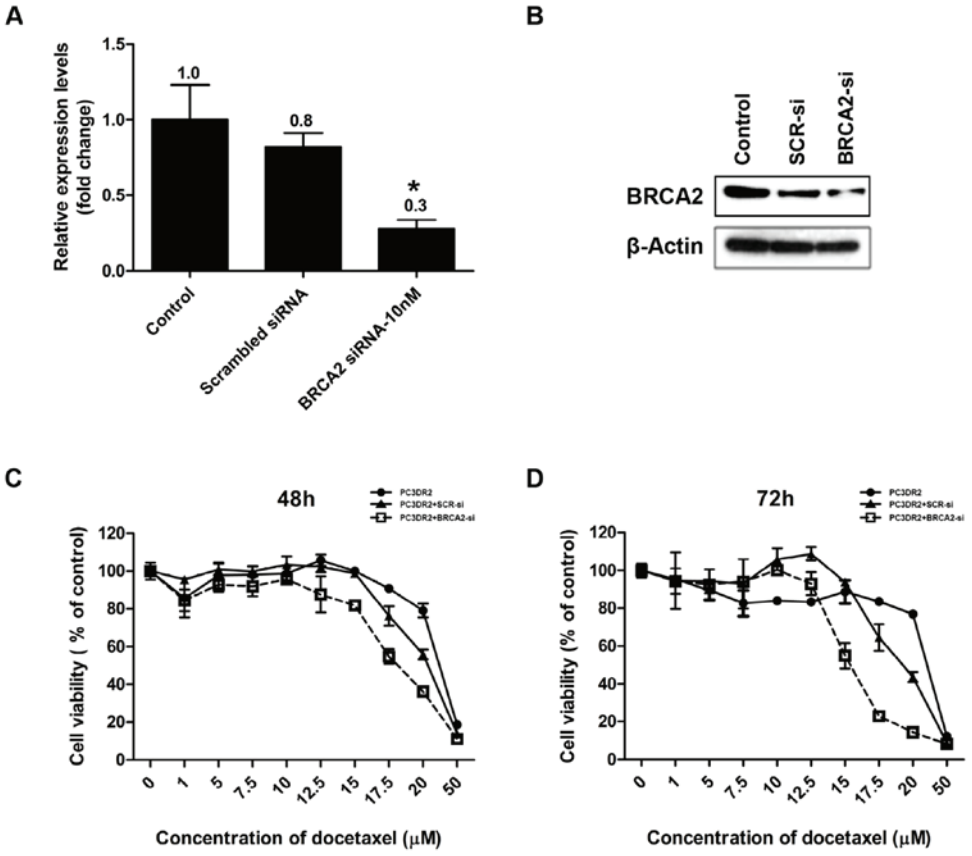

Figure 6. BRCA2 knockdown with siRNA abolished docetaxel resistance in PC3DR2 cells. BRCA2 expression in prostate cancer cells transfected with BRCA2 siRNA was demonstrated to be decreased using (A) qPCR and (B) western blot analysis. The qPCR data are presented as relative values normalized to those of the internal control (GAPDH). The cell viability of PC3DR2 cells transfected with BRCA2 siRNA for (C) 48 and (D) 72 h was then assessed. Results represent the mean ± standard deviation (n=3). *P<0.05 compared with PC3DR2 cells by t-test. BRCA2, breast cancer 2; siRNA, small interfering RNA; qPCR, quantitative polymerase chain reaction.

docetaxel resistance in PC3DR2 cells. Collectively, these results may appear to be conflicting; however, this effect is expected when considering that the major anticancer mechanism for docetaxel is to stabilize microtubules during mitosis to induce cell cycle arrest at G2-M phase, leading to apoptosis (5). BRCA proteins are also involved in the mitotic spindle assembly process. The normal DNA repair functions of BRCA1 and BRCA2 serve a critical function in cell cycle processes during G2-M phase. When BRCA2 expression is low, malfunction of the DNA repair system may retard the function of the mitotic spindle to slow or arrest the G2-M process (23). Thus, it can be speculated that tumors with low BRCA expression may be more sensitive to docetaxel treatment, indicating that docetaxel may exert a greater effect on prostate cancer cells where the function of mitotic spindles is already partially retarded due to low BRCA2 expression, in accordance the results of the present study.

Tumor suppressor genes may regulate the sensitivity of cancer cells to chemotherapy (24). Previous studies have demonstrated that decreased BRCA1 expression following siRNA transfection may increase cell sensitivity to platinum compounds and topoisomerase inhibitors (25-27). Clinical studies also indicated that BRCA1 may be a suitable biomarker for the clinical prognosis of ovarian, lung and breast cancer treatment after DNA-damage-based targeted therapy, as reviewed by Stordal and Davey (28). An *in vitro* and *in vivo* study demonstrated that low BRCA1 expression, potentially leading to defects in the DNA damage repair mechanism, was associated with the high sensitivity to DNA-damaging drugs including cisplatin and PARP inhibitors (29). Preclinical and clinical studies have revealed that the loss of BRCA1 may also result in resistance to other types of chemotherapeutic agent, including the anti-microtubule agents paclitaxel and docetaxel, and molecularly targeted agents (30,31). Therefore, BRCA1 expression affects chemosensitivity differently depending on the type of agent.

However, in the present study, BRCA1 was not identified to be significantly altered in PC3DR2 cells. This is noteworthy, as BRCA1 and BRCA2 are breast cancer-susceptibility genes that have been identified through linkage analysis of families susceptible to breast cancer (16,17). However, it has also been reported that sporadic breast cancer may exhibit decreased BRCA1 mRNA levels, whereas BRCA2 mRNA levels were variable, compared with normal breast tissue (32-34). These results may be caused by the hypermethylation of BRCA1 promoter, which explains the downregulation in sporadic breast cancer, whereas the promoter for BRCA2 is not hypermethylated (34). BRCA2 may have different operating system

from BRCA1 and it may be possible for only BRCA2 to be involved in docetaxel resistance.

In conclusion, the results of the present study suggest the novel hypothesis that BRCA2 may be associated with docetaxel resistance in human prostate cancer cells. To clarify this suggestion, further study with an *in vivo* model is required.

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