Analysis of cabazitaxel-resistant mechanism in human castration-resistant prostate cancer

Hiroshi Hongo | Takeo Kosaka | Mototsugu Oya

Department of Urology, Keio University School of Medicine, Tokyo, Japan

Correspondence: Takeo Kosaka, Department of Urology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo, 160-8582, Japan Email: (takemduro@gmail.com).

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Abstract
Cabazitaxel (CBZ) is approved for docetaxel-resistant castration-resistant prostate cancer (CRPC). However, efficacy of CBZ for CRPC is limited and there are no effective treatments for CBZ-resistant CRPC. In order to investigate the CBZ-resistant mechanism, the establishment of a CBZ-resistant cell line is urgently needed. We established CBZ-resistant CRPC cell lines DU145CR and PC3CR by incubating DU145 and PC3 cells with gradually increasing concentrations of CBZ for approximately 2 years. We analyzed the gene expression profiles and cell cycle changes using microarray and flow cytometry. Pathway analysis revealed DU145CR cells had enhanced gene clusters of cell division and mitotic nuclear division. Enhancement of ERK signaling was detected in DU145CR cells. DU145CR cells had resistance to G2/M arrest induced by CBZ through ERK signaling activation. The MEK inhibitor PD184352 significantly inhibited cell proliferation of DU145CR. In contrast to DU145CR, PC3CR cells had enhancement of PI3K/AKT signaling. The PI3K/mTOR inhibitor NVP-BEZ 235 had a significant antitumor effect in PC3CR cells. Cabazitaxel-resistant CRPC cells established in our laboratory had enhancement of cell cycle progression signals and resistance to G2/M arrest induced by CBZ. Enhancement of ERK signaling or PI3K/AKT signaling were detected in the cell lines, so ERK or PI3K/AKT could be therapeutic targets for CBZ-resistant CRPC.

KEYWORDS
cabazitaxel resistance, castration-resistant prostate cancer (CRPC), cell cycle, ERK signaling, PI3K/AKT signaling

INTRODUCTION
Prostate cancer is the most prevalent cancer and second leading cause of cancer among men in the USA.1,2 The most common treatment for metastatic prostate cancer is androgen deprivation therapy using luteinizing hormone-releasing hormone analogues and non-steroidal antiandrogens. Although metastatic prostate cancer initially responds well to androgen deprivation therapy, most of the patients acquire resistance to this therapy and develop CRPC. Treatment options for CRPC have expanded with the addition of next-generation antiandrogens such as abiraterone acetate and enzalutamide, autologous immunotherapy with sipuleucel-T, internal radiotherapy treatment with radium223, and chemotherapeutic agents.3 The current standard first-line chemotherapy for metastatic CRPC is DOC. Cabazitaxel was approved by the US FDA for docetaxel-resistant CRPC in 2010. However, the duration of the response to CBZ is limited to only several months for DOC-resistant
CRPC and there are no effective treatments for CBZ-resistant CRPC.

The taxanes exert antitumor effects by stabilizing microtubules, thereby blocking cells in the G2/M phase of the cell cycle. There have been several reports on prostate cancer cell line models that showed resistance to PAC or DOC. One of the mechanisms of PAC/DOC resistance is P-gp drug transporter. Cabazitaxel was found by screening for a taxane with poor affinity for P-gp and effective for PAC/DOC resistant CRPC. However, acquired CBZ resistance is clinically inevitable and the mechanisms of CBZ resistance are still unclear.

In order to analyze the mechanisms of CBZ resistance, we incubated CRPC cell lines with gradually increasing concentrations of CBZ and established CBZ-resistant CRPC cell lines. We analyzed the gene expression profiles of the cell line and identified a therapeutic target for CBZ-resistant CRPC.

2 MATERIALS AND METHODS

2.1 Reagents

We used rabbit mAb for pan AKT, phosphorylated AKT (Ser473) (Cell Signaling Technology, Danvers, MA, USA), rabbit polyclonal antibody for ERK 1/2, phosphorylated ERK 1/2 (Thr202/Tyr204) (Cell Signaling Technology), and mouse mAb for α-tubulin and β-actin (Sigma-Aldrich, St. Louis, MO, USA). WST reagents (Takara Bio, Kyoto, Japan) were also used.

2.2 Cell lines and culture

The CRPC cell lines DU145 and PC3 were obtained from ATCC (Manassas, VA, USA). To develop resistance, DU145 and PC3 cells were grown in RPMI-1640 supplemented with 10% FBS containing CBZ at 37°C in a humidified 5% CO2 atmosphere. Starting at a concentration of 0.3 nmol/L, the exposure dose was increased by 30%-50% until it reached a final concentration of 3 nmol/L. These cells were passaged after reaching confluence over a 24-month period. These new cell lines were named DU145CR and PC3CR. Parental DU145 and PC3 cells were cocultured without drug for the 2 years as control cells. DU145, PC3, DU145CR, and PC3CR were routinely maintained in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (Dainippon Pharmaceutical, Tokyo, Japan) at 37°C in a humidified 5% CO2 atmosphere, and further examinations were carried out after 3 months without CBZ exposure.

2.3 WST cell viability assay

DU145, PC3, DU145CR, and PC3CR cells were seeded on 96-well plates, allowed to attach for 24 hours, and then treated with different concentrations of CBZ, PD184352 (Wako, Osaka, Japan), and NVP-BEZ235 (BEZ235) (kindly provided by Novartis, Basel, Switzerland) for 48 hours. At the end of the incubation period, WST reagents were added to each well and the cells were incubated for 1 hour. Cell viability was estimated by colorimetry by reading color intensity in a plate reader at 570 nm.

2.4 Murine xenograft prostate cancer model

Six-week-old male athymic nude BALB-C mice were castrated by scrotal incision under anesthesia and were used to create a xenograft model with DU145 cells and DU145CR cells. The cells (2 × 10^6) were suspended in 100 μL Matrigel (Becton Dickinson Labware, Lincoln Park, NJ, USA) were s.c. inoculated in the mice. The mice were monitored and tumors were measured every 2 days. To investigate the sensitivity to CBZ in vivo in each cell, the mice were assigned to control and CBZ treatment groups. Treatment with CBZ was started when the average tumor volume was approximately 100 mm^3. Cabazitaxel (10 mg/kg) was given to the CBZ treatment group i.p. in a single dose on day 1. Saline solution (200 μL) was given to the control group on day 1. On day 13, the mice were killed and s.c. tumors were harvested. Animal care was carried out in accordance with Keio University (Tokyo, Japan) guidelines for animal experiments.

2.5 Immunohistochemistry

We immunostained the xenograft tumors treated by CBZ. Sections (4 mm) of formalin-fixed, paraffin-embedded material were analyzed. After antigen retrieval with citric acid (pH 6.0), endogenous peroxidase activity was blocked with 1% hydrogen peroxide. Primary antibodies (monoclonal anti-Ki67 antibody, dilution 1:200, and monoclonal anti-p-ERK 1/2 [Thr202/Tyr204] antibody, dilution 1:100) were applied and incubated with secondary antibodies conjugated to peroxidase labeled dextran polymer. The immunoreaction was visualized using diaminobenzidine and counterstaining was undertaken with 1% hematoxylin. The percent of cancer cells with nuclei stained for Ki67 (the Ki67 index) was calculated for each section based on more than 1000 cancer cell nuclei. Apoptosis was measured by TUNEL assay using an in situ apoptosis detection kit (Takara Bio). For the TUNEL assay, we used control slides from the apoptosis detection kit as a positive control, and control slides without terminal deoxynucleotidyl transferase enzyme as a negative control. The average number of stained cells was counted and the apoptosis index was calculated as the average number in 5 areas in a 400× field.

2.6 Immunofluorescence

To measure immunofluorescence, 1 × 10^4 cells were seeded on 14-mm coverslips in 8-well plates. After treatment was completed the cells were washed with PBS, fixed in 4% paraformaldehyde-PBS for 20 minutes at room temperature, and permeabilized in cold PBS with 0.2% Triton X-100 for 10 minutes at room temperature. Blocking was carried out with Dako blocking reagent (X0909; Agilent Technologies, Santa Clara, CA, USA) and 10% BSA for 40 minutes at room temperature. The slides were then incubated with primary antibody (α-tubulin antibody, dilution 1:500) for 1 hour at room temperature and thereafter with anti-rabbit Alexa 555 antibody (dilution...
1:200). Coverslips were mounted on glass slides with DAPI containing Vectashield mounting medium (ProLong™ Gold Antifade Mountant with DAPI; Thermo Fisher Scientific, Waltham, MA, USA) and visualized by confocal microscopy.

2.7 | Microarray gene expression analysis

Total RNA was isolated from cell lines using an RNeasy Mini kit (Qiagen, Valencia, CA, USA). Gene expression profiles were determined using the Affymetrix GeneChip Human Gene 1.0 ST array according to the manufacturer's instructions. After generating the single-stranded cDNA, fragmentation and sense-strand cDNA labeling was carried out with an Affymetrix GeneChip WT Terminal Labeling Kit (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's protocol. After hybridization, a GeneChip Fluidics Station 450 (Affymetrix) was used for washing the arrays, and scanning was undertaken with a GeneChip Scanner 3000 7G (Affymetrix). The raw intensity data from scanned images of the microarrays were preprocessed using Affymetrix Expression Console software. Expression intensity data from scanned images of the microarrays were preprocessed with an Affymetrix GeneChip Human Gene 1.0 ST array according to the manufacturer’s instructions. After generating the single-stranded cDNA, fragmentation and sense-strand cDNA labeling was carried out with an Affymetrix GeneChip WT Terminal Labeling Kit (Affymetrix, Santa Clara, CA, USA) according to the manufacturer’s protocol. After hybridization, a GeneChip Fluidics Station 450 (Affymetrix) was used for washing the arrays, and scanning was undertaken with a GeneChip Scanner 3000 7G (Affymetrix). The raw intensity data from scanned images of the microarrays were preprocessed using Affymetrix Expression Console software. Expression intensities were stored as cell intensity files, and the files werenor-

2.10 | Cell cycle assay by flow cytometry

DU145 cells and DU145CR cells were incubated for 6 or 12 hours with CBZ. The cells were harvested by exposing them to a 0.25% trypsin-EDTA solution for 5 minutes. They were centrifuged, washed in PBS, fixed in 3 mL ice-cold 70% ethanol, and then incubated overnight at −20°C. After overnight incubation, the cells were cen-

2.11 | Statistics

Experiments were carried out with 3 or more replicates. Statistical analysis was undertaken using Student’s t test and the Tukey-Kramer method for multiple comparison test, with P < .05 considered significant.

3 | RESULTS

3.1 | Establishment of CBZ-resistant cell lines

We incubated DU145 and PC3 cells with gradually increasing concentrations of CBZ for 24 months and established CRPC sublines, which were named DU145CR and PC3CR (Figure 1A). DU145CR and PC3CR had significantly more resistance to CBZ than DU145 and PC3 in WST assay (Figures 1B and S1A). Direct cell counting showed that the number of DU145CR and PC3CR cells increased against CBZ, whereas those of DU145 and PC3 cells significantly decreased at the same dose of CBZ (Figure S2). DU145CR and PC3CR cells had as much sensitivity for DOC as DU145 and PC3 cells (Figure S3). We treated DU145 and DU145CR xenograft mice with CBZ (10 mg/kg). Although CBZ (10 mg/kg) significantly suppressed tumor growth in DU145 cells, the same dose of CBZ did not significantly suppress tumor growth in DU145CR cells (Figure 1C,D). We evaluated Ki67 expression by immunohistochemistry as an index of proliferation. The Ki67 index of DU145 tumors was significantly decreased in the CBZ treatment group (35.3% ± 1.8%) compared to that of the control group (50.4% ± 2.0%), whereas there were no significant difference in the Ki67 index between the CBZ group (51.2% ± 1.3%) and control group (50.0% ± 1.1%) in DU145CR tumors (Figure 1E,F). We next evaluated apoptosis by immunohistochemistry using the TUNEL assay. The apoptosis index of DU145 tumors in the CBZ group (3.63% ± 0.57%) was significantly higher than that of the control group (0.63% ± 0.50%). The
(A) 
DU145

Incubated with gradually increasing concentration of CBZ for 2 y

DU145CR

(B) 

IC50:

|          | DU145  | DU145CR |
|----------|--------|---------|
| CBZ (nmol/L) |        |         |
| 0        | 0.1    | 0.1     |
| 2        | 0.2    | 0.2     |
| 4        | 0.3    | 0.3     |
| 6        | 0.4    | 0.4     |
| 8        | 0.5    | 0.5     |
| 10       | 0.6    | 0.6     |
| 12       | 0.7    | 0.7     |

***P < .001 compared to DU145 cells at the same dose of CBZ

(C) 

DU145

Tumor volume (mm³)

(D) 

DU145CR

Relative tumor volume

(E) 

DU145

DU145CR

Cont

CBZ

(F) 

Ki67 index (%)

(G) 

DU145

DU145CR

Cont

CBZ

(H) 

Apoptosis index (%)

† † † P < .001 compared to Control
apoptosis index of DU145CR tumors treated with CBZ (0.75% ± 0.08%) was not increased compared to that of the control group (0.64% ± 0.14%) (Figure 1G,H).

3.2 | Enhancement of cell cycle progression signaling and remodeling of the microtubule network in CBZ-resistant cells

We analyzed the gene expression profiles by microarray to investigate the mechanisms of CBZ resistance. Functional annotation clustering analysis using DAVID showed cell division (Gene Ontology: 0007067) and mitotic nuclear division (Gene Ontology: 0051301) were the most enhanced clusters in DU145CR compared with DU145 (FAC enrichment score 21.4) (Figure 2A). Fluorescence activated cell sorting analysis revealed G2/M arrest by CBZ was inhibited in DU145CR cells, although CBZ induced G2/M arrest in DU145 cells (Figure 2B), indicating that DU145CR had resistance to cell cycle changes by CBZ through enhancement of cell cycle progression signaling. We analyzed the microtubule network in CBZ-sensitive and CBZ-resistant cell lines by confocal microscopy. In the DMSO treated state, microtubule filaments in DU145 cells orderly radiated to the cell periphery (Figure 3A,B). Treatment with CBZ reduced the density of microtubule filaments and de-organized these networks (Figure 3C,D). In DU145CR cells, the density of microtubules were lower than that in parental cells (Figures 3E,F and S3). Treatment with CBZ did not affect the organization or the density of the microtubule network in DU145CR (Figure 3G,H).

3.3 | Enhancement of MAPK signaling as a potential therapeutic target for CBZ-resistant CRPC

We investigated the status of MAPK signaling and PI3K signaling as cell cycle-related pathways by western blotting. Phosphorylation of ERK was enhanced in DU145CR cells, whereas AKT phosphorylation was not (Figure 4A,B). DU145CR xenograft tumors also had higher levels of phosphorylate ERK than DU145 tumors (Figure S4). No significant differences of P-gp expression were detected between DU145 and DU145CR cells (Figure S5). To determine whether enhancement of ERK signaling was a potential therapeutic target for CBZ-resistant CRPC, we examined and compared the efficacy of the MEK1/2 inhibitor PD184352 in DU145 and DU145CR cells. The relative viability of DU145CR cells treated with PD184352 was significantly less than that of DU145 cells (Figure 4C). Phosphorylation of ERK was inhibited in DU145CR 24 hours after treatment with PD184352 (Figure 4D,E).

4 | DISCUSSION

In this study, we describe the gene expression profiles and cell cycle patterns of a CBZ-resistant cell line originally established in our laboratory and identify enhanced ERK signaling as a therapeutic target for CBZ-resistant CRPC. A previous report described cross-resistance to CBZ of DOC-resistant CRPC cell lines. On this point, we also reported the sensitivity of a DOC-resistant cell line, C4-2AT6, to CBZ. Contrary to the report by Lombard et al, C4-2AT6 had higher sensitivity for CBZ than the DOC-sensitive cell line C4-2 in our study. In this study, we report the establishment of CBZ-resistant cell lines by continuously exposing taxane-naive cell lines to CBZ. Our established CBZ-resistant cell lines derived from DOC-naive ones had no resistance to DOC (Figure S3). This result suggests that DOC might be useful in the post-CBZ setting although, based on findings from the FIRSTANA clinical trial, CBZ is currently not going to be used before DOC.

As a result of the DAVID FAC analysis, based on microarray data of the CBZ-resistant cell line, the clusters of cell division and mitotic nuclear division are strongly enhanced. These clusters involved cyclin A2, B1, CDC25A, CDK14, and MCAK. Cyclin A2, B1, CDC25A, and CDK14 are associated with regulation of the G2/M phase in the cell cycle. As a member of kinesin family, MCAK modulates microtubule detachment. These results suggested that modification of cell cycle regulation and microtubule dynamics might contribute to acquired CBZ resistance.

The ERK pathway is evolutionarily conserved and regulates cell proliferation, growth, apoptosis, and differentiation. Enhancement of ERK signaling has been reported to be a risk factor for various cancers such as pancreas, lung, or colorectal carcinomas. However, the relationship between ERK signaling and CBZ-resistant CRPC progression is still unclear. In this study, the CBZ-resistant cell line showed enhancement of ERK signaling and an MEK inhibitor was effective in the cell line, suggesting that ERK signaling is also involved in CBZ-refractory CRPC.

Prostate cancer patients are often reported to be PTEN deficient, so PI3K/AKT signaling has attracted attention as a prostate cancer risk and a therapeutic target. We also reported that enhancement of PI3K/AKT signaling was related with castration-refractory and DOC-resistant CRPC. Enhancement of PI3K/AKT signaling

FIGURE 1 | A, Schema for establishing cabazitaxel (CBZ)-resistant cell lines. B, DU145 cells and DU145CR cells were treated with CBZ and viability was measured by WST assay. DU145CR cells showed significantly lower sensitivity to CBZ than DU145 cells. C, Time course changes of DU145 and DU145CR xenograft tumors in female nude mice by treatment with CBZ. D, Comparison of relative tumor volume of DU145 and DU145CR tumors at day 13. E, Representative immunohistochemical staining for Ki67 in DU145 and DU145CR tumors treated with or without 10 mg/kg CBZ. F, CBZ treatment significantly reduced the Ki67 index in DU145 tumors, but not in DU145CR tumors. G, Representative TUNEL staining in DU145 and DU145CR tumors treated with or without 10 mg/kg CBZ. H, Significant increase in the apoptosis index was observed in DU145 tumors treated with CBZ, but not in DU145CR tumors. Cont, control.
FIGURE 2  A, DAVID functional annotation clustering analysis of microarray data in DU145CR cells compared with DU145 cells. As a result of the DAVID functional annotation clustering analysis based on microarray data of the cabazitaxel (CBZ)‐resistant cell line, the clusters of cell division and mitotic nuclear division are strongly enhanced. These clusters involved cyclin A2, B1, CDC25A, CDK14, and MCAK. Cyclin A2, B1, CDC25A, and CDK14 are associated with regulation of G2/M phase in the cell cycle. B, Cell cycle analysis by flow cytometry with propidium iodide of DU145 and DU145CR treated with 10 nmol/L CBZ. CBZ‐induced cell cycle arrest at G2/M was significantly inhibited in DU145CR 12 hours after treatment with CBZ.
was not detected in DU145CR cells, most likely because DU145 had intact PTEN, which inhibited PI3K. We also established a CBZ-resistant cell line derived from PC3 cells, which have a PTEN deletion (Figure S1A). This cell line, named PC3CR, also had enhancement of the cell division and mitotic nuclear division clusters in the DAVID FAC analysis (Figure S1B). In confocal microscopy images, the microtubule network in PC3 cells was deorganized by CBZ (Figure S6A-D). However, the microtubule network in PC3CR cells was not affected by CBZ (Figure S6E-H). PC3CR cells had enhancement of AKT phosphorylation (Figure S7A,B), whereas enhancement of P-gp expression was not detected (Figure S5). We examined and compared the efficacy of the PI3K/mTOR inhibitor BEZ235 in PC3 and PC3CR cells. The relative viability of PC3CR cells treated with BEZ235 was significantly less than that of PC3 cells (Figure S7C). These results suggested that PI3K signaling could also be a therapeutic target for CRPC, especially with PTEN deletion. Although we analyzed the expression and phosphorylation of upstream molecules of the MAPK and PI3K/Akt pathways, no specific molecule was detected as the activator of these pathways. It was thought that the mechanism of CBZ resistance was not explained by a single molecule.

**FIGURE 3** Confocal microscopy images of microtubules in DU145 (A-D) and DU145CR (E-H) cells at 12 hours after treatment with DMSO (control) (A, B, E, F) or 3 nmol/L CBZ (C, D, G, H). Scale bar, 20 μm.
in these signaling pathways. In addition, we incubated DU145 and PC3 cells with conditioned medium from DU145CR or PC3CR cells. However, neither the MAPK nor the PI3K/AKT pathway was enhanced in DU145 or PC3 cells (Figure S8).

There are some limitations to our CBZ-resistant models. First, DU145CR and PC3CR cells lack ARs. The majority of CRPC patients still express AR. We are now trying to establish a CBZ-resistant AR-positive prostate cancer model from another cell line. Second, a 3-fold increase of IC50 in DU145CR and PC3CR cells in vitro could be relatively mild resistance compared with that in the reported DOC-resistant cell lines. In WST assays, the cell viability of DU145CR and PC3CR cells were twice as high as that of DU145 and PC3 cells under exposure to 6 nmol/L CBZ. Direct cell counting showed that DU145CR and PC3CR cells proliferated under 3 nmol/L CBZ, which inhibited DU145 and PC3 proliferation. In addition, the growth of CBB-resistant xenograft tumors was not suppressed by treatment with 10 mg/kg CBZ, whereas CBZ-sensitive xenograft tumors were significantly inhibited at the same dose of CBZ in vivo. These results ensure that our cell line models have significant resistance to CBZ.

We previously reported on cisplatin-resistant urothelial cancer cell lines, which had an IC50 3 times higher than parental cells. Xenograft tumors of these cell lines also grew to twice the size of that of their parental cell lines. In addition, some researchers have reported that cell lines derived from chemoresistant cancer patients had an IC50 2-3 times higher than those from chemotherapy naïve cancer patients. As can be surmised from the above, the observable degree of resistance may be different among in vitro, in vivo, and clinical settings.

In conclusion, we established CBZ-resistant CRPC cell lines. The CBZ-resistant cell line derived from DU145 cells had enhancement of cell cycle progression. Enhancement of ERK or PI3K/AKT signaling

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**Figure 4**

A, AKT, phosphorylated (p)AKT, ERK and phosphorylated (p) ERK protein expressions in DU145 and DU145CR cells. B, Quantitative results of western blotting revealed DU145CR had enhancement of ERK phosphorylation. C, Relative viability of DU145CR cells treated with PD184352 was significantly less than that of DU145 cells in WST assay. D, Western blotting of ERK and pERK in DU145CR after treatment with DMSO (control [Cont]) and 10 μmol/L PD184352. E, Quantitative results of western blotting revealed inhibition of ERK phosphorylation by PD184352.
were detected in the cell lines, so ERK or PI3K/AKT could be therapeutic targets for CBZ-resistant CRPC.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

ORCID

Takeo Kosaka http://orcid.org/0000-0002-4371-4594

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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