IDENTIFICATION OF DOCETAXEL RESISTANCE GENES IN CASTRATION-RESISTANT PROSTATE CANCER

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Abbreviation list:

Cq         Quantification cycle
CRPC       Castration-resistant prostate cancer
FFPE       Formalin-Fixed Paraffin-Embedded
IPA        Ingenuity Pathways Analysis
OCT        Optimal Cutting Temperature
PC         Prostate cancer
QRT-PCR    Quantitative-retrotranscribed PCR

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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 2 models of docetaxel-resistant CRPC cell lines, and to test for the most differentially expressed genes in tumor samples from CRPC patients. 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 4 cell lines. Results showed differential expression of 243 genes (P<0.05, Bonferroni-adjusted P-values and LogRatio>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-beta superfamily in conferring drug resistance. Changes in expression of 18 selected genes were validated by real-time quantitative RT-PCR in all 4 cell lines and tested in a set of 11 FFPE and 5 OCT tumor samples. Analysis in patients showed a noteworthy down-expression 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 CRPC patients that can circumvent such resistance to treatment.
INTRODUCTION

Prostate cancer (PC) is the second most common cancer among men worldwide (1). Approximately 85% of newly diagnosed PC cases are localized to the prostate, while the remainder are invasive or metastatic disease (2). Metastatic PC patients 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 in order 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 RT-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 nM docetaxel and culturing surviving cells during 1 yr and 6 mo, 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% fetal bovine serum. No further authentication of the cell lines was done by the authors. Docetaxel (Sigma) was dissolved in DMSO (10mM). IC 50 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 performed according to manufacturer’s instructions.

Raw expression measures were summarized after background correction and normalization steps using the rma methodology in the affy (10) package from the Bioconductor project (11). Unsupervised cluster 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 LogRatio changes with the limma package (13), according to adjusted \( P \)-values <0.05 for the comparison of interest. Special attention was given to the statistically significant genes that showed the largest changes (\( |\text{LogRatio}| >1.2 \)).

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 metastatic PC patients 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 1-2 ml of Optimal Cutting Temperature (OCT) medium immediately after surgery. The OCT samples were stored at -80ºC until processing. The institutional review board approved this study and written informed consent was obtained from all patients.

FFPE samples belonged to 11 CRPC patients, 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 CRPC patients 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 PSA-response criteria (15) and RECIST (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 (\(| \text{LogRatio} | \geq 2\)), but also by function, ie, 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 μg of total RNA was reverse transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems), following manufacturer’s instructions. QRT-PCR was performed in a 7500 Real Time PCR system (Applied Biosystems), according to the manufacturer’s recommendations. Data was acquired using SDS Software 1.4. Expression values were based on the quantification cycle (Cq) from target genes relative to \( \Delta Cq \). Relative expression with respect to each reference group studied was reported as LogRatio. 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), DLC1 (deleted in liver cancer 1-Hs00183436_m1), GJB2 (gap junction protein, beta 2-Hs00955889_m1), GSPT2 (G1 to S phase transition 2-Hs00250696_s1), IFIH1 (interferon 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), MX1 (myxovirus resistance 1-Hs00895608_m1), MYO6 (myosin VI-Hs00192265_m1), S100A4 (S100 calcium binding protein A4-Hs00243202_m1), SERPINA1 (serpin peptidase inhibitor, clade A-Hs01097800_m1), SYK (spleen tyrosine kinase-Hs00895377_m1), EPCAM (epithelial cell adhesion molecule-Hs00901885_m1), NEAT1 (nuclear paraspeckle assembly transcript 1-Hs01008264_s1), and TNFAIP3 (tumor necrosis factor, alpha-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 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 GUSB endogenous genes.

Viability assay

TGFB1 (transforming growth factor, beta 1) (Sigma-Aldrich) was dissolved in sterile 4mM HCl containing 0.1% endotoxin-free recombinant human serum albumin (50 ug/ml). Parent cells (DU-145 and PC-3) were maintained during 7 consecutive days with TGFB1 (5 ng/ml) in the medium. Parental cells with and without sustained treatment were then seeded at a density of 3200 cells/well in a 96-well microtiter plate in the corresponding medium with 10% fetal bovine serum. After 24h, cells were exposed to docetaxel with/without TGFB1 for an additional 72h. Finally, cell proliferation/viability was assessed by using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] 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 10-15
nM and 20-22 nM, respectively, while DU-145 and PC-3 registered 4-5 nM and 3-5 nM, respectively (data not shown).

**Differentially expressed genes in resistant cells**

Initially, unsupervised clustering analysis of microarrays was performed 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 based on expression values (data not shown). Subsequent differential expression analysis revealed 1064 and 1361 differentially expressed genes (Bonferroni<0.05 and LogRatio>1.2), equivalent to 1710 and 2117 Affymetrix probe sets, between DU-145R vs DU-145 and PC-3R vs PC-3, respectively (Supplementary Table I). Top 20 over- and down-expressed genes from docetaxel-resistant cells with respect to their parent cells according to LogRatio expression values, and their main function, are summarized in supplementary table II. When comparing differentially expressed genes between both resistant cell lines (DU-145R and PC-3R), 243 genes overlapped (Supplementary Table III). From those genes, 172 were over- or down-expressed in both DU-145R and PC-3R cells vs their parent cells. The top 10 commonly over- and down-expressed 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 based on this gene selection with a score >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 *PPARA* (peroxisome proliferator-activated receptor alpha). This gene directly interacts with an important complex for cell survival and proliferation, nuclear factor kB (*NFKB*), which in turn interacts with *TNFAIP3* (tumor necrosis factor, alpha-induced protein 3), *HMGA1* (high mobility group AT-hook 1) and *ISG15* (ISG15 ubiquitin-like modifier). Other deregulated genes directly related to *PPARA* 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, beta 2), and TGFBR3 (transforming growth factor, beta receptor III, also known as betaglycan) (Fig. 1a). The last, is over-expressed in both DU-145R and PC-3R cells. Interestingly, other TGF-beta members appear deregulated in the same network, such as TGFB2 which is differentially regulated in both cell lines and TGF-beta ligand, which is included by the software as a link between the former TGF-beta elements and LTBP2 (latent transforming growth factor beta binding protein 2).

Network 2 (Fig. 1b) is mainly centered on the CDH1 gene (E-cadherin), which codifies a classical calcium dependent cell-cell adhesion glycoprotein. MYO6 (myosin VI), ID2 (inhibitor of DNA binding 2), PTPRM (protein tyrosine 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 biological functions with overrepresentation, when considering together the differentially expressed genes in docetaxel-resistant vs 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 two 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 over-expressed individual genes were enzymes, cell surface receptors, transcription regulators, and structural proteins involved in cell transport, signaling, binding, and cytoskeletal organization. Similarly, proteins from down-expressed 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-beta family**

The TGF-beta superfamily was represented at network 1 and was over-expressed 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 TGFB1 (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 TGFB1 (10 ng/ml) for 72 h, cell proliferation was until 27% greater in PC-3 cells at 7.5 nM of docetaxel than in cells without TGFB1 treatment. Cell viability of DU-145 cells was not affected by TGFB1 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 vs parent cells lines (see materials and methods) was selected for being commonly over- or down-expressed.

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) vs -sensitive (n=6) FFPE tumor samples, and docetaxel-resistant (n=3) vs -sensitive (n=2) OCT tumor samples from metastatic CRPC patients. This analysis showed a significant down-expression 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 Figure 4, 7 of these marker genes were commonly down-expressed in docetaxel-resistant cell lines and in FFPE and OCT samples from CRPC patients resistant to docetaxel: AREG (amphiregulin), CDH1 (E-cadherin), DLC1 (deleted in liver cancer 1), GJB2 (gap junction protein, beta 2), IFIH1 (interferon
induced with helicase C domain 1), MX1 (myxovirus resistance 1, interferon-inducible protein p78) and EPCAM (epithelial cell adhesion molecule) (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 performed. Docetaxel resistance was achieved with nanomolar concentrations of the drug (10-15 nM and 20-22 nM 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 nM (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 LogRatio>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 (G1 to S phase transition 2) and NEAT1 (nuclear paraspeckle assembly transcript 1) were within the top 10 over-expressed genes, and TACSTD2 (tumor-associated calcium signal transducer 2), JPH1 (junctophilin 1), and GPR87 (G protein-coupled receptor 87) within the top 10 down-expressed 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 nonprotein coding RNA that seems to regulate mRNA export. Such involvement of docetaxel with mRNA regulation is not surprising since, 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 which 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 infraexpression in resistant cell lines and the potential role of docetaxel in methylation-based regulation need to be further explored.

Another down-expressed gene was JPH1, which mediates cross-talk between the cell surface and intracellular ion channels, modulating electrochemical gradients which 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 physiological processes, including neurotransmission, immunity, and inflammation. This gene seems to be over-expressed 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 down-regulate 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 de-regulated 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 taxenes in CRPC, as they are the primary target of these drugs (9;28). In the present study we observed that TUBB2B (tubulin, beta 2B) was down-expressed in PC-3R cells vs. their parent cell line (see Supplementary Table I), 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 (peroxisome proliferator-activated receptor alpha), which was over-expressed 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 down-regulated by androgens. It has been suggested that its over-expression in advanced PC indicates a role in tumor progression, with the potential involvement of dietary factors (29). As shown in Figure 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 PC (31). However, no link between \textit{ETS1} expression and docetaxel resistance has been described to date.

\textit{ETS1} directly interacts with \textit{VDR} (vitamin D receptor) and \textit{ITGB2} (integrin, beta 2), which also are down-expressed. \textit{VDR} is a trans-acting 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 demonstrated (32;33). Down-regulation of \textit{ITGB2} has been previously associated with transition between prostatic intraepithelial neoplasia and PC, playing a role in cell adhesion of invasive PC cells (34).

A second focus of interest in network 1 is \textit{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 \textit{NFKB}, whereas its activity is low in the androgen-sensitive LNCaP and LAPC-4 cells, which is consistent with the role of \textit{NFKB} in progression of PC. Moreover, higher levels of NFκB 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 prostate cancer (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-beta 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, TGFBRIII acts as a co-receptor of TGF-beta ligand, being a positive or negative regulator of TGF-beta signals depending on the cellular context (42;43). Recently, it has been demonstrated that TGFBRIII may act as a protective factor in the apoptotic process of fibroblasts by negative regulation of TGF-beta signaling (44). Our results
further suggest that TGFB1 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-beta/TGFBR3 protects DU-145 and PC-3 cells from this drug remains to be elucidated.

The epithelial cell adhesion molecule CDH1, which is significantly down-expressed 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 over-expressed in resistant cells, and enzymes like OCLN (occludin), which is also involved in cell-cell adhesion and is down-expressed 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 down-expressed 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. Down-expression 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, in order 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 vs 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 PC (48), was represented in both models. Methodological 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 vs 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 (deleted in liver cancer 1), GJB2 (gap junction protein, beta 2), IFIH1 (interferon induced with helicase C domain 1), MX1 (myxovirus resistance 1, interferon-inducible protein p78) and EPCAM (epithelial cell adhesion molecule), all of them consistently down-expressed 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 down-expressed in docetaxel-resistant tumors but was within the top 10 over-expressed 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 cells 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 performed 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-beta 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 CRPC patients.

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REFERENCES


(38) Domingo-Domenech J, Oliva C, Rovira A, Codony-Servat J, Bosch M, Filella X, et al. Interleukin 6, a nuclear factor-kappaB target, predicts resistance to docetaxel in hormone-independent prostate cancer and


### Table 1: Top 10 commonly over- and down-expressed genes in DU-145R and PC-3R vs their parent cells, with their respective main function and LogRatio expression.

<table>
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<tr>
<th>Gene Symbol</th>
<th>LogRatio</th>
<th>Main function</th>
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<th>LogRatio</th>
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FIGURES LEGENDS

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.

Figure 2: Cell viability assay of docetaxel treatment for 72 h in DU-145 and PC-3 cells, a) after a sustained treatment with TGFB1 5 ng/ml, during 7 days plus additional 72 h, and b) after 72 h of treatment with TGFB1 10 ng/ml. Cell viability is expressed as the relative colorimetric signal calculated from the untreated wells (ctrl.). Each experimental condition was performed by triplicate (standard deviation is showed).

Figure 3: Validation of microarrays data by qRT-PCR in DU-145, DU-145R, PC-3 and PC-3R cell lines. Expression data is represented by a LogRatio calculated by comparing $\Delta$Cqs from resistant cells to $\Delta$Cqs from parent cells. $\Delta$Cq was calculated as the difference between Cq of target genes and Cq of the endogenous control gene ACTB.

Figure 4: Differential gene expression by qRT-PCR in docetaxel-resistant vs -sensitive FFPE and OCT tumor samples from patients with metastatic CRPC. Expression data is represented by a LogRatio calculated comparing $\Delta$Cqs from docetaxel resistant patients to the median of $\Delta$Cqs from docetaxel-sensitive patients. $\Delta$Cq was calculated as the difference between Cq of target genes and the mean of Cq of the endogenous control genes ACTB, B2M and B-GUS. *: $P<0.05$. 

*Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.*
1a

1b

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Figure 4

FFPE tumor samples

OCT tumor samples

Genes
Molecular Cancer Therapeutics

IDENTIFICATION OF DOCETAXEL RESISTANCE GENES IN CASTRATION-RESISTANT PROSTATE CANCER

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

Mol Cancer Ther Published OnlineFirst October 25, 2011.

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