Award Number: W81XWH-05-1-0102

TITLE: The Double-Edged Sword of Prostate Cancer: 14-3-3(sigma)

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REPORT DATE: December 2005

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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In the first year of study, we tested the hypothesis that advanced and hormone refractory prostate cancers may have increased 14-3-3σ expression, which in turn may contribute to drug resistance in advanced and hormone-refractory prostate cancers. We found that, indeed, the expression level of 14-3-3σ in androgen-independent prostate cancer cell lines DU145 and PC3 are much higher than that in the androgen-dependent cell line LNCaP and that the androgen-independent cells are more resistant to mitoxantrone and Adriamycin than the androgen-dependent cells. Down-regulating 14-3-3σ expression in the androgen-independent cell line DU145 by small interference RNA significantly sensitized these cells to mitoxantrone and Adriamycin by causing failure in maintaining G2/M checkpoint and increasing apoptosis. We also showed that 14-3-3σ deficiency caused nuclear localization of Cdc2 and dephosphorylation of the Tyr15 residue upon DNA damage. Based on these studies, we propose that therapeutic intervention targeting 14-3-3σ may prove to be useful for sensitizing hormone-refractory prostate cancers to chemotherapy by both G2/M checkpoint abrogation and enhancing apoptotic cell death.
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INTRODUCTION

Use of anticancer agents in appropriate combinations has led to major improvements in the treatment of malignant tumors. Previously fatal diseases, such as testicular cancer, are now curable while others can undergo meaningful remission. Despite such successes, resistance to chemotherapy frequently occurs and it is a major obstacle in the successful systematic treatment of cancers. Studies with model tumor cell lines have revealed that multidrug resistance (MDR) can develop and cause chemotherapy failure.

We recently found that cancer cell lines over-expressing 14-3-3σ are resistant to anticancer drugs such as Adriamycin and mitoxantrone (unpublished observations). 14-3-3σ is a member of a highly conserved family of 14-3-3 proteins that are present in high abundance in all eukaryotic organisms studied so far. Currently, there are 7 known 14-3-3 isotypes (β, ρ, ε, η, ζ, σ and τ/θ) in human and they are highly conserved (1,2). The 14-3-3 proteins play important roles in many biological activities by directly binding to and altering the subcellular localization and/or stability of key molecules in several signaling cascades (3,4). 14-3-3 proteins can interact with more than 100 cellular proteins at their Ser phosphorylation sites and the target proteins include various protein kinases, receptor proteins, enzymes, structural and cytoskeletal proteins, proteins involved in cell cycle control and apoptosis (1,4,5).

Recently, it has also been reported that 14-3-3σ expression is increased in pancreatic ductal adenocarcinoma and colorectal cancers and that the increased 14-3-3σ expression is an independent prognostic marker for poor survival of these cancer patients (6-10), suggesting that the patients who have higher level of 14-3-3σ in their pancreatic and colorectal caners responded poorly to therapeutics compared to the ones with lower level. It was also found that higher level of 14-3-3σ is associated with the higher grade of colorectal cancers. It is possible that the tumors with higher level of 14-3-3σ will respond poorly to chemotherapy in a clinical setting. It is also possible that chemotherapy treatment selects the resistant cancer cells with higher expression levels of 14-3-3σ which then causes relapse of the disease and eventual failure of chemotherapy. Indeed, it has been found that 14-3-3σ expression is increased in the drug resistant pancreatic adenocarcinoma cell lines (11).

Prostate cancer and treatment: Prostate cancer progression and the development of androgen-independency have been largely related to a number of genetic abnormality that affect not only the androgen receptor but also crucial molecules involved in the regulation of survival or apoptotic pathways. The series of events leading to apoptosis may be ordered into receipt of the apoptotic stimulus, recognition and intracellular signal transduction of the stimulus, down regulation of anti-apoptotic and cell cycle regulatory proteins, recruitment of the effector apoptotic machinery and removal of the apoptotic body in vivo. Involved in this series of events are the two broadly classified functional groups, intracellular and cell surface functions. Identifying these signaling events of apoptosis, particularly how deregulation of such events contributes to apoptotic resistance, is fundamental for designing novel approaches for treating prostate cancer.

14-3-3σ and cancer: 14-3-3σ was originally characterized as a human mammary epithelium marker 1 (HME1) (12). Recent evidence suggests that 14-3-3σ may be a tumor suppressor gene. 14-3-3σ has been found frequently lost or decreased in human cancers of breast (13,14), liver, (15), vulva (16), mouth (17), neuroendocrine (18), small and non-small cell lung (19) (20) cancers. The decrease was found to be due to hypermethylation of the CpG islands of the 14-3-3σ gene (13,19,21,22). Thus, 14-3-3σ may be a tumor suppressor of epithelial cells of multiple origins.

Function of 14-3-3σ: Although the mechanism how 14-3-3σ functions as a tumor suppressor is unknown, it has been shown that 14-3-3σ is strongly up-regulated upon exposure to ionizing radiation and DNA-damaging agent and appears to be essential for maintaining the G2/M checkpoint (23). It has also been shown that 14-3-3σ negatively regulates cell cycle progression by interacting with cyclin-dependent kinase 2 (24). Thus, likely p53 and BRCA1 co-activates 14-3-3σ expression which in turn inhibits cyclin-dependent kinase 2 and leads to G2 arrest (25).

BODY

In the original application, we proposed to accomplish two specific objectives: (1) to determine whether expression of 14-3-3σ causes resistance to DNA-damaging anticancer drugs and androgen-independent growth advantage for prostate cancer cells and (2) to determine whether the lost or reduced expression is one of the causes of prostate carcinogenesis.
In the first year of support, we have accomplished part of the first specific aim. We have demonstrated that the expression of 14-3-3σ in androgen-independent prostate cancer cell lines causes resistance of these cells to DNA-damaging anticancer drugs such as Adriamycin and mitoxantrone. We have also demonstrated that the mechanism of action for 14-3-3σ in drug resistance is due to their increased ability to resist DNA-damage induced apoptosis. The following describes in detail on this work.

14-3-3σ is expressed in both normal prostate epithelial cells and hormone-independent prostate cancer cell lines. To examine the expression pattern of 14-3-3σ in androgen-dependent and -independent prostate cancer cells, we tested three commonly-used prostate cancer cell lines, LNCaP, PC-3 and DU145 as well as an immortalized normal prostate epithelial cell line RWPE-1. Cell lysates were prepared from these cells and analyzed for 14-3-3σ expression by Western blot. As shown in Fig. 1A, 14-3-3σ was not detected in LNCaP, an androgen-dependent cancer cell line (lane 1), whereas it was detected in the androgen-independent cell lines DU145 and PC-3 (lanes 3 and 4) as well as in the normal RWPE-1 cells (lane 2). We also tested another androgen-independent prostate cancer cell line CWR22RV and found that it also expresses 14-3-3σ to a similar level as DU145 and PC-3 cells (data not shown).

We next determined the 14-3-3σ mRNA level in these cells using real-time RT-PCR. As shown in Fig. 1B, the mRNA level in RWPE-1, DU145 and PC3 cells was 195, 36, 24-fold higher than that in LNCaP cells, respectively. Based on both the western and real-time RT-PCR results, we conclude that 14-3-3σ expression is reduced in the prostate cancer cell lines compared with the normal cells, consistent with previous findings that prostate tumors have reduced expression of 14-3-3σ compared to normal tissues (26-29). However, 14-3-3σ expression in the androgen-independent cell lines DU145 and PC3 is considerably high despite the fact that its expression is completely lost in the androgen-dependent LNCaP cells.

To determine whether DNA methylation plays any role in reduced 14-3-3σ expression in these prostate cell lines, we performed methylation specific PCR analysis of genomic DNAs isolated from these cells using a pair of methylation-specific primers and a pair of unmethylation-specific primers targeting the CpG dinucleotides known to be hypermethylated (Fig. 1C, see also (30)). As shown in Fig. 1D, the CpG islands in LNCaP cells were partially methylated (lane 3). However, no methylation was detected in other cell lines. Thus, the diminished 14-3-3σ expression in LNCaP cells is partially due to methylation of the CpG islands in its gene whereas the methylation plays no role in the reduced expression of 14-3-3σ in DU145 and PC3 cells compared to normal cells.

14-3-3σ expression is induced by DNA damage and may account for mitoxantrone resistance in androgen-independent prostate cancer cells. Because the androgen-independent cell lines DU145 and PC3 express significant amount of 14-3-3σ whereas the androgen-dependent cell line LNCaP does not, we
hypothesized that the androgen-independent cells may have a higher level of resistance to anticancer drugs such as mitoxantrone in addition to their resistance to androgen ablation treatment. To test this hypothesis, we first compared the growth inhibition and apoptosis induced by mitoxantrone in DU145 and LNCaP cell lines. As shown in Fig. 2A, the two cell lines displayed different mitoxantrone susceptibilities. LNCaP cells were completely killed at 48 hrs after treatment with 2 µM mitoxantrone whereas DU145 cells largely survived the treatment. We next performed a dose-response analysis of these cells using MTT assay. Again, LNCaP cells were more sensitive to growth inhibition induced by mitoxantrone compared to DU145 cells (Fig. 2B).

To analyze whether apoptosis is the mechanism that mediates mitoxantrone-induced cell killing, lysates of mitoxantrone-treated cells were prepared and analyzed for cleavage of PARP, a 115-kDa protein substrate of caspases during execution of apoptosis. The cleavage of this protein by caspases yields an 85-kDa product. As shown in Fig. 2C, the level of the full-length PARP decreased with an appearance of the 85-kDa fragment in LNCaP but not in DU145 cells following a 24-hr treatment with 1 µM mitoxantrone. Taken together, we conclude that the androgen-independent DU145 cells which express significant amount of 14-3-3σ are more resistant to mitoxantrone-induced apoptosis compared to the androgen-dependent LNCaP cells which do not express 14-3-3σ.

To explore the potential role of 14-3-3σ in mitoxantrone resistance in DU145 cells, we examined the expression level of 14-3-3σ before and after mitoxantrone treatment. As shown in Fig. 2D, the expression of 14-3-3σ was significantly increased at 7 hrs after treatment with 0.1 µM mitoxantrone. This finding suggests that 14-3-3σ expression in DU145 cells is inducible by anticancer drugs that in turn mediate resistance to these drugs. Furthermore, it is likely that the induction of 14-3-3σ by mitoxantrone in DU145 cells is through a p53-independent pathway because these cells do not express a functional wild type p53 (31).

RNA interference of 14-3-3σ enhances apoptosis induced by mitoxantrone. Because the sensitivity of LNCaP cells to mitoxantrone can also be due to the expression of wild-type p53 in these cells, whereas PC3 and DU145 cells have mutated p53, we decided to determine the contribution of 14-3-3σ to intrinsic drug resistance by manipulating the expression level of 14-3-3σ in DU145 cells. For this purpose, we designed four siRNAs to specifically suppress the 14-3-3σ expression by targeting the coding region of 14-3-3σ mRNA. These siRNAs showed no homology with other known genes in the GeneBank. A siRNA with a scrambled sequence which has no homology to any known mammalian sequence was also synthesized and used as a negative control. After transient transfection of these siRNAs into the androgen-independent DU145 cells, lysates were prepared for Western blot analysis to evaluate the efficacy of these siRNAs. As shown in Fig. 3A, the siRNA (#365) showed the strongest activity to suppress 14-3-3σ expression. In contrast, the level of Chk1 was unaffected by any of the 14-3-3σ siRNAs.

Figure 2. 14-3-3σ expression and response to mitoxantrone in androgen-dependent and –independent cell lines. (A) Morphology of LNCaP and DU145 cells following mitoxantrone treatment. Cultured LNCaP and DU145 cells were treated with or without 2 µM mitoxantrone for 48 h followed by viewing on a microscope. (B) Effect of mitoxantrone on proliferation of LNCaP and DU145 cells. Both LNCaP and DU145 cells were treated continuously with different concentrations of mitoxantrone for 48 h followed by MTT assay. The data was a representative of three independent experiments. (C) Western blot analysis of PARP. DU145 and LNCaP cells were treated with or without 1 µM mitoxantrone for 48 h followed by cell lysate preparation and western blot analysis of PARP. The 116- and 85-kDa bands correspond to intact and cleaved product of PARP, respectively. (D) Western blot analysis of 14-3-3σ following mitoxantrone treatment. DU145 cells were treated with 0.1 µM mitoxantrone for 0, 7, and 24 h followed by cell lysate preparation for western blot analysis of 14-3-3σ and β-actin.
To facilitate further analysis of the role of 14-3-3σ in mitoxantrone resistance, we constructed the expression plasmid pSilencer-σ that can express the small hairpin RNA (shRNA) of 14-3-3σ using the H1 promoter (Fig. 3B). The shRNA expressed from this plasmid is expected to be processed into the corresponding siRNA (#365) inside the cell. The plasmid pSilencer-SCR that expresses the scrambled siRNA was used as the negative control. Transient transfection of 14-3-3σ shRNA but not the SCR shRNA effectively suppressed the expression of 14-3-3σ (data not shown). We next established stable DU145 clones that express the shRNA of 14-3-3σ and control cell clones that express the control shRNA with scrambled sequence. As shown in Fig. 3C, two stable DU145 clones transfected with shRNA of 14-3-3σ (σ1- and σ2-) have significant decreases in 14-3-3σ expression compared with the control clones (Scr1 and Scr2) (compare lanes 3-4 with 1-2).

We next determined mitoxantrone sensitivity of these stable DU145 clones. The cells were first treated with 1 µM mitoxantrone for 24 hrs and then processed for western blot analysis of PARP cleavage. As shown in Fig. 3D, both σ1- and σ2- clones showed significant levels of PARP cleavage to generate the 85-kDa fragment as compared with the control Scr1 and Scr2 clones (compare lanes 6 and 8 with lanes 2 and 4). More PARP cleavage product was observed with the σ1- than the σ2- clone (compare lane 6 with 8), likely because the σ1- clone expressed a lower level of 14-3-3σ than the σ2- clone (compare lane 3 with 4 in Fig.3C, also compare lane 5 with 7 in Fig. 3D). In addition, we also found that the 14-3-3σ protein level increased at 24 hrs after mitoxantrone treatment with these stable clones (Fig. 3D), consistent with the previous finding that the 14-3-3σ expression is up-regulated by mitoxantrone treatment (Fig. 1D).

To rule out the possibility that the shRNA-mediated mitoxantrone sensitization was due to the specific cell clones selected, we performed an experiment on pooled cells transiently transfected with siRNA (#365). DU145 cells were first transiently transfected with the siRNA of 14-3-3σ (#365) or siRNA with scrambled sequence (SCR). Twenty-four hrs following transfection, the cells were treated with 1 µM mitoxantrone for 6 to 24 hrs followed by preparation of cell lysates for analysis of PARP cleavage. As shown in Fig. 3E, cells transiently transfected with the siRNA (#365) generated the 85-kDa cleavage product of PARP with decreases in the level of the full-length PARP, whereas the cells treated with the negative scrambled siRNA control did not. Thus, reducing 14-3-3σ expression under both stable and transient conditions sensitized the androgen-independent DU145 cells to mitoxantrone-induced apoptosis.

We next performed a survival study of the DU145 cells with reduced expression of 14-3-3σ using MTT assay. For this purpose, the σ1- clone was used because it has a better suppressed expression of 14-3-3σ. The
σ1- cell displayed no difference in proliferation rate from the control Scr2 cell under normal growth conditions (data not shown). However, the σ1- cell was much more sensitive to growth inhibition induced by mitoxantrone (Fig. 4A). Majority of the σ1- cells were killed at 48 hrs after treatment with 2 µM mitoxantrone while few control Scr2 cells were killed under the same condition (Fig. 4B). The PARP cleavage was observed at 5 hrs following mitoxantrone treatment in σ1- cells (Fig. 4C) and the death of these cells was observed as early as 5 hrs following mitoxantrone treatment (data not shown). Similarly, the σ1- cells were also more significantly sensitive to apoptosis induced by another anticancer drug Adriamycin (Fig. 4D). At 9 hrs following Adriamycin treatment, approximately half of the PARP in σ1- cells was cleaved and no intact PARP was observed at 24 hrs of treatment. In contrast, significant cleavage of PARP was not observed in the control Scr2 cells (Fig. 4C and 4D). Thus, the expression of 14-3-3σ in DU145 cells clearly causes resistance to treatment by anticancer drugs mitoxantrone and Adriamycin.

**Figure 4. Effect of reducing 14-3-3σ expression on drug resistance.** (A). Effect of 14-3-3σ expression on drug resistance. DU145 cells stably transfected with 14-3-3σ shRNA (σ1-) and control scrambled siRNA (Scr2) were treated with different concentrations of mitoxantrone for 48 h followed by MTT assay. The data were representative of three independent experiments. (B) Morphology of σ1- and Scr2 cells following mitoxantrone treatment. Scr2 and σ1- cells were treated with or without 1 µM mitoxantrone for 48 h followed by viewing on a microscope. (C) and (D), Time course of drug-induced PARP cleavage. Scr2 and σ1- cells were treated with 1 µM mitoxantrone or 2 µM Adriamycin for various times followed by cell lysate preparation and western blot analysis of PARP.

To further confirm if the sensitized killing of σ1- cells is due to facilitation of drug-induced apoptosis by silencing 14-3-3σ expression, we performed co-staining of mitoxantrone-treated cells with Annexin V and propidium iodide (PI) which detect apoptosis and necrosis, respectively (32,33). Within the apoptotic cell population, cells in the early stage of apoptosis were Annexin V-positive and PI-negative. The population of cells in the late stage of apoptosis were Annexin V-positive and PI-positive. As shown in Fig. 5, approximately 12.8% and 3.5% of σ1- cells were in the process of early and late apoptosis, respectively, at 16 hrs following treatment with 320 nM mitoxantrone. In contrast, the control Scr2 cells at the same time following mitoxantrone treatment showed no significant difference in Annexin V and PI staining compared with the untreated cells.

**Figure 5. Effect of reducing 14-3-3σ expression on mitoxantrone-induced apoptosis and cell cycle distribution.** DU145 cells stably transfected with 14-3-3σ shRNA (σ1-) and control scrambled siRNA (Scr2) were treated with 320 nM mitoxantrone for 16 h followed by double staining with Annexin V and PI and FACS analysis.

The androgen-independent DU145 cells with depleted 14-3-3σ expression could not maintain G2/M arrest and Cdc2 inactivation. It has been suggested that 14-3-3σ expression is important for maintaining the G2/M arrest induced by DNA damages (34). To determine whether the increased drug sensitivity in the σ1- cell was due to the loss of G2/M check point, we analyzed cell cycle profiles for cells treated with 320 nM mitoxantrone for 16 hrs. As shown in Fig. 6A, significantly less σ1- cells were arrested at G2/M compared with the control Scr2 cells. Thus, it is possible that DU145 cells with depleted 14-3-3σ expression could not effectively maintain G2/M arrest following drug treatment.

To further determine whether the G2/M checkpoint control was affected by 14-3-3σ depletion in DU145 cells, the σ1- and Scr2 control cells were treated with 50 nM mitoxantrone and harvested for cell cycle analysis.
at different time points. As shown in Fig. 6B, the control Scr2 cells were arrested at S and G2/M at 24 hrs post mitoxantrone treatment. At 48 hrs, majority of the control Scr2 cells were arrested at G2/M phase and the G2/M arrest was maintained thereafter until 96 hrs. Although there is significant G2/M arrest of σ1- cells at 24 hrs following mitoxantrone treatment, there is a significant increase in population in G1 and sub-G1 phases for σ1- cells at 48 and 96 hrs following mitoxantrone treatment. This observation suggests that some σ1- cells were able to go through G2/M and re-enter G1 phase. These results demonstrate that DU145 cells with reduced 14-3-3σ expression could not stably maintain G2/M arrest induced by mitoxantrone which in turn led to apoptosis.

**Figure 6. Effect of 14-3-3σ expression on mitoxantrone-induced G2/M arrest and Cdc2 inactivation and localization.** (A) and (B) Cell cycle analysis. DU145 cells stably transfected with 14-3-3σ shRNA (σ1-) and control scrambled siRNA (Scr2) were treated with 320 nM mitoxantrone for 16 h (A) or with 50 nM mitoxantrone for various times (B) followed by cell cycle analysis. (C) Cdc2 cellular localization. DU145 cells stably transfected with 14-3-3σ shRNA (σ1-) and control scrambled siRNA (Scr2) were cultured on coverslips in the presence or absence of 50 nM mitoxantrone for 72 h, and then processed for immunostaining with antibody against Cdc2 followed by confocal imaging. (D) Cdc2 phosphorylation. DU145 cells stably transfected with 14-3-3σ shRNA (σ1-) and control scrambled siRNA (Scr2) were treated with mitoxantrone for various times followed by cell lysate preparation and western blot analysis using antibody against total and phospho-Cdc2 (Tyr15). The histogram shows the relative ratio of phosphor-Cdc2/total Cdc2 of the gel determined using Scion image software.

Entry of all eukaryotic cells into M-phase is regulated by Cdc2 kinase. Activation of Cdc2 is a complex process that requires multiple steps. The Cdc2 protein forms a complex with cyclin B and is localized in cytoplasm during the interphase but enter the nucleus during mitosis. However, Cdc2/cyclin B complex was retained in cytoplasm when cells encounter DNA damage by checkpoint control systems, which prevent mitosis of cells with damaged DNA. To determine if the subcellular localization of Cdc2 is affected by depleting 14-3-3σ expression, we performed an indirect immunofluorescence staining of Cdc2 in σ1- and control Scr2 cells following mitoxantrone treatment. As shown in Fig. 6C, Cdc2 accumulates in cytoplasm in both treated and untreated control Scr2 cells. In contrast, the σ1- cells displayed a less clear cytoplasmic localization under normal growth conditions, and Cdc2 accumulates significantly in the nucleus when the σ1- cells were treated with 50 nM mitoxantrone for 72hrs. This result suggests that 14-3-3σ was essential for cytoplasmic localization of Cdc2.

To further correlate the Cdc2 activity with cell cycle progression in these two stable clones, we performed Western blot analysis to determine the level of phosphorylation of Cdc2 on Tyr15. Since this phosphorylation blocks the ATP binding site and, thus, inactivates the kinase activity of Cdc2, the phosphorylation level of Tyr15 inversely correlates with Cdc2 activity (35). As shown in Fig. 6D, the level of Cdc2 with phosphorylated Tyr15 in the control Scr2 cells is drastically increased with prolonged mitoxantrone treatment, suggesting that Cdc2 was inactivated upon mitoxantrone treatment. However, the level of Cdc2 with phosphorylated Tyr15 in σ1- cells slightly increased at 24 hrs but significantly decreased at 48 hrs following mitoxantrone treatment (Fig. 6D), suggesting that 14-3-3σ expression contributes to Cdc2 phosphorylation. Taken together, our cell cycle and Cdc2 analysis indicated that the 14-3-3σ-depleted cells could not maintain the cell cycle arrest at G2/M following drug treatment due to the lack of effective ability to inhibit the Cdc2 activity.

**KEY RESEARCH ACCOMPLISHMENTS**

1. 14-3-3σ expression was found higher in the androgen-independent prostate cancer cell lines DU145 and PC3 compared with the androgen-dependent LNCaP cells.
2. The higher expression level of 14-3-3σ appears to cause the androgen-independent cells resistant to anticancer drugs such as mitoxantrone and Adriamycin.
3. Down-regulating 14-3-3σ expression sensitized these cells to drug treatment likely by decreasing the ability of cells to maintain G2/M arrest and increasing apoptosis upon exposure to anticancer drugs.

REPORTABLE OUTCOMES
1. Han, B.; Xie, H.; Chen, Q.; Zhang, J. T. Sensitizing hormone-refractory prostate cancer cells to drug treatment by targeting 14-3-3σ. (submitted for publication).

CONCLUSIONS
In conclusion, we found that 14-3-3σ expression is higher in the androgen-independent prostate cancer cell lines DU145 and PC3 compared with the androgen-dependent LNCaP cells. The higher expression level of 14-3-3σ appears to cause the androgen-independent cells resistant to anticancer drugs such as mitoxantrone and Adriamycin. Down-regulating 14-3-3σ expression sensitized these cells to drug treatment likely by decreasing the ability of cells to maintain G2/M arrest and increasing apoptosis upon exposure to anticancer drugs. These findings implicate that the androgen refractory prostate cancers are likely resistant to chemotherapy due to its potential higher 14-3-3σ expression and 14-3-3σ may be developed as a target for sensitizing hormone refractory prostate cancers to chemotherapy.

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