Epithelial-to-Mesenchymal Transition Mediates Docetaxel Resistance and High Risk of Relapse in Prostate Cancer

Mercedes Marín-Aguilera, Jordi Codony-Servat, Óscar Reig, Juan José Lozano, Pedro Luis Fernández, María Verónica Pereira, Natalia Jiménez, Michael Donovan, Pere Puig, Lourdes Mengual, Raquel Bermudo, Albert Font, Enrique Gallardo, María José Ribal, Antonio Alcaraz, Pere Gascón, and Begona Mellado

Abstract

Molecular characterization of radical prostatectomy specimens after systemic therapy may identify a gene expression profile for resistance to therapy. This study assessed tumor cells from patients with prostate cancer participating in a phase II neoadjuvant docetaxel and androgen deprivation trial to identify mediators of resistance. Transcriptional level of 93 genes from a docetaxel-resistant prostate cancer cell lines microarray study was analyzed by TaqMan low-density arrays in tumors from patients with high-risk localized prostate cancer (36 surgically treated, 28 with neoadjuvant docetaxel + androgen deprivation). Gene expression was compared between groups and correlated with clinical outcome. VIM, AR and RELA were validated by immunohistochemistry. CD44 and ZEB1 expression was tested by immunofluorescence in cells and tumor samples. Parental and docetaxel-resistant castration-resistant prostate cancer cell lines were tested for epithelial-to-mesenchymal transition (EMT) markers before and after docetaxel exposure. Reversion of EMT phenotype was investigated as a docetaxel resistance reversion strategy. Expression of 63 (67.7%) genes differed between groups (P < 0.05), including genes related to androgen receptor, NF-κB transcription factor, and EMT. Increased expression of EMT markers correlated with radiologic relapse. Docetaxel-resistant cells had increased EMT and stem-like cell markers expression. ZEB1 siRNA transfection reverted docetaxel resistance and reduced CD44 expression in DU-145R and PC-3R. Before docetaxel exposure, a selected CD44+ subpopulation of PC-3 cells exhibited EMT phenotype and intrinsic docetaxel resistance; ZEB1/CD44+ subpopulations were found in tumor cell lines and primary tumors; this correlated with aggressive clinical behavior. This study identifies genes potentially related to chemotherapy resistance and supports evidence of the EMT role in docetaxel resistance and adverse clinical behavior in early prostate cancer. Mol Cancer Ther; 13(5); 1–15. ©2014 AACR.

Introduction

Prostate cancer is the most common malignancy in the Western world and the second most common cause of cancer-related mortality in men (1). Although most patients with metastatic prostate cancer respond to androgen deprivation therapy, virtually all of them eventually develop castration-resistant prostate cancer (CRPC). In 2004, the combination of docetaxel and prednisone was established as the new standard of care for patients with CRPC (2). More recently, two hormonal agents, abiraterone and enzalutamide, and a new taxane, cabazitaxel, have been approved for the treatment of CRPC (3–5). However, current therapies are not curative and research is needed to identify predictors of benefit and mechanisms of resistance for each agent.

To date, several factors have been associated with docetaxel resistance, including expression of isoforms of β-tubulin (6), activation of drug efflux pumps (7), PTEN loss (8), and expression and/or activation of survival factors (i.e., PI3K/AKT1 and MTOR; refs. 9, 10). Previous work by our group and others correlated the activation of NF-κB/interleukin (IL)-6 pathways with docetaxel resistance in CRPC models and in patients (11–13). Other studies support a role of JUN/AP-1, SNAI1, and
NOTCH2/Hedgehog signaling pathways in the development of resistance to docetaxel or paclitaxel (14, 15). Moreover, it has been shown that the inhibition of androgen receptor (AR) nuclear translocation and AR activity may be an important mechanism of taxane action in prostate cancer (9).

In previous work, we identified 243 genes with differential expression in CRPC docetaxel-sensitive versus docetaxel-resistant cell lines (16). In the present study, 73 genes from that study together with 20 genes from the literature were tested in tumor specimens of patients with high-risk localized prostate cancer included in a clinical trial of neoadjuvant hormone chemotherapy (17), and compared with nontreated specimens with similar clinical characteristics. This approach was based on the notion that residual tumor cells in prostatectomy specimens after neoadjuvant systemic therapy are likely enriched for resistant tumor cells and their molecular characterization may provide important information on mechanisms of resistance (18). Our key findings were then tested in two models of docetaxel-resistant prostate cancer cell lines.

Patients and Methods

Patients and samples

The study included 28 patients with high-risk localized prostate cancer from a previously published, multicenter, phase II trial of neoadjuvant docetaxel plus androgen deprivation followed by radical prostatectomy and 36 control patients with high-risk prostate cancer treated with radical prostatectomy without neoadjuvant treatment. Of the 57 participants in the clinical trial (17), 29 were not included in this study: 23 patients did not consent to participation in the molecular substudy and insufficient material for molecular analysis was available for 6 patients, 3 of whom had a pathologic complete response (pCR) and 3 had microscopic residual tumor (near pCR) in the prostate specimen.

Inclusion criteria were histologically confirmed adenocarcinoma of the prostate with any of the following three risk criteria: (i) clinical stage T3; (ii) clinical stage T1c or T2 with serum prostate-specific antigen (PSA) >20 ng/mL and/or Gleason score sum of 8, 9, or 10; or (iii) a Gleason sum of 7 with a predominant form of 4 (i.e., Gleason score 4 + 3). Clinical characteristics are shown in Table 1.

Treatment consisted of three cycles every 28 days of docetaxel 36 mg/m² on days 1, 8, and 15 concomitant with androgen blockade, followed by radical prostatectomy. Patients were followed from the time of study inclusion until death or last visit. Median follow-up time was 82 months (range, 10–135). PSA relapse was defined as two consecutive values of 0.2 ng/mL or greater (19). Radiologic progression was defined as the progression in soft tissue lesions measured by computed tomography or MRI, or by progression to bone (20).

The study was approved by the Institutional Ethics Committee of each participating hospital and written informed consent was obtained from all participants. Formalin-fixed paraffin-embedded (FFPE) specimens were collected after radical prostatectomy. A representative tumor area was selected for each block and, according to its size, between 2 and 12 sections were cut, 10-μm thick, and used for RNA isolation. Hematoxylin and eosin (H&E)–stained sections from tumors and adjacent tissues were prepared to confirm the histologic diagnosis.

RNA extraction

Total RNA was isolated from tumor specimens using the RecoverAll Total Nucleic Acid Isolation Kit (Life Technologies) according to the manufacturer’s protocol. Total RNA was quantified with a spectrophotometer (NanoDrop Technologies).

Gene selection

In total, 93 target genes that could potentially be related to docetaxel resistance and two endogenous control genes (ACTB and GUSB) were selected for further analysis in tumors. A set of 73 target genes was selected for their relative expression in docetaxel-resistant cells (DU-145R and PC-3R) versus parental cells (DU-145 and PC-3; ref. 16) using DAVID (21) and Ingenuity Pathway Analysis software (http://www.ingenuity.com). Twenty genes highlighted in the literature as potential targets of docetaxel resistance were also selected.

Reverse transcription and preamplification

A High-Capacity cDNA Reverse Transcription Kit (Life Technologies) was used to reverse transcribe 1 μg of total RNA in a 50 μL reaction volume. cDNA preamplification was performed by multiplex PCR with the 93 selected genes (Supplementary Table S1) and the stem-like cell markers CD24 and CD44, following the manufacturer’s instructions for the TaqMan PreAmp Master Mix Kit (Life Technologies), except that final volume of the reaction was 25 μL.

Gene expression analysis in FFPE samples

Preamplified cDNA was used for gene expression analysis using 384-Well Microfluidic Cards (Life Technologies). Preamplified samples were diluted 1:20 in TE 1X buffer before use. Each card was configured into four identical 96-gene sets (95 selected genes plus an endogenous control gene, RNA18S, by default). The reaction was carried out following the manufacturer’s instructions on an ABI 7900HT instrument (Life Technologies). Array cards were analyzed with RQ Manager Software for manual data analysis.

Gene expression of CD24 and CD44 markers was studied by amplifying with TaqMan Gene Expression Master Mix in a StepOnePlus Real-Time PCR system (Life Technologies), according to the manufacturer’s recommendations. Relative gene expression values were calculated on the basis of the quantification cycle values obtained with SDS 2.4 software (Life Technologies). Expression values were relative to the GUSB endogenous gene. Samples from
patients who did not receive neoadjuvant treatment were used for calibration.

**Cell culture conditions**

The CRPC cell lines DU-145 and PC-3 were purchased from the American Type Culture Collection in October 2009. The docetaxel-resistant cell lines DU-145R and PC-3R were developed and maintained as previously described (12). No further authentication of the cell lines was done by the authors.

**Cell proliferation assays**

Cell viability in response to docetaxel was assessed by an MTT assay with the CellTiter 96 Aqueous Proliferation Table 1.

<table>
<thead>
<tr>
<th>Clinical characteristics of patients</th>
<th>All</th>
<th>Neoadjuvant treatment</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number</td>
<td>64</td>
<td>28</td>
<td>36</td>
</tr>
<tr>
<td>Median age, y</td>
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<td>64 (range, 48–70)</td>
<td>64.5 (range, 46–74)</td>
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<td></td>
<td></td>
<td></td>
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<td>4 (14.3%)</td>
<td>15 (41.7%)</td>
</tr>
<tr>
<td>T2</td>
<td>33 (51.6%)</td>
<td>14 (50%)</td>
<td>18 (50%)</td>
</tr>
<tr>
<td>T3</td>
<td>12 (18.8%)</td>
<td>10 (35.7%)</td>
<td>3 (8.3%)</td>
</tr>
<tr>
<td>Pathologic stage</td>
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<td></td>
<td></td>
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<td>T0</td>
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<td>1 (3.6%)</td>
<td>0</td>
</tr>
<tr>
<td>T1</td>
<td>1 (1.6%)</td>
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<tr>
<td>T2</td>
<td>26 (40.6%)</td>
<td>13 (46.4%)</td>
<td>13 (36.1%)</td>
</tr>
<tr>
<td>T3</td>
<td>36 (56.3%)</td>
<td>13 (46.4%)</td>
<td>23 (63.9%)</td>
</tr>
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<td>Gleason score (biopsy)</td>
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<td></td>
</tr>
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<td>≤6</td>
<td>10 (15.6%)</td>
<td>2 (7.1%)</td>
<td>8 (22.2%)</td>
</tr>
<tr>
<td>7 (3 + 4)</td>
<td>22 (34.4%)</td>
<td>7 (25%)</td>
<td>15 (41.7%)</td>
</tr>
<tr>
<td>7 (4 + 3)</td>
<td>16 (25%)</td>
<td>8 (28.6%)</td>
<td>8 (22.2%)</td>
</tr>
<tr>
<td>7 (N/A(^a))</td>
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<td>—</td>
<td>1 (2.8%)</td>
</tr>
<tr>
<td>8</td>
<td>11 (17.2%)</td>
<td>8 (28.6%)</td>
<td>3 (8.3%)</td>
</tr>
<tr>
<td>9</td>
<td>4 (6.3%)</td>
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<td>1 (2.8%)</td>
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<tr>
<td>Gleason score (prostatectomy)</td>
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<td></td>
<td></td>
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<tr>
<td>N/A(^b)</td>
<td>18 (28.3%)</td>
<td>18 (64.3%)</td>
<td>0</td>
</tr>
<tr>
<td>≤6</td>
<td>7 (15.2%)</td>
<td>7 (25%)</td>
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</tr>
<tr>
<td>7 (3 + 4)</td>
<td>13 (20.3%)</td>
<td>1 (3.6%)</td>
<td>12 (33.3%)</td>
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<tr>
<td>7 (4 + 3)</td>
<td>17 (26.6%)</td>
<td>—</td>
<td>17 (47.2%)</td>
</tr>
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<td>8</td>
<td>2 (4.3%)</td>
<td>0</td>
<td>2 (5.6%)</td>
</tr>
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<td>9</td>
<td>7 (15.2%)</td>
<td>2 (7.1%)</td>
<td>5 (13.9%)</td>
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<td>Median PSA (ng/mL)</td>
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<td>12.2 (range, 4.7–41)</td>
<td>8.2 (range, 2.01–19.2)</td>
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<td>PSA (ng/mL)</td>
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<tr>
<td>&lt;20</td>
<td>56 (87.5)</td>
<td>20 (71.4%)</td>
<td>36 (100%)</td>
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<tr>
<td>&gt;20</td>
<td>8 (12.5%)</td>
<td>8 (28.6%)</td>
<td>0 (0%)</td>
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<td>Postoperative radiotherapy</td>
<td></td>
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<td>13 (54.2%)</td>
<td>22 (61.1%)</td>
</tr>
<tr>
<td>Yes</td>
<td>24 (40%)</td>
<td>10 (41.7%)</td>
<td>14 (38.9%)</td>
</tr>
<tr>
<td>N/A(^a)</td>
<td>1 (1.7%)</td>
<td>1 (4.2%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Biochemical relapse</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>30 (46.9%)</td>
<td>11 (39.3%)</td>
<td>19 (52.8%)</td>
</tr>
<tr>
<td>Yes</td>
<td>34 (53.1%)</td>
<td>17 (60.7%)</td>
<td>17 (47.2%)</td>
</tr>
<tr>
<td>Median biochemical relapse–free survival (mo)</td>
<td>31.7 (range, 4–81)</td>
<td>29.3 (range, 4–59)</td>
<td>34.1 (range, 8–81)</td>
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<td>Clinical relapse</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>No</td>
<td>58 (90.6%)</td>
<td>22 (78.6%)</td>
<td>36 (100%)</td>
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<tr>
<td>Yes</td>
<td>6 (9.4%)</td>
<td>6 (21.4%)</td>
<td>0 (0%)</td>
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<tr>
<td>Median clinical relapse–free survival (mo)</td>
<td>51.2 (range, 31–84)</td>
<td>51.2 (range, 31–84)</td>
<td>—</td>
</tr>
<tr>
<td>Follow-up (mo)</td>
<td>82 (range, 10–135)</td>
<td>91 (range, 81–96)</td>
<td>69 (range, 10–135)</td>
</tr>
</tbody>
</table>

Abbreviation: N/A, not available

In some cases Gleason score could not be assessed because of tissue changes related to neoadjuvant treatment.

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Assay Kit (Promega) and by the trypan blue exclusion method using a Neubauer hemocytometer chamber.

**Western blot analysis**

Whole-cell extracts were prepared and Western blot analysis performed as described previously (22). Antibodies used were anti-PARP. Antibody was purchased from Roche (ref. 11835238001); β-catenin (683; CTNNB1) antibody (ref. 9582); CD44 (156-3C11) Mouse mAb (monoclonal antibody; ref. 3570); E-cadherin (CDH1) antibody (ref. 4065); Snail (C15D3) Rabbit mAb (ref. 3879); TCF8/ZEB1 (D80D3) Rabbit mAb (ref. 3396); and Vimentin (R28; VIM) antibody (ref. 3932) were purchased from Cell Signaling Technology. Monoclonal anti-α-tubulin clone B-5-1-2 (ref. T5168) was purchased from Sigma-Aldrich.

**Real-time qRT-PCR in cell lines**

Total RNA was isolated from cell lines using the RNeasy Micro Kit (Qiagen), and quantified using a spectrophotometer (Nanodrop Technologies). cDNA was generated from 1 μg of total RNA using the High Capacity cDNA Archive Kit (Life Technologies), following the manufacturer’s instructions. Real-time quantitative reverse transcription PCR (qRT-PCR) was carried out in a StepOnePlus Real-Time PCR system (Life Technologies) according to the manufacturer’s recommendations. Data were acquired using SDS Software 1.4. Amplification reactions were performed in duplicate. Expression values were relative to the ACTB endogenous gene. Target genes were amplified using commercial primers and probes (Life Technologies; Supplementary Table S1).

**Immunohistochemistry**

Tissue sections were deparaffinized in xylene and rehydrated in graded alcohols. For AR and VIM staining, the sections were placed in a 97°C solution of 0.01 mol/L EDTA (pH 9.0) for antigen retrieval. Primary mouse mononuclear antibody for AR (DAKO; Agilent Technologies) was applied for 20 minutes at room temperature at dilution 1:150. FLEX Monoclonal Mouse anti-VIM, Clone V9 (DAKO) was used for VIM staining. Detection was accomplished with the DAKO Envision System followed by diaminobenzidine enhancement. For RELA, the sections were placed in a 97°C solution of 0.01 mol/L sodium citrate (pH 6.0) for antigen retrieval. Then, samples were incubated with a rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc.) at dilution 1:400. Detection was performed with Bond Polymer Refine Detection (DAKO; Agilent Technologies) for the automated Bond system.

AR and RELA were evaluated throughout the semi-quantitative method histologic score (H-score), which measures both the intensity and proportion of staining. The H-score for each sample was calculated by multiplying the percentage of stained tumor cells by the intensity (0, nonstained; 1, weak; 2, moderate; 3, strong). VIM was evaluated in the same way but scoring the percentage of staining on a scale of 0 to 4 (0, 0%; 1,<1%; 2, 1%-9%; 3, 10%-50%; 4, >50%). Nuclear and cytoplasmatic stains were scored separately for AR and RELA proteins. The assessment of all samples was done by a senior pathologist (P.L. Fernández) who was blinded to all clinical information.

**Immunofluorescence staining in cell lines and tumor samples**

Cell pellets were collected in a 1% agarose solution, fixed in 4% PBS-buffered formaldehyde, and then FFPE. Sections of 5 μm were analyzed with a multiplex immunofluorescent assay. They were stained with H&E for histopathologic assessment and stained using immuno-fluorescence with Alexa fluorochrome-labeled antibodies. Briefly, both control and resistant prostate cancer cell lines were evaluated with a series of simplex and duplex immunofluorescence assays to quantify the level of selected antibody–antigen complexes from specific regions of interest (ROI).

The FFPE prostate tissue sections also were assessed by immunofluorescence using a single multiplex assay with two differentially labeled antibodies (ZEB1 and CD44). For all specimens the H&E images were used to guide and register immunofluorescence image capture with a maximum of four ROIs per cell pellet and six per tissue section. Alexa fluorochrome dyes were Vimentin (ref. M0725; Dako), CD44 (ref. 156-3C11; Cell Signaling Technology), ZEB1 (ref. sc-25388; Santa Cruz Biotechnology). The ROIs were acquired from the cells and tumor tissue sections, blinded to outcome, with a CRI Nuance imaging system, and then analyzed with fluorescent image analysis software to derive quantitative features from cellular/tissue compartments. Quantitative assessment was performed using a pixel-area function, normalized to the ROI under investigation.

**siRNA transfection**

Dharmacon SMART pool control and ZEB1 siRNA were used with lipofectamine according to the manufacturer’s protocol (Thermo Scientific) to inhibit ZEB1 in DU-145/R cells. Commercial Silencer Select siRNA of ZEB1 (s229971; Life Technologies) was transfected to PC-3/R cell lines. Cells were incubated with the siRNA complex for 24 hours, treated with docetaxel, then harvested to study protein expression changes of ZEB1 and CDH1 by Western blot analysis. Apoptosis was studied at 24 and 48 hours by PARP analysis (Western blot analysis), and cell viability was measured by MTT at 72 hours as described before.

**Fluorescence-activated cell sorting**

For flow cytometry, cells were dissociated with Accutase (Invitrogen) and washed twice in a serum-free medium. Cells were stained live in the staining solution containing bovine serum albumin and fluorescein isothiocyanate-conjugated monoclonal anti-CD44 (15 min at 4°C). A minimum of 500,000 viable cells per sample were analyzed on a cytometer. For fluorescence-activated cell sorting (FACS), 2 × 10^6 cells were similarly stained for CD44 and used to sort out CD44^- and CD44^+ cells. For the positive
Table 2. Multivariate analysis of gene expression and patient outcomes

<table>
<thead>
<tr>
<th>Gene</th>
<th>FC</th>
<th>Progression</th>
<th>Multivariatea HR (95% CI)</th>
<th>Gene</th>
<th>FC</th>
<th>Progression</th>
<th>Multivariatea HR (95% CI)</th>
<th>Gene</th>
<th>FC</th>
<th>Progression</th>
<th>Multivariatea HR (95% CI)</th>
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<tbody>
<tr>
<td>Differentially expressed genes</td>
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<tr>
<td>TGFBR3</td>
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<td>ABCB1</td>
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<td></td>
<td>NFKB1</td>
<td>1.76</td>
<td>bPFS</td>
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<tr>
<td>SERPINB5</td>
<td>4.43</td>
<td>—</td>
<td></td>
<td>CDH2</td>
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<td>AR</td>
<td>1.73</td>
<td>rPFS</td>
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<td>PTPRM</td>
<td>1.71</td>
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<td>CLDN11</td>
<td>3.69</td>
<td>rPFS, bPFS</td>
<td>3.056 (1.169–7.988)</td>
<td>TIMP2</td>
<td>2.38</td>
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<td>GOSR2</td>
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<td>EGFR</td>
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<td>BTBD11</td>
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<td>bPFS</td>
<td>0.321 (0.143–0.720)</td>
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<td>—</td>
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<td>CCNB1</td>
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(Continued on the following page)
population, only the top 10% mostly brightly stained cells were selected. The CD44\(^+\) cells selected were cultured as an individual clone in 96-well plates and expanded.

**Statistical analysis**

TaqMan low-density arrays (TLDA) gene expression data were evaluated by the Wilcoxon rank-sum test and receiver operating characteristic (ROC) analysis. Time to PSA progression and radiologic progression were calculated from the time of prostate cancer diagnosis until PSA or radiologic progression, respectively. The log-rank test was used in univariate survival analyses. Multivariate analysis of gene expression was evaluated by Cox proportional hazards regression, including stage, Gleason, PSA, and neoadjuvant treatment as clinical covariates; backward stepwise likelihood was used for selection. Real-time qRT-PCR experimental data were expressed as mean ± SEM and were analyzed by the Student \(t\) test. All the statistical tests were conducted at the two-sided 0.05 level of significance.

**Results**

**Differential gene expression between treated and nontreated tumors**

Among the 93 genes analyzed (Supplementary Table S1), we observed differential expression \( (P < 0.05) \) in 63 (67.7%) genes (Table 2); 53 genes were overexpressed and 10 underexpressed in tumor specimens from patients treated with neoadjuvant docetaxel plus androgen deprivation. Genes of the NF-κB pathway (such as \( NFKB1, REL, \) and \( RELA \)), AR, and epithelial-to-mesenchymal transition (EMT)–related genes (such as \( ZEB1, VIM, CDH2, \) and \( TGFBR3 \)) were overexpressed in treated tumors. Among the downregulated genes in treated tumors, were the metastasis-suppressor gene \( NDRG1 \) (23) and the adhesion molecule \( EPCAM \), a regulator of the alternative splicing of \( CD44 \) \((ESRP1; \text{ref. 24})\) and \( ST14 \) (a negative regulator of the EMT mediator \( ZEB1 \); Table 2; Fig. 1A; ref. 25).

**Gene expression and clinical outcome**

We tested the possible prognostic impact of the 93 genes studied by TLDA (Supplementary Table S1). Individually, the expression of several genes was related to time-to-PSA and/or clinical relapse (Table 2). Time to radiologic progression and PSA progression curves are shown in Fig. 1B and C and Supplementary Figs. S1 and S2. Of note, the overexpression of \( AR \), and the EMT-related genes \( TGFBR3, ZEB1, \) and \( VIM \) was correlated with a shorter time of radiologic progression (Fig. 1B).

We then performed a multivariate analysis, including the genes with individual prognostic value, clinical prognostic factors (PSA, Gleason, and clinical stage), and neoadjuvant treatment. Results are shown in Table 2A and B. In the multivariate analysis, the reduced expression of \( CLDN7 \) was an adverse-independent prognostic factor for clinical relapse. Loss of \( CLDN7 \) has been correlated with adverse prognostic variables in prostate cancer and
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A. Nontreated vs. Treated

B. Proportion of biochemical relapse-free survival

C. CD24

D. IHC score (arbitrary units)

E. Cytoplasmatic AR

F. Treated patients vs. Controls (nontreated)
with EMT (26). Of note, the low expression of CDH1 was an independent prognostic factor for time to PSA relapse.

We also analyzed the prognostic impact of the stem-like cell markers CD24 and CD44, which were underexpressed and overexpressed, respectively, in treated tumors (PC CD24: 0.59, \( P = 0.07 \); FC CD44: 1.63, \( P < 0.000 \); Supplementary Table S1 and Supplementary Figs. S1 and S2). Of note, low expression of CD24 was correlated with shorter time of biochemical progression (Fig. 1C).

**Immunohistochemistry in treated versus nontreated tumors**

We explored the expression of VIM and both cytoplasmic and nuclear RELA and AR in tumor samples from neoadjuvant-treated and -nontreated patients. Staining of cytoplasmatic RELA was significantly higher in the treated versus nontreated patients [immunohistochemistry (IHC) score 181.9 vs. 148.3, respectively; Fig. 1D and F]. Moreover, nuclear RELA was significantly related to worse clinical relapse (Fig. 1E). Vimentin expression was nonsignificantly higher in treated tumors (IHC score 2 vs. 1, respectively; Fig. 1D). No differences were found in the expression of nuclear AR; however, cytoplasmatic AR expression was significantly higher in the treated tumors (IHC score 102.5 vs. 14.5) and correlated with radiologic progression survival (Fig. 1D–F).

**Docetaxel-resistant prostate cancer cells express EMT and stem-like cell markers**

On the basis of the results described above, we studied the link between EMT and docetaxel resistance in four prostate cancer cell lines models (parental DU-145 and PC-3R cells, and their docetaxel-resistant partners DU-145R and PC-3R, respectively). As shown in Fig. 2A and B, the docetaxel-resistant cells phenotype was consistent with EMT, i.e., decreased expression of epithelial markers (CDH1 and CTNNB1) and increased expression of mesenchymal markers (VIM and ZEB1) at the protein level. Consistent results were found at mRNA level, except for CTNNB1 (data not shown).

Recent studies have shown that cells with EMT phenotype share characteristics of stem-like cancer cells (14, 27). For that reason, we tested the expression of stem-like cell markers and showed that docetaxel-resistant cells, both DU-145R and PC-3R, exhibit transcriptional features of cancer-stem cells, such as increased expression of CD44 and the loss of CD24 (Fig. 2C).

Moreover, in cell lines, we detected by immunofluorescence analysis a subset of cells coexpressing CD44 and ZEB1. Scattered cells with these features were detectable in the parental cell lines; however, this population was highly enriched in the resistant cells (Fig. 2D). By FACS, we then isolated from the parental PC-3 cells a subpopulation of cells with high expression of CD44. We selected a derived CD44+/PC-3 clone that showed an increased expression of VIM and ZEB1 and decreased CDH1 expression (Fig. 2E). This clone from the parental cells was significantly more resistant to docetaxel than the parental cell line, PC-3 (Fig. 2F).

Dose–response experiments in both parental and resistant cells showed that docetaxel exposure significantly increased the expression of VIM in PC-3 and PC-3R cells, of ZEB1 in PC-3 cells, and of SNAI2 in DU-145, PC-3 and PC-3R cells. TWIST1 expression increased in all cell lines after docetaxel treatment. In contrast, no significant differences were observed in the expression of SNAI2 and CDH1 with docetaxel exposure (Fig. 3A). About stem-like cell markers, inconsistent results were obtained for CD24 expression after docetaxel exposure because CD24 expression increased in PC-3 cells but decrease in DU-145 cells. In contrast, CD44 significantly increased in PC-3 cells with docetaxel treatment (Fig. 3B).

**EMT mediates docetaxel resistance in prostate cancer cells**

To test whether inhibition of EMT could revert docetaxel resistance, we downmodulated the expression of ZEB1, a key inducer of EMT. siRNA ZEB1 transfected DU-145R and PC-3R cells had an increased expression of CDH1 (Fig. 4A) and decreased CD44 (Fig. 4B), confirming the link between EMT and stem-like cell phenotype. Moreover, siRNA ZEB1 transfected cells showed significantly increased sensitivity to docetaxel compared with control cells (\( P < 0.05 \); Fig. 4B). The magnitude of the reversion of chemoresistance was more pronounced in DU-145R and PC-3R cells than in the parental cells. Docetaxel-induced apoptosis was more pronounced in the ZEB1–siRNA transfected cells (Fig. 4B).

**ZEB1/CD44 expression in tumor samples**

On the basis of preclinical findings, we decided to investigate whether CD44+/ZEB1+ cells were present in primary prostate cancer specimens. Twenty-two FFPE tumors from patients with high-risk prostate cancer
treated with docetaxel and androgen suppression and 15 control patients with sufficient remaining material were available for immunofluorescence studies. All samples were positive for CD44 staining but only 7 of 15 controls (46.7%) and 7 of 22 treated patients (31.8%) had a ZEB1 signal. Overall, there were no differences between the control and treated groups in the expression of ZEB1 (0.0059 vs. 0.013 mean intensity, respectively) or CD44 (1.27 vs. 1.01 mean intensity, respectively). Tumor cells that coexpressed ZEB1 and CD44 were observed in 3 (13.6%) of the 22 patients in the neoadjuvant group. However, none of the control patients presented with coexpression of both markers (Fig. 4D). Notably, ZEB1/CD44 coexpression was associated with aggressive clinical behavior: At the time of outcome analysis, all patients had relapsed, 2 had developed liver metastasis, and 1 had died due to disease progression (Fig. 4E).

Discussion

In this study, we confirm that some of the molecular alterations associated with docetaxel resistance in a previously described in vitro model of CRPC cell lines are present in residual cells of prostatectomy specimens treated with neoadjuvant docetaxel plus androgen deprivation. Our findings may be especially relevant in clinical practice because most patients receive androgen deprivation prior and concomitantly to the administration of docetaxel. The observed deregulated pathways may translate common mechanisms of resistance to both therapies.
Different neoadjuvant studies have been designed to identify pathways involved in resistance to androgen deprivation or chemotherapy in prostate cancer. In one study of neoadjuvant androgen deprivation, the authors observed that many androgen-responsive genes, including AR and PSA, were not suppressed; this suggests that suboptimal suppression of tumoral androgen activity may lead to adaptive cellular changes to allow prostate cancer cells survival in a low-androgen environment (28).

Another group analyzed prostate tumors removed by radical prostatectomy after 3 months of androgen deprivation. Gene expression analysis revealed that PSA and other androgen-responsive genes were overexpressed in tumors from patients who relapsed (29). Our data are in concordance with these reports. We observed that the expression of AR and several AR-regulated genes (i.e., ZEB1, IL6, TGFBR3, KLF9) increased in treated tumors, even though serum PSA levels decreased under therapy in most cases, as we previously reported (17). Moreover, high levels of AR correlated with high risk of clinical relapse. These data suggest that persistence of AR signaling may be related to treatment resistance and/or to eventual disease progression.

We observed no differences in AR nuclear staining between treated and nontreated samples. However, cytoplasmic expression was significantly higher in residual tumor cells after androgen deprivation and docetaxel exposure. Prior reports have shown that taxanes inhibit AR nuclear translocation and that patients treated with taxanes may have lower nuclear expression than treatment-naive patients (30). This was not observed in our study, likely because our patients were treated with combined therapy. Prior studies have shown that androgen deprivation increased full-length AR protein levels in CRPC cells, but decreased its nuclear localization (31).

Other studies have used a similar approach in patients treated with neoadjuvant chemotherapy alone (32, 33). One group performed microarray analysis of tumor specimens from 31 patients treated with docetaxel plus mitoxantrone (33). The comparison of pre- and posttreatment samples showed increased expression of cytokines regulated by the NF-κB pathway. These data are in concordance with our

Figure 3. Effect of docetaxel exposure on EMT and stem-like gene expression markers in prostate cancer cell lines. A, EMT markers gene expression in a docetaxel dose–response manner. B, stem-like cell markers gene expression in a docetaxel dose–response manner. Geometrical symbols represent significant differences in the corresponding cell line; data from DU-145 0 nmol/L were considered the reference for all the other measures (i.e., fold change, 1).
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A

B

C

D

E

ZEB1 and CD44 Colocalization

Proportion of radiologic progression-free survival

Colocalization

No colocalization

P = 0.371

P = 0.273
results showing an increased expression in treated tumors of NF-κB subunits and NF-κB–regulated cytokines, such as IL-6, adding support to a body of evidence on the involvement of this pathway in resistance to chemotherapy in prostate cancer (11). On the other hand, NF-κB activation may induce EMT in prostate cancer (34). Although our study did not investigate the potential causal relationship between NF-κB activation and EMT, this last phenomenon was found to be highly relevant in resistance to therapy. Moreover, increased nuclear NF-κB (RELA staining) correlated with a shorter time to clinical relapse, confirming the prognostic value of this pathway activation in prostate cancer (22).

In the present study, we analyze the transcriptional profile of residual tumor cells after combined neoadjuvant androgen deprivation and docetaxel treatment. Because macrodissected tumor tissues were used for gene expression studies, our results may translate expression patterns from both tumor and surrounding nontumor cells. However, a prior study using the macrodissection strategy reported only minor interference of nontumor cells with the overall gene expression profile (35). Moreover, we considered stroma and benign cells contamination to be homogeneous in both the treated and nontreated patient groups. Among the 93 genes analyzed, we observed differential expression between treated and nontreated tumors in 63 (67.7%) genes. Of note, the over expression of the EMT genes correlated with a shorter time to clinical relapse.

In the EMT process, cells lose epithelial characteristics and gain mesenchymal properties to increase motility and invasion, allowing tumor cells to acquire the capacity to infiltrate surrounding tissues and to metastasize in distant sites. EMT is typically characterized by the loss of epithelial (i.e., CDH1) and the gain of mesenchymal (i.e., VIM, CDH2) markers expression (36). Several reports suggest that AR activation, as well as androgen deprivation therapy, may induce changes characteristic of EMT that may be involved in prostate cancer progression (37–39). The expression of the transcription factor ZEB1 may be induced by dihydrotestosterone and is mediated by two androgen-response elements (40). Recently, Sun and colleagues showed that androgen deprivation causes EMT in animal models and in tumor samples of patients treated with hormone therapy (41). Moreover, the presence of AR-truncated isoforms, which are increased in the castration-resistant progression, regulate the expression of EMT (42).

On the other hand, there are molecular similarities between cancer stem-like cells and EMT phenotypic cells. Moreover, cells with an EMT phenotype induced by different factors are rich sources for stem-like cancer cells (14, 27). We observed in the DU-145 in vitro model that docetaxel-resistant cells expressed high levels of the stem cell marker CD44 and decreased levels of CD24. Moreover, docetaxel treatment increased CD44 expression in tumor cells. Likewise, RT-PCR results in tumor samples showed an increased expression of CD44 and a decreased expression of CD24 in tumors treated with neoadjuvant androgen deprivation plus docetaxel. Our results are in accordance with those of Puhr and colleagues, who detected an increased CD24low–CD44high cell population in docetaxel-resistant prostate cancer models (43). Similarly, Li and colleagues detected CD24low–CD44high breast cancer cells that were resistant to neoadjuvant chemotherapy (44). In a preclinical study, CD44 and CD147 enhanced metastatic capacity and chemoresistance of prostate cancer cells, potentially mediated by activation of the phosphoinositide 3-kinase and mitogen-activated protein kinase pathways (45).

In the present work, we identified a population of prostate cancer cells exhibiting an EMT phenotype that are primarily resistant to docetaxel. The presence of an intrinsic resistant cell population was supported by the isolation of docetaxel-resistant clonal cells in the parental cell line PC-3, before docetaxel exposure, with a high expression of CD44 and EMT markers and the loss of CDH1. ZEB1+/CD44− cells were identified at a very low frequency in the two parental cell lines, DU-145 and PC3, before docetaxel exposure but their frequency massively increased in docetaxel-resistant cells. Similarly, a small percentage of ZEB1+/CD44+ cells were also observed in primary high-risk localized prostate cancer tumors. ZEB1+/CD44− cells were present only in tumors that had previously received neoadjuvant androgen deprivation plus docetaxel (13.6%). Both in vitro and tumor sample findings support the presence of primary resistant cells harboring EMT/stem cell–like characteristics and suggest that the exposure to docetaxel may eliminate sensitive cells resulting, however, in the selective out-growth of this resistant cell population.

In our model, docetaxel also induced EMT changes in the parental and resistant cell lines. On the basis of our findings, both mechanisms, the existence of a primary resistant cell with an EMT phenotype and the induction of EMT changes induced by docetaxel, are possible. In recent work on docetaxel-resistant PC-3– and DU-145–derived cell lines, the authors reported that docetaxel-resistant cells underwent an EMT transition associated with a reduction of microRNA (miR)-200c and miR-205, which
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regulate the epithelial phenotype. Their study also showed reduced CDH1 expression in tumors after neoadjuvant chemotherapy (43). Another study showed that paclitaxel DU-145–resistant cells have greater ZEB1, VIM, and SNAI1 expression (46).

We tested whether EMT played a causal role in docetaxel chemoresistance by interfering with the expression of the transcription factor ZEB1, a key mediator of EMT, in prostate cancer cell lines. We observed that ZEB1 genetic downmodulation restored CDH1 but suppressed CD44 expression, which was consistent with a reversion of EMT and stem-like cell features. We also observed that ZEB1 inhibition caused prostate cancer cell mortality independently of docetaxel. This effect was previously described and is consistent with the known role of ZEB1 in cell proliferation related, which is related to the expression of cell cycle inhibitory cyclin-dependent kinase inhibitors (47). Furthermore, ZEB1 inhibition restored sensitivity to docetaxel, supporting a mechanistic role of EMT and stem-like cell phenotype in resistance to therapy. In a previous study of an adenocarcinoma lung cancer model, inhibition of ZEB1 significantly enhanced the chemosensitivity of docetaxel-resistant cells in vitro, and in vivo the ectopic expression of ZEB1 increased chemoresistance (48).

Several reports have provided evidence that EMT is critical for invasion and migration and is involved in tumor recurrence, which is believed to be tightly linked to cancer stem cells. CD44 and VIM expression in primary tumors has been correlated with adverse prognosis (34, 49). Notably, the few patients in our series with ZEB1+/CD44+ tumor cells in primary tumors showed extremely aggressive clinical behavior.

In summary, we observed a differential expression of NF-kB, AR, EMT, and stem-like cell markers between treated and nontreated tumors. Moreover, they were related to a higher risk of PSA and/or clinical relapse. Because the neoadjuvant population may be of higher risk than the surgical patients, we cannot exclude the possibility that the expression of these markers is more related to the characteristics of the disease than to the therapy. However, none of the clinical factors (PSA, Gleason, clinical stage, or the presence of prior neoadjuvant therapy) correlated with clinical outcome in the univariate or multivariate analysis in our series.

Overall, our findings support a role of EMT in resistance to prostate cancer therapy and progression. Our clinical data were generated in the neoadjuvant setting and cannot be extrapolated to patients with CRPC. However, both in vitro and clinical results support the investigation of the role of EMT in resistance to chemotherapy in CRPC. Moreover, novel strategies to revert or prevent EMT are warranted to improve the outcome of CRPC or to increase the probabilities of cure for patients with high-risk prostate cancer.

Disclosure of Potential Conflicts of Interest

M. Donovan is a consultant/advisory board member for Althia. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Marín-Aguilera, J. Codony-Servat, O. Reig, J. J. Lozano, P. L. Fernández, N. Jiménez, M. Donovan, L. Mengual, A. Font, P. Gascon, B. Mellado

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References


Epithelial-to-Mesenchymal Transition Mediates Docetaxel Resistance and High Risk of Relapse in Prostate Cancer

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