Exploration and Identification of Potential Therapeutic Targets and Biomarkers for Docetaxel Resistant Prostate Cancer

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Research Article

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Abstract

Background

Prostate cancer has become the third most common cancer, and the death rate of advanced patients due to metastasis and invasion is high. Approximately 40–50% of castration resistant prostate cancer responding to docetaxel did not show a substantial and sustained prostate specific antigen decline, and the median response duration was limited to 6 to 9 months. To understand the pathogenesis of docetaxel-resistant prostate cancer (DRPC), and to search for prognostic markers and new therapeutic targets.

Methods

RNA sequencing data of GSE36135 and GSE33455 from the Gene Expression Synthesis Database were used to search for co-expressed genes. Univariate and multivariate Cox regression analyses were performed to identify independent prognostic factors, which were validated by expression and survival analyses. Subsequently, their ability to jointly predict patient prognosis was evaluated. Then, the lasso Cox regression model was established to evaluate the correlation of DEGs with immune score, ferroptosis, methylation, and OCLR score. And predicted for targeted small molecule drugs and validated the effect of small molecule drugs.

Results

EZH2 and SRC were identified as potential therapeutic targets and effective prognostic markers for docetaxel-resistant prostate cancer, and were found to be significantly associated with the immune score, ferroptosis, methylation, and OCLR score. Targeted small molecule drugs were predicted and validated for EZH2 and SRC.

Conclusions

This study helps to fully explain the mechanism of docetaxel-resistant prostate cancer formation and provides new immune-related therapeutic targets and biomarkers for it. Preliminary exploration of the effects of docetaxel in combination with urapidil or roxithromycin in prostate cancer cells

Introduction

In 2020 Global Cancer Statistics, prostate cancer has become the third most common cancer, accounting for 7.3% of new cancers, after breast cancer and lung cancer, and its mortality rate accounts for about 7% of cancer-related deaths in men worldwide^[1]. The standard treatment for metastatic prostate cancer is endocrine therapy, but it develops into castration resistant prostate cancer (CRPC) after 18 to 24 months

of treatment^[2, 3]. Docetaxel is a taxane antimitotic drug that binds to β -tubulin and stabilizes microtubule assembly, which inhibits spindle formation, inhibits cell mitosis, and promotes cell apoptosis^[3, 4, 5]. In further studies, docetaxel induces phosphorylation of Bcl-2, which leads to apoptosis of cancer cells and tumor regression^[6, 7]. Docetaxel inhibits the transcriptional activity of androgen receptor(AR) by promoting the binding of forkhead box protein O1(FOXO1) to AR in the nucleus of prostate cancer cell line 22RV1. Down-regulating the expression of AR and prostate-specific antigen (PSA), which promotes the apoptosis of tumor cells^[7, 8].

Since 2004, docetaxel combined with prednisone has been the standard treatment for CRPC because of its ability to improve overall survival in CRPC patients^[3, 4]. However, lots of patients develop resistance to docetaxel after treatment due to a variety of factors, such as tubulin- β 3 abnormal expression^[9], over-expression of P-glycoprotein^[10], phosphatidylinositol 3-kinase / protein kinase B / mammalian target of rapamycin signaling pathway activation^[11, 12], abnormal activation of hedgehog signaling pathway^[13], epithelial-mesenchymal transformation^[14], AR abnormal transcription^[15], and abnormal expression of glucocorticoid receptor^[16]. Acquired resistance to docetaxel is usually fatal due to the limited availability of effective therapies in this condition. Currently, the biomarkers and prognostic indicators for docetaxel-resistant prostate cancer (DRPC) are not clear, and the mechanism of DRPC still needs to be further clarified.

In this study, we identified co-expressed genes in DRPC by comprehensive bioinformatics analysis of GSE33455^[17] and GSE36135^[18], screened potential target genes, and established independent prognostic genes by univariate and multifactorial Cox regression analysis, analysis of variance, and survival analysis. Finally, prognostic genes were assessed for their correlation with immune infiltration and predicted targeting small molecule drugs. This study will help us to gain insight into the molecular mechanisms, therapeutic approaches and prognostic biomarkers of DRPC.

Materials And Methods

Microarray Data Analysis and Screening of DEGs

To compare differential expressed genes (DEGs) in docetaxel-resistant (DR) and docetaxel-sensitive (DS) prostate cancers, the GSE36135 and GSE33455 dataset were selected for subsequent analyses (Supplement Table 1). DEGs were screened using the limma package in the R software^[19]. The cut-of conditions were set to an adjusted P-value < 0.05, and the absolute value of log-fold change $|\log_2FC| \geq 1$ as statistically significant for the DEGs. Use ImageGP to create volcano maps and venn maps online.

Functional Enrichment Analysis of DEGs

Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of DEGs, including biological process (BP), cellular component (CC), molecular function (MF), was performed by ClusterProfiler software package^[20–21] to explore functional annotation and enrichment pathways.

Identification of Independent Prognostic Genes

Independent prognostic factors were clarified by univariate and multifactorial Cox regression analyses and further validated by differential expression and survival analyses through survival Package^[22].

Construction and validation of the DEGs-related prognostic model

Multivariate Cox regression analysis was performed to construct a prediction model based on DEGs, and EZH2 and SRC were independent prognostic factors. Signatures were established based on the coefficients corresponding to independent prognostic genes. Patients from TCGA-PRAD dataset were divided into low and high risk groups weighted by the risk score obtained from the multivariate Cox regression. t-distributed stochastic neighbor embedding(t-SNE) and principal-component analysis (PCA) were used to explore the distribution characteristics of different groups by R packages. Finally, the effectiveness of prognostic indicators was evaluated by the area under curve (AUC) of "time receiver operating characteristic curve (ROC)".

Construction of the Nomogram

Nomogram and calibration curves were obtained and independent prognostic hub genes for 3, 5 and 7 year OS were evaluated.

Assessment of the Immune Microenvironment in DRPC

Relationship between DEGs and immune microenvironment

Using xCell algorithm in "immunedeconv" R package, the relationship between DEGs expression levels and immune cells and immune score was analyzed, and the effects of gene expression levels on 8 immune checkpoint-related genes were analyzed in "ggplot2" R package^[23].

OCLR Scores of Independent Prognostic Hub Genes

Tumor-associated RNA-seq data were obtained from TCGA-PRAD, mRNAs was calculated by OCLR algorithm, and the dryness index was obtained.

Immunohistochemistry Staining of Independent Prognostic Hub Genes

Immunochemical staining of SRC and EZH2 in normal kidney tissues and prostate cancer and their distribution in different prostate cells obtained by HPA (www.proteinatlas.org).

Relationship between methylation and ferroptosis with DEGs

The third-order RNA sequencing data of genes were obtained based on TCGA data set, and analyzed association with ferroptosis-related genes and m6A-related genes in "ggplot2" R package.

Screening of Small Molecule Therapeutic Drugs

The independent prognostic hub genes were used for drug prediction in Connectivity Map (Cmap), which is currently the most comprehensive transcriptome database for drug interference therapy and is commonly used to explore potential drugs for the treatment of diseases. Therefore, enrichment < 0.7 and $P < 0.01$ were used for screening. The PubChem22 database was used to retrieve the molecular structure of identified drugs.

Cell Culture, Drug IC₅₀ and CCK8 Experiments

DU145 was grown in RPMI medium 1640 basic containing 10% FBS. Cells were maintained in an incubator at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. The 96 empty plates were spread at a concentration of 10000/ml and left for 24h. The target drugs were added separately and the absorbance was measured after 48h. The data were then analyzed to obtain the IC₅₀ of each drug. Then the drugs were combined two by two and the absorbance was measured after 24h, 48h and 96h of addition, respectively.

Statistical Analysis

The statistical analysis of this study were carried out automatically by the relevant database. When $p < 0.05$ or log rank $p < 0.05$, the difference was statistically significant.

Results

Identification of DEGs

By analyzing the GEO database of DRPC (Figs. 1A-B), 439 DEGs were obtained in GSE36135, including 184 up genes and 255 down genes. In GSE33455, 708 DEGs were obtained, including 243 up genes and 465 down genes. Venn diagram obtained by ImageGP showed that the two GSE databases finally obtained 33 co-expressed DEGs, including 10 up genes and 23 down genes (Figs. 2C-D). To further explore the biological functions of DEGs in DRPC, GO and KEGG enrichment analysis revealed that the DEGs were mainly enriched in "ErbB signaling pathway", "ERBB2 signaling pathway" and "cell-cell junction" (Fig. 2).

Identification of Independent Prognostic Genes

By univariate and multifactorial Cox regression analysis, it was clear that SRC and EZH2 were independent prognostic factors (Figs. 3A-B). Using the TCGA-PRAD database, we verified the difference in expression of SRC and EZH2 in paraneoplastic and carcinoma samples and found that EZH2 and SRC was up-regulated in PRAD (Figs. 4A-B). By Overall Survival (OS), Disease specific Survival (DFS) and

Progress Free Interval (PFU) analysis, high expression of SRC and EZH2 was found to be associated with poor prognosis of patients (Figs. 4C-H).

Construction and validation of the DEGs prognostic risk model

Use lasso cox regression to construct a prognostic model of DEGs-related risks, Risk Score= $(0.824)*EZH2+(1.3857)*SRC$ (Fig. 5A, Fig. 5B). According to the median risk score (50%), patients were divided into high-risk and low-risk groups. It can be seen in the t-SNE and PCA heat maps that EZH2 and SRC are highly expressed in the high-risk group (Fig. 5C). HR = 4.38, the prognosis model can be considered as a risk factor model. The median survival time of the high-risk group was significantly lower than that of the low-risk group (Fig. 5C). We used ROC to evaluate the prognostic prediction efficiency of the model, and the results showed that the AUC was 1 (1-year OS), 0.838 (3-year OS), and 0.8 (5-year OS) (Fig. 5C).

The construction nomogram

Subsequently, the potential predictive value of PRAD patient age, PAS, residual tumor, Gleason score, SRC and EZH2 levels for OS at 3-, 5- and 7- years was predicted by nomogram with C-index of 0.844 (0.771–0.918) and $p = 0.017$ (Figs. 6A). Finally, the assessment by calibration curve suggested the combined prediction probability of SRC and EZH2 was in good agreement with the calibration curve and could better evaluate the OS of patients (Figs. 6B).

Correlation between risk score of DEGs-related risks prognostic model and immune score

To further investigate the relationship between the DEGs-related risks prognostic model and expression levels at the immune cell level, Spearman correlation analysis was made between risk score and immune score. The results(Fig. 7) showed that the risk score was significantly associated with expression of B cell ($p = 2.56*e-08$), macrophage cell ($p = 1.33*e-04$), NK cell ($p = 1.88*e-07$), Myeloid cell ($p = 0.010$), uncharacterized cell ($p = 0.002$) and T cell CD4+ ($p = 7.73*e-07$).

Correlation between the expression of immune infiltrating cells in PRAD tissues and DEGs

The PRAD population in the TCGA-PRAD database was divided into DEGs low expression group (G1) and DEGs high expression group (G2), and the correlation between the expression of DEGs and immune-infiltrating cells was analyzed. The results show that EZH2 and SRC were highly correlated with the expression levels of a variety of immune infiltrating cells, and the expression levels of macrophage, T cell CD4 + Th2 and B cell plasma are significantly correlated with EZH2 and SRC. It suggests that these cells may be related to the progression of PRAD (Fig. 8).

OCLE Scores and Immunohistochemistry Staining of Independent Prognostic Genes

Through dryness index, it was found that there were significant differences in dryness degree between hub genes with different expression levels and PRAD (Fig. 9A-B). These results suggested that SRC and EZH2 might affect the degree of similarity between PRAD cells and stem cells, thereby affecting tumor biological processes and degree of dedifferentiation. Then, spearman correlation analysis revealed a significant association between SRC and EZH2 ($r = 0.200$, $p < 0.001$) (Fig. 9C). HPA database was used to compare hub genes expression differences between normal prostate and PRAD tissue of similar age male patients (Supplement Table 2). The results showed that SRC was high expressed (Fig. 9D-E) and EZH2 was highly expressed in PRAD (Fig. 9G-H). This conclusion was consistent with the mRNA expression results obtained in TCGA. Finally, SRC was also found to be highly expressed in urothelial cells, glandular cells and macrophages (Fig. 9F), while EZH2 was mainly highly expressed in macrophages, T-cells and fibroblasts (Fig. 9I) in the prostate tissue by HPA database.

Relationship between methylation, ferroptosis and expression of DEGs

We analyzed the correlation between m6A methylation-related genes and DEGs and found that EZH2 and SRC were significantly correlated with multiple methylated genes (Fig. 10). In particular, METTL3, HNRNPA2B1, RBM15B, HNRNPC, RBMX, VIRMA, YTHDF3, FTO, YTHDC2, YTHDC1, RBM15, YTHDF1 and YTHDF2 were significantly associated with both EZH2 and SRC ($p < 0.05$).

The results in Fig. 11 show that the expression of DEGs is correlated with the expression levels of multiple ferroptosis related genes.

Results of Enrichment Analysis and Small Molecule Therapeutic Drugs

In the gene database of DRPC (GSE36346, GSE33455), we collated the co-expressed gene probes of mRNAs (EZH2, SRC). The Cmap database was used to import probes to obtain 4 potential treatments for DRPC, namely atropine, urapidil, tolafenamic acid and roxithromycin (Figs. 12).

Targeted Small Molecule Drug Experiments

The IC_{50} of docetaxel, roxithromycin and uradil in DU145 cells was measured by IC_{50} assay as 130 nM, 63.73 μ M and 75.07 μ M, respectively (Figure 13). subsequently, it was suggested that docetaxel combined with roxithromycin or uradil could enhance the inhibitory effect on cells to some extent in docetaxel combined with roxithromycin or uradil CCK8 assay alone. It is suggested that roxithromycin and uradil can be used as adjuvants of docetaxel or even as immunotherapy to enhance the efficacy of the drug.

Discussion

Prostate cancer has become the third most common cancer, with a mortality rate of about 7% of the world's male cancer-related deaths^[1]. Patients in the early stage have a good prognosis^[24], while patients in the late stage have a high mortality rate due to metastasis and invasion^[25]. Approximately 40–50% of CRPC do not show substantive and sustained PSA decreases in response to a taxane and the median duration of response is limited at 6 to 9 months^[3, 4]. Therefore, this study identified potential genes affecting DRPC formation and predicts therapeutic targets, biomarkers and targeted small analysis drugs through comprehensive bioinformatics analysis, which is expected to provide new ideas and insights for the treatment of DRPC.

EZH2 is an important component of polycomb-repressive complex 2 (PRC2), and its increased expression is related to the occurrence of CRCP^[26]. It has been found that EZH2 can interact with AR methylation to provide survival signal for CRPC cells^[26]. Xiaofu Qiu et al discovered that EZH2 is a necessary condition to cause docetaxel resistance in PC cells^[27]. Current mechanisms have shown that miR-124, miR-101-3p and miR-185-5p inhibit the development and drug resistance of prostate cancer by down-regulating EZH2, SRC, and AR splice patterns^[27, 28]. SRC, a tyrosine kinase, regulates complex signaling networks that drive the development of castration resistance and bone metastasis^[29]. Studies have shown that the over-expression of AR and SRC in naive prostate epithelia increases the activity of SRC, thus inducing the transformation to invasive adenocarcinoma^[30]. In DRPC, the use of a SRC multikinase inhibitor, dasatinib, did not improve efficacy^[31].

As an inert immunogenic tumor that produces a complete antitumor immune response in sufficient time^[32], prostate cancer has made significant advances in the treatment of both cancer vaccines and checkpoint inhibitors in prostate cancer^[33]. In this study, SRC and EZH2 were identified as potential immunotherapeutic targets for docetaxel-resistant prostate cancer, with significant association with immune cell infiltration. At the level of bioinformatic analysis SRC and EZH2 showed significant responses to immune cell infiltration, ferroptosis, methylation, and OCLR score. Available studies suggested that SRC tyrosine kinase inhibitors (STKIs), such as dasatinib and saracatinib, had been employed in the treatment of castration-resistant or bone metastatic prostate cancer^[34~35]. While targeted agents against EZH2 have shown promising antitumor effects in cellular assays, in vivo preclinical models and patient clinical trials, e.g. Tazemetostat^[36] and GSK126^[37] have been shown to improve poor prognosis in patients with CRPC. With the rise of immune checkpoint blocker therapies such as anti-CTLA4 antibody, anti-PD1 (PDCD1) antibody and anti-PDL1 (CD274) antibody^[38], the combination of SRC and EZH2-targeted drugs will play a key role in DRPC.

Interestingly, using the independent prognostic hub genes EZH2 and SRC, we identified four drugs for the treatment of DRPC: eatropine, urapidil, tolfenamic acid and roxithromycin. This study focused on exploring the role of urapidil and roxithromycin in combination with docetaxel in prostate cancer. Urapidil, as an α -2 adrenergic receptor antagonist, can be used in hypertrophic prostate adenoma^[39] and

pancreatic cancer^[40]; Roxithromycin, as a macrolide antibiotic, can induce the activation of nuclear factor kappaB by inhibiting the production of VEGF, thereby inhibiting the proliferation of human hepatocellular carcinoma cells^[41–42]. The IC50 of docetaxel, roxithromycin and uradil were 130nM, 63.73uM, 75.07uM, respectively, as measured by the IC50 assay in RM1 cells. The combination CCK8 trial suggested that docetaxel combined with roxithromycin or uradil could enhance the inhibitory effect on cells to some extent. These results suggest that roxithromycin and uradil can be used as adjuvants to docetaxel and even immunotherapy to enhance the effect of the drug.

Conclusion

Based on a comprehensive bioinformatics analysis, this study successfully predicted that SRC and EZH2 may be potential therapeutic targets and biomarkers for docetaxel-resistant prostate cancer. Subsequently, SRC and EZH2 were found to be significantly correlated with immune infiltration, which suggested their possible use as adjuvant targets for DRPC immunotherapy. Finally, small molecule drugs targeting SRC and EZH2 were predicted, and it was found that uradil and roxithromycin inhibited significantly in DU145 cells, and the combination with docetaxel enhanced their inhibitory effect. This study provides new ideas for therapeutic targets of DRPC, biomarkers, and its adjuvant drugs such as immunotherapy.

Abbreviations

castration resistant prostate cancer (CRPC)

forkhead box protein O1 (FOXO1)

androgen receptor (AR)

prostate-specific antigen (PSA)

docetaxel-resistant prostate cancer (DRPC)

differential expressed genes (DEGs)

Gene ontology (GO)

Kyoto Encyclopedia of Genes and Genomes (KEGG)

biological process (BP)

cellular component (CC)

molecular function (MF)

Declarations

Ethics approval and consent to participate: Our article is bioinformatics analysis. We do not need ethics approval and consent to participate

Consent for publication: All authors agree for publication.

Availability of data and material: Publicly available datasets were analyzed in this study. This data can be found here: <https://tcga.xenahubs.net>

Competing interests: There is no conflicts of interest.

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Supplement Table

Supplement Table 1 | The detailed information of the two datasets.

Dataset	Number of samples	Array types	Cell lines / Tissue
(Sensitive/Resistant)			
GSE36135	6 sensitive and 6 resistant samples	GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array GPL571 [HG-U133A_2] Affymetrix Human Genome U133A 2.0 Array	DU145, 22Rv1
GSE33455	6 sensitive and 6 resistant samples	GPL570[HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array	DU145, PC3

Supplement Table 2 | Basic immunohistochemical information.

Hub DEGs	Tissue types	Age	Gender	Antibody
SRC	Normal tissue	51	Male	CAB004023
SRC	Adenocarcinoma	53	Male	CAB004023
EZH2	Normal tissue	76	Male	CAB009589
EZH2	Adenocarcinoma	73	Male	CAB009589

Figures

Figure 1

Screening of differential expressed genes. (A, B) Volcano maps of the GSE33455 and GSE36135 dataset. (C, D) Common up-regulated and down-regulated DEGs in the GSE33455 and GSE36135 datasets. (E, F) Results of GO enrichment analysis of the DEGs.

Figure 2

Results of GO and KEGG enrichment analysis of the DEGs.

Figure 3

Independent prognostic factors were identified. (A, B) Univariate and multivariate analyses of DEGs.

Figure 4

Expression analysis of independent prognostic genes, including EZH2 (A) and SRC (B). Overall Survival analysis of independent prognostic genes, including EZH2 (C) and SRC (D). Disease specific Survival analysis of EZH2 (E) and SRC (F). Progress Free Interval analysis of EZH2 (G) and SRC (H). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Figure 5

Construction of a prognostic model for the risks associated with DEGs

The calculations for the model according to the multivariate Cox regression analyses (A-B). The prognostic model was analyzed by survival time, survival status, target gene expression heat map, and 1/3/5-year overall survival (C). $\lambda_{\min}=0.0022$. $\text{Risk score}=(0.8243)*\text{EZZH2}+(1.3857)*\text{SRC}$

A

B

Figure 6

Nomogram for predicting 3-, 5-, and 7-year OS in entire TCGA cohort (A). Calibration curves of nomogram on consistency between predicted and observed 3-, 5- and 7-year OS in entire TCGA cohort (B).

Figure 7

Spearman correlation analysis between risk score and immune score

A

B

Figure 8

The difference of expression of immune infiltration cells in PRAD tissues with high and low EH2 (A) and SRC (B) gene expression. G1 is a low expression group and G2 is a high expression group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Figure 9

OCLR scores and expression validation of independent prognostic DEGs.

The OCLR scores of low vs high expression of SRC (A) and EZH2 (B). (C) Correlation between SRC and EZH2. Immunohistochemistry of SRC and EZH2 (E is normal^{SRC}, D is cancer^{SRC}, G is normal^{EZH2} and H is

cancer^{EZH2}. Expression of SRC (F) and EZH2 (I) in different cells of prostate tissue. *p<0.05, **p<0.01, ***p<0.001

Figure 10

The difference of expression of methylation of m6A related genes in PRAD tissues with high and low EZH2 (A) and SRC (B) gene expression. G1 is a low expression group and G2 is a high expression group.

*p<0.05, **p<0.01, ***p<0.001

A

B

Figure 11

The difference of expression of ferroptosis related genes in PRAD tissues with low and high EZH2 (A) and SRC (B) gene expression. G1 is a high expression group and G2 is a lowexpression group. *p<0.05, **p<0.01, ***p<0.001

Figure 12

Prediction results of potential small molecule drugs for the treatment of docetaxel-resistant prostatic adenocarcinoma. Prediction results of targeted drugs, including atropine, urapidil, tolfenamic acid, roxithromycin.

Figure 13

IC₅₀ and CCK8 for key small molecule drugs. (A) Docetaxel. (B) Roxithromycin. (C) Urapidil. (D) CCK8 trial of combination drugs.