PLCε knockdown enhances the radiosensitivity of castration-resistant prostate cancer via the AR/PARP1/DNA-PKcs axis

JUN PU[1], TING LI[2], NANJING LIU[2], CHUNLI LUO[2], ZHEN QUAN[1], LUO LI[2] and XIAOHOU WU[1]

[1]Department of Urology, The First Affiliated Hospital of Chongqing Medical University;
[2]Key Laboratory of Diagnostics Medicine Designated by The Ministry of Education, Chongqing Medical University, Chongqing 400016, P.R. China

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Abstract. Radiotherapy (RT) has been used as a therapeutic option for treatment of prostate cancer (PCa) for a number of years; however, patients frequently develop RT resistance, particularly in castration-resistant PCa (CRPC), although the underlying mechanisms remain unknown. Understanding the underlying mechanism of RT resistance in CRPC may potentially highlight novel targets to improve therapeutic options for patients with PCa. In the present study, the expression levels of phospholipase Cε (PLCε), androgen receptor (AR) and DNA-dependent protein kinase catalytic subunit (PKcs) were examined in PCa tissue samples and PCa cells, and the effects of PLCε knockdown on AR and DNA damage repair (DDR)-related molecules were determined. The association between PLCε, AR and Poly (ADP-ribose) polymerase 1 (PARP1), as well as their respective roles in radiation resistance, were assessed using gene knockdown and pharmaceutical inhibitors or activators. A chromatin immunoprecipitation assay was used to determine the epigenetic regulatory effects of PLCε on PARP1. Animal experiments were performed to assess whether the mechanisms observed *in vitro* could be replicated *in vivo*. The expression levels of PLCε, AR and DNA-PKcs were significantly upregulated in PCa, particularly in CRPC. PLCε knockdown reduced the viability and increased apoptosis of cells subjected to radiation. Additionally, PLCε deficiency suppressed DDR progression by downregulating an AR and PARP1 positive feedback loop and the associated downstream molecules following radiation. PLCε depletion also increased the presence of histone H3 lysine 27 trimethylation in the PARP1 promoter region, suggesting increased methylation of the PARP1 gene and thus resulting in reduced expression of PARP1. *In vivo*, PLCε knockdown significantly potentiated the effects of radiation on tumor growth. Taken together, the results of the present study demonstrated that PLCε knockdown enhanced the radiosensitivity of CRPC by downregulating the AR/PARP1/DNA-PKcs axis.

Correspondence to: Professor Xiaohou Wu, Department of Urology, The First Affiliated Hospital of Chongqing Medical University, 1 YouYi Road, Yuzhong, Chongqing 400016, P.R. China
E-mail: wuxiaohou@hospital.cqmu.edu.cn

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Introduction

Prostate cancer (PCa) is one of the most prevalent malignant tumors in males worldwide. In 2017, the estimated number of new cases and deaths from PCa in the United States were 161,360 and 26,730, respectively (1). Cancer cells often exhibit altered epigenetic signatures, which results in dysregulation of expression of genes involved in processes such as transcription, proliferation, apoptosis and DNA repair (2). Radiation therapy (RT) is an important treatment approach for PCa; it can be performed at any stage of PCa as a curative monotherapy or as an adjuvant with other therapeutic options (3). However, 30-50% patients with high-risk PCa exhibit local relapse following radiation therapy (4). Therefore, determining the mechanisms underlying the development of resistance to RT and a means of enhancing the sensitivity of PCa to RT may improve patient outcomes.

Phospholipase Cε (PLCε) is a critical signaling hub that regulates a variety of biological processes in cells (5). In contrast to the other members of the PLC family, PLCε exhibits PLC enzyme activities and serves a role as a guanine nucleotide exchange factor (6). Previous studies have reported that high expression levels of PLCε are closely associated with the initiation and progression of various types of cancer, particularly in the upper gastrointestinal tract (7,8). This finding has been further verified by two genome-wide studies demonstrating that the PLCε gene is an essential tumor promoter in esophageal squamous cell carcinoma (9). Our previous study demonstrated that PLCε expression was significantly upregulated in PCa tissue compared with normal prostate tissue and that PLCε was capable of enhancing the proliferative ability and metastatic potential of PCa cells via various pathways (10-12). However, the association between PLCε and RT resistance in PCa has not been determined.

Androgen receptor (AR) is crucial for the development of PCa (13,14). Following ligand binding, the AR-ligand complex translocates to the nucleus, binds to the DNA and subsequently alters downstream gene transcription (15). Biologically, androgen deprivation therapy (ADT) abrogates the ability of AR to promote cellular DNA damage repair (DDR) (16,17). ADT-mediated blocking of the AR pathway results in an inability of PCa cells to effectively activate DDR; therefore, a combination of ADT and RT may promote the lethality of RT and initiate DNA damage (16-18). Poly (ADP-ribose) polymerase 1 (PARP1) is a member of the PARP superfamily, and it exerts a large number of cellular activities, such as gene transcription, chromatin remodeling and cell death (19,20). In addition, PARP1 can be recruited to AR functional sites and facilitate AR occupancy and function (21).

The present study explored the underlying mechanisms between PLCε and radiosensitivity in primary PCa (PPC) and castration-resistant PCa (CRPC) using *in vitro* and *in vivo* models. The aim of the present study was to establish whether PLCε knockdown enhanced the radiosensitivity of CRPC via the AR/PARP1/DNA-PKcs axis.

Materials and methods

Patients and specimens. A total of 30 samples of benign prostatic hyperplasia (BPH), 35 samples of PPC and 27 samples of CRPC were collected from patients who underwent prostate biopsy, TURP or radical prostatectomy at the Department of Urology at the First Affiliated Hospital of Chongqing Medical University (Chongqing, China) between September 2015 and August 2017. All patients provided written informed consent, and the protocol was approved by the Ethical Committee of the First Affiliated Hospital of Chongqing Medical University. PCa samples were histologically graded according to the criteria of EAU guidelines (22). Adjacent normal prostate tissues (10 mm from the malignant locus) were also collected from patients with unifocal lesions and were verified by pathologists. BPH samples were verified by histological examination. Specimens were stored in liquid nitrogen until further use.

Immunohistochemistry (IHC). Prostate tumor and BPH tissues were fixed in 10% neutral buffered formalin (Sigma-Aldrich; Merck KGaA) diluted with 0.01 M PBS buffer (pH 7.4; OriGene Technologies, Inc.) for 24 h at 4˚C, embedded in paraffin and cut into 4-μm sections. Antigen retrieval was performed in citrate buffer (pH 6.0) for 15 min at 98˚C, and the sections were subsequently blocked with normal goat serum (Beyotime Institute of Biotechnology) for 30 min at 37˚C. The sections were incubated overnight with the primary antibody targeting PLCε (1:50; cat. no. sc-28402; Santa Cruz Biotechnology, Inc.), AR (1:400; cat. no. 5153; CST Biological Reagents Co., Ltd.) and DNA-dependent protein kinase catalytic subunit (DNA-PKcs; 1:100; cat. no. ab168854; Abcam) at 4˚C, washed with PBS and subsequently incubated with the biotin-streptavidin horseradish peroxidase labeled Goat anti-rabbit IgG (1:300; cat. no. SP-9001; Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.) for 1 h at room temperature. Signals were visualized using a peroxidase substrate and hematoxylin counterstaining for 1 min at room temperature. Sections were semi-quantitatively scored for staining intensity as follows: 0, no staining; 1, weak staining; 2, light staining; 4, moderate staining; and 6-8, strong staining. Staining scores ≥2 were regarded as positive expression, whereas scores <2 were considered negative.

Cell lines, transfection and agents. PPC cell line LNCaP was purchased from American Type Culture Collection. Mycoplasma testing was performed using a Mycoplasma Detection kit (Beijing Solarbio Science & Technology Co., Ltd.). Bicalutamide®-resistant cells (Bica-R) and Enzalutamide®-resistant cells (Enza-R) were developed by treating LNCaP cells with Bicalutamide® and Enzalutamide® in our laboratory between October 2016 and February 2017 and were used as CRPC cell lines. To develop resistance, LNCaP cells were cultured with 1, 5, 10 or 25 μM bicalutamide or enzalutamide. After a month of screening, a dose of 10 μM was selected as the optimal concentration for subsequent induction of CRPC cells. All cells were cultured in DMEM/F-12 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 2 mM L-glutamine and 100 U/ml penicillin at 37˚C with 5% CO_2 in a humidified incubator. The lentiviral vectors non-targeting LV-control (LV-Ctrl) and LV-short hairpin (sh)RNA targeting PLCε (shPLCε) were purchased from Shanghai GenePharma Co., Ltd. Other reagents used were as follows: DMSO (Sigma-Aldrich; Merck KGaA); AR inhibitor ARN-509 (38 nM; Medchem Express); PARP1 inhibitor AZD2281 (0.5 μM; Selleck Chemicals); AR activator DHT (10 nM; Sigma-Aldrich; Merck KGaA) and enhancer of zeste homolog 2 (EZH2) inhibitor 3-deazaneplanocin A (DZNeP; 1 μM; Selleck Chemicals).

Cell irradiation and MTT assays. LNCaP, Enza-R and Bica-R cells ($3x10^3$ cells/well) were plated in a 96-well plate in 100 μl medium. Following adhesion, radiation was delivered at room temperature using an x-6 MV photon linear accelerator (CD2300; Varian Corporation). A total single dose of 2, 4, 6 or 8 Gy was delivered with a dose rate of 300 cGy/sec using a source-to-surface distance of 100 cm. Irradiated cells were incubated for 24 h at 37˚C; subsequently, 5 mg/ml MTT (Sigma-Aldrich; Merck KGaA) was added to each well, and the cells were further incubated for 4 h at 37˚C. After removing the medium, 100 μl DMSO was added to each well, and the plates were agitated on a rotator for 10 min at room temperature. Absorbance was recorded using an ultraviolet spectrophotometric reader at a wavelength of 490 nm. The MTT experiments were repeated five times.

Flow cytometric analysis. LNCaP, Enza-R and Bica-R cells ($1x10^6$ cells/well) were plated in 6-well plates and cultured to 60% confluence for cell cycle analysis. Serum-free medium was added, and the cells were cultured for a further 24 h at 37˚C. Following the administration of 6 Gy radiation, cells were collected and fixed with 75% ethanol overnight at 4˚C. The distribution of cells in different stages of the cell cycle was detected using a PN B49007AD flow cytometer (Beckman Coulter Co., Ltd.) and analyzed using FlowJo version 7.6.2 (FlowJo, LLC). Cell cycle distribution analysis experiments were performed in triplicate.

Apoptosis analysis. LNCaP, Enza-R and Bica-R cells ($1x10^6$ cells/well) were plated in 6-well plates and cultured to

60% confluence for apoptosis analysis. Cells were irradiated at room temperature as described above. After 24 h, cells were washed with PBS and detached with 2.5 mM EDTA in PBS. Annexin V and 7-amino-actinomycin D (7-AAD; both from Sungene Biotech Co., Ltd) staining was performed according to the manufacturer's instructions. Staining was quantified using flow cytometry (CytoFLEX; Beckman Coulter, Inc.) by analyzing Annexin V-fluorescein isothiocyanate (FITC) and 7-AAD fluorescence using phycoerythrin emission signal detectors and FlowJo V10 software (FlowJo LLC).

Colony formation assay. LNCaP, Enza-R and Bica-R cells (400 cells/well) were seeded in 6-well plates, and the culture medium was changed every 2 days. Following 14 days of culture, cells were washed twice with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. Colonies were stained with Giemsa solution for 15 min at room temperature, washed twice with PBS and air-dried. The number of colonies were counted using a light microscope (magnification, x40). Colony formation efficiency (CFE) was calculated as follows: CFE (%)=(number of colonies/400) x100. Colony formation assays were performed in triplicate.

Immunofluorescence. Enza-R and Bica-R cells ($1x10^5$ cells/well) were seeded on polylysine coated coverslips and cultured for 24 h. Cells were washed with PBS, fixed in 4% paraformaldehyde for 15 min at 4˚C, permeabilized with PBS/0.1% Triton X-100 and blocked with 5% BSA containing 1% Tween-20 for 30 min at 37˚C. Immunostaining was performed with the primary antibody targeting AR (1:200; cat. no. 5153; CST Biological Reagents Co., Ltd.) overnight at 4˚C and a goat anti-rabbit IgG-AlexaFluor 488 secondary antibody diluted in goat serum (1:500; cat. no. SR134; Beijing Solarbio Science & Technology Co., Ltd.) for 1 h at 37˚C. Immunofluorescence images were acquired using fluorescence microscopy using a Nikon Eclipse 80i microscope (Nikon Corporation; magnification, x400).

Western blotting. Patient tissues were ground repeatedly and lysed using RIPA buffer (Beyotime Institute of Biotechnology) containing 0.1% PMSF. LNCaP, Enza-R and Bica-R cells were lysed using RIPA lysis buffer containing protease inhibitors and 1 mM Na_3VO_4. Nuclear and cytoplasmic proteins were extracted with the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Institute of Biotechnology). Proteins were quantified using the bicinchoninic acid assay (Beyotime Institute of Biotechnology) and 3 μg protein/lane was resolved using SDS-PAGE (NuPAGE 3-8% Tris-Acetate or NuPAGE 4-12% BisTris; Invitrogen; Thermo Fisher Scientific, Inc.) and transferred to PVDF membranes (EMD Millipore). Following blocking with 5% skimmed milk for 2 h at room temperature, the membranes were incubated with primary antibodies targeting PLCε (1:500; cat. no. sc-28402; Santa Cruz Biotechnology, Inc.), AR (1:1,000; cat. no. 5153; CST Biological Reagents Co., Ltd.), PARP1 (1:1,000; cat. no. ab32138; Abcam), DNA-dependent protein kinase catalytic subunit (DNA-PKcs; 1:1,000; cat. no. ab168854; Abcam), H2AX (1:1,000; cat. no. 7631; CST Biological Reagents Co., Ltd.), γ-H2AX (1:1,000; cat. no. 7631; CST Biological Reagents Co., Ltd.) and GAPDH (1:1,000; cat. no. 5174; Cell Signaling Technology) overnight at 4˚C, washed with TBS + 0.05% Tween-20 (Beijing Solarbio Science & Technology Co., Ltd.), and subsequently incubated with a horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G secondary antibody (1:2,000; cat. no. TA130015; OriGene Technologies, Inc.) for 1 h at room temperature. The signals were visualized using the Enhanced Chemiluminescence kit (EMD Millipore), and the intensity of each band was quantified on X-ray films and densitometry analysis was performed using imaging software Quantity One version 4.6.2 (Bio-Rad Laboratories, Inc.).

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from LNCaP, Enza-R and Bica-R cells and ground tissue using TRIzol® (Takara Bio, Inc.) according to the manufacturer's protocol. RNA quality was determined using a 2100 BioAnalyzer (Agilent Technologies GmbH). cDNA synthesis was performed using a PrimeScript® RT reagent kit (Takara Bio, Inc.) according to the manufacturer's protocol, and the reverse transcription temperature protocol was 15 min at 37˚C and 5 sec at 85˚C. SYBR PremixExTaq™ II kit (Takara Bio, Inc.) was used for RT-qPCR with the CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.). The sequences of the primers were as follows: PLCε forward, 5'-GCAACT ACAACGCTGTCATGGAG-3' and reverse, 5'-GCAACT ACAACGCTGTCATGGAG-3'; AR forward, 5'-CCTACG GCTACACTCGG-3' and reverse, 5'-CTGGCAGTCTCC AAACG-3'; H2AX forward, 5'-AGTGCTGGAGTACCTCAC CG-3' and reverse, 5'-CACGGCCTGGATGTTGG-3'; PARP1 forward, 5'-TTTCCATCAAACATGGGCGAC-3' and reverse, 5'-CGGAGTCTTCGGATAAGCTCT-3'; DNA-PKcs forward, 5'-CGGACCTACTACGACTG-3' and reverse, 5'-AGACAA AGGGTGGAAA-3'; and GAPDH forward, 5'-ACGGAT TTGGTCGTATTGGGCG-3' and reverse, 5'-CCTCCTGGA AGATGGTGATGG-3'. The thermocycling conditions were as follows: 95˚C for 30 sec, followed by 41 cycles of 95˚C for 10 sec, 60˚C for 30 sec and 72˚C for 30 sec. The mRNA expression levels were calculated using the comparative $2^{-\Delta\Delta Cq}$ method (10) and GAPDH served as the reference. All gene expression experiments were repeated at least three times.

Chromatin immunoprecipitation (ChIP). A SimpleChIP® Enzyme Chromatin IP kit (Magnetic Beads; cat. no. 9003; CST Biological Reagents Co., Ltd.) was used according to the manufacturer's protocol. Enza-R cells (~$4x10^6$) were cross-linked with 1% formaldehyde and uncross-linked using glycine. Chromatin was digested to ~500 bp fragments and centrifuged at 9,400 x g for 10 min at 4˚C. Chromatin fragments were mixed with antibody targeting histone H3 lysine 27 (H3K27) trimethylation (H3K27me3; 1:50; cat. no. 9733) and control antibody (rabbit IgG; 1:100; cat. no. both from 2729; CST Biological Reagents Co., Ltd.), and incubated overnight at 4˚C. After DNA purification and elution, DNA enrichment was detected using RT-qPCR.

Subcutaneous xenograft assay. Male 6-week-old NOD. Cg-Prkdc^{scid} mice were housed in a barrier facility, and the experiments were approved by the Chongqing Medical University Institutional Animal Care and Use Committee and the Animal Ethics Committee. A total of 5 mice/group were used, which was determined using power analysis. For the xenograft assay,

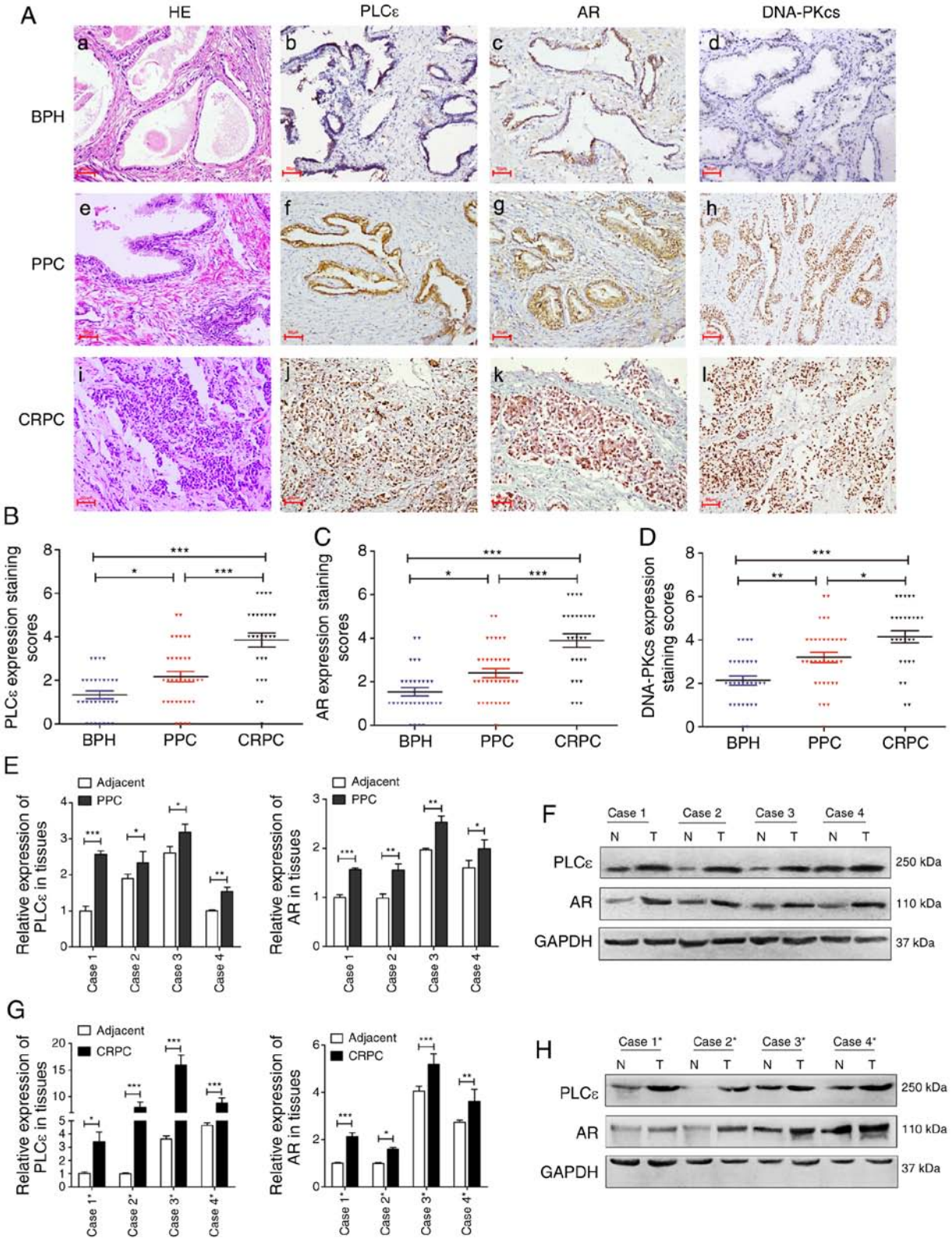

Figure 1. Expression of PLCε, AR and DNA-PKcs in PCa. (A) Hematoxylin and eosin and immunohistochemistry staining of BPH, PPC and CRPC tissues. Magnification, x200. (B-D) Staining scores for (B) PLCε, (C) AR and (D) DNA-PKcs. (E and F) Expression of PLCε and AR in four PPC specimens. (G and H) Expression of PLCε and AR in four CRPC specimens. *P<0.05, **P<0.01 and ***P<0.001. PCa, prostate cancer; PLCε, phospholipase Cε; AR, androgen receptor; BPH, benign prostatic hyperplasia; PPC, primary PCa; CRPC, castration-resistant PCa; DNA-PKcs, DNA-dependent protein kinase catalytic subunit.

$1x10^6$ Bica-R cells were injected subcutaneously into the right flank of the mice. When the tumors reached 1 mm^3 in volume, mice were irradiated with 2 Gy/day for 5 days. Body weight and tumor size were measured using calipers twice a week for 20 days after irradiation. At the end of the experiment, the mice were sacrificed by carbon dioxide asphyxiation.

Table I. Clinicopathological characteristics of patients with prostate cancer.

Variable	Overall	PLCε			AR		
		Negative	Positive	P-value	Negative	Positive	P-value
Total, n (%)	62	12	50		14	48	
Age, years				0.375			0.647
Median	75	76	73		75	74	
IQR	65-79	64-80	61-77		64-79	63-78	
PSA in PPC, μg/l	35	7/35 (20)	28/35 (80)	0.658	9/35 (26)	26/35 (74)	0.865
Median	93.56	74.49	142.53		76.34	150.86	
IQR	32.46-243.79	16.53-175.26	75.38-250.16		18.61-197.95	86.32-264.78	
PSA in CRPC, μg/l	27	5/27 (19)	22/27 (81)	0.386	6/27 (22)	21/27 (78)	0.452
Median	40.32	42.27	37.85		41.87	38.65	
IQR	20.18-156.97	22.45-187.35	18.94-100.32		20.98-169.76	19.67-97.48	
Gleason score in PPC	35			0.610			>0.999
<7	25/35 (71)	3/5 (60)	22/30 (70)		7/9 (77)	18/26 (69)	
≥7	10/35 (29)	2/5 (40)	8/30 (30)		2/9 (23)	8/26 (31)	
Gleason score in CRPC	27			0.011[a]			0.017[a]
<7	8/27 (30)	5/7 (71)	3/20 (15)		4/5 (80)	4/22 (18)	
≥7	19/27 (70)	2/7 (29)	17/20 (85)		1/5 (20)	18/22 (82)	

[a]P<0.05. PLCε, phospholipase Cε; AR, androgen receptor; IQR, interquartile range; PSA, prostate-specific antigen; PPC, primary prostate cancer; CRPC, castration-resistant prostate cancer.

Statistical analysis. Statistical analysis was performed using SPSS version 22.0 (IBM Corp.). Data are presented as the mean ± standard deviation of at least three independent experiments. For analysis of the differences between two groups, unpaired Student's t-test were used to determine statistical significance. For multiple comparisons, ANOVA and Tukey-Kramer (comparisons among multiple groups) and Dunnett's (comparisons of multiple groups with the control group) post-hoc analysis were used. Correlations were calculated using Spearman's correlation coefficient. Mann-Whitney test was used for the analysis of two independent variables. P<0.05 was considered to indicate a statistically significant difference.

Results

PLCε, AR and DNA-PKcs expression in PCa. To identify potential associations among PLCε, AR and DNA-PKcs during the development of PCa, the expression levels of these proteins were determined in clinical samples of BPH, PPC and CRPC by IHC. The PPC and CRPC tissues exhibited positive staining for PLCε, AR and DNA-PKcs (Fig. 1A). The staining scores of PLCε, AR and DNA-PKcs were significantly higher in CRPC compared with BPH and PPC (Fig. 1B-D). A total of four PPC and CRPC samples each, with their corresponding adjacent normal tissues serving as controls, were used to determine the mRNA and protein expression levels. The mRNA expression levels of PLCε and AR were significantly increased in all PPC and CPRC samples compared with the adjacent tissues (Fig. 1E and G). The western blotting results demonstrated that the expression levels of PLCε and AR in PPC and CRPC samples were upregulated compared with the adjacent normal tissues (Fig. 1F and H). The differences between the clinicopathological parameters and the expression of activated PLCε or AR were determined; significant differences in Gleason score between the positive and negative staining groups of PLCε and AR were observed (10) in CRPC; no differences were observed in the other clinicopathological characteristics (Table I).

Upregulated expression levels of PLCε and AR are associated with RT resistance in PCa. To verify the characteristics of the CRPC (Bica-R and Enza-R) cells, MTT assays were performed to detect the half maximal inhibitory concentration (IC_{50}) of Bicalutamide or Enzalutamide in Bica-R and Enza-R cells. The results demonstrated that Bica-R cells exhibited a 43-fold increased resistance to Bicalutamide, and Enza-R cells exhibited a 53-fold increased resistance to Enzalutamide compared with the parental LNCaP cells (Fig. 2A). The mRNA and protein expression levels of PLCε and AR were determined by RT-qPCR and western blot analysis in LNCaP, Bica-R and Enza-R cells; the results revealed that the levels of PLCε and AR were increased in the two CRPC cell lines compared with the LNCAP PPC cell line (Fig. 2B and C). To investigate the molecular mechanisms by which RT resistance occurred in CRPC, LNCaP, Bica-R and Enza-R cells were treated with increasing doses (0-8 Gy) of radiation. MTT assays were performed after 24 h of irradiation. The results demonstrated that irradiation reduced cell survival, and cell survival of Bica-R and Enza-R cells was significantly increased compared with that of LNCaP cells at each dose of radiation (Fig. 2E). To probe the molecular mechanism underlying this phenomenon, RT-qPCR was

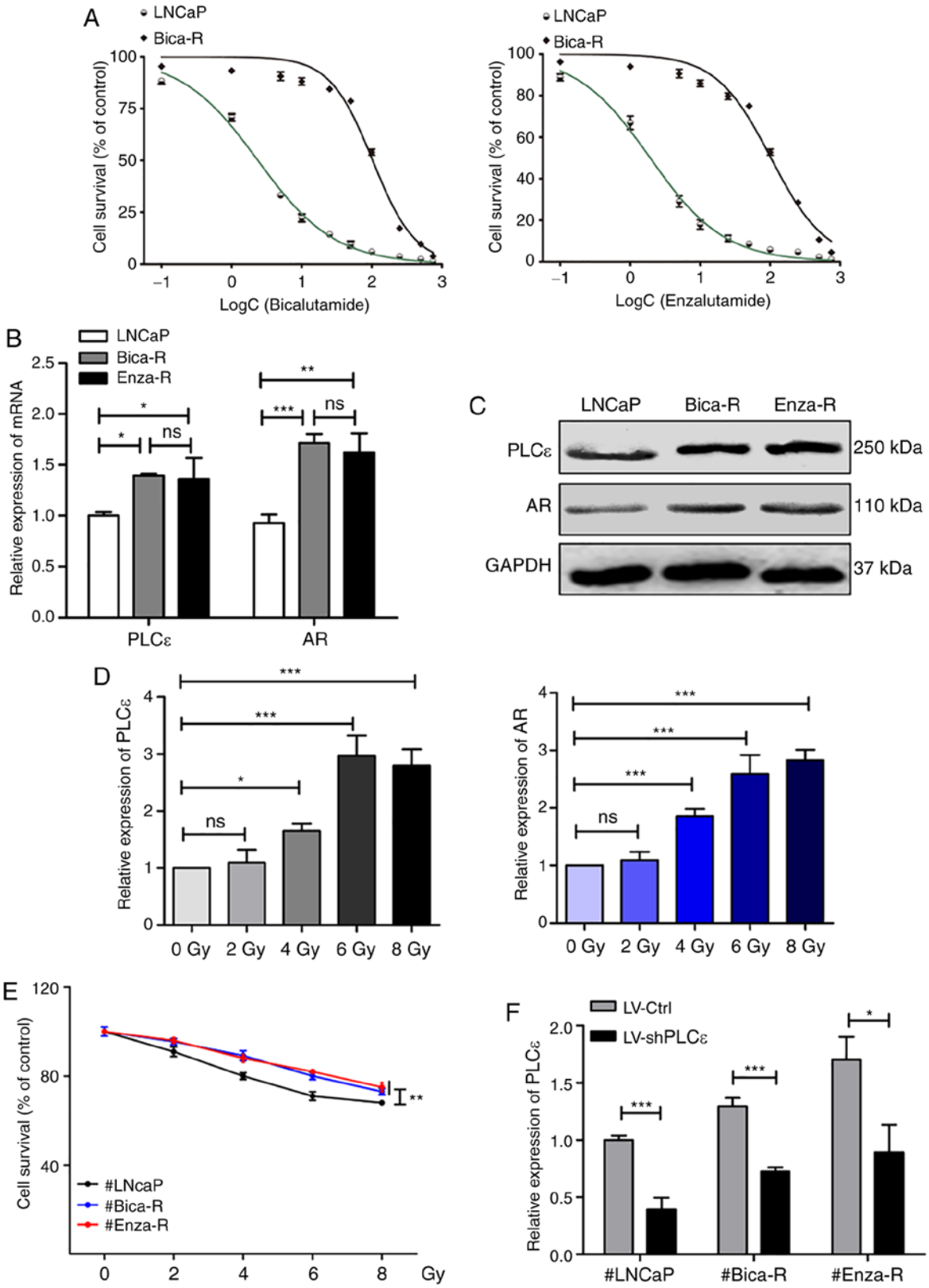

Figure 2. PLCε and AR expression are associated with RT resistance. (A) Half maximal inhibitory concentrations of PCa cell lines to bicalutamide and enzalutamide were determined using an MTT assay. (B and C) Expression of PLCε and AR in PCa cell lines. (D) Expression of PLCε and AR in cells treated with increasing doses of radiation. (E) Proliferation of cells treated with increasing doses of radiation measured using an MTT assay following 24 h of irradiation. (F) Efficiency of PLCε knockdown.

performed to measure the expression levels of PLCε and AR. The results showed that expression of PLCε and AR increased as the dose of radiation increased (Fig. 2D). Based on the results of MTT and RT-qPCR assays, 6 Gy was selected as the optimal dose of radiation for subsequent experiments. To confirm the role of PLCε in RT resistance, PLCε was

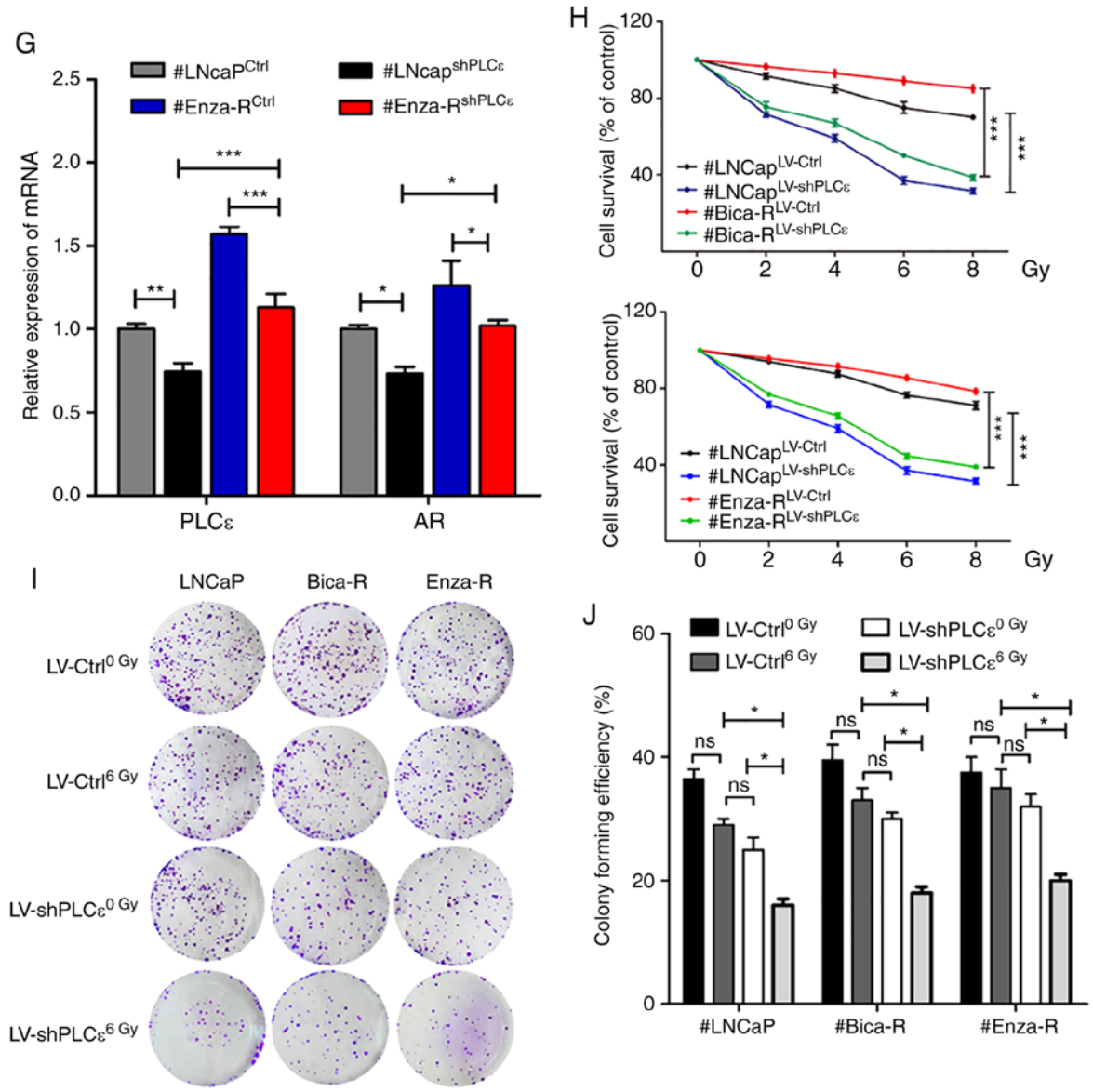

Figure 2. Continued. PLCε and AR expression are associated with RT resistance. (G) PLCε and AR expression levels in cells transfected with lentiviral shPLCε of control following treatment with 6 Gy of radiation. (H) Viability of cells transfected with lentiviral shPLCε or control treated with increasing doses of radiation. (I and J) Colony formation of PLCε-knockdown cells following treatment with or without radiation. '#' indicates irradiated cells. *P<0.05, **P<0.01 and ***P<0.001. PCa, prostate cancer; PLCε, phospholipase Cε; AR, androgen receptor; RT, radiotherapy; sh, short hairpin.

silenced in LNCaP, Bica-R and Enza-R cells using shPLCε, and PLCε mRNA levels were measured to evaluate the gene knockdown efficiency (Fig. 2F). Additionally, the mRNA levels of AR were assessed, and the results demonstrated that the mRNA levels of AR were decreased following shPLCε transfection in Enza-R, but not LNCaP cells (Fig. 2G). Inhibition of cell viability in the shPLCε-transfected cells compared with the control cells following radiation was also observed (Fig. 2H). In addition, PLCε knockdown or radiation alone inhibited cell colony formation, and PLCε knockdown enhanced the inhibitory effect of radiation on cell colony formation (Fig. 2I and J). Flow cytometry analysis revealed similar results; either shPLCε knockdown or radiation alone resulted in an increase in the percentage of cells in the G2/M phase (Fig. 3A) and increased apoptosis (Fig. 3B) compared with the control, and the combination of the two treatments exhibited an enhanced effect. Therefore, it was concluded that PLCε served a crucial role in RT resistance of CRPC cells.

PLCε deficiency impairs DNA damage repair by preventing unclear translocation of AR and inhibiting the AR/DNA-PKcs pathway in CRPC. To determine whether PLCε down-regulation enhanced the sensitivity of CRPC to radiotherapy through DDR, the mRNA and protein expression levels of AR, DNA-PKcs and H2AX were determined following PLCε knockdown in CRPC cells. The results of RT-qPCR demonstrated that PLCε knockdown significantly reduced AR and DNA-PKcs expression, but exhibited no significant effects on H2AX (Fig. 3C). Western blotting revealed similar alterations in the expression of AR, DNA-PKcs and H2AX at the protein level, but the level of γ-H2AX was significantly downregulated in the shPLCε group compared with the control group (Fig. 3D). Immunofluorescence results demonstrated that

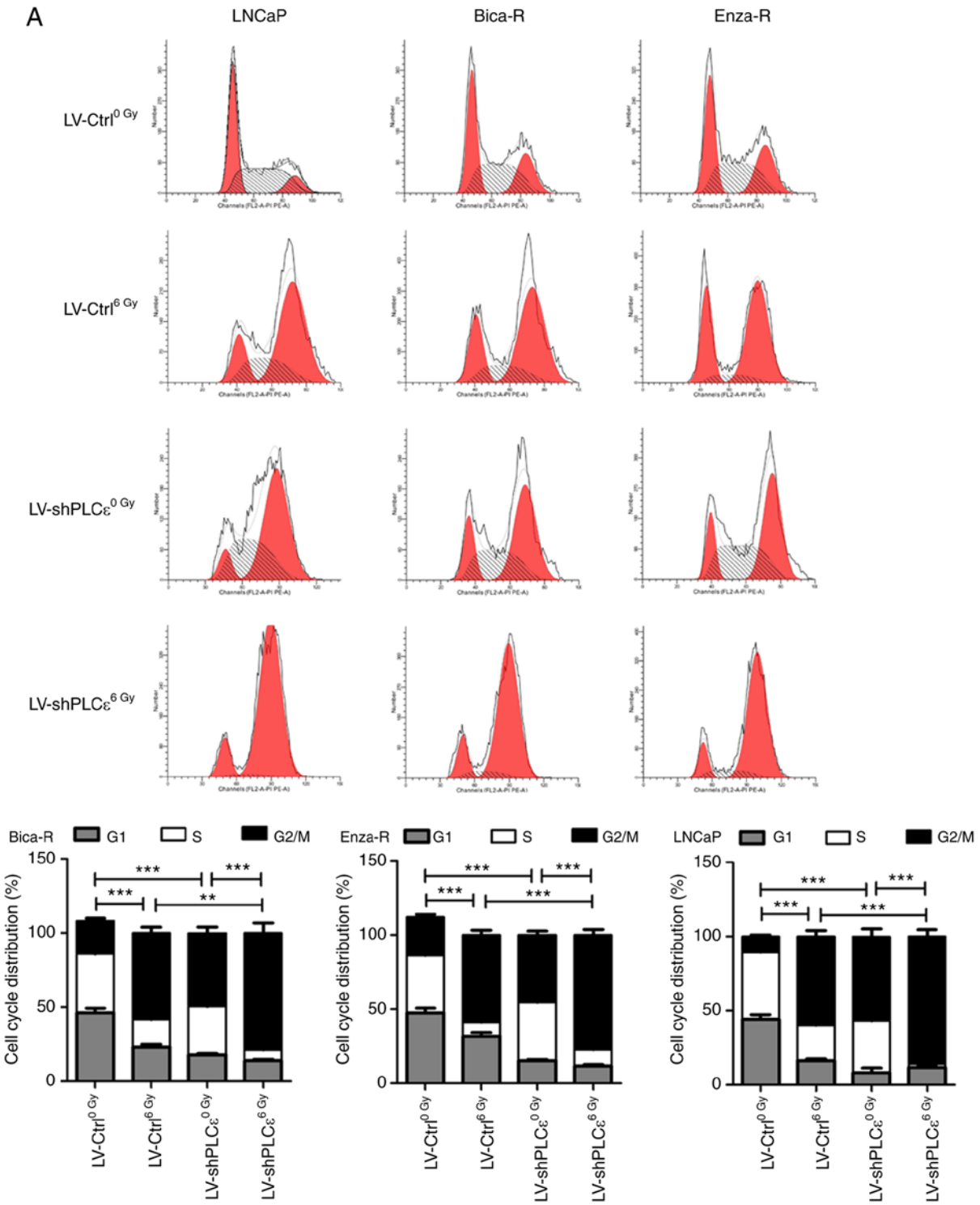

Figure 3. PLCε regulates DNA damage repair by altering the AR/DNA-PKcs pathway. (A) Cell cycle distribution in PCa cells.

shPLCε prevented AR translocation from the cytoplasm to the nucleus in irradiated cells (Fig. 4A). Western blotting results also demonstrated a decrease in AR expression in the nucleus in the shPLCε-transfected cells compared with the control cells (Fig. 4B).

An AR/PARP1 positive feedback loop serves a vital role in DDR in CRPC. To verify whether expression of PARP1 and DNA-PKcs were regulated by AR, CRPC cells were treated with the AR activator DHT or the AR inhibitor ARN-509. Colony formation assay results revealed that DHT increased the number of colonies formed by CRPC cells, whereas ARN-509 decreased the number of colonies (Fig. 5A and B). A significant increase in apoptosis was observed in CRPC cells treated with ARN-509 compared with those treated with DHT (Fig. 5C). RT-qPCR results demonstrated that DHT treatment increased the expression of AR, PARP1 and DNA-PKcs in CRPC cells compared with the corresponding control groups; by contrast, treatment with ARN-509 resulted in a decrease in AR, PARP1 and DNA-PKcs expression levels (Fig. 5D). Western blotting

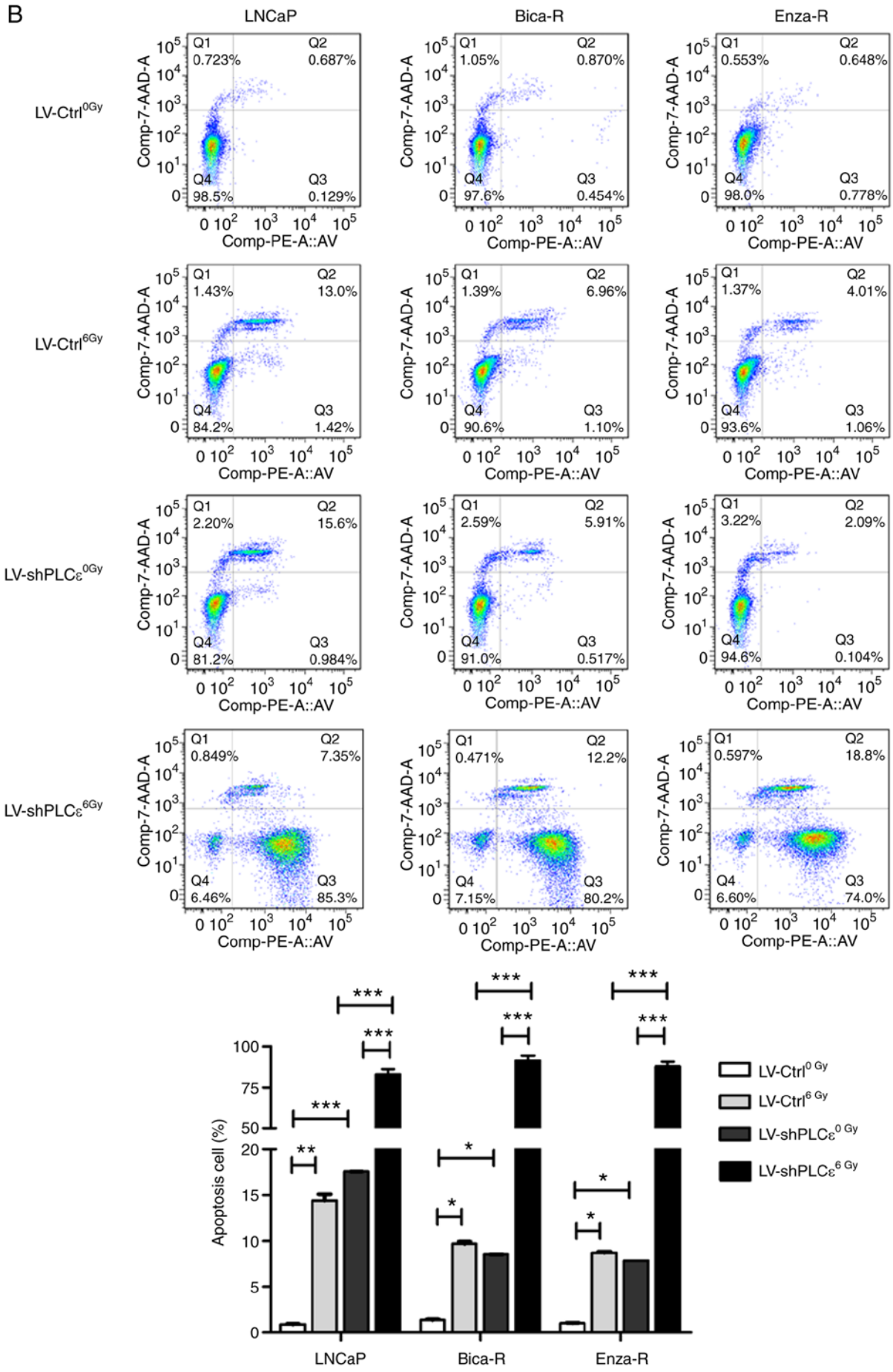

Figure 3. Continued. PLCε regulates DNA damage repair by altering the AR/DNA-PKcs pathway. (B) Measurement of the proportion of apoptotic cells in PCa cells.

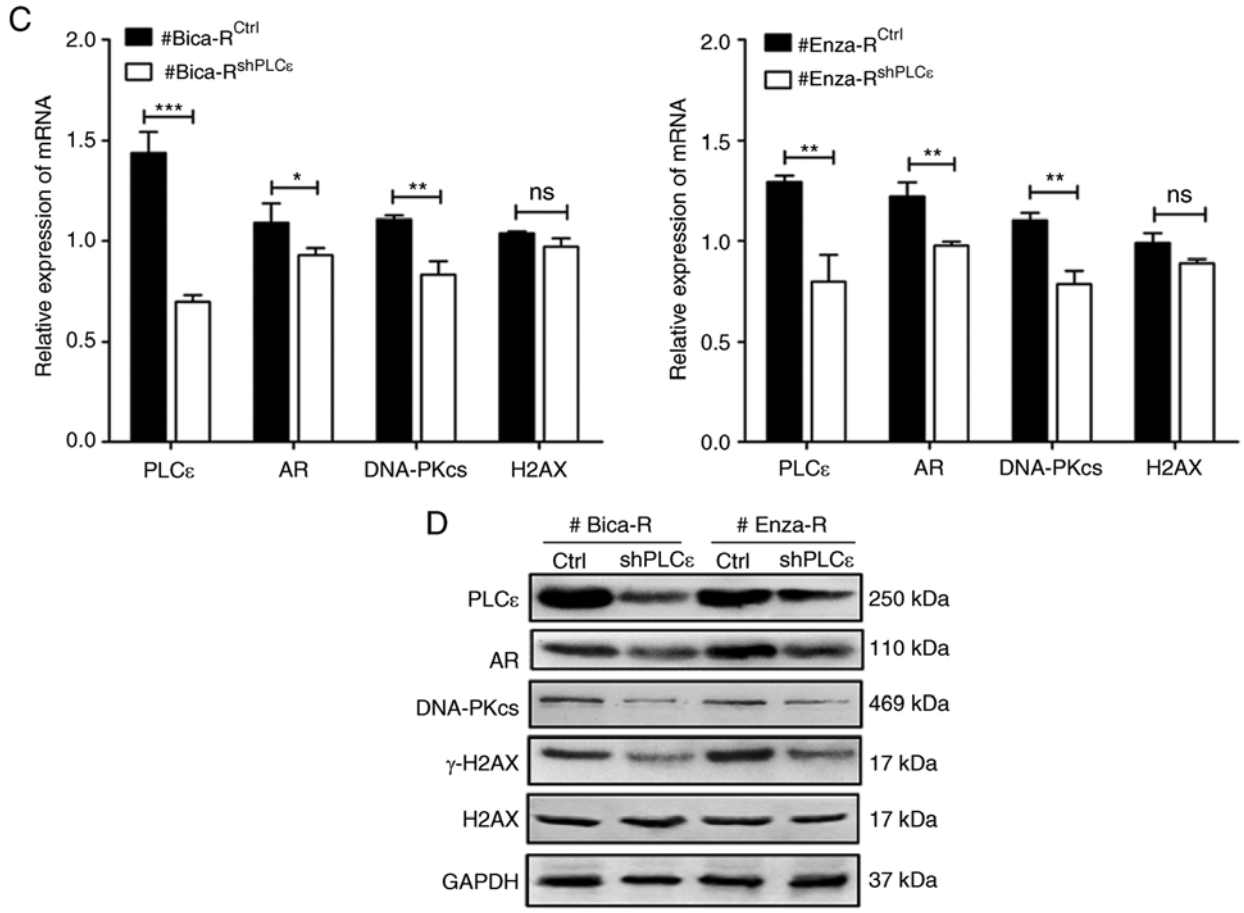

Figure 3. Continued. PLCε regulates DNA damage repair by altering the AR/DNA-PKcs pathway. (C) mRNA and (D) protein expression levels of PLCε, AR, DNA-PKcs and H2AX in PCa cells. '#' indicates irradiated cells. *P<0.05, **P<0.01 and ***P<0.001. PCa, prostate cancer; PLCε, phospholipase Cε; AR, androgen receptor.

exhibited similar results (Fig. 5E). To determine the association between AR and PARP1, CRPC cells were treated with the PARP1 inhibitor AZD2281, and the expression levels of AR, PARP1 and DNA-PKcs were assessed. The levels of AR were decreased in cells treated with AZD2281, as was the expression of PARP1, DNA-PKcs (Fig. 5F) and γ-H2AX (Fig. 5G).

PLCε enhances the radiosensitivity of CRPC by regulating H3K27 trimethylation levels in the PARP1 promoter region. EZH2, a histone-lysine N-methyltransferase enzyme that participates in histone methylation, alters gene expression patterns by specifically contributing to the H3K27me3 (23). It was hypothesized that the regulatory effects of PLCε on PARP1 were associated with the levels of H3K27me3 in the PARP1 promoter region. Bica-R cells were transfected with shPLCε or treated with DZNeP (a specific inhibitor of EZH2), and RT-qPCR results demonstrated that shPLCε reduced the expression of PARP1, AR and DNA-PKcs, whereas DZNeP reversed the effects of PLCε knockdown in Bica-R cells at the mRNA (Fig. 6A) and protein (Fig. 6B) levels. PLCε knockdown resulted in an increase in the levels of EZH2 and H3K27me3 compared with the control, and this effect was reversed by DZNep (Fig. 6B). ChIP-PCR was performed using a H3K27me3 antibody, and the results demonstrated that shPLCε increased the levels of H3K27me3 in the PARP1 promoter region compared with the control group, and DZNep abrogated this effect (Fig. 6C).

PLCε knockdown inhibits CRPC growth in vivo. To further evaluate the tumor-promoting role of PLCε in CRPC and its association with RT, a mouse xenograft model was used, where mice were injected with Bica-R cells or Bica-R cells transfected with shPLCε. As demonstrated in Fig. 7A and B, PLCε-knockdown resulted in a significant inhibition of tumor growth in animals following radiation compared with the irradiated and non-irradiated control groups. In addition, IHC results revealed that shPLCε transfection significantly reduced the protein expression levels of PLCε, AR, PARP1, DNA-PKcs and γ-H2AX in the tumors, evidenced by the positive rate of IHC (Fig. 7C and D), which was similar to the results obtained from analysis of clinical samples.

Discussion

The prostate is highly dependent on the proper functioning of AR (24). AR signaling is essential for the development and physiological functions of the prostate, which has been reported to be one of the most important initiating and supporting factors underlying the carcinogenesis and progression of PCa (13,15). Therefore, ADT, which targets the AR signaling pathway, has become a valuable and fundamental therapeutic method for treatment of PCa (25). If PCa progresses to CRPC or develops resistance to second-generation agents, such as enzalutamide, abiraterone acetate or other therapeutics, clinicians typically

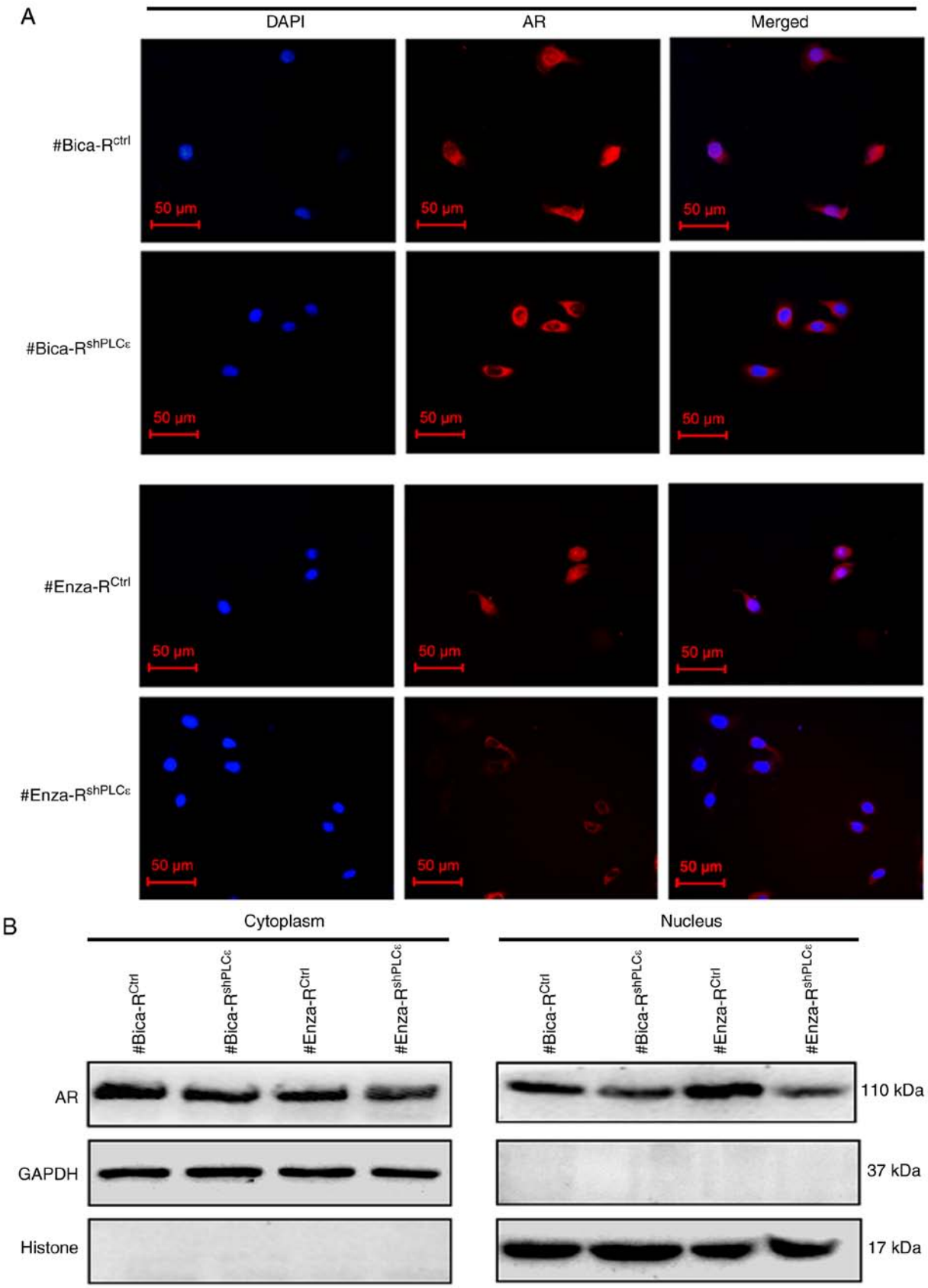

Figure 4. PLCε knockdown inhibits nuclear translocation of AR in castration-resistant prostate cancer cells after radiation. (A) Immunofluorescence staining of AR in PCa cells. Magnification, x400. (B) Protein expression levels of AR in the nucleus and cytoplasm of PCa cells. '#' indicates irradiated cells. PLCε, phospholipase Cε; AR, androgen receptor; PCa, prostate cancer; shPLCε, short hairpin RNA targeting PLCε.

resort to alternative therapeutics that were initially hypothesized to function through AR-independent mechanisms, among which RT has exhibited great potential (14). RT alone has been demonstrated to be effective in patients with PCa, and patients treated

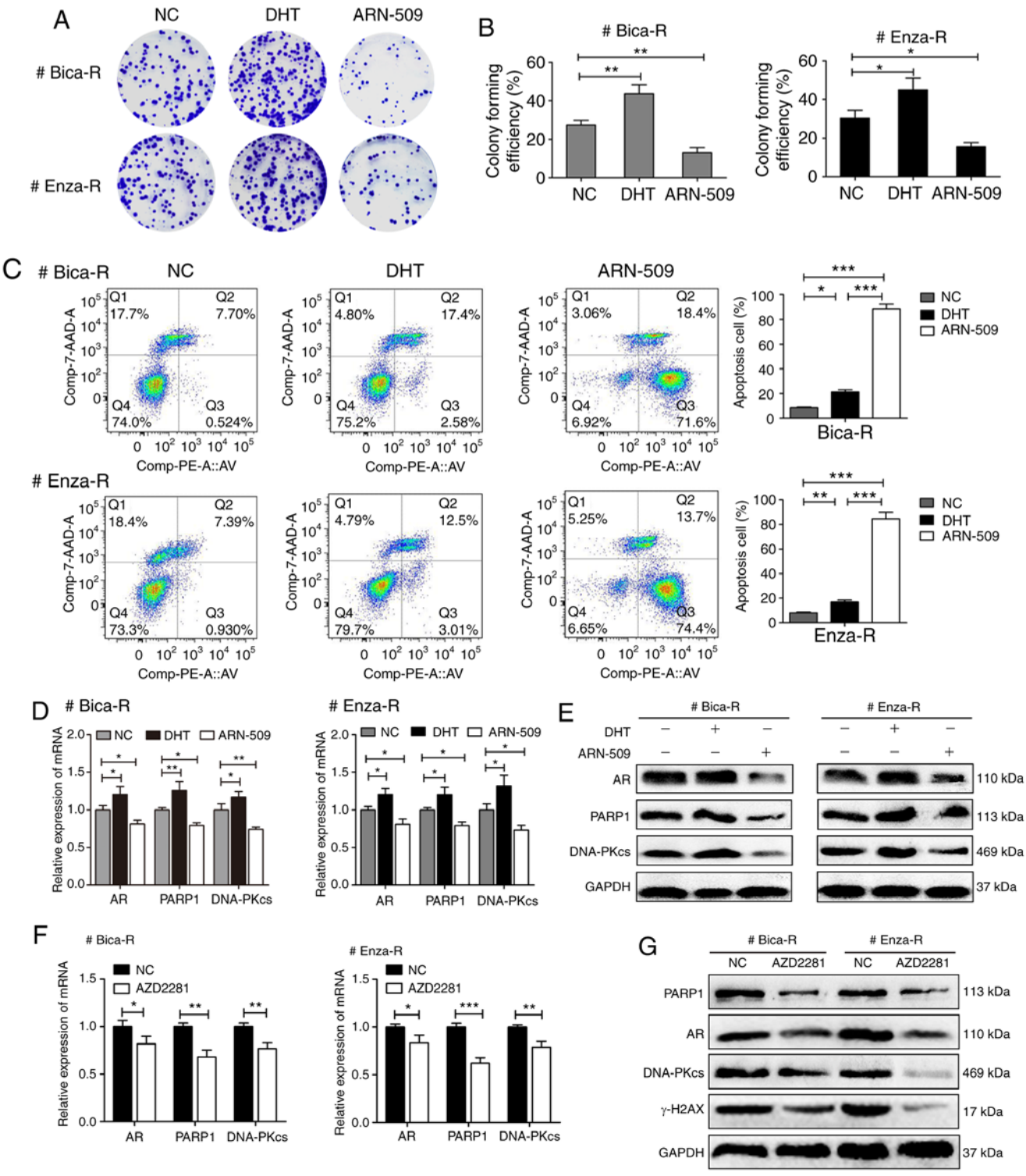

Figure 5. Role of an AR/PARP1 positive feedback loop in DNA damage repair in CRPC. PCa cells were treated with different inhibitors, and subsequently treated with 6 Gy of radiation. (A and B) Colony forming efficiency and (C) apoptosis analysis in treated PCa cells. (D and F) mRNA and (E and G) protein expression levels of AR, PARP1 and DNA-PKcs. *P<0.05, **P<0.01 and ***P<0.001. PCa, prostate cancer; AR, androgen receptor; PARP1, Poly (ADP-ribose) polymerase 1; CRPC, castration-resistant PCa.

with a combination of ADT and RT exhibit favorable survival outcomes (26-28). The increased survival times were previously hypothesized to be the result of the synergistic effects of mechanistically different anti-cancer strategies (26). However, PCa of different pathological grades or clinical stages displays heterogeneity in AR expression levels, thus exhibiting varying levels of sensitivity to ADT and different clinical benefits from combined ADT and RT (28). In addition, resistance to RT is frequently recurrent, particularly in CRPC (29). The results of the present study suggested possible involvement of AR signaling in RT resistance. A previous study has demonstrated that AR signaling is capable of enhancing cellular DDR and consequently serves a pivotal role in resistance to radiotherapy (30). However, the underlying mechanism remains unclear.

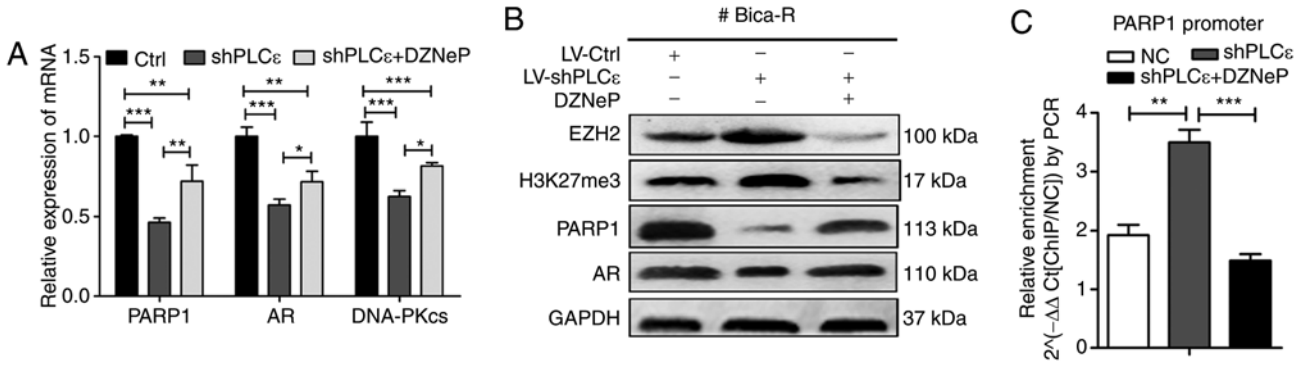

Figure 6. PLCε enhances the radiosensitivity of CRPC by regulating H3K27me3 levels in the PARP1 promoter region. (A) mRNA levels of PARP1, AR and DNA-PKcs in Bica-R cells were transfected with shPLCε or treated with DZNeP. (B) Protein expression levels of EZH2, H3K27me3, PARP1 and AR in Bica-R cells. (C) ChIP-PCR was used to analyze the H3K27me3 levels in the PARP1 promoter region. *P<0.05, **P<0.01 and ***P<0.001. PLCε, phospholipase Cε; CRPC, castration-resistant prostate cancer; Bica-R, Bicalutamide-resistant; sh, short hairpin; EZH2, enhancer of zeste homolog 2; PARP1, poly (ADP-ribose) polymerase 1; AR, androgen receptor; ChIP, chromatin immunoprecipitation.

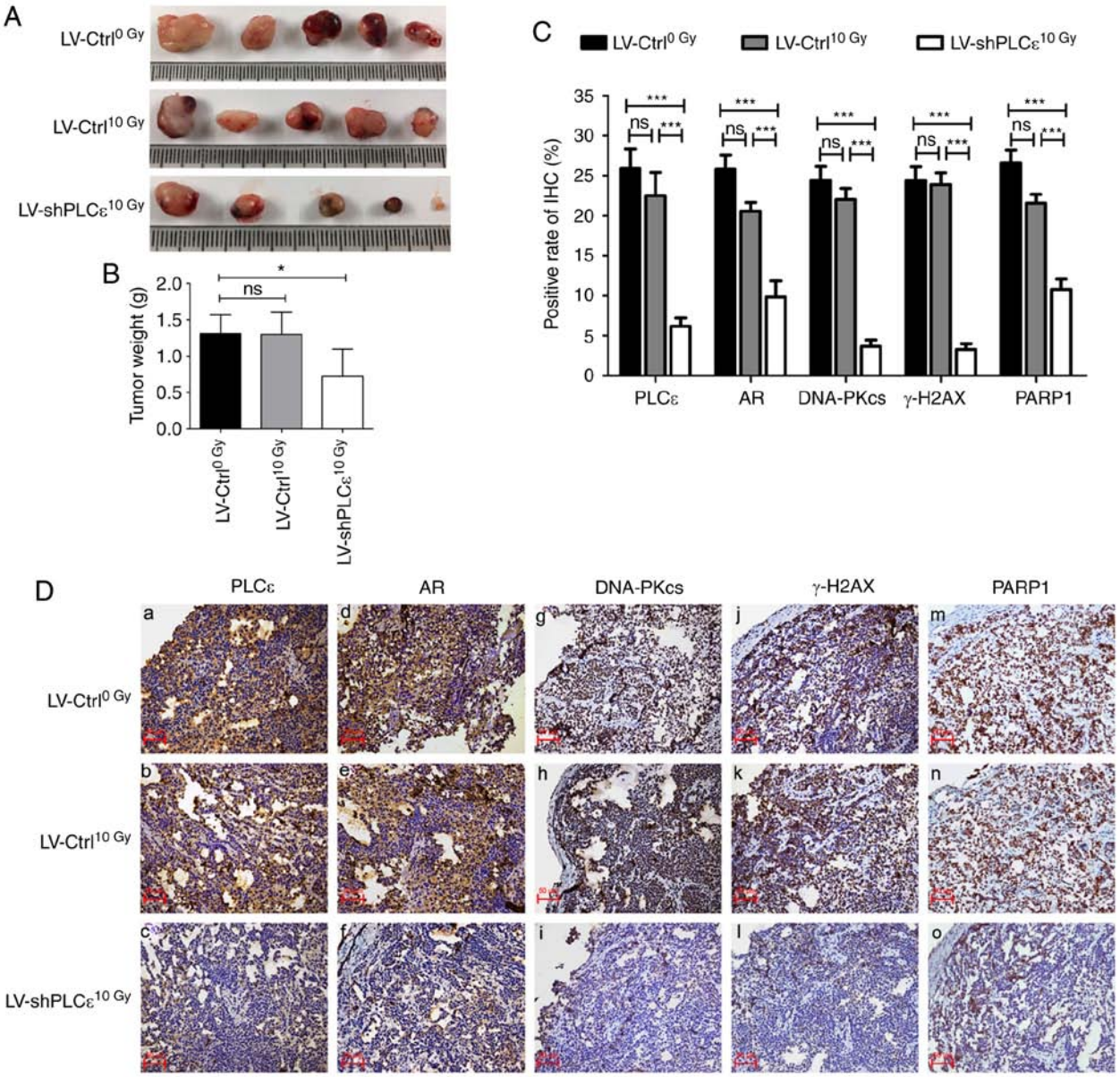

Figure 7. PLCε knockdown inhibits the growth of CRPC *in vivo*. (A and B) Immunocompromised mice were subcutaneously injected with PLCε-knockdown or control Bica-R cells, and the tumor sizes and weights of the two groups are presented. (C) Comparison of positive rate of IHC in each group. (D) Expression of PLCε, AR, PARP1, DNA-PKcs and γ-H2AX in each group. Magnification, x200. *P<0.05 and ***P<0.001. PLCε, phospholipase Cε; CRPC, castration-resistant prostate cancer; Bica-R, Bicalutamide-resistant; AR, Androgen receptor; PARP1, poly (ADP-ribose) polymerase 1; H2AX, H2A histone family member X.

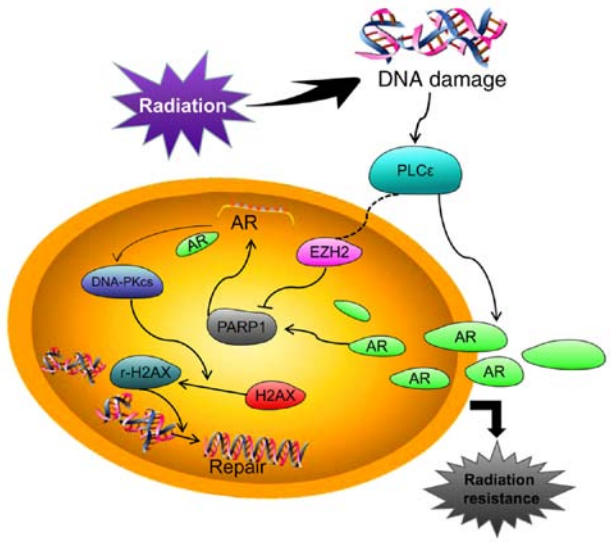

Figure 8. Signaling pathways discussed in the present study.

In the present study, a novel mechanism for the acquisition of RT resistance in CRPC was determined. RT induced the upregulation of PLCε, which subsequently promoted AR translocation. The increased presence of nuclear AR recruited PARP1 to specific regions of the DNA, and PARP1 in turn facilitated AR transcriptional activity and upregulated AR-mediated signaling, constituting a positive feedback loop that enhanced AR signaling. In addition, RT-induced increases in expression of PLCε reduced the expression levels of EZH2, resulting in epigenetic upregulation of PARP1 via the inhibition of H3K27me3 in its promoter region, which further potentiated the AR/PARP1 loop. Finally, the elevated activity of AR signaling induced alterations in the expression of DDR-associated molecules towards a RT-resistant phenotype. The clinical and *in vivo* data revealed that PLCε, AR and DNA-PKcs were simultaneously increased in prostate cancer samples, providing preliminary evidence for this mechanism. In the cytological experiments, CRPC cell lines were used to confirm the crucial role of the AR/PARP1/DNA-PKcs pathway in the development of resistance to RT.

In the present study, PLCε was considered to be the initiator of this novel signaling pathway in PCa following RT. PLCε is the primary member of the PLC family of enzymes that catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate (5). PLCε is a unique member of this family due to its ability to receive signal inputs from both the Ras and Rho families of proteins and other heterotrimeric G proteins, thus functioning as a hub in the regulation of signaling pathways involved in tumor development and progression (5,31). Numerous studies have demonstrated that PLCε is involved in the initiation and progression of tumors, particularly in upper gastrointestinal tract cancers (9,32-36). Two genome-wide studies identified single nucleotide polymorphisms in the PLCε gene in esophageal squamous cell carcinoma, providing additional evidence that PLCε acts as an oncogene leading to cell transformation (9,37). Our previous study demonstrated that PLCε expression was significantly higher in PCa tissues compared with normal prostate tissue, and PLCε knockdown resulted in reduced proliferation of PCa through downregulation of AR expression (10), and this result was confirmed in the present study. Additionally, the results of the present study revealed that PLCε regulation of AR may underlie the development of RT resistance in PCa.

Since AR signaling has been implicated in RT resistance, it was hypothesized in the present study that preservation of AR functionality or AR-mediated signaling pathways may promote the development of RT resistance. The results demonstrated that RT upregulated the expression of PLCε and AR, whereas RT combined with PLCε knockdown significantly decreased the expression of AR and enhanced the radiosensitivity of LNCaP, Bica-R and Enza-R cells. These results were further verified by the data demonstrating that the expression of DDR-related molecules, DNA-PKcs and γ-H2AX were reduced in cells or tissues following PLCε knockdown.

Mechanistically, it was hypothesized that PARP1 was involved in the AR signaling regulation of RT resistance based on previous studies. PARP1 is recruited to AR functional sites on DNA and consequently increases the transcriptional activities of AR and promotes AR-dependent cellular biofunctions in PCa (20). Enhanced AR signaling activity has also been demonstrated to be a prominent stimulator of PARP1 in certain types of cancer (38). Furthermore, increased expression of PARP1 is associated with enhanced chromatin remodeling, DNA replication and cell survival, all of which are related to phenotypic alterations observed in RT-resistant cancer, including ovarian, breast and prostate cancer (39). The results of the present study demonstrated that activation of AR signaling using DHT increased PARP1 expression, whereas inhibition of AR signaling, both by knockdown of PLCε expression or treatment with an AR antagonist, reduced PARP1 expression. In addition, a PARP1 inhibitor attenuated AR signaling. These results suggested an intricate association between PLCε, AR and PARP1; PLCε knockdown directly impaired AR expression and translocation or reduced PARP1 expression, disrupting the positive feedback loop that exists between PARP1 and AR, which also resulted in the downregulation of AR. The attenuation of AR was, in turn, capable of decreasing the expression of PARP1. Therefore, upregulated expression of PLCε, as a tumor promoter in PCa and CRPC, may act as an indispensable protector of proper functioning of the AR/PARP1 loop.

However, the possibility that PLCε knockdown may downregulate PARP1 expression via an AR-independent manner cannot be ruled out. In the present study, EZH2 was demonstrated to be upregulated following PLCε knockdown. Histone methylation serves an important role in DNA repair and consequent cell survival associated with RT resistance (23,40), and EZH2 can directly initiate trimethylation of H3K27, a type of histone methylation of the amino (N) terminal tail of the core histone H3, which is associated with downregulation of nearby genes through the formation of heterochromatic regions (41). Expression of H3K27me3 is associated with radiation, and the loss of H3K27me3 is frequently observed in radiation-associated angiosarcoma of the breast (42). In addition, the loss of H3K27me3 expression is a sensitive marker for the detection of malignant tumors of peripheral nerve sheaths induced by radiation (43). Therefore, radiation or radiotherapy may attenuate H3K27me3 expression and induce the expression of genes associated with oncogenesis.

The results of the present study suggested that PLCε knockdown mediated an increase in the presence of H3K27me3 in the PARP1 promoter region by increasing EZH2 levels and reducing the expression of PARP1, which may have disrupted

the AR/PARP1 loop and impaired downstream target genes, including the DDR associated proteins, DNA-PKcs and γ-H2AX; this in turn resulted in a reduction in DDR in CRPC, which serves a critical role in radiotherapy (Fig. 8). However, the mechanism by which PLCε regulated H3K27me3 levels in the PARP1 promoter region remains unclear.

In conclusion, the results of the present study suggested that PLCε knockdown enhanced the radiosensitivity of CRPC by preventing nuclear translocation of AR and consequently inhibiting AR signaling. In addition, PLCε knockdown impaired the AR/PARP1 positive feedback loop by suppressing AR signaling or epigenetically through regulating PARP1 expression, which ultimately resulted in the downregulation of the DNA-PKcs axis and reduction of DDR. This novel mechanism may represent a potential therapeutic approach for treating patients with CRPC with RT resistance.

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Availability of data and materials

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

Authors' contributions

JP and XW designed the experiments. JP and TL collected the specimen and analyzed the clinical data. JP, TL, NL and LL performed the experiments. JP, TL and ZQ wrote the manuscript. XW and CL provided technical support during the research project and supervised the progress of the experiments. TL and ZQ analyzed the statistical data. JP and TL created the figures. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University (Chongqing, China). Informed consent was obtained from the patients or their family members. The xenograft experiments were approved by the Chongqing Medical University Institutional Animal Care and Use Committee and the Animal Ethics Committee.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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