Expression of Androgen and Estrogen Signaling Components and Stem Cell Markers to Predict Cancer Progression and Cancer-Specific Survival in Patients with Metastatic Prostate Cancer

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Abstract

Purpose: Genes of androgen and estrogen signaling cells and stem cell–like cells play crucial roles in prostate cancer. This study aimed to predict clinical failure by identifying these prostate cancer–related genes.

Experimental Design: We developed models to predict clinical failure using biopsy samples from a training set of 46 and an independent validation set of 30 patients with treatment-naïve prostate cancer with bone metastasis. Cancerous and stromal tissues were separately collected by laser-captured microdissection. We analyzed the association between clinical failure and mRNA expression of the following genes androgen receptor (AR) and its related genes (APP, FOX family, TRIM36, Oct1, and ACSL3), stem cell–like molecules (Klf4, c-Myc, Oct 3/4, and Sox2), estrogen receptor (ER), Her2, PSA, and CRP.

Results: Logistic analyses to predict prostate-specific antigen (PSA) recurrence showed an area under the curve (AUC) of 1.0 in both sets for Sox2, Her2, and CRP expression in cancer cells, AR and ERα expression in stromal cells, and clinical parameters. We identified 10 prognostic factors for cancer-specific survival (CSS): Oct1, TRIM36, Sox2, and c-Myc expression in cancer cells; AR, Klf4, and ERα expression in stromal cells; and PSA, Gleason score, and extent of disease. On the basis of these factors, patients were divided into favorable-, intermediate-, and poor-risk groups according to the number of factors present. Five-year CSS rates for the 3 groups were 90%, 32%, and 12% in the training set and 75%, 48%, and 0% in the validation set, respectively.

Conclusions: Expression levels of androgen- and estrogen signaling components and stem cell markers are powerful prognostic tools. Clin Cancer Res; 20(17); 4625–35. ©2014 AACR.

Introduction

The pioneering work of Huggins and Hodges (1) showed that prostate cancer is sensitive to androgen deprivation therapy (ADT); however, at least two problems are associated with ADT. First, durability of ADT varies among prostate cancer patients (2). For example, the median survival time was 13 months for patients with prostate-specific antigen (PSA) nadirs of >4 ng/mL, 44 months for patients with PSA nadirs of 0.2–4 ng/mL, and 75 months for patients with PSA nadirs of ≤0.2 ng/mL (2). Second, ADT is initially effective as a treatment for advanced prostate cancer, but not when prostate cancer acquires a castration-resistant status. A recent study proposed that stem cell–like prostate cancer cells with a pluripotent phenotype are involved in castration-resistant prostate cancer (CRPC; ref. 3). In cases with stem cell–like prostate cancer cells, ADT may actually stimulate cancer progression (3). Therefore, evaluation of both the durability of ADT and presence of stem cell–like cell components in prostate needle biopsy samples before ADT prescription is the first step in personalized medicine for patients with metastatic prostate cancer.

Determination of the therapeutic strategy for breast cancer commonly depends on the expression patterns of estrogen receptor (ERα), progesterone receptor (PR), and Her 2 in needle biopsy samples (4). However, pretreatment diagnosis by estimating gene expression is not yet prevalent for patients with prostate cancer. The growth-inhibitory effects on prostate cancer cells are associated with the status of steroid nuclear receptors and related genes of prostate cancer–related molecules, such as androgen receptor (AR; refs. 5, 6), AR-related genes (5, 6), amyloid precursor...
Translational Relevance
Androgen and estrogen signaling play crucial roles in prostate cancer. Stem cell–like cells are also known to be involved in prostate cancer progression. In the present study, we investigated the expression of androgen and estrogen signaling components and stem cell markers to predict prostate-specific antigen (PSA) recurrence and cancer-specific survival (CSS) in patients with metastatic prostate cancer. Discriminant analysis using the mRNA expression of AR, ERα, Sox2, Her2, CRP, and clinical parameters highly predicted PSA recurrence. We identified 10 prognostic factors for CSS: Oct1, TRIM36, Sox2, and c-Myc, AR, Klf4, and ERα expression as well as PSA, Gleason score, and extent of disease. On the basis of these factors, we propose a new risk classification for CSS. Taken together, the expression pattern of androgen and estrogen signaling components and stem cell markers predicted PSA recurrence and CSS in patients with prostate cancer characterized by bone metastasis.

Materials and Methods
Tissue selection and patient characteristics of the training set
Formalin-fixed, paraffin-embedded sections of the primary tumors were obtained from 46 treatment-naive consecutive patients (age, 58–87 years; mean age, 74 years) diagnosed with bone metastatic prostate cancer between 2001 and 2009 by transrectal ultrasound–guided biopsy. This study was approved by our institutional ethics committee. Before treatment, serum PSA levels were 8.6 to 13,700 ng/mL (mean, 219 ng/mL). The sections were evaluated by two pathologists, and the tumors were assigned to Gleason scores (GSs) of ≤7 (n = 8), 8 (n = 11), 9 (n = 26), and 10 (n = 1). The clinical primary tumor (cT) stages were 2 (n = 6), 3a (n = 15), 3b (n = 14), and 4 (n = 11). The clinical regional lymph node (cN) stages were 0 (n = 21) and 1 (n = 25). To diagnose bone metastasis, we performed bone scintigraphy using technetium-99m-methylene diphosphonate in all the patients. Computed tomography was used in 3 of 46 patients to distinguish from bone degenerative changes. On the basis of the number of or extent of metastases, the scans were divided into five extent of disease (EOD) grades as follows: 0, normal or abnormal due to benign bone disease; 1, number of bony metastases less than 6, each of which is less than 50% of the size of a vertebral body (one lesion about the size of a vertebral body would be counted as two lesions): 2, number of bone metastases between 6 and 20, size of lesions as described above; 3, number of metastases greater than 20 but fewer than a “super scan”; and 4, “superscan” or its equivalent (i.e., more than 75% of the ribs, vertebrae, and pelvic bones; ref. 16). EOD was 1 (n = 25), 2 (n = 10), 3 (n = 9), and 4 (n = 2).

All patients received ADT by medical or surgical castration with or without the administration of antiandrogen agents, bicalutamide (n = 22), chlormadinone (n = 3), flutamide (n = 3), and estramustine phosphate (n = 2; Table 1). PSA relapse was defined by consecutive increase in serum PSA levels to above the patient’s PSA nadir (17). If PSA relapse occurred during initial ADT, either new antiandrogen agent was added or switched to another. When patients were switched to other antiandrogen agents, antiandrogen withdrawal syndrome was checked. From September 2008, systemic chemotherapy by docetaxel was also administered every 3 or 4 weeks. If prostate cancer became hormone- and chemotherapy-refractory, patients received best supportive care. A total of 37 patients (80%) experienced relapse. The mean time to PSA relapse was 972 ± 1,193 days (range, 5–4,616 days). The mean follow-up duration was 1,650 ± 1,319 days (range, 79–5,961 days). At the end of the follow-up period, 9 patients (19%) were alive without PSA relapse, whereas 15 (33%) were alive with biochemical or clinical recurrence. Twenty-two patients (48%) died of prostate cancer during the follow-up period.

Patient characteristics of the validation set
An independent cohort of 30 patients with prostate cancer with bone metastasis (age, 59–91 years; mean age, 68 years) between 2001 and 2011 were enrolled. Before treatment, serum PSA levels were 5.8 to 8,428 ng/mL (mean, 498 ng/mL). The tumors were assigned to Gleason scores of ≤7 (n = 4), 8 (n = 4), 9 (n = 16), and 10 (n = 6). The cT stages were 2 (n = 4), 3a (n = 9), 3b (n = 8), and 4 (n = 9). The cN stages were 0 (n = 5) and 1 (n = 25). EOD was 1 (n = 11), 2 (n = 9), 3 (n = 4), and 4 (n = 6; ref. 16).

All patients received ADT by medical or surgical castration with or without the administration of antiandrogen agents, bicalutamide (n = 27), and estramustine phosphate (n = 3). A total of 25 patients (83%) experienced relapse. The mean time to PSA relapse was 571 ± 877 days (range, 6–4,616 days). The mean follow-up duration was 1,143 ± 1,226 days (range, 71–6,395 days). At the end of the follow-up period, 5 patients (16%) were alive without PSA relapse, whereas 13 (43%) were alive with biochemical...
dissection (Leica 6500). To obtain sufficient materials, we
into groups of cancer cells and stromal cells by laser micro-
blue solution (WAKO), the tissue sections were separated
hydrated using graded ethanol and rinsed in diethylpyrocar-
tate cancer during the follow-up period.

Twelve patients (40%) died of pros-

or clinical recurrence. Twelve patients (40%) died of pro-
tate cancer during the follow-up period.

Laser-captured microdissection
Tissue sections (10 μm) were deparaffinized and rehy-
hydrated using graded ethanol and rinsed in diethylpyrocar-
bone-treated water. After staining with 0.05% toluidine
blue solution (WAKO), the tissue sections were separated
into groups of cancer cells and stromal cells by laser micro-
dissection (Leica 6500). To obtain sufficient materials, we
collected 30 to 460 acini of epithelium within an area of
3,865 mm² in average, and all of the surrounding stroma
were collected. Tissues were collected into Eppendorf cap
containing 25 μL of ISOGEN reagent (Nippon Gene) and
tissues were stored at –80°C before RNA isolation.

Quantitative reverse-transcription PCR
Total RNA was extracted using ISOGEN PB kit (Nippon
Gene). Tissue samples were incubated 15 minutes with
protease K and extraction buffer at 50°C then added to
an ISOGEN-LS (Nippon Gene). Bound RNA was purified in a
series of wash steps to remove cellular components. Residual
dNA was digested by incubating the eluate with DNase. RNA
quality and RNA quantity was assessed using a NanoDrop
ND-1000 spectrophotometer (Japan SCRIM Inc). The ratio
of absorbance at 260 nm and 280 nm was 1.7:2.0, and a total
of 40 ng RNA was used. First-strand cDNA was generated
using PrimeScript RT Master Mix (Takara). Quantitative
reverse-transcription PCR (qRT-PCR) was performed with
150 nmol/L primers using 7300 Real-Time PCR system
(Applied Biosystems); one cycle at 50°C for 10 minutes, 40 cycles at 95°C for 15 seconds
and at 50°C for 1 minute, one cycle at 95°C for 15 seconds,
one cycle at 59°C for 30 seconds, and one cycle at 95°C for 15
seconds. mRNA expression was normalized relative to
GAPDH and the average relative expression of 4 times
examination was adopted.

Primers
The size of PCR amplicons (base pair: bp) and the
sequences of the PCR primers used are shown below:

| AR (59 bp) | AR forward: 5'- CCTGCAAGGCTCTCTCTAAAGA-3' |
| AR reverse: 5'- GCTGGCCGCACAGGTACTCT-3' |
| Oct1 (130 bp) | Oct1 forward: 5'- CTCGCTTCTTTCCGGTAG-3' |
| Oct1 reverse: 5'- GTCCTGTTT GCCCCAACCAT-3' |
| FOXO1 (128 bp) | FOXO1 forward: 5'- CTCGATCCATGGCACAAC-3' |
| FOXO1 reverse: 5'- AGGCCATTTGGAAAATGTG-3' |
| FOXA1 (78 bp) | FOXA1 forward: 5'- ACCGGCTCCATGGGAAT-3' |
| FOXA1 reverse: 5'- CGTGTCAGCTCTCCCGTATT-3' |
| APP (121 bp) | APP forward: 5'- GAGGACACATTGGGAGTAGA-3' |
| APP reverse: 5'- CTTGACGTCCTGCTCTCCCT-3' |
| ACSL3 (110 bp) | ACSL3 forward: 5'- GACACAGGGGCGATATCT-3' |
| ACSL3 reverse: 5'- AGGTGGGCAATAGTGACG-3' |
| TRIM36 (136 bp) | TRIM36 forward: 5'- CTGCTGCTGCTCTTCCAA-3' |
| TRIM36 reverse: 5'- GACACACGGCTGACAGA-3' |
| Oct3/4 (110 bp) | Oct3/4 forward: 5'- AGTGAGAGGCAACCTTGAGA-3' |

Table 1. Correlation between men with and without PSA recurrence in patients with bony metastatic prostate cancer (n = 46)

<table>
<thead>
<tr>
<th>Clinical findings</th>
<th>Groups</th>
<th>PSA recurrence (n = 37)</th>
<th>Without PSA recurrence (n = 9)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>70 ± 8.0</td>
<td>72 ± 4.3</td>
<td>0.20</td>
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<tr>
<td>Serum PSA (ng/mL)</td>
<td>1,109 ± 2,421</td>
<td>1,170 ± 2,777</td>
<td>0.15</td>
<td></td>
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<tr>
<td>Gleason score</td>
<td>≤7</td>
<td>7</td>
<td>1</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>7</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>22</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Clinical T stage</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>0.27</td>
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<tr>
<td></td>
<td>3a</td>
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<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3b</td>
<td>13</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>9</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Clinical N stage</td>
<td>0</td>
<td>14</td>
<td>5</td>
<td>0.61</td>
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<tr>
<td></td>
<td>1</td>
<td>23</td>
<td>4</td>
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<tr>
<td>EOD</td>
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<td>2</td>
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<tr>
<td></td>
<td>3</td>
<td>9</td>
<td>0</td>
<td></td>
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<tr>
<td></td>
<td>4</td>
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<td></td>
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<tr>
<td>MAB</td>
<td>No</td>
<td>14</td>
<td>3</td>
<td>0.58</td>
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<td></td>
<td>Yes</td>
<td>21</td>
<td>6</td>
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<tr>
<td>Initial antiandrogen agent</td>
<td>Bicalutamide</td>
<td>17</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chlormadinone</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Flutamide</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Estrogens</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>PSA nadir</td>
<td>&lt;0.01</td>
<td>1</td>
<td>9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>0.01–0.1</td>
<td>16</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1≤</td>
<td>20</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Time to PSA nadir after ADT (days)</td>
<td>424 ± 286</td>
<td>419 ± 303</td>
<td>0.31</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: MAB, maximum androgen blockade with antiandrogen agents.
Oct3/4 reverse: 5'-ACACCTGGACACATCCITT-3'
Sox2 (95 bp) Sox2 forward: 5'
CAAGATCCACACTGGAGA-3'
Sox2 reverse: 5'-GCTTACCTGCGATGTAAC-3'
Klf4 (127 bp) Klf4 forward: 5'
ACTCGCCTTGCTGATTGTCT-3'
Klf4 reverse: 5'-AGTTAACGCAAGGTGGTG-3'
c-Myc (123 bp) c-Myc forward: 5'
TCAAGGACACACACAC-3'
c-Myc reverse: 5'-TAACATACCTGGCGCCCTT-3'
CRP (107 bp) CRP forward: 5'
TGCTCTGACGACCCCTT-3'
CRP reverse: 5'-CGGTGCTTGAAGGGGATCAT-3'
Her2 (132 bp) Her2 forward: 5'
ACCAAGGCTCTGCCCACACT-3'
Her2 reverse: 5'-ACTGGCTGAGTGTACACAC-3'
ERβ (175 bp) ERβ forward: 5'
AAGAGATCCCGGCTTGT-3'
ERβ reverse: 5'-CTTCTACCTGCTCTTCA-3'
Klf5 (81 bp) Klf5 forward: 5'
CACCTCTATCCTATGCTGTC-3'
Klf5 reverse: 5'-AGTTAACGCAAGGTGGTG-3'
Erα (153 bp) Erα forward: 5'
AGCACCCTGATGCTCCTGGA-3'
Erα reverse: 5'-GATGTGGGAGAGGATGAGGA-3'
GAPDH (80 bp) GAPDH forward: 5'
GGTCTGCTCTGCTGACTTACAC-3'
GAPDH reverse: 5'-GGTCTGCTGACTTACACGATTG-3'

We have previously uncovered the AR transcriptional network in prostate cancer cells by chromatin immunoprecipitation (ChIP) combined with DNA microarray (ChIP-chip) and cap analysis gene expression (CAGE) (18). In addition, on the basis of previous studies, we selected APP (7), FOX family proteins (FOXO1, FOXA1, and FOXP1; refs. 9–11), tripartite molecule 36 (TRIM; ref. 18), Oct1 (12), and ACSL3 (18) for this study. Stem cell–like markers [Oct3/4, Sox2, Krüppel-like factor (Klf4), and c-Myc; refs. 19, 20] and prostate cancer–related genes (CRP, Her2, ERβ, Klf5, and Erα) were also evaluated (21–23).

Antibodies

We performed immunohistochemistry of AR and Klf4 to evaluate a correlation with its mRNA expression.

Because only a small amount of the biopsy samples was available, we chose two antibodies; AR (AR441; ref. 24) and Klf4 (ab72543; ref. 25) which had been reported to be adequate for immunohistochemical analysis. Mouse monoclonal antibody for AR (AR441) and rabbit polyclonal antibody for Klf4 (ab72543) were purchased from Dako and Abcam, respectively.

Immunohistochemical analysis

We have previously uncovered the AR transcriptional network in prostate cancer cells by chromatin immunoprecipitation (ChIP) combined with DNA microarray (ChIP-chip) and cap analysis gene expression (CAGE) (18). Oct3/4 and Sox2, which were previously described (12, 26). The primary antibody against AR and Klf4 (1:50 dilution) was applied and incubated at room temperature for 1 hour. The sections were then washed in PBS and incubated at room temperature with EnVision + for 1 hour. The antigen–antibody complex was visualized with 3,3′-diaminobenzidine (DAB) solution [1 mmol/L DAB, 50 mmol/L Tris-HCl buffer (pH 7.6), and 0.006% H2O2].

Immunohistochemical assessment

The sample slides were evaluated for staining intensity (ref. 26; none, weak, moderate, and strong) based on labeling index (LI; ref. 27). LIs were determined by counting the percentage of cells with positive immunoreactivity in 1,000 cells (27). Two pathologists (T. Fujimura and S. Takahashi) independently evaluated the tissue sections, and the average LI was used. We defined positive immunoreactivity as showing moderate or strong immunoreactivity.

Statistical analyses

Correlations between age, pretreatment serum PSA levels, mRNA levels, and PSA relapse were evaluated using the Wilcoxon test. Associations between PSA relapse, Gleason scores, clinical stage, and antiandrogen therapy were assessed using χ2 tests. Correlations between mRNA expression, PSA relapse, and clinical parameters were statistically analyzed using logistic regression analyses. Appropriate variables indicating ≥2 F values were selected by stepwise method. CSS curves were plotted using the Kaplan–Meier method and verified using the log-rank test. Cox-hazard proportional analysis was used for estimating the relationship between mRNA expression and CSS. A correlation between the mRNA expression and LI was evaluated using Spearman rank-correlation coefficient. JMP 9.0 software (SAS Institute) was used for statistical analyses, and P < 0.05 was considered statistically significant.

Results

Relationship between PSA relapse and clinicopathologic data

We divided the patients into two groups according to PSA recurrence during the follow-up period: the PSA recurrence group (n = 37) and the no-recurrence group (n = 9; Table 1). No significant correlations were found between PSA recurrence and clinicopathologic characteristics such as age, pretreatment serum PSA levels, Gleason scores, clinical stage, EOD, or therapeutic regimens (Table 1). However, all patients without PSA recurrence achieved a PSA nadir of <0.01, which is the measurement limit in our institute, a proportion significantly higher than that in the PSA recurrence group (P < 0.0001). Time to PSA nadir following ADT among the PSA recurrence and no-recurrence groups was 424 ± 268 days and 419 ± 303 days, respectively (P = 0.31). The CSS rate was significantly worse in men with PSA recurrence than in the other patients (P = 0.0045).
Relative mRNA expression of AR, AR-related genes, stem cell–like markers, and prostate cancer-related genes between the PSA recurrence and no-recurrence groups

The relative mRNA expression of AR, AR-related genes, stem cell–like markers, and prostate cancer–related genes is shown in Table 2. Expression of AR in both cancer and stromal cells was significantly stronger in men without PSA recurrence than in the other group ($P = 0.0026$ and 0.013, respectively). Expression of APP, TRIM36, Klf4, c-Myc, and ERβ in stromal cells from men without PSA recurrence was significantly increased ($P = 0.0018$, 0.047, 0.032, 0.044, and 0.032, respectively).

Comparison of area under the curve for clinical parameters and gene expression in prostate needle biopsy samples for predicting PSA recurrence

Logistic regression analyses for predicting PSA recurrence using age, serum PSA levels, Gleason scores, T stage, N stage, and EOD revealed relatively high area under the curves (AUCs; 0.83; Fig. 1A); however, 11 patients (23%) were misclassified by discriminant analyses. In contrast, the AUCs for Sox2, Her2, and CRP mRNA expression in cancer cells; AR and ERα mRNA expression in stromal cells; and clinical parameters were 1.0 in men with PSA recurrence. Only 2 patients (4%) were misclassified by discriminant analyses (Fig. 1B).

Correlation between CSS and gene expression

We compared prognostic clinical parameters and gene expression profiles using Cox proportional hazard analyses (Table 3). Cutoff values for age, serum PSA levels, T stage, EOD, and relative mRNA expression for each gene were determined using receiver operating characteristic (ROC) curves. Decreased expression of Oct1, TRIM36, Sox2, and c-Myc in cancer cells and decreased expression of AR, Klf4, and ERα in stromal cells were significant prognostic factors in univariate hazard analyses (HR: 2.6, 2.9, 3.0, 2.7, 3.8, 4.1, and 2.5, respectively; $P = 0.031$, 0.0015, 0.045, 0.022, 0.0067, 0.0014, and 0.0034, respectively). Increased serum PSA levels ($\geq 335$ ng/mL), increased Gleason scores ($\geq 8$), and high EOD ($\geq 2$) were also correlated with CSS (HR: 2.7, 3.5, and 2.9; $P = 0.027$, 0.046, and 0.016, respectively). Multivariate analyses were not performed because of the following reasons. To avoid multicollinearity problem, a correlation matrix was constructed among 10 prognostic parameters. The Spearman rank-correlation coefficient test showed that relative expression of Oct1, TRIM36, c-Myc, and SOX2 in cancer cells has significant correlation with one another. Relative expression of AR, ERα, and Klf4 in stromal cells has also significant correlation with one another. Therefore, we used 10 factors to classify 3 prognostic groups because of lacking independent variables. Furthermore, the number of cancer-specific death ($n = 24$) events was small relative to 10 prognostic factors.

Risk classification according to gene expression and correlation with CSS

According to the number of cancer-specific risk factors described above (Oct1, TRIM36, Sox2, and c-Myc expression in cancer cells; AR, Klf4, and ERα expression in stromal cells; serum PSA levels $\geq 335$ ng/mL, Gleason scores $\geq 8$, and EOD $\geq 2$), we divided the patients into favorable-, intermediate-, and poor-risk groups, which had $0–3$, $4–7$, and $8–10$ risk factors, respectively. Significant differences were observed in CSS rates among the 3 groups (favorable vs. intermediate, $P = 0.0013$; favorable vs. poor; $P < 0.0001$; and intermediate vs. poor, $P = 0.0059$; Fig. 1C). Five-year CSS rates for the favorable-, intermediate-, and poor-risk groups were 90%, 32%, and 12%, respectively.

Validation study in an independent cohort of 30 patients with bony metastatic prostate cancer

To validate the reproducibility, we performed logistic regression analyses, discriminant analyses for predicting PSA recurrence, and made new risk classification for CSS in an independent cohort of 30 patients with prostate cancer with bone metastasis. Logistic regression analyses for predicting PSA recurrence using clinical findings showed relatively high AUCs (0.95; Fig. 2A); however, 9 patients (30%) were misclassified by discriminant analyses. In contrast, the AUCs for Sox2, Her2, and CRP mRNA expression in cancer cells; AR and ERα mRNA expression in stromal cells; and clinical parameters were 1.0 in men with PSA recurrence. Four patients (13%) were misclassified by discriminant analyses (Fig. 2B). According to the above classification, we also divided the patients into favorable-, intermediate-, and poor-risk groups. Clinical significance was shown in CSS rates among the 3 groups (favorable vs. intermediate, $P = 0.11$; favorable vs. poor; $P = 0.0025$; and intermediate vs. poor, $P = 0.033$; Fig. 2C). Five-year CSS rates for the favorable-, intermediate-, and poor-risk groups were 75%, 48%, and 0%, respectively.

Immunohistochemistry and correlation between immunoreactivity and mRNA expression of AR and Klf4

Among seven prognostic genes, we selected AR and Klf4 to investigate the relationship between immunoreactivity and mRNA expression of these genes. Figure 2D–I shows the results of immunohistochemical analyses for AR and Klf4 in prostate cancer. Immunostaining of AR was identified in the nuclei of cancer and stromal cells (Figure 2D–F), whereas that of Klf4 was localized in both nuclei and cytoplasm in cancer and stromal cells (Figure 2G–I). The LIs of AR in cancer and stromal cells were $55 \pm 41$ (0–100) and $19 \pm 29$ (0–100), respectively. The LIs of Klf4 in cancer and stromal cells were $50 \pm 39$ (0–100) and $12 \pm 17$ (0–70), respectively (Supplementary Fig. S1A–S1D). Two pathologists independently evaluated the tissue sections and the average LI was used. The SEs of LIs AR in cancer and stromal cells were 0.35 and 0.06, respectively. The SEs of LIs of Klf4 in cancer and stromal cells were 1.25 and 0.99, respectively. The Spearman rank-correlation coefficient ($r$) between immunoreactive score and mRNA expression was shown as follows: AR
in cancer and stromal cells ($\rho = 0.73$, $P < 0.0001$ and $\rho = 0.56$, $P = 0.0001$, respectively), Klf4 in cancer and stromal cells ($\rho = 0.08$, $P = 0.61$ and $\rho = 0.56$, $P < 0.0001$, respectively; Supplementary Fig. S1).

**Discussion**

The most common initial systemic therapy for metastatic prostate cancer is ADT; however, the durability of ADT varies. Among patients with metastatic prostate cancer who received ADT, the median survival time was 13 months for patients with PSA nadirs of $>4$ ng/mL, 44 months for patients with PSA nadirs of 0.2–4 ng/mL, and 75 months for patients with PSA nadirs of $\leq 0.2$ ng/mL (2). Several relevant nomograms are used to predict progression-free survival and CSS in patients with prostate cancer (28). These nomograms are estimated according to age, serum PSA levels, Gleason scores, and clinical stage. Clinical parameters showed a degree of predictive power in the present study. These clinical parameters do not sufficiently reflect cancer aggressiveness or durability of treatments such as ADT, radiation, or chemotherapy because clinical parameters do not accurately correlate with prostate cancer cell behavior.

The durability of ADT may be influenced by AR and AR-dependent gene profiles in prostate cancer cells. ADT has an

<table>
<thead>
<tr>
<th>Gene</th>
<th>PSA recurrence ($n = 37$)</th>
<th>Without PSA recurrence ($n = 9$)</th>
<th>$P$</th>
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</thead>
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<tr>
<td>Androgen-related genes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AR</td>
<td>Cancer 20 ± 47</td>
<td>33 ± 24</td>
<td>0.0026</td>
</tr>
<tr>
<td></td>
<td>Stroma 11 ± 14</td>
<td>38 ± 31</td>
<td>0.013</td>
</tr>
<tr>
<td>Oct1</td>
<td>Cancer 4.7 ± 8.0</td>
<td>6.6 ± 12</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>Stroma 3.4 ± 5.3</td>
<td>9.9 ± 20</td>
<td>0.35</td>
</tr>
<tr>
<td>FOXO1</td>
<td>Cancer 2.6 ± 5.1</td>
<td>8.3 ± 22</td>
<td>0.30</td>
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<tr>
<td></td>
<td>Stroma 3.1 ± 6.4</td>
<td>0.78 ± 1.1</td>
<td>0.29</td>
</tr>
<tr>
<td>FOXA1</td>
<td>Cancer 0.64 ± 1.9</td>
<td>0.30 ± 0.35</td>
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</tr>
<tr>
<td></td>
<td>Stroma 0.79 ± 2.1</td>
<td>0.11 ± 0.23</td>
<td>0.15</td>
</tr>
<tr>
<td>FOXP1</td>
<td>Cancer 0.66 ± 1.7</td>
<td>0.33 ± 0.57</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>Stroma 0.94 ± 4.2</td>
<td>0.081 ± 0.15</td>
<td>0.37</td>
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<tr>
<td>APP</td>
<td>Cancer 10 ± 21</td>
<td>21 ± 32</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>Stroma 38 ± 99</td>
<td>39 ± 38</td>
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<tr>
<td>ACSL3</td>
<td>Cancer 4.8 ± 12</td>
<td>1.6 ± 2.0</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>Stroma 3.4 ± 7.2</td>
<td>8.3 ± 16</td>
<td>0.09</td>
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<tr>
<td>TRIM36</td>
<td>Cancer 0.78 ± 1.6</td>
<td>0.92 ± 1.6</td>
<td>0.25</td>
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<td></td>
<td>Stroma 2.7 ± 12</td>
<td>3.4 ± 6.9</td>
<td>0.047</td>
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<tr>
<td>Oct3/4</td>
<td>Cancer 9.8 ± 13</td>
<td>18 ± 36</td>
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<tr>
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<td>Stroma 9.2 ± 12</td>
<td>22 ± 32</td>
<td>0.31</td>
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<tr>
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<td>0.61 ± 1.1</td>
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<tr>
<td></td>
<td>Stroma 2.7 ± 9.1</td>
<td>1.0 ± 1.1</td>
<td>0.12</td>
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<tr>
<td>Klf4</td>
<td>Cancer 4.5 ± 8.5</td>
<td>5.5 ± 8.7</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>Stroma 9.2 ± 23</td>
<td>9.4 ± 13</td>
<td>0.032</td>
</tr>
<tr>
<td>c-MyC</td>
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<tr>
<td></td>
<td>Stroma 17 ± 37</td>
<td>26 ± 40</td>
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<td>Prostate cancer–related genes</td>
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<tr>
<td>CRP</td>
<td>Cancer 4.8 ± 9.7</td>
<td>2.6 ± 3.0</td>
<td>0.53</td>
</tr>
<tr>
<td></td>
<td>Stroma 8.3 ± 33</td>
<td>3.9 ± 5.9</td>
<td>0.54</td>
</tr>
<tr>
<td>Her2</td>
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<td>0.83</td>
</tr>
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<td>ERβ</td>
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<td>8.0 ± 15</td>
<td>0.28</td>
</tr>
<tr>
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<td>Stroma 6.7 ± 17</td>
<td>8.0 ± 9.3</td>
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<tr>
<td>Klf5</td>
<td>Cancer 7.2 ± 11</td>
<td>6.7 ± 8.2</td>
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<td>Stroma 25 ± 68</td>
<td>12 ± 11</td>
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<tr>
<td>ERα</td>
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<td>0.18 ± 0.38</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>Stroma 0.18 ± 0.27</td>
<td>1.9 ± 3.7</td>
<td>0.16</td>
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Inhibitory effect on prostate cancer; however, ADT negatively selects stem cell–like prostate cancer cells, an important component of CRPC (3, 29, 30). Germann and colleagues found that castration induced the expression of 4 essential transcription factors required for reprogramming, self-renewal, and pluripotency in differentiated somatic cells, namely Oct4, Sox2, Klf4, and NANOG (3). Therefore, before ADT is prescribed, an accurate prediction is critical for treatment decisions, which range from ADT alone to initial combination therapy with other therapeutic agents such as docetaxel, cabazitaxel, zoledronic acid, and denosumab (31), that are tightly linked to prognoses. In this respect, we determined molecular indicators of ADT stability and cancer progression in patients with metastatic prostate cancer by evaluating AR, AR-related genes, stem cell–related genes, and prostate cancer–related genes.

Molecular diagnosis using immunohistochemistry, FISH, and DNA, RNA, or miRNA analyses is discussed in a recent article (32–38). The studies in which gene sets of stem cell–like cells, micro-RNA, or cell-cycle progression markers reflect more aggressive disease are limited to localized prostate cancer (33–35). Although several reports have shown that certain gene expression profiles in primary prostate cancers correlate with poor prognosis, no consensus has been reached regarding specific prognostic markers (32). In addition, controversial data may reflect contamination of samples by other tissue elements such as aggressive tumors, normal prostate epithelium, and normal stromal components. Therefore, LCM techniques may influence the accuracy of the molecular diagnosis of prostate cancer. A recent study using frozen samples showed that low PSA/HK3 mRNA expression in prostate cancer was associated with increased risk of biochemical recurrence in patients with intermediate preoperative serum PSA levels (2–10 ng/mL; ref. 36). Fresh frozen samples are preferable for RNA analysis of prostate biopsy samples. However, a refined LCM technique efficiently provided mRNA and miRNA from formalin-fixed biopsy samples (37, 38). In this study, we showed that the durability of ADT is a cancer-specific prognostic factor in patients with metastatic prostate cancer using paraffin-embedded needle biopsy samples.

Gene expression profiles have been successfully used to define breast cancer subclasses with different biologic behaviors and responses to therapy (4). Gene expression-based diagnostic tests are currently in clinical use for assessing the risk of recurrence and for predicting the benefits of adjuvant chemotherapy in patients with localized ER-positive and lymph node-negative breast cancers (39). In contrast to breast cancers, in which the status of estrogen receptors in primary tumors is commonly used to make therapeutic and prognostic decisions, the status of AR protein expression does not seem to be as useful as in prostate cancer (32). One possible explanation for this is that AR expression is heterogeneous and changes over time (31). Therefore, measurement of the expression of selected AR downstream targets can provide information on the individual functional status of ARs in prostate cancer cells. We previously defined an AR transcriptional network in prostate cancer cells using ChIP–chip and CAGE assays (18) and also analyzed the AR-related genes APP, Oct1, and FOXP1 in prostate cancer (7, 11, 12). In these experiments, APP and Oct1 were correlated with prostate cancer aggressiveness and both were considered therapeutic targets (7, 12). Some FOX proteins that are involved in cell growth and differentiation as well as in embryogenesis and longevity are known to be AR-related genes (8). In the current study, we showed relationships between biochemical recurrence and AR, ERα, Sox2, CRP, and Her2 expression and between prognoses and expression of AR, Oct1, TRIM36, Sox2, Klf4, c-Myc, and ERα. Functional analysis of TRIM36 proteins, a subfamily of RING type E3 ubiquitin ligases, remains unresolved, and further investigation of the TRIM family is required in...
association with prostate cancer. The correlation between suppressed Oct1, Sox2, and c-Myc expression in cancer cells and poor prognosis may reflect the sensitivity of these genes to ADT. Recent studies determined the localization and function of Klf4 in prostate cancer cells (40, 41). In these studies, Klf4 was downregulated in prostate cancer cell lines and metastatic prostate cancer tissue (41), and RNA activator-mediated overexpression of Klf4 inhibited prostate cancer cell growth/survival and arrested cell-cycle progression (41). Together with the present data, Klf4 appears to exert a powerful inhibitory effect in prostate cancer.

Recent therapeutic strategies for advanced and metastatic prostate cancer include ADT combined with antiandrogen agents such as bicalutamide or flutamide, secondary
hormonal manipulation using adrenal testosterone inhibitors, low-dose diethylstilbestrol therapy, steroid therapy, somatostatin analog therapy, and chemotherapy (31). In the present study, we identified patients in whom the durability of ADT was poor or moderate on the basis of molecular diagnoses. We also validated the models of both PSA recurrence and CSS in an independent metastatic prostate cancer cohort. Further investigations in a relative large cohort prove the reproducibility, molecular diagnosis of needle biopsy samples may be generalized for strategic interventions. Immuno-histochemical analysis using biopsy samples may be easier to perform in clinical settings than estimating mRNA expression using LCM. Therefore, patients predicted to have CRPC in the early phase of disease may be given other therapeutic interventions before the progression of aggressive phenotypes.

This study found that genes expressed in stromal cells, such as AR, Klf4, and ERa, were correlated with cancer progression. Expression of these genes may contribute to the stromal–epithelial interactions in prostate cancer progression. The stromal cells in the prostatic tissue consist of myofibroblasts, fibroblasts, and smooth muscle cells within a connective tissue matrix. Numerous growth factors produced by the stromal cells, including transforming growth factors, platelet-derived growth factors, fibroblast growth factors, and EGFs, are crucial for prostate cancer growth (42,
43). These growth factors change microenvironment around the stromal cells (43). Clark and colleagues created a bioengineered microenvironment using tissue recombination that involved mixing of stromal and epithelial cell populations (43). In coculture with cancer-associated fibroblasts (CAF), BPH-1 cells showed a more aggressive phenotype with increased motility and a more direct way of cell migration (43). In addition, the secretion of growth factors is influenced by steroid hormones because stromal cells express AR, ERα, and ERβ that play significant roles in prostate epithelial cell growth (42). For example, Risbridger and colleagues investigated prostatic tissue recombinants established from wild-type ERα and its knockout (KO) mice (44). Presence of squamous metaplasia induced by estrogen was evaluated in each combination of tissue recombinants, such as wt-stroma (S) + wt-epithelia (E), αERKO-S + αERKO-E, wt-S + αERKO-E, and αERKO-S + wt-E. Squamous metaplasia induced by estrogen was found only in the wt-S + wt-E group, which suggested that both stromal and epithelial ERα are required to achieve full response to estrogen in terms of developing squamous metaplasia (44). Stromal AR also influences oncogene epithelium cell growth (45). In Lai and colleagues (45), the authors established an animal model with AR deletion in stromal fibrous cells in PTEN deleted from chromosome 10 mouse. Prostatic intraepithelial neoplasia (PIN) was developed via changing tumor microenvironment, such as the alteration of angiogenesis and immune cells infiltration in the model (45). Moreover, AR degradation enhancer, ASC-J9, suppresses PIN development via stromal AR degradation (45). These findings suggest that modulators of stromal–epithelial interactions may be useful as future therapeutic agents.

In conclusion, we demonstrated a predictive model of PSA recurrence and CSS in patients with prostate cancer with bone metastasis by measuring the expression of prostate–related cancer–related genes in needle biopsy samples. Intermediate- or poor-risk patients with bony metastatic prostate cancer would be candidates for further clinical trials in addition to ADT.

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

Authors’ Contributions
Conception and design: T. Fujimura, S. Takahashi, T. Urano, Y. Yamada, S. Inoue, Y. Homma
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Fujimura, T. Sugihara
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Fujimura, S. Takahashi, K. Takayama, S. Inoue
Writing, review, and/or revision of the manuscript: T. Fujimura, S. Takahashi, T. Urano, K. Takayama, J. Kumagai, S. Inoue, Y. Homma
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Fujimura, K. Takayama, D. Obinata, H. Kume, Y. Ouchi, Y. Homma
Study supervision: S. Takahashi, J. Kumagai, S. Inoue, Y. Homma

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References
Androgen and Estrogen Signaling and Stem Cell Markers in Prostate Cancer

Clinical Cancer Research

Expression of Androgen and Estrogen Signaling Components and Stem Cell Markers to Predict Cancer Progression and Cancer-Specific Survival in Patients with Metastatic Prostate Cancer

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