

ORIGINAL ARTICLE

Gene amplification of YB-1 in castration-resistant prostate cancer in association with aberrant androgen receptor expression

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Abstract

Although Y-box binding protein-1 (YB-1) is known to be overexpressed in prostate cancer, especially castration-resistant prostate cancer (CRPC), the mechanism of its overexpression remains unclear. We aimed to elucidate the mechanism of YB-1 overexpression in CRPC. Gene amplification in CRPC cells and tissues was examined by public database analysis, and digital PCR. The significance of YB-1 amplification for the YB-1/androgen receptor (AR) axis and prognosis was examined by public database analysis and immunohistochemistry. YB-1 amplification was mainly observed in CRPC tissues by public database analysis and confirmed in CRPC cells and tissues by digital PCR. Expression of YB-1 was increased in CRPC tissues compared with treatment-naïve tissues. Furthermore, YB-1 and phosphorylated YB-1 levels were associated with AR and AR V7 expression levels. Finally, YB-1 amplification was associated with poor outcomes in CRPC. Taken together, the present findings suggest that YB-1 amplification contributes to progression to CRPC through regulation of AR and AR V7 expressions, and that YB-1 is a promising therapeutic target in CRPC.

KEYWORDS

amplification, androgen receptor, castration resistance, prostate cancer, YB-1

1 | INTRODUCTION

Most prostate cancers are initially dependent on androgens for tumor growth, and show good response to androgen-deprivation therapy (ADT).¹ However, most of these cancers eventually recur during ADT in a castration-resistant manner and are defined as castration-resistant prostate cancer (CRPC).² Despite the recognition of a variety of molecular mechanisms for progression to castration resistance, androgen

receptor (AR)-dependent mechanisms, such as AR overexpression and AR variant expression, are considered critical.^{3,4}

Y-box binding protein-1 (YB-1) has pleiotropic roles in the nucleus and cytoplasm as a transcription and splicing factor that modulates the expression of its target genes.^{5,6} Y-box binding protein-1 was shown to be overexpressed in various cancers and involved in their treatment resistance.^{5,6} In prostate cancer, YB-1 expression was reported to be increased in ADT and CRPC, and associated with resistance to ADT

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and AR-pathway inhibitors (ARPIs) like abiraterone and enzalutamide in preclinical models.⁷⁻¹¹ As a molecular mechanism, YB-1 was indicated to regulate AR and AR variant expressions, leading to progression to CRPC.^{7,11,12} Furthermore, YB-1 expression was correlated with AR expression in ADT-naïve prostate cancer.⁷ Consistently, YB-1 expression was increased in highly malignant tumors and associated with poor prognosis.¹³⁻¹⁶ Furthermore, a genetic polymorphism in YB-1 was correlated with probability of progression to CRPC.¹¹

Thus, YB-1 overexpression in prostate cancer plays a critical role in disease progression and treatment resistance to ARPIs. However, the expression status of YB-1 and the mechanism for YB-1 overexpression in human CRPC tissues remain to be elucidated. Copy number alteration of genes is one possible mechanism that can affect gene expression, especially in malignant tumors.¹⁷ In this study, we aimed to determine the mechanism for YB-1 overexpression in CRPC, focusing on gene amplification.

2 | MATERIALS AND METHODS

2.1 | Amplification and expression profile using a public database

The public datasets of Grasso et al,¹⁸ Beltran et al,¹⁹ Abida et al,²⁰ and Liu et al²¹ were downloaded from cBioPortal (<https://www.cbioportal.org>).²² The frequency of YB-1 amplification, and the associations of YB-1 amplification with YB-1 mRNA expression and prognosis were analyzed. The prognostic impact of YB-1 amplification on overall survival was determined using International Cancer Genome Consortium (ICGC) data in the UCSC Xena browser (<https://xena.ucsc.edu/>).²³ The correlation between YB-1 and AR mRNA expressions was analyzed in the GEPIA browser (<http://gepia.cancer-pku.cn/index.html>).²⁴

2.2 | Cell culture

Human prostate cancer PC-3 (MEM), LNCaP (RPMI-1640), and C4-2 (RPMI-1640) cells were cultured in medium (Life Technologies) containing 10% FBS. LNCaP cells were obtained from ATCC. C4-2 cells were kindly provided by Dr Martin Gleave (Vancouver Prostate Centre). LNCaP cells propagated approximately 10-40 times were used. Castration-resistant derivatives of LNCaP cells, designated LNCaP-CxR cells (CxR cells hereafter), were established and maintained as described previously.²⁵ All cell lines were maintained in a 5% CO₂ atmosphere at 37°C.

2.3 | Patients

Eight CRPC tissue samples from patients who had progressed to CRPC were obtained by transurethral resection (n = 7) and metastatic lymph node dissection (n = 1). Among them, treatment-naïve tissues were available in five cases (needle biopsy, n = 4; metastatic lymph

node dissection, n = 1). The study was carried out in accordance with the principles described in the Declaration of Helsinki and the Ethical Guidelines for Epidemiological Research enacted by the Japanese Government and was approved by our institutional review board.

2.4 | Digital PCR

Genomic DNA (40 ng) was analyzed on a droplet digital PCR (ddPCR) platform QX200 Droplet Digital PCR System (Bio-Rad Laboratories) using specific primers and probes (FAM, target gene; HEX, reference gene) and ddPCR Supermix for Probes (no dUTP) according to the manufacturer's protocol. The primers and probes for ddPCR were YB-1 (dHsaCNS346181577) or AR (dHsaCP2500359) for the target gene and RPP30 (dHsaCP2500350) for the reference gene. Droplets were generated using a QX200 droplet generator (Bio-Rad Laboratories). The PCR protocol comprised a 10-minute incubation at 95°C, followed by 40 cycles at 94°C for 30 seconds and 55°C for 1 minute, one cycle at 98°C for 10 minutes, and a 4°C hold. Droplet fluorescence was assessed in a QX200 droplet reader. Analysis of ddPCR data for allele calling and calculation of absolute copy numbers were undertaken using QuantaSoft software (Bio-Rad Laboratories).

2.5 | Immunocytochemistry

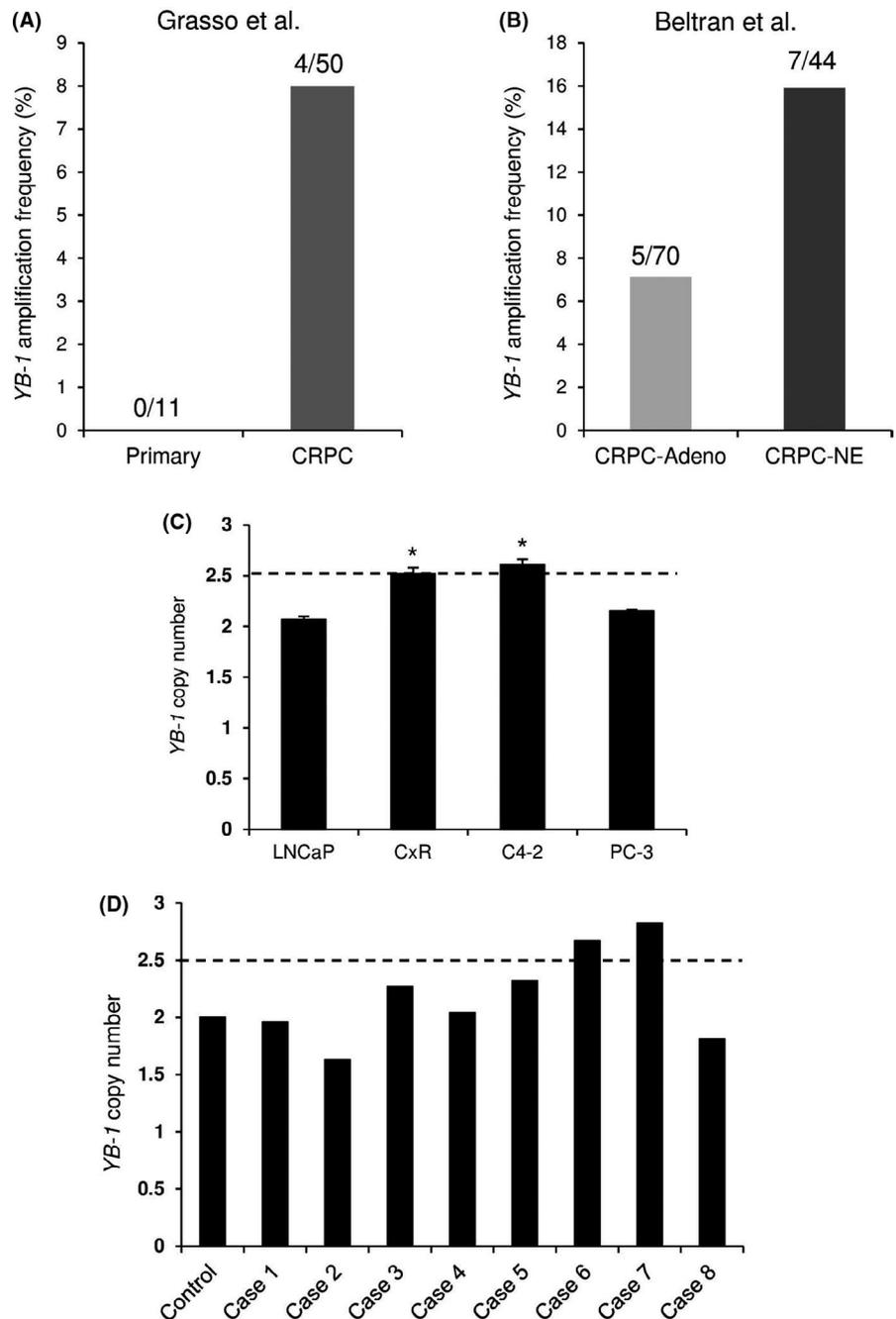
Antibodies against YB-1 (2397-1), phosphorylated YB-1^{Ser102} (p-YB-1; #2900), AR (N-20; sc-816), and AR V7 (AG10008) were purchased from Epitomics, Cell Signaling Technology, Santa Cruz Biotechnology, and Precision Antibody, respectively.

Immunocytochemical staining was carried out using an EnVision Plus detection system (Dako) with the primary Abs against YB-1 (1:250), p-YB-1 (1:50), AR (1:100), and AR V7 (1:50). After fixation of the cells on the culture slides by incubation in methanol/acetone at 4°C for 20 minutes, endogenous peroxidase activity was blocked in methanol containing 0.3% hydrogen peroxidase for 20 minutes. The cells were then permeabilized by incubation with 0.2% Triton X-100 (Sigma) for 15 minutes and subjected to antigen retrieval by microwave heating with sodium citrate buffer (pH 6.0) for 5 minutes. Next, the cells were incubated with a primary Ab at room temperature for 60 minutes, followed by incubation with secondary Ab/peroxidase-linked polymers (EnVision+; Dako) for 30 minutes at room temperature. The reaction products were visualized with diaminobenzidine tetrahydrochloride as a chromogen. Finally, the sections were counterstained with hematoxylin.

2.6 | Immunohistochemical analysis

Nuclear expression of YB-1 and AR and cytoplasmic expression of p-YB-1 and AR V7 was assessed. For scoring of the expression levels, the staining intensity was initially divided into four groups: 0, negative; 1, weak; 2, intermediate; and 3, strong intensity. The

FIGURE 1 Y-box binding protein-1 (YB-1) amplification in prostate cancer. A, YB-1 amplification was observed in tissues from patients with primary prostate cancer (n = 11) and castration-resistant prostate cancer (CRPC) (n = 50), using data obtained from the public datasets of Grasso et al.¹⁸ B, YB-1 amplification was observed in tissues from patients with adenocarcinoma-type CRPC (CRPC-Adeno) (n = 70) and neuroendocrine-type CRPC (CRPC-NE) (n = 44), using data obtained from the public datasets of Beltran et al.¹⁹ C, YB-1 copy numbers were determined in LNCaP, CxR, C4-2, and PC-3 cells. Data are representative of at least three independent experiments and represent means \pm SD. * $P < 0.05$ vs LNCaP cells. D, YB-1 copy numbers were determined in CRPC tissues from eight patients. The threshold for gene amplification was set at 2.5



proportions of stained cells were then counted among at least 1000 tumor cells selected from the areas with the greatest accumulations of positive signals (hotspots). Finally, the intensities and proportion scores were multiplied and added together as $([\% \text{ of weak}] \times 1) + ([\% \text{ of intermediate}] \times 2) + ([\% \text{ of strong}] \times 3)$, giving a range of 0-300.¹⁴

2.7 | Statistical analysis

All statistical analyses were undertaken using JMP14 software (SAS Institute). Continuous data were examined by the Wilcoxon rank sum test. Correlations between groups were analyzed by the Pearson correlation coefficient. Time on treatment and overall survival were

determined by the Kaplan-Meier method, and the log-rank test was used for comparisons of survival between groups. The Cox proportional hazards model was used to estimate the hazard ratio (HR) and 95% confidence interval (CI). All P values are two-sided. The level of statistical significance was set at $P < 0.05$.

3 | RESULTS

3.1 | YB-1 amplification in prostate cancer

First, we explored the public database for YB-1 gene alterations in prostate cancer. In the study by Grasso et al.,¹⁸ which investigated

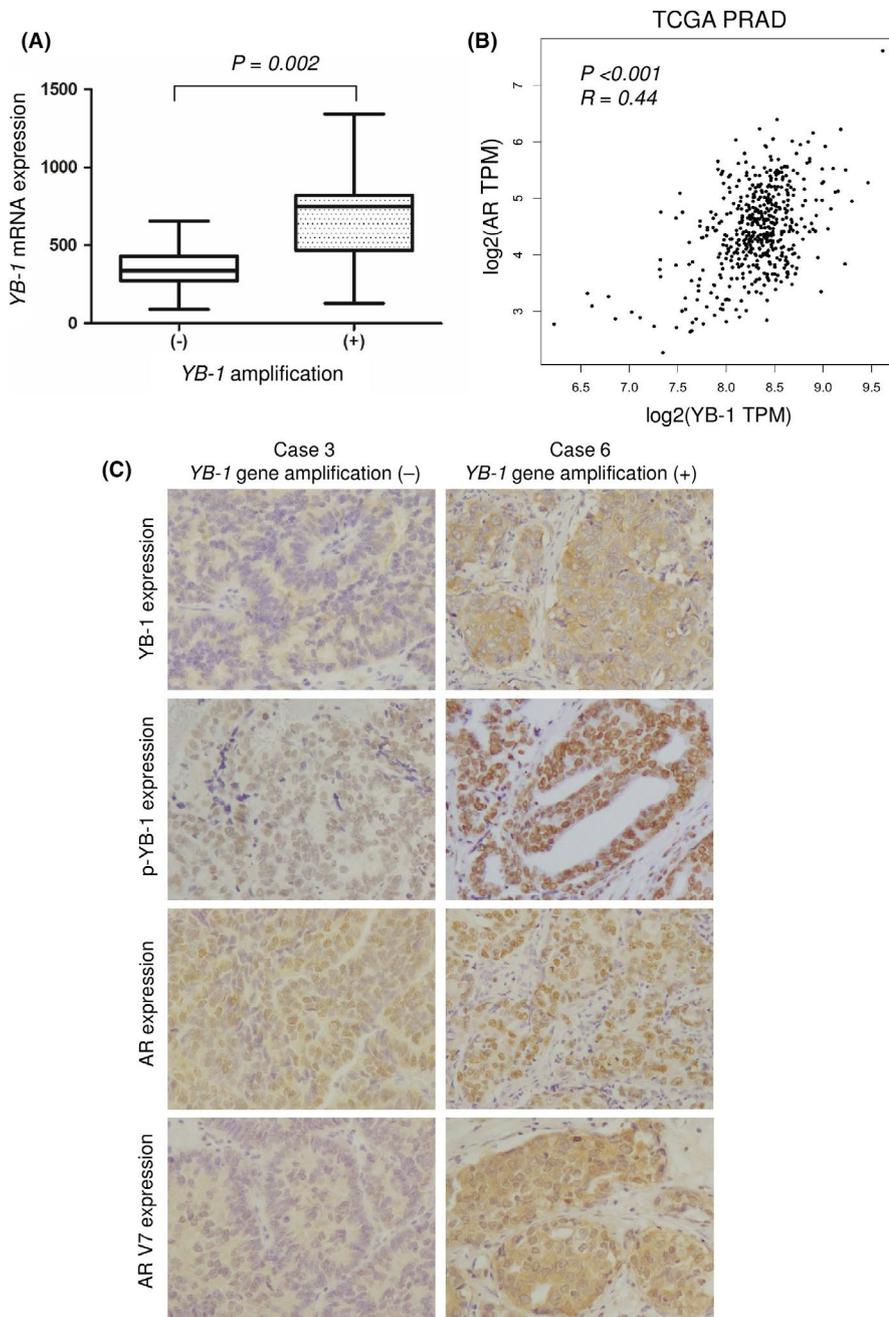


FIGURE 2 Expression levels of Y-box binding protein-1 (YB-1) and its related molecules in tissues. A, YB-1 mRNA expression levels were determined in tumors without YB-1 amplification (n = 197) and with YB-1 amplification (n = 15), using data obtained from the public datasets of Abida et al.²⁰ Box-and-whisker plots depict the median with the first and third quartiles. Data represent means \pm SD. B, Correlations between YB-1 and AR mRNA expression levels were determined using data obtained from public datasets in the GEPIA browser (<http://gepia.cancer-pku.cn/index.html>). PRAD, prostate adenocarcinoma; TPM, transcripts per million; TCGA, The Cancer Genome Atlas. C, Expression levels of YB-1, p-YB-1, androgen receptor (AR), and AR V7 were determined in castration-resistant prostate cancer tissues. Representative images of high-magnification fields (200 \times) are shown. Weak and strong staining of YB-1, p-YB-1, AR, and AR V7 were observed in Case 3 without YB-1 amplification and Case 6 with YB-1 amplification, respectively

the landscape of genomic alterations between primary prostate cancer and CRPC, YB-1 amplification was observed in four of 50 CRPC cases compared with zero of 11 treatment-naïve localized prostate cancer cases (Figure 1A). In the study by Beltran et al,¹⁹ which investigated the landscape of genomic alterations between adenocarcinoma-type CRPC (CRPC-adeno) and neuroendocrine-type CRPC (CRPC-NE), YB-1 amplification was observed in five of 70 CRPC-adeno cases compared with seven of 44 CRPC-NE cases (Figure 1B). Further analysis of the public database revealed that YB-1 amplification was observed in various cancers, including ovarian cancer, uterine cancer, and bladder cancer, according to The Cancer Genome Atlas (TCGA) Pan-Cancer Atlas Studies (Figure S1).

Thus, the presence of YB-1 amplification was suggested by the data in the public database. Accordingly, we examined YB-1 amplification in CRPC cell sublines (CxR and C4-2 cells) established from androgen-dependent LNCaP cells and AR-null PC-3 cells. As shown in Figure 1C, aneuploidy of the YB-1 gene was detected in both CxR and C4-2 cells, compared with diploidy in parental LNCaP cells. YB-1 amplification was defined as greater than 25% increased ploidy. Subsequently, we examined YB-1 amplification in tissues derived from patients with CRPC. The patient background characteristics are shown in Table S1. Among the eight CRPC tissues, YB-1 amplification (more than 25% increased ploidy) was observed in the tissues from Case 6 and Case 7 (Figure 1D). Meanwhile, AR amplification (more than triploidy) was observed in four patients

TABLE 1 Immunohistochemical staining of Y-box binding protein-1 (YB-1), p-YB-1, androgen receptor (AR), and AR V7 in hormone-sensitive prostate cancer (HSPC) and castration-resistant prostate cancer (CRPC) tissues

Variable	HSPC (n = 5)	CRPC (n = 8)	P value
	Median (range)	Median (range)	
YB-1	0.04 (0.03-0.24)	0.19 (0.06-0.69)	0.019*
p-YB-1	0.84 (0.12-0.98)	0.92 (0.67-0.99)	0.46
AR	0.92 (0-1.83)	1.61 (0.79-1.81)	0.14
AR V7	0.16 (0-1.03)	1.67 (0-2.71)	0.12

*Significant difference.

TABLE 2 Correlations among Y-box binding protein-1 (YB-1), p-YB-1, androgen receptor (AR), and AR V7 determined by immunohistochemical staining

Variable	YB-1	p-YB-1	AR	AR V7
YB-1	-	0.046 (P = .88)	0.44 (P = .14)	0.56 (P = .047*)
p-YB-1	-	-	0.48 (P = .098)	0.30 (P = .32)
AR	-	-	-	0.13 (P = .66)
AR V7	-	-	-	-

*Significant difference. Dash, blank.

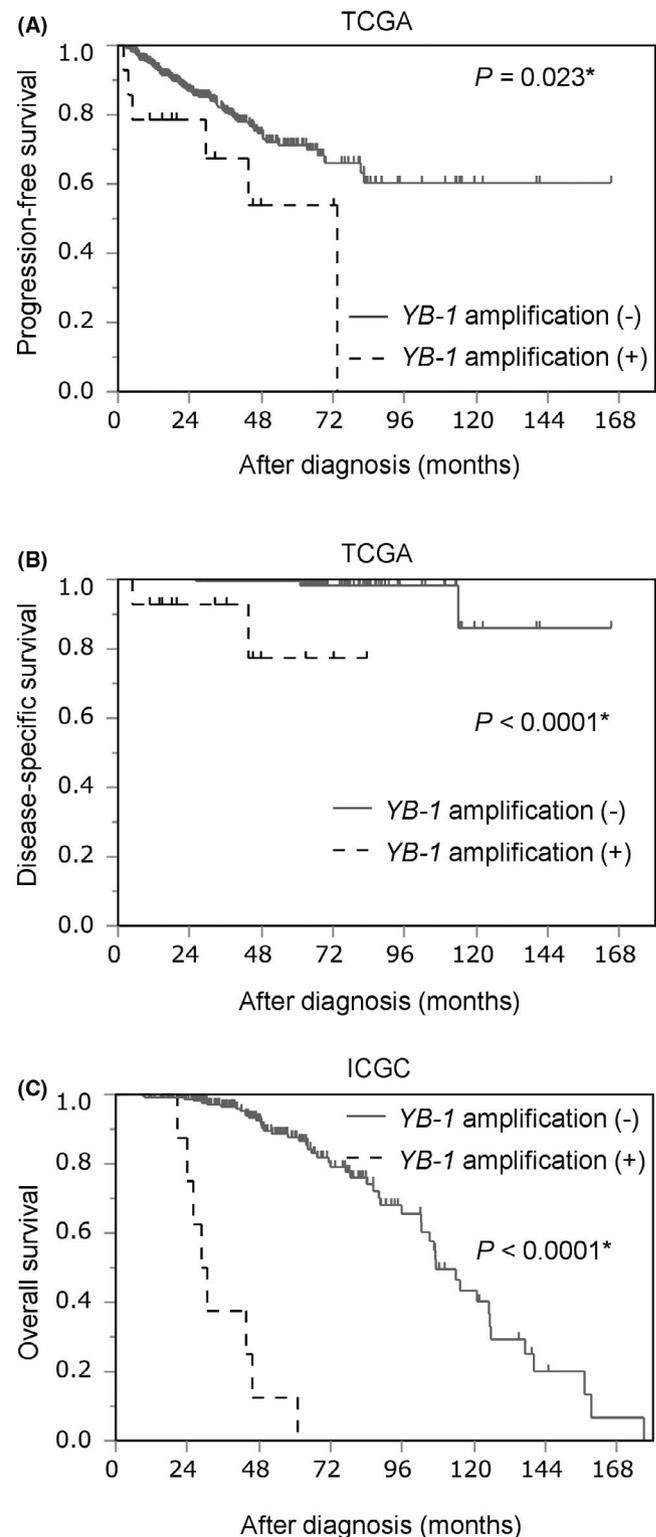
(50%) with CRPC, but not in prostate cancer cells, except PC-3 cells (Figure S2). AR amplification was observed in both cases with YB-1 amplification.

3.2 | Association between YB-1 and AR expressions in metastatic prostate cancer tissues

Using the public database, we investigated the association between YB-1 amplification and YB-1 mRNA expression. In the study by Abida et al,²⁰ data on mRNA expressions were available in 212 patients with metastatic CRPC. As shown in Figure 2A, YB-1 mRNA expression was higher in 15 cases with YB-1 amplification. In addition, a positive correlation between YB-1 mRNA and AR mRNA expressions in multiple cohorts was observed using the public database (Figure 2B).

FIGURE 3 Association between Y-box binding protein-1 (YB-1) amplification and clinical outcomes in hormone-naïve prostate cancer. A, B, Kaplan-Meier curves showing (A) progression-free survival and (B) disease-specific survival in patients with YB-1 amplification (n = 15) and without YB-1 amplification (n = 474 in A; n = 473 in B) when copy numbers of YB-1 with $\log_2 > 0.1$ were defined as positive amplification, using data obtained from the public datasets of Liu et al.²¹ C, Kaplan-Meier curves showing the overall survival in patients with YB-1 amplification (n = 8) and without YB-1 amplification (n = 238) when copy numbers of YB-1 with $\log_2 > 0$ were defined as positive amplification, using data obtained from public datasets in the UCSC Xena browser (<https://xena.ucsc.edu/>). ICGC, International Cancer Genome Consortium; TCGA, The Cancer Genome Atlas

To further elucidate the significance of YB-1 amplification in clinical samples, we investigated the protein levels of YB-1 and its related molecules including p-YB-1, AR, and AR V7 (Figure 2C). The YB-1 expression in CRPC tissues was significantly increased compared with the levels in treatment-naïve tissues (Table 1). The levels of p-YB-1, AR, and AR V7 also tended to be increased in CRPC tissues, but statistical significance was not reached, probably because of the small sample number (Table 1). Positive correlations



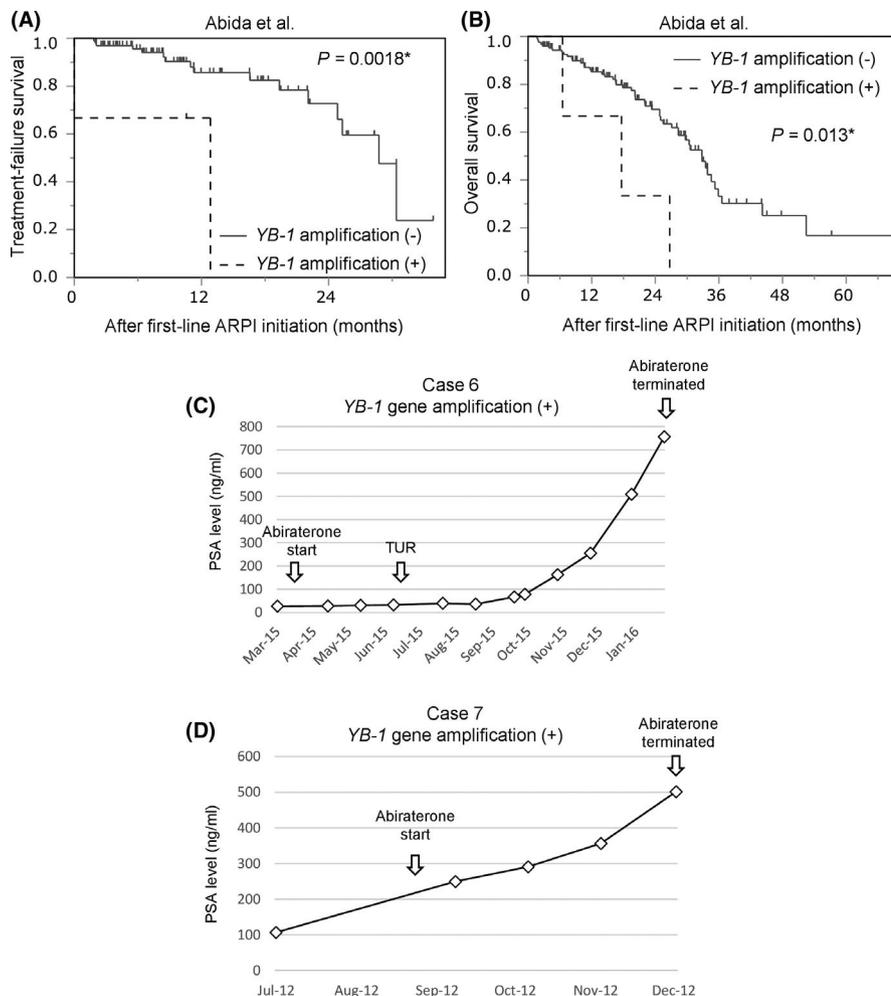


FIGURE 4 Association between Y-box binding protein-1 (YB-1) amplification and clinical outcomes in castration-resistant prostate cancer. A, B, Kaplan-Meier curves showing (A) survival with a first-line androgen receptor pathway inhibitor (ARPI) and (B) overall survival after initiation of a first-line ARPI in patients with YB-1 amplification ($n = 3$) and without YB-1 amplification ($n = 105$ in A; $n = 125$ in B), using data obtained from the public datasets of Abida et al.²⁰ C, D, Kinetics of prostate-specific antigen (PSA) levels during abiraterone treatment in (C) Case 6 (C) and (D) Case 7. TUR, transurethral resection

between YB-1 or p-YB-1 and AR or AR V7 expressions were also observed (Table 2).

3.3 | Association between YB-1 amplification and clinical outcomes in prostate cancer

Subsequently, we investigated the prognostic impact of YB-1 amplification in hormone-naïve prostate cancer and CRPC. In the TCGA study,²¹ YB-1 amplification was associated with worse progression-free survival (HR, 2.53; 95% CI, 1.10-5.79; $P = .029$) and disease-specific survival (HR, 28.55; 95% CI, 4.00-203.7; $P = .0008$) (Figure 3A,B). In the ICGC study,²³ YB-1 amplification was associated with poor overall survival (HR, 22.53; 95% CI, 9.66-52.53; $P < .0001$) (Figure 3C).

The study by Abida et al²⁰ included 128 patients who were taxane-naïve and initiating therapy with a first-line ARPI (abiraterone or enzalutamide) for metastatic CRPC. Time on treatment and overall survival data were available in 108 and 128 patients, respectively. YB-1 amplification was observed in three cases, among whom significantly worse time on treatment with a first-line ARPI and overall survival after a first-line ARPI were observed (Figure 4A,B).

Both Case 6 and Case 7 with YB-1 amplification carried a low-volume metastatic burden defined by the CHARTED criteria,²⁶ and time to CRPC was relatively long. Docetaxel chemotherapy was given before abiraterone, but terminated with two cycles due to adverse events in both patients. In Case 6, enzalutamide was given, but the patient stopped treatment due to fatigue. No other life-prolonging agent, including cabazitaxel and radium-223, was utilized in both patients. Surprisingly, however, both patients showed primary resistance to abiraterone, as indicated by the continuous prostate-specific antigen rise after abiraterone treatment, and the disease became lethal within 4 months after termination of abiraterone treatment (Figure 4C,D).

4 | DISCUSSION

To date, despite various experimental data showing increased YB-1 expression in CRPC, there have been no clinical data on YB-1 expression in human CRPC tissues. To our knowledge, we have shown for the first time that YB-1 expression was increased in CRPC tissues compared with tissue-naïve tissues. Thus, YB-1 overexpression in CRPC was robustly shown in both preclinical and clinical settings.

The mechanism of YB-1 overexpression in CRPC has also remained unclear. The present study showed that YB-1 amplification is one of the mechanisms responsible for YB-1 overexpression. This novel finding might be applicable to other cancers with YB-1 amplification (Figure S1). However, the degree of YB-1 amplification (approximately 25% increase) was not very large compared with the increase in YB-1 expression in CRPC tissues. In the CRPC cell sublines examined, YB-1 mRNA levels were increased approximately 8-fold in CxR cells and approximately 2-fold in C4-2 cells, in which consistently increased YB-1 and p-YB-1 levels were observed.^{7,9,11} In addition, YB-1 overexpression was observed in CRPC tissues from patients without YB-1 amplification. These data suggest that other mechanisms, including epigenetic regulation, are also involved in YB-1 overexpression. Actually, several signal transduction pathways, such as Akt, ERK, and ribosomal S6 kinase (RSK), as well as transcription factors, such as Twist1, were shown to regulate YB-1 expression in prostate cancer.^{9,11-14} Collectively, both genetic and epigenetic mechanisms are considered to contribute to YB-1 overexpression in CRPC.

Previously, we showed that YB-1 regulated the expression of AR and AR variants at the transcription and splicing levels.^{7,11} Consistently, increased expressions of YB-1 and AR were observed in CRPC preclinical models.^{7,9,11,26} Furthermore, a positive association between nuclear YB-1 and AR was found in nonmetastatic prostate cancer specimens obtained by radical prostatectomy.⁷ In line with these findings, the present study showed a robust association between YB-1 and AR signaling in metastatic prostate cancer samples from patients, as well as an association between YB-1 and AR variants in patients. Higher prevalence of YB-1 amplification was also observed in CRPC-NE tumors (Figure 1B), which usually lack AR activity. Other important roles of YB-1 in non-AR signaling have been suggested. Actually, YB-1 has been shown to regulate multiple key functions in cancer such as epithelial-mesenchymal transition, angiogenesis, cell proliferation, and cell survival, which are critical in CRPC progression.^{5,6,27} Thus, YB-1 is thought to play critical roles in the progression to CRPC through both AR and non-AR signaling.

Collectively, the present findings indicated that YB-1 amplification plays a critical role in progression to CRPC as well as therapeutic resistance to ARPIs through regulation of AR and AR variants.²⁸⁻³² Therefore, YB-1 is a promising therapeutic target in prostate cancer. We previously showed that a small-molecule inhibitor for RSK, a kinase that regulates YB-1 function, had excellent anticancer effects, especially in combination with ARPI and taxane.^{9,11,33} Consistently, fisetin targeting the RSK2/YB-1 axis exerted strong anticancer effects in a melanoma model.³⁴ Furthermore, a class I HDAC inhibitor, MS-275, enhanced YB-1 acetylation, and showed effective anticancer activity in a sarcoma model.³⁵ Currently, we are investigating the therapeutic efficacy of a novel YB-1 inhibitor in prostate cancer as a preclinical study (data not shown). Therefore, among the YB-1 inhibitors, some agents can be expected to proceed to clinical trials and bring benefits to patients.

5 | CONCLUSION

In conclusion, the present study revealed frequent occurrence of YB-1 amplification in CRPC, thereby leading to activation of the YB-1/AR axis responsible for resistance to hormone therapy, including ADT and ARPI therapy. Thus, YB-1 is a promising therapeutic target, and YB-1 amplification could be helpful to identify putative candidates for treatment with YB-1 inhibitors.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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