Heterogeneous Responses to Combination Therapy of Anti-AR Antagonists and mTORi: Analysis of Gene Expression Profiles Across Prostate Cancer Cell Lines

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Article

Heterogeneous Responses to Combination Therapy of Anti-AR Antagonists and mTORi: Analysis of Gene Expression Profiles Across Prostate Cancer Cell Lines

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Shortened title: Drug Sensitivity and Gene Profiles in PC

Simple Summary: This study investigated the drug responsiveness and metastasis ability of prostate cancer cell lines, and associated differences in susceptibility to combination therapy with gene expression patterns. This identified genes that affect drug sensitivity and metastasis ability, providing important information that can contribute to the development of customized prostate cancer treatment strategies.

Abstract: Background: Combination therapies targeting different pathways, such as mTOR inhibitors and AR antagonists, have been explored for treating castration-independent prostate cancer. However, their success has been limited. **Materials and Methods**: Four prostate cancer cell lines with varying metastatic potential were analyzed for sensitivity to combination therapy using proliferation, western blot, and viability assays. Gene set expression profiles were compared to identify correlations with drug sensitivity. Gene set enrichment profiles of malignant versus benign cell lines were also analyzed using CCLE data. **Results**: The cell lines showed varying sensitivity to the combination therapy, which correlated with distinct gene expression and enrichment profiles. These patterns were linked to drug resistance and metastatic potential. Additionally, several genes were identified as promising therapeutic targets for prostate cancer. **Conclusions**: This study demonstrates significant heterogeneity in drug resistance and metastatic potential among prostate cancer cell lines. Unique gene expression patterns linked to these characteristics were identified, providing insights for developing targeted therapies and advancing prostate cancer treatment.

Keywords: PC Cell Line; Androgen Receptor Antagonists; mTOR Inhibitors; Gene Expression Profiling

1. Introduction

With the onset of an aging society, prostate cancer is the second diagnosed solid tumor and the fifth leading cause of cancer-related mortality in men worldwide. It is caused by the accumulation of genetic mutations and/or epigenetic alterations over time. Most are adenocarcinomas, which are generally indolent and dependent on androgens for growth and survival. During long-term androgen deprivation therapy (ADT), hormone-refractory cells arise and develop resistance to ADT [1,2]. They become increasingly resistant to treatment and reach an incurable stage, ultimately leading to castration-refractory prostate cancer (CRPC). Based on many clinical trials in the past decades, new strategies of combining ADT and chemotherapy have been established as the standard of care for CRPC patients [3–5]. Although the combination treatment of advanced-stage prostate

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cancer patients showed improvement in disease-free survival and quality of life, there have been many reports suggesting the limited value of combination treatment. All these previous clinical results from different treatment strategy of CRPC indicate that there is still lack of clear understanding on the biological nature of prostate cancer at different stages including individual variation[6–8].

Here we used four different prostate cancer cell lines with different genetic lesions, PC3, DU145, LNCaP[9] and 22Rv1[10] to test their sensitivity to the combination treatment of mTOR inhibitor (CCI-779)[11] and anti-androgen receptor inhibitor (enzalutamide)[12]. Our results show that the LNCaP cell line, which expresses the wild-type androgen receptor, is the only cell line that shows the synergistic effect upon combination treatment of mTOR inhibitor and androgen antagonist. We also compared gene expression profiles of these cell lines to characterize gene set enrichment patterns that characterize sensitivity to the combination drug treatment. In addition, we compared gene set enrichment profiles of the cell lines to find gene expression signatures that characterize the aggressive behavior leading to hormone independence and metastasis. Our results show that certain gene sets are enriched in the cell line sensitive to the combination treatment compared to the other cell lines. We were able to show that certain gene sets are enriched only in cell lines with high metastatic potential. In addition, we found that these gene sets contain many promising drug targets that are specifically expressed in malignant cell lines. We believe our results will be valuable in the development of novel combination drug therapy protocols for the treatment of prostate cancer with a malignant phenotype.

2. Materials and Methods

2.1. Cell Culture

PC-3, DU145, and LNCap cell lines were purchased from KCLB (Korean Cell Line Bank, Seoul, South Korea), and 22Rv1 was purchased from ATCC (American Type Culture Collection, Rockville, MD, USA). RPMI 1640 (Gibco) supplemented with 10% FBS (Gibco;26140) and 1% Pen/Strep (Gibco) was used for normal culture conditions. For androgen deprivation experiments and inhibitor treatment conditions, phenol red-free 5% charcoal-stripped FBS medium was used to avoid non-specific hormonal effects.

2.2. Antibodies and Western Blot

Cells were lysed in RIPA buffer (Thermo) containing protease and phosphatase inhibitors (Roche). Protein concentrations were determined using the Bradford assay (Bio-Rad). Equal amounts of protein lysate (20 μg) were separated by SDS-PAGE on 8% polyacrylamide gels and transferred to PVDF membranes (Millipore). Membranes were blocked with 5% skimmed milk in Tris-buffered saline with Tween-20 (TBST) for 1 hour at room temperature and then incubated with primary antibodies overnight at 4°C with the following primary antibodies: Anti-androgen receptor (AR; 1:200 dilution; sc-7305; Santa Cruz Biotech), Anti-androgen receptor variant 7 (ARv7; 1:1000 dilution; ab198394; Abcam), mTOR (1:1000 dilution; #2983; Cell Signaling Technology), p-mTOR (Ser2448; 1:1000 dilution; #5536; Cell Signaling Technology), Protein kinase B (AKT; 1:1000 dilution; #9272; Cell Signaling Technology), p-AKT (Ser473; 1:1000 dilution; #9271; Cell Signaling Technology), with β-actin (1:5000 dilution; ab8266; Abcam) as a loading control. Membranes were then washed three times with TBST for 15 minutes each and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000 dilution; anti-rabbit IgG HRP; Cell Signaling Technology) for 1 hour at room temperature. Finally, the membranes were washed again three times with TBST and developed using ECL reagents (Thermo). Protein bands were visualized using a ChemiDoc and Image Lab system (Bio-Rad).

 doi:10.20944/preprints202504.1855.v1

2.3. Inhibitors and Viability Assay

The mTOR inhibitors CCI-779 (10–20 μM; Selleckchem) and RAD001 (10–20 μM; Sellekchem) were dissolved in DMSO and diluted in culture medium immediately before treatment. Cells were adjusted to phenol red-free RPMI with 5% charcoal-stripped FBS and 1% Pen/Strep three days before the experiment. The concentration of DHT was fixed at 10nM. Enzalutamide was applied in a dose-dependent manner.

2.4. GSEA Analysis

The Hallmark gene sets (H) from the Molecular Signatures Database (MSigDB; http://www.broad.mit.edu/gsea/) were used for GSEA analysis. The GSEA software, version 4.3.2[29], was employed for the enrichment plot, significant gene list and heatmap analysis. The significance threshold for enrichment or depletion was set at a nominal p-value < 0.05, and the results were further adjusted for multiple testing using the False Discovery Rate (FDR) method[30]. Cell line gene expression data for PC3, DU145, LNCap, and 22rv1 were obtained from the Cancer Cell Line Encyclopedia(CCLE) database (CCLE_RNAseq_genes_counts_20180929; https://depmap.org/portal/download/all). Four prostate cancer cells were analyzed in one and three, and one and two ways for variable pairings.

2.5. MTT Assay

Equal density groups of cells were seeded on 96wells plates. mTOR inhibitor Temsirolimus [CCI-779] and Everolimus [RAD001] were treated with serial dilutions for 24~72 hours. DMSO treatment set up as vehicle control. The effect of inhibitors on cell viability and growth were evaluated using the MTTassay (Sigma-Aldrich). Absorbance was measured at a 550nm/wavelength with the microplate reader (Bio-rad). 1x10(4) cells were seeded in 96 well plates before serial diluted inhibitors were treated.

2.6. Statistical Analysis

Data analysis was performed, and bar graphs were generated using GraphPad Prism 10 software (Boston, MA, USA). Data were analyzed by one-way analysis of variance (ANOVA) or two-way ANOVA with Bonferroni post-hoc tests. Statistical significance is shown in the figure legends. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

3. Results

3.1. Differences in Proliferation Ability and Protein Expression in Four Prostate Cancer Cell Lines

We examined growth rates to understand the basic characteristics of commonly used prostate cancer cell lines (Figure 1a). In the hormone-independent prostate cancer cell lines DU145 and PC3, proliferation rates were two to three times higher than in the hormone-dependent lines LNCaP and 22Rv1 after 72 hours in normal culture medium. Androgen receptor (AR) expression was detectable in LNCaP and 22Rv1 cells, but not in DU145 and PC3 cells (Figure 1b). The transcript variant ARv7 (AR-V3) was only detected in 22Rv1. AR was detectable in the hormone-dependent lines LNCaP and 22Rv1, which require androgens for proliferation, but not in the hormone-independent lines DU145 and PC3, which can proliferate without androgen stimulation. Since the PI3K/mTOR/AKT pathway is known to be involved in the aggressive behavior of prostate cancer cells, such as cell proliferation and metastasis, we also examined the levels of mTOR, p-mTOR, and PTEN expression in four cell lines. As shown in Figure 1b, mTOR and p-mTOR were detected in all prostate cancer cell lines. In contrast, PTEN expression was detectable in DU145 and 22Rv1, while it was almost undetectable in PC3 and LNCaP cell lines.

 doi:10.20944/preprints202504.1855.v1

3.2. Dose-Dependent Sensitivity to Androgen Receptor Inhibition in Four Prostate Cancer Cell Lines

To test sensitivity to the AR inhibitor in four prostate cancer cell lines, we used different doses of enzalutamide, a non-steroidal androgen receptor inhibitor, in a cell proliferation assay. Cells were seeded in phenol red-free RPMI containing 5% charcoal-stripped hormone-free FBS and cultured for three days, and viable cells were counted. Different doses of enzalutamide were administered on day 0 with 10 nM DHT (dihydrotestosterone) as a positive control. Cells were harvested and counted after 72 hours. Regardless of the dose of enzalutamide, PC3 and DU145 grew rapidly in hormone-free medium (Figure 2a, 2b). The LNCaP cells, which express normal levels of androgen receptor, show sensitivity to enzalutamide starting at 1 μM concentration (Figure 2c). Despite the expression of wild-type androgen receptor, 22Rv1 was insensitive to enzalutamide up to the concentration of 1μM, probably due to the ARv7-dependent escape mechanism (Figure 2d).

3.3. Dose-Dependent Sensitivity to mTOR Inhibition in Four Prostate Cancer Cell Lines Treated with CCI-779

To determine the sensitivity to mTOR inhibition in four cell lines, we first compared the sensitivity to the mTOR inhibitor CCI-779 for mTOR phosphorylation. As shown in Figure 3a, all four cell lines were sensitive to CCI-779 in terms of mTOR phosphorylation. Among the cell lines, LNCap cell line was less sensitive to CCI-779 for mTOR phosphorylation compared to the other cell lines. In cell viability assay, all four cell lines showed sensitivity to CCI-779 starting from 1nM concentration as measured by cell viability at 72 hours after drug treatment (Figure 3b).

3.4. The Effect of Combination Treatment with CCI-779 and Enzalutamide on the Growth of Different Prostate Cancer Cell Lines

To test whether combined treatment of mTOR inhibitor and androgen receptor inhibitor cause synergistic effect on the prostate cancer cell lines, we treated four different cell lines with suboptimal dose of CCI-779 (1 nM) and enzalutamide (100 nM). The results show that the synergistic effect of the combined treatment was evident only in the LNCap cell line, which has a functional androgen receptor. The synergistic effect of combined treatment on cell proliferation was not evident in other three cell lines (Figure 4).

3.5. The Characteristics of Gene Expression Profile of LNCap Cell Line and Metastatic Prostate Cancer Cell Lines

To test whether the cell line showing the synergistic effect in the combined treatment of mTOR inhibitor and androgen receptor inhibitor has a characteristic gene expression profile, we performed gene set enrichment analysis using the RNAseq database of the four prostate cancer cell lines. As shown in Figure 5, the LNCaP cell line has a higher representation of the ANDROGEN RESPONSE gene set in addition to the OXIDATIVE PHOSPHRYLATION and WNT BETA CATENIN SIGNALING gene sets. The first 30 genes from each gene set are shown in Figure 5. We believe that some of these genes are very attractive potential drug targets, which will be discussed later in the discussion session. We also compared the gene expression profiles of the DU145 and PC3 metastatic malignant prostate cancer cell lines with those of the LNCaP and 22rv1 cell lines, which are known to be more benign than the DU145 and PC3 cell lines, to find any specific gene expression profile(s) that may be responsible for the aggressive behavior of the cell lines. As shown in Figure 6, the EPITHELIAL TO MESENCHYMAL TRANSITION and IL6-JAK-STAT3 PATHWAY gene sets are significantly enriched in both DU145 and PC3 compared to the LNCap and 22Rv1 cell lines. On the other hand, the OXYDATIVE PHOSPHORYLATION and PI3K-AKT-MTOR SIGNALING gene sets are significantly less represented, which partly explains the unresponsiveness to mTOR inhibitors in these cell lines.

 doi:10.20944/preprints202504.1855.v1

4. Discussion

Although long-term androgen deprivation therapy (ADT) has been the first choice of prostate cancer treatment with fairly successful results in the clinic, hormone-refractory cells arise, leading to castration-resistant prostate cancer (CRPC) in many patients. Although drug combination strategies of combining ADT and chemotherapy brought some success in treating CRPC patients[3-5], the success rate is still less than adequate, indicating that the better understanding of the biological mechanism of drug resistance and malignancy of cancer cells.

Here we investigated the sensitivity of four different prostate cancer cell lines to combination treatment with CCI-779 and enzalutamide. CCI-779, called Temsirolimus. In this experiment, we observed a synergistic effect of the combination treatment only in the LNCaP cell line. We included PC3 and Du-145 cell lines in this assay because there is some uncertainty about the complete absence of androgen receptor[13,14]. The 22Rv1 cell line expresses androgen receptor, although at lower levels than the LNCaP cell line, as shown in Figure 1.

Since LNCaP is the only cell line showing the significant synergistic effect after combination treatment with CCI-791 and enzalutamide (Figure 3), we further analyzed and compared the gene expression profile of the LNCaP cell line with the rest of the cell lines to characterize any unique gene expression patterns. Three gene sets, Androgen Response Gene Set, Oxidative Phosphorylation Gene Set, and Beta Catenin Signaling Gene Set are the gene sets that are significantly upregulated compared to the rest of the cell line. The increased representation of the Androgen Response Gene Set in the LNCaP cell line is not surprising given the high level of androgen protein expression and sensitivity to enzalutamide treatment. The MAF gene is the most highly expressed in this gene set. The MAF gene is involved in driving the cells to a malignant phenotype, making the molecule interesting as a potential drug target[15,16]. In addition to MAF, the BMPR1B gene is also involved in cancer maintenance as recently reported[17,18]. The BMPR1B protein molecule is located on the plasma membrane, making the molecule more valuable as a potential drug target. The increased representation of the oxidative phosphorylation gene set in LNCaP may indicate that the cell line uses the aerobic glycolysis pathway less than the highly malignant cells such as PC3 and DU-145. Interestingly, the LNCaP cell line also shows increased expression of the BETA CATENIN SIGNALING PATHWAY gene set. As research on the development of novel drugs targeting the BETA CATENIN SIGNALING PATHWAY is currently progressing, the treatment of prostate cancers with these drugs may be another option in the future[19,20].

The gene set enrichment analysis with the malignant (PC3 and DU145) versus benign (LNCaP and 22Rv1) prostate cancer cell lines, shown in Figure 6, revealed that the malignant cell lines show enhanced representation of the EPITHELIAL TO MESENCHYMAL TRANSITION (EMT) and IL-6/JAK STAT3 PATHWAY gene sets. The EMT gene set has been the target of intense research for the development of novel drugs for cancer therapy[21,22]. From the list of genes among the top 30 in the list (Supplementary Table 1, 2), MMP14[23], BASP[24,25], SDC[26] are membrane proteins involved in matrix degradation and metastasis. CD59[27] is another membrane molecule that suppresses complement activation on the cellular plasma membrane. These are all membrane proteins that are accessible to the antibody, making them better drug targets. The increased representation of the IL-6/JAK STAT3 PATHWAY gene set in malignant cell lines is not surprising as this pathway plays an important role in cell cycle progression and cell proliferation.

The malignant cell lines also have less representation of the gene sets OXIDATIVE PHOSPHORYLATION and PI3K/MTOR AKT PATHWAY. The down regulation of the OXIDATIVE PHOSPHORYLATION gene set probably indicates that aerobic glycolysis is active in these cell lines. The down regulation of the PI3K/MTOR AKT PATHWAY gene set in malignant cell lines is unexpected since the PI3K/MTOR AKT PATHWAY has been known to play important role in cell proliferation and cell surviva[28].

In conclusion, we showed that comparing the cancer cell lines with different characteristics from the same tissue origin both in terms of cellular behavior and gene expression profiles generates important information that are useful in drug development through novel drug targets and in

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designing an appropriate combination therapy avoiding the potential resistance to the particular set of drugs.

5. Conclusion

We showed that comparing the cancer cell lines with different characteristics from the same tissue origin both in terms of cellular behavior and gene expression profiles generates important information that are useful in drug development through novel drug targets and in designing an appropriate combination therapy avoiding the potential resistance to the particular set of drugs.

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