Title

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Activation of the ABCB1-amplicon in docetaxel and cabazitaxel resistant prostate cancer cells

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Abstract

Docetaxel and cabazitaxel based taxane chemotherapy are critical components in the management of advanced prostate cancer. However, their efficacy is hindered due to de novo presentation with or the development of resistance. Characterizing models of taxane resistant prostate cancer will lead to creation of strategies to overcome insensitivity. We've previously characterized docetaxel resistant C4-2B and DU145 cell line derivatives, TaxR and DU145-DTXR, respectively. In the present study, we characterize cabazitaxel resistant derivative cell lines created from chronic cabazitaxel exposure of TaxR and DU145-DTXR cells, CabR and CTXR, respectively. We show that CabR and CTXR cells are robustly resistant to both taxanes but retain sensitivity to anti-androgens. Both CabR and CTXR cells possess increased expression of ABCB1, which is shown to mediate resistance to treatment. Interestingly, we also present evidence for coordinated overexpression of additional genes present within the 7q21.12 gene locus where ABCB1 resides. This locus, known as the ABCB1-amplicon, has been demonstrated to be amplified in multidrug resistant tumor cells, but little is known regarding its role in prostate cancer. We show that two ABCB1-amplicon genes other than ABCB1, RUNDC3B and DBF4, promote cellular viability and treatment resistance in taxane resistant prostate cancer models. We present evidence that coordinated amplification of ABCB1-amplicon genes is common in a subset of prostate cancer patients. These data together suggest that ABCB1-amplicon activation plays a criticle role in taxane resistance.

Keywords

docetaxel; cabazitaxel; ABCB1-amplicon; prostate cancer; resistance

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Introduction

Castration-resistant prostate cancer (CRPC) remains an incurable, significant challenge facing clinicians. Taxane-based chemotherapy using docetaxel was the first treatment shown to extend survival in CRPC patients [1]. However, only ~50% of patients respond, and all eventually fail treatment due to resistance. Our previous studies detailed creation and characterization of two models of docetaxel-resistant CRPC; TaxR from androgen receptor (AR)-positive C4-2B cells and DU145-DTXR from AR-negative DU145 cells [2]. We demonstrated that ABCB1 was a principle mechanism involved in acquired resistance to docetaxel treatment and that inhibition of ABCB1 using small molecule inhibitors or anti-androgens could re-sensitize resistant cells to treatment [2, 3]. Cabazitaxel is a second-generation taxane designed for increased efficacy in the docetaxel-resistant state [4, 5]. The TROPIC clinical trial demonstrated the utility of cabazitaxel in patients previously exposed to docetaxel [6]. Despite this finding, response rates and survival improvements are modest, and the development of resistance remains inevitable. Interestingly, we've shown that docetaxel resistant TaxR and DU145-DTXR cells display only a modest cabazitaxel response due to ABCB1 mediated taxane cross-resistance, which can be overcome using ABCB1 blockade [7].

It was hypothesized that cabazitaxel may outperform docetaxel in the first-line treatment setting. The FIRSTANA clinical trial was designed to test this hypothesis but surprisingly, the study found no difference in efficacy between the two drugs [8]. Thus, cabazitaxel currently remains in the second line setting based upon the TROPIC trial. This makes it necessary to study cabazitaxel-resistance post-docetaxel. To address this unmet need, we developed two models of cabazitaxel-resistant CRPC, CabR and CTXR, derived from the docetaxel-resistant TaxR and DU145-DTXR cell lines respectively [9]. These novel models mimic the clinical progression of acquired resistance to subsequent cabazitaxel post-docetaxel treatment. Characterization of these models demonstrates that they are robustly resistant to cabazitaxel while retaining insensitivity to docetaxel.

Evidence presented in this study suggests that acquired cabazitaxel resistance post-docetaxel failure is partially dependent on further augmented levels of ABCB1 in both CabR and CTXR cells. However, it is well known that efforts to target ABCB1 to enhance taxane efficacy in the clinic have not been successful, likely due to two reasons; 1) lack of adequate patient stratification based on ABCB1 expression and 2) the presence of compensatory co-mechanisms of resistance [10]. It is known that the ABCB1 gene exists within a larger gene locus, 7q21.12, which is thought to house several genes shown to be repeatedly co-amplified/co-expressed along with ABCB1 [10]. Co-overexpression of 7q21.12 (aka ABCB1-amplicon) is widely thought to be associated with tumor progression and taxane resistance. However, the direct role of the ABCB1-amplicon beyond ABCB1 in mediating resistance is less understood. Furthermore, the role of the ABCB1-amplicon in prostate cancer is poorly defined.

In the present study, we provide evidence for activation of the ABCB1-amplicon in our models of taxane resistance. We functionally test the role of two ABCB1-amplicon genes, RUNDC3B and DBF4, and show that each regulates tumor cell viability and resistance to

taxanes. Lastly, we present clinical data from the cBioPortal demonstrating evidence for the presence of the ABCB1-amplicon in prostate cancer patients.

Materials and Methods

Cell Culture and Reagents

C4-2B cells were kindly provided and authenticated by Dr. Leland Chung (Cedars-Sinai Medical Center, Los Angeles, CA). DU145 cells were obtained from the American Type Culture Collection (ATCC). ATCC uses short tandem repeat profiling for testing and authentication of cell lines. All cell lines are routinely tested for mycoplasma using ABM mycoplasma PCR detection kit (Cat#: G238). All experiments with these cell lines and their derivatives were conducted within 6 months of receipt or resuscitation after cryopreservation. Cells were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum, 100 IU penicillin and 0.1 mg/ml streptomycin. Docetaxel-resistant C4-2B cells (TaxR) and DU145 cells (DU145-DTXR) were characterized and described previously and maintained in complete RPMI 1640 supplemented with 5nM docetaxel [2]. Cabazitaxel-resistant CabR and CTXR cells were derived from TaxR and DU145-DTXR cells respectively through chronic exposure to increasing doses of cabazitaxel up to 25nM over a period of 4 months. CabR and CTXR cells are maintained in complete RPMI 1640 supplemented with 5nM cabazitaxel. C4-2B, DU145, TaxR, and DU145-DTXR cells were cultured alongside cabazitaxel resistant derivative cell lines as appropriate controls. All cells were maintained at 37°C in a humidified incubator with 5% carbon dioxide. Docetaxel (Ca#: RS019) was purchased from TSZ CHEM. Cabazitaxel (Cat#: S3022) and enzalutamide (Cat#: S1250) were purchased from Selleckchem. Bicalutamide (Cat#: B3209) was purchased from LKT Laboratories. Abiraterone Acetate (Cat#: X6144) was purchased from AK Scientific, Inc. Elacridar (Cat#: 143664-11-3) was purchased from Sigma-Aldrich. RNAi was performed using Dicer-Substrate siRNAs (DsiRNA) purchased from IDT. The following DsiRNA's were used; Negative Control (NC) = Cat#: 51-01-14-04, RUNDC3B = Cat#: hs.Ri.RUNDC3B.13.1, DBF4 = Cat#: hs.Ri.DBF4.13.1. Transfection of DsiRNA's was done using Lipofectamine RNAiMAX (Cat#: 56532) purchased from ThermoFisher Scientific according to manufacturer's protocol.

Cell Growth Assay

Cells were plated at a density of 25,000 cells/well in 24-well plates in complete RPMI 1640 media without any selection agent. After 24 hours, cells were subjected to indicated treatments. For experiments combining RNAi with drug, transfection of DsiRNA was performed 24 hours after plating and drug was administered as indicated the following day. Total cells were counted via coulter counter 72–120 hours post treatment. Alternatively, cell count was determined using Cell Counting Kit-8 (Cat#: CK04-20) purchased from Dojindo Molecular Technologies, Inc. Data is displayed as percent of control cell growth. All conditions were performed either in triplicate or quadruplicate. All experiments were performed at least twice.

Colony Formation Assay

Cells were plated at 500 cells/well in 6-well plates in complete RPMI 1640 with no selection agent. Plated cells were subsequently treated 24 hours later as indicated. Colonies formed for 14 days. At the completion of each assay, cell colonies were fixed and stained using the following solution for 20 minutes; 0.05% w/v crystal violet, 1% of 37% formaldehyde, 1% methanol, 1X PBS. After staining, colonies were rinsed, allowed to air dry, and counted. Data is displayed as a percent of control cell colony growth. All conditions were performed in duplicate or triplicate. All experiments were performed at least twice.

Cell Death ELISA

Cells were plated at 25,000 cells/well in 24-well plates in complete RPMI 1640 with no selection agent. Cells were treated as indicated 24 hours after plating. Mono- and oligonucleosomes indicative of apoptosis released into the media were detected using the Cell Death Detection ELISA kit (Cat#: 11544675001) purchased from Roche according to the manufacturer's instructions. Apoptotic cell death was measured at 405 nm absorbance. A cell growth assay on cells in the wells was also performed and apoptosis absorbance values were normalized to cell count. All conditions were performed in triplicate. Experiment was performed twice.

Preparation of Whole Cell Lysates

Cells were harvested, washed with PBS, and lysed in RIPA buffer supplemented with 5mM EDTA, 1mM NaV, 10mM NaF, and 1X Halt Protease Inhibitor Cocktail (Cat#: 78430) purchased from ThermoFisher. Protein concentration was determined with Pierce Coomassie Plus (Bradford) Assay Kit (Cat#: 23236) purchased from ThermoFisher.

Western Blot

Protein extracts were resolved by SDS-PAGE and indicated primary antibodies were used. ABCB1 antibody (SC-8313, rabbit-polyclonal, 1:500 dilution) was purchased from Santa Cruz Biotechnology. AR-441 antibody (SC-7305, mouse-monoclonal antibody, 1:1000 dilution) was purchased from Santa Cruz Biotechnology. Tubulin (T5168, mouse monoclonal antibody, 1:6000 dilution) was purchased from Sigma-Aldrich. GAPDH (MAB374, mouse monoclonal antibody, 1:10000) was purchased from EMD Millipore. Tubulin or GAPDH was used to monitor the amounts of samples applied. Proteins were visualized with a chemiluminescence detection system (Cat#: WBLUR0500) purchased from Millipore.

Quantitative PCR (qPCR)

Total RNAs were extracted using TRizol reagent (Cat#: 15596018) purchased from ThermoFisher. RNA was digested with RNase-free DNase 1 (Cat#: EN05216101) purchased from ThermoFisher. cDNAs were prepared using ImProm-II reverse transcriptase (Cat#: M314C) purchased from Promega. The cDNAs were subjected to quantitative-PCR (qPCR) using SsoFast EvaGreen Supermix (Cat#: 172-5205) purchased from Bio-Rad according to the manufacturer's instructions. Triplicates of samples were run on default settings of Bio-Rad CFX-96 real-time cycler. Each reaction was normalized by co-amplification of

Actin. Data was calculated using the efficiency corrected method. Primers used for qPCR are below;

ABCB1: 5’ - ATATCAGCAGCCCACATCAT, 3’ - GAAGCACTGGGATGTCCGGT

RUNDC3B: 5’ - TGCTGGCATGCTTCTAGGAC, 3’ - AGCAGGAAAACTGCCATCCA

DBF4: 5’ - AACTCCGGAGCCATGAGGAT, 3’ - TCTGGCCTGTTATCAGTTTTCAGA

Actin: 5’ - CCCAGCCATGTACGTTGCTA, 3’ - AGGGCATACCCCTCGTAGATG

RNA-Sequencing (RNA-Seq) Analysis

RNA samples were submitted to the UC Davis Comprehensive Cancer Center’s Genomics Shared Resource (GSR) for RNA-Seq analysis. Stranded mRNA-Sequencing (RNA-Seq) libraries were prepared from 100 ng total RNA using the NEBNext Ultra Directional RNA Library Prep Kit (New England BioLabs, Ipswich, MA) according to the manufacturer’s standard protocol, and as previously described [11]. Subsequently, libraries were combined for multiplex sequencing on an Illumina HiSeq 4000 System (2 × 150 bp, paired-end, ≥20 ×10^6 reads per sample). De-multiplexed raw sequence data (FASTQ) was analyzed with a HISAT-StringTie-Cufflinks pipeline [12, 13] for read mapping to the human reference genome assembly (Dec. 2013, GRCh38/hg38)[14], followed by transcript assembly and quantification of expression values as FPKM (fragments per kilobase of exon per million mapped sequence reads)[15]. Gene- and transcript-level FPKM expression values were then passed onto Cuffdiff [13] for differential expression analysis.

Gene Set Enrichment Analysis (GSEA)

GSEA was performed using the Java desktop software (http://software.broadinstitute.org/gsea/index.jsp) [16, 17]. HALLMARK genesets downloaded from the Molecular Signature Database were used for the analysis.

cBioPortal and Oncomine Analysis

Prostate cancer patient data sets were interrogated for the presence of co-amplification of 7q21.12 genes using the cBioPortal online resource. Only those samples which were assessed for copy-number alterations were included in each analysis. Results were downloaded and are displayed to show patients which harbor amplifications of 7q21.12 genes. The corresponding percentage of patients positive for alterations is displayed next to each gene. RUNDC3B was specifically analyzed in the cBioPortal Prostate Adenocarcinoma (MSKCC, Cancer Cell 2010) dataset. Gene amplification and overexpression was correlated with disease free survival in samples harboring mRNA data. Overexpression cutoff was placed at Z-Score cutoff 1.5. Data for DBF4 expression in benign prostate tissue, localized disease tissue, and mCRPC tissue was downloaded from the Grasso Prostate Dataset in the ONCOMINE database.

Statistics

All quantitated data is displayed as percent of control mean ± standard deviation. Significance was assessed using a two tailed two sample equal variance students t-test. A p-value ≤ 0.05 was accepted as significant.

Results

Characterization of cabazitaxel-resistant prostate cancer models

Cabazitaxel is currently a CRPC second line therapy necessitating the development of models which mimic this setting. Our previous work created and characterized two models of docetaxel-resistant CRPC; TaxR from AR-positive C4-2B cells and DU145-DTXR from AR-negative DU145 cells [2]. We also showed that while cross-resistance existed between docetaxel and cabazitaxel in these models, each remained relatively sensitive to cabazitaxel [7]. To properly study prostate cancer cabazitaxel-resistance, we developed models from each docetaxel-resistant cell line through chronic exposure to increasing doses of cabazitaxel over a 4-month period; CabR from TaxR cells and CTXR from DU145-DTXR cells (Fig. 1A).

Initial characterization of these models using cell growth assays demonstrates robust resistance to cabazitaxel versus parental cells (Fig. 1B). IC_{50} values for cabazitaxel treatment were 8.6nM and 22.4nM for TaxR and CabR, respectively and 2.5nM and 21.5nM for DU145-DTXR and CTXR, respectively. Both CabR and CTXR cells retain complete insensitivity to high dose (5nM) docetaxel similar to parental docetaxel-resistant TaxR and DU145-DTXR cells (Fig. 1C). Colony formation assays confirm a high degree of insensitivity to cabazitaxel in CabR and CTXR cells versus parental cells (Fig. 1D). We also show that cell death response is blunted in cabazitaxel-resistant cells versus parental cells in response to 5nM cabazitaxel (Fig. 1E). Taken together, these data show that CabR and CTXR cells are resistant to cabazitaxel in addition to docetaxel and possess great utility for studying the cabazitaxel-resistant state post-docetaxel treatment, similar to the clinical setting.

CabR cells remain sensitive to AR-directed therapies

Despite advances in the treatment of CRPC, the optimal treatment sequencing for maximum patient benefit remains elusive and is further complicated by the potential for cross-resistance between available therapies. Whether there exists cross-resistance between taxanes and anti-androgen therapies is a topic of intense study. We've previously shown no cross-resistance between anti-androgen drugs and taxanes using TaxR cells and models of enzalutamide and abiraterone resistance [18]. To continue addressing the critical issue of taxane/anti-androgen cross-resistance, we subjected AR-positive C4-2B, TaxR, and CabR cells to increasing doses of either enzalutamide or abiraterone, two currently approved AR-targeting treatments for CRPC (Fig. 2A) [19–22]. Interestingly, we found no decrease in sensitivity in either docetaxel- or cabazitaxel-resistant cells versus parental C4-2B cells. We've previously presented evidence that AR-variants and most notably, AR-v7, play a role in mediating resistance to anti-androgen therapies but not taxanes [18, 23, 24]. We've also shown that AR-variant expression is augmented in models of enzalutamide and abiraterone

resistance but not docetaxel resistance [18]. Here, western blots for AR demonstrate no significant increases in either full-length or variant expression in both TaxR and CabR cells versus C4-2B cells (Fig. 2B). Our data suggest that taxane resistance does not alter AR expression nor does it induce cross-resistance to AR-targeting therapies. These data suggest that subsequent treatment of patients with anti-androgen therapies post taxane treatment is feasible without threat of diminished activity.

Small-molecule inhibition of ABCB1 re-sensitizes CabR and CTXR cells to cabazitaxel treatment

We sought to understand how cabazitaxel resistance arises post docetaxel treatment. To investigate mechanisms of resistance, we performed RNA-seq to compare gene expression profiles between cabazitaxel resistant and docetaxel resistant cells. GSEA was performed to assess general hallmark pathway alterations which may be involved in the progression to cabazitaxel resistance (Fig. S1). CabR cells demonstrated significant enrichment in cell cycle and metabolism related pathways while CTXR cells showed significant alteration in KRAS related signaling. Interestingly, CTXR cells showed significant negative enrichment of several cell cycle pathways, in contrast to CabR cells. CabR cells displayed downregulation of varying pathways including EMT signaling and interestingly, decreased androgen signaling, in line with previously shown data showing lower AR expression (Fig. 2B). Notably, no significant hallmark pathways were similarly altered between the two cell lines. These data suggest that cabazitaxel resistance may be characterized by significant heterogeneity.

Based on our previous work, we hypothesized that ABCB1 may play a role [7]. qPCR demonstrated that ABCB1 expression was further increased in both CabR and CTXR cells versus docetaxel resistant models (Fig. 3A). This was confirmed by western blots which show increased ABCB1 protein levels exhibited by docetaxel resistant TaxR and DU145-DTXR and further increased levels in both CabR and CTXR cells (Fig. 3B). To determine whether augmented levels of ABCB1 in CabR and CTXR cells mediate cabazitaxel resistance, we performed cell growth assays treating CabR and CTXR cells with a combination of elacridar, a small-molecule inhibitor of ABCB1, and cabazitaxel versus control and single agent treatment (Fig. 3C). We found that combination treatment significantly re-sensitized both lines to cabazitaxel. Interestingly, we found that elacridar did not enhance cabazitaxel treatment in parental C4-2B and DU145 cells to the degree observed in resistant cells, demonstrating specificity of the mechanism (Fig. S2). Colony formation assays confirmed the finding that ABCB1 inhibition sensitizes to cabazitaxel (Fig. S3). We previously demonstrated that the anti-androgen drugs bicalutamide and enzalutamide have a secondary function in blocking ABCB1 function by inhibiting its ATPase activity [3]. Using this strategy, we have shown that combination therapy utilizing anti-androgen therapies can re-sensitize docetaxel-resistant cells to either docetaxel or cabazitaxel [3, 7]. We sought to test whether this strategy would work for advanced taxane-resistance represented by CabR and CTXR cells. Cell growth assays demonstrate that enzalutamide can re-sensitize either cell line to cabazitaxel (Fig. 3D). Colony formation assays were also performed and similarly demonstrate that either enzalutamide or bicalutamide can re-sensitize cabazitaxel-resistant cells to treatment (Fig. S4). These data suggest that therapies already available for

prostate cancer treatment could be combined with taxanes to re-sensitize ABCB1 dependent taxane-resistant tumors to treatment.

ABCB1-amplicon is overexpressed in taxane resistant cells

Our collective evidence suggests that ABCB1 represents a potent mechanism of chemotherapy resistance. However, efforts to translate this knowledge into effective therapies has proven difficult. We believe this to be due to two key reasons; 1) failure to stratify patients by ABCB1 status and 2) additional, co-present mechanisms of resistance. Interestingly, the ABCB1 gene is known to exist within a larger genomic locus, 7q21.12, which is alternatively referred to as the ABCB1-amplicon (Fig. 4A). Several reports have shown overexpression/amplification of this region in taxane resistant tumor cells, leading to increased levels not only of ABCB1, but also of other genes present within the locus [10]. It is thought that activation of the ABCB1-amplicon, encompassing both amplification and/or transcriptional upregulation of genes within the locus, is a key contributor to taxane insensitivity and acquisition of the multi-drug resistant state.

While activation of the ABCB1-amplicon is poorly characterized in prostate cancer, we hypothesized it may be responsible for taxane resistance beyond that mediated by ABCB1 alone. To determine whether the ABCB1-amplicion is activated in our models, we further analyzed our RNA-seq data. Indeed, analysis revealed coordinated overexpression of ABCB1-amplicon genes in both C4-2B and DU145 based taxane resistant model series (Fig. 4B). However, the degree to which these genes were co-expressed varied between series. ABCB1 was the most overexpressed in both model series. While 10/11 genes were overexpressed in the DU145 based models, only 7/11 were overexpressed in the C4-2B based models. More interestingly, of the 6 genes other than ABCB1 overexpressed in C4-2B based models, RUNDC3B expression was increased much more than that of the other genes. In contrast, the degree of overexpression of genes other than ABCB1 appeared more even in the DU145 based models. These data suggest that activation of the ABCB1-amplicon is associated with taxane resistance in prostate cancer. Furthermore, the manner in which the locus is activated appears to greatly vary between models, indicating alternate routes to taxane resistance.

Downregulation of RUNDC3B and DBF4 expression decrease cell viability and increase sensitivity to taxane therapy in prostate cancer cells

While ABCB1 is well characterized to mediate taxane resistance, less is known regarding the function of the other genes within the ABCB1-amplicon. However, several groups have described coordinated expression of these genes being associated with taxane resistance, suggesting they are involved in the resistant state. We sought to determine whether ABCB1-amplicon genes other than ABCB1 may play a functional role in taxane sensitivity. We chose to focus on RUNDC3B in the C4-2B series as it was the most overexpressed gene besides ABCB1. qPCR confirmed increased RUNDC3B expression in TaxR and further increased levels in CabR versus parental C4-2B cells (Fig. 5A). Cell growth assays demonstrated that siRNA mediated inhibition of RUNDC3B reduces cellular viability in all three cell lines, with more pronounced reduction in the viability of taxane resistant derivatives (Fig. 5B–C). However, inhibition of RUNDC3B had no effect on

docetaxel resistance and only sensitized TaxR and CabR cells to relatively high doses of cabazitaxel (Fig. 5D–E). These data suggest that RUNDC3B is a significant contributor to cellular survival in taxane resistant cells but marginally influences direct taxane sensitivity. Interestingly, we found that high RUNDC3B expression correlates with decreased patient disease free survival in a cBioPortal dataset (Prostate Adenocarcinoma (MSKCC, Cancer Cell 2010)) (Fig. 5F).

We focused on DBF4 in the DU145 series based upon a previous report detailing its overexpression in a PC3 based model of prostate cancer docetaxel resistance [25]. qPCR revealed increased expression of DBF4 in DU145-DTXR and further increased expression in CTXR versus parental DU145 cells (Fig. 6A). siRNA mediated inhibition of DBF4 had relatively little effect on viability of all three cell lines (Fig. 6B–C). Interestingly, DBF4 inhibition did significantly sensitize DU145-DTXR cells to docetaxel and cabazitaxel treatment, and also sensitized CTXR cells to cabazitaxel (Fig. 6D–E). Furthermore, we found increased expression of DBF4 in mCRPC samples versus benign and local disease samples in an ONCOMINE dataset (Fig. 6F). These data suggest that in contrast to RUNDC3B, DBF4 may play a more significant role in mediating direct taxane resistance in advanced tumors. While not comprehensive, these data together suggest that ABCB1-amplicon genes other than ABCB1 may promote viability of prostate tumor cells and resistance to taxane treatment. Thus, coordinated overexpression of the genes within the ABCB1-amplicon may be responsible for mediating taxane resistance and confounding efforts to overcome this difficult to treat state.

Clinical evidence suggests activation of the ABCB1-amplicon in a subset of prostate cancer patients

Whether ABCB1-amplicon activation is present in prostate cancer patients is unknown. To begin to investigate this possibility, we analyzed well annotated, publicly available datasets via cBioPortal (Fig. 7A, Fig. S5). We found evidence for co-amplification of ABCB1-amplicon genes in all interrogated datasets. Frequency of the amplification varied from 3–22% of patients/study. Interestingly, not all ABCB1-amplicon positive patients were known to have been exposed to chemotherapy, suggesting that some patients present de novo with putative ABCB1-amplicon dependent resistance mechanisms. These data suggest that it is not uncommon for prostate cancer patients to harbor activation of the ABCB1-amplicon. Our findings together suggest these patients may fare poorly on taxane treatment and may benefit from novel treatment strategies.

Discussion

The current CRPC clinical landscape necessitates the creation and study of novel models which accurately depict treatment progression. Such models will provide the tools with which to better understand the development of resistance. In this study, we characterize novel models of cabazitaxel-resistant CRPC (CabR and CTXR) derived from our previously described docetaxel-resistant cell lines (TaxR and DU145-DTXR); thus, these models replicate the clinical use of cabazitaxel in the post-docetaxel treatment setting. We show that

both CabR and CTXR cells exhibit robust resistance to cabazitaxel and remain insensitive to docetaxel.

We found that increased ABCB1 expression was common in both CabR and CTXR cells versus parental control cells. We've previously demonstrated that inhibition of ABCB1 re-sensitizes docetaxel-resistant cells to cabazitaxel treatment [7]. Here we show that inhibition of ABCB1 also sensitizes both CabR and CTXR cells to cabazitaxel. These data support the role of ABCB1 in mediating advanced taxane resistance, even in the dual taxane-resistant state. A previous study by Machioka et al characterized two models of cabazitaxel-resistant prostate cancer using DU145 and PC3 docetaxel-resistant cell line derivatives, similar to the models characterized in this study [26]. Interestingly, they also found that ABCB1 was a key mechanism of taxane resistance in the dual resistant setting. Our current findings in conjunction with our previous work continues to support the critical role of ABCB1 in mediating resistance to taxanes [2, 3, 7, 18].

While we and other groups have demonstrated the potential of reversing taxane resistance by blocking ABCB1 activity, clinical translation of these findings has been elusive. Clinical trials seeking to target ABCB1 and improve chemotherapy efficacy have been undertaken with mixed results [10, 27]. Several generations of ABCB1 inhibitdrors have largely failed most likely due to two causes; 1) lack of definitive clinical ABCB1 detection methods to properly stratify patients for ABCB1 targeting treatments and 2) additionally present, uncharacterized mechanisms of resistance. ABCB1 is known to reside within the 7q21.12 gene locus, which encompasses a number of genes. Co-amplification of the genes within the 7q21.12 locus (aka ABCB1-amplicon) has been widely reported to be associated with tumorigenesis and the multi-drug resistant phenotype [10]. It has also been demonstrated that genes within the locus other than ABCB1 may confer resistance to treatment. However, the role of the ABCB1-amplicon in prostate cancer is poorly understood. In the present study, we show evidence for increased expression not only of ABCB1, but of neighboring genes of the larger ABCB1-amplicon, suggesting activation of the ABCB1-amplicon in taxane resistant prostate cancer. Activation of the ABCB1-amplicon, defined as co-amplification and/or coordinated overexpression of the genes within the locus, has been shown to be associated with acquisition of chemotherapy resistance in a number of studies [10]. Furthermore, it has been postulated that co-overexpression of these genes promotes tumor progression and resistance beyond that mediated by ABCB1 alone, thus providing mechanisms which may explain failure of efforts to target only ABCB1.

Our study highlights the putative role of RUNDC3B and DBF4, members of the ABCB1-amplicon, in promoting tumor cell viability and resistance to taxanes. This work demonstrates that genes co-overexpressed with ABCB1 may be critical for mediating prostate cancer progression and insensitivity to treatment. Currently, the function of RUNDC3B remains elusive, but its overexpression has been associated with poor prognosis in breast cancer and taxane resistance in ovarian cancer cells [28, 29]. Study of its structure suggests it may function in the RAS-like GTPase signaling pathway [30, 31]. DBF4 is a regulatory subunit of CDC7 kinase and plays a central role in cell cycle regulation. Like RUNDC3B, it is associated with tumor progression and taxane resistance in a number of studies in cancers of the breast, lung, and bladder [32, 33]. Furthermore, DBF4 was shown

to be up regulated in a PC3 prostate cancer cell line model of taxane resistance [25]. In addition to functional characterization, we present data from the cBioPortal showing coordinated amplification of ABCB1-amplicon genes in prostate tumors, supporting the hypothesis that it may be a clinically meaningful indicator of tumor aggression and putative taxane resistance [34, 35]. The clinical implications of our findings are summarized in the schematic found in Fig. 7B. In the sensitive setting, taxanes are very effective. ABCB1 may confer robust taxane insensitivity which can be reversed using an inhibitor such as elacridar. However, if the ABCB1-amplicon becomes active, inhibiting ABCB1 may be insufficient to reverse resistance, necessitating the need for an additional agent. Further work is needed to fully understand the contribution of the ABCB1-amplicon to prostate cancer disease progression.

While our findings support the role of the ABCB1-amplicon in mediating advanced taxane resistance, additional mechanisms of resistance have been implicated and must be accounted for when strategizing how best to improve the use of taxanes. The study by Machioka et al demonstrated several similarly differentially expressed genes in both of their models of dual taxane-resistant CRPC, suggesting these genes may be involved in the development of cabazitaxel resistance [26]. A similar study using only a DU145 based dual taxane-resistant model demonstrated that epigenetic regulation of a subset of genes may mediate resistance to cabazitaxel [36]. Hongo et al utilized two models of primary cabazitaxel resistant CRPC using DU145 and PC3 cells that were not first made to be resistant to docetaxel [37]. Interestingly, they found that the DU145 based-model displayed increased ERK signaling while the PC3 based-model displayed increased PI3K/AKT signaling. Thus, while ABCB1-amplicon activation appears important, additional study and characterization may uncover novel resistance mechanisms and additional vulnerabilities in taxane resistant tumors.

Whether cross-resistance exists between taxanes and next-generation anti-androgen drugs is not completely understood. We've previously shown a lack of cross-resistance between these two drug classes utilizing TaxR cells and additional models of enzalutamide and abiraterone resistance [18]. In the present study, we show that both TaxR and CabR cells retain complete sensitivity to both enzalutamide and abiraterone, suggesting that even in an advanced dual taxane-resistant setting, CRPC cells remain sensitive to next-generation anti-androgens. Our in vitro data support a number of clinical studies. Pezaro et al demonstrated robust activity of cabazitaxel post docetaxel and either enzalutamide or abiraterone [38]. A separate study demonstrated that PSA response in a cohort of patients treated with cabazitaxel post docetaxel and abiraterone was similar to that obtained in the TROPIC trial testing cabazitaxel in patients only pre-treated with docetaxel [39]. We also show that neither TaxR nor CabR augment AR or AR-variant expression versus parental C4-2B cells, suggesting that the AR is not involved in the acquisition of a taxane-resistant state. Indeed, other studies have shown a lack of association between AR and AR-variants in mediating taxane resistance [18, 40]. The collective body of work suggests that taxanes and next-generation anti-androgens can be safely sequenced without risk of cross-resistance. In contrast, our previous study did find significant, ABCB1 mediated cross-resistance between taxanes and olaparib [9]. Thus, data presented in this study suggest that a subset of patients positive for ABCB1-amplicon activation may also fail a recently approved PARP inhibitor treatment [41]. The clinical significance of this possibility deserves further study.

In conclusion, our study characterizes novel models of advanced taxane resistant prostate cancer and showcases the putative role of the ABCB1-amplicon in mediating multi-faceted insensitivity to taxanes used to treat prostate tumors. This work expands our current understanding of the development of taxane resistance in this disease and suggests that a more comprehensive approach may be needed to overcome taxane resistance in prostate cancer patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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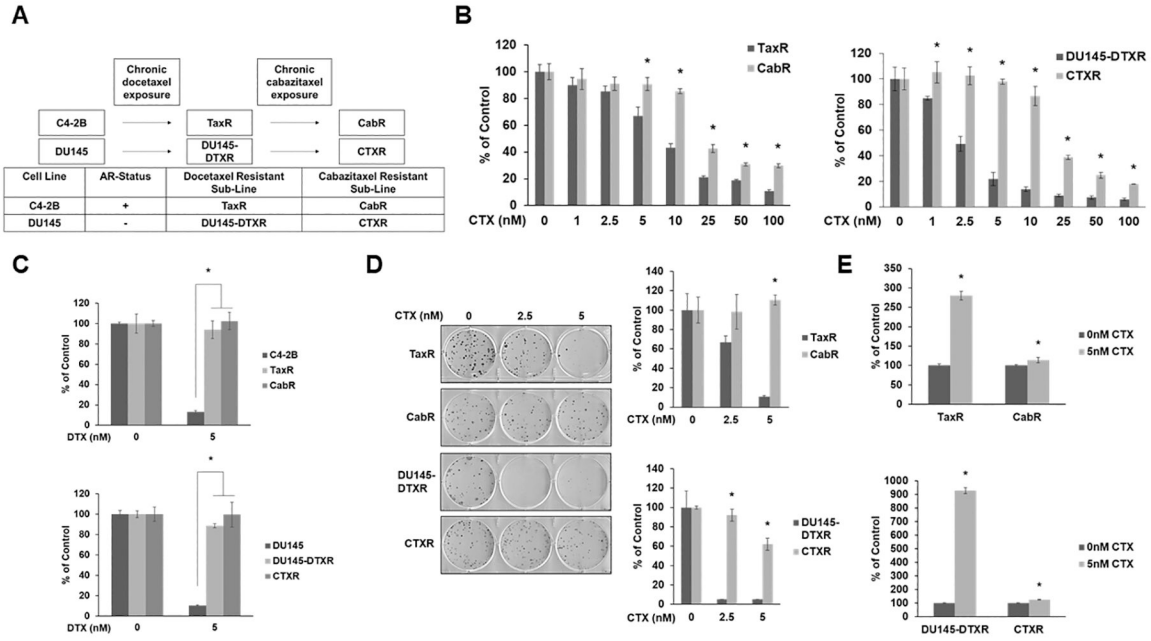


Figure 1: Characterization of cabazitaxel resistant CabR and CTXR cells.

A. Schematic for creation of C4-2B and DU145 derived taxane resistant cell line series. B. Cell growth assays were used to test response of taxane resistant cell lines to increasing doses of cabazitaxel. C. Cell growth assays were used to test response of taxane resistant cell lines versus parental control cells (C4-2B and DU145) to 5nM docetaxel. D. Colony formation assays were used to test response to cabazitaxel in taxane resistant cell lines. E. ELISA's were used to test cell death response to 5nM cabazitaxel in taxane resistant cell lines. DTX = docetaxel, CTX = cabazitaxel. * = p-value ≤ 0.05.

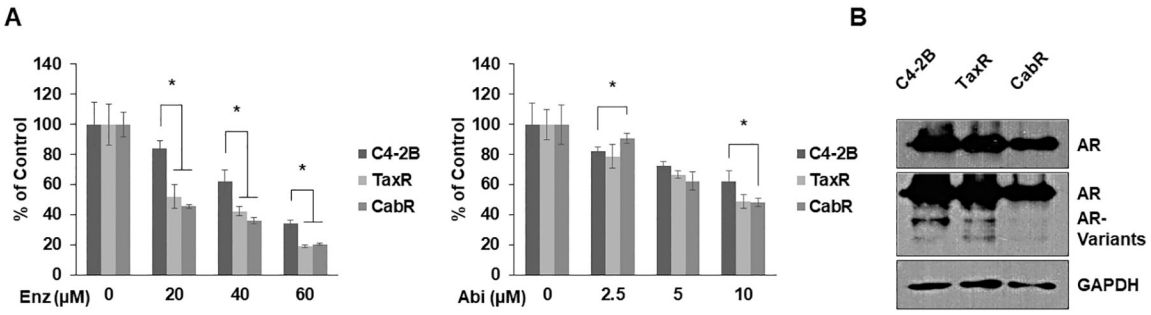


Figure 2: Taxane resistant prostate cancer cells retain sensitivity to anti-androgen drugs.

A. Cell growth assays were used to test response of taxane resistant cell lines versus parental C4-2B cells to increasing doses of enzalutamide or abiraterone acetate. B. Western blot was used to assess androgen receptor (AR) and AR-variant expression in taxane resistant cells versus parental C4-2B cells. GAPDH served as loading control. Enz = enzalutamide, Abi = abiraterone. * = p-value ≤ 0.05.

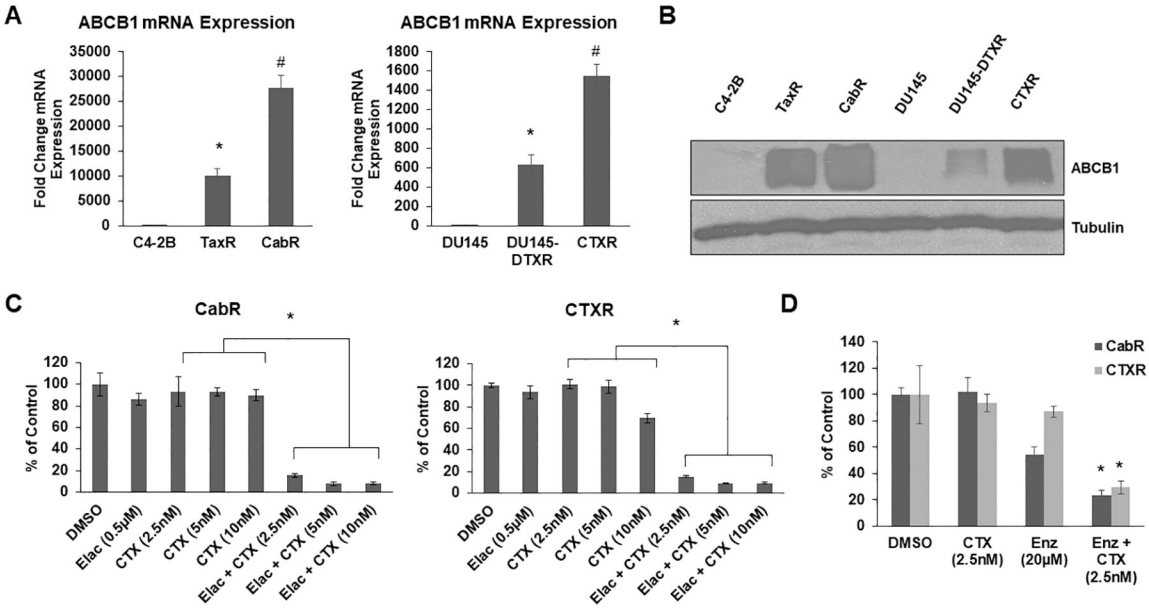
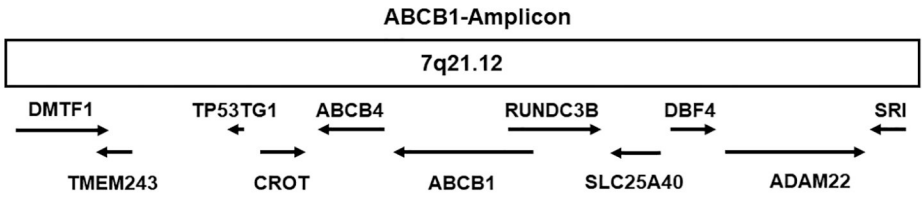


Figure 3: ABCB1 overexpression mediates cabazitaxel resistance in CabR and CTXR cells.

A. qPCR was used to assess ABCB1 mRNA expression in taxane resistant cells versus parental C4-2B and DU145 cell. * = significant from C4-2B, # = significant from both C4-2B and TaxR. B. Western blot was used to assess ABCB1 protein expression in taxane resistant cells versus parental C4-2B and DU145 cell. Tubulin served as a loading control. C. Cell growth assays were used to test the combination of elacridar with varying doses of cabazitaxel in CabR and CTXR cells. D. Cell growth assays were used to test the combination of enzalutamide with cabazitaxel in CabR and CTXR cells. Elac = elacridar, CTX = cabazitaxel, Enz = enzalutamide. */# = p-value ≤ 0.05.

A



B

Gene	C4-2B (FPKM)	TaxR (FPKM)	CabR (FPKM)
SRI	20.1	23.1	29.0
ADAM22	1.8	3.8	3.3
DBF4	14.0	17.7	22.3
SLC25A40	21.8	17.0	20.0
RUNDC3B	0.1	11.2	20.7
ABCB1	0.3	517.6	946.8
ABCB4	0.2	0.5	0.6
CROT	12.8	22.5	34.9
TP53TG1	36.7	38.8	38.3
TMEM243	21.3	14.2	14.4
DMTF1	26.9	20.6	23.0

Gene	DU145 (FPKM)	DU145-DTXR (FPKM)	CTXR (FPKM)
SRI	40.5	485.4	557.8
ADAM22	8.1	47.6	50.8
DBF4	16.3	167.3	120.7
SLC25A40	18.8	161.9	158.1
RUNDC3B	2.3	8.0	4.5
ABCB1	0.3	67.1	95.3
ABCB4	0.0	0.0	0.2
CROT	4.9	30.7	57.1
TP53TG1	3.0	20.0	50.7
TMEM243	16.7	123.8	198.5
DMTF1	56.9	229.9	244.1

Figure 4: RNA-seq reveals coordinated overexpression of ABCB1-amplicon genes in taxane resistant prostate cancer models.

A. Schematic representation of the ABCB1-amplicon (7q21.12 gene locus). **B.** RNA-seq demonstrates increased expression of ABCB1-amplicon resident genes in models of taxane resistant prostate cancer versus parental C4-2B and DU145 cells. FPKM = fragments per kilobase of transcript per million mapped reads.

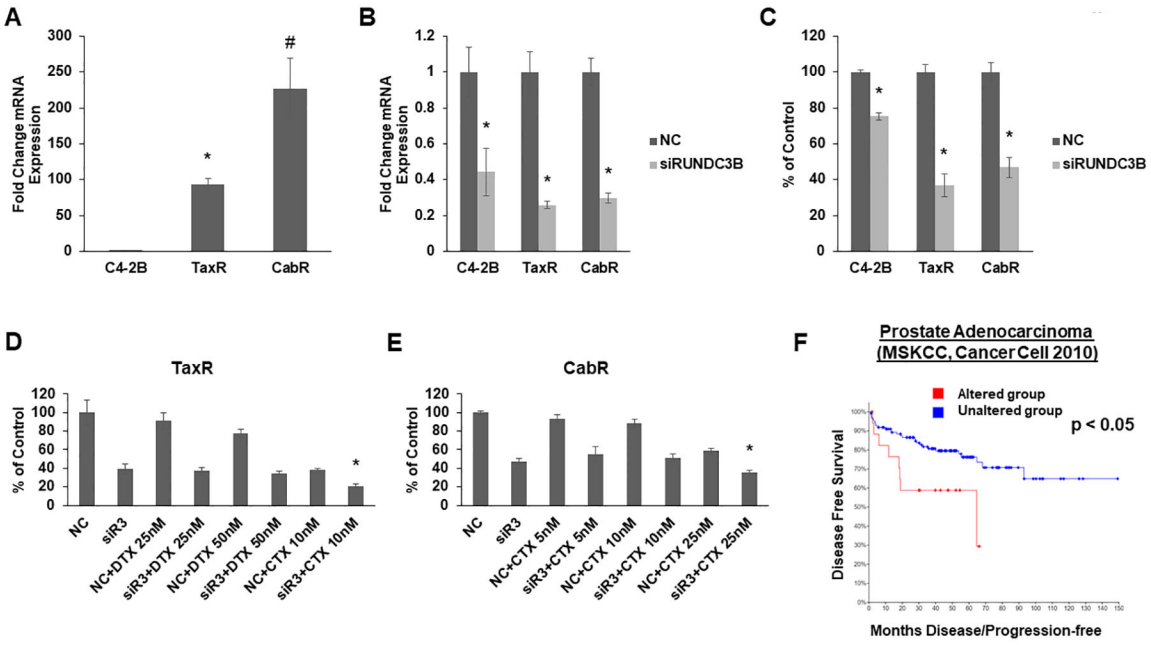


Figure 5: Inhibition of RUNDC3B decreases cellular viability and enhances cabazitaxel sensitivity.

A. qPCR was used to assess RUNDC3B mRNA expression in taxane resistant cell lines versus parental C4-2B cells. * = significant from C4-2B, # = significant from both C4-2B and TaxR. B. qPCR was used to determine RUNDC3B mRNA expression in response to siRNA mediated inhibition of RUNDC3B. C. Cell growth assays were used to test the effect of RUNDC3B knockdown on cellular viability. D. Cell growth assays were used to test the effect of RUNDC3B siRNA mediated inhibition in combination with indicated doses of docetaxel or cabazitaxel in TaxR cells. E. Cell growth assays were used to test the effect of RUNDC3B siRNA mediated inhibition in combination with cabazitaxel in CabR cells. F. Interrogation of cBioPortal dataset demonstrates that overexpressed RUNDC3B expression correlates with decreased disease-free survival. NC = non-targeting control siRNA, siR3 = RUNDC3B targeting siRNA, DTX = docetaxel, CTX = cabazitaxel. */# = p-value ≤ 0.05.

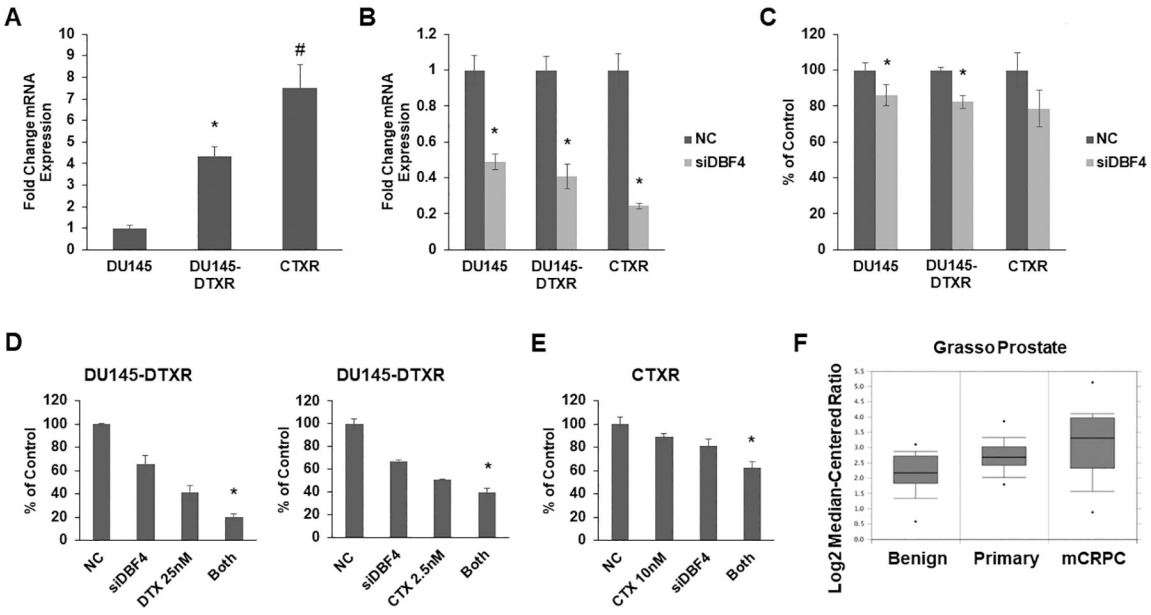


Figure 6: Inhibition of DBF4 decreases cellular viability and sensitizes to docetaxel and cabazitaxel.

A. qPCR was used to assess DBF4 mRNA expression in taxane resistant cell lines versus parental DU145 cells. * = significant from DU145 cells, # = significant from DU145 and DU145-DTXR cells. B. qPCR was used to determine DBF4 mRNA expression in response to siRNA mediated inhibition of DBF4. C. Cell growth assays were used to test the effect of DBF4 knockdown on cellular viability. D. Cell growth assays were used to test the effect of DBF4 siRNA mediated inhibition in combination with indicated doses of docetaxel and cabazitaxel in DU145-DTXR cells. E. Cell growth assays were used to test the effect of DBF4 siRNA mediated inhibition in combination with cabazitaxel in CTXR cells. F. DBF4 expression in prostate cancer progression series from an Oncomine dataset. NC = non-targeting control siRNA, siDBF4 = DBF4 targeting siRNA, DTX = docetaxel, CTX = cabazitaxel. */# = p-value ≤ 0.05.

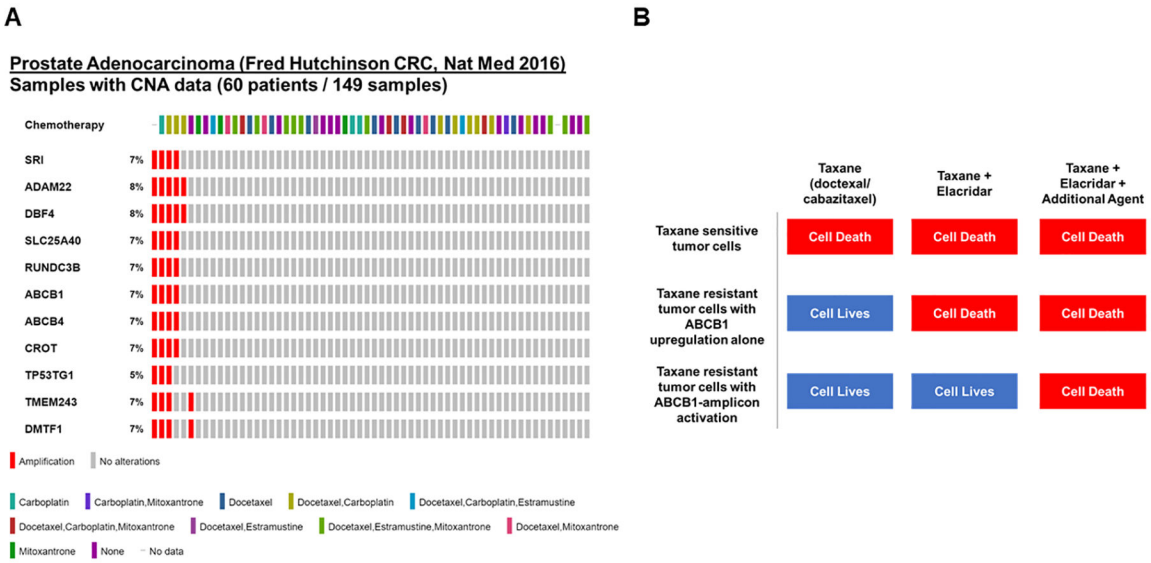


Figure 7: Assessment of ABCB1-amplicon gene expression via cBioPortal.

A. Interrogation of the Prostate Adenocarcinoma dataset from Fred Hutchinson CRC, Nat Med 2016 for the presence of amplification of ABCB1-amplicon genes. B. Schematic diagram detailing study findings. Taxane efficacy may be restored by ABCB1 inhibition (ie. Elacridar), but if the ABCB1-amplicon is active, additional targeting agents may be needed to overcome resistance.