Mutual Regulation between Raf/MEK/ERK Signaling and Y-Box–Binding Protein-1 Promotes Prostate Cancer Progression

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Abstract

Purpose: Y-box–binding protein-1 (YB-1) is known to conduct various functions related to cell proliferation, anti-apoptosis, epithelial-mesenchymal transition, and castration resistance in prostate cancer. However, it is still unknown how YB-1 affects cancer biology, especially its correlations with the mitogen-activated protein kinase (MAPK) signaling pathway. Therefore, we aimed to examine the interaction between YB-1 and the MAPK pathway in prostate cancer.

Experimental Design: Quantitative real-time PCR, Western blotting, and co-immunoprecipitation assay were conducted in prostate cancer cells. YB-1, phosphorylated YB-1 (p-YB-1), and ERK2 protein expressions in 165 clinical specimens of prostate cancer were investigated by immunohistochemistry. YB-1, p-YB-1, and ERK2 nuclear expressions were compared with clinicopathologic characteristics and patient prognoses.

Results: EGF upregulated p-YB-1, whereas MEK inhibitor (U0126, PD98059) decreased p-YB-1. Inversely, silencing of YB-1 using siRNA decreased the expression of ERK2 and phosphorylated MEK, ERK1/2, and RSK. Furthermore, YB-1 interacted with ERK2 and Raf-1 and regulated their expressions, through the proteasomal pathway. Immunohistochemical staining showed a significant correlation among the nuclear expressions of YB-1, p-YB-1, and ERK2. The Cox proportional hazards model revealed that high ERK2 expression was an independent prognostic factor [HR, 7.947; 95% confidence interval (CI), 3.527–20.508; P < 0.0001].

Conclusion: We revealed the functional relationship between YB-1 and MAPK signaling and its biochemical relevance to the progression of prostate cancer. In addition, ERK2 expression was an independent prognostic factor. These findings suggest that both the ERK pathway and YB-1 may be promising molecular targets for prostate cancer diagnosis and therapeutics.

Introduction

Prostate cancer is the most common noncutaneous cancer and the second leading cause of male cancer-related mortality in developed countries. Screening using prostate-specific antigen (PSA) has dramatically improved the early detection of prostate cancer and has been proved to improve cancer-specific as well as overall survival (1). However, 20% to 30% of patients with localized prostate cancer who received surgical or radiation therapy experience biochemical recurrence as defined by a PSA increase (2, 3). In addition, many patients with prostate cancer are still only diagnosed at an advanced stage of disease. As the majority of cases of prostate cancer are androgen-dependent at diagnosis, androgen deprivation therapy (ADT) prevents further growth and leads to tumor regression in most patients. However, most tumors will relapse in a castration-resistant manner after less than 24 months from initiating ADT and are thus designated castration-resistant prostate cancer (CRPC) tumors (4); these tumors remain a serious obstacle to overcome due to the dearth of therapeutic options for CRPC.

Y-box–binding protein-1 (YB-1) plays various biologic roles in both the nucleus and cytoplasm (5). As a transcription factor in nucleus (6), YB-1 regulates the expression of various genes, including proliferating cell nuclear antigen (PCNA), EGF receptor (EGFR), DNA topoisomerase II (7), CD44 (8), phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA; ref. 9), and MET
Translational Relevance

Y-box–binding protein-1 (YB-1) is known to conduct various functions related to cell proliferation, anti-apoptosis, and epithelial–mesenchymal transition as well as castration resistance in prostate cancer. It is still unknown how YB-1 affects cancer biology, especially its correlations with the mitogen-activated protein kinase (MAPK) signaling pathway. We revealed that YB-1 interacted with and regulated ERK2 and Raf-1 through the proteosomal pathway, whereas the ERK pathway regulated YB-1 activity. Furthermore, mutual regulation between the ERK pathway and YB-1 as well as the prognostic significance of this inter-regulation was confirmed in human prostate cancer tissues. Thus, this study indicated the cancer biologic relevance of the interactions between the ERK pathway and YB-1, suggesting their diagnostic and therapeutic significances in prostate cancer.

(10). As well, YB-1 was shown to regulate gene expression at a translational level (11). It is well known that YB-1 expression is closely associated with cell growth, antiapoptosis, and epithelial–mesenchymal transition (EMT) as well as clinical outcome in various cancers (5, 6, 11, 12). 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 androgen-dependent prostate cancer into CRPC (13). In addition, we reported that YB-1 regulates androgen receptor (AR) transcription and promotes the pathogenesis of CRPC (14).

Intracellular signaling pathways, which mediate the levels of various extracellular stimuli, including growth factors and cytokines, to initiate a wide range of gene expressions through transcription factors including YB-1, are known to play a critical role in the pathogenesis of various cancers including prostate cancer. Among several intracellular signaling pathways thus far been identified, the phosphoinositide-3-kinase (PI3K)/Akt/mTOR (15, 16) and Ras/mitogen-activated protein kinase (MAPK)/RSK pathways (11, 17–21) have been reported to be involved in YB-1 signaling by several studies. In addition, both the PI3K/Akt/mTOR and Ras/MAPK/RSK signaling pathways are well known to play a key role in the pathogenesis of various types of cancer, including prostate cancer (22, 23). Especially, aberrant activation of ERK has been shown in prostate cancer tissues (24), which was supported by various experimental studies indicating a tumor-promoting role of extracellular signal-regulated kinase (ERK) signaling (22, 23).

Because intracellular signaling pathways and YB-1 have both been shown to play roles in cancer pathology, we designed the present study to examine possible links between the actions of YB-1 and the Ras/MEK/ERK pathway in the pathology of various cancers, with a focus on prostate cancer.

Materials and Methods

Cell culture

Human prostate cancer PC-3 [Eagle’s Minimal Essential Medium (MEM)] and LNCaP cells (RPMI)-1640 medium) were cultured in the media recommended by the supplier. The media were purchased from Invitrogen and contained 10% FBS. Cell lines were maintained in a 5% CO2 atmosphere at 37°C.

Antibodies

Antibodies against A-Raf (sc-408), B-Raf (sc-166), Raf-1 (sc-133), and ERK2 (sc-1647) were purchased from Santa Cruz Biotechnology. Anti-EGFR (#2232), anti-phosphorylated EGFR\(^{\text{Y1068}}\) (p-EGFR; #3777), anti-HER2 (#2165), anti-phosphorylated HER2\(^{\text{Y1212/1222}}\) (p-HER2; #2243), anti-HER3 (#4754), anti-phosphorylated YB-1\(^{\text{Ser102}}\) (p-YB-1; #2900), anti-\(\alpha\)-tubulin (#2125), anti-MEK1/2 (#9122), anti-phosphorylated MEK1/2\(^{\text{Ser217/221}}\) (p-MEK1/2; #9121), anti-ERK1/2 (#9102), anti-phosphorylated ERK1/2\(^{\text{Thr202/Tyr204}}\) (p-ERK1/2; #4376), anti-STAT3 (#9139), anti-phosphorylated STAT3\(^{\text{Ser727/722}}\) (p-STAT3; #9134), anti-JNK (#9258), anti-phosphorylated JNK\(^{\text{Thr183/Tyr185}}\) (p-JNK; #4668), anti-p38 (#9212), anti-phosphorylated p38\(^{\text{Thr202/Tyr204}}\) (p-p38; #4511), anti-p90RSK (RSK; #9355), anti-phosphorylated p90RSK\(^{\text{Thr359/366}}\) (p-RSK; #9344), anti-Akt (#9272), and anti-phosphorylated Akt\(^{\text{Ser473}}\) (p-Akt; #4060) antibodies were obtained from Cell Signaling Technology. Antibodies against Lamin B1 (ab16048), YB-1 (2397-1), and \(\beta\)-actin (A3854) were purchased from Abcam, Epitomics, and Sigma, respectively.

Western blotting analysis

Whole-cell, nuclear, and cytoplasmic extracts were prepared as described previously (25). The protein concentration was quantified with a protein assay (Bio-Rad) based on the Bradford method. Similar amounts of extract were separated by 4% to 20% SDS-PAGE and transferred onto polyvinylidene difluoride microporous membranes (GE Healthcare Bio-Science) using a semidy blotter. Blotted membranes were incubated for 1 hour at room temperature with the above-mentioned primary antibodies. The membranes were then incubated for 40 minutes 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).

Immunocytochemistry

Immunocytochemical staining was conducted by using an EnVision Plus detection system (DAKO), with the primary antibodies against p-YB-1 (1:50 dilution), p-ERK1/2Thr202/Tyr204 (p-ERK1/2; #4376), anti-STAT3 (p-STAT3; #9134), anti-JNK (#9258), anti-phosphorylated JNK\(^{\text{Thr183/Tyr185}}\) (p-JNK; #4668), anti-p38 (#9212), anti-phosphorylated p38\(^{\text{Thr202/Tyr204}}\) (p-p38; #4511), anti-p90RSK (RSK; #9355), anti-phosphorylated p90RSK\(^{\text{Thr359/366}}\) (p-RSK; #9344), anti-Akt (#9272), and anti-phosphorylated Akt\(^{\text{Ser473}}\) (p-Akt; #4060) antibodies were purchased from Cell Signaling Technology. Antibodies against Lamin B1 (ab16048), YB-1 (2397-1), and \(\beta\)-actin (A3854) were purchased from Abcam, Epitomics, and Sigma, respectively.

Reagents

Recombinant human EGF (236-EG), cycloheximide, and PD98059 (#9900) were purchased from R&D Systems, nacalai tasque, and Cell Signaling Technology, respectively. MG132 and U0126 were purchased from Calbiochem.

Materials and Methods

Cell culture

Human prostate cancer PC-3 [Eagle’s Minimal Essential Medium (MEM)] and LNCaP cells (RPMI)-1640 medium) were cultured in the media recommended by the supplier. The media were purchased from Invitrogen and contained 10% FBS. Cell lines were maintained in a 5% CO2 atmosphere at 37°C.
(1:100), and YB-1 (1:250). We fixed cultured cells on a culture slide for 20 minutes in methanol/acetone at 4°C. Endogenous peroxidase activity was blocked for 20 minutes by methanol containing 0.3% hydrogen peroxide. Next, we permeabilized the cells for 15 minutes with 0.2% Triton X-100 (Sigma). Antigen retrieval was achieved by microwave heating for 5 minutes with sodium citrate buffer (pH 6.0). The sections were incubated for 60 minutes with primary antibody at room temperature, followed by staining for 30 minutes with the secondary antibody/peroxidase-linked polymers (EnVision + DAKO) at room temperature. The reaction products were visualized by diaminobenzidine tetrahydrochloride as a chromogen. Finally, the sections were counterstained with hematoxylin.

**Knockdown analysis using siRNAs**

The following double-stranded RNA 25-base-pair oligonucleotides were commercially generated (Invitrogen): 5'-UGGAIAGCCGUCUAAUUGGUUACC-3' (sense) and 5'-CCGUAACCAUIAAUCAGCGCUAACCA-3' (antisense) for YB-1 #1; 5'-UUGCUGGUAUUGCGUGAGGCC-3' (sense) and 5'-GGUUCUCCACCGAAUUIAACCAGAAA-3' (antisense) for YB-1 #2; 5'-GGUCACCIACAACACAGACGGUUIII-3' (sense) and 5'-AAACGUGUCUUGUGUGUGAGGUGC-3' (antisense) for HER2; and 5'-CCAIAAGAACAIUAGAACCAAIII-3' (sense) and 5'-AUUGUUGGGAUUGGUCUCUAUGGC-3' (antisense) for Raf-1. LNCaP and PC-3 cells were transfected with siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

**RNA isolation, reverse transcription, and quantitative reverse transcriptase PCR**

These procedures were conducted as previously described (25). Quantitative reverse transcriptase (RT)-PCR was carried out using TaqMan Gene Expression Assays for ERK2 (Hs01046830_m1), HER2 (Hs01001580_m1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Hs02758991_g1; Applied Biosystems) with the TaqMan Gene Expression Master Mix (Applied Biosystems) on a 7900HT PCR system (Applied Biosystems). The transcript levels of the indicated genes were corrected according to the corresponding GAPDH transcript levels. All values represent the results of at least 3 independent experiments.

**Co-immunoprecipitation assay**

Coimmunoprecipitation assays were conducted as previously described (26). Briefly, LNCaP and PC-3 were lysed in buffer X containing 50 mmol/L Tris-HCl (pH 8.0), 1 mmol/L EDTA, 120 mmol/L NaCl, 0.5% (v/v) Nonidet P-40, 10% (v/v) glycerol, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L dithiothreitol. The lysates were centrifuged at 21,000 × g for 10 minutes at 4°C, and the supernatants (500 µg) were incubated for 2 hours at 4°C with 2.0 µg of rabbit immunoglobulin G (IgG) or anti-YB-1 antibody with 20 µl protein A/G agarose (Santa Cruz Biotechnology). The immunoprecipitated samples were washed 3 times with buffer X, and the immunoprecipitated samples (50 µg) were subjected to Western blotting with the indicated antibodies.

**Patient characteristics and tissue collection**

Clinical specimens were obtained from 165 patients who had received radical prostatectomy without neoadjuvant chemotherapy or hormonal therapy at Kyushu University Hospital (Japan) between 1998 and 2006. The median age of the patients was 67 years (range, 47–78) and the median PSA value was 8.6 ng/mL (range, 0.61–51 ng/mL). Twenty-nine of the patients had tumors graded as Gleason score (GS) 3 + 3, 67 as GS 3 + 4, 46 as GS 4 + 3, and 23 as GS ≥ 8. There were 109 tumors in stage T2 and 56 in stage T3. Recurrence-free survival was defined as the time from radical prostatectomy to the date of PSA recurrence (PSA level ≥ 0.2 ng/mL) or last follow-up. Slides were prepared from prostate blocks that contained the largest and most representative area of the tumors whose areas were large enough for immunohistochemical evaluation.

The present study was conducted in accordance with the principles embodied in the Declaration of Helsinki. The study was also approved by the Ethics Committee of Kyushu University (No. 24–96) and conducted according to the Ethical Guidelines for Epidemiological Research enacted by the Japanese Government.

**Immunohistochemistry**

Immunohistochemical staining was conducted by using the EnVision Plus detection system, with the primary antibodies against YB-1 (1:250), p-YB-1 (1:50), and ERK2 (1:100). Four-micrometer-thick sections, from 10% formalin-fixed, paraffin-embedded material, were deparaffinized in xylene and rehydrated through ethanol. Endogenous peroxidase activity was blocked for 30 minutes by methanol containing 0.3% hydrogen peroxide. Antigen retrieval was conducted by microwave heating for 20 minutes with sodium citrate buffer (pH 6.0). Then, the sections were incubated at 4°C overnight with primary antibody, followed by staining for 40 minutes with the secondary antibody/peroxidase-linked polymers at room temperature. The reaction products were visualized by diaminobenzidine tetrahydrochloride as a chromogen. Finally, the sections were counterstained with hematoxylin.

**Immunohistochemical analysis**

Immunohistochemical results were judged independently by 3 investigators (K. Imada, K. Kohashi, and Y. Oda) who were blinded to the clinicopathologic variables. Nuclear expressions of YB-1, p-YB-1, and ERK2 were assessed by counting the stained nuclei in at least 1,000 tumor cells, selected from the areas of greatest accumulation of positive signals (hotspots). For YB-1, p-YB-1, and ERK2 nuclear expression, cut-off values of 10% (14, 27), 41% (median), and 62% (median) were used, respectively. For scoring YB-1 cytoplasmic expression, Allred score was used (28). The proportion of stained cells divided into 6 categories as follow: 0, completely negative; 1, <1%; 2, 1%–10%; 3, 11%–33%; 4,
34%–66%; 5, 67%–100%, and staining intensity was divided into 4 categories as follow: 0, negative; 1, weak; 2, intermediate; 3, strong. Finally, high cytoplasmic YB-1 expression was defined as total score (TS) of ≥ 7.

Statistical analysis
We used the χ² test to analyze correlations between YB-1, p-YB-1, and ERK2 expression levels and clinicopathologic parameters. The survival curves were depicted by the Kaplan–Meier method, and survival analyses were conducted by using the log-rank test. Cox proportional hazards model was used to estimate the HRs and 95% confidence intervals (CI). Values of P < 0.05 were considered to be statistically significant. The results were analyzed with JMP 9.0.2 software (SAS Institute Inc.).

Results
EGF stimulates Raf/ERK/RSK signaling, resulting in YB-1 phosphorylation
First, we confirmed the effects of EGF on intracellular signaling as well as YB-1 activity using androgen receptor (AR)-positive LNCaP and AR-null PC-3 cells. As expected, EGF induced rapid activations of EGF receptor (EGFR) and its downstream factors, including Raf-1, MEK, and ERK1/2, in both LNCaP and PC-3 cells (Fig. 1A). After activations of these molecules, RSK and YB-1, which are known to be downstream molecules of ERK1/2 (18, 29), were phosphorylated in both LNCaP and PC-3 cells (Fig. 1A). Concurrently, Akt phosphorylation was induced slightly in LNCaP cells and prominently in PC-3 cells (Fig. 1A). To reveal the correlation between phosphorylation status and

![Figure 1. EGF stimulates Raf/ERK/RSK signaling, resulting in YB-1 phosphorylation. A, LNCaP and PC-3 cells were incubated with serum-free media for 24 hours and treated with 50 ng/mL of EGF for the indicated duration. Whole-cell extracts were subjected to SDS-PAGE and Western blotting. B, LNCaP cells were incubated with serum-free media for 24 hours and treated with 50 ng/mL of EGF for 30 minutes. After fractionation, nuclear and cytoplasmic extracts were subjected to SDS-PAGE and Western blotting. C, LNCaP cells were incubated with serum-free media for 24 hours and treated with 50 ng/mL of EGF for the indicated duration. After the cells were fixed, immunocytochemistry was conducted. Representative images of high-magnification fields (×400) are shown. D, LNCaP cells were incubated with serum-free media for 24 hours and treated with 10 μmol/L of U0126 or 10 μmol/L of PD98059 for 1 hour before 50 ng/mL of EGF for the indicated duration. Whole-cell extracts were subjected to SDS-PAGE and Western blotting. N, nuclear; C, cytoplasm.](https://www.aacrjournals.org/files/figure1.png)
intracellular localization, we conducted fractionation of cells treated with or without EGF. The result showed that phosphorylated ERK1/2, RSK, and YB-1 were localized in both the nucleus and cytoplasm, although total ERK1/2, RSK, and YB-1 were mainly located in the cytoplasm, suggesting that phosphorylated ERK1/2, RSK, and YB-1 prefer nuclear localization (Fig. 1B). A similar result that phosphorylated ERK1/2 and YB-1 localized mainly in nucleus whereas total YB-1 localization was little affected by EGF was obtained in LNCaP cells by immunocytochemistry (Fig. 1C). In addition, it was confirmed that this stimulation of YB-1 by EGF was mediated by the MEK/ERK pathway using the MEK inhibitor U0126 and PD98059 in LNCaP cells, inhibiting MEK1/2 and MEK1, respectively (Fig. 1D, Supplementary Fig. S1).

**YB-1 expression affects Raf/ERK/RSK signaling**

We next examined the effect of YB-1 on ERK/RSK signaling in LNCaP and PC-3 cells. Both of the YB-1–specific siRNAs described above successfully suppressed YB-1 expression in both LNCaP and PC-3 cells (Fig. 2A). In addition, YB-1 knockdown downregulated the phosphorylation levels of MEK, ERK1/2, and RSK in both LNCaP and PC-3 cells and the phosphorylation levels of STAT3 in LNCaP cells (STAT3 was not detectable in PC-3 cells; Fig. 2A). Although total ERK1 expression was unaffected, total ERK2 expression was downregulated by YB-1 silencing. On the other hand, other signaling pathways, including the JNK and p38 signaling pathways, were not affected by YB-1 knockdown (Fig. 2A). Furthermore, stimulation of the MAPK pathway by EGF in LNCaP and PC-3 cells was blunted by YB-1 knockdown, as indicated by the downregulated phosphorylation levels of Raf-1, MEK, ERK1/2, and RSK, but not EGFR (Fig. 2B, Supplementary Fig. S2A and S2B).

**YB-1 regulates ERK2 protein levels, at least in part, via an increase in protein stability**

Because ERK2 expression was downregulated by YB-1 knockdown, we next tried to elucidate the mechanism of ERK2 downregulation. First, quantitative RT-PCR was carried out against ERK2 mRNA expression after YB-1 suppression. As shown in Fig. 3A, ERK2 mRNA expression was not affected by YB-1 suppression. Because YB-1 is known to

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**Figure 2.** YB-1 expression affects Raf/ERK/RSK signaling. A, LNCaP and PC-3 cells were transfected with 40 nmol/L of the indicated siRNA, and incubated for 72 hours. Whole-cell extracts were subjected to SDS-PAGE and Western blotting. B, LNCaP and PC-3 cells were transfected with 40 nmol/L of the indicated siRNA and incubated for 72 hours, then incubated with serum-free media for 24 hours, and treated with 50 ng/mL of EGF for the indicated duration. Whole-cell extracts were subjected to SDS-PAGE and Western blotting.
regulate the translation of various genes (30), we examined the possibility of translational regulation of ERK2 by YB-1. The decrease in the ERK2 protein level by cycloheximide was accelerated by YB-1 knockdown in both LNCaP and PC-3 cells, indicating that posttranslational regulation was involved in the ERK2 regulation by YB-1 although the possibility involving translational effect of YB-1 cannot be denied (Fig. 3B, Supplementary Fig. S3A). Subsequently, regulation of the protein level by the degradation pathway was examined. We conducted an immunoprecipitation assay to analyze the interaction between YB-1 and ERK proteins. After YB-1 protein was immunoprecipitated by a YB-1–specific antibody using whole-cell lysates from LNCaP and PC-3 cells, we examined whether YB-1 would form an immunocomplex with MEK and ERK1/2. As shown in Fig. 3C, ERK2 protein was specifically detected in complex with YB-1, although MEK and ERK1 were not, suggesting direct or indirect interaction between YB-1 and ERK2. Finally, we examined the ERK protein level with or without YB-1 knockdown after using proteasome inhibitor MG132. The results showed that the ERK2 protein levels remained detectable after YB-1 knockdown when MG132 was used (Fig. 3D, Supplementary Fig. S3B).

**YB-1 specifically regulates Raf-1 protein stability, affecting an activity of the MAPK pathway**

Although the previous assay indicated that YB-1 regulated the MAPK pathway by regulating the ERK2 protein, this result cannot account for the effect of YB-1 on the Raf/MEK/ERK activity, as shown in Fig. 2. Therefore, we tried to elucidate the mechanism underlying the effect of YB-1 on Raf/MEK/ERK activity. First, we considered that YB-1...
regulated the MEK/ERK pathway via the expression of EGFR family proteins, because the regulation of EGFR, HER2, and HER3 by YB-1 and the correlated expression between the EGFR family proteins (EGFR, HER2, and HER3) and YB-1 have been reported in various cancers other than prostate cancer (31–37). Therefore, we examined the expression levels of EGFR, HER2, and HER3 after YB-1 knockdown, and the results indicated that HER2 expression was downregulated in both LNCaP and PC-3 cells (Supplementary Fig. S4A). Furthermore, the mRNA expression level of HER2 was also downregulated by YB-1 silencing in both LNCaP and PC-3 cells, indicating that YB-1 transcriptionally regulates HER2 expression in prostate cancer (Supplementary Fig. S4B). However, HER2 shutdown using HER2-specific siRNA showed no apparent downregulation of phosphorylated MEK and ERK1/2, indicating that HER2 is not involved in the regulation of MEK/ERK signaling by YB-1 (Supplementary Fig. S4C).

Next, we examined the Raf family proteins (A-Raf, B-Raf, and Raf-1) as downstream factors of EGFR family. As shown in Fig. 4A, Raf-1 protein expression was specifically decreased by YB-1 knockdown, although A-Raf and B-Raf were unaffected. To elucidate the mechanism underlying these results, we examined the interactions between the Raf family proteins (A-Raf, B-Raf, and Raf-1) as downstream factors of EGFR family.
family proteins and YB-1 using the same membrane as used in Fig. 3C. As shown in Fig. 4B, Raf-1 specifically interacted with YB-1 directly or indirectly but A-Raf and B-Raf did not. Subsequently, the mechanism regulating Raf-1 protein by YB-1 was examined using the same membrane as used in Fig. 3B and D. The results showed that Raf-1 protein expression was decreased by cycloheximide, although it was not affected by MG132 when YB-1 was knocked down, indicating that the Raf-1 protein is also regulated by YB-1, at least in part, through the proteasomal pathway although translational involvement of YB-1 cannot be ruled out (Fig. 4C, Supplementary Fig. S5A and S5B). Subsequently, we investigated the effect of Raf-1 on the MEK/ERK pathway. As shown in Fig. 4D, the phosphorylation levels of MEK, ERK1/2, RSK, and YB-1 induced by EGF were blunted by Raf-1 shutdown, indicating that YB-1 regulated the activity of the MEK/ERK pathway, at least in part, by regulating the levels of Raf-1 protein (Fig. 4D, Supplementary Fig. S6). On the basis of interactions of YB-1 with ERK2 and Raf-1, we examined the effect of EGF on these interactions. As shown in Fig. 4E, coimmunoprecipitation of Raf-1 and ERK2 protein by YB-1 was decreased after EGF stimulation, indicating that Raf-1 and ERK2 uncoupled with YB-1 after EGF stimulation. This finding suggested that EGF stimulation caused a phosphorylation cascade of Raf-1/ERK2/YB-1, resulting in sequential divorce of protein complex.

The protein expressions of YB-1 and ERK2 as well as phosphorylated YB-1 in human prostate cancer tissues correlate with adverse clinicopathological parameters and recurrence-free survival

We summarized the correlations between clinicopathologic parameters and expressions of YB-1, p-YB-1, and ERK2 (Supplementary Table S1). High nuclear expressions of YB-1 and ERK2 were correlated with the advanced pT stage ($P = 0.0485$ and 0.0417). In addition, high nuclear expressions of p-YB-1 and ERK2 were correlated with PSA recurrence ($P = 0.0488$ and $P < 0.0001$). However, high nuclear expressions of YB-1, p-YB-1, and ERK2 were not correlated with pre-operative PSA or Gleason score. On the other hand, significant correlations were observed between nuclear expressions of YB-1 and p-YB-1 ($P = 0.0031$), YB-1 and ERK2 ($P = 0.0157$), and p-YB-1 and ERK2 ($P = 0.0003$; Supplementary Table S2), suggesting a functional correlation in human prostate cancer. In addition, patients with high nuclear expressions of ERK2 ($P < 0.0001$) and p-YB-1 ($P = 0.0287$) showed a lower PSA recurrence-free survival rate during the median follow-up of 5.01 years although patients with high nuclear ($P = 0.3098$) and high cytoplasmic ($P = 0.0877$) expressions of YB-1 did not (Fig. 5A).

Furthermore, the Cox proportional hazards model revealed that high nuclear ERK2 expression (HR, 7.947; 95% CI, 3.527–20.508; $P < 0.0001$) was an independent prognostic factor and one of the most significant contributors, together with 2 well-known prognostic factors, higher Gleason score (HR, 2.851; 95%CI, 1.504–5.589; $P = 0.0012$) and positive resection margin (HR, 3.724; 95% CI, 1.803–7.821; $P = 0.0004$; Table 1).

Discussion

In several previous studies, it has been postulated that Ras/ERK/RSK signaling regulates YB-1 activation represented by YB-1 phosphorylation. Ras was indicated to be involved in YB-1 phosphorylation induced by irradiation in breast cancer (21). In addition, ERK was also shown to regulate YB-1 phosphorylation, resulting in the regulation of VEGF (18). Jürchott and colleagues indicated that the Ras/ERK pathway regulated YB-1 activity, and their findings were further supported by the correlation between MEK/ERK pathway target genes and YB-1 expression in colorectal cancer (19). Furthermore, Stratford and colleagues have shown that RSK was responsible for the phosphorylation of YB-1 at Ser102 in breast cancer, which was inhibited by the RSK inhibitor SL0101 (20). In line with these previous findings, the present study showed that the Ras/ERK/RSK pathway activated by EGF also regulated YB-1 phosphorylation in prostate cancer.

Inversely, we clearly showed here that YB-1 regulated the activity of the MEK/ERK/RSK pathway as represented by the phosphorylation wave of this signaling. Evdokimova and colleagues reported that the YB-1–induced EMT was blocked by the MEK inhibitor PD98059, suggesting that YB-1 regulates the MEK pathway, resulting in an EMT induction in breast cancer (11). However, in their study, a direct regulation of the MEK pathway by YB-1 was not investigated. Recently, Astaneh and colleagues showed that a MAPK downstream target, MAPK-interacting kinase (MNK), was regulated by YB-1 in breast cancer (17), suggesting that YB-1 is involved in the MAPK pathway by interacting with MNK. In addition, Lee and colleagues indicated that YB-1 knockdown decreased STAT3 phosphorylation at Ser727 in breast cancer, accompanied by decreases of ERK and mTOR phosphorylation (16), although a mechanical investigation was not conducted. Furthermore, Feng and colleagues reported that YB-1 induced rat mesangial cell proliferation through ERK1/2 signaling, and ERK regulated YB-1 expression under inflammatory conditions (38). For the first time, the present study elucidated the mechanism of MEK/ERK/RSK regulation by YB-1. That is to say, YB-1 regulated the proteasome degradation of key molecules of the MAPK pathway, such as Raf-1 and ERK2, although there remained the possibility that YB-1 regulated MAPK pathway through translation. Recently, in silico analysis has raised the possibility that YB-1 may not function as a transcription factor in vivo (39), although this theory needs further evidence and discussion. Therefore, the present study may have revealed the undiscovered function of YB-1 as a proteasomal regulator. The specificity between YB-1 and ERK2, and YB-1 and Raf-1 can be supported by the previous findings that ERK2 phosphorylated YB-1 (11) and Raf-1 regulation by YB-1 (38). Furthermore, considering the distinct role of Raf and ERK isoforms, the selective binding of YB-1 to Raf-1 and ERK2, but not to A-Raf, B-Raf and ERK1, makes us curious on this specificity. It has been reported that Raf-1 played a role in transient ERK activity resulting in cell proliferation, whereas wild-type B-Raf induced sustained ERK activity and cell differentiation, suggesting a
tumor-promoting role of Raf-1 (40). Similarly, numerous studies have shown that ERK2 was a mediator of proliferation signal, whereas ERK1 had an antagonistic function of ERK2 (41–43). Therefore, the specificity of YB-1 to Raf-1 and ERK2 may represent the robust tumor-promoting role of YB-1, as well a promise of YB-1 as a therapeutic target. So far, 2 major intracellular signaling pathways, the Akt and MAPK pathways, have been shown to interact with YB-1 (11, 15, 16, 18, 19, 21). In line with these previous findings, the present study indicated that ERK signaling was closely correlated with YB-1, as indicated by the mutual regulation in different cell lines. In addition, the interaction between

Figure 5. Immunohistochemical staining of YB-1, p-YB-1, and ERK2 in prostate cancer tissue. A, PSA recurrence-free survival curves of patients in the low and high YB-1, p-YB-1, and ERK2 nuclear expression and YB-1 cytoplasmic expression groups. B, nuclear expression of YB-1, p-YB-1, and ERK2 and cytoplasmic expression of YB-1 in prostate cancer. Representative images of high-magnification fields (×400) are shown.
YB-1 and ERK signaling was shown both in AR-positive LNCaP and in AR-null PC-3 cells, suggesting that AR expression does not affect the interaction between YB-1 and ERK signaling.

In human prostate cancer samples, Gimenez-Bonafe and colleagues reported that increased YB-1 expression was correlated with a higher Gleason score, and YB-1 was accumulated in the nucleus after ADT (13). In agreement with these results, we have also shown that YB-1 expression was correlated with the Gleason score using a specific antibody against YB-1 (44) and that YB-1 expression was induced by ADT (45). Furthermore, nuclear YB-1 was found to be correlated with the Gleason score as well as with AR expression, in a study using prostate cancer tissues obtained from radical prostatectomy (14). The present study showed that higher nuclear YB-1 and p-YB-1 levels were correlated with advanced pathologic stage and higher PSA recurrence, respectively, indicating higher malignant potential, although there was no correlation between the nuclear YB-1 level and Gleason score. Curiously, in the present study, higher nuclear p-YB-1 level represented poor prognosis, whereas higher nuclear and cytoplasmic YB-1 levels did not. These findings suggested that YB-1 phosphorylation, but not localization may play a tumor-promoting role, leading to worse prognosis. On the other hand, ERK expression is well known to be upregulated in various cancers including prostate cancer (46, 47). In addition, the phosphorylated ERK levels have also been shown to be increased in prostate cancer, especially after ADT, and to be correlated with the Gleason score and tumor stage (48, 49). Also, an increase in nuclear ERK has been correlated with poor survival (50). Similarly, in the present study, nuclear ERK2 expression was correlated with advanced pathologic stage and higher PSA recurrence, respectively, indicating higher malignant potential.

Table 1. Relationship between PSA recurrence-free survival and clinicopathologic characteristics

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N = 165</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;70</td>
<td>108</td>
<td>1</td>
</tr>
<tr>
<td>≥70</td>
<td>57</td>
<td>1.054 (0.559–1.915)</td>
</tr>
<tr>
<td>Pre-operative PSA, ng/mL</td>
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</tr>
<tr>
<td>&lt;10</td>
<td>102</td>
<td>1</td>
</tr>
<tr>
<td>≥10</td>
<td>63</td>
<td>2.345 (1.304–4.274)</td>
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<tr>
<td>Gleason score</td>
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<td></td>
</tr>
<tr>
<td>≤6 or 7 (3 + 4)</td>
<td>96</td>
<td>1</td>
</tr>
<tr>
<td>7 (4 + 3) or &lt;8</td>
<td>69</td>
<td>3.018 (1.657–5.697)</td>
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<tr>
<td>Pathologic stage</td>
<td></td>
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<tr>
<td>pT2</td>
<td>109</td>
<td>1</td>
</tr>
<tr>
<td>pT3</td>
<td>56</td>
<td>1.878 (1.030–3.379)</td>
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<tr>
<td>Resection margin</td>
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<tr>
<td>Negative</td>
<td>98</td>
<td>1</td>
</tr>
<tr>
<td>Positive</td>
<td>67</td>
<td>2.483 (1.377–4.559)</td>
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<tr>
<td>Seminal vesicle invasion</td>
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<tr>
<td>Negative</td>
<td>155</td>
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<tr>
<td>Positive</td>
<td>10</td>
<td>1.397 (0.338–3.848)</td>
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<tr>
<td>Lymph node metastasis (n = 162)</td>
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<tr>
<td>Negative</td>
<td>159</td>
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<tr>
<td>Positive</td>
<td>3</td>
<td>8.201 (1.292–29.054)</td>
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<td>YB-1 expression (nucleus)</td>
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<tr>
<td>Low (≤10%)</td>
<td>132</td>
<td>1</td>
</tr>
<tr>
<td>High (≥10%)</td>
<td>33</td>
<td>1.415 (0.684–2.705)</td>
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<td>YB-1 expression (cytoplasm)</td>
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<tr>
<td>Low (TS ≤ 6)</td>
<td>60</td>
<td>1</td>
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<tr>
<td>High (TS ≥ 7)</td>
<td>105</td>
<td>1.754 (0.931–3.538)</td>
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<td>Phosphorylated YB-1 expression</td>
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<tr>
<td>Low (≤41%)</td>
<td>83</td>
<td>1</td>
</tr>
<tr>
<td>High (≥41%)</td>
<td>82</td>
<td>1.926 (1.064–3.590)</td>
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<tr>
<td>ERK2 expression</td>
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<td>Low (≤62%)</td>
<td>86</td>
<td>1</td>
</tr>
<tr>
<td>High (≥62%)</td>
<td>79</td>
<td>7.265 (3.451–17.788)</td>
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</table>

*Statistically significant.

YB-1 and ERK signaling was shown both in AR-positive LNCaP and in AR-null PC-3 cells, suggesting that AR expression does not affect the interaction between YB-1 and ERK signaling.

In human prostate cancer samples, Gimenez-Bonafe and colleagues reported that increased YB-1 expression was correlated with a higher Gleason score, and YB-1 was accumulated in the nucleus after ADT (13). In agreement with these results, we have also shown that YB-1 expression was correlated with the Gleason score using a specific antibody against YB-1 (44) and that YB-1 expression was induced by ADT (45). Furthermore, nuclear YB-1 was found to be correlated with the Gleason score as well as with AR expression, in a study using prostate cancer tissues obtained from radical prostatectomy (14). The present study showed that higher nuclear YB-1 and p-YB-1 levels were correlated with advanced pathologic stage and higher PSA recurrence, respectively, indicating higher malignant potential, although there was no correlation between the nuclear YB-1 level and Gleason score. Curiously, in the present study, higher nuclear p-YB-1 level represented poor prognosis, whereas higher nuclear and cytoplasmic YB-1 levels did not. These findings suggested that YB-1 phosphorylation, but not localization may play a tumor-promoting role, leading to worse prognosis. On the other hand, ERK expression is well known to be upregulated in various cancers including prostate cancer (46, 47). In addition, the phosphorylated ERK levels have also been shown to be increased in prostate cancer, especially after ADT, and to be correlated with the Gleason score and tumor stage (48, 49). Also, an increase in nuclear ERK has been correlated with poor survival (50). Similarly, in the present study, nuclear ERK2 expression was correlated with advanced pathologic stage and higher PSA recurrence, respectively, indicating higher malignant potential.
expression was correlated with increased malignant potential of prostate cancer, such as advanced pathologic stage and higher PSA recurrence. Moreover, surprisingly, the present study showed that nuclear ERK2 levels made a more prominent contribution to the PSA recurrence than did well-known factors such as the Gleason score and resection margin, suggesting that nuclear ERK2 could be a promising prognostic biomarker. Furthermore, the correlation between YB-1, p-YB-1, and ERK2 supported the in vitro experimental result that YB-1 and ERK2 mutually regulated each other.

In prostate cancer, YB-1 has been shown to play a role in promoting various cancerous characteristics including cell proliferation (14, 51), drug resistance (44), and castration resistance (14), similar to solid cancers in other organs (5, 12). Similarly, ERK is well known to promote prostate cancer growth (52) and progression (53) as well as castration resistance (50, 54). Inversely, inhibition of ERK signaling has been shown to exert therapeutic effects on prostate cancer (55). Also, YB-1 suppression has been shown to decrease prostate cancer proliferation (14, 51). Accordingly, a YB-1-targeting therapy using adenovirus vector (56) and decoy peptide (57) is now under development. However, compared with ERK signaling, less attention has been paid to YB-1 as a therapeutic target. Nonetheless, YB-1 appears to be a more promising therapeutic target because, as a nodal factor, YB-1 regulates several factors possessing cancer-promoting functions, such as PCNA, EGFR, DNA topoisomerase II (7), CD44 (8), PIK3CA (9), and MET (10).

In summary, YB-1 and ERK signaling mutually regulated one another’s activity in prostate cancer cells in the present study. This was supported by the finding in human prostate cancer tissues that nuclear YB-1 expression was correlated with nuclear ERK2 expression, which was correlated with advanced pathologic stage and shorter recurrence-free survival. On the basis of these findings in prostate cancer, we revealed the functional mechanism and biologic relevance of the interaction between YB-1 and ERK signaling.

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

Authors’ Contributions
Conception and design: Y. Oda
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Shiota, K. Kuroiwa
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Imada, M. Shiota
Writing, review, and/or revision of the manuscript: K. Imada, M. Shiota
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Kohashi, Y. Song, M. Sugimoto, Y. Oda
Study supervision: S. Naito, Y. Oda

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References
Mutual Regulation between Raf/MEK/ERK Signaling and Y-Box-Binding Protein-1 Promotes Prostate Cancer Progression

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