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CA916798 affects growth and metastasis of androgen-dependent prostate cancer cells

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Abstract. – OBJECTIVE: Abnormal activation of androgen receptor (AR) signaling pathway is a critical pathogenic mechanism and therapeutic target for prostate cancer (PCa). The CA916798 is a tumor-associated gene and may be regulated by the androgen-AR pathway. This study aims to investigate the function of CA916798 in the growth and metastasis of androgen-dependent PCa cells.

MATERIALS AND METHODS: CA916798 expression in PCa cell lines was investigated. LNCap cells were divided into 4 groups: LNCap, LNCap+ Dihydrotestosterone (DHT), LNCap+DHT+siCA916798, and LNCap+DHT+siRA group. CA916798 expressions in LNCap cells treated with siCA917698 or siAR were examined. The viability, apoptosis, migration, and invasion of PCa cells were examined. Dual luciferase and ChIP assays were used to examine the interaction between the AR and CA916798.

RESULTS: Endogenous CA916798 mRNA levels in PC3 cells were significantly higher than those in LNCap cells ($p < 0.05$). However, CA916798 was androgen-sensitive in LNCap cells, but not in PC-3 cells. Dual luciferase and ChIP assays showed that AR could specifically bind to the promoter regions of the CA916798. Knockdown of CA916798 (LNCap+DHT+siCA916798) and AR (LNCap+DHT+siAR) resulted in decreased cell viability, migration, and invasion, while it induced apoptosis and G1 cell cycle arrest in LNCap cells.

CONCLUSIONS: DHT could initiate the transcription of CA916798, which further mediates the androgen-AR signaling pathway-dependent cell growth and metastasis of the prostate cancer cell line LNCap.

Key Words:
Androgen receptor, CA916798, Growth, Prostate cancer.

Introduction

Prostate cancer (PCa) has become one of the most severe health problems for men worldwide; PCa tumors are responsible for the highest morbidity in American males[1]. PCa is the second-leading cause of cancer-related deaths, and its incidence has kept rising in recent years[2,3]. Rich-fat diets, smoking, physical activity, or working in the rubber industry have been proven to be exogenous factors that contribute to the oncogenesis of PCa[4,5]. Except for the exogenous factors, a few endogenous risk factors, such as aging, oxidative stress, family history, and race also affect the incidence of PCa[5]. It is believed that these endogenous and exogenous factors play significant roles in PCa by altering the metabolism of androgen, which determines development of PCa[5]. Moreover, due to the unsuitable radical treatment for PCa patients, androgen deprivation therapy remains the first line of treatment for advanced PCa patients. The central role of androgen in development of PCa has been pinpointed by numerous studies[5,6]. Previous studies[5,6] have reported that androgen regulates the transcription of downstream targeting genes by activating the androgen receptor (AR). Therefore, androgen plays critical roles in the proliferation of PCa. Therapies based on the deficient function of androgen have been shown to have considerable effects in ceasing the growth and inducing apoptosis of androgen-dependent PCa cells[7-9].

Action of the androgen in androgen-dependent PCa cells relies on the function of AR. AR is a member of the nuclear receptor superfamily and consists of three functional domains[6]. Sustained aberrant activity of AR is associated with the survival and progression of PCa cells, even post the treatment with androgen deprivation therapy[10,11]. The downstream genes mediated by the AR are closely associated with the cell growth, differentiation, metabolism, and apoptosis. Therefore, the AR signaling pathway is important and significant for normal prostate tissues and PCa tissues. Discovering the downstream targeting genes would

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provide novel theories and thus, evidence for the pathogenic mechanism and therapy of PCa. Song et al[3] discovered a common DNA amplification site in recurrent, hormone-refractory tumors at Xq11-q13, which is a site on the AR gene. Cucchiara et al[6] found that the regulation of AR signaling plays necessary roles in the carcinogenesis of PCa cells. AR also serves as a modulator of PCa resistance to androgen-deprivation therapy. It has been verified that the AR F876L mutation clone is resistant to enzalutamide[12] and AR splice variant 7 is associated with resistance to abiraterone and enzalutamide[13]. The downstream pathways regulated by these molecules are complicated and remain only partially understood[14]. Identification of the genes with altered expression in response to androgen administration is critical for understanding of proliferation, survival, and hormonal progression in PCa cells.

Although Romanuik et al[14] discovered the C19orf48 as a newly identified gene responding to the presence of androgen in year 2009. However, our team[15] was the first to discover the CA916798 in SPC-A-1/CDDP cells; it was included in the GenBank database in 2002. Additionally, Zhou et al[16] cloned the CA916798. Actually, the CA916798 is the C1904f48; both these genes are composed of 117 amino acid (aa) and encode the same protein. CA916798 is a drug resistance-related gene discovered in a human adenocarcinoma multi-drug resistance cell line[15,16]. Meanwhile, the CA916798 is closely related to the function of Akt in certain types of cancers[17-19]. The CA916798 is seldom expressed in normal tissues or cells; however, aberrantly high levels of CA916798 can be detected in some tumor tissues or cells[16]. Meanwhile, the CA916798 is involved in the growth and cisplatin resistance of lung carcinoma cells[16]. Overexpression of CA916798 also leads to multidrug resistance in breast cancer cells[16]. All the above details suggest that the CA916798 may be a tumor-associated gene and participate in the development of tumors. However, the pro-tumor function of CA916798 has not been evaluated in PCa cells. Additionally, given the response of this gene to the administration of androgen, it was reasonable to explore the role of CA916798 in the tumorigenesis of androgen-dependent PCa. Therefore, we believe that whether the CA916798 has anti-apoptotic functions needs to be clarified.

In the present study, the effects of CA916798 knockdown on the viability, migration, and invasiveness of the human androgen-dependent PCa cell line, LNCap, were investigated. To verify the modulation of CA916798 by androgen, the interaction between CA916798 and AR was also evaluated by using dual luciferase assay and chromatin immunoprecipitation assay (ChIP). Moreover, the expression of AR in PCa cells was also inhibited for the confirmation of the upstream regulating effect of androgen on CA916798.

Materials and Methods

Chemicals and Reagents

Antibodies against CA916798 (Cat. No. ab122764) and AR (Cat No. ab2769) were purchased from Abcam Biotech. (Cambridge, MA, USA). Antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from KANGCHEN BioTech. (Cat No. RC-5G5, Shanghai, China). Fetal bovine serum (FBS, Cat. No. 10099-141) was purchased from Gibco (Grand Island, NJ, USA). Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Cat. No. SH30809.01B) was obtained from HyClone (South Logan, UT, USA). Annexin V/PI apoptosis kit (Cat. No. CCS012) and Cell cycle staining kit (Cat. No. CCS012) were purchased from MultiSciences Biotech Co., Ltd. (Beijing, China). Transwell chamber (Cat. No. 3422) and Matrigel basement membrane matrix (Cat. No. 356234) were purchased from Corning Costar (Corning, MA, USA). Lipofectamine™ 2000 (Cat. No. 52887) was obtained from Invitrogen/Life Technologies (Carlsbad, CA, USA). Dihydrotestosterone (DHT) was purchased from Sigma-Aldrich (St. Louis, MO, USA). TRIzol was purchased from TaKaRa (Dalian, China). Reverse transcription and real-time polymerase chain reaction (PCR) kits were purchased from DBI (Houston, TX, USA). Protein Concentration Determination kit (Cat. No. #23227) was purchased from Thermo Scientific Pierce (Rockford, IL, USA).

Cell Culture

The human prostate epithelial cell lines RWPE-1, PC3, DU145, and LNCap were purchased from American Tissue Culture Collection (ATCC) Cell Bank (Manassas, VA, USA). The human 293T cells were purchased from Chinese Academy of Sciences cell bank (Shanghai, China). The cells were maintained in RPMI-1640 supplemented with 10% FBS and 1% antibiotic mixture (v/v) (penicillin and streptomycin) at

37°C in an atmosphere containing 5% CO_2 and 95% air. Expression of CA916798 at the messenger RNA (mRNA) and protein levels were determined using reverse transcription Real-time PCR (RT-PCR) and western blotting assays, respectively. Finally, we selected the DHT-stimulated cell lines, which expressed the highest levels of CA916798 for subsequent assays.

Levels of Endogenous CA916798 Determination

The RWPE-1, PC3, DU145, and LNCap cells were cultured using RPMI-1640 medium at conditions of 37°C, 5% CO_2 and 95% air. To observe the endogenous levels of CA916798, both the RT-PCR and Western blot assays were used as described.

Dihydrotestosterone (DHT) Stimuli

In order to observe the effects of DHT stimuli on CA916798 expression, the RWPE-1, PC3, DU145, and LNCap cells were stimulated with 10 nmol/l DHT for 12 h. Then, the CA916798 levels were examined using the RT-PCR assay.

Plasmid Construction and Transfection

Specific siRNA targeting CA916798 (5′-UGGAUUUGUACCAUUCUUCU GUU-3′) and AR (5′-GCUGCAAGGUCUUCUUCAAUU-3′), and NC siRNA (5′-UUCUCCGAACGUGUCACGUTT-3′) were purchased from GenePharma (Shanghai, China). siRNAs were targeted to genes in LNCap cells. For the determination of the interaction between CA916798 and AR, wild and mutant sequences of the CA916798 promoter were ligated to the pGL-3 plasmid, and the coding sequence of the AR was ligated to the pcDNA3.0 plasmid. Transfection was performed using Lipofectamine™ 2000, according to the manufacturer's instructions.

Cell Counting Kit 8 (CCK-8) Assay

Viabilities of the cells transfected with different plasmids were determined by the CCK-8 assay. The exponentially growing LNCap cells (5×10^5 cells/ml) were seeded into one well of a 96-well plate and incubated for 72 h (each treatment was represented by at least nine replicates). Every 24 h, CCK-8 solution (10 µl/ml medium) was added to three randomly selected wells, and the cells were incubated at 37°C for another 60 min. Cell viability was represented by using optimal density (OD) values detected at 450 nm with a Microplate Reader.

Scratch Assay

Cells (5×10^5/ml) were seeded into a six-well plate and the reference points were marked to guarantee that the area of image acquisition was the same. After being allowed to grow into a confluent monolayer at 37°C for two days, the cell layer was scratched to generate a cell-free straight line. Then, the cells were washed thrice with phosphate buffer saline (PBS) to remove the debris at the edges of the scratch. Cell migration towards the midline of the scratch was recorded in reference with reference points. For each well, two images (at 0 h and 48 h) were captured using a phase-contrast microscope (Motic, China). The relative area between the gaps were measured and analyzed by using the image-proplus (IPP) software 6.0 (Media Cybernetics Inc., Silver Spring, MD, USA).

Transwell Assay

For the determination of the invasion potential of LNCap cells, 100 µl medium containing 2×10^4 cells was seeded into the upper chamber of a transwell system (BSA-coated porous polycarbonate membrane with a pore size of 8 µm, which was pre-coated with 40 µl matrigel). The lower chamber of the transwell system contained 700 µl complete medium supplemented with 10% FBS. Cells were incubated and allowed to migrate through the porous membrane at 37°C for 48 h. The cells in the upper surface of the chamber were completely removed, and the chambers were then fixed with 4% paraformaldehyde for 20 min. After being washed thrice with PBS, the chambers were stained with 1% (w/v) crystal violet for 5 min. The cell numbers in each chamber were determined by using a microscope (Model: CX41, Olympus, Tokyo, Japan) and analyzed by using image-proplus (IPP) software 6.0 (Media Cybernetics Inc., Silver Spring, MD, USA).

Hoechst 33258 Staining

The morphological changes in the cell nuclei were detected by using a Hoechst staining kit according to the manufacturer's instructions. The cells that underwent different treatments were first incubated with 50 µl of the fix solution for 30 min at room temperature, and then stained with Hoechst 33258 (20 µM) for 5 min at room temperature. The results were observed under a fluorescence microscope at a wavelength of 460 nm.

Flow Cytometry

Cell cycle was examined using cell cycle staining kit, according to the manufacturer's instructions. 300 µl of propidium iodide (PI) were added to the cells in the dark to stain the DNA. After a 15-min incubation at room temperature, the DNA contents were analyzed using a FACS flow cytometer (Accuri C6, BD, USA). Apoptotic processes in LNCap cells were detected using an Annexin V/PI apoptosis kit, according to the manufacturer's instructions. The apoptotic rates were analyzed using a FACScan flow cytometer (Accuri C6, BD, USA). The total apoptotic rate was equal to the sum of the late apoptotic rate (UR, upper right quadrant-advanced stage apoptosis) and the early apoptotic rate (LR, lower right quadrant-prophase apoptosis).

RT-PCR Assay

Total RNA was extracted using the TRIzol method, according to the manufacturer's instructions. cDNA templates were obtained by reverse transcription of RNA using the reverse transcription kit, according to the manufacturer's instructions. The final reaction mixture volume was 20 µl; it contained 10 µl Bestar SybrGreen qPCR master Mix, 0.5 µl of each primer (CA916798, forward: 5′-TCCTCAACCTCGTCCTCTG-3′, reverse: 5′-CCCACTTATC CACCTTCTCC-3′. 5′-TGTTCGTCATGGGTGTGA AC-3′, GAPDH, forward: reverse: 5′-ATGGCATGGACTGTGGTCAT-3′), 1 µl cDNA template, and 8 µl RNase-free H_2O. Thermal cycling parameters for the amplification were set as follows: denaturation step at 94°C for 2 min, followed by 40 cycles of amplification of 94°C for 20 s, 58°C for 20 s and 72°C for 20 s. The melting curves were analyzed between 62°C and 95°C, and the relative expression levels of the target genes were calculated by the Real-time PCR Detection System (Mx3000P, Agilent Technologies, Santa Clara, CA, USA), using the $2^{-\Delta\Delta ct}$ method.

Western Blotting

Total cellular protein was extracted by using Total Protein Extraction Kit, according to the manufacturer's instructions. The concentrations of the protein samples were determined using the BCA method, according to the manufacturer's instructions. Then, a total of 40 µg of protein from each sample was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After the proteins were transferred onto polyvinylidene di difluoride (PVDF) membranes, the membranes were rinsed with Tris-buffered saline and Tween 20 (TBST-20) and incubated with the primary antibodies against CA916798 (1:2000), androgen receptor (AR, 1:1000), GAPDH (1:10000) (internal reference protein for total protein), and Histone H3 (1:1000) (internal reference protein for nuclear protein) for 1 h at room temperature. After three washes with Tris-buffered saline and Tween 20 (TBST-20), the membranes were incubated with secondary horse radish peroxidase (HRP)-conjugated IgG antibodies (1:20000) for 40 min at 37°C. Following six washes with TTBS, the blots were developed using the Beyo ECL Plus reagent, and the images were recorded in the Gel Imaging System.

Dual Luciferase Assay

The direct interaction between CA916798 and AR in 293T cells was determined using the dual luciferase assay. Luciferase activity was detected 48 h after transfection of different combinations of plasmids, using the Dual Luciferase Assay kit, according to the manufacturer's instructions. Co-transfection with the Renilla luciferase plasmid (pRL-SV40) was used as an internal control for the determination of transfection efficiency. The fluorescence intensity was detected using a Microplate Reader (GloMax, Promega, Madison, WI, USA).

ChIP-PCR Assay

LNCap cells were centrifuged and then sonicated in lysis buffer to generate chromatin fragments about 500 bp in length. The material was clarified by centrifugation, diluted 10-fold using buffer, and pre-treated with protein A-agarose beads. The pre-cleared chromatin-containing supernatant was used for performing immunoprecipitation reaction with antibodies against AR or with normal rabbit IgG, which was the negative control. The immunoprecipitated genomic DNA was cleared of protein and RNA by digestion with proteinase K and RNase H, respectively. The DNA was then extracted, cleared, and subjected to gene-specific ChIP-PCR analysis using primers against CA916798 (forward: 5′-TCCTCAACCTCGTCCTCTG-3′, reverse: 5′-CCCACTTATCCACCTTC TCC-3′) to determine the enrichment of the immunoprecipitated materials relative to that of the input material.

Statistical Analysis

The data were expressed as mean ± SD (n=3). Difference between the two groups were ana-

lyzed using Student' *t*-test, at a significance level of 0.05 (two-tailed *p*-value). All the statistical analyses were conducted using SPSS version 19.0 (IBM, Armonk, NY, USA).

Results

Endogenous CA916798 Levels in Human Prostate Epithelial Cell Lines

The endogenous CA916798 mRNA and protein levels in human prostate epithelial cells, including RWPE-1, PC3, DU145, and LNCap cells, were examined by the RT-PCR and western blot assay, respectively. The results indicated that the endogenous CA916798 mRNA levels in PC3 cells were higher significantly compared to those in RWPE1, LNCap, and DU145 cells (Figure 1A, $p < 0.01$). The endogenous CA916798 protein levels in PC3 cells were also significantly higher than those in the other cell lines (Figure 1B, $p < 0.05$).

Dihydrotestosterone (DHT) Induced CA916798 Expression in LNCap Cells

In order to investigate the sensitivity of human prostate epithelial cell lines to the DHT stimuli, the RWPE-1, PC3, DU145, and L1NCap cells were stimulated with 10 nmol/l DHT for 12 h. The results showed that the DHT stimuli significantly induced the enhancement of CA916798 levels in LNCap cells, compared to the case for the unstimulated LNCap cells (Figure 1C, $p < 0.01$). Therefore, we believed that the CA916798 expression in the LNCap cells is androgen-dependent.

CA916798 Knockdown Suppressed the Viability of DHT-Stimulated LNCap Cells

In order to clarify the effects of CA916798 expression on the proliferation of LNCap cells, the viability and apoptosis of those cells were observed. Firstly, the CA916798 in LNCap cells was knocked out by treatment with LNCap cell-specific siRNA. The results illustrated that

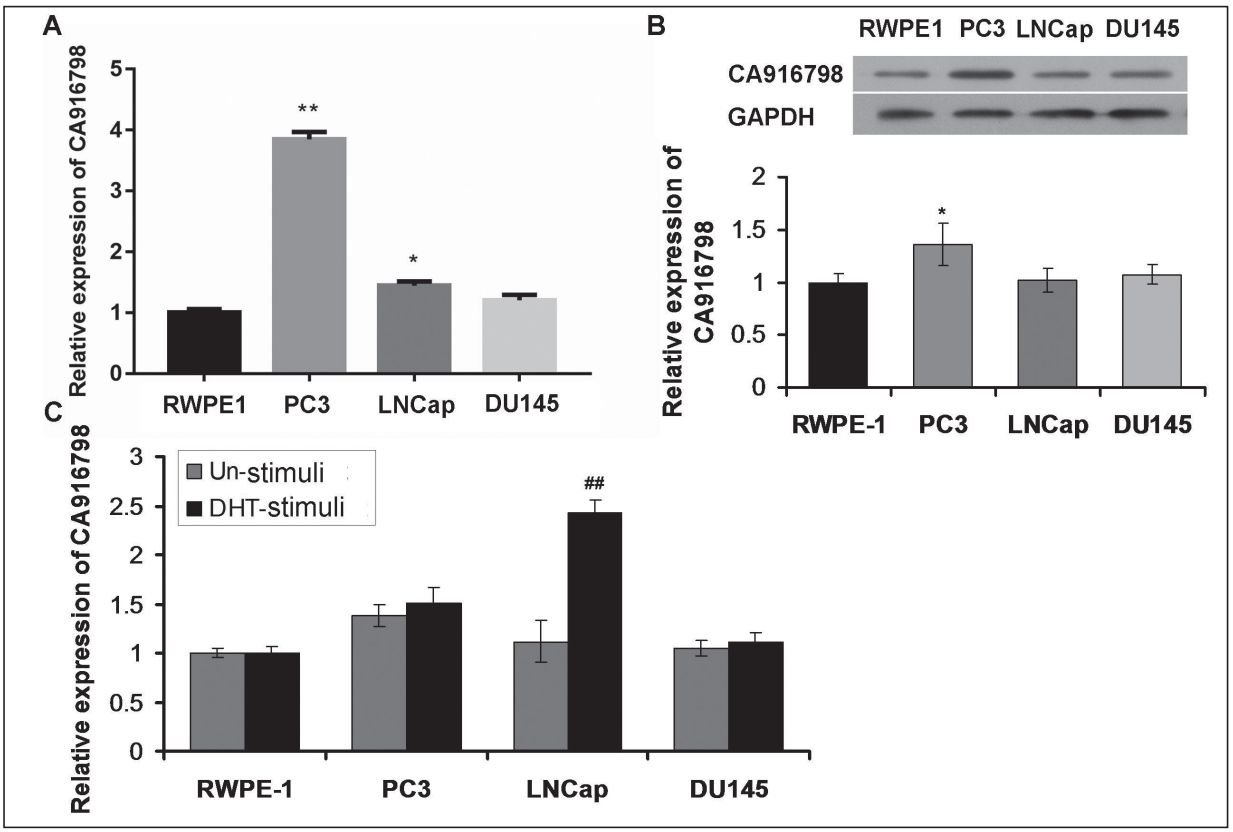

Figure 1. Examination of the endogenous CA916798 mRNA and protein levels using the RT-PCR and Western blot assay, respectively. A. CA916798 mRNA expression in each cell line. ***B.*** CA916798 protein expression in each cell line. ***C.*** CA916798 mRNA expression in each cell line after the DHT stimulation. $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* RWPE1 and DU145 cell groups. $^{\#\#}p < 0.01$ *vs.* Untreated cell group.

CA916798 mRNA levels (Figure 2A) and protein levels (Figure 2B) in siCA916798-treated and DHT-stimulated LNCap cells were significantly lower than those in the untreated LNCap cells ($p < 0.01$).

Post the CA916798 knockdown, the viability of DHT-stimulated LNCap cells was significantly suppressed, compared to the case for untreated LNCap cells; it followed a time-course dependent curve (Figure 2C, $p < 0.01$). The cell cycle results indicated that the S-phase cells in siCA916798-treated and DHT-stimulated LNCap cells were significantly decreased, and G0/G1-phase cells (68.8%) were significantly increased, compared to the case for the untreated/DHT-stimulated LNCap cells (Figure 2D, $p < 0.05$).

CA916798-Knockdown Induced Apoptosis in DHT-Stimulated LNCap Cells

According to the results of the Hoechst 33258 staining, plenty of the DHT-stimulated LNCap cells with bright blue staining (representing stronger apoptotic processes) were discovered post the CA916798 knockdown (Figure 3A). However, there were no bright blue stained untreated DHT-stimulated LNCap cells (Figure 3A). Meanwhile, the flow cytometry data showed that the average apoptotic rate of the siCA916798 -treated, DHT-stimulated LNCap cells was 24.6%, which was significantly higher than that of the untreated/DHT-stimulated LNCap cells, which was 16.7% (Figure 3B, $p < 0.05$).

CA916798 Knockdown Inhibited the Migration and Invasion of DHT-Stimulated LNCap Cells

To investigate the potential function of CA916798 in determining the metastasis of DHT-stimulated LNCap cells, the scratch and transwell assays were conducted. The results indicated that the siCA916798 suppressed CA916798 activity significantly and prevented the DHT-stimulated LNCap cells from penetrating the polycarbonate membranes in the transwell assay. The number of siCA916798-treated

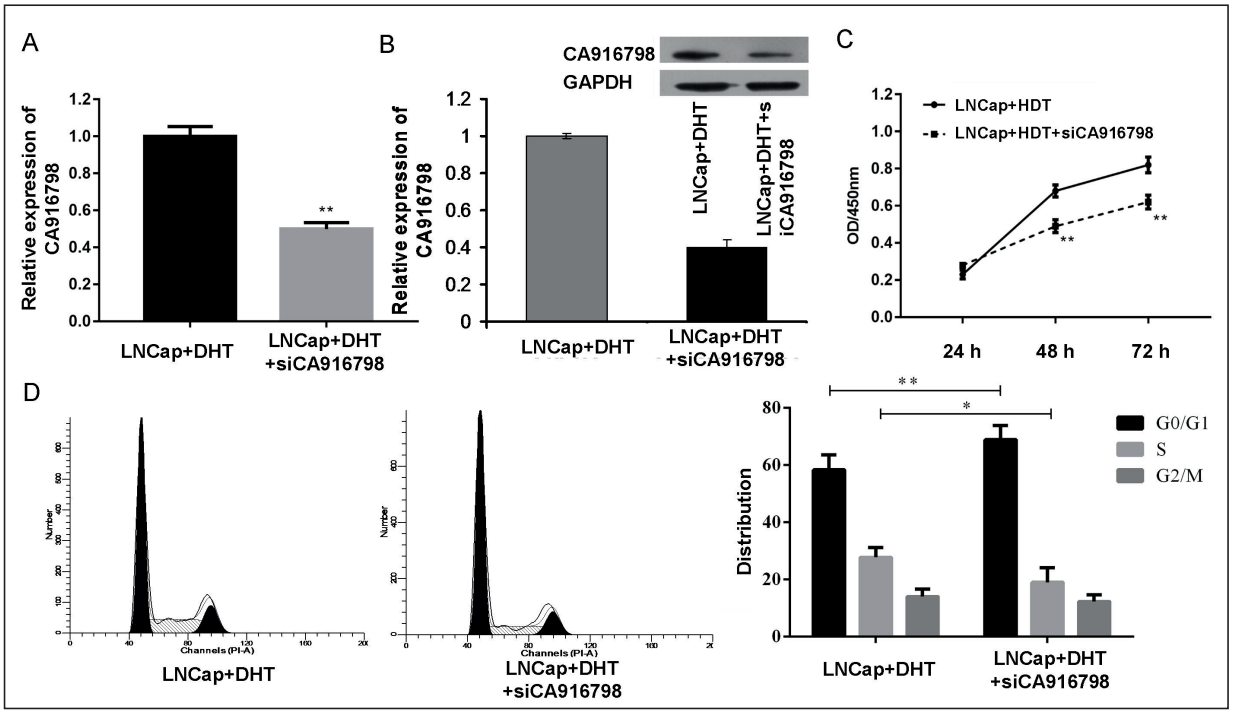

Figure 2. Observing the effects of CA916798 knockdown on the viability, apoptosis, and cell cycle in DHT-stimulated LNCap cells. ***A.*** CA916798 mRNA expression in DHT-stimulated siCA916798-treated LNCap cells. ***B.*** CA916798 protein expression in DHT-stimulated siCA916798-treated LNCap cells. ***C.*** OD_{450} value of CCK-8 assay was decreased after siCA916798 treatment. ***D.*** A larger proportion of DHT-stimulated LNCap cells stayed at the G1-phase, and a smaller proportion of LNCap cells were distributed in the S-phase, after siCA916798 treatment. *$p < 0.05$, **$p < 0.01$ *vs.* Untreated LNCap cell group (Cell + NC group).

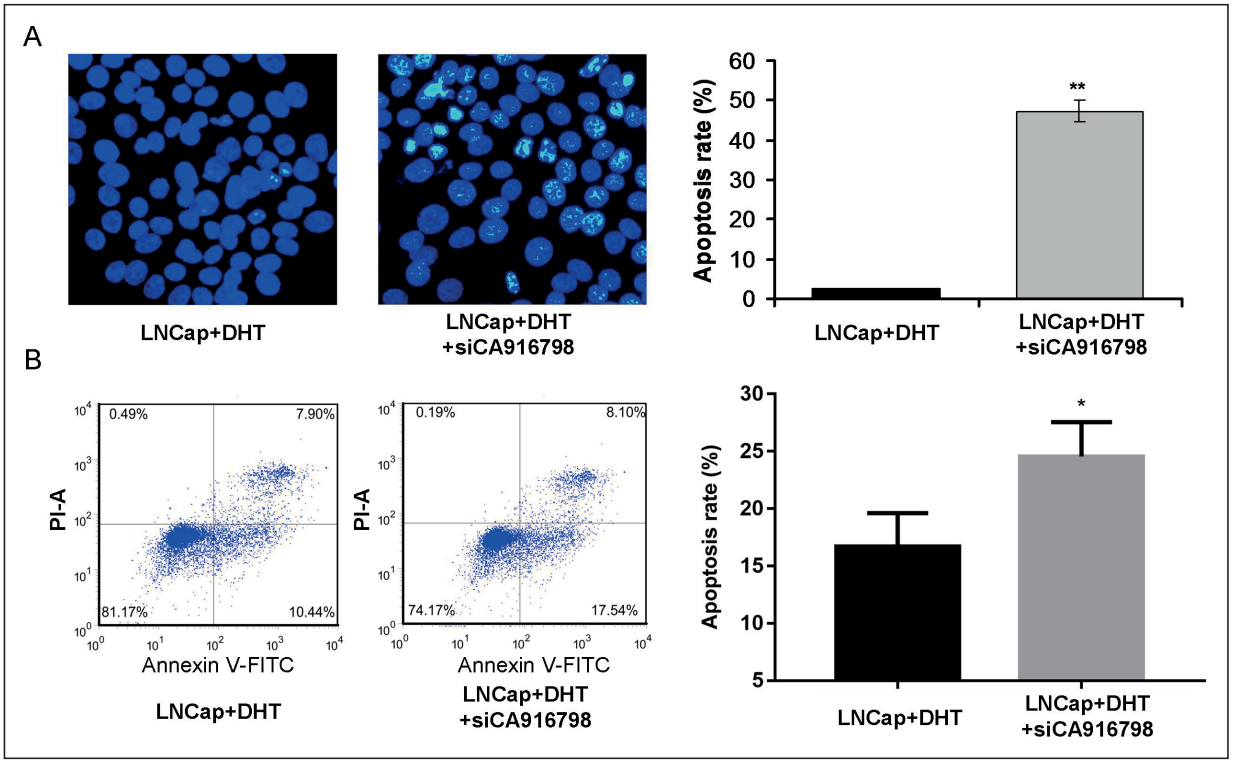

Figure 3. CA916798 knockdown induces apoptosis in DHT-stimulated LNCap cells. ***A.*** Apoptosis determination using Hoechst staining (the positively stained cells are bright blue). ***B.*** Apoptosis determination using flow cytometry assay. (the early apoptosis plus the late apoptosis). $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* DHT-stimulated LNCap cell group (LNCap+DHT group).

DHT-stimulated LNCap cells stained with crystal violet was lower than the number of untreated/DHT-stimulated LNCap cells (Figure 4A, $p < 0.01$). Meanwhile, the relative wound area in case of siCA916798-treated DHT-stimulated LNCap cells was significantly smaller than that in case of untreated LNCap cells at both 0 h and 48 h (Figure 4B, $p < 0.05$).

AR Knockdown Decreased the Viability, Induced Apoptosis, and Suppressed Migration in DHT-Stimulated LNCap Cells

The results indicated that the siAR-treated DHT-stimulated LNCap cell viabilities were significantly decreased, compared to the case for untreated DHT-stimulated LNCap cells (Figure 5A, $p < 0.01$). Some bright blue stained DHT-stimulated LNCap cells were discovered in the siAR-treated DHT-stimulated LNCap group. However, there were even no bright blue stained cells in the untreated DHT-stimulated LNCap group (Figure 5B). The number of DHT-stimulated LNCap cells stained with crystal violet was lower in case of the siAR-treated DHT-stimulated LNCap group, than in case of the untreated LNCap group (Figure 5C, $p < 0.01$).

AR Knockdown Inhibited CA916798 Activities

The results indicated that the AR and CA916798 mRNA levels in siAR-treated DHT-stimulated LNCap cells was significantly decreased (Figure 6A), compared to the case for the untreated DHT-stimulated LNCap cells ($p < 0.01$). Furthermore, the western blot assay showed that the CA916798 protein levels in siAR-treated DHT-stimulated LNCap cells were significantly decreased, compared to the case for the untreated DHT-stimulated LNCap cells (Figure 6B, $p < 0.05$).

AR Functions by Initiating CA916798 Expression

To further investigate the role of CA916798 in androgen-dependent PCa, the ChIP-PCR assay and a dual luciferase assay were performed to evaluate the possible interaction between AR and

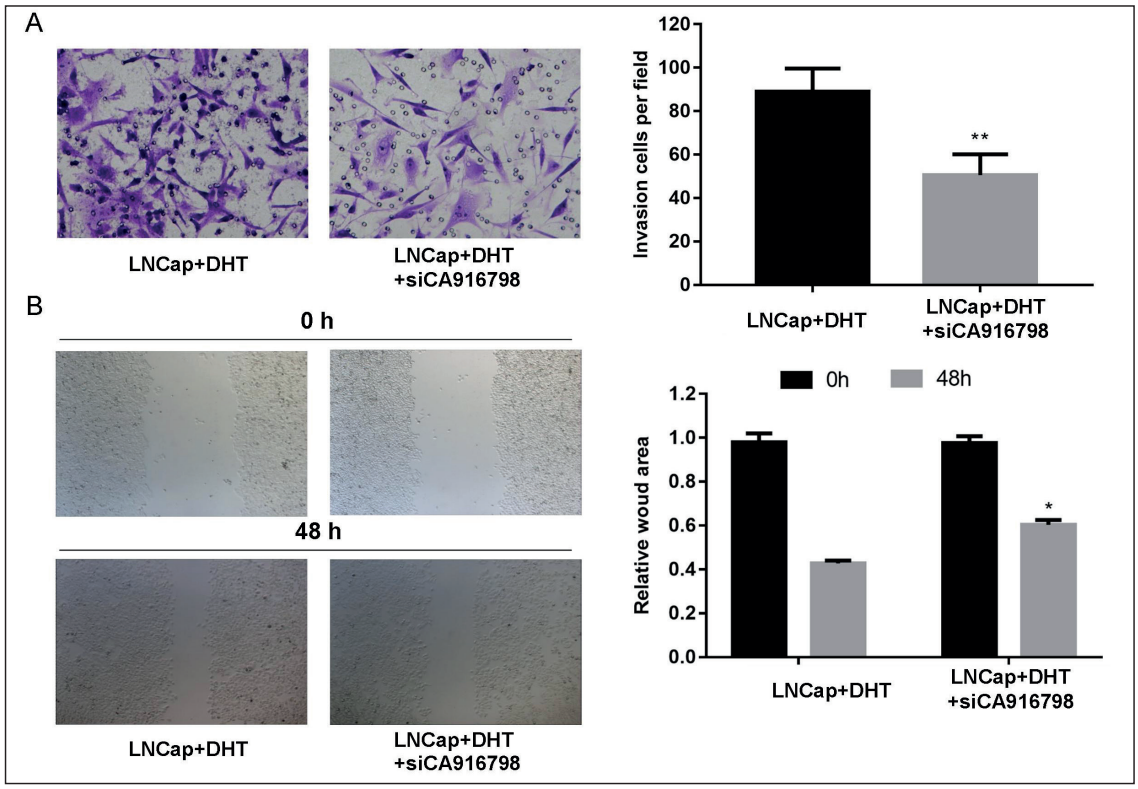

Figure 4. Evaluation of the migration and invasiveness of the DHT-stimulated LNCap cells after CA916798 knockdown. ***A.*** Representative images and quantitative analysis results of the transwell assay. ***B.*** Representative images and quantitative analysis results of the scratch assay. *$p < 0.05$, **$p < 0.01$ *vs.* DHT-stimulated LNCap cell group (LNCap+DHT group).

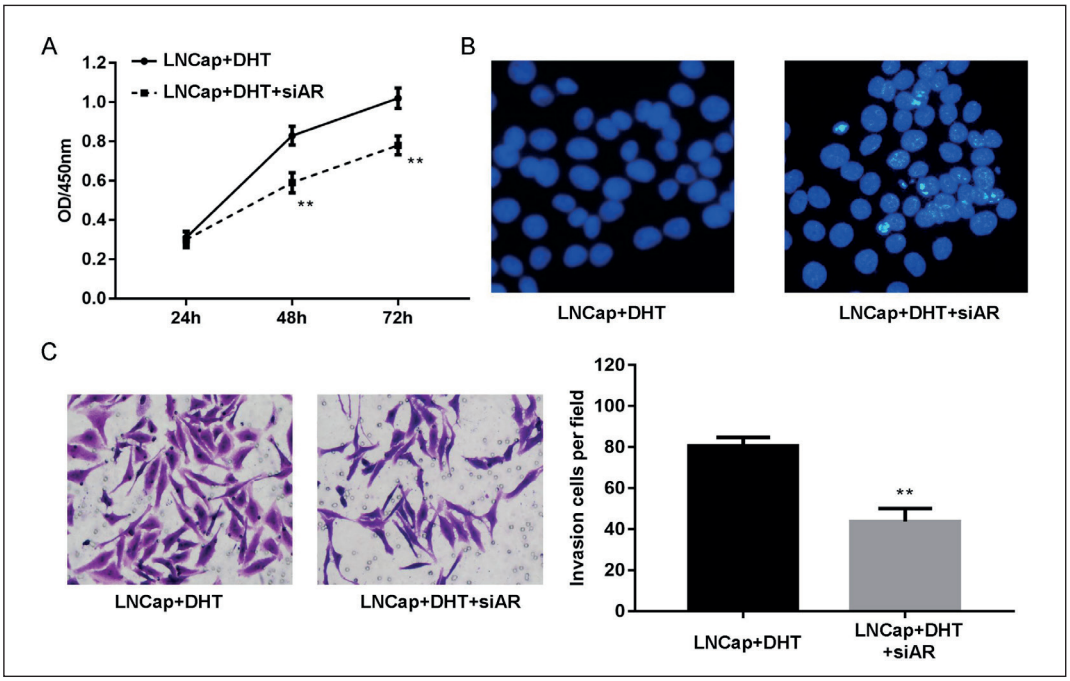

Figure 5. Observing the viability, apoptosis, and migration of siAR-treated DHT-stimulated LNCap cells. ***A.*** OD450 value of CCK-8 assay decreased by after siAR treatment. ***B.*** A larger proportion of DHT-stimulated LNCap cells stayed as apoptotic cells (bright blue staining cells). ***C.*** Representative images and quantitative analysis results of the transwell assay. **$p < 0.01$ *vs.* DHT-stimulated LNCap cell group (LNCap+DHT group).

CA916798. The promoter region of CA916798 was cloned into a pGL-3 plasmid. Meanwhile, a vector containing a mutant sequence of the CA916798 promoter was also constructed. Co-transfection with the AR expression vector significantly increased the relative luciferase activity in CA916798-overexpressed cells, compared to the case for cells transfected with the empty pGL-3 plasmid ($p < 0.05$, Figure 7A). When co-transfected with the mutant CA916798 promoter, no change in luciferase activity was detected; this represents the specific binding of AR to the CA916798 promoter. The results of dual luciferase assay were further verified by ChIP-PCR assay using antibodies against AR and specific primer for CA916798 (Figure 7B), representative of the fact that AR directly bound to the promoter region of CA916798.

Discussion

CA916798 was firstly identified from cisplatin-resistant human lung adenocarcinoma cells, and it has been proven to be a novel gene modulating the multi-drug resistance (MDR) of lung cancer[16,20]. Apart from the MDR-associated functions, CA916798 also encodes a minor histocompatibility antigen recognized by $CD8^+$ cytotoxic T cells and may contribute to tumor regression in patients with renal cell carcinoma[21]. Moreover, Romanuik et al[14] reported that CA916798 is an androgen-responsive gene. Given the key role of androgen in the progression of PCa and the multiple functions of CA916798 relevant to oncogenesis of certain types of cancer, it was reasonable to evaluate the potential of CA916798 as a target for therapies or as biomarkers of PCa. In the current study, with the loss of function assays, it was demonstrated that CA916798 is a critical modulator in the growth and metastasis of androgen-dependent PCa.

We first evaluated the endogenous CA916798 levels in the RWPE-1, PC3, DU145, and LNCap cells. We found that the endogenous expression levels of CA916798 were significantly higher in PC3 cells, than in the other cell lines, which is consistent with the results from a previous study[16]. Post the DHT treatment for 12 h, the CA916798 levels significantly increased in LNCap cells. However, there were no effects of DHT treatment on the CA916798 levels in PC3 cells, which suggests that the PC3 cell line represents androgen-resistant PCa.

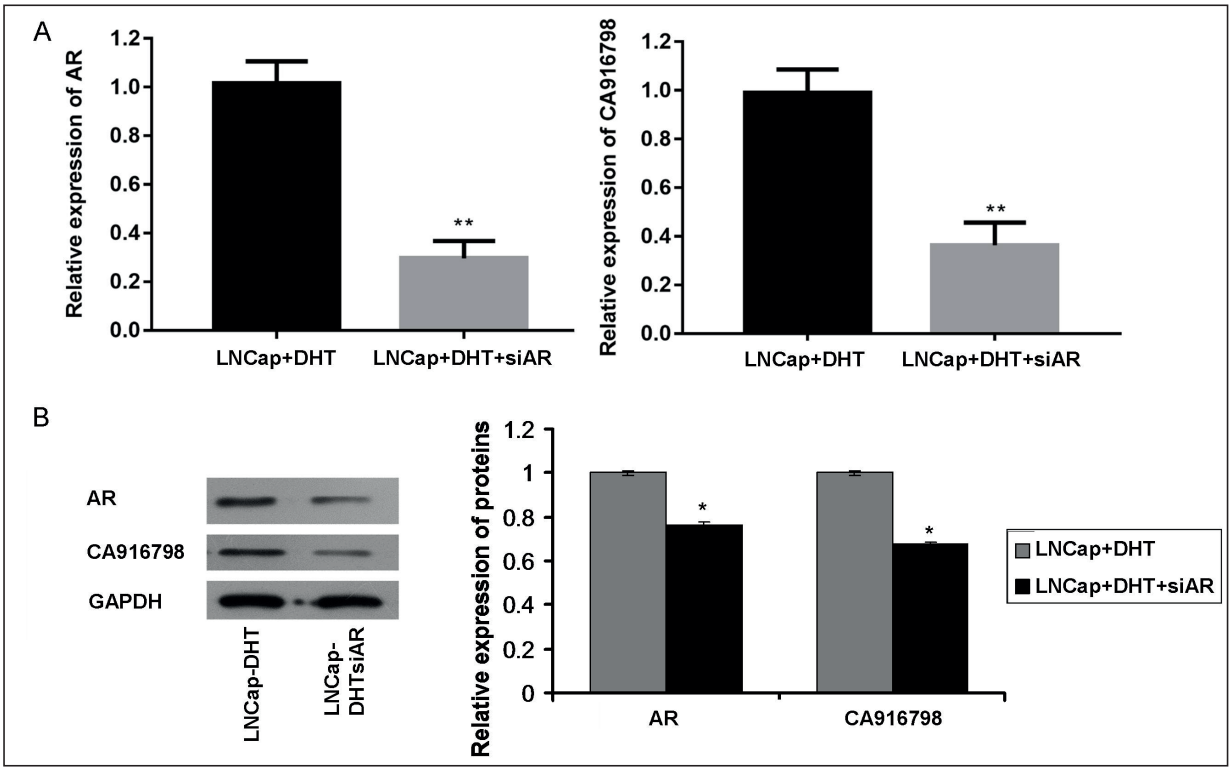

Figure 6. Observing the CA916798 expression in siAR-treated DHT-stimulated LNCap cells. ***A.*** AR and CA916798 mRNA expression in siAR-treated DHT-stimulated LNCap cells. ***B.*** AR and CA916798 protein expression in siAR-treated DHT-stimulated LNCap cells. $^{**}p < 0.01$ *vs.* DHT-stimulated LNCap cell group (LNCap+DHT group).

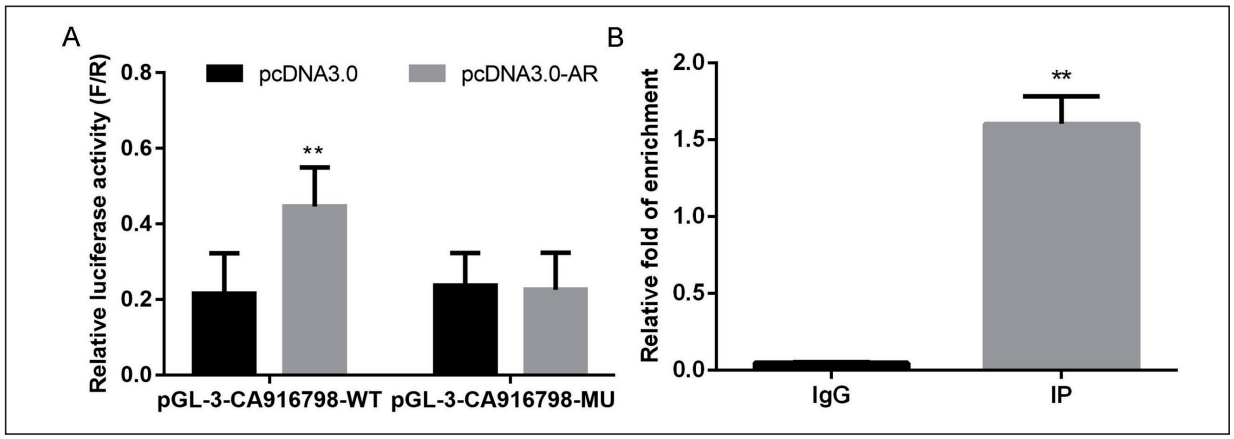

Figure 7. Interaction between the AR and the promoter of the *CA916798*. ***A.*** Dual luciferase assay for detecting the direct binding of AR to the promoter sequence of the *CA916798*. ***p* < 0.01 *vs.* pGL-3-CA916798-WT group. ***B.*** ChIP-PCR assay for detecting the interaction between AR and the *CA916798* in LNCap cells. ***p* < 0.01 *vs.* IgG group.

Romanuik et al[14] reported that androgen triggers the overexpression of the CA916798 in the PCa cell line, LNCap. Therefore, we speculated that the CA916798 may be regulated by the androgen-AR signaling pathway. AR is a type of ligand-dependent transcription factor and a member of the nuclear hormone receptor superfamily[22]. It plays a key role in the onset and progression of PCa[23]. Scholars[24,25] showed that AR and its co-activators increasingly bind to prostate-specific antigen (PSA) in presence of androgens. PSA is a common biomarker for monitoring the development of or the therapeutic response in PCa[24]. The present study originally discovered a direct initiating effect of AR on CA916798 transcription, which represents a novel mechanism driving the AR's contribution to the carcinogenesis of prostate cells. According to the results of both the dual luciferase assay and ChIP assay, AR could bind the promoter sequence of the CA916798, thus serving as a cis-regulatory element for the gene. This finding is critical to the androgen-dependent gene regulation by AR, because so far, only a few AR cis-regulatory sites across the genome have been explored in detail.

In this work, we also investigated the effects of the CA916798 on the growth, apoptosis, and migration of LNCap cells by knocking out both the CA916798 and *AR* (using the small interfering RNA method). Results showed that the siCA916798 treatment significantly induced the apoptosis of the DHT-stimulated LNCap cells, decreased their growth activity, and inhibited their migration. Interestingly, the siAR treatment first inhibited CA916798 expression, which is consistent with the effects of siCA916798 treatment. Meanwhile, the siAR treatment also significantly suppressed the growth and migration of the DHT-stimulated LNCap cells, and induced their apoptosis. We thus suggest that the possible signaling pathways mediating the LNCap cell growth and apoptosis may be similar. Therefore, the potential downstream pathway regulated by CA916798 in PCa cells (LNCap) was investigated in our study.

Actually, there were no investigations focusing on the involvement of the CA916798 gene in PCa. In this study, we found that the CA916798 affects the growth and metastasis of the PCa cell line, LNCap. It is logical for explaining the androgen-resistance of PC3 cells, just because CA916798 is overexpressed even without the DHT treatment. Therefore, when PCa develops from the androgen-sensitive type to the androgen-resistant type, the CA916798 expression was upregulated even without DHT stimuli. However, the specific mechanisms for these phenomena are also clarified, which need to be further investigated in the future studies.

Conclusions

We observed the role of CA916798 on the growth and metastasis of the PCa cell line, LNCap, and showed that the CA916798 plays a pro-PCa role. AR is closely associated with the CA916798 and participates in the growth of androgen-dependent PCa cells (androgen-sensitive or -dependent LNCap cells). The interaction between the CA916798 and AR is critical for the progression and the oncogenesis of PCa. Meanwhile, the CA916798 resolves problems of the pathogenesis

of androgen-dependent PCa. Therefore, this study would merit the development of novel therapeutic strategies that could be beneficial for the prevention and treatment of PCa tumors in the future.

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Conflict of Interest
The Authors declare that they have no conflict of interests.

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