14-3-3ζ, a novel androgen-responsive gene, is upregulated in prostate cancer and promotes prostate cancer cell proliferation and survival

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Running Title: Role of 14-3-3ζ in prostate cancer

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Abstract

Purpose: Androgen receptor (AR) is an essential transcriptional factor that contributes to the development and progression of prostate cancer. In this study, we investigated the androgen regulation and functional analysis of 14-3-3ζ in prostate cancer.

Experimental Design: Using chromatin immunoprecipitation (ChIP) combined with DNA microarray (ChIP-chip) analysis in LNCaP cells, we identified a functional AR-binding site in the downstream region of the 14-3-3ζ gene. Androgen regulation was examined by quantitative reverse transcription polymerase chain reaction and western blot analysis. Prostate cancer cells stably expressing 14-3-3ζ and small interfering RNA knock down were used for functional analyses. We further examined 14-3-3ζ expression in clinical samples of prostate cancer by immunohistochemistry and qRT-PCR.

Results: Androgen-dependent upregulation of 14-3-3ζ was validated at the mRNA and protein levels. The 14-3-3ζ gene is favorable for cancer-cell survival, as its ectopic expression in LNCaP cells contributes to cell proliferation and the acquired resistance to etoposide-induced apoptosis. 14-3-3ζ expression was associated with AR transcriptional activity and prostate specific antigen (PSA) mRNA expression. Immunoprecipitation indicated that 14-3-3ζ was associated with AR in the nucleus. Clinicopathological studies further support the relevance of 14-3-3ζ in prostate cancers, as its higher expression is associated with malignancy and lymph node metastasis.

Conclusions: 14-3-3ζ is a novel androgen-responsive gene that activates proliferation, cell survival, and AR transcriptional activity. 14-3-3ζ may facilitate the progression of prostate cancer.
Translational Relevance

Androgen receptor (AR) is an essential transcriptional factor that contributes to the development and progression of prostate cancer. Characterization of AR targets is the first step toward understanding the molecular mechanisms underlying primary and advanced prostate cancers. We have previously uncovered the AR transcriptional network in prostate cancer cells by ChIP-chip and cap analysis gene expression (CAGE) analysis. In the present study, we identified an AR-binding site in the downstream region of the 14-3-3ζ gene, and androgen treatment induced 14-3-3ζ expression in prostate cancer. Functional analysis indicated that 14-3-3ζ is associated with cell proliferation and survival in prostate cancer. We also found 14-3-3ζ enhances AR transcriptional activity by interacting with AR in the nucleus. Furthermore, 14-3-3ζ expression was upregulated in prostate cancer compared to the corresponding benign tissues. Taken together, 14-3-3ζ would have a significant role in the development of prostate cancer and could be a therapeutic target for advanced prostate cancer.
Introduction

Prostate cancer is one of the most common malignancies in developed countries, with high morbidity and mortality rates. The androgen receptor (AR) is known to play a role in the development and progression of prostate cancer [1,2]. The majority of primary prostate cancers are androgen-dependent, and therefore androgen-deprivation therapy (ADT) is a standard treatment [3]. Although ADT is initially effective for hormone-sensitive cancers, long-term treatment often results in the recurrence of castration-resistant prostate cancer. Tissue levels of testosterone and dihydrotestosterone (DHT) are reported to be sufficient for AR activation in recurrent prostate cancer during ADT, despite castrate levels of serum androgens [4, 5]. Thus, the identification and functional analysis of AR target genes should facilitate an understanding of the molecular mechanisms underlying the progression of advanced prostate cancer, as well as the development of primary prostate cancer.

Recent advances in high-throughput techniques have enabled the development of chromatin immunoprecipitation (ChIP) analysis combined with DNA microarray (ChIP-chip), which determines genome-wide transcription factor-binding sites [6, 7]. Using ChIP-chip analysis, we previously identified several new AR target genes, including $UGT1A$, $CDH2$ [8], and amyloid precursor protein ($APP$) [9]. In a subsequent study, we also reported 2872 androgen-dependent AR-binding sites (ARBSs), and in their vicinity a number of candidate AR target genes that are involved in cancer development, including $CAMKK2$ and $ARFGAP3$ [10]. In addition, using cap analysis gene expression (CAGE) analysis, which is a high-throughput sequencing technique to map the 5' termini of transcripts (CAGE tags) expressed in cells, we identified
androgen-regulated promoters in the whole genome of prostate cancer cells [10].

The 14-3-3 protein family is ubiquitously expressed and highly conserved in all eukaryotic organisms [11]. In humans, 7 different 14-3-3 isoforms have been identified [12]. Although 14-3-3 proteins do not function as enzymes, they function in the form of homo/heterodimers and bind to phosphorylated serine/threonine motifs on their target proteins [13]. Following binding, 14-3-3 can regulate target proteins by changing the conformation of the protein, affecting protein activity/stability, facilitating protein complex formation, or altering protein subcellular localization. 14-3-3 proteins can interact with hundreds of binding partners [14,15]. Through modulation of their binding partners, 14-3-3 proteins have been implicated in the regulation of diverse cellular processes, including apoptosis, mitogenic and stress signaling, cell-cycle progression, transcription, metabolism, and maintenance of cytoskeletal integrity [16, 17].

In terms of cancer biology, 14-3-3 proteins also contribute to the development and progression of malignant tumors. For example, 14-3-3σ is known to be a tumor suppressor in breast cancer, as its expression is frequently abolished by epigenetic and proteolytic regulation [18, 19]. We previously demonstrated that 14-3-3σ expression is downregulated in prostate cancer [20]. 14-3-3σ positively regulates p53 and sustains the G2/M checkpoint in epithelial cells following DNA damage [21,22]. Other 14-3-3 isoforms may have oncogenic roles. Some 14-3-3 isoforms have been shown to be involved in chemoresistance [23,24]. Although the upregulation of other 14-3-3 isoforms has also been implicated in certain cancer types, the clinical and biological significance of this remains unclear [17].

In the present study, we investigated the biological role of 14-3-3ζ (also designated as YWHAZ in RefSeq) in prostate cancer cells. Using ChIP-chip and CAGE analysis,
we identified an ARBS in the downstream region of the 14-3-3ζ gene and an androgen-regulated CAGE tag cluster (TC) around the 14-3-3ζ gene promoter. We confirmed that 14-3-3ζ expression was upregulated by androgen treatment in LNCaP cells. The 14-3-3ζ gene is favorable for prostate cancer cell survival, as its ectopic expression in LNCaP cells contributes to cell proliferation, motility, and acquired resistance to etoposide-induced apoptosis. Clinicopathological studies further support the relevance of 14-3-3ζ in prostate cancer, as its higher expression is associated with malignancy and lymph node metastasis. We propose that 14-3-3ζ is an androgen-regulated tumor-promoting factor in prostate cancer cells.
Materials and methods

Cell culture and transfection

Human prostate cancer LNCaP, 22Rv1, and VCaP cells were purchased from the American Type Culture Collection (Rockville, MD, USA). The cells were maintained as described [8-10]. Methyltrienolone 17β-hydroxy-17α-methyl-estra-4,9,11-trien-3-one (R1881; NEN Life Science Products, Boston, MA, USA) and dihydrotestosterone (DHT; Wako, Saitama, Japan) were used for androgen stimulation. Bicalutamide was purchased from Sigma (St Louis, MO). Transfection was performed using FuGENE6 (Roche Applied Science, Indianapolis, IN, USA), according to the manufacturer’s instructions. Small interfering RNA (siRNA) was transfected using siPORT NeoFX Transfection Agent (Applied Biosystems, Austin, TX, USA).

ChIP assay

ChIP was performed as described previously [8-10]. LNCaP cells were fixed with 1% formaldehyde for 5 min at room temperature. Lysates were rotated at 4°C overnight with anti-AR antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The primer sequences for 14-3-3ζ ARBS are shown below. The experiments were performed 3 times.

Forward: 5’-GAATCCCTCCAGCTGCTTTT-3’
Reverse: 5’-GCCACCACAGGGTGAAATA-3’
Western blot analysis

Western blot analysis was performed as described previously [8-10]. The membranes were incubated with 5 mL each of 1:1000-diluted anti-14-3-3ζ antibody (Santa Cruz Biotechnology), 1:1000-diluted anti-FLAG M2 antibody (Sigma), or 1:1000-diluted anti-β-actin antibody (Sigma) at room temperature for 1 h.

Quantitative reverse transcription polymerase chain reaction (qRT–PCR)

Total RNA was extracted using ISOGEN reagent (Nippon Gene, Tokyo, Japan). First-strand cDNA was generated using PrimeScript (Takara, Kyoto, Japan). Androgen responsiveness was analyzed by qRT-PCR as described previously [8-10]. The sequences of PCR primers are shown below.

GAPDH forward: 5’-GGTGGTCTCCTCTGACTTCAACA-3’
GAPDH reverse: 5’-GTGGTCGTTGAGGGCAATG-3’
14-3-3ζ forward: 5’-GGTTGCCGCTGGTGATG-3’
14-3-3ζ reverse: 5’-TCTTGGTATGCTTGTTGTGACTGA-3’
PSA forward: 5’-CAGGAACAAAAGCGTGATCTTG-3’
PSA reverse: 5’-GCTGTGGCTGACCTGAAATACC-3’

Cell proliferation assay

Cell growth rate was measured with an 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) proliferation assay. The Cell Titer 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) was performed according to the
manufacturer’s instructions. We seeded 4000 cells in 96-well plates and cultured them in RPMI supplemented with 10% FBS. Each time point was performed in quadruplicate, and each experiment was performed at least 3 times.

TUNEL assay

A TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) assay was performed using the DeadEnd Fluorometric TUNEL System (Promega), according to the manufacturer’s instructions. Cells were seeded in 24-well plates and cultured in RPMI supplemented with 10% FBS. The following day, etoposide (Sigma; 100 μM) was added to the culture medium. The assay was performed 24 h after the addition of etoposide. Fluorescein-12-dUTP-stained cells were counted in at least 4 fields at a magnification of 200× under a laser scanning microscope (VH-8000; Keyence, Osaka, Japan). The mean (SD) of ratio of fluorescein-12-dUTP-stained cells to total cells was calculated. Each experiment was performed at least 3 times.

siRNA transfection

A control siRNA (siRNA Control) and a specific siRNA targeting 14-3-3ζ were purchased from Applied Biosystems (Foster City, CA, USA) and transfected into LNCaP cells using siPORT NeoFX Transfection Agent (Applied Biosystems).

Immunoprecipitation

For immunoprecipitation with anti-FLAG M2 antibody, cell lysate (2 mg/mL) was mixed with anti-FLAG affinity gel (Sigma) and rotated for 4 h at 4°C. The affinity gel
was washed 4 times with Nonidet P-40 lysis buffer, and FLAG-tagged proteins were eluted with 0.3 M glycine HCl at pH 3.5. The elution was removed, neutralized with 2 M Tris-HCl (pH 8.8), boiled in sample buffer (0.1 M Tris-HCl, pH 6.8, 2% SDS, 0.1 M dithiothreitol, 10% glycerol, and 0.01% bromophenol blue) for 5 min, and analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE).

**Immunofluorescence staining**

Cells were grown on 12-mm circular coverslips (Fisher Scientific, Waltham, MA, USA) in 24-well plates. Living cells were washed 3 times with PBS, fixed with 4% paraformaldehyde/0.1 M phosphate buffer for 5 min at room temperature, washed once with PBS, and permeabilized with 0.2% Triton-X 100 in PBS for 10 min. After a further washing step with PBS and blocking in 3% bovine serum albumin (BSA)/TBST (100 mM Tris-HCl pH 8.0, 150 mM NaCl, and 0.05% Tween 20) for 30 min, the cells were incubated with rabbit anti-14-3-3ζ antibody (1:250; Santa Cruz Biotechnology) and mouse anti-AR antibody (1:250; Santa Cruz Biotechnology) in 3% BSA/TBST for 1 h at room temperature. The cells were then washed 3 times with PBS and incubated with anti-mouse IgG Alexa Fluor 546 (1:2000), and anti-rabbit IgG Alexa Fluor 488 (1:2000; Invitrogen, Carlsbad, CA) in 3% BSA/TBST for 1 h at room temperature. Nuclei were stained with DAPI (4’,6-diamidino-2-phenylindole). After washing the cells 3 times with PBS, coverslips were mounted in 1.25% DABCO, 50% PBS, and 50% glycerol, and the cells were visualized using a confocal laser scanning microscope (Olympus, Tokyo, Japan).
Luciferase assay

Luciferase reporter constructs containing the PSA promoter and enhancer regions were generated using the pGL3 basic plasmid (Promega). Luciferase assay was performed as described previously [8-10]. Data represent means (SD) from triplicate sets.

Cell-migration assay

Cell-migration assay was performed using a cell culture insert with an 8.0-μm-pore-size polyethylene terephthalate (PET) filter (Becton Dickinson, Franklin Lakes, NJ). RPMI 1640 medium (700 μL) containing 10% FBS was added to the lower chamber. Then, 5 × 10^4 cells were suspended in 300 μL of RPMI 1640 medium containing 10% FBS and added to the upper chamber. After incubation for 4 h at 37°C in a humidified atmosphere of 5% CO₂, the cells on the upper surface of the filter were completely removed by wiping with cotton swabs. The cells on the lower surface of the filter were fixed in methanol for 30 min, washed with PBS, and stained with Giemsa (Muto Pure Chemicals, Tokyo, Japan) for 30 s. After washing 3 times with PBS, the filters were mounted on a glass slide. The cells on the lower surface were counted in at least 4 fields under a microscope at a magnification of 200×. Student’s t-test was used to analyze the data from these experiments.

Patients and tissue samples

We obtained 90 prostate cancer samples from surgeries performed at the University of Tokyo Hospital (Tokyo, Japan). The study was approved by the ethics committee of Tokyo University, and informed consent was obtained from each patient before surgery.
The prostate tissue sections analyzed by immunohistochemical analysis contained 90 cancerous foci. Twenty benign tissues were also obtained from different patients who underwent radical prostatectomies and had no cancerous tissues. The age of those benign patients ranged from 57 to 79 years (mean, 67.8 ± 5.3 years), and pretreatment serum PSA levels ranged from 3.1 to 33 ng ml⁻¹ (mean, 10.7 ± 9.7 ng ml⁻¹). In addition, we collected both prostate cancer tissues and benign prostate tissues from 15 prostate cancer patients by laser capture microdissection (LCM) for qRT-PCR analysis. The clinical and histopathological characteristics of prostate cancer patients are presented in Supplementary Table 1.

Immunohistochemistry

Immunohistochemical analysis for 14-3-3ζ was performed using the streptavidin-biotin amplification method with an EnVision™ visualization kit (Dako, Carpinteria, CA, USA). Tissue sections (6 μm) were deparaffinized, rehydrated through graded ethanol, and rinsed in PBS. To retrieve the antigens, the sections were autoclaved at 121°C for 10 min in citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dehydrate, pH 6.0). The sections were treated with 0.3% hydrogen peroxide to block endogenous peroxidase, and incubated in 10% bovine serum for 30 min. The primary antibody, a polyclonal antibody for 14-3-3ζ (Santa Cruz Biotechnology) (1:1000 dilution), was applied and incubated overnight at 4°C. The sections were rinsed in PBS and incubated with EnVision⁺ and anti-rabbit polyclonal antibody for 1 h at room temperature. The antigen-antibody complex was visualized with 3,3′-diaminobenzidine solution (1 mM 3,3′-diaminobenzidine, 50 mM Tris-HCl
buffer [pH 7.6], and 0.006% hydrogen peroxide). Anti-rabbit IgG was used instead of the primary antibodies, as a negative control. Sections of healthy human livers (Dako) were immunoassayed as positive controls using the primary antibodies, as described above.

**Immunohistochemical assessment**

Slides were evaluated for the proportion (0, none; 1, <1/100; 2, 1/100–1/10; 3, 1/10–1/3; 4, 1/3–2/3; and 5, >2/3) and staining intensity (0, none; 1, weak; 2, moderate; and 3, strong) of positively stained cells. The total immunoreactivity (IR) score (0, 2–8) was determined as the sum of the proportion and intensity values. Two investigators (T.M. and T.F.) independently evaluated the tissue sections. If the IR score differed between the 2 investigators, a third investigator (S.T.) evaluated the tissue sections, and the average IR score was used. When the 2 investigators had difficulty in evaluating an IR score of heterogeneous cancerous lesions, the third investigator estimated and decided the IR score. We defined an IR score of 4 as the cut-off for low 14-3-3ζ IR when identifying a potential association between 14-3-3ζ expression in the malignant epithelium and the clinicopathological characteristics, because almost all benign foci showed IR scores of ≤4 for 14-3-3ζ.

**Statistical analysis**

Correlations between the IR score and the clinicopathological characteristics (benign or prostate cancer, pretreatment serum prostate specific antigen (PSA) level, Gleason score (GS), and lymph node metastasis) were evaluated using chi-square test...
(Supplementary table 2). The comparisons between OD, apoptosis, luciferase activity, and mRNA level of LNCaP clones were evaluated using Student’s t-test. The comparison of \(14\text{-}3\text{-}3\zeta\) mRNA levels between prostate cancer tissues and benign prostate tissues by qRT-PCR were evaluated using Wilcoxon test. Statistical assessment was performed using the Stat View-J 5.0 software (SAS Institute, Cary, NC, USA), with \(P < 0.05\) considered statistically significant.
Results

Androgen-responsive expression of 14-3-3ζ in prostate cancer cells

We previously reported an integrative analysis using ChIP-chip and CAGE to characterize ARBSs and androgen-regulated promoters in the whole genome of prostate cancer cells [10]. We identified one ARBS in the 3′ downstream region of 14-3-3ζ (Fig. 1A, upper panel) by ChIP-chip, although no AR binding could be observed with the other 14-3-3 isoforms. In addition, an androgen-regulated CAGE tag cluster (TC) was identified in the 14-3-3ζ promoter region. These results indicate that 14-3-3ζ may be regulated by androgen in LNCaP cells.

First, we validated DHT-dependent AR recruitment to the ARBS using ChIP assay (Fig. 1A, lower panel). Next, to investigate the effect of androgen on the regulation of 14-3-3ζ mRNA expression, we stimulated LNCaP cells with R1881 (10 nM) or control vehicle and isolated total RNA from the cells (Fig. 1B, upper panel). qRT-PCR analysis revealed that 14-3-3ζ mRNA levels were increased by androgen stimulation relative to control treatment. Western blot analysis also showed that 14-3-3ζ protein expression was upregulated by androgen stimulation (Fig. 1B, middle and lower panels). In addition, DHT treatment (10 nM) for 24 h also increased 14-3-3ζ expression in LNCaP cells (Fig. 1C). This DHT-induced upregulation was inhibited by treatment with bicalutamide, an AR inhibitor. We further analyzed the regulation of 14-3-3ζ by androgen in other AR-positive prostate cancer cell lines. DHT treatment induced 14-3-3ζ expression in 22Rv1 and VCaP cells (Fig. 1D). Thus, we inferred that 14-3-3ζ is an androgen-responsive gene regulated by AR binding.

14-3-3ζ promotes the proliferation, survival, and motility of LNCaP cells
To investigate the function of 14-3-3ζ in prostate cancer cells, we generated LNCaP clones stably expressing human FLAG-14-3-3ζ protein and LNCaP vector clones (LNCaP-vector clones #1 and #2). We selected LNCaP-FLAG-14-3-3ζ clones #1 and #2, which expressed the FLAG-14-3-3ζ protein as confirmed by western blotting using anti-FLAG M2 antibody (Fig. 2A). Proliferation of LNCaP cells was determined using an MTS assay. The cells were maintained in RPMI 1640 medium supplemented with 10% FBS. According to a previous report [25], the total testosterone is at the castrate level under this condition. LNCaP cells are considered to metabolize testosterone to produce a physiological (i.e., 10 nM) level of DHT [25]. The proliferation rate of LNCaP-FLAG-14-3-3ζ stable clones was significantly increased after 72-h and 96-h incubations, compared to that of the vector clones ($P < 0.05$; Fig. 2B). This indicates that stable expression of 14-3-3ζ increases the proliferation of cultured prostate cancer cells.

Next, we evaluated etoposide-induced apoptosis using MTS and TUNEL assays. Etoposide-induced inhibition of cell proliferation rate was reduced in 14-3-3ζ-overexpressing LNCaP cells at 24 h and 48 h after the addition of etoposide to the medium, compared to control cells (Fig. 2C). The TUNEL assay was performed 24 h after etoposide treatment. Apoptotic cells were stained with fluorescein-12-dUTP. The percentage of fluorescein-12-dUTP-stained cells was significantly lower in 14-3-3ζ-overexpressing LNCaP cells than in control cells (Fig. 2D). These results suggest that stable expression of 14-3-3ζ promotes LNCaP cell survival.

Furthermore, motility of LNCaP cells was determined using a cell migration assay. Cell migration was significantly enhanced in 14-3-3ζ-overexpressing LNCaP cells, compared with control vector cells ($P < 0.05$; Supplementary Fig. 1).
14-3-3ζ gene silencing reduces LNCaP cell growth and resistance to apoptosis

We confirmed that an siRNA targeting 14-3-3ζ reduced the level of endogenous 14-3-3ζ in LNCaP cells compared to that in control siRNA-transfected cells (Fig. 3A). We investigated the effect of 14-3-3ζ siRNA in LNCaP cells. MTS assay showed that LNCaP cell growth was reduced at day 4 after transfection with the 14-3-3ζ siRNA (Fig. 3B). To examine the effect of 14-3-3ζ gene silencing on etoposide-induced apoptosis, 14-3-3ζ siRNA- and control siRNA-transfected cells were treated with etoposide at 24 h post-transfection and TUNEL assay was performed 24 h after etoposide treatment. The percentage of fluorescein-12-dUTP-stained cells was significantly higher in 14-3-3ζ siRNA-transfected LNCaP cells than in control siRNA-transfected LNCaP cells (Fig. 3C). Thus, we confirmed that LNCaP cells became more sensitive to apoptosis following 14-3-3ζ suppression.

14-3-3ζ binds to AR in the nucleus and promotes PSA promoter activation

Next, a luciferase assay was performed to evaluate the effect of 14-3-3ζ on AR transcriptional activity. Control cells and LNCaP cells overexpressing FLAG-tagged 14-3-3ζ were transfected with the PSA-Luc vector after 72 h of hormone depletion. After 24 h, the cells were treated with R1881 (10 nM) or vehicle (0.1% ethanol) for 24 h. The PSA-Luc assay showed that the transcriptional activity of AR was higher in LNCaP cells overexpressing 14-3-3ζ than in control cells (Fig. 4A, left). LNCaP cells were co-transfected with the PSA-Luc vector and siRNA after 72 h of hormone depletion. After 24 h, they were treated with R1881 (10 nM) or vehicle (0.1% ethanol) for 24 h. 14-3-3ζ knockdown by siRNA repressed the transcriptional activity of AR (Fig. 4A,
right).

In addition, we evaluated PSA mRNA expression using qRT-PCR. LNCaP cells overexpressing 14-3-3ζ exhibited higher levels of PSA mRNA than control cells (Fig. 4B, left). By contrast, knockdown of 14-3-3ζ by siRNA decreased PSA expression in LNCaP cells (Fig. 4B, right). These results confirm that 14-3-3ζ promotes AR transcriptional activity of the PSA promoter.

To investigate the function of 14-3-3ζ in AR activation, we used an immunoprecipitation assay to examine the interaction between AR and 14-3-3ζ. Control cells and LNCaP cells overexpressing FLAG-tagged 14-3-3ζ were treated with R1881 (10 nM) or vehicle (0.1% ethanol) for 48 h after 72 h of hormone depletion, and total cellular proteins were extracted. Immunoprecipitation with anti-FLAG antibody was performed, and anti-AR antibody was used for immunoblotting. FLAG-tagged 14-3-3ζ associated with AR in the presence of androgen (Fig. 4C).

We further performed immunofluorescence staining of the cultured cells and examined the subcellular distribution of endogenous 14-3-3ζ and AR by confocal laser scanning microscopy. Cells were immunostained with 14-3-3ζ and AR antibodies in the presence of androgen (Fig. 4D). Although endogenous 14-3-3ζ was mainly localized in the cytoplasm of the LNCaP cells, we also observed 14-3-3ζ expression in the nucleus and colocalization of AR with 14-3-3ζ.

**14-3-3ζ is overexpressed in prostate cancer tissues**

We analyzed the immunoreactivity of 14-3-3ζ in formalin-fixed paraffin-embedded sections of benign prostate tissues and prostate cancer tissues obtained from 20 and 90
cases, respectively (Table 1 and Supplementary Table 1). Heavy and diffuse 14-3-3ζ immunoreactivity was observed in both the nucleus and cytoplasm of prostate cancer epithelial cells (Fig. 5A). However, 14-3-3ζ immunoreactivity was weak in the benign prostate tissue (Fig. 5B), and was observed only in the nucleus of benign prostatic epithelial cells. 14-3-3ζ IR scores were high (>5) in 50 of 90 prostate cancer cases (55.6%; Table 1). By contrast, only 10% of benign prostate tissue specimens exhibited strong expression of 14-3-3ζ protein (Table 1). Comparison of 14-3-3ζ IR scores within a single specimen revealed markedly higher scores in cancerous foci than in benign lesions ($P < 0.001$). We analyzed the association between 14-3-3ζ IR in prostate cancer and clinicopathological characteristics using chi-square test (Supplementary Table 2). 14-3-3ζ IR was significantly higher in cases positive for lymph node metastasis than in those negative for lymph node metastasis ($P = 0.03$).

Next, we analyzed 14-3-3ζ mRNA levels in frozen human prostate tissue specimens using qRT-PCR. We collected both prostate cancer tissues and benign prostate tissues from 15 other patients by using laser capture microdissection (LCM) (Supplementary Table 1). 14-3-3ζ mRNA levels were significantly higher in the prostate cancer tissue specimens than in the benign prostate tissue specimens ($P < 0.05$; Fig. 5C). Moreover, we also confirmed 14-3-3ζ overexpression in the Oncomine database of expression profiles of clinical cancer samples (Oncomine) [26]. In 4 analyses [27-30], significant overexpression of 14-3-3ζ was observed in cancer tissues compared to benign tissues (Fig. 5D and Supplementary table 3); by contrast, none of the analyses revealed underexpression in cancers relative to normal tissues. These results indicate that 14-3-3ζ expression is upregulated at the mRNA and protein levels in prostate cancer.
Discussion

Prostate cancer develops via androgen-mediated AR activation. Furthermore, it is reported that AR is activated or amplified, and plays an important role in castration-resistant prostate cancer [31]. In a previous study [10], we identified ARBSs in LNCaP cells and searched for new AR target genes. We identified one ARBS in the 3′ downstream region of 14-3-3ζ. We previously reported that 14-3-3σ is downregulated in prostate cancer [20]. Among the 14-3-3 genes, only 14-3-3ζ was shown to have an ARBS in the adjacent region by ChIP-chip analysis. 14-3-3σ expression is inhibited in LNCaP cells via promoter DNA methylation [20]. Moreover, in the promoter region of 14-3-3ζ, histone H3 acetylation, which is one of the markers of histone activation, was detected by our ChIP-chip analysis (Fig. 1A). Taken together, such epigenetic changes, in addition to the regulation by AR, may be associated with the 14-3-3 isoform differences in prostate cancer.

14-3-3 proteins regulate the activity of target proteins by specifying their location or by transporting them [32]. For example, under survival-signaling conditions, binding of 14-3-3 proteins inactivates numerous pro-apoptotic proteins, including BAD (Bcl-xL/Bcl-2-Associated Death Promoter), BAX (BCL2 Associated X-protein), and the FOXO (Forkhead box O) transcription factors, by sequestering them from their sites of action, such as the mitochondria and nucleus. Under stress-signaling conditions, activated JNK (c-JUN N-terminal kinase) disrupts the binding of 14-3-3 to several pro-apoptotic proteins by directly phosphorylating the 14-3-3 proteins themselves, thereby enabling the apoptosis regulators to localize to their sites of action [33-38].
Thus, deregulation or dysfunction of 14-3-3 proteins leads to the disruption of cellular processes, and is associated with the occurrence and development of cancer.

14-3-3σ is considered a tumor suppressor. 14-3-3σ has been identified as a normal mammary epithelial cell marker that is downregulated during neoplastic development [39]. A large number of primary breast cancers fail to express 14-3-3σ [40,41]. On the other hand, some studies have reported an oncogenic function for 14-3-3ζ. It was reported that 14-3-3σ and 14-3-3ζ play opposite roles in cell growth inhibition mediated by TGF-β1 [42]. Increased expression of 14-3-3ζ has been observed in several human tumors, including human hepatocellular carcinoma, stomach cancer, seminoma, squamous carcinoma, pancreatic adenocarcinoma, breast cancer, and several types of lung carcinoma [43-45]. In addition, the gene encoding 14-3-3ζ maps to a chromosome region (8q23) that is frequently amplified in metastatic cancer [46,47]. Moreover, downregulation of 14-3-3ζ suppresses anchorage-independent growth of lung cancer cells [48]. Downregulation of 14-3-3ζ also sensitizes cells to stress-induced apoptosis, and enforces cell-cell contact and expression of adhesion proteins [43].

In the present study, we showed that 14-3-3ζ expression was increased by androgen stimulation and this promoted the proliferation and motility of LNCaP cells. Expression of 14-3-3ζ also enhanced the survival of LNCaP cells. It has been shown that 14-3-3ζ interacts with the tumor suppressor tuberin via the Akt phosphorylation site [49], thereby inducing hyperactivation of the PI3-kinase/Akt pathway and downregulation of p53 [50]. An increase in 14-3-3ζ may promote p53 degradation via activation of the PI3-kinase/Akt pathway, and thus contribute to the occurrence of prostate cancer. Furthermore, 14-3-3ζ proteins may bind to and inactivate apoptosis-associated proteins.
T-cadherin, E-cadherin, and γ-catenin have been shown to be significantly higher in 14-3-3ζ-downregulated cells [43]. Thus, 14-3-3ζ overexpression may downregulate cell adhesion proteins in LNCaP cells, thereby promoting LNCaP motility.

Using co-immunoprecipitation, we demonstrated that 14-3-3ζ associated with AR in an androgen-dependent manner in LNCaP cells. Although Titus et al. (2009) reported that 14-3-3ζ did not bind to AR [51], their study examined a combination of 14-3-3ζ and exogenous AR in COS cells. It is speculated that cell-specific factors [52] may participate in the interaction between 14-3-3ζ and AR in prostate cancer cells. Furthermore, in the present study, immunofluorescence staining showed that 14-3-3ζ colocalized with AR in the presence of androgen. These results suggest that 14-3-3ζ associates with AR in the nucleus following the androgen-induced upregulation of 14-3-3ζ and translocation of AR.

The relationship between 14-3-3 proteins and AR or androgen-regulated genes has been previously investigated. Haendler et al. (2001) found that 14-3-3η increased AR transcriptional activity following androgen stimulation [53]. In the presence of androgen, 14-3-3η bound to AR and, in the recurrent human prostate cancer cell line CWR-R1, transcriptional activation of AR was stimulated by 14-3-3η at low DHT concentrations [51]. On the other hand, 14-3-3σ increases AR transcriptional activity in an androgen-independent manner [53,54,55]. In the present study, 14-3-3ζ promoted PSA promoter activation in an androgen-dependent manner. This suggests that 14-3-3ζ increases AR transcriptional activity by binding to AR in the nucleus. AR transcriptional activation may induce many types of AR target genes that promote the proliferation and survival of prostate cancer cells.
Using immunohistochemistry and qRT-PCR analysis, we evaluated 14-3-3ζ expression in human prostate tissue. The mRNA and protein levels of 14-3-3ζ were significantly higher in prostate cancer tissue than in normal prostate tissue. Immunohistochemical analysis revealed strong 14-3-3ζ immunoreactivity only in the nuclei of benign prostate tissues. This result suggests that 14-3-3ζ increases AR transcriptional activity by binding to AR in the nucleus of benign prostate epithelial cells. However, heavy and diffuse 14-3-3ζ immunoreactivity was observed in both the cytoplasm and nucleus of prostate cancer cells. 14-3-3ζ promotes cell proliferation and inhibits apoptosis by activating PI3-K in the cytoplasm [56]. It is speculated that the functions of 14-3-3ζ may be activated in the cytoplasm in prostate cancer. Moreover, AR transcriptional activity is increased in prostate cancer. Thus, the increase in nuclear localization of 14-3-3ζ may be related to the activation of AR transcriptional activity. In addition, array data from Oncomine indicated 14-3-3ζ overexpression in prostate cancers, which is consistent with our mRNA expression data. This indicates that 14-3-3ζ may play an important role in tumorigenesis. Interestingly, 14-3-3ζ expression was significantly higher in cases positive for lymph node metastasis than in those negative for lymph node metastasis. Thus, 14-3-3ζ may be an important clinical factor in tumorigenesis and prostate cancer development.

In summary, we used ChIP-chip analysis to identify 14-3-3ζ as a new androgen-responsive gene. Increased 14-3-3ζ expression promoted cell proliferation and motility. Moreover, 14-3-3ζ associated with AR in an androgen-dependent manner and promoted AR transcriptional activity. These data suggest that 14-3-3ζ plays an important role in the progression of prostate cancer. Further studies may facilitate the
development of 14-3-3ζ as a new diagnostic marker and/or a therapeutic target for prostate cancer.
Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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Figure Legends

Figure 1. Expression of 14-3-3ζ/YWHAZ is upregulated by androgen stimulation in LNCaP cells. (A) (Upper panel) Genomic view of the 14-3-3ζ/YWHAZ gene in the UCSC genome browser: Chromatin immunoprecipitation (ChIP) combined with DNA microarray (ChIP-chip) analysis identified an androgen receptor (AR)-binding site (ARBS) in the downstream region of 14-3-3ζ/YWHAZ. Location of the androgen-regulated cap analysis gene expression (CAGE) tag cluster (TC) is indicated by an arrow. (Lower panels) Validation of ligand-dependent AR recruitment to the 14-3-3ζ ARBS using ChIP assay. LNCaP (left) and VCaP (right) cells were treated with dihydrotestosterone (DHT) or vehicle for 24 h. (B) Induction of 14-3-3ζ by androgen treatment in LNCaP cells. (Upper panel) Quantitative reverse transcription polymerase chain reaction (qRT-PCR) shows androgen-dependent upregulation of 14-3-3ζ mRNA in LNCaP cells. LNCaP cells were treated with 10 nM R1881 or vehicle. 14-3-3ζ mRNA levels are plotted relative to that of the vehicle control. (Middle and lower panels) Androgen-mediated induction of 14-3-3ζ protein expression in LNCaP cells. Protein levels were analyzed by western blot analysis (middle panel). β-actin was used as a loading control. 14-3-3ζ protein levels were quantified by densitometry and normalized to β-actin levels (lower panel). (C) Bicalutamide inhibits DHT-mediated upregulation of 14-3-3ζ. LNCaP cells were treated with DHT (10 nM), DHT + bicalutamide (1 or 10 μM), or vehicle for 24 h. (D) Androgen-mediated induction of 14-3-3ζ in 22Rv1 (left) and VCaP (right) cells. Cells were treated with DHT (10 nM) or vehicle. VCaP cells were treated for 24 h.
Figure 2. The 14-3-3ζ gene promotes LNCaP cell proliferation and survival. (A) LNCaP cells were transfected with pcDNA3-FLAG-14-3-3ζ or control vector. Two clones of 14-3-3ζ-overexpressing LNCaP cells were selected and used in the following assays (upper panel). 14-3-3ζ protein levels were quantified by densitometry and normalized to β-actin levels (lower panel) (B) The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay shows that LNCaP cells overexpressing 14-3-3ζ exhibit a higher cell proliferation rate than control cells expressing the empty vector. Data represent optical density (OD) values at 490 nm in the MTS assay. *P < 0.05, compared to vector controls. (C) Etoposide-induced inhibition of cell proliferation rate is reduced in LNCaP cells overexpressing 14-3-3ζ. Data represent optical density (OD) values at 490 nm in the MTS assay. *P < 0.05, compared to vector controls. (D) Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay on LNCaP cells at 24 h after etoposide treatment (100 μM). (Left panel) Apoptotic cells were stained with fluorescein-12-dUTP. (Right panel) Percentage of TUNEL-stained cells. *P < 0.05, compared to vector controls. Scale bar: 20 μM.

Figure 3. Knockdown of 14-3-3ζ reduces the growth and apoptosis resistance of LNCaP cells. (A) Western blot analysis of 14-3-3ζ protein levels in LNCaP cells 48 h after transfection with control or 14-3-3ζ-specific small interfering RNA (siRNA). (B) Effect of 14-3-3ζ siRNA (5 nM) on LNCaP cell growth and etoposide resistance. Data represent optical density (OD) values at 490 nm in the MTS assay. **P < 0.01, *P < 0.05, compared to control siRNA. (C) TUNEL assay on LNCaP cells at 24 h after
etoposide treatment (100 μM). Apoptotic cells were stained with fluorescein-12-dUTP. Control or 14-3-3ζ-specific siRNA was transfected 24 h before etoposide treatment. The percentage of fluorescein-12-dUTP-stained cells in 4 representative fields imaged using a confocal laser scanning microscope is shown. *P < 0.05, compared to control siRNA.

Figure 4. The 14-3-3ζ gene binds to AR in the nucleus and activates the PSA promoter. (A) Luciferase assay shows that the androgen-dependent transcriptional activity of AR is regulated by 14-3-3ζ expression levels. (Left panel) Hormone-deprived control and 14-3-3ζ-overexpressing LNCaP cells were transfected with the PSA-luc vector (50 ng / well) for 24 h. Luciferase assay was performed 24 h after ligand treatment. *P < 0.05, compared to vector controls. (Right panel) LNCaP cells were co-transfected with the PSA-luc vector (150 ng / well) and siRNA. **P < 0.01, *P < 0.05, compared to control siRNA. (B) Expression of PSA mRNA is regulated by 14-3-3ζ expression levels. (Left panel) 14-3-3ζ-overexpressing LNCaP cells express higher levels of PSA mRNA than control cells. *P < 0.05, compared to vector controls. (Right panel) siRNA-mediated knockdown of 14-3-3ζ downregulates endogenous PSA mRNA in LNCaP cells. *P < 0.05, compared to vector controls. (C) Interaction of ectopic FLAG-tagged 14-3-3ζ with endogenous AR in LNCaP cells. Cells were cultured in hormone-depleted medium for 72 h and treated with R1881 (10 nM) or vehicle for 24 h. Whole cell extracts were immunoprecipitated with anti-FLAG antibody and subjected to immunoblotting with anti-AR antibody. (D) Immunocytochemical analysis showing the subcellular distribution of endogenous 14-3-3ζ and AR in LNCaP cells treated with 10 nM R1881 for 24 h. Cells were observed using a confocal laser scanning microscope.
Figure 5. The 14-3-3ζ gene is overexpressed in prostate cancer tissues.

(A) Representative image of 14-3-3ζ expression in prostate cancer tissue (IR score = 8). Scale bar: 100 µm (25 µm for inset). (B) Representative image of 14-3-3ζ expression in benign prostate tissue (IR score = 2). Scale bar: 100 µm (25 µm for inset). (C) 14-3-3ζ mRNA is upregulated in prostate cancer, compared to benign prostate tissue. Thirteen benign prostate tissue samples and 15 malignant prostate tissue samples were obtained by laser capture microdissection (LCM). Expression of 14-3-3ζ was evaluated by qRT-PCR. *P < 0.05, compared to benign prostate tissue. (D) A representative analysis of 14-3-3ζ expression in prostate cancer using the Oncomine database. Other results obtained from the Oncomine search are summarized in Supplementary Table 3.
Figure 1

[Image of a figure showing experimental results related to androgen-regulated CAGE TC, qRT-PCR analysis, and other related experiments.]
Figure 2
Figure 3
Figure 4
Figure 5
14-3-3ζ, a novel androgen-responsive gene, is upregulated in prostate cancer and promotes prostate cancer cell proliferation and survival

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