Sensitizing hormone-refractory prostate cancer cells to drug treatment by targeting 14-3-3σ

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Abstract

Advanced and hormone-refractory prostate cancer has long been considered as a chemoresistant disease. Recently, it was found that 14-3-3σ expression increases as prostate tumor progresses, and that 14-3-3σ contributes significantly to drug resistance in breast cancers. We, thus, hypothesized that advanced and hormone-refractory prostate cancers may have an increased level of 14-3-3σ, which in turn may contribute to drug resistance in advanced and hormone-refractory prostate cancers. In this study, we tested this hypothesis and found that, indeed, the expression level of 14-3-3σ in androgen-independent prostate cancer cell lines DU145, PC3, and CWR22RV 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. Depleting 14-3-3σ expression in DU145 and CWR22RV by RNA interference significantly sensitized these cells to mitoxantrone and increasing apoptosis, whereas restoring 14-3-3σ expression in LNCaP cells enhanced drug resistance. 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 be useful for sensitizing hormone-refractory prostate cancers to chemotherapy by both G2-M checkpoint abrogation and apoptosis enhancement. [Mol Cancer Ther 2006;5(4):903–12]

Introduction

Prostate cancer is the most commonly diagnosed invasive cancer and a leading cause of death in men in western countries. Most patients who develop metastatic disease initially respond to androgen deprivation but ultimately develop androgen-independent disease that results in progressive clinical deterioration and death (1). As a well-tolerated treatment regimen and one of the few chemotherapeutic approaches, mitoxantrone combined with low-dose prednisone showed some benefits on disease-related symptoms and quality of life (2). However, chemotherapy with drugs, such as mitoxantrone, eventually also fails. Examining the mechanism of mitoxantrone resistance is, thus, critical for improving treatment of prostate cancers with advanced and hormone-refractory prostate cancers.

Using proteomics, we recently found that up-regulated 14-3-3σ expression may be a cause of selected Adriamycin resistance in breast cancer cell lines.1 14-3-3σ is a member of the 14-3-3 family that include seven isoforms (α/β, γ, ε, ζ, τ/θ, and η) in mammal and play diverse roles in intracellular signaling (3, 4). 14-3-3 proteins are known to negatively regulate cell cycle progression and inhibit onset of apoptosis at multiple steps (4). Among all seven isoforms, 14-3-3σ is the most divergent member and directly linked to cancer (5). Recent evidences suggest that 14-3-3σ is a critical G2-M regulator and that σ−/− HCT116 colorectal cancer cells could not maintain G2-M arrest after DNA damage and died by mitotic catastrophe (6, 7). 14-3-3σ seems to be critical for sequestration of Cdc2/cyclin B1 complex in cytoplasm, thereby preventing cells from entering mitosis (6, 7).

Thus, 14-3-3σ likely plays an important role in response to chemotherapeutic drugs in epithelial cancer cells. In this study, we investigated the expression profile of 14-3-3σ in commonly used prostate cancer cell lines and tested the hypotheses that the androgen-independent prostate cancer cells have a higher level of 14-3-3σ expression than the androgen-dependent cells and that the higher level of 14-3-3σ in these cells is a cause of resistance to DNA-damaging anticancer drugs, such as mitoxantrone and Adriamycin.

Materials and Methods

Materials

Restriction enzymes were purchased from New England Biolabs (Ipswich, MA). The vector pSilencer3.1-H1neo vector and pSilencer-Scr were from Ambion (Austin, TX). Green PCR Master Mix for real-time PCR was purchased...
from Applied Biosystems (Foster City, CA). The oligonucleotide primers for PCR, LipofectAMINE plus, LipofectAMINE 2000, G418, fetal bovine serum, and Opti-MEM I were from Invitrogen (Carlsbad, CA). Annexin V-FITC apoptosis detection kit was from Calbiochem (La Jolla, CA). The EDTA-free protease inhibitor cocktail was from Roche Diagnostics (Indianapolis, IN). Enhanced chemiluminescence reagent was from Amersham (Piscataway, NJ). Adriamycin, mitoxantrone, sulforhodamine B, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), anti-β-actin antibody, and horseradish peroxidase–conjugated and FITC-conjugated IgG molecules were from Sigma (St. Louis, MO). Anti-Cdc2 and p-Cdc2 (Tyr15) antibodies were from Cell Signaling (Danvers, MA), and antibodies against 14-3-3 and poly(ADP-ribose) polymerase (PARP) were from Neomarkers (Fremont, CA). All other chemicals were of molecular biology grade from either Fisher (Chicago, IL) or Sigma.

Cell Culture and Transient Small Interfering RNA Transfection

LNCaP, PC-3, CWR22RV, and DU145 cells were cultured in RPMI supplemented with 10% fetal bovine serum. RWPE-1 were grown in keratinocyte serum-free medium in RPMI supplemented with 10% fetal bovine serum and 0.05 mg/mL bovine pituitary growth factor and 0.05 mg/mL bovine pituitary. RWPE-1 were grown in keratinocyte serum-free medium in RPMI supplemented with 10% fetal bovine serum. Transfection

either Fisher (Chicago, IL) or Sigma. 

Role of 14-3-3 in Drug Resistance

The pSilencer-Scr and the control vector pSilencer-Scr were transfected into DU145 and CWR22RV cells, whereas pDNA3- HA-14-3-3o and pDNA3 vector were transfected into LNCaP cells using LipofectAMINE plus according to the vendor’s manual. At 24 hours following transfection, the cells were split at an appropriate dilution into medium supplemented with 1 mg/mL G418. Individual stable clones were selected and expanded for further analysis.

Western Blot Analysis and Immunofluorescence Imaging

Cell survival was determined using MTT (11) or sulforhodamine B (8) assays. For MTT assay, cells were seeded on 96-well plates at 10,000 per well for CWR22RVDerived, 6,000 per well for LNCaP-derived, or 3,000 per well for DU145-derived cells. The cells were treated next day with various concentrations of mitoxantrone or Adriamycin. At 48 or 72 hours after drug treatment, the culture medium was replaced with 100 μL fresh medium containing 0.5 mg/mL MTT followed by incubation in darkness at 37 °C for 4 hours and addition of 10% SDS containing 0.01 N HCl to dissolve the formazan product for overnight. A570 nm was then measured in a plate reader (MRX, Dynex Technologies, Chantilly, VA). For sulforhodamine B assay, the cells were stained with 0.4% (w/v) sulforhodamine B in 1% acetic acid for 30 minutes. Unbound sulforhodamine B was removed by washing in 1% acetic acid, and bound sulforhodamine B was solubilized with 10 mmol/L Tris. The sulforhodamine B concentration was measured at A570 nm in a plate reader. The data analysis was done using GraphPad PRISM3.02.

Cell Cycle and Apoptosis Analyses

Cell cycle analysis was done using propidium iodide staining analyzed with fluorescence-activated cell sorting. Briefly, the cells were trypsinized, washed in cold PBS, fixed in 4 mL cold 70% ethanol (−20°C), centrifuged, and resuspended in 0.5 mL PBS containing RNase A (50 μg/mL) and propidium iodide (100 μg/mL) before fluorescence-activated cell sorting analysis. Cell cycle profiles were determined using CELL Quest and ModFit programs.
Annexin V and propidium iodide double staining were used to quantify apoptosis. Cells were treated with 320 nmol/L mitoxantrone for 16 hours and then harvested for analysis using Annexin V-FITC apoptosis detection kit according to the vendor’s protocol and analyzed with fluorescence-activated cell sorting.

**Bisulfite Treatment of Genomic DNA and Methylation-Specific PCR**

Genomic DNA was isolated using QIAamp DNA Mini kit (Qiagen). For bisulfite treatment, 1 μg of genomic DNA was denatured in 0.2 mol/L NaOH for 10 minutes at 37°C in 50 μL. The denatured DNA was mixed with 30 μL of 10 mmol/L hydroquinone and 520 μL of 3.5 mol/L sodium bisulfite (pH 5) and incubated for 16 hours at 50°C followed by purification and further incubation in 0.3 mol/L NaOH for 5 minutes at room temperature. The treated DNA was precipitated and dissolved in TE for methylated DNA template.

Methylation-specific PCR analysis was done using a primer set that covers CG dinucleotide numbers 3, 4, 8, and 9 (see Fig. 1C and ref. 12). Methylation-specific PCR using these primers (for methylated DNA: M-forward, 5'-TGGTAGTTTTATGAAAGGCGTC-3' and M-reverse, 5'-CCCTCTAACCCACCACCAACG-3'; for unmethylated DNA: U-forward, 5'-TTATGAGGGTGCGTGGATTGT-3' and U-reverse, 5'-CAACCCCAACACCAACACATAT-3') were expected to yield 105- and 107-bp products, respectively, which were separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining.

**Real-time Reverse Transcription-PCR**

Total RNAs were isolated from cells using RNeasy mini kit (Qiagen), and real-time reverse transcription-PCR was performed as previously described (9). The PCR primers used for 14-3-3-α were 5'-TAGCGTGCTTCTTGTCCCAA-3' (forward) and 5'-ACCAGTGGTTAGTGGCCCTCA-3' (reverse) and for glyceraldehyde-3-phosphate dehydrogenase were 5'-GAAGGTGAAGGTCGGAGTC-3' (forward) and 5'-GAAGATGGTGATGGAGATTTCTC-3' (reverse). Real-time PCR was done using an ABI Prism® 7000 Sequence Detection System (Applied Biosystems). Homogeneity of products was confirmed by melt curve analysis. The threshold cycle (Ct) was defined as the PCR cycle number at which the reporter fluorescence crosses the threshold, reflecting a statistically significant point above the calculated baseline. The Ct of 14-3-3-α was determined and normalized against that of glyceraldehyde-3-phosphate dehydrogenase. The 14-3-3-α level in each cell line relative to that in LNCaP was calculated using the formula $2^{-\Delta\Delta Ct}$.

**Results**

**14-3-3-α Is Expressed in Both Normal Prostate Epithelial Cells and Hormone-Independent Prostate Cancer Cell Lines**

To examine the expression pattern of 14-3-3-α in androgen-dependent and androgen-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 14-3-3-α expression by Western blot. As shown in Fig. 1A, 14-3-3-α 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 14-3-3-α to a similar level as DU145 and PC-3 cells (data not shown, but see Fig. 6A).

We next determined the 14-3-3-α mRNA level in these cells using real-time reverse transcription-PCR. As shown

Figure 1. Analysis of 14-3-3-α expression in prostate cell lines. A, Western blot analysis. Cell lysates of LNCaP, RWPE-1, DU145, and PC3 were separated by SDS-PAGE followed by Western blot analysis of 14-3-3-α and β-actin. B, real-time reverse transcription-PCR analysis. Total RNAs from LNCaP, RWPE-1, DU145, and PC3 cells were used for real-time reverse transcription-PCR analysis as described in Materials and Methods. The 14-3-3-α level was normalized to that of LNCaP cells. C, schematic diagram illustrating primers for methylation-sensitive PCR analysis. The transcription (TC) and translation (TL) start sites were marked with arrows. M, methylation-specific amplicon. The position of CpG dinucleotides were boxed and numbered. D, methylation-sensitive PCR analysis. Methylation-sensitive PCR analysis was done as described in Materials and Methods using primers indicated in C and products were separated by agarose gel electrophoresis. U, unmethylated DNA template; M, methylated DNA template.
in Fig. 1B, the mRNA level in RWPE-1, DU145, and PC3 cells was 195-, 36-, and 24-fold higher than that in LNCaP cells, respectively. Based on both the Western and real-time reverse transcription-PCR results, we conclude that 14-3-3\(\alpha\) 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 14-3-3\(\alpha\) compared with normal tissues (13–16). However, 14-3-3\(\alpha\) 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 14-3-3\(\alpha\) expression in these prostate cell lines, we did methylation-specific PCR analysis of genomic DNAs 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 ref. 12). 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 14-3-3\(\alpha\) 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 14-3-3\(\alpha\) in DU145 and PC3 cells compared with normal cells.

14-3-3\(\alpha\) Expression Is Induced by DNA Damage and May Account for Mitoxantrone Resistance in Androgen-Independent Prostate Cancer Cells

Given that the androgen-independent cell lines DU145 and PC3 express significant amount of 14-3-3\(\alpha\), whereas the androgen-dependent cell line LNCaP does not, we hypothesized that the androgen-independent cells may be more resistant 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 between DU145 and LNCaP cells. As shown in Fig. 2A, the two cell lines displayed different mitoxantrone sensitivities. LNCaP cells were completely killed after mitoxantrone treatment (2 \(\mu\)mol/L, 48 hours), whereas DU145 cells largely survived the treatment. We next did a dose-response analysis of these cells using MTT assay. Again, LNCaP cells were more sensitive to growth inhibition induced by mitoxantrone compared with 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 PARP by caspases yields an 85-kDa fragment. As shown in Fig. 2C, the level of the full-length PARP decreased with an appearance of the 85-kDa product in LNCaP but not in DU145 cells following mitoxantrone treatment (1 \(\mu\)mol/L, 24 hours). Thus, we conclude that the androgen-independent DU145 cells, which express significant amount of 14-3-3\(\alpha\), are more resistant to mitoxantrone-induced apoptosis compared with the androgen-dependent LNCaP cells, which do not express 14-3-3\(\alpha\).

To explore the potential role of 14-3-3\(\alpha\) in mitoxantrone resistance in DU145 cells, we examined the expression level of 14-3-3\(\alpha\) before and after mitoxantrone treatment. As shown in Fig. 2D, the expression of 14-3-3\(\alpha\) was significantly increased at 7 hours after treatment with 0.1 \(\mu\)mol/L.
mitoxantrone. This finding suggests that 14-3-3-σ 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 14-3-3-σ by mitoxantrone in DU145 cells is through a p53-independent pathway because these cells do not express a functional wild-type p53 (17).

**Relationship between 14-3-3-σ Expression and Drug Resistance**

Because LNCaP cells express wild-type p53, whereas PC3 and DU145 cells have mutated p53, and the different mitoxantrone sensitivity may be due to the expression of different p53, we decided to determine the contribution of 14-3-3-σ to intrinsic drug resistance by manipulating its expression level using siRNA or overexpression strategies. We first designed four siRNAs to specifically suppress the 14-3-3-σ expression by targeting the coding region of 14-3-3-σ mRNA in DU145 cells. These siRNAs have no homology with other known genes in the Genbank. A siRNA with a scrambled sequence that has no homology to any known mammalian sequence was also synthesized and used as a negative control. After transient transfection of these siRNAs into 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 14-3-3-σ expression. In contrast, the level of a control protein (Chk1) was unaffected by any of the siRNAs tested.

To further analyze the role of 14-3-3-σ in mitoxantrone resistance, we constructed the expression plasmid pSilencer-α that can express the small hairpin RNA (shRNA) of 14-3-3-σ using the H1 promoter (Fig. 3B), which is expected to be processed into the corresponding siRNA (#365) inside cells. The plasmid pSilencer-SCR that expresses the scrambled siRNA was used as the negative control. We next established stable DU145 clones that express the shRNA of 14-3-3-σ and control shRNA with scrambled sequence. As shown in Fig. 3C, two stable DU145 clones transfected with shRNA of 14-3-3-σ (α1 and α2) have significant decreases in 14-3-3-σ expression compared with two control clones (Scr1 and Scr2; compare lanes 3-4 with lanes 1-2).

We next determined mitoxantrone sensitivity of these stable DU145 clones. The cells were first treated with 1 μmol/L mitoxantrone for 24 hours and then processed for Western blot analysis of PARP cleavage. As shown in Fig. 3D, both α1 and α2 clones showed significant levels of PARP cleavage to generate the 85-kDa fragment 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 α1 than the α2 clone (compare lane 6 with lane 8), likely because the α1 clone expressed a lower level of 14-3-3-σ than the α2 clone (compare lane 3 with lane 4 in Fig. 3C, also compare lane 5 with lane 7 in Fig. 3D). We also found that the 14-3-3-σ protein level increased at 24 hours after mitoxantrone treatment with these stable clones (Fig. 3D), consistent with the finding of transient transfection that 14-3-3-σ expression is up-regulated by mitoxantrone treatment (Fig. 2D).

To rule out the possibility that the shRNA-mediated mitoxantrone sensitization was due to the specific cell clones selected, we did an experiment on pooled cells transiently transfected with siRNA (#865). DU145 cells were first transiently transfected with the siRNA of 14-3-3-σ (#865) or siRNA with scrambled sequence (SCR). Twenty-four hours after transfection, the cells were treated with 1 μmol/L mitoxantrone and harvested and at 6 and 24 hours after drug treatment for cell lystate preparation and Western blot analysis of PARP.
transiently transfected with the siRNA (#365) generated the 85-kDa fragment with decreases in the level of the full-length PARP, whereas the cells treated with the negative scrambled siRNA control did not. Thus, reducing 14-3-3 expression under both stable and transient conditions sensitized the androgen-independent DU145 cells to mitoxantrone-induced apoptosis.

We next did a survival study of the DU145 cells with reduced expression of 14-3-3 using MTT assay. For this purpose, the $\sigma^1$ clone was used because it has a better suppressed expression of 14-3-3 than the $\sigma^1$ clone. The $\sigma^1$ cell displayed no difference in proliferation rate from the control Scr2 cell under normal growth conditions (data not shown). However, the $\sigma^1$ cell was much more sensitive to mitoxantrone treatment (Fig. 4A). Majority of the $\sigma^1$ cells were killed by mitoxantrone (2 $\mu$mol/L, 48 hours), whereas few control Scr2 cells were killed under the same condition (Fig. 4B). The PARP cleavage (Fig. 4C) and the death of $\sigma^1$ cells were observed as early as 5 hours following mitoxantrone treatment (data not shown). Similarly, the $\sigma^1$ cells were more sensitive to apoptosis induced by another anticancer drug Adriamycin (Fig. 4D). At 9 hours following Adriamycin treatment, approximately half of the PARP in $\sigma^1$ cells was cleaved, and no intact PARP was observed at 24 hours of treatment. In contrast, significant cleavage of PARP was not observed in the control Scr2 cells (Fig. 4C and D). Thus, 14-3-3 expression in DU145 cells clearly causes resistance to treatment by anticancer drugs mitoxantrone and Adriamycin.

To further confirm if the sensitized killing of $\sigma^1$ cells with depleted expression of 14-3-3 is due to facilitation of drug-induced apoptosis, we did costaining of mitoxantrone-treated cells with Annexin V and propidium iodide, which detect apoptosis and necrosis, respectively (18, 19). Within the apoptotic cell population, cells in the early stage of apoptosis were Annexin V positive and propidium iodide negative. The population of cells in the late stage of apoptosis were Annexin V positive and propidium iodide positive. As shown in Fig. 5, ~12.8% and 3.5% of $\sigma^1$ cells were in the process of early and late apoptosis, respectively, following mitoxantrone treatment (320 nmol/L, 16 hours). In contrast, the control Scr2 cells showed no significant difference in Annexin V and propidium iodide staining compared with the untreated cells.

To extend our observation with DU145 cells, we examined if reducing 14-3-3 expression in another androgen-independent cell line CWR22RV would sensitize the cells to drug treatment by establishing stable clones...
with severely reduced 14-3-3σ expression using shRNA (Fig. 6A). Survival study of these cells (Δ16) showed that reducing 14-3-3σ expression significantly sensitized CWR22RV cells to both mitoxantrone (~15-fold decrease in EC50) and Adriamycin (~3-fold decrease in EC50) compared with the control CWR22RV clone expressing shRNA of scrambled sequence (Scr1; Fig. 6B and C).

We next determined whether restoring 14-3-3σ expression in the androgen-dependent LNCaP cells could enhance drug resistance. For this purpose, we generated stable LNCaP clones that overexpress 14-3-3σ (σ+; see Fig. 6D). Survival study showed that enforced 14-3-3σ expression in LNCaP cells (σ+) significantly increased their resistance to mitoxantrone (~4-fold increase in EC50) and Adriamycin (~3-fold increase in EC50) compared with the control vector–transfected cells (Vec). Based on the above observations, we conclude that the expression of 14-3-3σ in prostate cancer cells causes drug resistance in all prostate cancer cell lines tested.

**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 (6). To determine whether the increased drug sensitivity in the DU145-derived α1− cell was due to the loss of G2-M check point, we analyzed cell cycle profiles for cells treated with 320 nmol/L mitoxantrone for 16 hours. As shown in Fig. 7A, significantly less α1− 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 (α1−) 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 α1− and Scr2 control cells were treated with 50 nmol/L mitoxantrone and harvested for cell cycle analysis at different time points. As shown in Fig. 7B, the control Scr2 cells were arrested at S and G2-M at 24 hours after mitoxantrone treatment. At 48 hours, majority of the control Scr2 cells were arrested at G2-M phase, and the G2-M arrest was maintained thereafter until 96 hours. Although there was significant G2-M arrest of α1− cells at 24 hours following mitoxantrone treatment, there was a significant increase in population in G1 and sub-G1 phases for α1− cells at later time points (48 and 96 hours). This observation suggests that some α1− cells were able to go through G2-M and reenter G1 phase. These results show that DU145 cells with reduced 14-3-3σ expression can not stably maintain G2-M arrest induced by mitoxantrone.

Entry of all eukaryotic cells into the 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 enters 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 did an indirect immunofluorescence staining of Cdc2 in DU145-derived α1− and control Scr2 cells following mitoxantrone treatment. As shown in Fig. 7C, Cdc2 accumulates in cytoplasm in both treated and untreated control Scr2 cells. In contrast, the α1− cells displayed a less clear cytoplasmic localization under normal growth conditions, and Cdc2 accumulates significantly in the nucleus when the α1− cells were treated with 50 nmol/L mitoxantrone for 72 hours. 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 did Western blot analysis to determine the level of phosphorylation of Cdc2 on Tyr15. Because 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 (20). As shown in Fig. 7D, 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 α1− cells slightly increased at 24 hours but significantly decreased at 48 hours following mitoxantrone treatment (Fig. 7D), suggesting that 14-3-3σ...

**Figure 6.** Effect of 14-3-3σ expression on drug resistance of CWR22RV and LNCaP cells. Stable clones derived from CWR22RV (A–C) and LNCaP (D–F) cells with reduced (ΔΔ) and increased (Δ+) expression of 14-3-3σ, respectively, and their corresponding control clones (Scr1 and Vec) were generated as described in Materials and Methods. Expression of 14-3-3σ in these clones were confirmed by Western blot analysis (A and D), and their survivals following treatment with mitoxantrone (B and E) and Adriamycin (C and F) were determined using sulforhodamine B assay as described in Materials and Methods. Representative of three independent experiments.
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.

Discussion

Hormone-refractory prostate cancers have long been considered a chemoresistant disease. In this study, we found that 14-3-3σ expression is higher in the androgen-independent prostate cancer cell lines DU145, PC3, and CWR22Rv compared with the androgen-dependent LNCaP cells. The higher level of 14-3-3σ seems 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 that 14-3-3σ may be developed as a target for sensitizing hormone-refractory prostate cancers to chemotherapy. 14-3-3σ was originally characterized as a human mammary epithelium-specific marker 1 primarily expressed in epithelial cells, and its expression increased during epithelial differentiation (5, 21). The expression of 14-3-3σ was found to be transcriptionally inactivated in several types of epithelial tumors by CpG island hypermethylation (22–24). However, it was recently reported that the lost 14-3-3σ expression in breast cancers was a sporadic event, and that 14-3-3σ expression was increased in some breast tumors (25). Similarly, 14-3-3σ expression has been reported to increase in pancreatic and colorectal cancers, and it is an independent prognosis factor for poor survival (26, 27).

Several recent reports also indicated a lost or reduced 14-3-3σ expression in prostatic intraepithelial neoplasia and invasive cancers (13–16). However, a significant fraction of prostate cancers (about 20% in one study) still displayed a positive 14-3-3σ staining possibly due to a heterogeneous and multifocal nature of metastatic prostate cancers (28). Furthermore, Cheng et al. found an increase in 14-3-3σ expression as prostate tumor progresses, and that islands of tumor cells with and without 14-3-3σ expression sometime coexisted in the same specimen (13). Hence, it is tempting to propose that cells retaining 14-3-3σ expression may be selected during disease progression and treatment. Indeed, we recently found that the expression of 14-3-3σ is elevated in drug-selected breast cancer cell lines.1 Previously, it has also been shown that withdrawal of estrogen increased 14-3-3σ expression in breast cancer cell line MCF7 by reduced Efp-mediated degradation (29). It is also possible that prostate tumor cells that initially lack 14-3-3σ expression may start expressing 14-3-3σ during tumor progression towards hormone-independent and advanced cancers. However, our examination of a hormone-independent C4-2 cells, derived from LNCaP (30), indicated that 14-3-3σ expression was not restored during establishment of androgen independence (data not shown). Taken together, we speculate that prostate cancers with advanced and metastatic nature and refractory to androgen ablation therapy express higher levels of 14-3-3σ and are resistant
to chemotherapy likely by selection. Our finding that reduction in 14-3-3σ expression significantly sensitizes the androgen-independent prostate cancer cells to anticancer drugs has a very profound implication to future treatment of advanced prostate cancers. 14-3-3σ may be developed as a future target to sensitize these prostate cancers to chemotherapy.

Previous studies have indicated that in p53-wild type HCT116 colorectal cancer cells, 14-3-3σ is critical to maintain G2-M checkpoint following DNA damage (6). 14-3-3σ expression was induced through a p53-dependent pathway, which led to a sustained sequestration of Cdc2/cyclin B complex in the cytoplasm following DNA damage. In this study, we showed that down-regulating 14-3-3σ expression with RNA interference in p53 mutant DU145 cells caused escape from stable G2-M checkpoint control after DNA damage. Similarly, depleting 14-3-3σ expression in p53-mutant PC3 cell line also abrogated G2-M arrest after DNA damage (14). Thus, 14-3-3σ is a critical regulator of G2-M checkpoint in both p53 wild type (HCT116) and mutant (DU145 and PC3) cancer cells, and it may be a good target for therapeutic development to abrogate G2-M checkpoint in prostate cancer cells.

In this study, we found that depleting 14-3-3σ expression inhibited the DNA-damage–induced cytoplasmic sequestration and Tyr15 phosphorylation of Cdc2 (Fig. 8). Intriguingly, 14-3-3σ depletion also led to Cdk2 activation in HCT116 cells upon DNA damage (31). Because 14-3-3σ interacts with and inhibits both Cdc2 and Cdk2 (7), depletion of 14-3-3σ may lead to activation of both kinases through a similar mechanism. The Tyr15 residue is the main target of Cdc2 in DNA damage–induced G2-M arrest (32). DNA damage–induced phosphorylation of Tyr15 on Cdc2 was thought to occur by the induced expression of Wee1, a protein kinase for Tyr15 of Cdc2 (33), and by 14-3-3σ-mediated relocation and cytoplasmic sequestration of Cdc25C, a phosphatase specific for Cdc2-Tyr15 (34). Because 14-3-3σ does not bind Cdc25C and does not heterodimerize with other 14-3-3 isoforms (35), 14-3-3σ deficiency is not expected to affect cellular localization of Cdc25C (6). Clearly, further studies are required to elucidate the mechanism underlying 14-3-3σ-mediated effect on Cdc2 phosphorylation.

We also observed a dramatic increase in DNA damage–induced apoptosis in 14-3-3σ-deficient DU145 cells. How 14-3-3σ prevents DNA damage–induced apoptosis in DU145 cells is currently unknown. Because Bax in DU145 cells is mutated, it is unlikely that 14-3-3σ prevents apoptosis through modulating Bax (36). However, it is possible that 14-3-3σ may suppress Bad-mediated apoptosis because DU145 cells contain high p-Bad under normal growth conditions (37). Bad is one of the BH3-only proapoptotic members of Bcl-2 family, and phosphorylation of Bad at Ser112, Ser136, and Ser155 has been shown to inactivate its proapoptotic function in a mechanism involving binding to 14-3-3 scaffold proteins that results in sequestering Bad from mitochondria and dissociation from Bcl-2 and/or Bcl-xL (5). Nevertheless, the binding of 14-3-3σ proteins with Bad could be impaired by phosphorylation on the Ser128 residue of Bad by Cdc2 (38). We found that depleting 14-3-3σ led to activation of Cdc2 upon DNA damage, which in turn may lead to phosphorylation of Bad at Ser128. Thus, it is possible that depleting 14-3-3σ sensitized DU145 cells to drug-induced apoptosis by failure to sequester Bad in cytoplasm due to phosphorylation of Bad at Ser128 in addition to reduced pool of 14-3-3 available for Bad binding (see Fig. 8). We are currently testing these hypotheses.

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References


Figure 8. Schematic model of DU145 cell responses to DNA damage mediated by 14-3-3σ. DNA damage induces 14-3-3σ expression in both p53 wild-type and mutant cells, which promotes cytoplasmic sequestration and Tyr15 phosphorylation of Cdc2, thereby inactivating the Cdc2 kinase activity and leading to G2-M arrest. The induced 14-3-3σ expression also binds to Bad and inactivates Bad-mediated apoptosis. Furthermore, inactivation of Cdc2 also prevents phosphorylation of Bad on Ser128, thus inactivating Bad-mediated apoptosis by an alternative mechanism.
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