Y-box binding protein-1 promotes castration-resistant prostate cancer growth via androgen receptor expression

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Abstract

The androgen receptor (AR) is well known to play a central role in the pathogenesis of prostate cancer (PCa). In several studies, AR was overexpressed in castration-resistant PCa (CRPC). However, the mechanism of AR overexpression in CRPC is not fully elucidated. Y-box binding protein-1 (YB-1) is a pleiotropic transcription factor that is upregulated in CPRC. We aimed to elucidate the role of YB-1 in castration resistance of PCa and identify therapeutic potential of targeting YB-1. Using immunohistochemistry, we found that nuclear YB-1 expression significantly correlated with the Gleason score and AR expression in PCa tissues. In PCa cells, YB-1 regulated AR expression at the transcriptional level. Furthermore, YB-1 expression and nuclear localization were upregulated in CRPC cells. Overexpression of AR, as well as YB-1, conferred castration-resistant growth in LNCaP and 22Rv1 cells. Conversely, knocking down YB-1 resulted in suppressed cell growth and induced apoptosis, which was more efficient than knocking down AR in LNCaP cells. In other types of PCa cells, such as CRPC cells, knocking down YB-1 resulted in a significant reduction of cell growth. In conclusion, these findings suggested that YB-1 induces castration resistance in androgen-dependent PCa cells via AR expression. Thus, YB-1 may be a promising therapeutic target for PCa, as well as CRPC.

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Introduction

Prostate cancer (PCa) is the most common non-cutaneous cancer and the second leading cause of male cancer-related mortality in developed countries. Androgen deprivation therapy (ADT) along with bilateral orchietomy or administration of LHRH agonists is the gold standard of treatment for recurrent or advanced PCa (Sharifi et al. 2010). Although about 90% of PCa is originally androgen dependent and responds well to ADT, PCa eventually circumvents the low circulating levels of androgen and re-grows in a castration-resistant manner during ADT. This is referred to as castration-resistant PCa (CRPC; Debes & Tindall 2002). There are currently very few successful therapies for CRPC, and thus progression of PCa into CRPC is a serious problem.

The androgen/androgen receptor (AR) signaling pathway is thought to play a key role in the development and progression of PCa. Several studies have shown that the progression of PCa into CRPC is associated with an increased level of AR expression (Gregory et al. 1998, Zegarra-Moro et al. 2002, Chen et al. 2004, Scher & Sawyers 2005). Indeed, the AR gene is overexpressed in most cases of CRPC, and in only 10–20% of these cases, AR gene amplification occurs (Linja et al. 2001). The mechanism by which AR is overexpressed in patients without AR amplification remains largely unknown. Therefore, unraveling the
mechanisms of AR overexpression in CRPC appears to be a promising strategy for developing novel therapeutics for CRPC, especially since AR downregulation suppresses tumor growth even in CRPC (Zegarra-Moro et al. 2002, Chen et al. 2004, Scher & Sawyers 2005).

Y-box binding protein-1 (YB-1) possesses various biological activities in both the nucleus and the cytoplasm (Kohno et al. 2003). YB-1 functions as a transcription factor in the nucleus, which binds to the nucleotide sequence 5′-ATTGG-3′ denoted as the Y-box (Kuwano et al. 2003). YB-1 also regulates the expression of various genes, including proliferating cell nuclear antigen, epidermal growth factor receptor, DNA topoisomerase II, thymidine kinase, DNA polymerase α, CD44 (Dhillon et al. 2010, To et al. 2010), PIK3CA (Astanehe et al. 2009), and MET (Finkbeiner et al. 2009). It is well known that YB-1 expression is closely associated with cell growth, drug resistance and clinical outcome in various cancers (Kohno et al. 2003, Kuwano et al. 2003, 2004). We previously showed that Twist1, which belongs to the family of basic helix–loop–helix transcription factors (Olson & Klein 1994, Maestro et al. 1999), is involved in both tumor growth and cisplatin resistance through YB-1 expression (Shiota et al. 2008). Conversely, YB-1 regulates Twist1 expression at the translational level (Evdokimova et al. 2009). Thus, there is a close relationship between Twist1 and YB-1 expression. We also recently found that Twist1, which can be induced by oxidative stress, regulates YB-1 as well as AR expression (Shiota et al. 2008, 2010a). Moreover, YB-1 is known to be upregulated during prostate tumor progression and androgen ablation in a mouse xenograft model, suggesting that YB-1 plays a role in the progression of PCa into CRPC (Giménez-Bonafé et al. 2004). Based on these previous findings, we speculate that YB-1 also interacts with androgen/AR signaling and has a role in the pathogenesis of PCa.

Given that the role of YB-1 in androgen/AR signaling and PCa pathology remains largely unknown, we intended to resolve the roles of YB-1 in androgen/AR signaling and PCa pathology, especially in PCa that progressively becomes castration resistant. Furthermore, we wanted to identify therapeutic potential of targeting YB-1 in PCa.

Materials and methods

Cell culture

Human PCa PC3, LNCaP, 22Rv1, and VCaP cells were cultured in Eagle’s minimal essential medium, RPMI1640, RPMI1640, and DMEM respectively. These media were purchased from Invitrogen and contained 10% fetal bovine serum. LNCaP cells were propagated roughly 10–40 times prior to use. Castration-resistant derivatives of LNCaP cells, specifically LNCaP-CxR cells (referred to as CxR cells), were established and maintained, as described previously (Shiota et al. 2010a). Stable transfectants were derived from LNCaP or 22Rv1 cells. Specifically, LNCaP-AcGFP (#1 and #2), LNCaP-pDS-Red1 (#1 and #2), LNCaP-AR-GFP (#1 and #2), LNCaP-GFP-YB-1 (#1 and #2), 22Rv1-AcGFP (#1 and #2), 22Rv1-pDS-Red1 (#1 and #2), 22Rv1-AR-GFP (#1 and #2), and 22Rv1-GFP-YB-1 (#1 and #2) cell lines were established. These cells stably express their corresponding proteins, as described previously (Yokomizo et al. 2011). Briefly, LNCaP or 22Rv1 cells were transfected with AcGFP, pDS-Red1, AR-GFP, or GFP-YB-1 expression plasmids using Lipofectamine 2000 (Invitrogen). The transfected cells were then cultured for 2 weeks in medium containing 300 µg/ml (LNCaP cells) or 600 µg/ml (22Rv1 cells) geneticin (Nacalai Tesque, Kyoto, Japan). Protein expression in the obtained clones was verified using western blotting and fluorescence microscopy (BIOZERO, Keyence, Tokyo, Japan; data not shown). Isolated clones were maintained in the presence of their corresponding geneticin concentration. Cell lines were maintained in a 5% CO₂ atmosphere at 37 °C.

Antibodies

Antibodies against AR (sc-815), PARP (sc-1561), β-tubulin (sc-5274), and GFP (sc-8334) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-YB-1 and anti-cleaved-PARP antibodies were obtained from Epitomics (Burlingame, CA, USA) and Promega respectively. Anti β-actin and anti-Lamin B1 antibodies were purchased from Sigma.

Patient characteristics and tissue collection

A total of 35 patients that had received radical prostatectomy without chemotherapy, hormonal therapy prior to surgery, and carcinoma areas large enough for immunohistochemical evaluation were recruited from the Kyushu University Hospital, Japan, between 1997 and 2001. Patient characteristics were described previously (Shiota et al. 2010b). All patients underwent surgery for clinically localized PCa, as determined by pre-operative prostate-specific antigen concentrations, digital rectal examination, and prostate needle biopsies. Slides were prepared from prostate blocks that contained the largest and most representative area of the tumor and adjacent normal epithelium.
Immunohistochemistry

Immunohistochemistry was performed using the streptavidin–biotin–peroxidase method with a Histofine streptavidin–biotin–peroxidase kit (Nichirei, Tokyo, Japan). The primary antibodies were anti-YB-1 antibody (rabbit monoclonal, dilution 1:250, Epitomics) and anti-AR antibody (mouse monoclonal (AR441), dilution 1:100, Dako, Glostrup, Denmark). Sections (4 μm thick) that were previously fixed with 10% formalin and embedded in paraffin were deparaffinized with xylene and rehydrated with ethanol. Then, endogenous peroxidase activity was blocked with methanol containing 0.3% hydrogen peroxidase for 30 min. Antigen retrieval was performed by heating sections in either citrate buffer (pH 6.0) for YB-1 antibody or Tris-buffered saline (pH 9.0) for AR antibody for 20 min in a microwave. After exposure to 10% non-immunized goat serum (for YB-1) or rabbit serum (for AR) in PBS for 10 min, sections were incubated at 4°C overnight with primary antibodies. The sections were then incubated with the second antibody for 20 min at room temperature. The reaction products were visualized by diaminobenzidine tetrahydrochloride as a chromogen. Finally, sections were counterstained with hematoxylin.

Immunohistochemical analysis

Whole cellular AR expression levels were assessed by evaluating the proportion and intensity of positively stained carcinoma cells. A score was assigned to represent the estimated percentage of positively stained carcinoma cells, where 0, none; 1, ≤1%; 2, 1–10%; 3, 10–33%; 4, 33–67%; and 5, ≥67%. An intensity score was assigned to represent the average estimated stain intensity in positive carcinoma cells, where 0, none; 1, weak; 2, intermediate; and 3, strong. Proportion and intensity scores were added to obtain a total score ranging from 0 to 8 (Allred et al. 1998). Immunohistochemistry results were classified according to the total scores with 0–4 classified as low expression and 5–8 as high expression. To assess nuclear YB-1 expression, nuclear YB-1 positive cells were counted. If the proportion of nuclear YB-1-positive cells was either greater than or less than 10%, then it was classified either as low or as high expression. To assess cytoplasmic YB-1 expression, an intensity score was assigned to represent the average estimated stain intensity in positive carcinoma cells, which was as follows: 0, none; 1, weak; 2, intermediate; and 3, strong. Then, a score of 0 or 1 was classified as low expression and a score of 2 or 3 was classified as high expression.

Knockdown analysis using siRNAs

The following double-stranded RNA 25 bp oligonucleotides were commercially generated (Invitrogen): 5′-UG-GAUAGCGUCUAUUAGGUUCG-3′ (sense) and 5′-CCGUAAACCUUUAUGACCGCUAACA-3′ (antisense) for YB-1 #1; 5′-UUUCGUGGAAUGCG-GUGGAGACC-3′ (sense) and 5′-GGUCUCGAACCAGAAA-3′ (antisense) for YB-1 #2; 5′-UAGAGAGCAAGGCUGCAAAGGAGUC-3′ (sense) and 5′-GACUCUUUGAGCCUUGUCUCUA-3′ (antisense) for AR siRNA #1; and 5′-CAUAGUGACACCCAGACGCUAUC-3′ (sense) and 5′-GAUGAGCUCUGGGUGUCACUAUG-3′ (antisense) for AR siRNA #2. PCa cells were transfected with siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol.

RNA isolation, reverse transcription, and quantitative real-time PCR

These procedures were performed as described previously (Shiota et al. 2010a,b). Quantitative real-time PCR was performed using TaqMan Gene Expression Assays for YB-1 (Hs00898625_g1), AR (Hs00907244_m1), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Hs02758991_g1; Applied Biosystems, Foster City, CA, USA) with the TaqMan Gene Expression Master Mix (Applied Biosystems) on a 7900HT PCR system (Applied Biosystems). The transcript levels of YB-1 and AR were corrected according to the corresponding GAPDH transcript levels. All values represent the results of at least three independent experiments.

Western blotting analysis

Whole-cell, nuclear, and cytoplasmic extracts were prepared, as described previously (Shiota et al. 2010a,b). Briefly, protein concentration was quantified with a Protein Assay (Bio-Rad) that is based on Bradford method. Similar extract amounts were separated by 4–20% SDS–PAGE and transferred onto polyvinylidene difluoride microporous membranes (GE Healthcare Bio-Science, Piscataway, NJ, USA) using a semi-dry blotter. Blotted membranes were incubated for 1 h at room temperature with the above-mentioned primary antibody. Membranes were then incubated for 40 min at room temperature with a peroxidase-conjugated secondary antibody. Bound antibody was visualized using an ECL kit (GE Healthcare Bio-Science) and membranes were exposed to X-ray film (GE Healthcare Bio-Science). Protein expression levels were numerically assessed using the Bradford method.
NIH Image program (National Institutes of Health, Bethesda, MD, USA). Expression levels of target proteins were corrected by comparing their expressions to corresponding β-actin, Lamin B1, or β-tubulin protein levels, which were used as internal controls. The results are representative of three experiments.

**Plasmid construction**

The AR-GFP plasmid was kindly provided by Dr Toshihiko Yanase (Fukuoka University, Fukuoka, Japan; Tomura et al. 2001). The pCMV-AR plasmid expressing wild-type AR was kindly provided by Dr Chawnshang Chang (University of Rochester, Rochester, NY, USA). AcGFP and pDS-Red1 plasmids expressing GFP and DsRed, respectively, were purchased from Clontech. The GFP-YB-1 plasmid expressing the N-terminally myc-tagged YB-1 protein was constructed, as described previously (Koike et al. 1997). To construct the pCMV-myc-YB-1 plasmid expressing the N-terminally myc-tagged YB-1 protein, PCR was carried out with the GFP-YB-1 plasmid as a template using the following primer pairs: 5′-CTCGAGTTATGAGCTGCAGGCGCCGGA-3′ and 5′-GGGCCCCTTACTCAGGCCCCGGCCT-3′. The PCR product was cloned into the Topo2.1 cloning vector (Invitrogen). Then, the XhoI-NotI fragment of YB-1 cDNA was ligated into the pCMV-myc (Invitrogen) plasmid. Construction of the pCMV-YB-1-myc-nuc and pCMV-YB-1-myc-cyto plasmids, which express the C-terminally myc-tagged YB-1 protein with or without nuclear-localizing signaling (NLS), respectively, was performed as follows. PCR was carried out with the GFP-YB-1 plasmid as a template using the following primer pairs: 5′-CTCGAGATGAGCAGCGAGGCCGAGA-3′ and 5′-GGGCCCCTTACTCAGGCCCCGGCCT-3′. The PCR product was cloned into the Topo2.1 cloning vector (Invitrogen). Then, the XhoI-NotI fragment of YB-1 cDNA was ligated into the pCMV-myc-nuc (Invitrogen) and pCMV-myc-cyto (Invitrogen) plasmids. Construction of the pCMV-YB-1-myc-nuc and pCMV-YB-1-myc-cyto plasmids, which express the C-terminally myc-tagged YB-1 protein with or without nuclear-localizing signaling (NLS), respectively, was performed as follows. PCR was carried out with the GFP-YB-1 plasmid as a template using the following primer pairs: 5′-CTCGAGATGAGCAGCGAGGCCGAGA-3′ and 5′-GGGCCCCTTACTCAGGCCCCGGCCT-3′. The PCR product was cloned into the Topo2.1 cloning vector (Invitrogen). Then, the XhoI-NotI fragment of YB-1 cDNA was ligated into the pCMV-myc-nuc (Invitrogen) and pCMV-myc-cyto (Invitrogen) plasmids.

Luciferase reporter plasmids containing various lengths of the wild-type AR gene (AR-Luc #1, AR-Luc #2, AR-Luc #3, and AR-Luc #4) promoter region were constructed, as described previously (Shiota et al. 2010a). The mutation in Y-box of AR-Luc Y-box MT. The underlined nucleotides indicate mutated sequences.

**Chromatin immunoprecipitation assay**

The chromatin immunoprecipitation assay (ChIP) assay was performed, as described previously (Shiota et al. 2010a,b). Briefly, soluble chromatin from 1 × 10⁶ LNCaP cells was incubated with anti-rabbit IgG or anti-YB-1 antibody and 20 μl protein A/G-PLUS agarose (Santa-Cruz Biotechnology). Purified DNA was dissolved in 20 μl dH₂O. The quantitative real-time PCR assay was performed using ABI 7900HT with 1 μl diluted DNA, the primer pairs describe previously (Shiota et al. 2010a), and SYBR Premix Ex Taq II (Takara Bio, Tsu, Japan). The results are representative of three experiments.

**Luciferase reporter assay**

The transient transfection and luciferase assay were performed, as described previously (Shiota et al. 2010a,b). Briefly, PCa cells were transfected with the indicated amounts of AR reporter plasmids and expression plasmids using Lipofectamine 2000, according to the manufacturer’s protocol. After 48 h, luciferase activity was detected using a Dual-Luciferase Reporter Assay System (Promega). Light intensity was measured using a plate reader (ARVOTM MX, Perkin Elmer, Inc., Waltham, MA, USA). Firefly luciferase activity was corrected for the corresponding Renilla luciferase activity. The results are representative of three experiments.

**Fluorescence microscopy**

Fluorescence microscopy was performed, as described previously (Shiota et al. 2010c). Briefly, LNCaP and CxR cells (1 × 10⁵) were transfected with 2.0 μg GFP-YB-1 expression plasmid and plated onto cover glasses in 6-well plates. After 48 h of incubation, cells were washed with PBS and stained with Hoechst 33342 (Nacalai Tesque) to visualize their nuclei. Imaging was performed using a BIOZERO fluorescence microscope (Keyence).

**Cell proliferation assay**

The cell proliferation assay was performed, as described previously (Shiota et al. 2010a,b). Briefly, 2.5 × 10⁵ PCa cells were seeded into 12-well plates and transfected with siRNA, as described above. After 12 h of post-transfection was referred to as 0 h. Cells were harvested with trypsin and counted daily using a cell counter (Beckman Coulter, Fullerton, CA, USA). The results were normalized to cell counts at 0 h and are representative of three experiments.

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**Mouse xenograft model**

Male NCr-nu/nu mice (6–8 weeks old; n = 20) were obtained from Charles River Laboratories in Japan (Yokohama, Japan). The lateral flanks of mice were inoculated s.c. with either LNCaP-AcGFP #1 (n = 10) or LNCaP-GFP-YB-1 #1 (n = 10) cells (5.0 × 10^7). After 7 days, each ten mice were randomly divided into two groups: castrated or sham operated. For both operations, mice were anesthetized with an i.p. injection of ketamine/xylazine. In the castrated group, both testes were surgically removed 7 days after tumor inoculation. Sizes of the inoculated tumors were measured and recorded every 2–3 days using calipers. Tumor volume was calculated using the following formula: \( V = \frac{A \times B^2}{2} \), where \( V \) is the volume (mm³), \( A \) is the long diameter (mm), and \( B \) is the short diameter (mm).

**Flow cytometry**

Flow cytometry was performed, as described previously (Shiota et al. 2010a,b). Briefly, 2.5 × 10^5 LNCaP cells were seeded into 6-well plates, transfected with siRNA and cultured for 72 h. Cells were harvested, washed twice with ice-cold PBS with 0.1% BSA, and resuspended in 70% ethanol. After washing twice with ice-cold PBS, cells were resuspended in PBS with 0.1% BSA, incubated with RNase (Roche Molecular Biochemicals) and stained with propidium iodide (Sigma). Cells were analyzed using a FACS Calibur (BD Biosciences, San Jose, CA, USA).

**Colony formation assay**

The colony formation assay was performed, as described previously (Shiota et al. 2008, 2010a). Briefly, LNCaP cells (2.5 × 10^5) were transfected with 20 nM of the indicated siRNA and 0.5 μg pCMV or pCMV-AR and then were seeded into 6-well plates. After 14 days of transfection, cells were washed with PBS and fixed in 100% methanol for 30 min and then stained with 2% Giemsa solution for 1 h. Wells were washed with H_2O and dried. Colonies with > 50 cells were counted on an inverted microscope (Olympus, Tokyo, Japan).

**Statistical analysis**

The \( \chi^2 \) test was used to analyze correlations between YB-1 and AR expression levels and clinicopathological parameters. The Mann–Whitney \( U \) test was used for other variables. Values of \( P < 0.05 \) were considered to be statistically significant.

**Results**

**Nuclear YB-1 expression correlates with Gleason score and AR expression in human PCa tissues**

Immunohistochemistry in human PCa tissues was performed to clarify the relationship between YB-1 and AR expression in human PCa. In human normal and malignant prostate tissues, YB-1 distributed into both the nucleus and the cytoplasm. Expression of YB-1 was weakly positive in both the nuclei of normal prostatic glands and stromal cells of normal prostate parenchyma. The expression of YB-1 was moderate to strong in PCa (Fig. 1A). In addition, nuclear YB-1 expression was significantly greater in PCa tissues from patients with a higher Gleason score compared with those with a lower Gleason score (\( P = 0.0427 \); Table 1 and Fig. 1B). These findings are comparable to those of previous reports, which found that YB-1 expression correlated with the degree of malignancy in various types of cancer (Kuwano et al. 2004).

Conversely, AR distribution was primarily located in the nucleus, and the expression of AR was similar between normal and malignant prostate tissues. Although AR staining was not associated with the Gleason score (\( P = 0.288 \); Table 1), a correlation

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**Figure 1** Nuclear YB-1 expression correlates with Gleason score and AR expression in human PCa tissues. (A) YB-1 expression in PCa tissues accompanied by adjacent normal tissues. (B) YB-1 and AR expression in PCa tissues from cases with low and high Gleason scores.
between nuclear YB-1 and AR expressions was found ($P=0.00731$; Table 2 and Fig. 1B), suggesting that nuclear YB-1 may regulate AR expression.

**YB-1 regulates AR transcription via binding to the Y-box in the AR promoter region**

Given that YB-1 is a transcription factor, we examined whether YB-1 regulates AR expression. When YB-1 expression was suppressed with YB-1-specific siRNAs in LNCaP cells, AR mRNA and protein expression were decreased (Fig. 2A). In addition, suppressing YB-1 expression in another AR-expressing PCa cell line, specifically in 22Rv1 cells, resulted in lowering of AR mRNA and protein expression (Supplementary Figure 1A, see section on supplementary data given at the end of this article). Furthermore, YB-1 in other AR-expressing PCa cell lines, specifically VCaP and CxR cells, also resulted in a decrease in AR protein levels (Supplementary Figure 1B and C, see section on supplementary data given at the end of this article).

To investigate the mechanism of AR transcription with YB-1, we performed a quantitative ChIP assay using the anti-YB-1 antibody and PCR primers against AR gene, as shown in Fig. 2B. YB-1 did not bind to the AR 5′-UTR region (i.e. AR #4), although it did bind to AR #1 and AR #3, the regions near and including the Y-box respectively (Fig. 2B).

Subsequently, we performed luciferase reporter assays using various lengths of AR-reporter plasmids (AR-Luc #1, AR-Luc #2, AR-Luc #3, and AR-Luc #4; Fig. 2C). Overexpressing of YB-1 results in an upregulation of luciferase activity in all AR-Luc, except AR-Luc #4, which does not contain the Y-box (Fig. 2C). To confirm whether the upregulation in AR transcription was induced by YB-1 binding to the Y-box, we introduced a mutation into the Y-box of the AR-promoter region (Fig. 2D). Introducing a mutation into the Y-box (AR-Luc Y-box MT), which is located in the proximal AR promoter region, decreased luciferase activity in LNCaP cells. In addition, this mutated reporter plasmid (AR-Luc Y-box MT) did not respond to YB-1 overexpression (Fig. 2D). Finally, YB-1 expression plasmids were constructed to express C-terminally myc-tagged YB-1 either with NLS (YB-1-myc-nuc) or without NLS (YB-1-myc-cyto) to confirm whether YB-1 regulates AR transcription. The luciferase reporter assay was conducted on these constructs. Overexpression of YB-1 with NLS, which localizes YB-1 expression within the nucleus, led to a more effective upregulation of AR luciferase activity in LNCaP cells than an overexpression of YB-1 without NLS (Fig. 2E).

### Table 1 Relationships between YB-1 and AR expression and clinicopathological parameters

| Variable      | n = 35 | Nuclear YB-1 expression | | Cytoplasmic YB-1 expression | | AR expression | | P value | Low | High | | P value | Low | High | | P value |
|---------------|--------|-------------------------|----------------|-----------------------------|----------------|----------------|----------------|--------|----------------|----------------|--------|----------------|----------------|
| Age <70       | 21     | Low: 13 High: 8          | 0.561          | Low: 7 High: 14             | 0.884          | Low: 9 High: 12| 0.678          |        |                |                |        |                |                |
| Age ≥70       | 14     | Low: 10 High: 4          |                | Low: 5 High: 9              |                | Low: 7 High: 7 |                |        |                |                |        |                |                |
| PSA <10       | 19     | Low: 16 High: 3          | 0.109          | Low: 7 High: 12             | 0.842          | Low: 10 High: 9| 0.552          |        |                |                |        |                |                |
| PSA ≥10       | 12     | Low: 7 High: 5           |                | Low: 4 High: 8              |                | Low: 5 High: 7 |                |        |                |                |        |                |                |
| Gleason score | <7 or 7 (=3+4) | Low: 23 High: 19 | 0.0427a        | Low: 10 High: 13            | 0.113          | Low: 12 High: 11| 0.288          |        |                |                |        |                |                |
| Gleason score | 7 (=4+3) or 7 < | Low: 12 High: 6 |                | Low: 2 High: 10             | 0.456          | Low: 11 High: 15| 0.492          |        |                |                |        |                |                |
| Stage pT2     | 26     | Low: 17 High: 9          | 0.49           | Low: 8 High: 18             | 0.456          | Low: 11 High: 15| 0.492          |        |                |                |        |                |                |
| Stage pT3–4   | 9      | Low: 7 High: 2           |                | Low: 4 High: 5              |                | Low: 5 High: 4 |                |        |                |                |        |                |                |
| Surgical margin | Negative | Low: 26 High: 16 | 0.128          | Low: 7 High: 19             | 0.119          | Low: 10 High: 16| 0.143          |        |                |                |        |                |                |
| Surgical margin | Positive | Low: 9 High: 8 |                | Low: 5 High: 4              |                | Low: 6 High: 3 |                |        |                |                |        |                |                |

PSA, prostate-specific antigen; YB-1, Y-box binding protein-1; AR, androgen receptor.

*Statistically significant ($\chi^2$ test).

### Table 2 Relationship between nuclear YB-1 and AR expression levels

| Nucleary YB-1 expression | AR expression | P value | Low | High | | P value | Low | High | | P value |
|--------------------------|--------------|---------|-----|-----|----------------|---------|-----|-----|----------------|---------|-----|-----|---------|---------|-----|
| Low                      | 25           | 15      | 10  |     |                |         |     |     |                | 0.00731a|     |     |         |         |     |
| High                     | 10           | 1       | 9   |     |                |         |     |     |                |         |     |     |         |         |     |

YB-1, Y-box binding protein-1; AR, androgen receptor.

*Statistically significant ($\chi^2$ test).
YB-1 expression and nuclear translocation increased in castration-resistant CxR cells

YB-1 and AR expression levels in castration-resistant CxR cells were investigated. AR mRNA and protein expression were upregulated in CxR cells compared with parental LNCaP cells (Fig. 3A). Given that YB-1 may play a distinct role in both the nucleus and the cytoplasm, nuclear and cytoplasmic YB-1 expression levels were examined. Surprisingly, YB-1 was abundantly distributed within the nucleus of CxR cells compared with parental LNCaP cells (Fig. 3B).

Similar results were obtained when GFP-YB-1 expression was induced in LNCaP and CxR cells (Fig. 3C).

AR, as well as YB-1 overexpression, circumvents growth retardation induced by androgen deprivation in androgen-dependent and androgen-sensitive PCa cells

The above-mentioned findings suggest that YB-1 expression may be implicated in the castration-resistant phenotype of PCa. Therefore, we investigated whether

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Figure 2 YB-1 regulates AR transcription via binding to the Y-box in the AR promoter region. (A) Following total RNA extraction and cDNA synthesis from LNCaP cells transfected with 40 nM of the indicated siRNA and incubated for 48 h, quantitative real-time PCR was performed for YB-1, AR, and GAPDH. Target transcript expression was adjusted to GAPDH expression. Each target transcript expression level in LNCaP cells was defined as 1. Boxes, mean; bars, ± s.d.; *P < 0.05 versus cells transfected with control siRNA. Whole-cell extracts from LNCaP cells transfected with 40 nM of the indicated siRNA and incubated for 72 h were subjected to SDS–PAGE and western blotting analysis. Each target protein expression level in the cells transfected with control siRNA was defined as 1. Boxes, mean; bars, ± s.d.; *P < 0.05 versus control siRNA. (B) Schematic representation of the promoter region of the AR gene. The black box indicates the Y-box (5'-ATTGG-3'). ChIP assays with nuclear extracts from LNCaP cells were performed using rabbit IgG or anti-YB-1 antibody and 20 µl Protein A/G PLUS-agarose. Quantitative real-time PCR was performed using immunoprecipitated DNAs, soluble chromatin, and specific primer pairs for the AR promoter region and 5' end of AR gene. Each target transcript expression level in the cells transfected with control siRNA was defined as 1. Boxes, mean; bars, ± s.d.; *P < 0.05 versus control siRNA. (C) Schematic representation of the promoter region of the AR gene, AR-Luc #1, AR-Luc #2, AR-Luc #3, and AR-Luc #4 used in (C–E). LNCaP cells were transiently transfected with the indicated amount of YB-1 expression plasmid, 0.5 µg of the various AR-Luc plasmids and 0.05 µg pRL-TK. The luciferase activity of AR-Luc #1 with myc expression plasmid was defined as 1. Boxes, mean; bars, ± s.d.; *P < 0.05 versus myc expression plasmid. LNCaP cells were transfected with 2.0 µg myc or myc-YB-1 expression plasmid and incubated for 48 h. Whole-cell extracts were subjected to SDS–PAGE and western blotting. (D) Schematic representation of the AR-Luc Y-box MT. LNCaP cells were transiently transfected with 0.5 µg of the indicated expression plasmid, 0.5 µg of the various AR-Luc plasmids, and 0.05 µg pRL-TK. The luciferase activity of AR-Luc #3 with myc expression plasmid was defined as 1. Boxes, mean; bars, ± s.d.; *P < 0.05 versus myc expression plasmid. (E) LNCaP cells were transiently transfected with 0.5 µg of the indicated expression plasmid, 0.5 µg AR-Luc #1 reporter plasmid, and 0.05 µg pRL-TK. The luciferase activity of AR-Luc #1 with the myc-nuc expression plasmid was defined as 1. Boxes, average; bars, ± s.d.; *P < 0.05 versus myc expression plasmid. LNCaP cells were transfected with 2.0 µg myc-nuc, YB-1-myc-nuc, or YB-1-myc-cyto expression plasmid, and incubated for 48 h. Nuclear and cytoplasmic extracts were subjected to SDS–PAGE and western blotting.
YB-1 overexpression contributes to castration-resistant tumor growth. After we established several LNCaP (androgen-dependent PCa cells) cell lines expressing GFP (LNCaP-AcGFP #1 and LNCaP-AcGFP #2), DsRed (LNCaP-pDS-Red1 #1 and LNCaP-pDS-Red1 #2), AR-GFP (LNCaP-AR-GFP #1 and LNCaP-AR-GFP #2), and GFP-YB-1 (LNCaP-GFP-YB-1 #1 and LNCaP-GFP-YB-1 #2), a cell proliferation assay with all of these cell lines was performed in the presence and absence of androgen. Proliferation of both LNCaP-AcGFP and LNCaP-pDS-Red1 cell lines was retarded by 30% with androgen deprivation (Fig. 4A). Conversely, proliferation of the LNCaP-AR-GFP cell line was not significantly affected by androgen starvation. Surprisingly, in the absence of androgen, the LNCaP-GFP-YB-1 cell line proliferated more rapidly than both the LNCaP-AcGFP and the LNCaP-pDS-Red1 cell lines. Furthermore, growth retardation rates in the LNCaP-GFP-YB-1 cell line were lower than those of the LNCaP-AcGFP and LNCaP-pDS-Red1 cell lines (Fig. 4A). In addition, the LNCaP-GFP-YB-1 cell line proliferated at a similar rate to the LNCaP-AcGFP and LNCaP-pDS-Red1 cell lines in the presence of androgen. Although, the growth rate of the LNCaP-AR-GFP cell line in the presence of androgen was lower than both the LNCaP-AcGFP and the LNCaP-pDS-Red1 cell lines. Similarly, we established several 22Rv1 (androgen-independent, but androgen-sensitive PCa cells) cell lines expressing GFP (22Rv1-AcGFP #1 and 22Rv1-AcGFP #2), DsRed (22Rv1-pDS-Red1 #1 and 22Rv1-pDS-Red1 #2), AR-GFP (22Rv1-AR-GFP #1 and 22Rv1-AR-GFP #2), and GFP-YB-1 (22Rv1-GFP-YB-1 #1 and 22Rv1-GFP-YB-1 #2), and a cell proliferation assay with all of these cell lines was performed. Similar results were obtained in the 22Rv1 cell lines as in the LNCaP cell lines, although the growth retardation rate with androgen starvation was lower compared with those of LNCaP cells (Fig. 4B). Subsequently, we performed similar experiments in an in vivo system using the mouse xenograft model. Without castration, tumor growth in the LNCaP-GFP-YB-1 #1 cell line was similar or slightly greater than that of the LNCaP-AcGFP #1 cell line. Conversely, tumor growth in the LNCaP-AcGFP #1 cell line was significantly retarded by castration. However, tumor growth in the LNCaP-GFP-YB-1 #1 cell line was not affected by castration (Fig. 4C).

Figure 3 YB-1 expression and nuclear localization increased in castration-resistant CxR cells. (A) Following total RNA extraction and synthesis cdNA from LNCaP and CxR cells, quantitative real-time PCR was performed for YB-1, AR, and GAPDH. Target transcript expression was adjusted to GAPDH expression. Each target transcript expression level in LNCaP cells was defined as 1. Boxes, mean; bars, ± s.d.; *P < 0.05 versus LNCaP cells. Whole-cell extracts from LNCaP and CxR cells were subjected to SDS–PAGE and western blotting analysis. Each target protein expression level in LNCaP cells was defined as 1. Boxes, mean; bars, ± s.d.; *P < 0.05 versus LNCaP cells. (B) Nuclear and cytoplasmic extracts from LNCaP and CxR cells were subjected to SDS–PAGE and western blotting. Each YB-1 protein expression level of both the nucleus and the cytoplasmic fraction of LNCaP cells was defined as 1. Boxes, mean; bars, ± s.d.; *P < 0.05 versus LNCaP cells. (C) LNCaP and CxR cells transfected with 2.0 μg GFP-YB-1 expression plasmid were incubated for 48 h and stained with Hoechst 33342. Stained cells were observed by fluorescence microscopy. Representative images of high-magnification fields are shown. Western blots against GFP, Lamin B1, and β-tubulin are also shown. Each GFP-YB-1 protein expression level of both the nucleus and the cytoplasmic fraction in LNCaP cells was defined as 1. Boxes, mean; bars, ± s.d.; *P < 0.05 versus LNCaP cells.
YB-1 knockdown induces growth retardation and apoptosis in various PCa cells

YB-1 was knocked down with YB-1-specific siRNAs and cell proliferation assays were conducted to determine whether there is any therapeutic potential of targeting YB-1 in PCa. LNCaP cell growth was retarded and LNCaP cell numbers were decreased after a 72 h incubation period with androgen (Fig. 5A). In addition, LNCaP cell growth was suppressed by the YB-1 knockdown in the absence of androgen, suggesting that knocking down YB-1 may affect cell viability independent of the androgen/AR signaling pathway (Fig. 5A). This may be due to the fact that YB-1 regulates other targets than AR. Furthermore, a cell cycle analysis with flow cytometry was conducted. Silencing of YB-1 expression led to a significant and reproducible increase in the proportion of LNCaP cells in the G1 fraction. Also, there was a significant increase in the proportion of sub-G1 fractions, indicating that the YB-1 knockdown induced cellular death (Fig. 5B). These data are consistent with our previous report, which found that knocking down YB-1 induces cell cycle arrest in the G1 phase in PC3 cells and human breast cancer MCF7 cells (Shiota et al. 2008). The mechanism by which YB-1 knockdown induces cellular death in LNCaP cells was found to be via cellular apoptosis, as determined by immunoblotting against cleaved PARP. YB-1 knockdown activated caspase and thereby resulted in cellular apoptosis, whereas AR knockdown had no apoptosis-inducing effects (Fig. 5C). Subsequently, rescue experiments were performed by overexpressing AR in a YB-1 knockdown. The colony formation assay revealed that when YB-1 was knocked down, colony formation was significantly reduced, whereas AR overexpression partially rescued the reduction in colony formation (Fig. 5D).

Finally, various AR-expressing PCa cells were transfected with YB-1-specific siRNAs, and cell proliferation assays were performed. All PCa cell proliferations were retarded with YB-1 knockdown. Furthermore, cell number in CxR cells was decreased, indicating that a YB-1 knockdown induced cellular apoptosis similarly in CxR cells as in LNCaP cells (Fig. 5E).

Discussion

In this study, YB-1 was found to regulate AR expression at transcriptional level. Using the ChIP-on-chip assay, Finkbeiner et al. (2009) found that YB-1 binds to the AR promoter in breast cancer cells (i.e. a fold change of 6.347), suggesting that AR is a direct

Figure 4 YB-1 overexpression circumvents growth retardation induced by androgen deprivation. (A and B) Stable transfectants of LNCaP (A) and 22Rv1 (B) cells plated into 12-well plates and incubated under charcoal-stripped medium with or without 1 nM dihydrotestosterone (DHT). After 120 h, cell numbers were counted. The results were normalized to cell numbers at 0 h. Relative growth rate was calculated as the ratio between relative cell count in the absence of DHT to the presence of 1 nM DHT. Boxes, mean; bars, ± s.d.; *P<0.05 versus AcGFP #1 cells. (C) Male NCr-nu/nu mice were injected s.c. with either LNCaP-AcGFP #1 or LNCaP-GFP-YB-1 #1 cells, and each (n=10) were either castrated or sham-operated 7 days following inoculation. Tumor volume of mice was inspected every 2–3 days. Bars, ± s.e.m.; *P<0.05 versus tumor in non-castrated mice.

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androgen deprivation induces YB-1 (Giménez-Bonafé et al. 2004) into castration-resistant cells. Although it is known that androgen-dependent and androgen-sensitive PCa cells C R P C c e l l s, a n d Y B - 1 o v e r e x p r e s s i o n c o n v e r t e d PCa cells. In this study, YB-1 was overexpressed in

of AR expression contribute to castration resistance in CRPC. However, it remains unclear whether regulators SRC-1, are also involved in the progression of PCa into PCa progression and evoke the castration-resistant expression correlates with AR expression.

YB-1, indeed, bound to the gene target of YB-1 (Finkbeiner et al. 2009). Furthermore, our analysis of the AR promoter region with a quantitative chromatin immunoprecipitation assay revealed that there are several Y-boxes and that YB-1, indeed, bound to the AR promoter. In addition, YB-1 was found to regulate AR transcription via the AR promoter, as determined with the luciferase reporter assay. This relationship between YB-1 and AR was also supported with immunohistochemistry against YB-1 and AR in PCa, which found that nuclear YB-1 expression correlates with AR expression.

AR overexpression is well known to occur during PCa progression and evoke the castration-resistant phenotype in PCa. Moreover, AR cofactors, including SRC-1, are also involved in the progression of PCa into CRPC. However, it remains unclear whether regulators of AR expression contribute to castration resistance in PCa cells. In this study, YB-1 was overexpressed in CRPC cells, and YB-1 overexpression converted androgen-dependent and androgen-sensitive PCa cells into castration-resistant cells. Although it is known that androgen deprivation induces YB-1 (Giménez-Bonafé et al. 2004), the role of YB-1 overexpression in the progression of PCa into CRPC remains unresolved. We revealed that both AR and YB-1, an inducer of AR, are responsible for the progression PCa into CRPC in two cell lines, as well as in in vitro and in vivo systems. Furthermore, YB-1 was found, for the first time, to be distributed more within the nucleus in CRPC cells.

Previously, cellular stress, such as u.v. irradiation, has been reported to cause YB-1 translocation into the nucleus (Koike et al. 1997). Recently, Akt and p90 ribosomal S6 kinases have been shown to be responsible for YB-1 phosphorylation, which thereby results in the nuclear translocation of YB-1 (Sutherland et al. 2005, Stratford et al. 2008). Thus, given that we found an increase in the nuclear translocation of YB-1 in CRPC, then it may be that cellular stress is increased in CRPC. An increase in cellular stress in CRPC may be related to oxidative stress caused by androgen deprivation (Shiota et al. 2010a). Moreover, the signaling pathway responsible for YB-1 phosphorylation appears to be involved in the development of castration resistance. Therefore, future studies are warranted to clarify these unresolved issues.

We also investigated the therapeutic potential of targeting YB-1 in PCa. In the presence of androgen, LNCaP cell growth was significantly reduced by a YB-1 knockdown. This growth inhibition appeared to be due to cell cycle arrest in the G1 phase and cellular apoptosis. To investigate whether the growth suppression induced by the YB-1 knockdown was dependent on the androgen/AR signaling pathway, we performed cell proliferation assays in androgen-deprived conditions, as well as rescue experiments with AR overexpression in YB-1 knockdown cells. It was found that the YB-1 knockdown suppressed LNCaP cell growth, in part, via AR suppression. However, this
was also likely, in part, due to the activation and suppression of other gene targets of YB-1, as we have previously shown that cell growth in AR-null PC3 cells was retarded by YB-1 knockdown (Shiota et al. 2008). However, YB-1 knockdown appears to be more effective in LNCaP cells compared to PC3 cells. This was demonstrated via flow cytometry, where there was a greater increase in the sub-G1 fraction in LNCaP cells than in PC3 cells (10.9 and 4.6% in LNCaP cells versus 4.1% in PC3 cells). These differences may result from the additive effect of AR suppression. Conversely, stable YB-1 overexpression in LNCaP and 22Rv1 cells had no effect on cell growth, indicating that the primary function of YB-1 is to negatively regulate apoptosis rather than to promote proliferation. Taken together, these findings suggest that YB-1 suppression may also be a promising therapeutic modality in CRPC.

Recently, several novel therapeutics targeting YB-1 have been reported, indicating the feasibility of targeting YB-1 as a therapeutic strategy for both PCa and CRPC. For example, integrin-linked kinase (ILK) was found to regulate the Twist1/YB-1 signaling pathway (Kalra et al. 2010). It was reported that a small molecular inhibitor of ILK, QLT0267, inhibited not only Twist1 but also YB-1 expression and had suppressive effects on aggressive breast cancer growth (Kalra et al. 2009, 2010). It has also been reported that QLT0267 has anticancer activity with or without conventional therapy in various cancers (Koul et al. 2005, Younes et al. 2005, Edwards et al. 2008). However, its activity in PCa is unknown and future studies investigating its effects are warranted, as this agent may exert a suppressive effect on YB-1, as well as AR expression, in PCa. Furthermore, a molecular decoy to YB-1 is also effective in both breast cancer and PCa cells. Although this decoy suppresses breast cancer and PCa cell growth, it does not exhibit suppressive effects on normal immortalized breast epithelial cells and primary breast epithelial cells and does not inhibit the differentiation of hematopoietic progenitors. In addition, its suppressive effects on cell growth were greater in LNCaP cells compared with PC3 cells (Law et al. 2010). These observations corroborate our findings in that the YB-1 knockdown was more effective in LNCaP cells versus PC3 cells. Recently, several AR splice variants lacking the ligand-binding domain of AR have been reported to be active without the ligand and thus play an important role in CRPC growth through aberrant AR transcriptional signaling (Dehm et al. 2008, Guo et al. 2009, Hu et al. 2009, Sun et al. 2010). Therefore, therapeutics that target these AR splice variants need to be developed. Give that the expression of AR splice variants seems to be regulated by somewhat similar mechanisms as the expression of ordinary AR, then therapeutic strategies to target YB-1 may be applicable in cases with AR splice variants.

In summary, nuclear YB-1 expression correlated with the Gleason score and AR expression in PCa tissues. In PCa cells, we have found that AR is a gene target of YB-1. Moreover, YB-1 expression and nuclear localization increased in CRPC cells, and YB-1 overexpression induced castration-resistant growth. Based on these findings, we revealed that YB-1 may be a promising and feasible molecular target for the treatment of both androgen-dependent PCa and CRPC.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-11-0017.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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