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# Molecular Mechanism Mediating Cytotoxic Activity of Cabazitaxel in Docetaxel-resistant Human Prostate Cancer Cells

HIROMITSU WATANABE, ASUKA KAWAKAMI, RYO SATO,
KYOHEI WATANABE, YUTO MATSUSHITA and HIDEAKI MIYAKE

*Department of Urology, Hamamatsu University School of Medicine, Hamamatsu, Japan*

**Abstract.** *Background/Aim: Cabazitaxel is known to be effective in patients with castration-resistant prostate cancer (CRPC) showing resistance to docetaxel. The objective of this study was to investigate the molecular mechanism mediating cytotoxic activity of cabazitaxel in docetaxel-resistant human CRPC cells. Materials and Methods: Parental human CRPC cell line PC3 (PC3/P) was continuously exposed to increasing doses of docetaxel, and a cell line resistant to docetaxel, PC3/R, was developed. Phenotypic differences between these cell lines were investigated. Results: There were no significant differences in sensitivity to cabazitaxel between PC3/P and PC3/R. In PC3/P, both docetaxel and cabazitaxel markedly inhibited the phosphorylation of AKT serine/threonine kinase 1 (AKT) and p44/42 mitogen-activated protein kinase (MAPK). In PC3/R, however, phosphorylation of AKT and p44/42 MAPK were maintained following treatment with docetaxel, whereas treatment with cabazitaxel resulted in the marked down-regulation of phosphorylation of AKT but not that of p44/42 MAPK. Furthermore, additional treatment of PC3/R with a specific inhibitor of AKT significantly enhanced the cytotoxic activity of docetaxel but not that of cabazitaxel. Growth of PC3/R in nude mice after treatment with cabazitaxel was significantly inhibited compared with that after treatment with docetaxel. Conclusion: Antitumor activity of cabazitaxel in docetaxel-resistant CRPC cells was explained, at least in part, by the inactivation of persistently phosphorylated AKT even after treatment with docetaxel.*

Docetaxel was the first agent shown to have a prognostic benefit for patients with metastatic castration-resistant prostate cancer (mCRPC) and, thus, was previously widely used as the only standard therapeutic option for these patients (1). In recent years, however, multiple novel agents with different mechanisms of action against mCRPC have been approved, resulting in marked changes in the therapeutic strategy for patients with mCRPC (2). Although the optimal sequence for using these agents has not been well-established because of the absence of clinical studies directly comparing different patterns of sequential treatment for mCRPC, docetaxel still plays an important role as one of the key agents during sequential treatments against mCRPC (3). In fact, docetaxel-based therapy involving novel androgen receptor axis-targeted agents and cabazitaxel in pre- and post-docetaxel settings, respectively, may be currently regarded as the most preferable sequential regimen for mCRPC (3).

Despite its powerful activity against mCRPC, docetaxel generally yields approximately 10 months of progression-free survival for patients with mCRPC (1, 4, 5), suggesting the acquisition of a phenotype resistant to this agent by mCRPC within a comparatively short period following its introduction. To date, however, there have been few studies investigating the molecular mechanisms underlying the acquisition of resistance to docetaxel by CRPC cells and the subsequent efficacy of cabazitaxel (6-10). In this study, therefore, a human CRPC cell line showing resistance to docetaxel was initially established, and changes in molecular profiles after treatment with docetaxel or cabazitaxel in this cell line were subsequently analyzed in order to identify potential targets for overcoming acquired resistance to docetaxel (11).

## Materials and Methods

*Tumor cell lines.* PC3, derived from human prostate cancer, was purchased from the American Type Culture Collection (Rockville, MD, USA). PC3 cells were maintained in RPMI (Life Technologies Inc., Gaithersburg, MD, USA) supplemented with 5% heat-inactivated fetal bovine serum.

A CRPC cell line resistant to docetaxel was established by growing parental PC3 cells (PC3/P) serially treated with an

*Correspondence to:* Hideaki Miyake, Department of Urology, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-Ku, Hamamatsu 431-3192, Japan. Tel: +81 534352306, Fax: 81 534352305, e-mail: hideakimiyake@hotmail.com

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increasing dose of docetaxel (Sigma-Aldrich, St. Louis, MO, USA) up to 1 nM. After continuous culture in complete medium supplemented with 1 nM docetaxel for >20 passages, these cells were used as a docetaxel-resistant CRPC cell line (PC3/R) for all subsequent experiments.

*Cell proliferation assay.* In order to compare the *in vitro* proliferation of PC3 sublines, $5\times10^3$ cells from each cell line were seeded in a 96-well plate, allowed to attach, and the number of cells in each well was then evaluated after 48-hour incubation with Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). The changes in the proliferation of PC3 sublines by treatment with docetaxel (1 nM), cabazitaxel (0.5 nM) (Sigma-Aldrich) with/without the selective inhibitor of PI3K LY294002 (2 μM) (SA Bioscience, Frederick, MD, USA) were also assessed following a 48-hour incubation.

*Western blot analysis.* Western blotting was performed as previously reported (12). Briefly, samples containing equal amounts of protein (15 μg) extracted from PC3 sublines cultured in either standard medium or that containing docetaxel or cabazitaxel were electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel and transferred to a nitrocellulose filter. The filters were incubated for 1 hour with antibodies against total and phosphorylated (p)-AKT serine/threonine kinase 1 (AKT), total and phosphorylated p44/42 mitogen-activated protein kinase (MAPK), total and phosphorylated signal transducer and activation of transcription 3 (STAT3) (Cell Signaling Technology, Danvers, MA, USA) and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and were then exposed for 30 minutes to horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). Specific proteins were detected with an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Arlington Heights, IL, USA).

*Assessment of in vivo tumor growth.* Nude mice (BALB/c-nu/nu male, 6-8 weeks old) used in this study were purchased from CLEA Japan (Tokyo, Japan). Animals were maintained based on the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Each experimental group consisted of 10 mice. Approximately $5\times10^6$ cells suspended in phosphate-buffered saline were subcutaneously injected into nude mice. One week after cell injection, 30 mice were randomly assigned to receive an intraperitoneal injection of vehicle, docetaxel or cabazitaxel at a dose of 10 mg/kg once weekly for 4 weeks. The volume of each tumor was measured with calipers, as previously described (13).

*Histopathological assessment of in vivo tumors.* Tumor specimens were immunohistochemically stained, as previously described (14). Paraffin-embedded sections were stained using antibodies against phospho (p)-AKT and Ki-67 (Cell Signaling Technology) for 1 h, and then incubated with a biotinylated secondary antibody (Santa Cruz Biotechnology, Inc.) for 30 min. After being incubated with an avidin–biotin peroxidase complex, the sections were exposed to diaminobenzidine tetrahydrochloride solution, stained with methyl green, and the expression levels of p-AKT and Ki-67 in each section were evaluated.

*Statistical analysis.* The unpaired *t*-test was used to analyze the differences between the two groups. All statistical analyses were conducted with Statview 5.0 software (Abacus Concepts, Berkley, CA, USA), and $p<0.05$ was considered significant.

## Results

*Establishment of PC3 cells resistant to docetaxel.* PC3/R cells were generated by growing PC3/P cells in the presence of increasing doses of docetaxel up to a final concentration of 1 nM. No significant difference in growth patterns was noted between PC3/P and PC3/R cells when cultured in standard medium without docetaxel (data not shown). However, PC3/R cells exhibited significantly higher resistance to docetaxel than PC3/P cells, showing an approximately 5-fold higher half-maximal (50%) inhibitory concentration (Figure 1A).

We then examined whether PC3/R cells acquire cross-resistance to cabazitaxel, another taxane drug, shown to be effective for docetaxel-refractory patients with mCRPC (15). As shown in Figure 1B, there was no significant difference in sensitivity to cabazitaxel between PC3 sublines.

*Changes in patterns of activation of key molecules involved in signal transduction in PC3 sublines after treatment with docetaxel or cabazitaxel.* Western blot analyses were used to investigate whether the acquisition of a phenotype resistant to docetaxel by PC3/P cells affects the expression levels of major molecules associated with signal transduction before and after treatment with docetaxel or cabazitaxel (Figure 2). Both docetaxel and cabazitaxel markedly inhibited the expression of p-AKT and p-p44/42 MAPK in PC3/P cells; however, in PC3/R cells, expression of p-AKT and p-p44/42 MAPK was maintained following treatment with docetaxel, while treatment with cabazitaxel resulted in the marked down-regulation of p-AKT but not of p-p44/42 MAPK. As for the expression of p-STAT3 after treatment with docetaxel or cabazitaxel, there were no significant differences between PC3 sublines.

*Changes in sensitivity of PC3 sublines to docetaxel and cabazitaxel by combined treatment with LY294002.* Based on the findings presented in Figure 2, the effects of additional treatment with LY294002, a selective inhibitor of PI3K, on the sensitivity of PC3 sublines to docetaxel and cabazitaxel were subsequently examined. As shown in Figure 3, treatment of PC3/R cells with LY294002 significantly enhanced the cytotoxicity of docetaxel, resulting in the lack of a difference in the sensitivity to docetaxel between the PC3 sublines; however, sensitivity to cabazitaxel in PC3 sublines were not affected by combined treatment with LY294002.

*Effects of treatment with docetaxel or cabazitaxel on the in vivo growth of PC3 sublines.* To compare the inhibitory effects of docetaxel and cabazitaxel on the *in vivo* growth of PC3 sublines, $5\times10^6$ cells of each subline were subcutaneously injected into nude mice, which were then

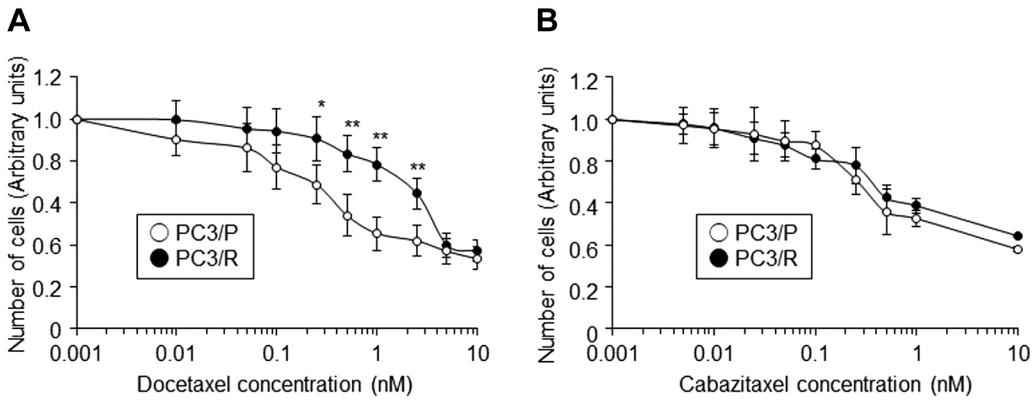


Figure 1. *The dose-dependent effect of treatment with docetaxel (A) and with cabazitaxel (B) on in vitro cell growth of PC3 sublines. PC3/P and docetaxel-resistant PC3/R cells were treated with the indicated doses of each taxane. After 48 hours of incubation, cell growth was determined in triplicate in three independent experiments. Data are the mean±standard deviation. Significantly different at *p<0.05 and **p<0.01 from PC3/P cells.*

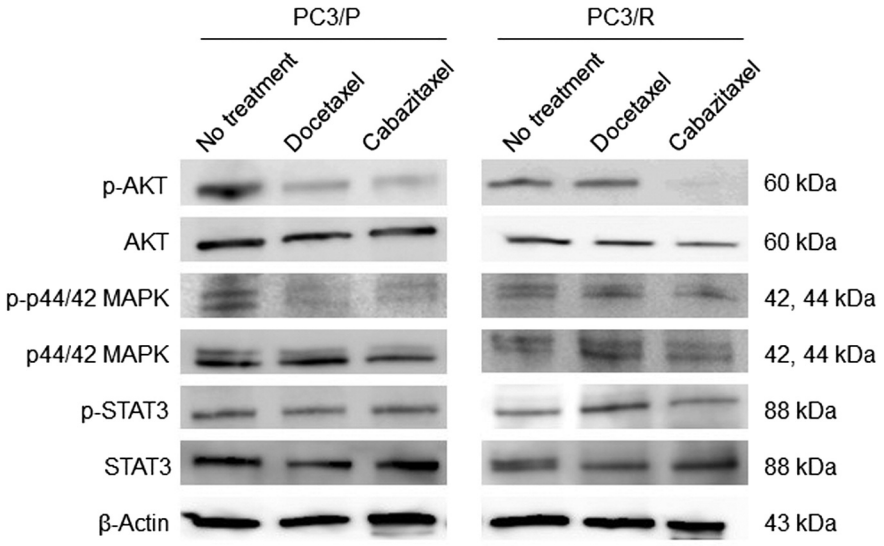


Figure 2. *Changes in the expression patterns of key molecules involved in major signal transduction pathways in PC3 sublines after treatment with 1 nM docetaxel or 0.5 nM cabazitaxel. PC3 sublines were treated with each taxane for 48 hours. Protein was extracted from cultured cells, and evaluated for phosphorylated (p)-AKT serine/threonine kinase 1 (AKT), AKT, p-p44/42 mitogen-activated protein kinase (MAPK), p44/42 MAPK, p-signal transducer and activation of transcription 3 (STAT3) and STAT3 levels by western blotting.*

randomly selected for treatment with either vehicle, docetaxel or cabazitaxel. As shown in Figure 4A, treatment with docetaxel and cabazitaxel significantly inhibited the growth of PC3/P tumors compared with those treated with with vehicle; however, there were no significant differences in the tumor growth patterns between those treated with docetaxel and cabazitaxel. Furthermore, treatment with cabazitaxel significantly inhibited the growth of PC3/R tumors compared with docetaxel, which had a similar effect on the growth of PC3/R tumors to the vehicle (Figure 4B).

We then compared the changes in the status of AKT phosphorylation and cell proliferative potential between PC3/P and PC3/R tumors after the administration of docetaxel or cabazitaxel, by immunohistochemical staining. As shown in Figure 5, expression of p-AKT was not detectable in PC3/P tumors after treatment with docetaxel and cabazitaxel; however, p-AKT was expressed in PC3/R tumors after treatment with docetaxel but not with

cabazitaxel. Similarly, there was no significant difference in Ki-67 expression between PC3/P tumors after treatment with docetaxel and cabazitaxel, while Ki-67 expression in PC3/R tumors treated with docetaxel was significantly higher than that in those treated with cabazitaxel.

## Discussion

In this study, we initially established a docetaxel-resistant human CRPC cell line, PC3/R, with an approximately 5-fold higher half-maximal inhibitory concentration than the parental cell line, PC3/P. Most previous studies analyzing the mechanism underlying drug resistance used a cell line showing high-level resistance to therapeutic agents (16); however, considering the concentration of each agent used during the generation of such a highly resistant cell line, it might be optimal to use a cell line with moderate resistance to the target drug, like PC3/R, in order to generate clinically relevant findings. In fact, there was no significant difference in sensitivity to cabazitaxel between PC3/P and PC3/R cells, as observed in clinical practice, showing the favorable effect of cabazitaxel on patients with docetaxel-refractory mCRPC (15). Accordingly, we believe that the current model might be useful for investigating the molecular mechanism involved in the resensitization of mCRPC to novel agents, including cabazitaxel, following the acquisition of resistance to docetaxel.

To date, persistent activation of signal transduction pathways has been shown to play an important role in the acquisition of a drug-resistant phenotype in various types of cancer model (7, 8, 16, 17). In this study, therefore, we evaluated changes in the activation status of major signal transduction pathways after exposure to either docetaxel or cabazitaxel in PC3 sublines. The expression of p-AKT and p-p44/42 MAPK in PC3/P cells was markedly inhibited by both docetaxel and cabazitaxel. In PC3/R, however, despite the constitutive activation of p44/42 MAPK after treatment with either taxane, phosphorylation of AKT was maintained after treatment with docetaxel but not with cabazitaxel. Moreover, acquired resistance to docetaxel in PC3/R was overcome by additional treatment with a selective inhibitor of PI3K, LY294002. Similarly to the current study, there have been several studies showing the involvement of the AKT pathway in the regulation of sensitivity to taxanes in prostate cancer cells (7, 8). For example, Schmidt *et al*. reported the induction of apoptosis in prostate cancer cells by acetyl-lupeolic acid via the inactivation of AKT signaling (8), while Liu *et al*. showed the association between the up-regulation of PI3K/AKT and resistance to microtubule-targeting drugs in prostate cancer cells (7). Considering these findings, persistent expression of p-AKT may be involved in the acquisition of resistance to docetaxel in CRPC cells, and it might be a promising approach to inactivate p-AKT using cabazitaxel with/without LY294002 to efficaciously inhibit the growth of docetaxel-resistant CRPC cells.

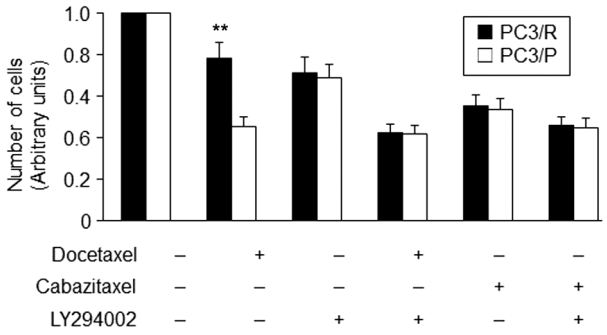


Figure 3. *The effects of treatment combining docetaxel (1 nM) or cabazitaxel (0.5 nM) with LY294002 (2 μM), a selective inhibitor of phosphoinositol 3-kinase, on the in vitro growth of PC3 sublines. Each cell line was treated with either taxane with/without LY294002 for 48 hours then cell numbers for each cell line were counted in triplicate. Bars, standard deviation. **Significantly different at p<0.01 from PC3//P.*

Despite markedly different effects of these two taxanes on PC3/P and PC3/R cells *in vitro*, it is still of interest to characterize the growth-inhibitory effects of these agents on both PC3/P and PC3/R tumors *in vivo*. Similarly to *in vitro* studies, there was no significant difference in the growth of PC3/P tumors between mice treated with the two taxanes; however, the administration of cabazitaxel significantly inhibited the growth of PC3/R tumors compared with that of docetaxel. Furthermore, histochemical studies confirmed changes in p-AKT and Ki-67 expression in PC3/P and PC3/R tumors, reflecting growth patterns after treatment with docetaxel and cabazitaxel. Collectively, these findings suggest that activation of the AKT pathway might be regarded as one of the major molecular events underlying acquired resistance to docetaxel in CRPC cells, considering its close association with *in vivo* growth patterns of CRPC tumors.

Here, we would like to emphasize the potential limitation of this study, which did not take into account the common mechanism mediating resistance to taxanes in mCRPC cells. For example, de Morrée *et al*. reported that solute carrier organic anion transporter family member 1B3, which determines intracellular concentrations of docetaxel and cabazitaxel, consequently influenced their efficacy, and that loss of this drug transporter may drive resistance to these taxanes in mCRPC cells (9). Lombard *et al*. also showed the mechanism mediating cross-resistance between docetaxel and cabazitaxel, though the up-regulation of adenosine triphosphate-binding cassette subfamily B member in prostate cancer cells (10). It will be necessary to consider mechanisms associated with acquired resistance as well as cross-resistance to taxanes in order to develop strategies for overcoming resistance to docetaxel in mCRPC.

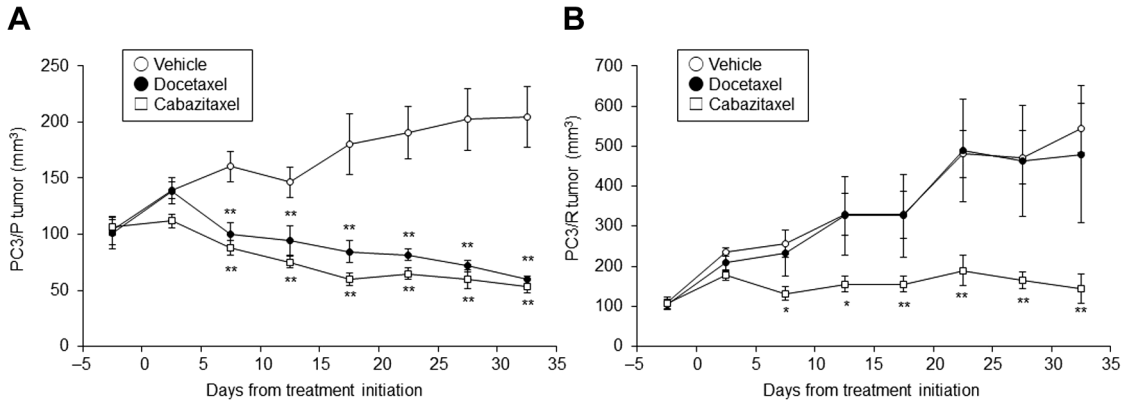


Figure 4. *The effect of treatment with docetaxel (A) and with cabazitaxel (B) on the in vivo growth of PC3 subline-derived tumors. Thirty nude mice were subcutaneously given 5×10⁶ cells of each PC3 subline, then randomly selected for weekly treatment with either 10 mg/kg vehicle, docetaxel or cabazitaxel for 4 weeks. The subcutaneous tumor volume was measured at intervals using calipers and calculated using the formula: Length × width × depth × 0.5236. Each data point represents the mean tumor volume in each experimental group including 10 mice. Bars, standard deviation. Significantly different at *p<0.05 and **p<0.01 from PC3/P cells.*

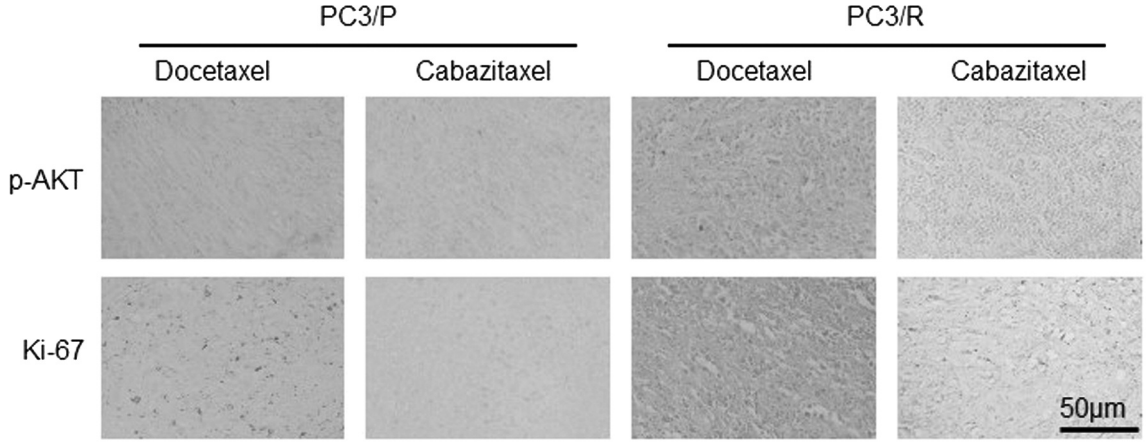


Figure 5. *Histopathological study for the evaluation of in vivo expression of phosphorylated (p)-AKT serine/threonine kinase 1 (AKT) and Ki-67 in PC3 sublines after treatment with docetaxel or cabazitaxel. In vivo subcutaneous tumors were harvested from nude mice undergoing treatment with either taxane for 4 weeks. Sections from each tumor tissue were examined by immunohistochemical staining with antibodies against p-AKT and Ki-67.*

## Conclusion

We successfully developed PC3/R, a human CRPC cell line with a phenotype resistant to docetaxel, while being shown to have a sensitivity to cabazitaxel similar to the parental cell line. We subsequently showed that despite a lack of p-AKT in both PC3 sublines after treatment with cabazitaxel, persistent phosphorylation of AKT in PC3/R cells was observed after treatment with docetaxel but not in PC3/P. Furthermore, additional treatment of PC3/R with a selective inhibitor of PI3K overcame resistance to docetaxel. Collectively, these findings suggest that sustained AKT activation during docetaxel treatment may be involved, at least in part, in the acquisition of a phenotype resistant to docetaxel in CRPC cells, and that combined treatment with docetaxel and a potential agent targeting AKT might be a promising approach for overcoming docetaxel resistance in patients with CRPC.

## Conflicts of Interest

The Authors have no conflicts of interest to declare.

## Authors' Contributions

The types of contribution by each Author are as follows: Study conception and design, Hideaki Miyake; acquisition of data, Asuka Kawakami, Ryo Sato, Kyohei Watanabe, Yuto Matsushita; analysis and interpretation of data, Hiromitsu Watanabe, Hideaki Miyake; drafting of article,Hiromitsu Watanabe and Hideaki Miyake.

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