Targeting the Metabolic Enzyme PGAM2 Overcomes Enzalutamide Resistance in Castration-Resistant Prostate Cancer by Inhibiting BCL2 Signaling

Zhen Li1,2,3, Kang Ning1,2, Diwei Zhao1,2, Zhaohui Zhou1,2, Junliang Zhao1,2, Xingbo Long1,2, Zhenyu Yang1,2, Dong Chen1,2, XinYang Cai4, Lexuan Hong4, Luyao Zhang4, Fangjian Zhou1,2, Jun Wang1,2, and Yonghong Li1,2

ABSTRACT

The next-generation androgen receptor (AR) inhibitor enzalutamide is the mainstay treatment for metastatic prostate cancer. Unfortunately, resistance occurs rapidly in most patients, and once resistance occurs, treatment options are limited. Therefore, there is an urgent need to identify effective targets to overcome enzalutamide resistance. Here, using a genome-wide CRISPR-Cas9 library screen, we found that targeting a glycolytic enzyme, phosphoglycerate mutase PGAM2, significantly enhanced the sensitivity of enzalutamide-resistant prostate cancer cells to enzalutamide both in vivo and in vitro. Inhibition of PGAM2 together with enzalutamide treatment triggered apoptosis by decreasing levels of the antiapoptotic protein BCL-xL and increasing activity of the proapoptotic protein BAD. Mechanically, PGAM2 bound to 14–3-3ζ and promoted its interaction with phosphorylated BAD, resulting in activation of BCL-xL and subsequent resistance to enzalutamide-induced apoptosis. In addition, high PGAM2 expression, which is transcriptionally regulated by AR, was associated with shorter survival and rapid development of enzalutamide resistance in patients with prostate cancer. Together, these findings provide evidence of a nonmetabolic function of PGAM2 in promoting enzalutamide resistance and identify PGAM2 inhibition as a promising therapeutic strategy for enzalutamide-resistant prostate cancer.

Significance: PGAM2 promotes resistance to enzalutamide by activating antiapoptotic BCL-xL and suppressing apoptosis, indicating that PGAM2 is a potential target for overcoming enzalutamide resistance in prostate cancer.

Introduction

The next-generation androgen receptor (AR) inhibitor, enzalutamide, is currently the mainstay treatment for metastatic prostate cancer (1). However, the disease can relapse and progress to an enzalutamide-resistant stage, for which treatment options are limited. The antitumor activity of enzalutamide mainly occurs through suppression of activated AR nuclear translocation to androgen response elements and the induction of cancer cell apoptosis (2). Hence, overexpression of antiapoptotic BCL2 protein family members BCL-xL and MCL1 in prostate cancer cells is a critical mechanism leading to enzalutamide resistance (3, 4).

The intrinsic or mitochondrial pathway of apoptosis is mediated by BCL2 family proteins through balancing proapoptotic BH3-only proteins (e.g., BIM, BID, BAD, PUMA, and NOXA) and antiapoptotic family members (e.g., BCL2, BCL-xL, MCL1, and BCL-w). The balance between pro- and antiapoptotic proteins determines whether effector proteins (BAX and BAK) are activated, leading to subsequent mitochondrial outer membrane permeabilization. Finally, the release of cytochrome c and activation of caspase-9 culminates in cell-wide proteolysis and cell death (5, 6). Upregulation or hyperactivation of antiapoptotic BCL2 proteins is associated with therapy resistance, and targeting BCL2 proteins may be a promising strategy to overcome enzalutamide resistance (6–8).

Phosphoglycerate mutase (PGAM), a glycolytic enzyme, is a dimeric protein consisting of subunits B-PGAM and M-PGAM, and catalyzes the conversion of 3-phosphoglycerate (3-PGA) to 2-phosphoglycerate (2-PGA), a crucial step in glycolysis (9, 10). PGAM2, a homodimer of M-PGAM, is also a glycolytic enzyme that has oncogenic capability attributed to its function in regulating glycolysis. Recent studies have revealed that metabolic enzymes are widely involved in a variety of biological processes through various nonmetabolic pathways and that they play important roles in the regulation of gene expression, DNA damage repair, and regulation of the cell cycle (11, 12). For instance, PGAM1 binds to phosphorylated wild-type p53-induced phosphatase 1 (WIP1) to prevent its translocation to the nucleus (13). Understanding the roles of noncanonical mechanisms of metabolic enzymes in the development of cancer may help to identify new vulnerabilities in cancer that can be therapeutically targeted.

In this study, we found that PGAM2 directly binds to tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein zeta (14–3-3ζ) and promotes its interaction with phosphorylated BAD, resulting in activation of BCL-xL and subsequent resistance to enzalutamide-induced apoptosis. Notably, inhibiting PGAM2 expression overcomes enzalutamide resistance, indicating...
a promising therapeutic strategy for enzalutamide-resistant prostate cancer.

Materials and Methods

Cell culture and reagents

Human castration-resistant prostate cancer cell lines C4-2 (RRID: CVCL_4782) and 22Rv1 (RRID: CVCL_1045) were obtained from ATCC in 2020. We established and maintained the enzalutamide-resistant prostate cancer cell line (C4-2R) in accordance with a previous method (14). All cell lines were maintained in RPMI1640 (C11875500BT, Gibco) supplemented with 10% FBS (35-081-CV, Corning), 100 U/mL penicillin–streptomycin (15140148, Gibco) at 37°C and 5% CO2. Short tandem repeat (STR) authentication was performed on all cell lines. Mycoplasma testing (D101–02, Vazyme) was conducted every 3 months. The cells were cryopreserved within the first five passages after generation and were used within 15 passages after thawing. We purchased small-molecule inhibitors, including PKM2-IN-1 (HY-103617), NG52 (HY-15154), Salermide (HY-101073), and the AR PROTAC ARD-266 (HY-133020, from MedChemExpress). Enzalutamide (SC0074) and anisomycin (SO132) were purchased from Beyotime. Dihydrotestosterone (DHT, S4757) was purchased from SelleckChem.

RNA interference, lentivirus, and plasmids

siRNAs targeting PGK1 and PKM2 were synthesized by Ribobio and transfected using Lipofectamine 3000 (L3000015, Invitrogen). Lentiviruses packaging PGAM2 short hairpin RNAs (shRNA) and ARs targeting lentiCRISPRv2-gRNA were synthesized by GeneChem. We infected C4-2R and 22Rv1 cells with the lentiviral vectors and treated them with 5 μg/mL puromycin (ST551, Beyotime) for 7 to 10 days. The plasmids were constructed with the help of GeneChem. The plasmids in our study included His-PGAM2, GST-14–3–3ζ (1–40 aa, 1–100 aa, 101–245 aa, 41–245 aa, 1–245 aa truncations, and 41–50 aa, 51–60 aa, 61–70 aa, 71–80 aa, 81–90 aa, 91–100 aa mutants), Flag-PGAM2, His-14–3–3ζ (WT and 91–100 aa MUT) and luciferase reporter plasmids (pGL3-PGAM2 WT, pGL3-PGAM2 MUT1, and pGL3-PGAM2 MUT2). All the primer sequences of plasmids were listed in Supplementary Table S1. The wild-type PGAM2 or acetylation mimetic K100Q mutant plasmids were synthesized by GeneChem. Plasmids were transfected using Lipofectamine 3000 transfection reagent.

Genome-wide CRISPR-Cas9 screen

The human Genome-scale CRISPR Knock-Out (GeCKO) v2.0 library targeting 19,050 genes (6 sgRNAs per gene) were used in this study. The sgRNAs were designed and then packaged into the lentCRISPR transfer plasmid as previously described by Zhang and colleagues (15). For virus production, 293T cells were cotransfected with 5 μg of the lentCRISPR plasmid library, 4 μg of pSPAX2, and 3 μg of pVSVG with 30 μL of Lipofectamine 3000. The transfected 293T cells were incubated at 37°C, and the transfection medium was replaced after 8 hours. Viral particles were harvested 48 to 72 hours after transfection and frozen at –80°C until further use. For the in vitro CRISPR-Cas9 knockout screen under enzalutamide treatment, 4.5 × 104 C4-2 cells were plated in 6-well plates to ensure sufficient coverage of sgRNAs (Complexity = 100) and infected at a low multiplicity of infection (MOI = 0.3) to ensure that most cells received only one viral construct with high probability. Forty-eight hours after infection, the infected C4-2 cells were selected with 5 μg/mL puromycin for 7 days to select the positively transduced cells and eliminate uninfected cells to obtain genome-edited cell pools. The selected cells were assigned into two groups, and treated with dimethylsulfoxide (DMSO; D2650, Sigma-Aldrich) or 20 μM/L enzalutamide. After a week, we performed genomic DNA extraction using a TIANamp Genomic DNA Kit (DP304, Tiangen Biotech). DNA fragments containing the sgRNA sequences were amplified by two-step PCR using NEB Next High-Fidelity 2X PCR Master Mix (M0541L, New England Biolabs) and the primers as previously listed in Zhang and colleagues (15). The PCR products containing the sgRNA sequences were gel extracted, quantified, mixed, sequenced, and analyzed as previously described.

Cell counting kit-8 assay

Cells were seeded into 96-well plates at a density of 3,000 cells/well and allowed to attach overnight. DMSO or enzalutamide (20 μM/L) was added to the medium for the following days. After adding CCK8 reagents (CK04, Dojindo) to each well, cells were incubated at 37°C for 2 hours. An automated plate reader was used to measure the absorbance at 450 nm.

Clonogenicity assay

 Cells were seeded in 6-well plates at 1,000 cells/well and incubated at 37°C for 14 days in media containing DMSO or enzalutamide (20 μM/L). At the end of the experiment, the colonies were counted after cells were fixed with methanol, and stained with crystal violet.

Western blot analysis

RIPA lysis buffer (P0013B, Beyotime) was used to extract proteins from cells, and Bicinchoninic Acid Protein Assay Kit (P0010, Beyotime) was used to determine protein concentration. Samples were separated by 10% SDS-PAGE and transferred to a methanol-activated polyvinylidene fluoride (PVDF) membrane under constant 300 mA in an ice bath for 2 hours (protein mass < 20 kDa, constant 300 mA for 1 hour). The PVDF membrane was blocked with 5% BSA for 1 hour at room temperature, and then incubated with primary antibody at 4°C overnight. At room temperature, the membrane was incubated for 60 minutes with 5% skim milk containing secondary antibodies. After three washes with Tris-buffered saline containing 0.1% Tween 20 (TBST), the bands were visualized using ChemiStar High-sig ECL Western blotting substrate (180–5001, Tanon). The primary antibodies used in western blot analysis were demonstrated in Supplementary Table S2.

Human tissues and qRT-PCR

We collected 41 prostate cancer biopsy tissues from patients with castration-resistant prostate cancer (CRPC) who were scheduled to receive enzalutamide. All the clinical parameters of patients were listed in Supplementary Table S3. In addition, the biopsy tissues or prostatectomy tissues before and after neoadjuvant next-generation AR inhibitor therapy were also collected and preserved in RNA Stabilization Reagent (76106, Qiagen). Ethics approval was obtained from the Institutional Review Board of Sun Yat-sen University Cancer Center (Guangzhou, China), and the Declaration of Helsinki was followed. Written informed consent was obtained from the patients before the study began.

Total RNA samples from the prostate cancer tissue specimens or cell lines in this study were extracted with TRIzol Reagent (15596026, Invitrogen) according to the manufacturer’s protocol, and quantified with Nanodrop 2000 (Thermo Fisher Scientific). Real-time PCR was
performed using ABI Prism 7500 FastSequence Detection System (Applied Biosystems) with ChamQ SYBR qPCR Master Mix (Q311–02, Vazyme). The sequences for the gene-specific primers used are listed in Supplementary Table S1. β-Actin was used as the internal control.

Apoptosis assays
To analyze cell apoptosis levels, cells were seeded into 6-well plates at \(2 \times 10^5\) cells/well. After 48-hour attachment, DMSO or enzalutamide (20 μM/L) was added to treat the cells for 24 hours. In accordance with the instructions, Annexin V-FITC Apoptosis Detection Kits (KGA108, KeyGEN) were used to test for apoptosis. Flow cytometry and FlowJo software were used to measure and analyze apoptosis rates. Then, the caspase-3/7 and propidium iodide (PI) fluorescent labeling dye (C11061–2, riboAPO) were used to detect the apoptosis of cells. Fluorescent microscopy was used to detect the fluorescence of cells.

IHC analysis
All tumors of nude mice and human tissues were fixed, embedded in paraffin, and subsequently sectioned. The sections were then subjected to hematoxylin and eosin staining and IHC analysis. IHC analysis was performed according to the manufacturer’s instructions. Detailed information regarding the primary antibodies can be found in Supplementary Table S2. IHC scoring was performed using the Chip software version 3.3 (IndicaLab), including tissue segmentation, cell segmentation, and phenotyping to assign each cell to a phenotypic category. Briefly, following the importation of IHC slide images acquired through scanning into the Chip software, automated recognition of tissue regions occurred, followed by further identification of individual cells and their corresponding degree of antibody staining within a 20× magnification field. The Chip software categorizes cells into four levels based on their staining intensity: negative, weak positive, moderate positive, and strong positive. Subsequently, the Chip software performs tissue-wide recognition and quantifies the proportions of cells belonging to each staining level. Multiplying these proportions by the corresponding numerical scoring weights yields the ultimate H-score rating. We used the median value of H-score as a threshold to define two groups: the PGAM2-high expression group (above the median value) and the PGAM2-low expression group (below the median value).

PGAM2 enzyme activity assay
PGAM2 enzyme activity was measured by the change of absorbance at 340 nm resulting from NADH oxidation in Multi-Mode Microplate Reader (BioTec Epoch) with a previous method (16). All the reagents contained triethanolamine (T0449, Sigma-Aldrich), ADP (A0180, Solarbio), NADH (ST358, Beyotime), 3-phosphoglycerate (P8877, Sigma-Aldrich), 2,3-diphosphoglycerate (D5764, Sigma-Aldrich), MgSO₄ (ST374, Beyotime), KCl (ST345, Beyotime), pyruvate kinase (P7768, Sigma-Aldrich), 1-lactate dehydrogenase (L3916, Sigma-Aldrich), and enolase (E6126, Sigma-Aldrich).

Extracellular acidification rate
Seahorse XF Glycolysis Stress Test Kit (103020–100, Agilent) was used to determine glycolysis capacity according to the instructions. In brief, \(5 \times 10^5\) cells were seeded in 96-well plates and incubated overnight. To measure the extracellular acidification rate (ECAR), 25 μM of each of 80 mM/L glucose, 9 μM/L oligomycin, and 500 mM/L 2-deoxy-glucose were added to the cells after they had been washed with Seahorse buffer. ECAR values were calculated and normalized to the cell number.

Chromatin immunoprecipitation
We used the Jaspar (https://jaspar.genereg.net/) to predict the AR-binding sites in the PGAM2 promoter. After treatment with 10 nmol/L DHT, enzalutamide-resistant C4–2R cells were immunoprecipitated with EZ-Magna ChIP A/G Chromatin Immunoprecipitation Kit (17–10086, Merck) according to the manufacturer’s instructions. We evaluated the occupancy by gel electrophoresis and quantitative PCR of samples precipitated with anti-AR (1:100, ab108341, Abcam) versus samples precipitated with Normal Rabbit IgG (#2729, Cell Signaling Technology) using the primers listed in Supplementary Table S1.

Dual luciferase reporter assay
Different fragments containing the promoter of the PGAM2-encoding gene were inserted into the pgL3 Basic Vector (Promega). Primer sequences were listed in Supplementary Table S1. pgL3-derived plasmids (contains pgL3-Basic, pgL3-PGAM2 WT, pgL3-PGAM2 MUT1, and pgL3-PGAM2 MUT2) and the pRL-TK Renilla plasmid were transiently cotransfected into C4–2R cells using Lipofectamine 3000 transfection reagent. The Dual-Luciferase Reporter Assay System (E1910, Promega) was used to detect dual-luciferase activities after 48 hours of treatment with 10 nmol/L DHT according to a protocol provided by the manufacturer.

Immunoprecipitation and mass spectrometry
Cells were collected and lysed using Western and IP lysis buffer (P0013, Beyotime) supplemented with a protease inhibitor cocktail (P8430, Sigma-Aldrich). After quantifying the protein using a BCA assay, 100 μg of proteins were incubated with His-PGAM2 protein according to the instructions of GST pulldown Kit (IEMed-K203, IEMed), and followed by analysis using immunoblotting.

GST-pulldown assay
Recombinant GST–14–3–3ζ (1–40 aa, 1–100 aa, 101–245 aa, 41–245 aa, and 41–245 aa truncations, and 41–50 aa, 51–60 aa, 61–70 aa, 71–80 aa, 81–90 aa, 91–100 aa mutants) proteins were respectively incubated with His-PGAM2 protein according to the instructions of GST pulldown Kit (IEMed-K203, IEMed), and followed by analysis using immunoblotting.

Xenograft mouse model
BALB/c (RRID: IMSR_APB:4790) nude mice used in this study were obtained from Charles River and housed in a pathogen-free animal barrier facility. To conduct the experiments, 6-week-old male nude mice were castrated. Then the mice were anesthetized and C4–2R cells were subcutaneously injected with Matrigel premixed at 1:2 into the right rear back region 2 weeks later. When tumors reached 100 mm³, mice were randomized into four groups (\(n = 6\) each) and treated with PGAM2 shNC + PBS, PGAM2 shNC + enzalutamide, shPGAM2 + PBS, or shPGAM2 + enzalutamide. Mice were orally administered with 30 mg/kg enzalutamide or PBS, while PGAM2
AAV-shRNA (2 × 10⁸ PFU) was intratumorally injected. We monitored tumor size and volume every week. The tumor volume was calculated as follows: volume = length × width²/2. All the mice were sacrificed at the appropriate time, and the tumors were dissected and embedded in paraffin for IHC analysis. All procedures were performed in compliance with ethical regulations. Sun Yat-sen University Cancer Center’s Animal Care and Use Committee approved all animal testing procedures.

Bioinformatic analysis

The results in this study were externally verified by published transcriptome data and single-cell sequencing data. We got three single-cell sequencing datasets from Tumor Immune Single-cell Hub 2 (TISCH2): GSE137829 (CRPC), GSE141445 (prostate cancer), GSE172301 (benign prostatic hyperplasia). The expression of PGAM2 in each cell type was analyzed and compared in those three single-cell sequencing datasets. Single-sample gene set enrichment analysis (ssGSEA) was used to evaluate apoptosis score in malignant tumors. The median of an apoptosis score was calculated using HALLMARK gene sets. Survival analysis was performed in CRPC cohort (GSE35988), where we used the median value of PGAM2 expression as a threshold to define two groups: the PGAM2-high expression group (above the median value) and the PGAM2-low expression group (below the median value).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.0 software and the R package V.4.1.0. Unpaired paired Wilcoxon test or one-way ANOVA was used to measure differences between groups. Kaplan–Meier method with log-rank test was used to plot overall survival curves and evaluate the difference in survival rates. P < 0.05 was considered statistically significant.

Data availability

All chromatin immunoprecipitation-sequencing (ChIP-seq) data were obtained from publicly available sequenced data of prostate cancer cell lines (Cistrome Data Browser: http://cistrome.org/db/#!). The data used for single-cell sequencing and prognosis analysis were sourced from the GEO database (GSE137829, GSE141445, GSE172301, GSE35988). The dataset for survival analysis of patients with CRPC is also available on GEO: GSE35988. The remaining data generated in this study can be obtained by contacting the corresponding author upon request.

Results

CRISPR screening identified that PGAM2 deficiency enhances the sensitivity of prostate cancer cells to enzalutamide

To identify potential targets to overcome enzalutamide resistance in prostate cancer, a genome-wide CRISPR-Cas9 screen was performed in prostate cancer cells treated with control or enzalutamide (Fig. 1A). Compared with the control, we detected that PGAM2 was the most significant gene deletion in the enzalutamide group (Fig. 1B and C), which increased the sensitivity of prostate cancer cells to enzalutamide. In addition, several genes such as EIF4F, BIRC6, and ID1, which have been reported to be involved in enzalutamide resistance in previous studies, were identified in our screen as well (Supplementary Table S4; refs. 17–19). It was reported that the overexpression or reactivation of AR is one of the main mechanisms of enzalutamide resistance (19). Unsurprisingly, AR was also identified in the screen.

We then investigated whether inhibition of PGAM2 sensitized enzalutamide-resistant prostate cancer cells to enzalutamide. Two enzalutamide-resistant prostate cancer cell lines, C4-2R and 22Rv1, were transfected with PGAM2 shRNA or shNC (Supplementary Fig. S1). Cell viability of transfected cells treated with enzalutamide or DMSO was evaluated by CCK-8 and colony-forming assays (Fig. 1D–G). Our results showed that PGAM2 inhibition combined with enzalutamide treatment decreased the cell proliferation rate as well as the number of colonies formed by enzalutamide-resistant prostate cancer cells. No significant difference was observed in the cell proliferation rate or in the number of cell colonies formed following PGAM2 inhibition alone, enzalutamide treatment alone, or control groups. These results indicate that PGAM2 inhibition enhanced the sensitivity of enzalutamide-resistant prostate cancer cells to enzalutamide.

PGAM2 inhibition promotes enzalutamide-induced apoptosis of enzalutamide-resistant prostate cancer cells

Induction of apoptosis is one of the mechanisms by which enzalutamide represses prostate cancer. Thus, resistance to enzalutamide-induced apoptosis is one of the key mechanisms through which enzalutamide resistance develops. We then explored whether inhibition of PGAM2 enhanced the apoptosis of enzalutamide-resistant prostate cancer cells following enzalutamide treatment. Immunofluorescent staining of caspase-3/7 was performed to evaluate the apoptosis of prostate cancer cells treated with shNC, shPGAM2, enzalutamide, or a combination of shPGAM2 and enzalutamide. A significant increase in caspase-3/7 staining was observed after treatment with both shPGAM2 and enzalutamide compared with single-agent treatment (Fig. 2A). Consistent with an enhanced effect on apoptosis induction, Annexin V-FITC and PI staining demonstrated that the combined treatment of shPGAM2 and enzalutamide resulted in significant apoptosis of enzalutamide-resistant prostate cancer cells (Fig. 2B and C). In contrast, shPGAM2 or enzalutamide alone did not induce apoptosis of C4-2R or 22Rv1 cells. Western blotting confirmed the substantial upregulation of apoptotic markers such as cleaved caspase-3 and cleaved PARP after combined shPGAM2 and enzalutamide treatment of C4-2R and 22Rv1 cells (Fig. 2D). Enzalutamide inhibits prostate cancer by regulating BCL2 signaling. We therefore analyzed BCL2 family proteins and found that the levels of BCL-xL (an antiapoptotic protein) were decreased in cells treated with shPGAM2 and enzalutamide. Although the levels of BAD (a proapoptotic protein) did not alter significantly, phosphorylated BAD levels were decreased in cells treated with shPGAM2 and Enzalutamide. These results demonstrated that PGAM2 inhibition promotes enzalutamide-induced apoptosis by inhibiting antiapoptotic BCL2 signaling.

Targeting PGAM2 overcomes enzalutamide resistance in enzalutamide-resistant prostate cancer xenografts

To investigate the efficiency of targeting PGAM2 in enzalutamide-resistant prostate cancer xenografts, we subcutaneously implanted enzalutamide-resistant C4-2R prostate cancer cells into castrated mice and then randomized and treated the mice with shNC, enzalutamide, shPGAM2, and shPGAM2 combined with enzalutamide (Fig. 3A). As expected, the combined shPGAM2 and enzalutamide treatment significantly reduced xenograft tumor growth and weight and achieved the best suppressive effects against tumorigenic activities compared with shNC, enzalutamide, or
shPGAM2 alone (Fig. 3B). The protein levels of PGAM2, AR, BAD, p-BAD, and BCL-xL in xenograft tumor tissues were determined by IHC (Fig. 3C). PGAM2 levels were reduced in groups receiving shPGAM2 treatment, while levels of AR and BAD were not significantly changed in any group (Supplementary Fig. S2A). Levels of p-BAD and BCL-xL were not significantly altered in the shNC, shPGAM2, or enzalutamide treatment groups, but were significantly reduced in the shPGAM2 and enzalutamide treatment groups (Fig. 3D). This was consistent with the above results and further supported PGAM2 as a promising target to overcome enzalutamide resistance in prostate cancer.

Inhibition of PGAM2 enhances enzalutamide-induced apoptosis independent of its metabolic activity

PGAM2 is a metabolic enzyme; therefore, we first speculated that the ability of PGAM2 to overcome enzalutamide resistance was dependent on its enzyme activity. To investigate the role of the enzymatic activity of PGAM2 in enzalutamide-resistant prostate cancer, we constructed a plasmid with a mutation in a residue of the PGAM2-active site that is modified by acetylation (K100Q). This mutation inactivates PGAM2 enzyme activity. We transfected PGAM2-K100Q or PGAM2-WT plasmids into two enzalutamide-resistant cell lines and then immunopurified to measure the enzyme.
Figure 2.
PGAM2 deficiency combines with enzalutamide (ENZ) treatment to induce apoptosis. **A**, The fluorescence intensity of PI, caspase-3/7, and DAPI by immunofluorescence staining assays. **B** and **C**, Flow cytometry analysis of cell apoptosis for cells transfected with PGAM2 shRNAs or control shRNA (shNC) under the treatment of DMSO or enzalutamide. **D**, Expression of caspase-3, cleaved-caspase-3, PARP, cleaved PARP, phosphorylated BAD, total BAD, and BCL-xL in shNC or shPGAM2 cells after treatment of enzalutamide. ***, P < 0.001.
Figure 3.
PGAM2 deficiency enhances the antitumor effect of enzalutamide (ENZ) in vivo. A, Experimental design in establishing a xenograft mouse model with C4-2R cells. B, Effects of knockdown of PGAM2 combined with enzalutamide treatment on tumor growth in a subcutaneous xenograft model. The size of tumors was measured at the indicated time points, and tumors were extracted and weighed after mice were sacrificed. C and D, Immunohistostaining image and quantification of IHC H-score of PGAM2, AR, BAD, p-BAD, and BCL-xL. Magnification, ×40. Scale bar, 100 μm. ns, not significant; ***, *P < 0.001. H&E, hematoxylin and eosin.
activity of PGAM2. Transfection of PGAM2-K100Q significantly inhibited PGAM2 enzymatic activity in both cell lines (Fig. 4A). Surprisingly, cell proliferation and cell colony counting assays demonstrated that inhibiting PGAM2 enzyme activity did not enhance the sensitivity of enzalutamide-resistant cell lines to enzalutamide (Fig. 4B and C; Supplementary Fig. S3A). We next examined whether inhibiting PGAM2 enzyme activity in cell lines promoted enzalutamide-induced apoptosis. As expected, the combination of PGAM2-K100Q transfection and enzalutamide did not lead to increased apoptosis of enzalutamide-resistant cell lines (Fig. 4D; Supplementary Fig. S3B). To further investigate the impact of endogenous PGAM2 enzymatic activity on the enzalutamide-resistant cell line, we used salermide, a SIRT1/SIRT2 inhibitor, which increases the K100AC modification of PGAM2 without affecting its protein expression level, to treat C4-2R and 22Rv1 cells. We found that increasing the K100AC of PGAM2 decreased its enzymatic activity (Supplementary Fig. S4A) and led to a slightly decrease in proliferation and an increase in apoptosis, although this difference remained statistically insignificant (Supplementary Fig. S4B–S4D).

To demonstrate that enhancement of enzalutamide sensitivity by inhibition of PGAM2 is independent of its enzyme activity, we investigated the effect of PGAM2 upstream and downstream substrates, 2-PG and 3-PG, respectively, on cell proliferation and enzalutamide-induced apoptosis. To alter the intracellular 2-PG and 3-PG, we used siRNA to knock down its metabolic enzymes, phosphoglycerate kinase 1 (PGK1) and pyruvate kinase 2 (PKM2; Fig. 4E; Supplementary Fig. S5A), respectively. In addition, we used small-molecule inhibitors, PKM2-IN-1 and NG52, to inhibit the enzyme activity of PKG1 and PKM2. Inhibiting the expression or enzyme activity of PKG1 and PKM2 did not influence cell proliferation under enzalutamide treatment or apoptosis induced by enzalutamide (Fig. 4F–K; Supplementary Fig. S5B–S5D; Supplementary Figs. S6A–S6C and S7A–S7C). Measurement of the ECAR confirmed that glucose metabolism was efficiently altered in siRNA-transfected and inhibitor-treated cells (Fig. 4L and M; Supplementary Fig. S5E). These results indicate that PGAM2 inhibition enhances enzalutamide-induced apoptosis through nonenzymatic activity.

**PGAM2 interacts with 14–3–3ζ to activate antipapoptotic BAD/BCL-xL signaling, which promotes resistance of prostate cancer cells to enzalutamide-induced apoptosis**

Metabolic enzymes have recently been shown to be involved in the regulation of cellular processes through nonmetabolic regulatory functions. To elucidate the mechanism by which PGAM2 regulates enzalutamide sensitivity, Flag-PGAM2 was constructed and transfected into C4-2R cells. Flag-PGAM2 was purified by anti-Flag affinity purification and subjected to mass spectrometry analysis. We identified 225 proteins that potentially interact with PGAM2, and overlapped these proteins with previously reported apoptosis-related proteins. This identified 14–3–3ζ, which participates in apoptosis regulated by BCL2 family proteins (Fig. 5A). To verify the interaction between PGAM2 and 14–3–3ζ, GST pulldown assays were performed using purified GST-14–3–3ζ and His-PGAM2 recombinant proteins. Various lengths of 14–3–3ζ truncations were designed to examine their association with PGAM2. Among these, interactions with PGAM2 were lost with truncations 1–40 aa and 101–245 aa, whereas the other truncations retained the association with PGAM2 (Fig. 5B), indicating that the 41–100 aa region is the interaction domain of 14–3–3ζ. We then constructed six GST-14–3–3ζ mutants and examined their interaction with PGAM2. The mutation of 91–100 aa, but not other mutations, abolished the interaction with PGAM2 (Fig. 5C), indicating that the 91–100 aa region of 14–3–3ζ is required for its interaction with PGAM2. To further verify the interaction between PGAM2 and 14–3–3ζ in an enzalutamide-resistant cell line, we performed coimmunoprecipitation assays by ectopically expressing Flag-tagged PGAM2, His-tagged 14–3–3ζ WT, and His-tagged 14–3–3ζ MUT (91–100 aa) in C4-2R cells. The result showed that the interaction between PGAM2 and 14–3–3ζ involved the 91–100 aa region (Fig. 5D). Immunoprecipitation of endogenous 14–3–3ζ using anti-PGAM2 antibody also pulled down endogenous 14–3–3ζ in C4-2R cells and vice versa for PGAM2 (Supplementary Fig. S8A). These results further confirmed the interaction between endogenous PGAM2 and 14–3–3ζ in enzalutamide-resistant cells.

To verify that the interaction between PGAM2 and 14–3–3ζ activates antipapoptotic BAD/BCL-xL signaling, we first examined the expression of BAD, p-BAD, and BCL-xL after treating C4-2R and 22Rv1 cells with ectopically expressed 14–3–3ζ WT or 14–3–3ζ MUT (91–100 aa) and enzalutamide (Fig. 5E; Supplementary Fig. S8B). Ectopically expressed 14–3–3ζ MUT (91–100 aa) combined with enzalutamide treatment decreased antipapoptotic p-BAD and BCL-xL expression and increased proapoptotic BAD expression. Accordingly, cell proliferation and colony cell assays and Annexin V-FITC apoptosis analysis demonstrated that the combination of 14–3–3ζ MUT (91–100 aa) transfection and enzalutamide treatment significantly enhanced the sensitivity of enzalutamide-resistant cell lines to enzalutamide (Fig. 5F–H; Supplementary Fig. S8C–S8E). Phosphorylation of 14–3–3ζ (Ser 184) by c-Jun N-terminal kinase (JNK) sustains dephosphorylation of BAD and leads to subsequent BAD-mediated apoptosis (20). We therefore examined whether the interaction between PGAM2 and 14–3–3ζ affected the phosphorylation status of 14–3–3ζ. C4-2R and 22Rv1 cells were transfected with Flag-PGAM2, sh-PGAM2, His-14–3–3ζ WT, or His-14–3–3ζ MUT (91–100 aa) and then cells were treated with either a JNK activator (anisomycin) or DMSO. As expected, inhibition of PGAM2 expression or mutation of 14–3–3ζ (91–100 aa) combined with anisomycin significantly enhanced the phosphorylation of 14–3–3ζ (Fig. 5I and J; Supplementary Fig. S8F and S8G). Collectively, these results indicate that PGAM2 directly interacts with 14–3–3ζ to activate antipapoptotic BAD/BCL-xL signaling in enzalutamide-resistant prostate cancer cells.

**PGAM2 is transcriptionally regulated by AR**

Androgen and AR play vital roles in tumorigenesis and in the development of prostate cancer. AR is a transcription factor that regulates the expression of a variety of genes; therefore, we first speculated that AR upregulates the transcription of PGAM2. The mRNA expression of AR and PGAM2 was evaluated in prostate cancer tissues before and after treatment with next-generation AR inhibitor (Fig. 6A). The result showed that the expression of AR and PGAM2 consistently decreased after treatment, which suggests a potential relationship between AR and PGAM2. To further investigate the link between AR and PGAM2, DHT, an AR agonist, was used to treat C4-2R sh-NC and C4-2R sh-PGAM2 cell lines (Fig. 6B). PGAM2 expression was respectively increased in C4-2R sh-NC and sh-PGAM2 cells by treatment with 20 μmol/L and 40 μmol/L DHT compared with DMSO control treatment. In addition, we utilized the CRISPR-Cas9 system or the PROTAC drug ARD-266 to knock out or degrade AR in C4-2R cells and confirmed that the expression of AR was inhibited. (Supplementary Fig. S9A and S9B). After treatment with DHT, the upregulation of PGAM2 mRNA expression was found to be blocked in C4-2R cells under conditions of AR inhibition, which is consistent with KLK3, an AR transcript. (Supplementary Fig. S9C and S9D).
Figure 4.
PGAM2 enzyme activity does not affect the sensitivity of enzalutamide-resistant cells to enzalutamide. A, The K100Q mutation decreases PGAM2 enzyme activity and the acetylation levels of the K100. B–D, Effects of PGAM2 K100Q mutation on cell proliferation and apoptosis of C4-2R and 22Rv1 cells under the treatment of enzalutamide (ENZ). E, The knockdown efficacy of siRNAs targeting PKM2 or PGK1 in C4-2R and 22Rv1 cells. F–H, Effects of knockdown of PGK1 and PKM2 expression on cell proliferation and apoptosis of C4-2R and 22Rv1 cells under the treatment of enzalutamide. I–K, Effects of inhibition of PGK1 and PKM2 by using small-molecule inhibitors on cell proliferation and apoptosis of C4-2R and 22Rv1 cells under the treatment of enzalutamide. L–M, ECAR from Seahorse analysis from C4-2R cells after treatment of indicated siRNAs or inhibitors. ns, not significant; *, P < 0.05; ***, P < 0.001.
Figure 5.

PGAM2 interacts with 14-3-3ζ. A, PGAM2-interacting proteins were purified by affinity purification using an anti-Flag antibody and visualized by silver staining. The proteins related to apoptosis were compared with the PGAM2-interacting proteins identified through mass spectrometry. B and C, Domain mapping of 14-3-3ζ amino acids that interact with PGAM2 detected by GST pulldown assays. D, Detection of protein interaction between Flag-PGAM2 and His-14-3-3ζ by coimmunoprecipitation. E, Expression of phosphorylated BAD, total BAD, and BCL-xL in His-14-3-3ζ-WT or His-14-3-3ζ-MUT (91-100 aa)-transfected C4-2R cells after treatment of enzalutamide (ENZ). F-H, Effects of 91-100 aa mutation of 14-3-3ζ on the phosphorylation status of 14-3-3ζ was examined in C4-2R cells treated with the JNK activator anisomycin. I-J, The impact of changes in PGAM2 expression and the 91-100 amino acid mutation of 14-3-3ζ on the phosphorylation status of 14-3-3ζ was examined in C4-2R cells treated with the JNK activator anisomycin. **, P < 0.01; ***, P < 0.001; ns, not significant.
Next, we used ChIP-seq public datasets to analyze AR binding to the PGAM2 promoter region. We identified an AR peak near the PGAM2 promoter present in several prostate cancer cells (Fig. 6C). To investigate the binding regions, we used Jaspar (https://jaspar.genereg.net/) to predict AR-binding sites in the PGAM2 promoter. Two binding sites were predicted (Fig. 6D) and were mutated in pGL3 plasmids containing 2,000 bp of sequence upstream of the first PGAM2 exon (pGL3-PGAM2 MUT 1 and pGL3-PGAM2 MUT 2). After transfection of C4-2R cells with pGL3-PGAM2 WT, pGL3-PGAM2 MUT 1, or pGL3-PGAM2 MUT 2, cells were treated with 10 nmol/L DHT. Fluorescein detection showed that transcriptional activity was significantly inhibited in C4-2R cells transfected with pGL3-PGAM2 MUT 1 or pGL3-PGAM2 MUT 2 compared with pGL3-PGAM2 WT transfaction (Fig. 6E). We next confirmed the presence of AR-binding sites in the PGAM2 promoter using ChIP-PCR in C4-2R cells. Specific primers for the region containing the predicted AR-binding sites were used to amplify the fragment pulled down by the anti-AR antibody (Fig. 6F). Our results demonstrated that AR upregulates PGMA2 expression in prostate cancer by directly binding to its promoter region and enhancing PGAM2 transcription.

**PGAM2 Knockdown Overcomes Enzalutamide Resistance**

To further examine the expression and clinical significance of PGAM2 in prostate cancer, the expression and prognostic value of PGAM2 were assessed in Sun Yat-sen University Cancer Center and public CRPC cohorts. Differences in PGAM2 expression were present in the CRPC cohorts (Fig. 7A). Patients with high PGAM2 expression rapidly progressed to enzalutamide resistance compared with patients with low PGAM2 expression (Fig. 7B). Analysis of single-cell datasets revealed that PGAM2 expression in malignant CRPC cells...
Figure 7. 
PGAM2 is associated with rapid resistance to enzalutamide (ENZ) and worse prognosis in patients with CRPC. 
A, Representative images showing IHC images of PGAM2 in patients with CRPC. Magnification, ×40 (left), ×200 (right). Scale bar, 100 μm. 
B, Kaplan-Meier analysis in SYSUCC cohort revealed that patients with CRPC with high PGAM2 expression had worse failure-free survival. 
C, PGAM2 was highly expressed in CRPC samples than that in benign prostate hyperplasia and was mainly enriched in malignant cells in published single-cell datasets. 
D, PGAM2 expression was negatively associated with apoptosis scores of malignant cells in a published single-cell dataset (GSE137829). 
E, In a published CRPC cohort (GSE35988), patients with higher PGAM2 expression had worse overall survival. 
F, The schematic illustrates the mechanism by which PGAM2 regulates resistance to enzalutamide in prostate cancer.
PRRX2 and BCL-2 were also enriched in our screen, although not the
tance via activation of the E2F and BCL2 pathways (24). Consistently,
guez and colleagues found that PRRX2 mediates enzalutamide resis-
ted in our genome-wide CRISPR screen. The use of different cell lines as
CRISPR screening identi ed enzalutamide resistance in prostate cancer. Previous kinome-wide
genome-wide CRISPR screen as a promising target to overcome
interest in cancer progression. In this study, we identi ed PGAM2 as a promising thera-
pic role for enzalutamide-resistant prostate cancer.

Discussion

Metabolic reprogramming is a hallmark of cancer and is believed to
provide new opportunities for cancer therapy (21). Recently, the
nonmetabolic activities of metabolic enzymes have aroused intense
interest in cancer progression. In this study, we identi ed PGAM2 in a
genome-wide CRISPR screen as a promising target to overcome
enzalutamide resistance in prostate cancer. Previous kinome-wide
CRISPR screening identi ed CK1 and BRAF as promising targets
to overcome enzalutamide resistance of prostate cancer (22, 23).
Although CK1 was not enriched in our screen, BRAF was identi ed
in our genome-wide CRISPR screen. The use of different cell lines as
well as different CRISPR libraries could be one of the reasons for this
discrepancy. Using a genome-wide CRISPR activation screen, Rodri-
guez and colleagues found that PRRX2 mediates enzalutamide resis-
tance via activation of the E2F and BCL2 pathways (24). Consistently,
PRRX2 and BCL-2 were also enriched in our screen, although not the
highest ranked.

Furthermore, our study provides the rst evidence of a non-
metabolic function of PGAM2 in the cellular processes of cancer.
By interacting with 14–3–3, PGAM2 promotes activation of
the antiapoptotic protein BCL-xL and subsequent resistance to
enzalutamide-induced apoptosis. Our study suggests that PGAM2
is a promising therapeutic target for enzalutamide-resistant prostate
cancer.

Previous studies of PGAM in tumorigenesis and cancer progression
have mainly focused on the metabolic activity of PGAM and its
associated metabolites (25–27). In triple-negative breast cancer,
PGAM1 promotes the proliferation of cancer cells, and inhibition of
its metabolic activity signi cantly inhibits cancer cell proliferation (25).
PGAM1 de ciency also promotes pancreatic adenocarcinoma growth
by inducing 3-PG accumulation and subsequent enhancement of
serine biosynthesis (27). PGAM1 is overexpressed in hepatocellular
carcinoma tissues, indicating an important role of PGAM1 in hepatic
carcinogenesis (26). We found that inhibiting PGAM2 mainly
enhances enzalutamide-induced apoptosis in enzalutamide-resistant
prostate cancer cells. In fact, most studies demonstrated that inhibiting
the metabolic activity of metabolic enzymes primarily in uences cell
proliferation, especially in cancer cells. In addition to metabolic
activity, metabolic enzymes play noncanonical roles in cellular pro-
cesses. The nonmetabolic functions of metabolic enzymes include:
(i) acting as protein kinases in posttranslational modi cations of down-
stream proteins (28); (ii) in uencing the functions of downstream
proteins through protein–protein interactions (12); (iii) Altering
subcellular localization so that its metabolites are directly involved
in nonmetabolic regulatory activities such as epi-modi cation and
transcription factor regulation (29); and (iv) Altering the tumor
microenvironment through secretion of factors from exosomes into
the extracellular compartment (30). By mutating the enzyme active site
or by using small-molecule inhibitors, we found that altering the
glycolysis rate or metabolites had no effect on enzalutamide sensitivity
in prostate cancer cells. Therefore, the nonenzymatic activity of
PGAM2 plays a critical role in enzalutamide resistance.

Targeting BCL2 proteins is an effective strategy to induce apoptosis
and sensitize CRPC cells to enzalutamide. Enzalutamide treatment
causes phosphorylation of BAD, the endogenous antagonist of BCL-
xL, and thereby disrupts its triggering of apoptosis. Inhibiting
the phosphorylation of BAD results in robust apoptosis in response to
enzalutamide in CRPC cell lines; therefore, inhibition of BAD phos-
phorylation may be a promising therapeutic strategy to overcome
enzalutamide resistance. BAD is normally phosphorylated by survival-
promoting kinases (AKT and PKA) and is sequestered in an inactive
form through interactions with 14–3–3 proteins (31). 14–3–3 proteins
play a critical role in the regulation of BAD phosphorylation in prostate
cancer cells. We found that the interaction of PGAM2 and 14–3–3 altered the balance of BAD/p-BAD, which revealed an intervention
target to induce apoptosis in enzalutamide-resistant prostate cells.
In addition, our results also showed that PGAM2 interacted with 14–3–3 to inhibit JNK-mediated phosphorylation of 14–3–3 and
apoptosis induced by enzalutamide. These results indicate a potential
therapeutic role for a JNK inhibitor in enzalutamide-resistant pro-
state cancer. Consistent with our results, a previous study found that
the JNK inhibitor AS602801 synergizes with enzalutamide to induce
apoptosis in prostate cancer both in vivo and in vitro (32). Our results
provide a new therapeutic strategy for the treatment of enzalutamide resistance, and clinical trials are needed to validate the safety and
effectiveness of such an approach.

AR reactivation, which includes AR amplification, mutation, spli-
cosome action, and steroid pathway activation of AR signaling, is
the main mechanism of prostate cancer resistance to next-generation AR
inhibitors (33). Our results suggest that AR regulates several metabolic enzymes at the transcriptional level. Our results showed that AR upregulates PGAM2 transcription.
This result deepens the understanding of the mechanisms of AR-
regulated enzalutamide resistance.

There are several limitations of our study. First, the expression of
PGAM2 before and after enzalutamide resistant could not be
assessed because tissue specimens were not available. In addition,
the lack of validated small-molecule PROTACs targeting PGAM2
makes it impossible to assess the efficacy of PGAM2 degradation for
the treatment of enzalutamide-resistant prostate cancer. Finally, our
studies are limited to xenograft mouse models, which are unable to
represent the biological heterogeneity of prostate cancer and there-
fore in uence the translation of our studies to humans.

In summary, our study reveals a nonenzymatic function of PGAM2
in enzalutamide resistance, and this function is associated with 14–3–
3 and BAD-mediated apoptosis of prostate cancer cells. We also
suggest that PGAM2 is a potential therapeutic target for improving the
antitumor ef icacy of enzalutamide in enzalutamide-resistant prostate
cancer. Future clinical studies targeting PGAM2 in patients with
enzalutamide-resistant prostate cancer are expected to further con-
firm our preclinical data.

Authors’ Disclosures

No disclosures were reported.
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**Authors’ Contributions**

Z. Li: Formal analysis, investigation, writing—original draft. K. Ning: Formal analysis, investigation, writing—original draft. D. Zhao: Formal analysis, investigation, writing—original draft. J. Zhao: Investigation. X. Long: Investigation. Z. Yang: Formal analysis. D. Chen: Data curation, formal analysis. X. Cai: Data curation. L. Hong: Investigation. L. Zhang: Investigation. F. Zhou: Conceptualization, resources, supervision. J. Wang: Conceptualization, resources, supervision, funding acquisition, writing—review and editing. Y. Li: Conceptualization, resources, supervision, funding acquisition, writing—review and editing.

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**Note**

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