Prostate cancer antigen-1 contributes to cell survival and invasion though discoidin receptor 1 in human prostate cancer

Keiji Shimada,[1] Mitsutoshi Nakamura,[1] Eiwa Ishida,[1] Tomonori Higuchi,[1] Hiroshi Yamamoto,[2] Kazutake Tsujikawa[2] and Noboru Konishi[1,3]

[1]Department of Pathology, Nara Medical University School of Medicine, 840 Shijo-cho, Kashihara, Nara 634-8521; [2]Department of Immunology, Osaka University, Graduate School of Pharmaceutical Sciences, Osaka University, Yamadaoka 1-6, Suita, Osaka 565-0871, Japan

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A novel gene, prostate cancer antigen (*PCA*)-*1*, was recently reported to be expressed in the prostate; however, its biological roles remain unclear. Knockdown of the *PCA-1* gene by small interfering RNA transfection induced apoptosis through reducing the expression of the anti-apoptotic molecule Bcl-xl and cytoplasmic release of cytochrome *c* in the androgen-independent prostate cancer cell line PC3. Moreover, *in vitro* matrigel and *in vivo* chorioallantoic membrane assays showed that silencing of *PCA-1* significantly downregulated discoidin receptor (*DDR*)-*1* expression, resulting in suppression of cancer-cell invasion. Transfection with *PCA-1* increased the levels of both Bcl-xl and DDR1, which made the cells more invasive through the upregulation of matrix metalloproteinase 9 in DU145. Interestingly, long-term culture using androgen-free medium increased the level of PCA-1 and the related expression of Bcl-xl and DDR-1 in the androgen-sensitive cancer cell line LNCaP, suggesting that PCA-1 signaling is associated with androgen independence. Immunohistochemical analysis in a series of 169 prostate carcinomas showed that PCA-1 and DDR1 were strongly expressed in prostate cancer cells, including preneoplastic lesions, but there was little or no expression in normal epithelium. Moreover, the expression of PCA-1 and DDR-1 was associated with a hormone-independent state of prostate cancer. Taken together, we propose that PCA-1–DDR-1 signaling is a new important axis involved in malignant potential prostate cancer associated with hormone-refractory status. (*Cancer Sci* 2008; 99: 39–45)

We have recently identified a novel gene, prostate cancer antigen (*PCA*)-*1*, to be highly and specifically expressed in prostate cancer.[1] The amino acid sequence of PCA-1 indicates a similarity to *Escherichia coli* AlkB, a DNA-alkylating damage-repair enzyme, and overexpression of PCA-1 makes COS-7 cells resistant to cell death due to an S_N2 alkylation agent, methylmethane sulfonate (MMS), acting primarily at the N7-position of guanine and the N3-position of adenine.[2,3] A number of reports have accumulated as to the molecular mechanisms by which MMS exhibits cytotoxicity in tumor cells. Kuo *et al*. reported that the *ras* oncogene alters the expression of Bcl-2 family members, resulting in suppression of MMS-induced apoptosis.[4] In addition, others have reported that apoptosis due to MMS is dependent on *c*-jun NH_2 terminal kinase activation, which is inhibited significantly by transfection with the anti-apoptotic Bcl2 family member Bcl-xl.[5] Taken together, we suggest that apoptosis-related molecules such as Bcl-2 family members might be involved in the mechanisms by which PCA-1 affects cell survival signals in human prostate cancer.

Discoidin domain receptor (DDR) is a tyrosine receptor kinase with an N-terminal domain that is homologous to the *Dictyostelium discoideum* protein discoidin I.[6] There are two types of DDR, DDR1 and DDR2, which are characterized by an approximately 155-amino acid discoidin homology domain in the extacellular region of the protein. DDR1 has been reported to be expressed in normal epithelial and tumor cells, including different carcinoma cell lines.[7–9] In contrast, DDR2 is expressed widely, particularly in skeletal muscle and heart muscle, kidney and skin.[7] Collagen types I–IV and VIII induce autophosphorylation of DDR1 with delayed kinetics. DDR1 and tyrosine kinase activity are well known to be key regulators of morphogenesis, differentiation, cell proliferation, and death,[10,11] but the effect is controversial in different cells and animal models: in DDR1 knock-out mice, migration of vascular smooth muscle cells in response to type I collagen is reduced by 33%.[12] DDR1 overexpression promotes glioma-cell invasion and adhesion in association with matrix metalloproteinase (MMP) 2,[13] and active DDR1 kinase mediates migration of smooth muscle cells,[14] or leukocytes through the endothelium,[15] whereas Wang *et al*. showed that DDR1 signals inhibit $\alpha2\beta1$-integrin-mediated cell migration.[16] Thus, DDR has opposite biological functions depending on the cell type-specific environments, and the upstream and downstream signals that regulate the physiological roles of DDR might be obscured in detail.

We have previously found that PCA-1 can enhance the invasion capacity and suppress the apoptosis induced by anticancer drugs (Shimada *et al*., unpubl. data, 2006), but the detailed mechanisms remain obscure. DDR1 is well known to be closely associated with not only cell migration but also cell survival. As to the latter function of DDR1, interesting reports have been published recently: upregulation of the anti-apoptotic molecule Bcl-xl or activation of nuclear factor κB can occur in response to DDR1 activation, resulting in protection from apoptosis induction by Fas ligand.[17] In the present study, we therefore investigated the biological roles of PCA-1 and its relationship to DDR1 in terms of survival and progression of human prostate cancer by *in vitro* and *in vivo* methods with immunohistochemical analysis. We found that PCA-1 functions as an upstream regulator of DDR1 and promotes prostate cancer development through enhancing cancer-cell survival, invasion, and resistance to chemotherapies including hormone deprivation.

Materials and Methods

Cell culture, plasmids and chemicals. The human prostate cancer cell lines PC-3, DU145, and LNCaP were purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI supplemented with 10% fetal bovine serum. An androgen-independent (AI) LNCaP cell line was established following long-term subculture in Dulbecco's modified Eagle's

[3]To whom correspondence should be addressed. E-mail: nkonishi@naramed-u.ac.jp

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medium (DMEM) and 5% charcoal-stripped fetal bovine serum.[18] Both the AI LNCaP and the parental LNCaP cell lines secreted prostate-specific antigen (PSA) in response to androgen stimulation (data not shown). Anti-Bcl-xl, anticytochrome *c*, and anti-poly ADP-ribose polymerase (PARP) antibodies were purchased from Cell Signaling (MA, USA); anti-actin, anti-DDR1, antiphosphotyrosine, and antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); etoposide and paclitaxel were from Calbiochem (San Diego, CA, USA).

Preparation of cell lysates, immunoprecipitation, and immunoblot analysis. Cells were washed once with phosphate-buffered saline (PBS) and suspended in lysis buffer (40 mM HEPES [pH 7.4] with 10% glycerol, 1% Triton X-100, 0.5% Nonidet P-40, 150 mM NaCl, 50 mM NaF, 20 mM β-glycerol phosphate, 1 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol bis(βaminoethyl ether)-N,N,N′,N′-tetra-acetic acid (EGTA), 1 mM phenylmethylsulfonyl fluoride, and 0.1 mM vanadate) with a protease inhibitor mixture. For immunoprecipitation, 5×10^7 cells were lysed and incubated with 5 μg anti-DDR-1 antibody for 18 h at 4°C, then precipitated with protein A and G sepharose (Amersham Pharmacia Biotech, Tokyo, Japan). Cell lysates or immunoprecipitates were resolved in sodium dodecylsulfate–polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA), which were blocked in TBST buffer (20 mM Tris-HCl [pH 7.5] containing 150 mM NaCl, 0.1% Tween-20) and 5% skim milk at room temperature for 1 h, then incubated with the indicated primary antibody for 1 h, washed with TBST, and incubated with horseradish peroxidase-conjugated antimouse or antirabbit IgG (Amersham Pharmacia Biotech). After further washing with TBST, peroxidase activity was detected on X-ray films using an enhanced chemiluminescence detection system.

Tissue samples and immunohistochemistry. One hundred and fifteen primary prostate carcinomas receiving no therapy, obtained at radical prostatectomy, and 31 cases with neoadjuvant androgen-deprivation therapy were examined in the present study. All fixation and tissue processing was as described in a previous report.[1,19] Slides were reviewed for Gleason score as demonstrated according to the Gleason system for prostate carcinomas. Informed consent was obtained from all patients before the collection of specimens. The study was approved by the ethics committee of Nara Medical University. The sections were incubated with the indicated primary antibodies for 16 h at 4°C and the reactions were visualized using a Histofine kit (Nichirei, Tokyo, Japan), with diaminobenzidine as the chromogen and hematoxylin counterstaining. The intensity of immunohistochemical staining was evaluated at a magnification of ×200 and scored as follows: no discernible staining (negative, 0); immunostaining only scarcely detectable (weak, 1); definite staining higher than weak but not strong (moderate, 2); and strong staining (strong, 3).

Preparation of antisera. Anti-PCA-1 antisera were prepared as described previously against a synthetic PCA-1 peptide (amino acids 64–76) as the antigen.[1] After a 0.5-mg aliquot of peptides was emulsified and injected into rabbits, blood was collected at 2-week intervals. The relative activity of antisera against the synthetic peptide was tested by enzyme-linked immunosorbent assay.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay. DNA cleavage, a characteristic of apoptosis, was detected using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay as described by the manufacturer (Roche Diagnostics, Tokyo, Japan). After small interfering RNA (siRNA) transfection, the cells were washed twice with PBS and fixed with 4% paraformaldehyde in PBS at room temperature for 20 min. The fixed cells were permeabilized with 0.1% Triton X-100 and 0.1% sodium citrate at 4°C for 2 min, and incubated for 1 h at 37°C with the TUNEL reaction mixture. Images of nuclear fluorescence typical of apoptotic cells were collected by fluorescence microscopy as described above. At least 600 cells from three different fields were examined in each experiment, and cell death was expressed as a percentage of TUNEL-positive cells.

Matrigel invasion assay. The *in vitro* invasion assay was carried out using a matrigel invasion chamber (BD Biosciences, MA, USA). At 48 h after transfection with or without PCA-1 siRNA, 2×10^4 cells were placed in the insert. After 36 h incubation at 37°C, the chambers were scrubbed with a cotton bud to remove non-invading cancer cells. The invading cells were fixed and stained with the Diff Quick staining kit (Sysmex, Kobe, Japan), then counted under a light microscope. The experiment was repeated three times.

Chick chorioallantoic membrane assay. The *in vivo* cancer-cell invasion and intravasation assays were carried out using 11-day-old chick embryos wherein 10^6 cancer cells transfected with the green fluorescent protein (GFP)-encoding vector pEGFP (Clontech, CA, USA) with or without PCA-1 or DDR1 siRNA. After 24 h cultivation, cells were seeded on the chorioallantoic membrane (CAM) for 3 days.[20] After collection of CAM samples, tissues were fixed and stained with anti-GFP; thereafter, the number of invading cancer cells positive for GFP was quantified in three or more randomly selected fields. At least three CAM were used for each experiment.

Gelatin zymography. The activity and expression of MMP9 was analyzed by gelatin zymography. Equal amounts of concentrated conditioned medium obtained from cell-culture experiments was mixed with sample buffer (0.25 M Tris-HCl [pH 6.8], 0.4% sodiumdodecyl sulfate, 40% glycerol, and bromophenol blue) and loaded onto a 7.5% sodium dodecylsulfate–polyacrylamide gel containing 1 mg/mL gelatin (Wako Pure Chemical Industries, Osaka, Japan). After electrophoresis, the gel was stained with Coomassie Blue solution using Quick-CBB plus (Wako Pure Chemical Industries) in accordance with the manufacturer's protocol, followed by destaining with destaining buffer (5% acetic acid, 10% ethanol in distilled water) until bands of gelatinolytic activity could be visualized.

Reverse transcription–polymerase chain reaction. Total RNA was extracted using Trizol reagent and subjected to reverse transcription (RT) and polymerase chain reaction (PCR) using a One-step RT-PCR Kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. The PCR conditions were 95°C for 30 s, 55–60°C for 30 s, and 72°C for 1 min, for a total of 35 cycles. The PCR primers were: MMP9 forward, 5′-TTCATCTTCC-AAGGCCAATC-3′; MMP9 reverse, 5′-ACATAGGGTACATG-AGCGCC-3′; Bcl-xl forward, 5′-GTAAACTGGGGTCGCA-TTGT-3′; Bcl-xl reverse, 5′-TGGATCCAAGGCTCTAGGTG-3′; DDR-1 forward, 5′-CTGGGGACTATTACCGTGTG-3′; DDR-1 reverse, 5′-CCTACAGAGCATCAGCACCT-3′; PCA-1 forward, 5′-TACCACTGCTAAGAGCCATCTCC-3′; PCA-1 reverse, 5′-ACCTGCTGAGGTTCTTTGAACAC-3′; glyceraldehyde-3-phosphate dehydrogenase (G3PDH) forward, 5′-ACCACA-GTCCATGCCATCAC-3′; and G3PDH reverse, 5′-TCCACC-ACCCTGTTGCTGTA-3′. The PCR products were analyzed on a 1.5% agarose gel and visualized by ethidium bromide staining.

Transfection of PCA-1 and DDR1 siRNA. The cells were seeded at 10^6 cells per well in 6-cm plates, then transfected with 100 nM control RNA (Santa Cruz), PCA-1 siRNA (horseradish peroxidase [HP]-validated siRNA 1027400; Qiagen) or DDR1 siRNA (Santa Cruz) in the presence or absence of the GFP-carrying vector pEGFP using lipofectamine (Invitrogen, Tokyo, Japan) in accordance with the manufacturer's protocol. After cultivation for the indicated time *in vitro* or *in vivo*, cells or tissue specimens were collected. The underlying samples were removed and homogenized, then the lysates were used for immunoblotting and RT-PCR to analyze PCA-1 and DDR1 expression.

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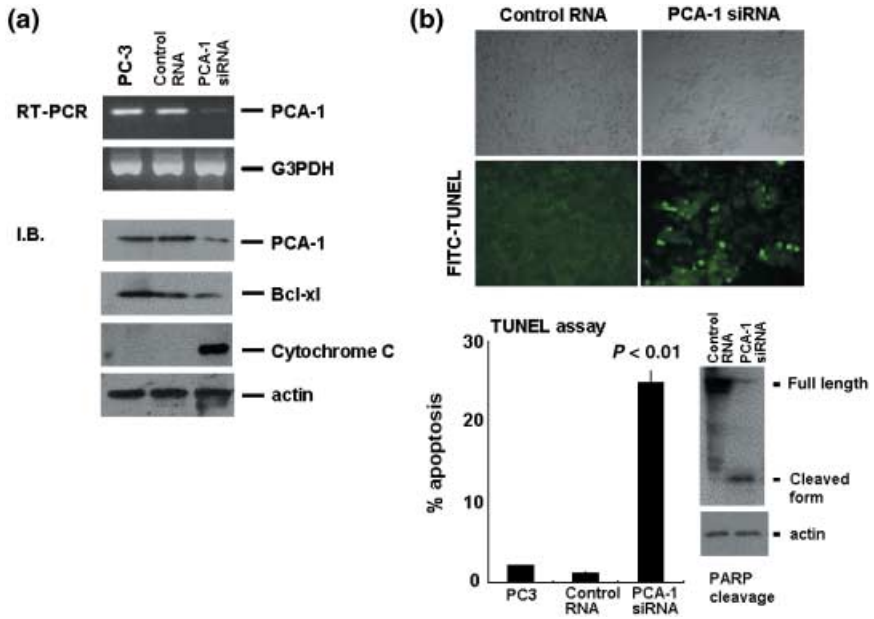

Fig. 1. Prostate cancer antigen (*PCA*)-*1* knockdown-induced apoptosis in PC3 cells. (a) PC3 cells were transfected with or without control RNA or PCA-1 small interfering RNA (siRNA). After 72 h cultivation, the mRNA and protein expression of PCA-1, Bcl-xl (whole-cell lysates), and cytochrome *c* (cytoplasmic fraction) were examined by reverse transcription–polymerase chain reaction (RT-PCR) and western blotting. (b) After transfection with control RNA or PCA-1 siRNA, cells were fixed and incubated with fluorescein isothiocyanate-conjugated terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) reaction mixture. Images of nuclear fluorescence typical of apoptotic features were collected by fluorescence microscopy and the percentages of TUNEL-positive apoptotic cells were calculated (upper and lower left panel). After the same treatment, cells were harvested, and the levels of full-length and cleaved poly ADP-ribose polymerase (PARP) were analyzed by western blotting using anti-PARP antibody (lower right panel). Glyceraldehyde-3-phosphate dehydrogenase or actin was used as a house-keeping gene in RT-PCR or western blotting.

Statistical analysis. The Wilcoxon's rank sum test was used to analyze the distribution. Statistical analyses were carried out using Fisher's exact test supplemented by the Bonferroni procedures as described previously.[21,22] Survival analyses for biochemical recurrence were evaluated using the Kaplan–Meier method and the log-rank test. Results were considered significant if P was < 0.05.

Results

***PCA-1* gene silencing induced apoptosis through Bcl-xl downregulation.** To clarify the role of PCA-1 in cancer cell survival signals, the effect of *PCA-1* gene silencing by transfection with siRNA on apoptosis induction was assessed using the AI human prostate cancer cell line PC-3. RT-PCR and immunoblotting data indicated that *PCA-1* gene expression was reduced by more than 85% by 100 nM siRNA transfection (designated by Qiagen) and cultivation for 72 h (Fig. 1a). As shown in Fig. 1b, knockdown of the *PCA-1* gene downregulated Bcl-xl expression and cytochrome *c* release into the cytoplasm, resulting in apoptosis as assessed by TUNEL staining and PARP-cleavage assays (% apoptosis: PC3 or control RNA transfection vs PCA-1 siRNA transfection, $P < 0.01$). We also observed cleavage of caspases 3 and 9 by *PCA-1* knockdown. In addition, treatment with caspase inhibitors such as z-vad-fmk could block apoptosis (data not shown). The results demonstrate that PCA-1 protects prostate cancer cells from mitochondrial pathway-mediated apoptosis through stabilizing Bcl-xl.

DDR1, downstream of PCA-1, contributes to prostate cancer invasion. Both mRNA and protein expression of the tyrosine kinase receptor DDR1, and the phosphorylated form, were decreased by *PCA-1* knockdown (Fig. 2a,b). As shown in Fig. 2c, *DDR1* gene silencing reduced Bcl-xl expression, resulting in induction of apoptosis to the same extent as with PCA-1 knockdown. DDR1 is well known to be involved in cell migration. Therefore, we herein examined whether cancer invasion was modified by *PCA-1* or *DDR1* gene knockdown. The *in vitro* matrigel invasion assay showed that the number of invaded cells in the cancer graft was significantly suppressed by knockdown of the *PCA-1* and *DDR1* genes (Fig. 2d) (number of invaded cells through matrix: control RNA transfection vs PCA-1 or DDR1 siRNA transfection, $P < 0.01$).

PCA-1 enhanced cancer invasion through upregulation of MMP9. To address the downstream signals of the PCA-1–DDR1-mediated pathway associated with cancer invasion, we constructed stable clones derived from the AI prostate cancer cell line DU145 overexpressing the *PCA-1* gene. DU145 cells were transfected with pcDNA4/HisMax-PCA-1, and were then incubated in the presence of 200 μg/mL zeocin (Invitrogen). After 2 weeks incubation, two clones were selected and the expression of His-tagged PCA-1 was analyzed by immunoblotting using anti-His antibody (Santa Cruz). Consistent with data from gene-silencing experiments, overexpression of PCA-1 resulted in increased expression of Bcl-xl and DDR1, and cancer invasion was also enhanced significantly (Fig. 3a). As shown in Fig. 3b, both the mRNA expression and gelatinolytic activity of MMP9 were low, but in cells overexpressing *PCA-1* they were increased significantly. This enhancement of MMP9 was strongly cancelled by siRNA transfection of PCA-1 or DDR1. We found no significant expression or activity of other MMP, including MMP1, -2, and -13, in the present study (data not shown). To assess the effect of *PCA-1* gene silencing or overexpression on cancer invasion *in vivo*, CAM assays were carried out. PC3 and DU145 cells were transfected with plasmids encoding GFP with or without PCA-1 or its siRNA, and were incubated on CAM for 3 days. The invasion capacity was assessed by examining the number of invaded cells positive for anti-GFP immunostaining (Fig. 3c). Cancer invasion was strongly suppressed by PCA-1 knockdown, whereas it was significantly enhanced by its overexpression (Fig. 3d) (number of invaded cells through matrix: PC3 or control RNA transfection vs PCA-1 siRNA transfection, $P < 0.01$; DU145 vs PCA-1 overexpression clone (A) or (B), $P = 0.022$ or $P = 0.048$). Reduction and enhancement of invasion was almost the same as the knockdown efficacy and overexpression level, respectively, of the *PCA-1* gene.

Amplified PCA-1 signaling in the process of androgen-resistance in prostate cancer cells. Next, we investigated whether PCA-1-related signaling is modified in the process of hormone resistance using LNCaP subculturing under androgen-free medium conditions (AI LNCaP). Expression of PCA-1 and the downstream molecules DDR1 and Bcl-xl was increased more in AI-LNCaP compared with the parental cells (Fig. 4a left panel). An increase in DDR1 and Bcl-xl expression was cancelled by silencing of the *PCA-1* and *DDR1* genes (Fig. 4a right panel). Interestingly, the *in vivo*

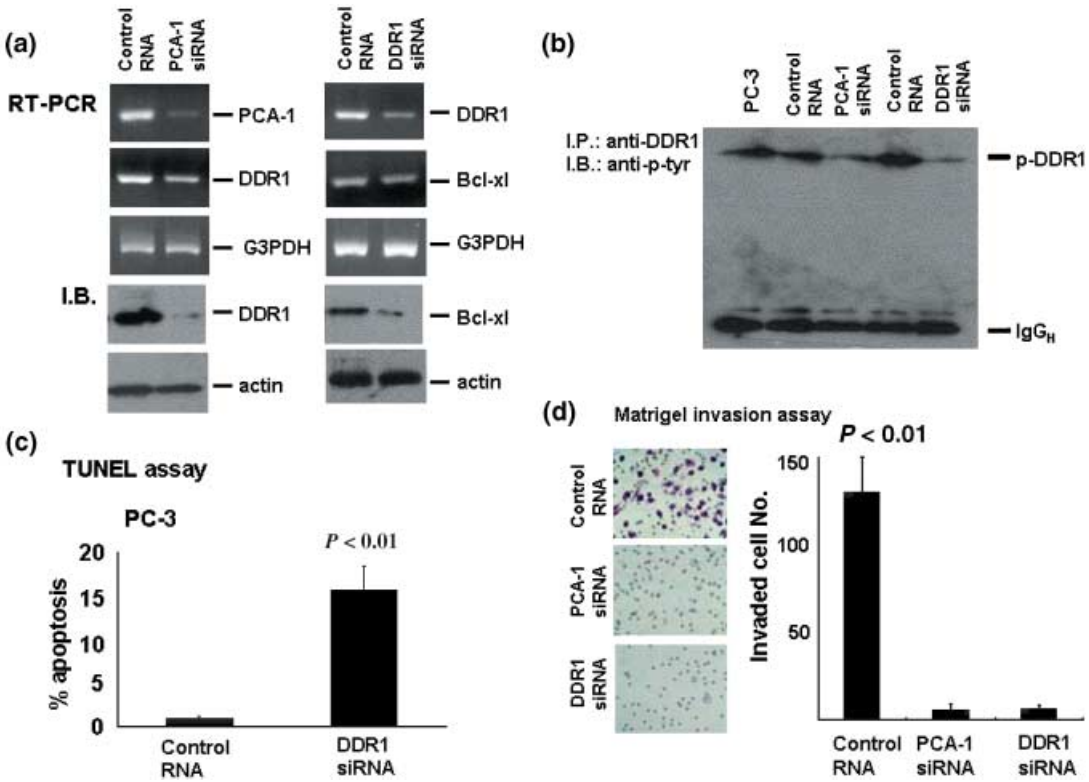

Fig. 2. Discoidin domain receptor (DDR) is downstream of prostate cancer antigen (PCA)-1 and contributes to prostate cancer invasion. (a) PC3 cells were transfected with control RNA, or PCA-1 or DDR1 small interfering RNA (siRNA). After 72 h cultivation, the mRNA and protein expression of PCA-1, DDR1, and Bcl-xl was examined by reverse transcription–polymerase chain reaction and western blotting. (b) After transfection with control RNA or siRNA as described in (a), the cells were lysed and immunoprecipitated with anti-DDR1 antibody. Phosphorylation of DDR1 was analyzed by western blotting using antiphosphotyrosine antibody. (c) After transfection with control RNA or DDR1 siRNA as described in (a), cells were fixed and incubated with fluorescein isothiocyanate-conjugated terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) reaction mixture. Images of nuclear fluorescence typical of apoptotic features were collected by fluorescence microscopy, and the percentages of TUNEL-positive apoptotic cells were calculated. (d) At 48 h after transfection with or without PCA-1 or DDR1 siRNA, cells were placed on matrigel. After 36 h incubation, invading cells were fixed and stained with the Diff Quick staining kit, then counted under a light microscope.

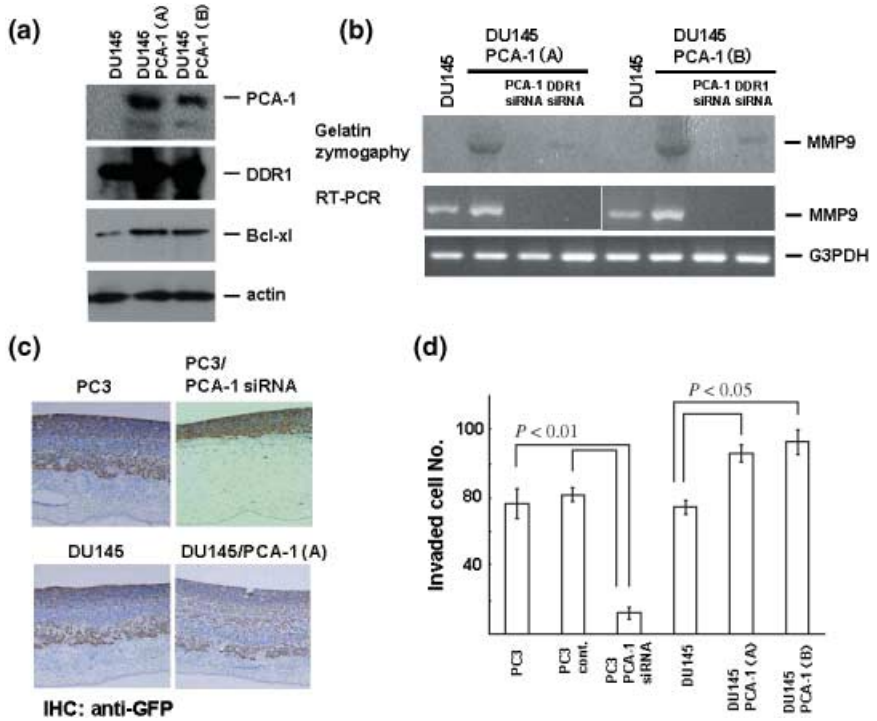

Fig. 3. Prostate cancer antigen (PCA)-1 overexpression enhanced cancer invasion through upregulation of matrix metalloproteinase (MMP) 9. (a) DU145-derived clones overexpressing the *PCA-1* gene were selected (DU145 PCA-1 [A] and [B]), and the protein expression of His-tagged PCA-1, discoidin domain receptor (DDR), and Bcl-xl was examined by western blotting using antibodies against His, DDR1, and Bcl-xl. (b) DU145 PCA-1 (A) and (B) cells were transfected with or without PCA-1 or DDR1 siRNA. After 72 h cultivation, the culture medium was concentrated and the gelatinolytic activity of MMP9 was examined. The mRNA expression of MMP9 was also investigated by reverse transcription–polymerase chain reaction. (c) PC3 cells were transfected with green fluorescent protein (GFP)-encoding plasmids in the presence of control RNA or PCA-1 siRNA. DU145 cells and DU145 PCA-1 (A) and (B) cells were similarly transfected with GFP-encoding plasmids. After 24 h cultivation, these cells were plated on chorioallantoic membrane, and grafts were collected at 3 days. Tissues were fixed and stained with anti-GFP; thereafter, the number of invading cancer cells positive for GFP was quantified in three or more randomly selected fields.

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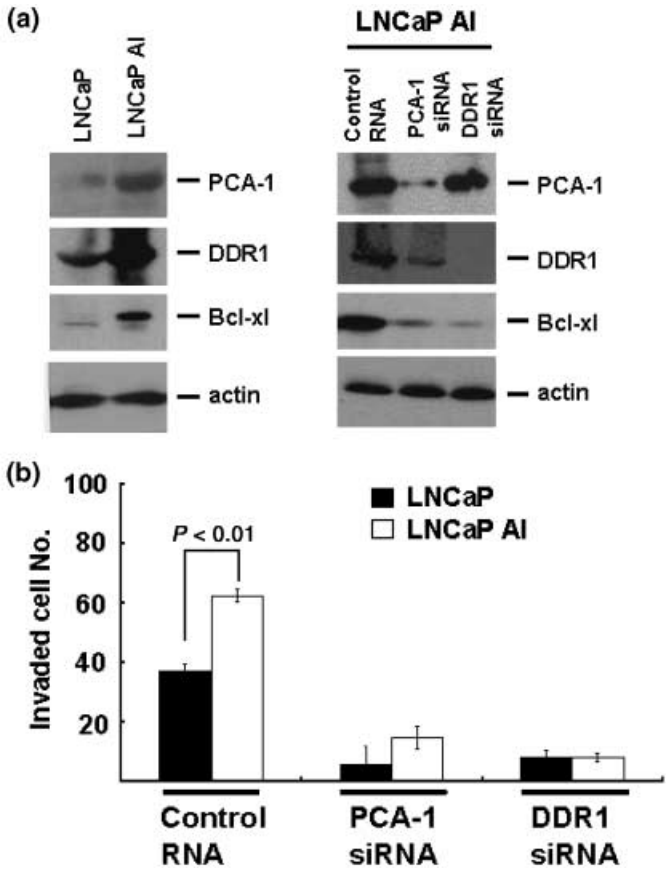

Fig. 4. Amplification of prostate cancer antigen (PCA)-1-mediated signals and androgen independence. (a) Androgen-independent (AI) LNCaP cells were developed under long-term culture in androgen-free medium. Protein expression of PCA-1, discoidin domain receptor (DDR), and Bcl-xl in LNCaP and AI LNCaP with or without transfection of control RNA or PCA-1 or DDR1 small interfering RNA (siRNA) (72-h incubation period) was examined by western blotting. (b) LNCaP and AI LNCaP cells were transfected with control or PCA-1 or DDR1 siRNA. After 24 h cultivation, cells were plated on chorioallantoic membrane and incubated for 72 h. Tissues were fixed and stained with anti-green fluorescent protein (GFP); thereafter, the number of invading cancer cells positive for GFP was quantified in three or more randomly selected fields.

CAM assay showed that cancer invasion was more prominent in androgen-resistant cells, but this was cancelled by silencing of the *PCA-1* and *DDR1* genes (Fig. 4b) (number of invaded cells through matrix: LNCaP vs LNCaP AI under control RNA transfection, $P < 0.01$; LNCaP vs LNCaP AI under PCA-1 siRNA transfection, $P = 0.058$; LNCaP vs LNCaP AI under DDR1 transfection, $P = 0.45$). These results clearly suggest that PCA-1 and the downstream signals are responsible for the enhancement of malignant potential associated with hormone resistance in human prostate cancer.

Association of PCA-1 and DDR1 with hormone-refractory prostate cancer. To characterize the expression of PCA-1 and DDR1, we carried out immunohistochemical analysis of 115 primary prostate carcinomas receiving no therapy, obtained from radical prostatectomy. We scored the intensity of immunostaining as follows: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining. As demonstrated in Fig. 5a,b, no or weak PCA-1 and DDR1 staining was present in normal prostate epithelial cells but these were increased in cancer cells, supported by western blot data using normal prostatic epithelial and cancer tissue specimens. As shown in Fig. 5c, box-plot data indicated that both PCA-1 and DDR1 were statistically increased in cancer cells and precancerous lesions compared with normal epithelial cells (PCA-1, 0.59 ± 0.09 in normal epithelial cells vs 1.61 ± 0.16 in cancer cells, $P < 0.001$ or 2.00 ± 0.18 in precancerous lesions, $P < 0.001$; DDR1, 0.51 ± 0.08 in normal epithelial cells vs 2.1 ± 0.12 in cancer cells, $P < 0.001$ or 1.92 ± 0.24 in precancerous lesions, $P < 0.001$). There were no statistical associations between PCA-1 and DDR1 expression levels and Gleason score, initial serum PSA levels, or regional lymph-node or distant metastasis (data not shown). Finally, correlation of PCA-1 and DDR1 expression and the hormone-responsive status was examined. The 31 cases were treated by complete androgen-deprivation therapy, and their diagnostic biopsies were analyzed for the expression of PCA-1 and DDR1 by immunohistochemistry. We classified these cancers into two groups: strong (score 3: $n = 17$ for PCA-1; $n = 18$ for DDR1) and moderate or weak (score 1 or 2: $n = 14$ for PCA-1; $n = 13$ for DDR1). We evaluated the time in days until the appearance of hormone-independent phenotype as the time variable (Fig. 5d). Free of biochemical recurrence curves were significantly different between the two groups, with a median free-time of 291 ± 38 days (95% confidence interval 215.8–366.2) and 229 ± 27 days (95% confidence interval 176.4–281.2) for cancers expressing high levels of PCA-1 and DDR1, respectively ($P < 0.001$).

Discussion

We demonstrated here for first time that PCA-1 contributes the survival and invasiveness of human prostate cancer by enhancing the downstream signals of Bcl-xl and DDR1. PCA-1 was specifically overexpressed in cancer cells, including precancerous lesions, and prostatic intraepithelial neoplasia; therefore, PCA-1 appears to affect the malignant potential of prostate cancer.[1] Knockdown of the *PCA-1* gene by siRNA transfection decreased the levels of Bcl-xl mRNA and protein, by which apoptosis is executed via the mitochondrial pathway. In contrast, PCA-1 overexpression resulted in the upregulation of Bcl-xl. These data demonstrate a major involvement of the *PCA-1* gene in the protection of cancer cells from apoptosis through stabilizing Bcl-xl. Bcl-xl is well known to be associated with resistance to cell death due to chemotherapy in various types of cancer cells, including prostate cancer. Yamanaka *et al*. reported that a reduction in the levels of Bcl-2 and Bcl-xl using antisense oligonucleotides induces apoptosis and enhances sensitivity to chemotherapeutic drugs, such as paclitaxel in the prostate cancer cell line LNCaP.[23] Moreover, Bcl-xl plays an important role in apoptosis due to proliferator-activated receptor γ agonists.[24] In line with these reports, silencing and overexpression of PCA-1 made prostate cancer cells more sensitive and resistant to anticancer drug-induced apoptosis, such as with etoposide (data not shown). The *in vitro* and *in vivo* invasion assays clearly showed that *PCA-1* gene silencing suppressed prostate cancer invasion and the effect was much more prominent than apoptosis induction, suggesting that PCA-1 has downstream signals involved in cancer invasion rather than the anti-apoptotic pathway. For a number of the cell migration-associated molecules examined, we found that DDR1 was closely regulated by PCA-1. Interestingly, DDR1 knockdown suppressed not only cancer invasion but also cell survival by reducing Bcl-xl expression to the same extent as with *PCA-1* gene silencing. Overexpression of PCA-1 increased the constitutive mRNA expression and gelatinolytic activity of MMP9 and enhanced cancer invasion *in vivo*, which was strongly cancelled by *DDR1* gene knockdown. Therefore, MMP9 is an effector molecule of PCA-1–DDR1-mediated signals in relation to cancer migration. Several reports have accumulated as to the role of DDR1 and DDR2 in cell migration and tumor invasion through induction of MMP1, -2 and -9.[13,14,17] We also investigated other MMP family members, but only MMP9 was modified in

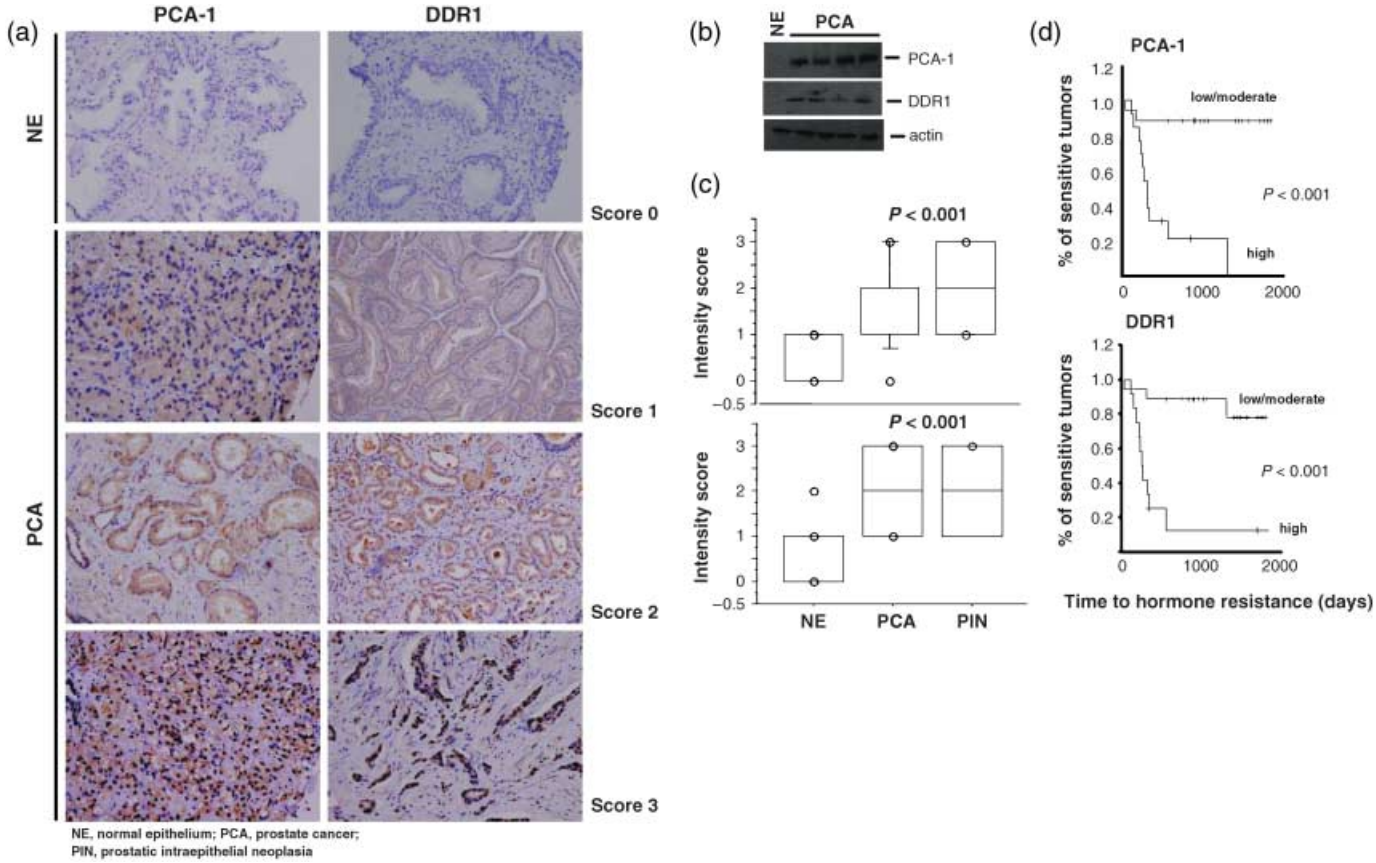

Fig. 5. Expression of prostate cancer antigen (PCA)-1 and discoidin domain receptor (DDR) in normal prostatic epithelial cells and cancer cells. (a) Immunohistochemical analysis of PCA-1 and DDR1 in prostatic epithelial and cancer tissues of no (score 0), mild (score 1), moderate (score 2), and high (score 3) intensities. Normal prostatic epithelial tissues were obtained from benign prostatic hypertrophy. NE, normal prostatic epithelial cells; PIN, prostatic intraepithelial neoplasm. (b) To characterize the specificity of antibodies used in the present study, western blotting analysis of PCA-1 and DDR1 was carried out in a sample of normal prostatic epithelial cells obtained from benign prostatic hypertrophy and four prostate cancer cells. (c) Statistical analysis of the intensities of PCA-1 and DDR1 in normal prostatic epithelial cells, prostate cancer cells, or precancerous cells (lesions). (d) Kaplan–Meier analysis of hormone-resistant prostate-specific antigen recurrence-free survival in groups of patients with low or moderate (less than score 2) and high (more than score 3) levels of PCA-1 or DDR1 expression. The follow-up period was 2000 days.

response to PCA-1 overexpression. In the PC3 and DU145 cell lines, MMP9 was expressed constitutively at low levels but was much increased following incubation on CAM. Induction of MMP9 upregulation during the *in vivo* invasion process was also inhibited by PCA-1 siRNA transfection (data not shown). Taken together, DDR1 is an important downstream molecule for PCA-1 and has divergent pathways associated with cell survival and cancer invasion, dependent on Bcl-xl and MMP 9, respectively.

One more important finding of the current study was that PCA-1 and its downstream signals are amplified in hormone-resistant prostate cancer. PCA-1 and DDR1 signals were enhanced in AI prostate cancer cells established by long-term culture in androgen-free medium compared with androgen-dependent LNCaP cells. Moreover, androgen independence resulted in an enhancement in cancer invasion, which was significantly blocked by *PCA-1* and *DDR1* knockdown. Expectedly, AI LNCaP cells were more resistant to anticancer drug-induced apoptosis than parental LNCaP due to increased expression of Bcl-xl (data not shown). Together with data using DU145, PC3, and AI LNCaP cells, we found that the PCA-1–DDR1 signal could be amplified during the process of hormone independence, resulting in enhancement of the malignant potential of prostate cancer cells. It is of great interest that expression of PCA-1 and DDR1 was statistically correlated with PSA recurrence assessed by immunohistochemical analysis using biopsy specimens prior to androgen-deprivation therapy. PSA recurrence doubling time is a prognostic factor for poor outcome of human prostate cancer;[25,26] therefore, PCA-1–DDR1-mediated signals are closely associated with the androgen independence and progression of human prostate cancer. The present data are supported by the fact that Bcl-xl has been reported to play an important role in androgen resistance and prostate cancer development.[27] Our previous and present studies demonstrate that PCA-1 and DDR1 are expressed significantly in cancer cells, including PIN, but not in normal epithelial cells. However, the expression profile was not correlated to clinicopathological parameters such as Gleason score and pretreatment PSA value. The present *in vitro* data clearly show a close association between PCA-1 expression and the enhancement of malignant potential of androgen-independent prostate cancer cells. Moreover, the expression was increased in response to long-term hormone withdrawal. The PCA-1 and DDR1 pathways might be involved in the early steps of tumorigenesis, and the signals are amplified in the process of hormone independence and promote malignant potential of androgen-independent prostate cancer. However, the pathways had no significant effects on progression of androgen-dependent or -sensitive cancer cells. Therefore, we can predict

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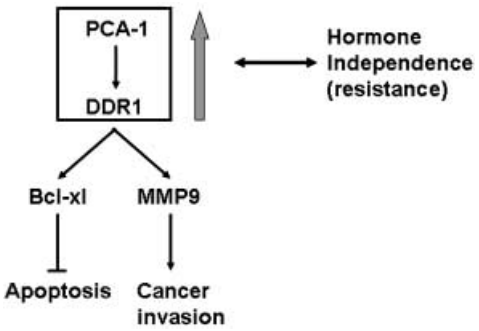

Fig. 6. Schematic presentation of the correlation of prostate cancer antigen (PCA)-1–discoidin domain receptor (DDR)-mediated signals with androgen independence (resistance) and cancer progression in human prostate cancer. PCA-1 and downstream DDR1 and Bclxl or matrix metaloprotease (MMP) 9 signals promoted cancer invasion and survival. PCA-1-mediated signals were amplified, associated with hormone resistance and enhance malignant potential of androgen-independent prostate cancer cells.

the possibility of PSA recurrence from the PCA-1–DDR1 expression profile of biopsy specimens, but the data cannot always reflect the malignant potential of prostate cancer cells prior to androgen-withdrawal therapy. The current study did not make clear how PCA-1 influences tumorigenesis, so further study is needed to examine that in detail.

In summary, PCA-1 is essential for prostate cancer progression through regulating DDR1-mediated Bcl-xl and MMP signals in human prostate cancer. We found that PCA-1 and DDR1 signals can be amplified in association with hormone resistance, are correlated with PSA recurrence by *in vitro* and immunohistochemical analyses, and play an important role in the progression of androgen-independent prostate cancer (Fig. 6). The present study shows for the first time that PCA-1 and DDR1 are useful pathological markers for poor prognosis and could be targets for effective therapy in human prostate cancer.

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