Identification of Docetaxel Resistance Genes in Castration-Resistant Prostate Cancer

Mercedes Marín-Aguilera1, Jordi Codony-Servat1, Susana G. Kalko2, Pedro L. Fernández3, Raquel Bermudo5, Elvira Buxó1, María José Ribal4, Pedro Gascón1, and Begoña Mellado1

Abstract

Docetaxel-based chemotherapy is the standard first-line therapy in metastatic castration-resistant prostate cancer (CRPC). However, most patients eventually develop resistance to this treatment. In this study, we aimed to identify key molecular genes and networks associated with docetaxel resistance in two models of docetaxel-resistant CRPC cell lines and to test for the most differentially expressed genes in tumor samples from patients with CRPC. DU-145 and PC-3 cells were converted to docetaxel-resistant cells, DU-145R and PC-3R, respectively. Whole-genome arrays were used to compare global gene expression between these four cell lines. Results showed differential expression of 243 genes ($P < 0.05$, Bonferroni-adjusted $P$ values and log ratio $> 1.2$) that were common to DU-145R and PC-3R cells. These genes were involved in cell processes like growth, development, death, proliferation, movement, and gene expression. Genes and networks commonly deregulated in both DU-145R and PC-3R cells were studied by Ingenuity Pathways Analysis. Exposing parental cells to TGFB1 increased their survival in the presence of docetaxel, suggesting a role of the TGF-β superfamily in conferring drug resistance. Changes in expression of 18 selected genes were validated by real-time quantitative reverse transcriptase PCR in all four cell lines and tested in a set of 11 FFPE and five optimal cutting temperature tumor samples. Analysis in patients showed a noteworthy downexpression of CDH1 and IFIH1, among others, in docetaxel-resistant tumors. This exploratory analysis provides information about potential gene and network involvement in docetaxel resistance in CRPC. Further clinical validation of these results is needed to develop targeted therapies in patients with CRPC that can circumvent such resistance to treatment.

Introduction

Prostate cancer is the second most common cancer among men worldwide (1). Approximately 85% of newly diagnosed prostate cancer cases are localized to the prostate, whereas the remainder are invasive or metastatic disease (2). Patients with metastatic prostate cancer respond initially to antiandrogen therapy. However, tumors eventually progress and transform themselves into castration-resistant prostate cancer (CRPC). Docetaxel improves survival of patients with metastatic CRPC and is considered a standard first-line therapy in such cases (3; 4). However, only approximately 50% of patients respond to docetaxel and most of them eventually develop resistance to this therapy.

Taxanes bind β-tubulin, stabilizing microtubules assembly and preventing depolymerization in the absence of GTP (5). Furthermore, docetaxel leads to Bcl-2 phosphorylation, which causes apoptosis of cancer cells that had previously blocked the apoptotic-inducing mechanism, leading to tumor regression (6). Some proteins such as stathmin, Aurora-A, and β-III tubulin have been described to be involved in resistance to antimicrotubule agents (7–9). However, mechanisms of resistance to docetaxel in CRPC need to be further elucidated to design targeted therapies that can circumvent treatment resistance.

cDNA microarrays technology has given us the ability to simultaneously examine the expression of thousands of genes and determine the molecular profile of clinical phenotypes, some of which can be involved in specific cell profiles, such as chemotherapy resistance. This study compared gene expression profiles of 2 models of docetaxel-resistant CRPC cell lines, and identified a set of genes involved in resistance to this chemotherapy. The most differentially expressed genes were validated by real-time quantitative reverse transcriptase PCR (qRT-PCR) in cell lines and, in an exploratory way, in CRPC tumor samples.
Materials and Methods

Cell culture
The human prostate carcinoma cell lines DU-145 and PC-3 (obtained from American Tissue Culture Collection) were converted to docetaxel-resistant cells by exposing them to an initial dose of 5 mmol/L docetaxel and culturing surviving cells during 1 year and 6 months, respectively, with increasing doses in an intermittent regimen. DU-145 and DU-145R cell lines were cultured in RPMI-1640 medium (Invitrogen), and PC-3 and PC-3R in F-12K nutrient mixture medium (Invitrogen), both supplemented with 10% FBS. No further authentication of the cell lines was done by the authors. Docetaxel (Sigma) was dissolved in dimethyl sulfoxide (10 mmol/L). IC50 values were estimated in triplicate from the dose–response curves.

Microarray hybridizations and differential expression analysis
Total RNAs were isolated from DU-145, DU-145R, PC-3, and PC-3R cell lines (in triplicate) using the TRIzol Reagent (Invitrogen) according to the manufacturer’s instructions. RNAs were purified using RNeasy Micro kit (Qiagen), and quality and quantity were assessed on a spectrophotometer. Fragmented, labeled, and amplified cDNA was hybridized to the Affymetrix Human Genome U133Plus2.0 array, which represents about 38,500 well-characterized genes. Washes and scanning of the arrays were carried out according to manufacturer’s instructions.

Raw expression measures were summarized after background correction and normalization steps using the robust multiarray analysis (RMA) methodology in the affy (10) package from the Bioconductor project (11). Unsupervised clustering analysis of high variability genes was done with dChip v1.3 software (12). Differential expression analysis was carried out by a linear model using the empirical Bayes method to moderate the standard errors of the estimated log ratio changes with the limma package (13), according to adjusted P values less than 0.05 for the comparison of interest. Special attention was given to the statistically significant genes that showed the largest changes (logratio > 1.2). CEL files and RMA values were deposited on Gene Expression Omnibus (GSE33455).

Network analysis
Gene interactions were studied using the Ingenuity Pathway Analysis (IPA; Ingenuity Systems) software (14), according to IPA instructions. First, genes differentially expressed between resistant and parent cell lines (DU-145R vs. DU-145 and PC-3R vs. PC-3) were listed. Interactions between common deregulated genes in DU-145R and PC-3R cells were then analyzed to understand resistance mechanisms at a molecular level. Network-eligible genes were placed by IPA into networks and ranked by the relevance of the network-eligible molecules.

Patients and samples
Tumor samples from patients with metastatic prostate cancer were collected to test microarray results in patients. We selected patients who were prescribed docetaxel-based therapy after the palliative transurethral desobstructive resection, or after being biopsied once the tumor had already spread. Samples were obtained before the start of docetaxel treatment.

We were able to collect 11 formalin-fixed, paraffin-embedded (FFPE) samples, and 5 tumor samples embedded in 1 to 2 mL of optimal cutting temperature (OCT) medium immediately after surgery. The OCT samples were stored at −80°C until processing. The institutional review board approved this study and written informed consent was obtained from all patients.

FFPE samples belonged to 11 patients with CRPC, all of whom had bone metastasis; 5 also had lymph node lesions, 2 had lung metastasis, and 2 others had liver and ureteral tumor growth. Six patients partially responded to docetaxel and the other 5 progressed during the treatment. On the other hand, OCT samples were extracted from 5 patients with CRPC with bone metastasis, of whom 4 also had a lymph node lesion and 1 had liver metastasis. Two patients partially responded to docetaxel and the other progressed during the treatment.

Treatment response was evaluated by prostate-specific antigen–based response criteria (15) and Response Evaluation Criteria in Solid Tumors (RECIST; ref. 16).

Gene validation in cell lines
Genes that could potentially be related with docetaxel resistance were selected for further validation in cell lines using qRT-PCR. Genes were selected according to their change in degree of relative expression (logratio > 1.2), but also by function, that is, genes involved in known pathways such as cell adhesion, cell signaling, or regulation of apoptosis, using DAVID (17; 18) and IPA softwares (14).

Total RNAs from cell lines were isolated using the TRIzol Reagent (Invitrogen) according to the manufacturer’s instructions. One microgram of total RNA was reverse transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems), following manufacturer’s instructions. qRT-PCR was carried out in a 7500 Real Time PCR system (Applied Biosystems), according to the manufacturer’s recommendations. Data were acquired using SDS Software 1.4. Expression values were based on the quantification cycle (Cq) from target genes relative to ACTB endogenous control gene (ΔCq). Relative expression with respect to each reference group studied was reported as log ratio. Commercial codes for primers and probes from target genes were AREG (amphiregulin-Hs00950669_m1), CDH1 (E-cadherin-Hs01023895_m1), CYBRD1 (cytochrome b-reductase 1-Hs00227411_m1), DLEC1 (deleted in liver cancer 1-Hs00183436_m1), GJB2 (gap junction protein, β 2-Hs00955889_m1), GSPT2 (G1 to S-phase transition 2-Hs00250696_s1), IFIHI (IFN induced with helicase C domain 1-Hs01070332_m1), IL8 (interleukin 8-Hs00174103_m1), MAPK13 (mitogen-activated protein
kinase 13-Hs00559623_m1), MPZL2 (myelin protein zero-like 2-Hs00170684_m1), MXI (myxovirus resistance 1-Hs00895608_m1), MYO6 (myosin VI-Hs00192265_m1), S100A4 (S100 calcium-binding protein A4-Hs00243202_m1), SERPINA1 (serpin peptidase inhibitor, clade A-Hs01978000_m1), SYK (spleen tyrosine kinase-Hs00895377_m1), EPCAM (epithelial cell adhesion molecule-Hs00901885_m1), NEAT1 (nuclear paraspeckle assembly transcript 1-Hs0108264_s1), and TNFAIP3 (tumor necrosis factor, α-induced protein 3-Hs00234713_m1).

Gene expression in tumor samples

Tissues from selected FFPE and OCT blocks were sectioned at 10 and 20 μm thicknesses, respectively, immediately before the RNA extraction. Total RNA from FFPE samples was obtained using the RecoverAll Total Nucleic Acid Isolation Kit for FFPE (Ambion). The RNAs from OCT samples were extracted using TRIzol Reagent (Invitrogen) according to the manufacturer’s instructions. Quality and quantity of total RNAs were measured by ND-1000 Spectrophotometer (Nanodrop Technologies).

The genes selected for microarrays validation in cell lines were also tested in tumor samples following the same qRT-PCR protocol except that the expression values of target genes were relative to the Cq mean of ACTB, B2M, and GLUSB endogenous genes.

Viability assay

TGFBI (Sigma-Aldrich) was dissolved in sterile 4 mmol/L HCl containing 0.1% endotoxin-free recombinant human serum albumin (50 μg/mL). Parent cells (DU-145 and PC-3) were maintained during 7 consecutive days with TGFBI (5 ng/mL) in the medium. Parental cells with and without sustained treatment were then seeded at a density of 3,200 cells per well in a 96-well microtiter plate in the corresponding medium with 10% FBS. After 24 hours, cells were exposed to docetaxel with/without TGFBI for an additional 72 hours. Finally, cell proliferation/viability was assessed by using MTT colorimetric assay (Promega).

Statistical analysis

Changes in gene expression comparing docetaxel-resistant and docetaxel-sensitive cell lines and tumor samples by qRT-PCR were analyzed with Wilcoxon rank-sum test, considering as positively validated those genes with significant expression changes (P < 0.05). SPSS 12.0 software was used for statistical analyses.

Results

Docetaxel-resistant cell lines

DU-145R and PC-3R cells acquired levels of resistance to docetaxel that was 2 to 5 times higher than their parent cells. IC50 values for DU-145R and PC-3R ranged from 10 to 15 and 20 to 22 nmol/L, respectively, whereas DU-145 and PC-3 registered from 4 to 5 and 3 to 5 nmol/L, respectively (data not shown).

Differentially expressed genes in resistant cells

Initially, unsupervised clustering analysis of microarrays was carried out using the probe sets that varied most throughout the whole experiment, and this exploration revealed a good segregation of the arrays in their respective classes on the basis of expression values (data not shown). Subsequent differential expression analysis revealed 1,064 and 1,361 differentially expressed genes (Bonferroni < 0.05 and log ratio > 1.2), equivalent to 1,710 and 2,117 Affymetrix probe sets, between DU-145R versus DU-145 and PC-3R versus PC-3, respectively (Supplementary Table SI). Top 20 over- and downexpressed genes from docetaxel-resistant cells with respect to their parent cells according to log ratio expression values, and their main function, are summarized in Supplementary Table SII. When comparing differentially expressed genes between both resistant cell lines (DU-145R and PC-3R), 243 genes overlapped (Supplementary Table SIII). From those genes, 172 were over- or downexpressed in both DU-145R and PC-3R cells versus their parent cells. The top 10 commonly over- and downexpressed genes, with their respective main function, are summarized in Table 1.

Ingenuity network analysis

Only considering probe sets common to both resistant cell lines, IPA qualified 252 genes as network and function eligible. A core analysis showed 12 networks on the basis of this gene selection with a score more than 2. The 2 top networks were detected with scores of 51 and 39 respectively, and were the same for DU-145R and PC-3R cell lines (Fig. 1). Specifically, network 1 (Fig. 1A) was centered on, amongst others, the nuclear receptor PPARα (peroxisome proliferator-activated receptor α). This gene directly interacts with an important complex for cell survival and proliferation, NFκB, which in turn interacts with TNFAIP3, HMGA1 (high mobility group AT-hook 1), and ISG15 (ISG15 ubiquitin-like modifier). Other deregulated genes directly related to PPARα are SOCS2 (suppressor of cytokine signaling 2), ASL (argininosuccinate lyase), ASNS (asparagine synthetase), ELOVL6 (ELOVL family member 6), IGFBP6 (insulin-like growth factor binding protein 6) and CITED2 (Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain 2). Other genes commonly deregulated in network 1 are SERPINA1 (serpin peptidase inhibitor, clade A), VDR (vitamin D receptor), ITGB2 (integrin, β 2), and TGFBR3 (TGF-β receptor III, also known as betaglycan; Fig. 1A). The last is overexpressed in both DU-145R and PC-3R cells. Interestingly, other TGF-β members appear deregulated in the same network, such as TGFBR2, which is differentially regulated in both cell lines, and TGF-β ligand, which is included by the software as a link between the former TGF-β elements and LTRP2 (latent TGF-β binding protein 2).

Network 2 (Fig. 1B) is mainly centered on the CDH1 gene, which codifies a classical calcium dependent cell–cell adhesion glycoprotein. MYO6 (myosin VI), ID2 (inhibitor of DNA binding 2), PTPRM (protein tyrosine kinase 13-Hs00559623_m1), MPZL2 (myelin protein zero-like 2-Hs00170684_m1), MXI (myxovirus resistance 1-Hs00895608_m1), MYO6 (myosin VI-Hs00192265_m1), S100A4 (S100 calcium-binding protein A4-Hs00243202_m1), SERPINA1 (serpin peptidase inhibitor, clade A-Hs01978000_m1), SYK (spleen tyrosine kinase-Hs00895377_m1), EPCAM (epithelial cell adhesion molecule-Hs00901885_m1), NEAT1 (nuclear paraspeckle assembly transcript 1-Hs0108264_s1), and TNFAIP3 (tumor necrosis factor, α-induced protein 3-Hs00234713_m1).
Table 1. Top 10 commonly over- and downexpressed genes in DU-145R and PC-3R versus their parent cells, with their respective main function and log ratio expression

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Log ratio</th>
<th>Main function</th>
<th>Gene symbol</th>
<th>Log ratio</th>
<th>Main function</th>
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<tr>
<td><strong>Overexpressed</strong></td>
<td></td>
<td></td>
<td><strong>PC-3R vs. PC-3</strong></td>
<td></td>
<td></td>
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<tr>
<td>DU-145R vs. DU-145</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSPT2</td>
<td>4.487</td>
<td>GTPase</td>
<td>NEAT1</td>
<td>3.468</td>
<td>Non-protein coding</td>
</tr>
<tr>
<td>ID2</td>
<td>4.050</td>
<td>Multicellular organism development; regulation of transcription</td>
<td>FLJ27352</td>
<td>3.287</td>
<td>Undefined</td>
</tr>
<tr>
<td>CXCR7</td>
<td>3.879</td>
<td>Signal transduction</td>
<td>TGFBR3</td>
<td>2.960</td>
<td>Epithelial to mesenchymal transition; signal transduction; cell cycle; cell migration</td>
</tr>
<tr>
<td>NDRG1</td>
<td>3.233</td>
<td>Stress and hormone responses, cell growth and differentiation</td>
<td>PLSCR4</td>
<td>2.552</td>
<td>Phospholipid scrambling</td>
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<tr>
<td>FRMD3</td>
<td>3.191</td>
<td>Structural protein</td>
<td>HTRA1</td>
<td>2.389</td>
<td>Regulation of cell growth</td>
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<td>TP53NP1</td>
<td>2.797</td>
<td>Cell-cycle arrest; induction of apoptosis</td>
<td>S100A4</td>
<td>2.217</td>
<td>Epithelial to mesenchymal transition; cell-cycle progression and differentiation.</td>
</tr>
<tr>
<td>GOSR2</td>
<td>2.434</td>
<td>Intracellular protein transport</td>
<td>CYBRD1</td>
<td>2.158</td>
<td>Transport; oxidation reduction</td>
</tr>
<tr>
<td>CITED2</td>
<td>2.372</td>
<td>Vasculogenesis; response to hypoxia; positive regulation of cell-cell adhesion; positive regulation of cell cycle</td>
<td>PPP2R2C</td>
<td>2.052</td>
<td>Signal transduction</td>
</tr>
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<td>KLLH24</td>
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<td>GSPT2</td>
<td>2.006</td>
<td>GTPase</td>
</tr>
<tr>
<td>NEAT1</td>
<td>2.267</td>
<td>Non-protein coding</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><strong>Downexpressed</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>SCEL</td>
<td>−6.428</td>
<td>Epidermis development; assembly or regulation of structural proteins</td>
<td>ESRP1</td>
<td>−6.612</td>
<td>Regulation of RNA splicing</td>
</tr>
<tr>
<td>EPCAM</td>
<td>−5.357</td>
<td>Calcium-independent cell adhesion</td>
<td>TACSTD2</td>
<td>−6.309</td>
<td>Signal transduction</td>
</tr>
<tr>
<td>C1orf116</td>
<td>−5.292</td>
<td>Undefined</td>
<td>EFEMP1</td>
<td>−6.079</td>
<td>Extracellular matrix glycoprotein</td>
</tr>
<tr>
<td>TACSTD2</td>
<td>−5.240</td>
<td>Signal transduction</td>
<td>GJB2</td>
<td>−5.984</td>
<td>Transport; cell-cell signaling</td>
</tr>
<tr>
<td>SERPINA1</td>
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<td>Response to hypoxia</td>
<td>MPZL2</td>
<td>−5.088</td>
<td>Cell adhesion</td>
</tr>
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<td>AIM1</td>
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<td>Undefined</td>
<td>RBM47</td>
<td>−4.333</td>
<td>Undefined</td>
</tr>
<tr>
<td>AREG</td>
<td>−4.864</td>
<td>EGF receptor signaling pathway; cell proliferation; cell-cell signaling</td>
<td>JPH1</td>
<td>−4.312</td>
<td>Calcium ion transport into cytosol</td>
</tr>
<tr>
<td>JPH1</td>
<td>−3.992</td>
<td>Calcium ion transport into cytosol</td>
<td>TXNIP</td>
<td>−4.224</td>
<td>Transcription; response to oxidative stress; regulation of cell proliferation</td>
</tr>
<tr>
<td>GPR87</td>
<td>−3.648</td>
<td>Signal transduction</td>
<td>GPR87</td>
<td>−4.192</td>
<td>Signal transduction</td>
</tr>
<tr>
<td>GALNT3</td>
<td>−3.501</td>
<td>Carbohydrate metabolic process</td>
<td>KAP2.1B</td>
<td>−4.181</td>
<td>Keratin-associated protein</td>
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</tbody>
</table>
Figure 1. Gene networks deregulated in resistant cell lines with respect to their parent cells. A, the most significant ($P < 0.05$) gene network deregulated in resistant cells, scored 51 by IPA software. B, the second most significant ($P < 0.05$) gene network deregulated in resistant cells, scored 39 by IPA software.
phosphatase, receptor type M), and OCLN (occludin) are other deregulated genes directly related with CDH1.

No overlapping genes were found between networks 1 and 2, although both are involved in gene expression.

**Functional in silico analysis**

IPA detected several biologic functions with overrepresentation, when considering together the differentially expressed genes in docetaxel-resistant versus their parent cells. Such functions were related with elemental metabolism of cell survival like gene expression, cell growth and proliferation, cell cycle, cell death, and cell movement. Specifically, and as expected, the 2 main networks were involved in cell growth and proliferation, but also in gene expression, cell movement and development, and the skeletal and muscular system.

The top overexpressed individual genes were enzymes, cell surface receptors, transcription regulators, and structural proteins involved in cell transport, signaling, binding, and cytoskeletal organization. Similarly, proteins from downexpressed genes were structural proteins, signal transducers, enzymes, growth factors, cell receptors, and transcription factors involved in cell structure, adhesion, transport, and cycle regulation.

**Cell viability assay for TGF-β family**

The TGF-β superfamily was represented at network 1 and was overexpressed in resistant cell lines. We selected this target for a functional study of docetaxel resistance. The results of MTT experiments showed that continuous treatment with TGFBI (5 ng/mL) increased DU-145 and PC-3 cell viability until 16.5% and 15.6%, respectively, with respect to cells that were not cultured with the ligand. Furthermore, when parental cells were exposed to TGFBI (10 ng/mL) for 72 hours, cell proliferation was until 27% greater in PC-3 cells at 7.5 nmol/L of docetaxel than in cells without TGFBI treatment. Cell viability of DU-145 cells was not affected by TGFBI under these experimental conditions (Fig. 2).

**Validation by qRT-PCR in cell lines and test in tumor samples**

A panel of 18 of the top 20 with the highest or lowest expression range in both DU-145R and PC-3R versus parent cells lines (see materials and methods) was selected for being commonly over- or downexpressed. It was possible to confirm by qRT-PCR significant differences in expression in all 18 (Wilcoxon test, \( P < 0.05 \)) in the 4 cell lines studied (Fig. 3). Moreover, their expression was analyzed in docetaxel-resistant \( (n = 5) \) versus docetaxel-sensitive \( (n = 6) \) FFPE tumor samples, and docetaxel-resistant \( (n = 3) \) versus docetaxel-sensitive \( (n = 2) \) OCT tumor samples from patients with metastatic CRPC. This analysis showed a significant downexpression of NEAT1 \( (p = 0.044) \) in FFPE tumors. Of the 18 markers studied, 10 markers in the FFPE samples and 13 in OCT samples were deregulated in the same way as in the *in vitro* models. Interestingly, as shown in Fig. 4, 7 of these marker genes were commonly downexpressed in docetaxel-resistant cell lines and in FFPE and OCT samples from patients with CRPC resistant to docetaxel: AREG, CDH1, DLC1, GJB2, IFIH1, MX1, and EPCAM (Fig. 4).

**Discussion**

This study revealed potential genes and networks involved in resistance to docetaxel in 2 CRPC cell lines, DU-145 and PC-3. Both cell lines were converted to
Docetaxel-resistant and then microarrays analysis of parent and resistant cells was carried out.

Docetaxel resistance was achieved with nanomolar concentrations of the drug (10–15 and 20–22 nmol/L for DU-145R and PC-3R, respectively). The fact that docetaxel IC50 in resistant cells was not substantially higher than in sensitive cells is consistent with other in vitro docetaxel resistance studies (19; 20). This issue may be partially explained by the fact that microtubule alterations induced by taxanes can occur even at concentration of 1 nmol/L (21).

Microarrays analysis was focused on the identification of commonly deregulated genes in the 2 different cell line models. We found 243 eligible genes (Bonferroni < 0.05 and log ratio > 1.2) that were similarly deregulated in DU-145R and PC-3R with respect to their parent cell lines. These genes were involved in survival functions such as gene expression and cell growth, proliferation, death, and movement. Interestingly, in both cell lines GSPT2 and NEAT1 were within the top 10 overexpressed genes, and TACSTD2 (tumor-associated calcium signal transducer 2), JPH1 (junctophilin 1), and GPR87 (G-protein–coupled receptor 87) within the top 10 downexpressed genes. Among these genes, only TACSTD2 and GPR87 have already been related with cancer in the literature (22; 23).

Briefly, GSPT2 encodes a GTPase that may be involved in mRNA stability and NEAT1 is a non–protein-coding RNA that seems to regulate mRNA export. Such involvement of docetaxel with mRNA regulation is not surprising because, as previously described, taxanes can promote transcription and mRNAs stabilization of some genes. Such stabilizing effects can be achieved by the stimulation of proteins that bind the AU-rich region of the 3’UTR region of target genes (24).

On the other hand, TACSTD2 encodes a carcinoma-associated antigen that is a cell surface receptor that transduces calcium signals. It has been described to be unmethylated in normal prostate cells and prostatic intraepithelial neoplasia but hypermethylated in primary prostate tumors (25), suggesting that methylation could be one of the mechanisms for silencing the expression of crucial genes, thus inactivating the apoptotic pathway in CRCP. The real significance of TACSTD2 intraexpression in resistant cell lines and the potential role of docetaxel in methylation-based regulation need to be further explored.
Another downregulated gene was *IPHI*, which mediates cross-talk between the cell surface and intracellular ion channels, modulating electrochemical gradients that are vital to the cell and a potentially strong influence on drug activity, according to previous work (26). According to the authors, the expression of several genes that encode subunits of sodium, chloride, potassium, and other cation channels also is correlated with drug activity.

**GPR87** encodes a G-protein–coupled receptor that plays an essential role in many physiologic processes, including neurotransmission, immunity, and inflammation. This gene seems to be overexpressed in diverse carcinomas and plays an essential role in tumor cell survival. A recent study revealed that a lack of **GPR87** triggers an increase in p53, concomitant with a decrease in AKT, which results in the sensitization of tumor cells to DNA damage–induced apoptosis and growth suppression (23). However, these results are in disagreement with the fact that resistant cells downregulate the expression of **GPR87** but remain able to survive and proliferate. The role of several other genes that contribute to docetaxel resistance must account for such discrepancy.

Interestingly, several well-known molecules already related with docetaxel resistance have been found to be deregulated in the present work. *ABCA13, ABCA8,* and *ABCC2,* all from the ABC (ATP-binding cassette) family of drug transporters, are significantly deregulated in DU-145R cells; only *ABCC2* was already known to be involved in multidrug resistance (27). None of these genes has been found to be deregulated in both resistant cell lines. On the other hand, there are growing evidences about the role of β-tubulin isotypes in resistance to taxanes in CRPC, as they are the primary target of these drugs (9; 28). In the present study we observed that TUBB2B (tubulin, β 2B) was downregulated in PC-3R cells versus their parent cell line (see Supplementary Table S1), but no differences were observed in the expression of β-tubulins in the DU-145 model. Furthermore, tubulin is one of the molecules included by the Ingenuity software as a link between EML1 and MAP2, actin and calmodulin proteins (Fig. 1B); however, its expression was not significantly deregulated. These results suggest that alternative pathways may be more important in generating docetaxel resistance than those directly related to tubulin alterations.

Apart from an individual view of deregulated genes, in this study we focused on IPA analysis that allowed us to obtain global and integrated molecular information about interactions between significant differentially expressed genes. Results from such analysis showed that the most significant network was centered on *PPARA,* which was overexpressed in docetaxel-resistant cells (Fig. 1A). *PPARA* is a nuclear receptor that regulates the expression of multiple genes involved in cell proliferation, differentiation, and immune and inflammation responses. The alpha form of this gene is functional in human prostate and is downregulated by androgens. It has been suggested that its overexpression in advanced prostate cancer indicates a role in tumor progression, with the potential involvement of dietary factors (29). As shown in Fig. 1A, *PPARA* interacts with *CITED2* (Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain 2), which has been involved in cisplatin resistance (30), and notably with the transcription regulators *ETS1* (v-ets erythroblastosis virus E26 oncogene homolog 1). Inappropriate expression of *ETS1* has been observed in a variety of human cancers and might play an important role in carcinogenesis and/or the progression of human prostate cancer (31). However, no link between *ETS1* expression and docetaxel resistance has been described to date.

**ETS1** directly interacts with *VDR* and *ITGB2,* which also are downregulated. *VDR* is a transacting transcriptional regulatory factor involved in a variety of metabolic pathways, but also in antineoplastic activities. In fact, calcitriol, the most active metabolite of vitamin D, showed to enhance antitumor activity of docetaxel, although an effect on patients survival has not been shown (32; 33). Downregulation of *ITGB2* has been previously associated with transition between prostatic intraepithelial neoplasia and prostate cancer, playing a role in cell adhesion of invasive prostate cancer cells (34).

A second focus of interest in network 1 is *NFKB.* This protein complex has been widely associated with oncogenesis due to its ability to regulate cell proliferation and protect cells from apoptosis (35). CRPC cell lines like DU-145 and PC-3 exhibit constitutive activation of *NFKB,* whereas its activity is low in the androgen-sensitive LNCaP and LAPC-4 cells, which is consistent with the role of *NFKB* in progression of prostate cancer. Moreover, higher levels of NfKb protein can be further enhanced in response to certain types of chemotherapy (36; 37). Our group previously found that the inhibition of this complex may be an attractive strategy to enhance docetaxel response in PC (38). This strategy has also been clinically tested through the use of bortezomib (39; 40).

The present study specifically explored the role of the TGF-β superfamily, which is also represented at network 1. The signaling pathway derived from this family of proteins has a principal role in growth control (41). As a member of this family, TGFBR3 acts as a coreceptor of TGF-β ligand, being a positive or negative regulator of TGF-β signals depending on the cellular context (42; 43). Recently, it has been shown that TGFBR3 may act as a protective factor in the apoptotic process of fibroblasts by negative regulation of TGF-β signaling (44). Our results further suggest that TGFβ1 acts as a protective factor against docetaxel for DU-145 and PC-3 cells and has a partial role in the development of docetaxel resistance in cultured cells. The specific mechanism through which TGF-β/TGFBR3 protects DU-145 and PC-3 cells from this drug remains to be elucidated.

The epithelial cell adhesion molecule *CDH1,* which is significantly downregulated in docetaxel-resistant cells, is included in the second most significant network obtained in this analysis (Fig. 1B). The loss of *CDH1* in
metastatic cells has been shown in a variety of in vitro and in vivo models, and has been related to the epithelial-mesenchymal transition process (45–47). However, its role in chemotherapy resistance should be further studied. The mere loss of CDH1 probably does not directly confer chemoresistance properties to the tumor cell, but signals may be conveyed that induce resistance to chemotherapy. According to network 2, CDH1 directly interacts with the transcription regulator ID2 (inhibitor of DNA binding 2), which is significantly overexpressed in resistant cells, and enzymes like OCLN, which is also involved in cell-cell adhesion and is downexpressed in resistant cells. Other CDH1-related genes in this network are the phosphatase PTPRM, the plakophilin PKP2, and the myosin MYO6.

Another interesting focal gene downexpressed in network 2 is IFI16 (interferon, gamma-inducible protein 16). The encoded protein contains domains involved in DNA binding, transcriptional regulation, and protein–protein interactions. It is known that this protein modulates P53 function and inhibits cell growth in the RAS/RAF signaling pathway, an antitumor activity. Downexpression of this gene in resistant cells seems to be related to their ability to grow despite the presence of docetaxel in the medium.

A recent study used microarray analysis to compare the PC-3R cell line with and without docetaxel in culture media, to identify genes responsible for the multinucleated process that PC-3 cells suffer as a result of docetaxel exposure. The authors also compared DU-145 with docetaxel-resistant DU-145 cells and showed a set of 10 genes present in both PC-3R and DU-145R cells (19). However, in contrast to our study only LAMC2, which increases more than 10% during hormone escape in prostate cancer (48), was represented in both models. Methodologic factors and differences in bioinformatics analysis between these studies could account for such high variability of commonly deregulated genes in docetaxel-resistant cells.

Eighteen genes were selected according to their function and degree of relative expression in resistant cells versus parent cells. They were further validated in cell lines and tested in docetaxel-sensitive and resistant CRPC tumor samples (11 FFPE and 5 OCT samples) by qRT-PCR. Seven of the 18 marker genes were deregulated in the same way in cell lines, FFPE and OCT samples. These included the CDH1 gene discussed above and also AREG (amphiregulin), DLC1, GJB2 (gap junction protein, β 2), IFI16, MX1, and EPCAM, all of them consistently downexpressed in docetaxel-resistant tumor samples.

Discordant results were observed in the expression of other genes such as NEAT1 (nuclear paraspeckle assembly transcript 1), which was significantly downexpressed in docetaxel-resistant tumors but was within the top 10 overexpressed genes in resistant cells. These conflicting results could be due to the limitations of the present study. First, gene panels derived from in vitro study cannot represent the complex biology and heterogeneity of tumor cells in patients. Moreover, molecular changes derived from tumor-stroma interaction are lost in in vitro models. On the other hand, the range of docetaxel concentrations in cultured cells differs from docetaxel levels in patients, a fact that also may cause differences in gene expression. Of note, we found molecular alterations in docetaxel-resistant cell lines after long-term exposure to docetaxel. It is not clear whether genetic alterations responsible for docetaxel resistance were already present in the initial cell population or were induced after docetaxel exposure. Future comparison of the present gene expression analysis with gene expression data from a de novo docetaxel-resistant cell line will be useful to further elucidate pathways involved in different resistance patterns. In patients, docetaxel resistance may exist before drug exposure (primary resistance) or may be developed after a number of chemotherapy cycles (acquired resistance). In our series, where tumor samples were obtained before chemotherapy exposure, we observed a concordance between some of the deregulated genes in the resistant tumors and previously observed results in resistant cell lines. These data suggest that basal gene expression alterations, not induced by treatment, may be responsible for the survival of cancer cells in the presence of docetaxel. Finally, in the present work we carried out an exploratory analysis in tumor samples that need to be further validated in a larger cohort of patients.

In summary, this exploratory analysis provides information about potential genes and networks involved in docetaxel resistance in CRPC, as well as a basis for the investigation of the specific mechanism through which the TGF-β family protects cultured cells from docetaxel. The identification of docetaxel resistance genes may be useful to select patients who may not benefit from therapy or to develop targeted therapies to overcome docetaxel resistance. Further clinical validation of these results is needed in patients with CRPC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Mercedes Marín-Aguilera, Jordi Codony-Servat, Susana G. Kalko, et al.

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