TP53 Inhibited AR Signaling Promote Enzalutamide Resistance in LNCaP cell

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Abstract

Background: To perform gene screening and search key knockout gene associated with enzalutamide resistance in LNCaP cells.

Methods: Genome-scale CRISPR-Cas9 transfection were done to screen genes associated with enzalutamide resistance in LNCaP cells. Bioinformatic analysis of TP53 and AR expression correlated with prostate adenocarcinoma by using TCGA database. Knockout interfering RNA (shRNA) were synthesized in lentivirus and transfected into LNCaP cells by using Lipofectamine 3000. TP53 and AR detection was performed by using conventional molecular biology methods, such as western blot, RT-PCR and flow cytometer et al.

Results: 27 high ($NES < -1.7$, $P < 0.01$) and 26 low ($NES > 1.7$, $P < 0.01$) expressed genes were identified in enzalutamide resistance Cells. TP53 interacted with AR may play an important role in the development of enzalutamide resistance from PPI and RNA-seq data. Data from TCGA support that patient with TP53&AR low expression endured higher pathology stage, Gleason grades, more disease progress, recurrence, positive lymphnodes examination numbers and more abnormal bones scan results. LNCaP cell dealt with enzalutamide or with TP53 knockout showed inhibited cell cycle at G0~G1, increased apoptosis percentage and also inhibited cell proliferation.

Conclusions: Current data support that TP53 protein inhabiting AR signaling promote enzalutamide resistance in LNCaP cell.

Background

According to 2015 cancer prevalence statistical in China, the incidence and mortality of prostate (PCa) were 60.3/100,000 and 26.6/100,000 respectively. The largest increase in incidence was seen for prostate cancers in men [1]. With the development of therapy drug, the overall survival of PCa increased, but the mortality has not significantly decline because of metastatic castration-resistant PCa (mCRPC) [2]. With mCRPC, the cancer stops completely responding to hormone treatments, and more importantly, it can spread to nearby lymph nodes, bones, et al [3]. Regarding the mechanism behind mCRPC, numerous researches have highlighted the role of androgen receptor (AR), showing that despite systemic androgen depletion, AR signaling remains active and support the survival and growth of PCa cells [4]. Therefore, novel anti-mCRPC agent, enzalutamide has been developed and applicated in clinical therapy. It aims to inhibit nuclear translocation, chromatin binding, and interactions with AR coregulators[5]. Results from clinical trials indicated that enzalutamide prolongs the survival and increases overall and progression free survival and delays the need for chemotherapy [6]. Even enzalutamide significantly improved patient survival, among the most common adverse events, seizures were reported in 0.6% of patients receiving enzalutamide therapy [5]. Still, the survival benefits of enzalutamide were achieved in only up to 50% of PCa patients, and patients who initially responded eventually developed resistance [7].

With the development of genome-scale CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats-CRISPR associated nuclease 9) knockout screening in human cells, researchers got alternative approach to illuminate the mechanism of enzalutamide resistance in mCRPC [8]. Such technology uses a single-guide RNA (sgRNA) to direct nuclease Cas9 that give rise to DNA editing. Applications of CRISPR-Cas9 in cancer research can quickly done cancer genetic research in whole genome scale [9]. In this study, we aim to screen genes associated with enzalutamide resistance in LNCaP cells by using genome-scale CRISPR-Cas9 methods, which may provide alternative perspective for mCRPC therapy.

Methods

LNCaP cell culturing and drug screening

Human LNCaP clone FGC cell lines were purchased from the Shanghai (Zhong Qiao Xin Zhou Biotechnology Co., Ltd, Lot#ZQ0039). LNCaP cell was cultured in 1640 medium supplemented with 10% FBS and 1% Glutamax (USA, Invitrogen, Lot#35050061). Cells were grown at 37°C in a humidified atmosphere containing 5%CO₂. Enzalutamide (MDV3100, Lot# PHB00235-1G) was obtained from Merk (USA, <https://www.sigmaaldrich.com/>). Transplant LNCaP cells into a 96-well plate (8000 cells/well) and then divided into 6 groups. One group was treated DMSO, and the other five groups were treated with enzalutamide at final concentrations of 5, 10, 20, 50 and 100 µM for 7 days, with a drug change on the 4th day. After the processing of each group is completed, Cell Counting Kit-8 (CCK-8) assay was performed for testing cell proliferation. Knockout interfering RNA (shRNA) were synthesized (Sangon Biotech Shanghai Co., Ltd. China) and transfected into LNCaP cells by using Lipofectamine 3000 (Invitrogen, USA), according to the manufacturer's protocol. For TP53 knockout gene assessment, primers were as follows:

shRNA-1: 5'- UUGCGGAGAUUCUCUUACU -3',

shRNA-2: 5'- UUCUCUUACUCCACACGCA -3'

shRNA-3: 5'- AUUCUCUUACUCCACACGC -3'.

CRISPR Cas9 sgRNA library construction and sequencing.

sgRNA library plasmid (20,000 genes, 6 sgRNA targets for each gene) was obtained from Addgene (<http://www.addgene.org>). sgRNA library plasmid was directly packed with lentivirus and transfected with 293T cells with the aid of Lipofectamine 2000 reagent (ThermoFisher, Lot#11668027). After 48 and 72 hours of transfection, the virus was collected, filtered with a 0.45µm membrane, and then concentrated to 6ml. The concentrated virus was aliquoted and stored at -80°C. Current drug screening experiment was divided into Enzalutamide treated group and DMSO group, and each group contains 1x10⁷ LNCaP cells (two 15cm dishes). The enzalutamide group was treated with the pre-experimentally determined drug (20µM) for 6 days, and the solution was changed once every 3 days. The DMSO group was added with the same volume of DMSO as the enzalutamide group. The surviving cells will continue to expand and

culture after 6 days, and half of them will be frozen and stored, and the remaining half of the cells will be added to the respective dose of drugs. This cycle is repeated for 3 times. After that, cell genomic DNA was extracted with kit of DP304 (TIANGEN, Lot#DP304-02). Then, PCR and sequencing were done by using illumina hiseq 3000. We use the RNAi Gene Enrichment Ranking (RIGER) algorithm to analyze the enrichment score of each gene. There is a total of 10048 enriched genes which at least 3 different sgRNA targets are enriched.

Western blot and RT-PCR

Total protein was extracted by using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) following the instruction. After measuring protein concentrations, 12% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels were conducted and transferred to polyvinylidene fluoride (PVDF) membranes (MerckMillipore, Darmstadt, Germany). Antibody applicated in current investigation list as following: p53 (1C12) Mouse mAb #2524, dilution ratio 1:1000 in western blot and 1:2000 in immunofluorescence. Androgen Receptor (D6F11) XP® Rabbit mAb #5153, dilution ratio 1:2000 in western blot and 1:600 in immunofluorescence. Total RNA was extracted using the RNAiso plus reagent (Takara Bio, Dalian, China). Quantitative real-time PCR was performed using SYBR Green mix (Toyobo, Osaka, Japan) in a StepOnePlus Real-Time PCR System (Life Technologies, Carlsbad, CA). For RT-PCR, primers were as follows:

AR fwd: 5'-ATGGTGAGCAGAGTGCCCTATC-3',

AR rev: 5'-ATGGTCCCTGGCAGTCTCCAAA-3',

GAPDH fwd: 5'-GAAGGTGAAGGTCGGAGTC-3',

GAPDH rev: 5'-GAAGATGGTGATGGGATTTC-3',

TP53 fwd: 5'-GAGCTGAATGAGGCCTTGGA-3',

TP53 rev: 5'- CTGAGTCAGGCC CTTCTGTCTT-3'.

Cell proliferation, apoptosis and cell cycle

Cell proliferation was assessed with Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. The optical density (OD) values at the wavelength of 450nm was measured on an enzyme-linked immunosorbent assay plate reader (Varioskan Flash, Thermo Scientific, Waltham, MA). Apoptosis and cell cycle detection was analyzed by a flow cytometer (MoFlo, Beckman, CA, USA). Cells were harvested 48 h after lentivirus transfection and incubated with annexin V-FITC and PI according to the manufacturer's instructions (BD, San Diego, CA, USA).

Database sources and statistical

PPI network analysis was done by using STRING (<https://string-db.org/>). RNA-seq data of LNCaP cell treated with enzalutamide and DMSO were obtained from GEO publications (GSE137833). Pathway analyses was conducted by using methods of GSEA (<https://www.gsea-msigdb.org/gsea/index.jsp>). PRAD data was download form publica database of The Cancer Genome Atlas (TCGA) (<https://portal.gdc.cancer.gov/>). All statistical or figures was dealt with software of GraphPad Prism v5.0 or R platform (<https://www.r-project.org/>).

Results

Genome-wide CRISPR screening of enzalutamide resistance-related genes

Considering genome-wide loss-of-function screens have provided a wealth of information in diverse cell model systems[10]. First and foremost, it must investigate drug lethal concentration of enzalutamide in LNCaP cells. LNCaP cells were treated with 6 sub-levels of enzalutamide (**Figure 1A**), and 5 μ M~100 μ M concentration of enzalutamide can obviously inhibit the proliferation of LNCaP cells (**Figure 1B**). Concentration of 20 μ M, 50 μ M and 100 μ M can directly kill LNCaP cells, but only at 20 μ M cells can continue to grow and expand after withdrawal, so the final working concentration was selected at 20 μ M. Secondly, CRISPR library transduction and sequencing were performed for screening enrichment genes, brief experimental pipeline was descripted in **Figure 1C**. Lastly, after sequencing, extremely significant genes were present according to the p-value ranking of significant differences (**Figure 1D**). 27 high (NES<-1.7, P<0.01) and 26 low (NES>1.7, P<0.01) expressed genes were identified in enzalutamide resistance Cells (**Figure 1E**).

Low expressed of TP53 correlated with PRAD progress

In order to find key knockout protein attributed to the development of enzalutamide resistance in LNCaP cell, PPI network (**Figure 2A**) was conducted for searching interaction with AR signaling pathways (**Supplementary table 1**) by using STRING database. We found key protein TP53 interacted with AR, which play an important role in the development of enzalutamide resistance. In addition, by searching GEO publications (GSE137833) [11], we found the RNA-seq expression difference between LNCaP cell treated with enzalutamide (n=3) and DMSO (n=3). 183 genes low (FC<-2.8, p<0.05) and 3 genes (FC<-2.8, p>0.05) high expressed in LNCaP cell treated with enzalutamide (**Figure 2B**). These genes enriched mainly in pathway associated with protein localization, protein folding, lipid modification, et al (**Figure 2C**). More importantly, GSEA analysis indicated these genes focus on pathway of Down-regulated by androgen (**Figure 2D**, NES=-1.63, FDR q = 0.006) and Stabilization of P53 (**Figure 2E**, NES=-1.58, FDR q = 0.022), which most of involved genes were down regulated (**Figure 2F**). To further make clearly the role of TP53 in the development of PRAD, we download and analysis data from TCGA database. TP53 expression demonstrated no significant difference among cohorts, age, TNM pathology stages, Gleason grades, therapy outcomes, biochemical recurrence, bone scan and lymphnodes examinations (**Supplementary Figure 1A-K**), but lower expressed in Frameshift mutations compared other mutation types (**Supplementary Figure 1L**). At the same time, RNA-seq data of AR showed no significant

association with diverse clinical characterize (**Supplementary Figure 2**). However, we focused on patients with TP53 and AR low expression simultaneously. 39 patients with TP53&AR high expression (TP53>1000 RPKM & AR>800 RPKM) and 37 patients with TP53&AR low expression (TP53<1000 RPKM & AR<800 RPKM) The results showed that patients with TP53&AR low expression endured higher pathology stage (**Figure 2G**), higher Gleason grades (**Figure 2H**), more disease progress (**Figure 2I**), more recurrence (**Figure 2J**), and more positive lymphnodes examination numbers (**Figure 2K**) and more abnormal bones scan results (**Figure 2L**) compared with patients with TP53&AR high expression.

Validation TP53 low expressed in LNCaP cell dealt with 20μM enzalutamide

To validate TP53 and AR protein expression in LNCaP cell treated with enzalutamide, immunofluorescence staining was performed. Double immunofluorescence staining of LNCaP cell demonstrated that AR receptor (red) was co-expressed with TP53 (green). Compared with that in LNCaP cell treated with DMSO, TP53 expression was significantly enhanced in LNCaP cell treated with enzalutamide (**Figure 3A-B, P<0.001**), but AR expression showed no difference between two kinds of cells (**Figure 3A-B**). That TP53 expression was decreased in LNCaP cell treated with enzalutamide was also supported by the results of western blot (**Figure 3C**) and RT-PCR (**Figure 3D, p<0.01**). However, even though AR receptor showed no difference between LNCaP cell treated with enzalutamide and DMSO in western blot (**Figure 3C**), RT-PCR indicated that AR mRNA was decreased significantly in LNCaP cell treated with enzalutamide (**Figure 3D, p<0.01**). In addition, LNCaP cell dealt with enzalutamide showed inhibited cell cycle at G0~G1 (**Figure 3E**), increased apoptosis percentage (**Figure 3F**) and also inhibited cell proliferation (**Figure 3G**).

Knockout of TP53 promote LNCaP cell enzalutamide resistance

Whether knockout of TP53 promote enzalutamide resistance in LNCaP cell or not still need validation. Three sets of lentivirus packed shRNA were designed to knock down the expression of TP53 protein. Among them, shRNA-3 has the highest knockdown efficiency in both western blot results (**Figure 4B**) and qRT-PCR (**Figure 4C**). Similarly, knock down TP53 in LNCaP cell showed inhibited cell cycle at G0~G1 (**Figure 4D**), increased apoptosis percentage (**Figure 4E**) and also inhibited cell proliferation (**Figure 4F**) which demonstrated same results with LNCaP cell treated with enzalutamide.

Discussion

In current studies, LNCaP cells treated with enzalutamide to identify resistance related genes by using CRISPR Cas9 sgRNA library screening. The result shows TP53, as a key transcript regulator, play an important role in enzalutamide resistance in LNCaP cells by inhabiting AR signaling pathways.

Regarding the mechanism behind mCRPC, it has been well-known that the expression of AR critically determines cancer progression [12]. Eliminating or lowering AR signaling determines the effects of prostate treatment. However, frequently observed phenomenon of resistance during enzalutamide or various other new treatment involve the alternative methods of generating AR. First important

mechanisms which must pay more attention to is AR receptor mutation, splice variants and truncations. Even genomic-scale AR variants might generate a stable form of mCRPC, it is also might relevant to the evolution of the PCa and it seems to be a transient response to therapy [13]. The second reason for independent of AR is intracrine synthesis of androgens by the tumor cells. It has been reported that glucocorticoid receptor could increase the expression of AR target genes in tumor cells contributing to progress[14].

In addition, alterations in expression and function of several AR coactivators balance, as well as multiple receptor-unrelated mechanisms have been described, which are also relevant for acquisition of resistance to anti-androgens in prostate cancer [15].

Among the frequently referred coactivators, most of them were acting as positive factors including p160 family, CREB binding protein, p300, BRCA-1 and c-Jun et al [16]. In contrast, relatively few proteins involving p53 protein have been identified and demonstrated to have negative activity on AR-mediated transcription. The function p53 protein, encoded by TP53 gene involve regulating expression of target genes, and inducing cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism [17]. About 23% of metastatic tumors endured p53 mutations and 24% p53 mutations was found with prostate cancer [18]. Our data show that both the TP53 and AR low expression measuring by RNA-seq associated with PCa progress. Although AR and TP53 gene alone have been shown no prognostic value, combined analysis of AR and TP53 RNA-seq studies simultaneously have demonstrated important progress value in PCa. In addition, synchronous profiling of all outcome-associated somatic alterations in AR and TP53 in liquid biopsies of patients with mCRPC prior to enzalutamide treatment demonstrates that TP53 is an independently associated negative response biomarker. Shenk et al point out that both the carboxyl-terminal region and DNA-binding domains of p53 are important in the inhibition of AR transactivation of the PSA promoter because either deletion of the former or disruption of the latter severely compromises the negative activity of p53 [19]. Evidence showed that p53 may have an indirect effect by blocking the activity of a protein mediating the AR N-to-C interaction because it is unable to detect a protein-protein interaction between p53 and AR by using in vitro precipitation methods and mammalian two-hybrid assays[20]. In vivo experiments, PCa cells harboring p53 mutations were selected for the establishment of mCRPC model and many of these late-stage and mCRPC cells have functional AR-dependent transcription. Interestingly the p53 mutation was also found in lymph node metastasis of prostate cancer [21]. Our data supported that knock out of TP53 and inhabit of AR signaling promote enzalutamide resistance in LNCaP cell which give us an alternative perspective for mCRPC.

In summary, by using genome-wide CRISPR screening, current data support that TP53 protein inhabiting AR signaling promote enzalutamide resistance in LNCaP cell which was also confirmed by analysis TCGA data of PRAD.

Abbreviations

mCRPC: metastatic castration-resistant prostate cancer, PCa: prostate cancer, LNCaP Lymph Node Carcinoma of the Prostate, sgRNA: single-guide RNA.

Declarations

Acknowledgements

Not applicable

Authors' contributions

(I) Conception and design: X Long, J Liu, (II) Administrative support: X Long, J Liu, (III) Provision of study materials or patients: X Long, B Jiang, J Liu, (IV) Collection and assembly of data: X Long, B Jiang, J Liu, (V) Data analysis and interpretation: X Long, B Jiang, J Liu, (VI) Manuscript writing: All authors, (VII) Final approval of manuscript: All authors.

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Conflicts of Interest

All authors have completed the ICMJE uniform disclosure form. The authors have no conflicts of interest to declare.

Ethical Statement

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

All authors noticed and consent for publishing the paper.

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Figures

Figure 1

Enzalutamide resistance gene filtering by using CRISPR-Cas9 sgRNA. (A) LNCaP cell treated with different concentration of Enzalutamide. (B) LNCaP cell proliferation in different concentration of Enzalutamide. (C) Pipelines of CRISPR-Cas9 sgRNA transduction. (D) Next generation sequencing analysis of sgRNA enrichment. (E) Volcano plot show 27 high expression genes (red color) and 26 low expression genes (blue color) in Enzalutamide treated LNCaP cells.

Figure 2

Low expression of TP53 and AR promote prostate cancer metastasis. (A) PPI network between Enzalutamide resistance genes and AR signaling pathway. (B) Volcano plot show 3 high expression (red) and 183 low expression genes (blue) between LNCaP cells treated with DMSO and Enzalutamide (n=3). (C) GO enrichment analysis of high and low expression genes (n=186) in Enzalutamide treated LNCaP cells. GSEA analysis show 2 key pathway changes in Enzalutamide treated LNCaP cells compared with DMSO treated LNCaP cells (D~E). Heatmap plot show 25 abnormal expression genes mainly distributed in AR signaling pathway (F). Patients with low expression of TP53 and AR show higher pathology stages

(G), higher Gleason grades (H), more disease progress (I), recurrence (J), positive lymphnodes examines (K) and abnormal bone scan results (L) compared with high expression of TP53 and AR.

Figure 3

Inactivation of TP53 in Enzalutamide treated LNCaP cells. (A) Immunofluorescence staining of TP53 and AR between Enzalutamide and DMSO treated LNCaP cells. (B) TP53 expression show lower in Enzalutamide treated LNCaP cells with immunofluorescence staining. Western blot demonstrated TP53, but not AR lower expressed in Enzalutamide treated LNCaP cells (C). RT-PCR demonstrated both of TP53 and AR lower expressed in Enzalutamide treated LNCaP cells (D). Cell cycle checking indicated

Enzalutamide treated LNCaP cells showing shorter G0~G1 stage (E). Apoptosis percentage of Enzalutamide treated LNCaP cells show higher than DMSO treated LNCaP cells (F). Cell proliferation was suppressed in Enzalutamide treated LNCaP cells (G).

Figure 4

Knock out of TP53 promote Enzalutamide resistance. (A) Fluorescent staining images between lentiviral interference RNA and normal controls. Both of Western blot (B) and RT-PCR (C) demonstrated higher efficient of lentiviral interference RNA (shRNA-3) in LNCaP cells. Knock out of TP53 in LNCaP cells show shorter G0~G1 stage (D), higher apoptosis percentage (E) and suppressed cell proliferation (F) compared with LNCaP cells treated with DMSO.

Supplementary Files

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