14-3-3ζ, a Novel Androgen-Responsive Gene, Is Upregulated in Prostate Cancer and Promotes Prostate Cancer Cell Proliferation and Survival

Taro Murata1,2, Ken-ichi Takayama1,3,5, Tomohiko Urano1,3,5, Tetsuya Fujimura2, Daisaku Ashikari1,4, Daisuke Obinata1,4, Kuniko Horie-Inoue5, Satoru Takahashi4, Yasuyoshi Ouchi3, Yukio Homma2, and Satoshi Inoue1,3,5

Abstract

**Purpose:** Androgen receptor 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 androgen receptor–binding site in the downstream region of the 14-3-3ζ gene. Androgen regulation was examined by quantitative reverse transcription PCR and Western blot analysis. Prostate cancer cells stably expressing 14-3-3ζ and siRNA knockdown were used for functional analyses. We further examined 14-3-3ζ expression in clinical samples of prostate cancer by immunohistochemistry and quantitative reverse transcription 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 androgen receptor transcriptional activity and prostate-specific antigen (PSA) mRNA expression. Immunoprecipitation indicated that 14-3-3ζ was associated with androgen receptor in the nucleus. Clinicopathologic 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 androgen receptor transcriptional activity. 14-3-3ζ may facilitate the progression of prostate cancer.

Clin Cancer Res; 18(20); 5617–27. ©2012 AACR.

Introduction

Prostate cancer is one of the most common malignancies in developed countries, with high morbidity and mortality rates. The androgen receptor 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 androgen receptor activation in recurrent prostate cancer during ADT, despite castrate levels of serum androgens (4, 5). Thus, the identification and functional analysis of androgen receptor 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 androgen receptor target genes, including UGT1A1, CDH2 (8), and amyloid precursor protein (APP; ref. 9). In a subsequent study, we also reported 2,872 androgen-dependent androgen receptor–binding sites (ARBS), and in their vicinity, a number of candidate...
Translational Relevance
Androgen receptor is an essential transcriptional factor that contributes to the development and progression of prostate cancer. Characterization of androgen receptor targets is the first step toward understanding the molecular mechanisms underlying primary and advanced prostate cancers. We have previously uncovered the androgen receptor transcriptional network in prostate cancer cells by chromatin immunoprecipitation (ChIP) combined with DNA microarray (ChIP-chip) and cap analysis gene expression (CAGE) analysis. In the present study, we identified an androgen receptor–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 that 14-3-3ζ enhances androgen receptor transcriptional activity by interacting with androgen receptor in the nucleus. Furthermore, 14-3-3ζ expression was upregulated in prostate cancer compared with 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.

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, 14-3-3ζ 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 showed that 14-3-3ζ expression is downregulated in prostate cancer (20). 14-3-3ζ 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 biologic significance of this remains unclear (17).

In the present study, we investigated the biologic 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. Clinicopathologic 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. The cells were maintained as described (8–10). Methyltrienolone 17β-hydroxy-17α-methyl-4,9,11-trien-3-one (R1881; NEN Life Science Products) and DHT (Wako) were used for androgen stimulation. Bicalutamide was purchased from Sigma. Transfection was conducted using FuGENE6 (Roche Applied Science), according to the manufacturer’s instructions. siRNA was transfected using siPORT NeoFX Transfection Agent (Applied Biosystems).

ChIP assay
ChIP was conducted as described previously (8–10). LNCaP cells were fixed with 1% formaldehyde for 5 minutes at room temperature. Lysates were rotated at 4°C overnight with anti-androgen receptor antibody (Santa Cruz Biotechnology), 1:1,000-diluted anti-FLAG M2 antibody (Sigma), or 1:1,000-diluted anti-α-methyl-estra-4,9,11-trien-3-one antibody (Sigma) at room temperature for 1 hour.

Western blot analysis
Western blot analysis was conducted as described previously (8–10). The membranes were incubated with 5 mL each of 1:1,000-diluted anti-14-3-3ζ antibody (Santa Cruz Biotechnology), 1:1,000-diluted anti-FLAG M2 antibody (Sigma), or 1:1,000-diluted anti-α-methyl-estra-4,9,11-trien-3-one antibody (Sigma) at room temperature for 1 hour.

Translational Relevance
Androgen receptor is an essential transcriptional factor that contributes to the development and progression of prostate cancer. Characterization of androgen receptor targets is the first step toward understanding the molecular mechanisms underlying primary and advanced prostate cancers. We have previously uncovered the androgen receptor transcriptional network in prostate cancer cells by chromatin immunoprecipitation (ChIP) combined with DNA microarray (ChIP-chip) and cap analysis gene expression (CAGE) analysis. In the present study, we identified an androgen receptor–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 that 14-3-3ζ enhances androgen receptor transcriptional activity by interacting with androgen receptor in the nucleus. Furthermore, 14-3-3ζ expression was upregulated in prostate cancer compared with 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.

Materials and Methods
Cell culture and transfection
Human prostate cancer LNCaP, 22Rv1, and VCaP cells were purchased from the American Type Culture Collection. The cells were maintained as described (8–10). Methyltrienolone 17β-hydroxy-17α-methyl-4,9,11-trien-3-one (R1881; NEN Life Science Products) and DHT (Wako) were used for androgen stimulation. Bicalutamide was purchased from Sigma. Transfection was conducted using FuGENE6 (Roche Applied Science), according to the manufacturer’s instructions. siRNA was transfected using siPORT NeoFX Transfection Agent (Applied Biosystems).

ChIP assay
ChIP was conducted as described previously (8–10). LNCaP cells were fixed with 1% formaldehyde for 5 minutes at room temperature. Lysates were rotated at 4°C overnight with anti-androgen receptor antibody (Santa Cruz Biotechnology). The primer sequences for 14-3-3ζ were purchased from the American Type Culture Collection. The cells were maintained as described (8–10). Methyltrienolone 17β-hydroxy-17α-methyl-4,9,11-trien-3-one (R1881; NEN Life Science Products) and DHT (Wako) were used for androgen stimulation. Bicalutamide was purchased from Sigma. Transfection was conducted using FuGENE6 (Roche Applied Science), according to the manufacturer’s instructions. siRNA was transfected using siPORT NeoFX Transfection Agent (Applied Biosystems).

ChIP analysis
ChIP was conducted as described previously (8–10). LNCaP cells were fixed with 1% formaldehyde for 5 minutes at room temperature. Lysates were rotated at 4°C overnight with anti-androgen receptor antibody (Santa Cruz Biotechnology). The primer sequences for 14-3-3ζ were purchased from the American Type Culture Collection. The cells were maintained as described (8–10). Methyltrienolone 17β-hydroxy-17α-methyl-4,9,11-trien-3-one (R1881; NEN Life Science Products) and DHT (Wako) were used for androgen stimulation. Bicalutamide was purchased from Sigma. Transfection was conducted using FuGENE6 (Roche Applied Science), according to the manufacturer’s instructions. siRNA was transfected using siPORT NeoFX Transfection Agent (Applied Biosystems).

Western blot analysis
Western blot analysis was conducted as described previously (8–10). The membranes were incubated with 5 mL each of 1:1,000-diluted anti-14-3-3ζ antibody (Santa Cruz Biotechnology), 1:1,000-diluted anti-FLAG M2 antibody (Sigma), or 1:1,000-diluted anti-α-methyl-estra-4,9,11-trien-3-one antibody (Sigma) at room temperature for 1 hour.
Quantitative reverse transcription PCR

Total RNA was extracted using ISOGEN reagent (Nippon Gene). First-strand cDNA was generated using PrimeScript (Takara). Androgen responsiveness was analyzed by quantitative reverse transcription PCR (qRT-PCR) as described previously (8–10). The sequences of PCR primers are shown below:

GAPDH forward: 5'-GGTGGTCTCCTCAGACTCACA-3'
GAPDH reverse: 5'-GTGTCGGTGGAGGCAATG-3'
14-3-3ζ forward: 5'-GGTGGCCGCTGATG-3'
14-3-3ζ reverse: 5'-TCTGGTTATGCTTGACTG-3'
Prostate-specific antigen (PSA) forward: 5'-CAGGAACAAGGCGTAGCTC-3'
PSA reverse: 5'-GCTGTGCGTACCTGAATTACC-3'

Cell proliferation assay

Cell growth rate was measured with an MTS proliferation assay. The Cell Titer 96 AQueous One Solution Cell Proliferation Assay (Promega) was conducted according to the manufacturer's instructions. We seeded 4,000 cells in 96-well plates and cultured them in RPMI supplemented with 10% FBS. Each time point was conducted in quadruplicate, and each experiment was carried out at least 3 times.

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay

A terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay was conducted 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 μmol/L) was added to the culture medium. The assay was conducted 24 hours 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 (Olympus). The mean (SD) of ratio of fluorescein-12-dUTP–stained cells to total cells was calculated. Each experiment was carried out at least 3 times.

siRNA transfection

A control siRNA (siRNA control) and a specific siRNA targeting 14-3-3ζ were purchased from Applied Biosystems 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 hours 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 mol/L glycine HCl at pH 3.5. The elution was removed, neutralized with 2 mol/L Tris-HCl, pH 8.8, boiled in sample buffer (0.1 mol/L Tris-HCl, pH 6.8, 2% SDS, 0.1 mol/L dithiothreitol, 10% glycerol, and 0.01% bromophenol blue) for 5 minutes, and analyzed by SDS-PAGE.

Immunofluorescent staining

Cells were grown on 12-mm circular coverslips (Fisher Scientific) in 24-well plates. Living cells were washed 3 times with PBS, fixed with 4% paraformaldehyde/0.1 mol/L phosphate buffer for 5 minutes at room temperature, washed once with PBS, and permeabilized with 0.2% Triton-X 100 in PBS for 10 minutes. After a further washing step with PBS and blocking in 3% bovine serum albumin (BSA)/TBST (100 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, and 0.05% Tween 20) for 30 minutes, the cells were incubated with rabbit anti-14-3-3ζ antibody (1:250; Santa Cruz Biotechnology) and mouse anti-androgen receptor antibody (1:250; Santa Cruz Biotechnology) in 3% BSA/TBST for 1 hour at room temperature. The cells were then washed 3 times with PBS and incubated with anti-mouse IgG Alexa Fluor 546 (1:2,000) and anti-rabbit IgG Alexa Fluor 488 (1:2,000; Invitrogen) in 3% BSA/TBST for 1 hour at room temperature. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). 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).

Luciferase assay

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

Cell migration assay

Cell migration assay was conducted using a cell culture insert with an 8.0-μm-pore size polycarbonate terephthalate (PET) filter (Becton Dickinson). RPMI-1640 medium (700 μL) containing 10% FBS was added to the lower chamber. Then, 5 × 10⁴ cells were suspended in 300 μL of RPMI-1640 medium containing 10% FBS and added to the upper chamber. After incubation for 4 hour 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 minutes, washed with PBS, and stained with Giemsa (Muto Pure Chemicals) for 30 seconds. 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. The Student t test was used to analyze the data from these experiments.

Patients and tissue samples

We obtained 90 prostate cancer samples from surgeries conducted 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 (mean, 10.7 ± 9.7 ng/mL). In addition, we collected both prostate cancer tissues and benign prostate tissues from 15 patients with prostate cancer by laser capture microdissection (LCM) for qRT-PCR analysis. The clinical and histopathologic characteristics of patients with prostate cancer are presented in Supplementary Table S1.

Immunohistochemistry

Immunohistochemical analysis for 14-3-3ζ was conducted using the streptavidin–biotin amplification method with an EnVision visualization kit (Dako). Tissue sections (6 μm) were deparaffinized, rehydrated throughgraded ethanol, and rinsed in PBS. To retrieve the antigens, the sections were autoclaved at 121°C for 10 minutes in citric acid buffer (2 mmol/L citric acid and 9 mmol/L 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 minutes. The primary antibody, a polyclonal antibody for 14-3-3ζ (Santa Cruz Biotechnology; 1:1,000 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 hour at room temperature. The antigen–antibody complex was visualized with 3,3′-diaminobenzidine (DAB) solution (1 mmol/L DAB, 50 mmol/L 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. Murata and T. Fujimura) independently evaluated the tissue sections. If the IR score differed between the 2 investigators, a third investigator (S. Takahashi) 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 cutoff point for low 14-3-3ζ IR when identifying a potential association between 14-3-3ζ expression in the malignant epithelium and the clinicopathologic characteristics because almost all benign foci showed IR scores of ≤4 for 14-3-3ζ.

Statistical analysis

Correlations between the IR score and the clinicopathologic characteristics (benign or prostate cancer, pretreatment serum PSA level, Gleason score, and lymph node metastasis) were evaluated using χ² test (Supplementary Table S2). The comparisons between OD, apoptosis, luciferase activity, and mRNA level of LNCaP clones were evaluated using the Student t test. The comparison of 14-3-3ζ mRNA levels between prostate cancer tissues and benign prostate tissues by qRT-PCR were evaluated using Wilcoxon test. Statistical assessment was conducted using the Stat View-J 5.0 software (SAS Institute), 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, top) by ChIP-chip, although no androgen receptor binding could be observed with the other 14-3-3ζ 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 androgen receptor recruitment to the ARBS using ChIP assay (Fig. 1A, bottom). Next, to investigate the effect of androgen on the regulation of 14-3-3ζ mRNA expression, we stimulated LNCaP cells with R1881 (10 nmol/L) or control vehicle and isolated total RNA from the cells (Fig. 1B, top). 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 bottom). In addition, DHT treatment (10 nmol/L) for 24 hours also increased 14-3-3ζ expression in LNCaP cells (Fig. 1C). This DHT-induced upregulation was inhibited by treatment with bicalutamide, an androgen receptor inhibitor. We further analyzed the regulation of 14-3-3ζ by androgen in other androgen receptor–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 androgen receptor 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 nmol/L) level of DHT (25). The proliferation rate of LNCaP-FLAG-14-3-3z stable clones was significantly increased after 72- and 96-hour incubations, compared with that of the vector clones (P < 0.05; Fig. 2B). This indicates that stable expression of 14-3-3z 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 14-3-3z-overexpressing LNCaP cells at 24 and 48 hours after the addition of etoposide to the medium, compared with control cells (Fig. 2C). The TUNEL assay was conducted 24 hours after etoposide treatment. Apoptotic cells were stained with fluorescein-12-dUTP. The percentage of fluorescein-12-dUTP–stained cells was significantly lower in 14-3-3z-overexpressing LNCaP cells than in control cells (Fig. 2D). These results suggest that stable expression of 14-3-3z promotes LNCaP cell survival.

Furthermore, motility of LNCaP cells was determined using a cell migration assay. Cell migration was significantly enhanced in 14-3-3z-overexpressing LNCaP cells, compared with control vector cells (P < 0.05; Supplementary Fig. S1).
**14-3-3ξ gene silencing reduces LNCaP cell growth and resistance to apoptosis**

We confirmed that an siRNA targeting 14-3-3ξ reduced the level of endogenous 14-3-3ξ in LNCaP cells compared with that in control siRNA–transfected cells (Fig. 3A). We investigated the effect of 14-3-3ξ siRNA in LNCaP cells. MTS assay showed that LNCaP cell growth was reduced at day 4 after transfection with the 14-3-3ξ siRNA (Fig. 3B). To examine the effect of 14-3-3ξ gene silencing on etoposide-induced apoptosis, 14-3-3ξ siRNA– and control siRNA–transfected cells were treated with etoposide at 24 hours posttransfection and TUNEL assay was conducted 24 hours after etoposide treatment. The percentage of fluorescein-12-dUTP–stained cells was significantly higher in 14-3-3ξ 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 androgen receptor in the nucleus and promotes PSA promoter activation

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

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

We further conducted immunofluorescent staining of the cultured cells and examined the subcellular distribution of endogenous 14-3-3ζ and androgen receptor by confocal laser scanning microscopy. Cells were immunostained with 14-3-3ζ and androgen receptor 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 androgen receptor 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 S1). 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 prostate epithelial cells. 14-3-3ζ IR scores were high (>5) in 50 of 90 prostate cancer cases (55.6%; Table 1). In 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 clinicopathologic characteristics using χ² test (Supplementary Table S2). 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 LCM (Supplementary Table S1). 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; ref. 26). In 4 analyses (27–30), significant overexpression of...
14-3-3ζ was observed in cancer tissues compared with benign tissues (Fig. 5D and Supplementary Table S3); in 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 androgen receptor activation. Furthermore, it is reported that androgen receptor 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 androgen receptor target genes. We identified one ARBS in the 3' downstream region of 14-3-3ζ. We previously reported that 14-3-3ζ 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. 14-3-3ζ 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 androgen receptor, may be associated with the 14-3-3ζ isoform differences in prostate cancer.

14-3-3ζ 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, binding of 14-3-3ζ to c-jun-NH2-kinase (JNKζ) disrupts the binding of 14-3-3ζ to several pro-apoptotic proteins by directly phosphorylating the 14-3-3ζ proteins themselves, thereby enabling the apoptosis regulators to localize to their sites of action (33–38). Thus, deregulation or dysfunction of 14-3-3ζ proteins leads to the disruption of cellular processes and is associated with the occurrence and development of cancer.

Table 1. Immunoreactivity of 14-3-3ζ protein in human prostate specimens

<table>
<thead>
<tr>
<th>Prostate status</th>
<th>Benign</th>
<th>Prostate cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Immunoreactivity</strong></td>
<td>Number of cases</td>
<td>Number of cases</td>
</tr>
<tr>
<td>Weak (score ≤ 4)</td>
<td>18</td>
<td>40</td>
</tr>
<tr>
<td>Strong (score ≥ 5)</td>
<td>2</td>
<td>50</td>
</tr>
<tr>
<td>P &lt; 0.001</td>
<td>20</td>
<td>90</td>
</tr>
</tbody>
</table>

The slides were evaluated for the proportion (0, none; 1, <1/100; 2, 1/100 to 1/10; 3, 1/10 to 1/3; 4, 1/3 to 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. Statistical significance was determined by chi-square test.

Figure 4. The 14-3-3ζ gene binds to androgen receptor in the nucleus and activates the PSA promoter. A, luciferase assay shows that the androgen-dependent transcriptional activity of androgen receptor is regulated by 14-3-3ζ expression levels. Left, hormone-deprived control and 14-3-3ζ-overexpressing LNCaP cells were transfected with the PSA-luc vector (100 ng/well) for 24 hours. Luciferase assay was conducted 24 hours after ligand treatment. *P < 0.05, compared with vector controls. Right, LNCaP cells were cotransfected with the PSA-luc vector (150 ng/well) and siRNA. **, P < 0.01, compared with control siRNA. B, expression of PSA mRNA is regulated by 14-3-3ζ expression levels. Left, 14-3-3ζ-overexpressing LNCaP cells express higher levels of PSA mRNA than control cells. *, P < 0.05, compared with vector controls. Right, siRNA-mediated knockdown of 14-3-3ζ downregulates endogenous PSA mRNA in LNCaP cells. **, P < 0.05, compared with vector controls. C, interaction of ectopic FLAG-tagged 14-3-3ζ with endogenous androgen receptor in LNCaP cells. Cells were cultured in hormone-depleted medium for 72 hours and treated with R1881 (10 nmol/L) or vehicle for 24 hours. Whole-cell extracts were immunoprecipitated (IP) with anti-FLAG antibody and subjected to immunoblotting with anti-androgen receptor antibody. D, immunocytochemical analysis showing the subcellular distribution of endogenous 14-3-3ζ and androgen receptor in LNCaP cells treated with 10 nmol/L R1881 for 24 hours. Cells were observed using a confocal laser scanning microscope. Scale bar, 10 μm. IB, immunoblotting.
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 14-3-3σ. 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 enhances 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 phosphoinositide 3-kinase (PI3K)/Akt pathway and downregulation of p53 (50). An increase in 14-3-3ζ may promote p53 degradation via activation of the PI3K/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 coimmunoprecipitation, we showed that 14-3-3ζ associated with androgen receptor in an androgen-dependent manner in LNCaP cells. Although Titus and colleagues reported that 14-3-3ζ did not bind to androgen receptor (51), their study examined a combination of 14-3-3ζ and exogenous androgen receptor in COS cells. It is speculated that cell-specific factors (52) may participate in the interaction between 14-3-3ζ and androgen receptor in prostate cancer cells. Furthermore, in the present study, immunofluorescent staining showed that 14-3-3ζ colocalized with androgen receptor in the presence of androgen. These results suggest that 14-3-3ζ associates with androgen receptor or androgen-regulated genes has been previously investigated. Haendler and colleagues found that 14-3-3ζ increased androgen receptor transcriptional activity following androgen stimulation (53). In the presence of androgen, 14-3-3ζ bound to androgen receptor and, in the recurrent human prostate cancer cell line CWR-R1, transcriptional activation of androgen receptor was stimulated by 14-3-3ζ at low DHT concentrations (51). On the other hand, 14-3-3ζ increases androgen receptor transcriptional activity in an androgen-independent manner (53–55). In the present study, 14-3-3ζ promoted PSA promoter activation in an androgen-dependent manner. This suggests that 14-3-3ζ increases androgen receptor transcriptional activity by binding to androgen receptor in the nucleus. Androgen receptor transcriptional activation may induce many types of androgen receptor target genes that promote the proliferation and survival of prostate cancer cells.

Using immunohistochemical 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 androgen receptor transcriptional activity by binding to androgen receptor 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

---

**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 with benign prostate tissue. Thirteen benign prostate tissue samples and 15 malignant prostate tissue samples were obtained by LCM. Expression of 14-3-3ζ was evaluated by qRT-PCR. D, 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 S3.
inhibits apoptosis by activating PI3K in the cytoplasm (56). It is speculated that the functions of 14-3-3ζ may be activated in the cytoplasm in prostate cancer. Moreover, androgen receptor transcriptional activity is increased in prostate cancer. Thus, the increase in nuclear localization of 14-3-3ζ may be related to the activation of androgen receptor transcriptional activity. In addition, array data from Onco-mine 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 was associated with androgen receptor in an androgen-dependent manner and promoted androgen receptor 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.

Disclosures of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References

Grant Support
This work was supported by Grants of the Cell Innovation Program and P-DIRECT (S. Inoue) from the MEXT, Japan; by Grants (S. Inoue, K. Takayama, T. Urano, and S. Takahashi) from the JSPS, Japan; by Grants-in-Aid (S. Inoue) from the MEXT, Japan; and by the Program for Promotion of Fundamental Studies in Health Sciences (S. Inoue). NIBIO, Japan.


14-3-3ζ, a Novel Androgen-Responsive Gene, Is Upregulated in Prostate Cancer and Promotes Prostate Cancer Cell Proliferation and Survival

Taro Murata, Ken-ichi Takayama, Tomohiko Urano, et al.

*Clin Cancer Res* 2012;18:5617-5627. Published OnlineFirst August 17, 2012.

Updated version

Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-12-0281

Supplementary Material

Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2012/08/17/1078-0432.CCR-12-0281.DC1

Cited articles

This article cites 56 articles, 21 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/18/20/5617.full.html#ref-list-1

Citing articles

This article has been cited by 1 HighWire-hosted articles. Access the articles at:
/content/18/20/5617.full.html#related-urls

E-mail alerts

Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.