Original Article
CYP19A1 is downregulated by BRD4 and suppresses castration-resistant prostate cancer cell invasion and proliferation by decreasing AR expression

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Abstract: Castration-resistant prostate cancer (CRPC) is the final stage of prostate cancer (PCa). As the main androgen in males, testosterone, and its androgen receptor (AR) play an important role in CRPC. The enzyme that catalyzes testosterone, aromatase, can be influenced by CYP19A1 activity - thus possibly affecting both *AR* expression and CRPC. However, the function of CYP19A1 in CRPC remains unclear. Using data derived from public databases and clinical samples, we analyzed the expression of *CYP19A1* in PCa and CRPC specimens. The effect of CYP19A1 on cell invasion and proliferation was investigated *in vitro* and *in vivo*; while its function in metabolizing testosterone was detected *in vitro*. The effect of BRD4 on CYP19A1 and AR was investigated by qRT-PCR and western blot; whereas the effect of JQ1 on cells was assessed based on the IC50 value. We found that CYP19A1 was downregulated in CRPC samples and cells which correlated with a decrease in CRPC cell invasion and proliferation, and an increase in *AR* expression. Inversely, CYP19A1 affected CRPC cell invasion and proliferation by suppressing the expression of *AR* which may be attributed to the metabolism of testosterone by CYP19A1. Moreover, the BRD4 inhibitor JQ1 induced the *CYP19A1* expression and suppressed the *AR* expression. Following *BRD4* knockdown, *CYP19A1* showed higher expression while *AR* expression was decreased. Our findings demonstrated that CYP19A1 could reduce CRPC cell invasion and proliferation by targeting AR, and this process could be regulated by BRD4. CYP19A1 may be a potential therapeutic target and enhance BRD4 inhibition in treating CRPC.

Keywords: CYP19A1, androgen, androgen receptor, castration-resistant prostate cancer, bromo-domain-containing-protein 4, JQ1

Introduction

Prostate cancer (PCa) remains one of the most common carcinomas in elderly men. It is the second leading cause of cancer-related deaths in Western countries [1]. There are various effective methods for treating PCa, including androgen deprivation therapy (ADT) [2]. As the first-line therapy for treating PCa, ADT is effective at the primary stage [3]. However, patients with PCa will inevitably develop castration-resistant prostate cancer (CRPC) after ADT in less than two years [4]. When patients relapse into the castration-resistant stage, the median survival rate is less than 20 months [5]. Moreover, there is still a lack of effective methods for treating CRPC.

Cytochromes P450 (CYPs) are one of the largest and most diverse superfamily of enzymes [6]. CYPs are involved in the metabolism of endogenous and exogenous substances, including drugs, and environmental compounds. As a terminal oxygenase, they are involved in sterol hormone synthesis in living organisms [7, 8]. The *CYP19A1* gene is a member of the CYP family, which can encode aromatase, a key enzyme that catalyzes the conversion of androgen to estrogen [9, 10]. Therefore, the expression of *CYP19A1* can affect the testosterone level and may be correlated with the occurrence of CRPC. However, the role of CYP19A1 in CRPC is still unclear.

Bromo-domain-containing protein 4 (BRD4) belongs to the bromo-domain and extraterminal (BET) family. It has been reported to exert carcinogenic functions in various cancers, including PCa [11-13]. As a nuclear protein, BRD4 can bind to histone-acetylated lysine res-

idues via its bromo-domains, and further recruit transcription factors to activate downstream gene expression [11, 14]. JQ1, specifically developed as a BRD4 inhibitor, plays an anti-tumor role [12, 14, 15]. The function of JQ1 in treating PCa has been demonstrated; however, the use of JQ1 in treating CRPC requires further investigation.

The androgen receptor (AR) is one of the key factors that promote CRPC [16, 17]. In addition, as the main component of androgen, testosterone can affect AR levels and cause CRPC [18]. In the prostate, when androgen binds to the AR, the AR-androgen complex translocates to the nucleus. Once in the nucleus, AR binds to androgen response elements upstream of target genes, leading to DNA transcription, and inducing prostate epithelial cell proliferation [17, 19]. Although the mechanisms of CRPC are still unclear, the abnormal amplification of AR by testosterone is one of the main underlying causes of CRPC [20].

In this study, we found that the expression of *CYP19A1* was lower in both PCa and CRPC specimens. *CYP19A1* overexpression decreased CRPC cell invasion and proliferation. The effect of CYP19A1 on cell invasion and proliferation may be attributed to its regulation of the level of AR. CYP19A1 could suppress AR levels because it could metabolize testosterone. Moreover, the expression of *CYP19A1* was increased when CRPC cells were subjected to JQ1 treatment or *BRD4* knockdown. Taken together, the findings suggest that BRD4 can influence the levels of AR by targeting CYP19A1. CYP19A1 may be a potential therapeutic target for treating CRPC, especially when combined with BRD4 inhibitors.

Materials and methods

Bioinformatic analysis

The starBase (http://starbase.sysu.edu.cn/starbase2/index) is a public database that provides gene expression data for different tumors based on The Cancer Genome Atlas (TCGA) database. UALCAN (http://ualcan.path.uab.edu/home) is another online web tool that contains sequence data from the TCGA database. The Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/) database is supported by the NCBI, which is used to collect gene expression chip data. We obtained GSE21034 from the GEO database, which had gene expression data from both PCa specimens and para-cancerous samples. The Chinese Prostate Cancer Genome and Epigenome Atlas (CPGEA) (http://www.cpgea.com/) database collects the sequence data of PCa patients in China. We downloaded the data and analyzed the gene expression using R v4.0.3 software.

Tissue samples

CRPC samples and para-cancerous samples were collected at Tongji Hospital, School of Medicine, Tongji University, China. A total of 18 paired PCa and 6 paired CRPC specimens were included in this study. Both tumor tissues and pan-cancer normal samples were collected. All specimens included in the study were tested by a professional pathologist to ensure the accuracy of subgroups. The methods used for collecting the samples were approved by the Ethics Committee of Tongji Hospital (SBKT-2021-220). Patients who provided the samples were familiar with the process of the experiment and gave informed consent.

Cell culture and drug treatment

Prostate cell lines were purchased from the Chinese Academy of Science Cell Bank (Shanghai, China). The human normal prostate epithelial cell line RWPE-1 and human PCa cell lines LNCaP, 22Rv1, and C4-2 were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (R8758; Sigma, Darmstadt, Germany) with 10% fetal bovine serum (FBS) (#10091; Gibco, Thermo Fisher Scientific, Waltham, MA, USA). The human PCa cell line VCaP was cultured in Dulbecco's modified Eagle's medium (DMEM) (D5796; Sigma) with 10% FBS. All cell lines were cultured in a humid environment with 5% CO_2 and 95% air at 37°C. JQ1 was purchased from SelleckChem (S7110; Houston, TX, USA). The PCa cell lines were treated with different drug concentrations following the manufacturers' instructions. In addition, PCa cells were cultured in culture medium without androgen.

Cell transfection and lentivirus production

Cell transfection assays were performed with Lipofectamine 3000 (#11668019; Ther-

Table 1. The shRNA sequence used in the study

Gene name	Targeting sequence
shCYP19A1#1	CUUUGGGAAUAAUAAUCGUUCAGGAUCCUGAACGAUUAUUAUUCCCAAAG
shCYP19A1#2	UCAGGAGUUCAUACAAGGUACAUUAUAAUGUACCUUGUAUGAACUCCUGA
shBRD4#1	GTACCGGTGAACCTCCCTGATTACTATACTCGAGTATAGTAATCAGGGAGGTTCATTTTTTG
shBRD4#2	CCGGCCTGGAGATGACATAGTCTTACTCGAGTAAGACTATGTCATCTCCAGGTTTTTG
shAR#1	CUACCGAGGAGCUUUCCAGAAUCUGUU
shAR#2	UUACACCAAAGGGCUAGAAGGCGAGAG

mo Fisher Scientific) following the manufacturer's instructions. The *CYP19A1* overexpression (oeCYP19A1) plasmid was constructed with *CYP19A1* pcDNA3.1-3xFlag-C by Youze Biotechnology Company (Guangzhou, China). shRNA lentivirus was constructed for specific gene knockdown. The shRNAs were purchased from Youze Biotechnology Company. In addition, blank normal control plasmid (oeControl) without overexpression of *CYP19A1* and blank control lentivirus (shControl) without specific gene knockdown were also constructed by Youze Biotechnology Company. The shRNA sequence is shown in **Table 1**.

Cell invasion assay

After 48 h transfection, the transfected 22Rv1 and C4-2 cells were digested and seeded into the upper chambers (N = 1×10^5) with a non-coated membrane and 200 µL 1640 medium + 2% FBS; 500 µL 1640 medium + 10% FBS was added to the lower chambers. Before the cells were added to the upper chambers, they were treated with mitomycin C (M5353; Sigma-Aldrich) to inhibit cell proliferation. After 48 h, cells on the upper filters were gently removed with a cotton swab. Cells that migrated to the lower chambers were fixed with carbinol for 30 min. Then, the cells were washed three times to remove the carbinol. The chambers were stained by crystal violet for 20 min and washed three times to remove the crystal violet. Finally, the membranes were dried with a blower and observed using a microscope (Olympus, Tokyo, Japan).

Cell proliferation assay

Cell proliferation was detected using the CCK-8 kit (CA1210; Solarbio, Beijing, China). In brief, cells were seeded in 96-well plates (3,000 cells/well), and cultured in 200 µL of 1640 medium + 10% FBS for 0, 24, 48, 72, and 106

h. After incubation, the cells were treated with CCK-8 reagents according to the manufacturer's instructions, and the absorbance was measured at 450 nm using a multi-mode reader (LD942, Beijing, China).

In vivo mouse xenografts

NOD-SCID mice (4 weeks old, 20-22 g) were used in the study. These mice were raised in our research center, at 22 ± 0.5°C with a relative humidity of 60 ± 2%. The mice were fed sufficient food and water every day by specialized feeding staff. Mice were randomly divided into designated groups. Stable shCYP19A1, and shControl 22Rv1 cell lines were constructed. Then, 5×10^6 22Rv1 cells were injected subcutaneously into the right flank of the mice. Tumor size was measured using a scale plate externally every 3 days after it can be gauged. The mice were sacrificed by Nembutal injection into the abdominal cavity on the 27th day after measurement. Then the xenograft tumors were removed and weighed. The animal experiment was approved by the Ethics Committee of Tongji Hospital of Tongji University.

RNA extraction and qRT-PCR

RNA was isolated from tissue samples and cell samples with TRIzol reagent (T9424; Sigma-Aldrich) according to the manufacturer's instructions. The RNA was reverse-transcribed to cDNA using a reverse transcription kit (Advantage® RT-for-PCR Kit, #639505; Takara Bio, Kusatsu, Japan). qRT-PCR was performed using the Applied Biosystems 7500 Sequence Detection System with qRT-PCR reagents and a kit (TB Green® Premix Ex Taq™ II, RR420A; Takara Bio) according to the manufacturer's instructions. GAPDH was used as the normal control. RNA expression was quantified according to the $2^{-\Delta\Delta Ct}$ method. The forward and reverse primer sequences are shown in **Table 2**.

Table 2. Primers used for the qRT-PCR

Gene name	Primer sequence
CYP19A1	Forward: 5'-TCATGCGAGTCGGATCTCTGGAG-3'
	Reverse: 5'-CAAGAAGAGCGTGTTAGAGGTGTCC-3
AR	Forward: 5'-GCTCCGCTGACCTTAAAGACATCC-3'
	Reverse: 5'-ACACCGACATGCCTTACACAAC-3'
BRD4	Forward: 5'-TAGCTCCTCGGACAGTGACAGTTC-3'
	Reverse: 5'-TGGCTCCTTCTTGCTCACATTGC-3'
GAPDH	Forward: 5-GGAGCGAGATCCCTCCAAAAT-3'
	Reverse: 5'-GGCTGTTGTCATACTTCTCATGG-3'

Western blot analysis

Total proteins from samples and cell lines were extracted with RIPA lysis buffer. Protein samples were treated with Dual Color Protein Loading Buffer (NP0007; Thermo Fisher Scientific). The proteins were separated on SDS-PAGE gels (7.5%, 10%), followed by transfer to nitrocellulose membranes (#71078; Merck, Darmstadt, Germany). Protein-Free Rapid Blocking Buffer (#37584; Thermo Fisher Scientific) was used to block the membranes. Then, the membranes were incubated overnight at 4°C with primary antibodies against CYP19A1 (1:1,000, ab18995), AR (1:1,000, ab198394), BRD4 (1:1,000, ab12887), and GAPDH (1:1,000, ab8245) (Abcam, Cambridge, UK). On the next day, 1×TBST was used to wash the membranes three times (10 min, each). Then, the membranes were incubated at 25°C for 1 h with a matched secondary antibody (HRP-labeled Goat Anti-Human IgG (H+L), A0216/A0208; Beyotime, Shanghai, China). Lastly, the membranes were exposed using X-ray irradiation.

Exogenous testosterone culture and test

Testosterone was purchased from Beyotime and added (1 nmol/mL) to the culture medium. At 0, 24, 48, and 72 h culture, the testosterone in the cells was analyzed using the Testosterone ELISA Kit (PT872; Beyotime) according to the manufacturer's instructions. The absorbance was measured at 450 nm using a multimode reader (LD942, Beijing, China).

Statistical analysis

The data represent the results of at least three independent experiments. The results are shown as the mean ± standard deviation (SD).

Statistical analysis was performed by one-way analysis of variance followed by Tukey's post-hoc test for multiple comparisons or Student's *t*-test for comparison between two groups. A *P* value less than 0.05 was considered statistically significant.

Results

CYP19A1 downregulation in PCa and CRPC specimens

To examine the expression of *CYP19A1* in PCa, we analyzed the CPGEA, GEO, and TCGA databases. We found that the mRNA level of *CYP19A1* was significantly downregulated in Chinese patients (**Figure 1A**). The result was similar for GSE21034, which included 130 tumor and 18 normal sample tissues (**Figure 1B**). Similar results were also obtained using the TCGA database. We found that *CYP19A1* was downregulated in PCa samples compared with normal samples in the starBase and UALCAN databases (**Figure 1C**, **1D**). Next, to investigate the expression of *CYP19A1* in CRPC specimens, we collected both CRPC tissue samples and para-cancerous normal tissues at our hospital from patients who underwent radical prostatectomy. The tissues included in the study were confirmed by professional pathologists. Similar to the results using the GEO, CPGEA, and TCGA databases, the expression of CYP19A1 was downregulated at both the mRNA and protein levels (**Figure 1E**, **1F**). We also determined the expression of *CYP19A1* in the normal prostate epithelial cell line RWPE-1 and PCa cell lines. We found that *CYP19A1* was downregulated in PCa cells compared with RWPE-1 cells. In addition, *CYP19A1* was lower in non-androgen-dependent PCa cells (**Figure 1G**, **1H**). The results demonstrated that *CYP19A1* was downregulated at both the mRNA and protein levels in PCa and CRPC samples.

Effect of CYP19A1 on CRPC cell invasion and proliferation in vivo and in vitro

As *CYP19A1* was downregulated in PCa and CRPC specimens, we hypothesized that CYP-19A1 may prevent CRPC occurrence. To verify our hypothesis, we constructed a *CYP19A1* overexpression (oeCYP19A1) plasmid and *CYP-19A1* knockdown (shCYP19A1) lentivirus. We
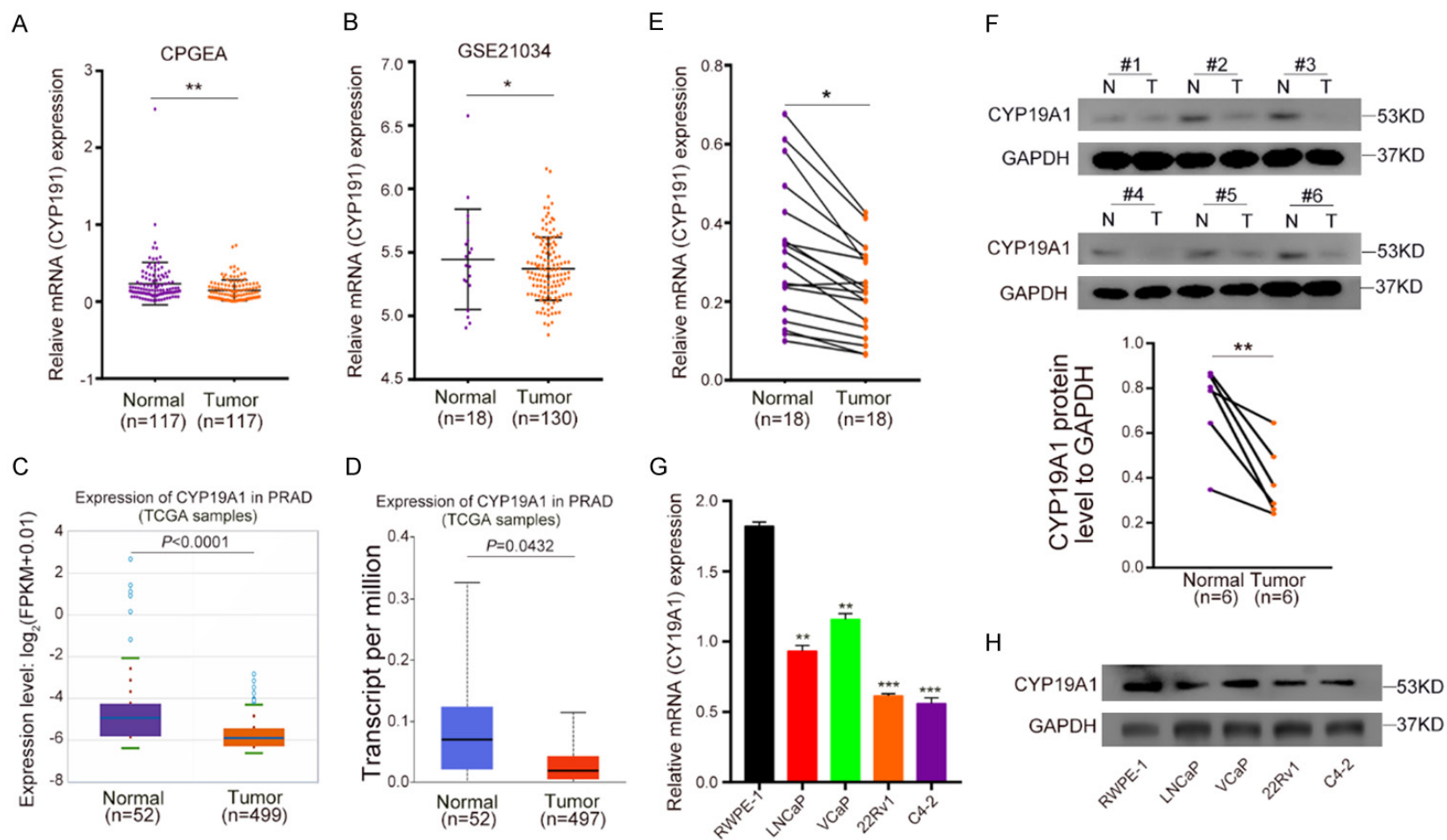
Figure 1. *CYP19A1* downregulates expression in PCa and CRPC specimens compared with normal prostate tissues. A. *CYP19A1* downregulates in Chinese PCa patients (data from CPGEA database). B. The expression of *CYP19A1* was lower in PCa samples than normal samples (data from GSE21034). C, D. The transcript expression of *CYP19A1* using the TCGA data depend on starBase and UALCAN databases. E. Comparation of the mRNA level of *CYP19A1* between CRPC and para-cancerous normal prostate tissues from Tongji Hospital. F. Protein level of CYP19A1 in CRPC patients. Protein was extracted from tumor tissues and para-cancerous normal tissues respectively. G. The mRNA expression of *CYP19A1* among normal prostate epithelial cell: RWPE-1, and human PCa cell lines: VCaP, LNCaP, 22Rv1, and C4-2. H. Protein level of CYP19A1 at both normal prostate epithelial cell line and PCa cell lines. The experiment of qRT-PCR and western blot used GAPDH as an inner control. * represents $P<0.05$, ** represents $P<0.01$, *** represents $P<0.001$. N: represents normal prostate tissues, T: represents CRPC tissues.

transfected the oeCYP19A1 plasmid and shCY-P19A1 lentivirus into 22Rv1 and C4-2 PCa cells, which are CRPC cell lines that can grow without androgen [21, 22]. After transfection, the effect of transfection and the function of *CYP19A1* in cell invasion and proliferation were examined. We found that after transfection with the oeCYP19A1 plasmid, the expression of *CYP19A1* was upregulated at both the mRNA and protein levels in 22Rv1 and C4-2 cells (**Figure 2A**, **2B**). On the other hand, after transfection with the shCYP19A1 lentivirus, the expression of *CYP19A1* was markedly down-regulated in 22Rv1 and C4-2 cells at both the mRNA and protein levels (**Figure 2C**, **2D**). As expected, CYP19A1 could affect cell invasion and proliferation. When transfected with the *CYP19A1* overexpression plasmid, invasion ability was decreased for both 22Rv1 and C4-2 cells. However, after transfection with the *CYP19A1* knockdown lentivirus, the invasion ability of 22Rv1 and C4-2 cells was increased (**Figure 2E**, **2F**). In addition, cell growth was slower after 22Rv1 and C4-2 cells were transfected with the oeCYP19A1 plasmid. On the other hand, when transfected with the shCY-P19A1 lentivirus, the cells showed faster growth (**Figure 2G**, **2H**). We injected stable shCYP19A1 and shControl 22Rv1 cells subcutaneously into NOD-SCID mice. Based on the *in vivo* results, shCYP19A1 increased tumor size and tumor growth (**Figure 2I-K**). Then, we detected the protein level of CYP19A1 in xenograft tumors from different groups of mice. As CYP19A1 may affect *AR* expression, we also detected the protein levels of AR in the xenograft tumors. We found that CYP19A1 protein level decreased while the AR level increased in the shCYP19A1 group mice (**Figure 2L**). Collectively the results indicated that CYP19A1 could suppress CRPC cell invasion and proliferation.

CYP19A1 mediated androgen-dependent regulation of AR expression

The main function of CYP19A1 in the human body is to metabolize androstenedione and testosterone. Therefore, the level of testosterone can affect the level of AR [23]. Since we found that CYP19A1 can decrease AR protein level in mice with the xenograft tumor (**Figure 2L**), we hypothesized that the regulation of AR by CYP19A1 may be attributed to the metabolism

of testosterone. To investigate the hypothesis, we added exogenous testosterone to the culture medium without androgen.

First, we measured the testosterone level in 22Rv1 and C4-2 cells after transfection with the oeCYP19A1 plasmid and shCYP19A1 lentivirus. We added exogenous testosterone to the culture medium of the oeCYP19A1 group and oeControl group for both CRPC cell lines. After 48 h culture, the level of testosterone was markedly lower in the cytoplasm of the oeCY-P19A1 group compared with the oeControl group (**Figure 3A**, **3B**). Following *CYP19A1* knockdown by the shCYP19A1 lentivirus, the level of testosterone was markedly increased (**Figure 3C**, **3D**). Subsequently, we determined whether the testosterone level would change with the addition of exogenous testosterone to the culture medium of the oeCYP19A1 group. We found that although *CYP19A1* overexpression could decrease the level of testosterone, the level in the cytoplasm was not significantly different when exogenous testosterone was added to the culture medium at 72 h (**Figure 3E**, **3F**). To determine whether the change in *AR* expression following the alteration of *CYP19A1* overexpression in CRPC cells is associated with a change in the testosterone level, we measured CYP19A1 and AR expression at both the mRNA and protein levels after exogenous testosterone was added in the oe*CYP19A1* group after 48 h. We found that testosterone increased the expression of CYP19A1 and AR at both the mRNA and protein levels. An increase in the level of testosterone reduced the effect of CYP19A1 on AR (**Figure 3G-J**). However, when we added dihydrotestosterone (DHT), a compound that cannot be metabolized by CYP19A1, to the culture medium, CYP19A1 did not affect *AR* expression (**Figure 3K**, **3L**).

Taken together, the results showed that CYP19A1 could regulate the expression of *AR* by metabolizing testosterone *in vitro*.

Tumor suppressive function of CYP19A1 via decreased AR expression

AR is well known to play an oncogenic role in the occurrence of PCa and even CRPC. Moreover, AR plays an essential role in the resistance to anti-androgen therapy. As an important member of the androgen metabolic pathway, AR is essential for the development of PCa
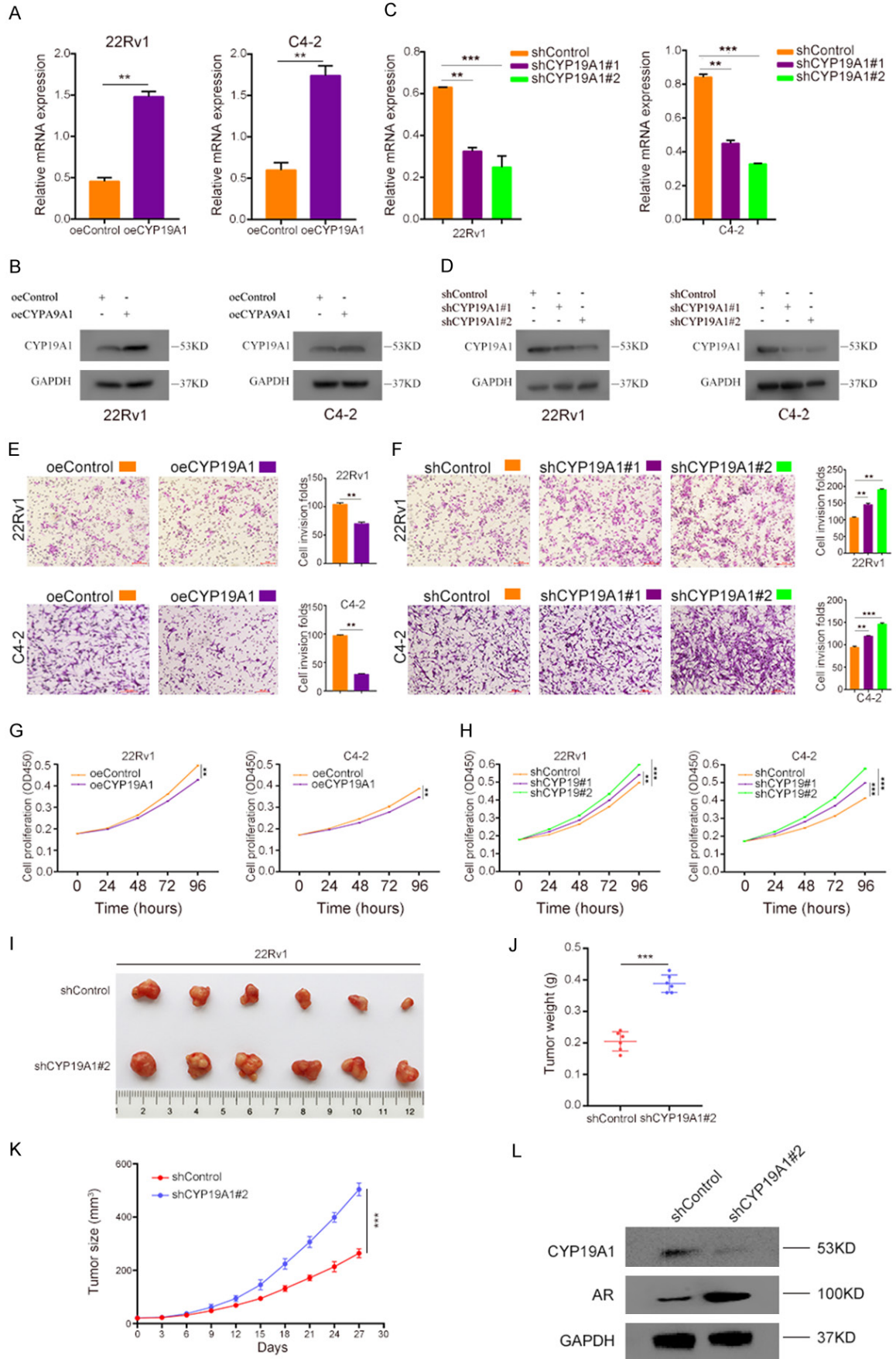
Figure 2. CYP19A1 decreases CRPC cell lines invasion and proliferation in vivo and vitro. A. The mRNA expression of *CYP19A1* after 22Rv1, and C4-2 cells transfected by oeCYP19A1 plasmids and oeControl plasmids. B. Protein level of CYP19A1 after oeCYP19A1 plasmids and oeControl plasmids transfected into CRPC cells. C. The mRNA level of CYP19A1 after shCYP19A1#1, shCYP19A1#2, and shControl lentivirus transfected into 22Rv1 and C4-2 cells, respectively. D. Protein level of CYP19A1 after 22Rv1 and C4-2 cells was transfected with different lentivirus, respectively. E. 22Rv1 and C4-2 cells which overexpressed *CYP19A1* had lower invasion ability than cells transfected with oeControl plasmids (scale bars: 100 μm, magnification: 100). F. *CYP19A1* knockdown 22Rv1 and C4-2 cells have higher invasion ability (scale bars: 100 μm, magnification: 100). G. Cell proliferative ability was decreased after oeCYP19A1 plasmids transfected into 22Rv1 and C4-2 cells. H. Cell proliferative ability was increased after shCYP19A1 lentivirus transfected into 22Rv1 and C4-2 cells. I-K. The volume, size and weight of tumors in NOD-SCID mice after injected with shCYP19A1 and shControl 22Rv1 cells, respectively. Every 3 days, the tumors size would be measured. At 27 days, the tumors were excised and the weight was measured. L. The protein level of CYP19A1 and AR in different group mice. * represents *P*<0.05, ** represents *P*<0.01, *** represents *P*<0.001. GAPDH as inner control at both qRT-PCR and western blot experiments.

and CRPC [24-26]. As shown previously, CYP19A1 can reduce the expression of *AR* by decreasing testosterone levels in CRPC cells. We hypothesized that the tumor suppression function of CYP19A1 may be correlated with AR.

To investigate our hypothesis, we observed the expression of *AR* after 22Rv1 and C4-2 PCa cells were transfected with the oeCYP19A1 plasmid or shCYP19A1 lentivirus. After transfection with the oeCYP19A1 plasmids, the expression of *AR* in 22Rv1 and C4-2 cells was decreased at both the mRNA and protein levels (**Figure 4A**, **4B**). In contrast, we found that the expression of *AR* was increased following *CYP19A1* knockdown (**Figure 4C**, **4D**). In addition, we also analyzed the correlation of *CYP19A1* and *AR* depending on the data from the GSE21034 dataset. We found that there is a negative correlation between *CYP19A1* and *AR* expression in PCa (**Figure 4E**). The results indicated that *CYP19A1* could reduce the expression of *AR*. Subsequently, we investigated whether CYP19A1 suppresses cell invasion and proliferation by regulating AR. We constructed an shAR (*AR* knockdown) lentivirus and found that the shRNA lentivirus can inhibit the expression of *AR* at both the mRNA and protein levels (**Figure 4F**, **4G**). As shown, following *CYP19A1* knockdown, 22Rv1 and C4-2 cell invasion was increased. Likewise, cell invasion ability was markedly reduced after the shAR was lentivirus transfected into 22Rv1 and C4-2 cells. However, there was no difference between shCYP19A1+shAR and shAR for both 22Rv1 and C4-2 cells (**Figure 4H**, **4I**). Similar results were also obtained for cell proliferation. In comparison with shControl cells, shCYP19A1-transfected 22Rv1 and C4-2 cells grew

faster. With shAR transfection, the proliferative ability of 22Rv1 and C4-2 cells was markedly decreased compared with that of shControl cells. However, the proliferative ability of CRPC cells was not significantly different between shCYP19A1+shAR and shAR (**Figure 4J**, **4K**).

Taken together, the results demonstrated that CYP19A1 could affect cell invasion and proliferation by targeting AR.

Upregulation of CYP19A1 by suppressing BRD4 and modulating the sensitivity of the CRPC cell response to JQ1 treatment

The BRD4 protein has been found to play an important role in the occurrence of PCa. JQ1 as a BRD4 inhibitor may be used to treat PCa. However, the mechanism of JQ1 in the treatment of PCa requires further investigation. In a previous study, researchers found that some anti-cancer compounds can collectively affect the function of BRD4 and aromatase [27]. BRD4 is involved in the regulation of downstream target gene expression [15]. Therefore, we hypothesized that *CYP19A1* may be a downstream target regulated by BRD4.

First, we measured the BRD4 protein level in prostate cell lines, and observed higher BRD4 levels in PCa cell lines (**Figure 5A**, **5B**). Next, we examined the expression of *BRD4* using the TCGA database. We found that *BRD4* was upregulated in tumor tissues compared with normal tissues (**Figure 5C**). Then, we examined the correlation between *BRD4* and *CYP19A1* in the GSE21034 dataset. We found that there was a negative correlation between *BRD4* and *CYP19A1* ($R = -0.32$, $P = 6.0 \times 10^{-5}$) (**Figure 5D**). To further confirm the correlation between *BDR4* and *CYP19A1,* we purchased a BRD4
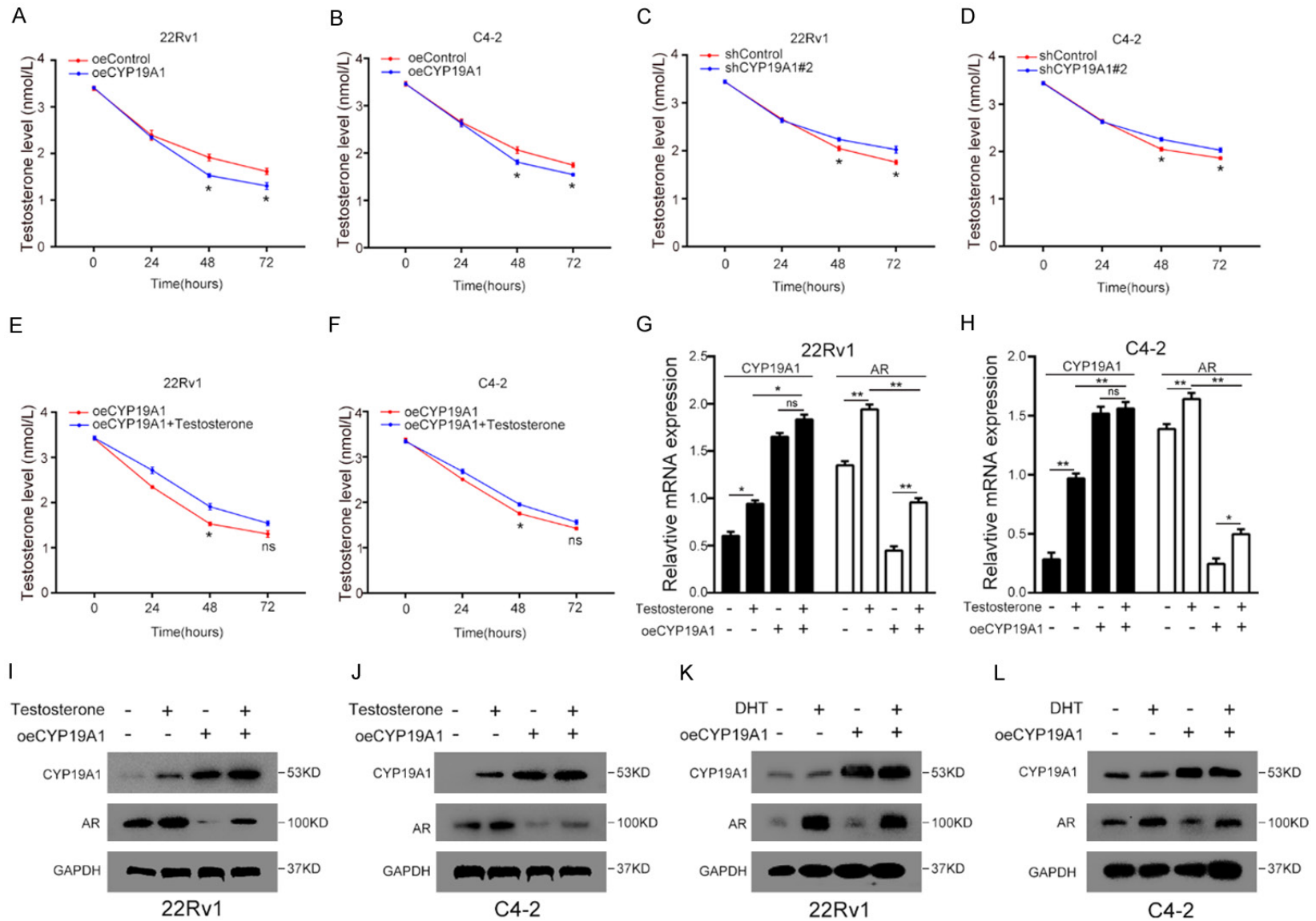
Figure 3. CYP19A1 regulates the expression of *AR* by metabolizing testosterone. A. The level of testosterone in cytoplasm after oeControl and oeCYP19A1 plasmids transfected into 22Rv1 cells. B. Testosterone level in cytoplasm after ocControl and oeCYP19A1 plasmids transfected into C4-2 cells. C. The level of testosterone in cytoplasm after shControl and shCYP19A1 lentivirus transfected into 22Rv1 cells. D. Testosterone level in cytoplasm after shControl and shCYP19A1 lentivirus

transfected into C4-2 cells. E. Testosterone level in cytoplasm after exogenous testosterone added into culture medium among different groups. F. Testosterone level in cytoplasm after exogenous testosterone added into culture medium cultured different C4-2 cells. G, H. The mRNA level of *CYP19A1* and *AR* after exogenous testosterone added into culture medium of different cells with or without *CYP19A1* overexpression. I, J. The protein level of CYP19A1 and AR after exogenous testosterone added into culture medium between different CRPC cell groups. K, L. CYP19A1 and AR protein expression after exogenous DHT added into culture medium between different CRPC cell groups. ns represents no significant, * represents *P*<0.05, ** represents *P*<0.01.
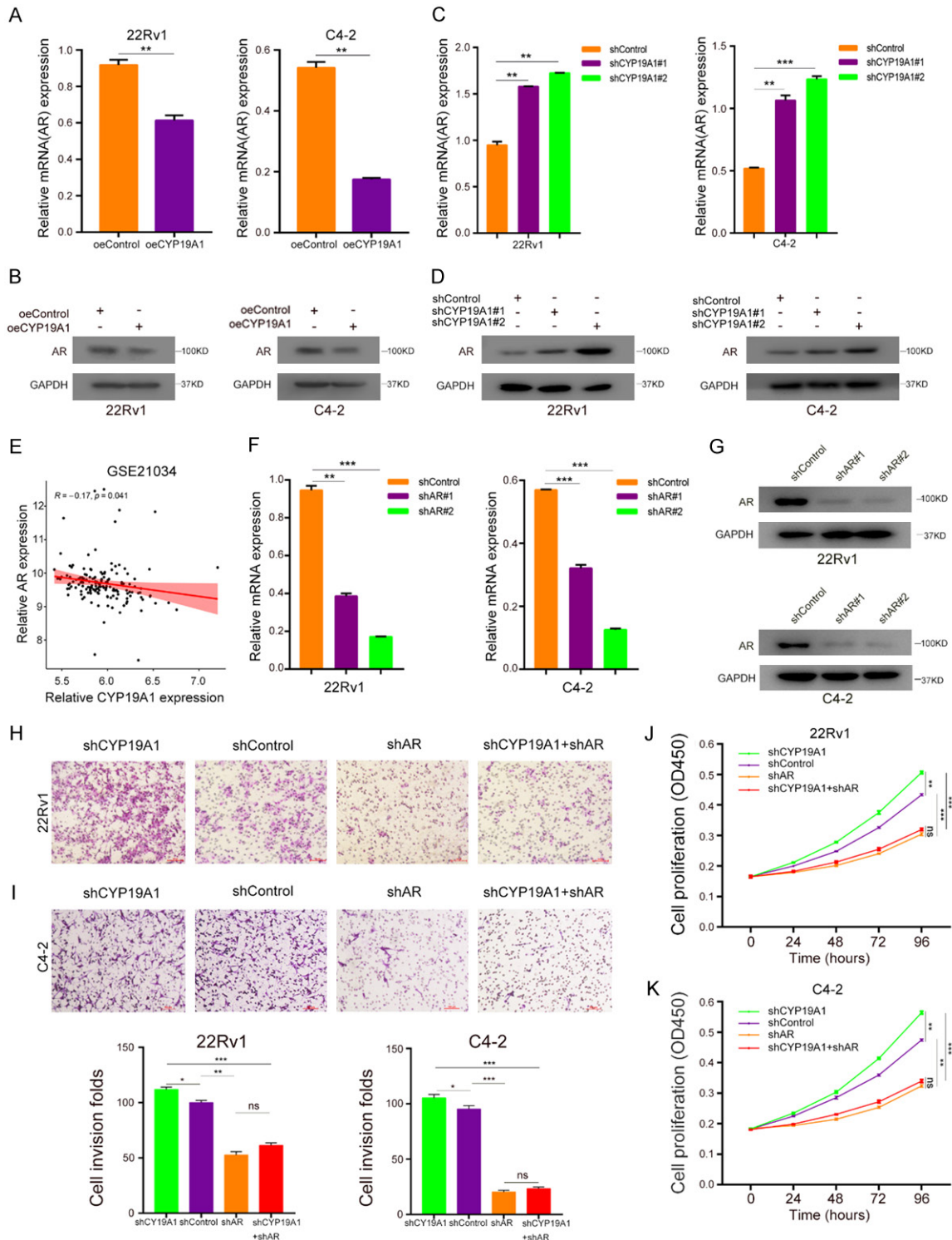
Figure 4. CYP19A1 depresses CRPC cell invasion and proliferation though inhibiting AR. A. The mRNA level of *AR* after 22Rv1 and C4-2 cells transfected with oeCYP19A1 and oeControl plasmids. B. Protein expression of AR after 22Rv1 and C4-2 cell were transfected by different plasmids. C. The mRNA expression of *AR* after shControl or shCYP19A1 lentivirus transfected into 22Rv1 and C4-2 cells. D. The protein level of AR after CYP19A1 knockdown in both 22Rv1 and C4-2 cells. E. The correlation of *CYP19A1* and *AR* in PCa samples from GSE21034 dataset. F, G. The efficiency of shAR lentivirus was verified at both the mRNA and protein levels in different cell lines. H, I. 22Rv1 and C4-2 cells invasion ability was detected after transfected with different lentivirus (scale bars: 100 μm, magnification: 100). J, K. After 22Rv1 and C4-2 cells transfected with different lentivirus, the cell proliferative ability was detected. ns represents no significant, * represents $P<0.05$, ** represents $P<0.01$, *** represents $P<0.001$. GAPDH as an inner control at both qRT-PCR and western blot experiments.
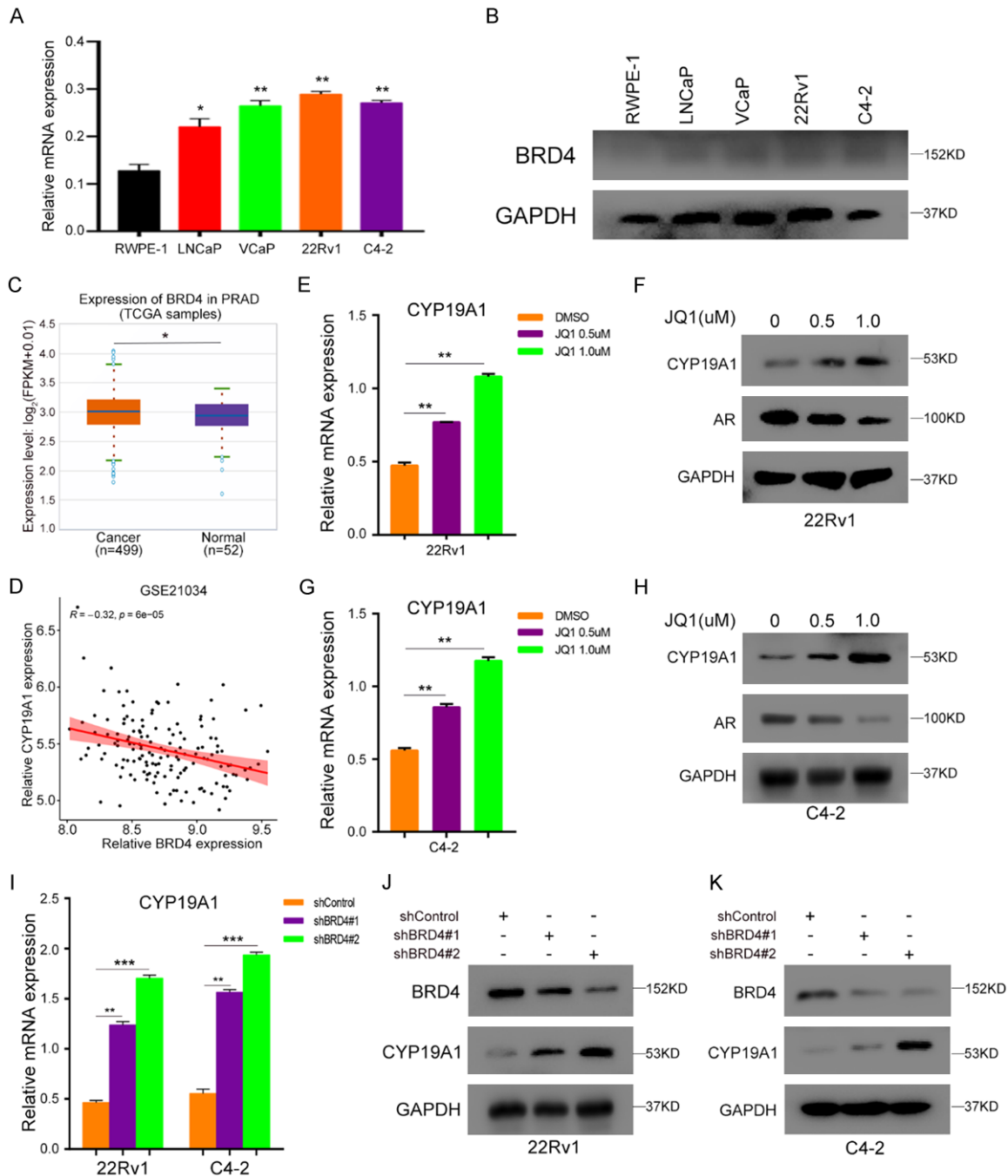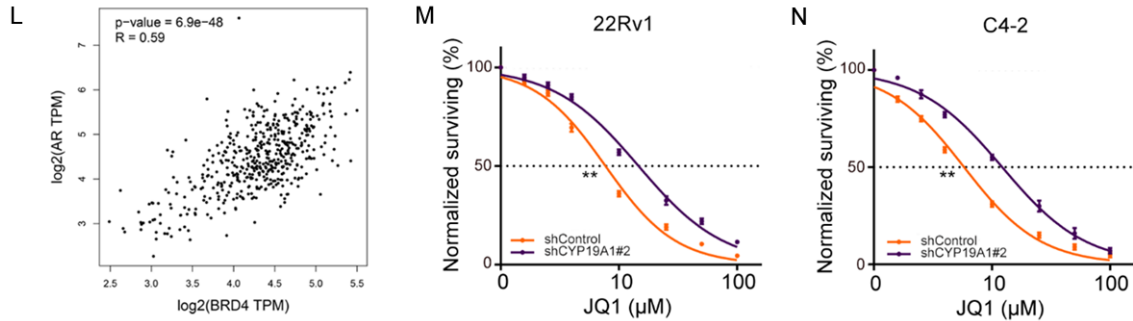
Figure 5. CYP19A1 is downregulated by BRD4 and affects the sensitivity of CRPC cell response to JQ1 treatment. A, B. The mRNA and protein level of BRD4 among RWPE-1 cells and PCa cell lines. C. The transcript expression level of *BRD4* in PCa using TCGA data. D. The mRNA expression correlation of *CYP19A1* and *BRD4* in human PCa samples was detected by GSE21034. E. The expression of *CYP19A1* at mRNA level after 22Rv1 cells treated by different dose of JQ1. F. Protein expression of CYP19A1 after 22Rv1 cells treated by different dose of JQ1. G. The mRNA level of *CYP19A1* in C4-2 cells after treated with different dose of JQ1. H. The expression of CYP19A1 and AR protein in C4-2 cells after treated with different dose of JQ1. I. The mRNA expression level of *CYP19A1* after 22Rv1 and C4-2 cells transfected with shBRD4 lentivirus. J, K. Protein level of CYP19A1 and AR after 22Rv1 and C4-2 cells transfected after shBRD4 lentivirus. L. The correlation of *BRD4* and *AR* in PCa from TCGA database. M, N. The dose-effect relation curves of JQ1 in 22Rv1 and C4-2 cell after transfected by different lentivirus (shCYP19A1 or shControl lentivirus). GAPDH as inner control at both qRT-PCR and western blot experiments. * represents *P*<0.05, ** represents *P*<0.01, *** represents *P*<0.001.

inhibitor (JQ1) and constructed a shBRD4 lentivirus. We found that after 22Rv1 and C4-2 cells were treated with JQ1, the expression of *CYP19A1* was increased at both the mRNA and protein levels; simultaneously, the expression of *AR* was decreased. In addition, the expression level of *CYP19A1* was increased with a higher concentration of JQ1 (**Figure 5E-H**). *BRD4* knockdown in 22Rv1 and C4-2 cells transfected with the shBRD4 lentivirus showed that the expression of *CYP19A1* was increased at both the mRNA and protein levels (**Figure 5I-K**). As we found that JQ1 can decrease *AR* expression, we speculated that there may be a correlation between *BRD4* and *AR* in PCa. Therefore, we detected the correlation of *BRD4* and *AR* depending on the TCGA data. We found a positive correlation between *BRD4* and *AR* in PCa (**Figure 5L**). The results indicated that BRD4 may be an oncogenic protein that contributes to PCa, and it may play a potential role in suppressing the expression of *CYP19A1* while increasing *AR* expression. Finally, we investigated whether the expression of *CYP19A1* modulates the effect of JQ1. We treated shCYP19A1-transfected and shControl-transfected CRPC cells with different concentrations of JQ1. We found that when *CYP19A1* was downregulated, the cells were more resistant to JQ1. The IC50 value of JQ1 was increased from 8.92 μM to 16.74 μM for 22Rv1 cells

and from 7.62 μM to 14.86 μM for C4-2 cells (**Figure 5M**, **5N**). These results demonstrated that CYP19A1 could be regulated by BRD4, which could affect the sensitivity of CRPC cells to treatment with JQ1.

CYP19A1 impacts BRD4-induced oncogenesis in CRPC cells

As we found that CYP19A1 can modulate the effect of JQ1 on CRPC cells, we next tried to find whether CYP19A1 can also influence the action of BRD4 on AR and cell function. Therefore, we first transfected both shCYP19A1 and shBRD4 lentivirus into 22Rv1 and C4-2 cells and then detected whether the expression of *AR* and cell function would change. We found that when CRPC cells were transfected by shBRD4 lentivirus, the protein level of AR would decrease sharply (**Figure 6A**, **6B**). In addition, we confirmed *CYP19A1* knockdown did not affect *BRD4* expression (**Figure 6A**, **6B**). We also found that though *CYP19A1* knockdown can increase the expression of *AR*, this effect would be offset with simultaneous *BRD4* knockdown (**Figure 6A**, **6B**). Then, we tried to confirm whether CYP19A1 can BRD4-induced oncogenesis in CRPC. We tested cell invasion and proliferation after these CRPC cells were transfected by different lentiviruses. For *BRD4* knockdown, we found a decrease in the inva-
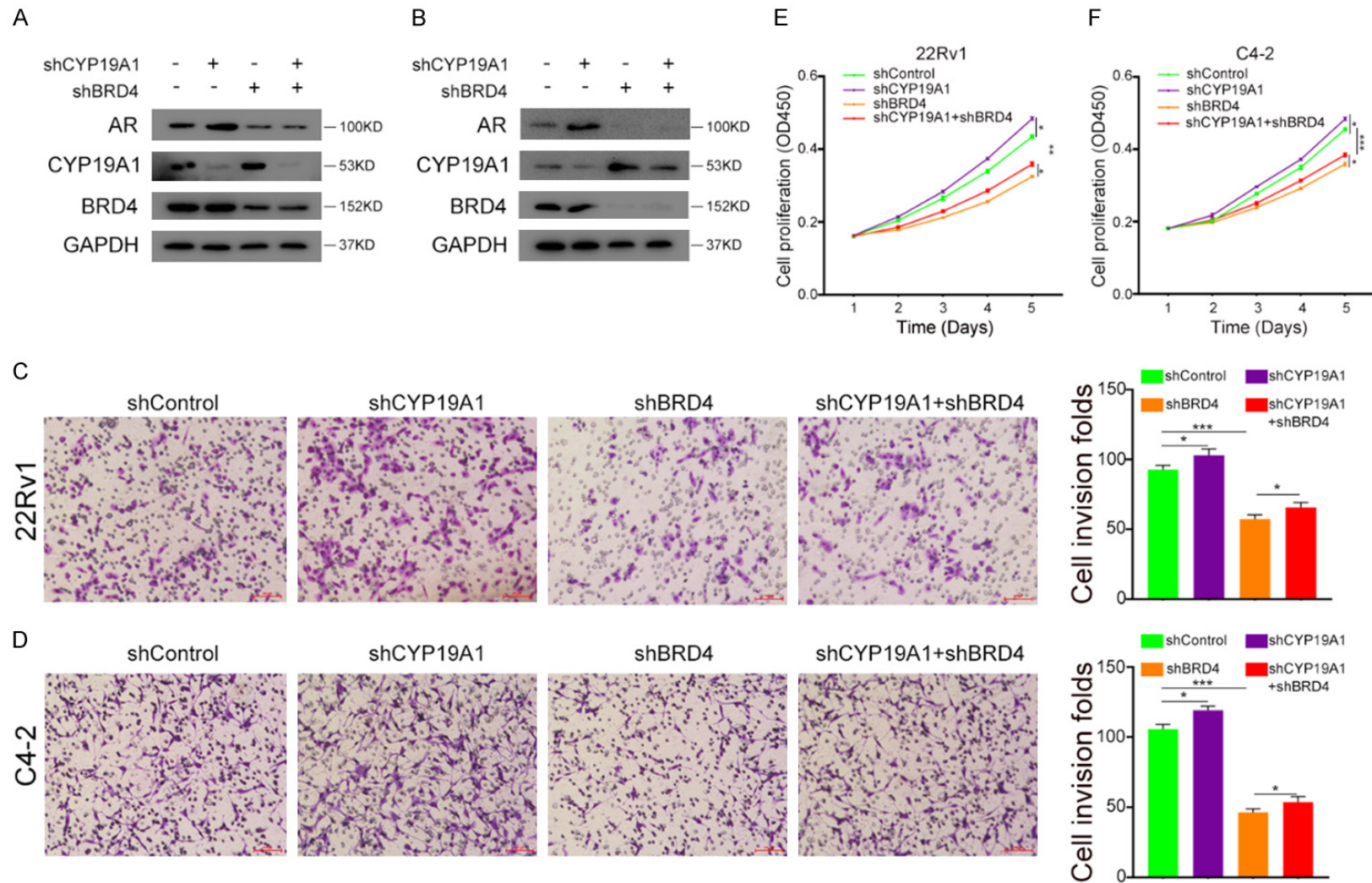
Figure 6. CYP19A1 enhances the degressive of CRPC cell invasion and proliferation after *BRD4* knockdown. (A, B) The protein expression of AR, CYP19A1, and BRD4 after 22Rv1 (A) and C4-2 (B) cells transfected by shCYP19A1 or shBRD4 lentivirus. (C, D) Cell invasion ability after CRPC cells transfected by shCYP19A1 or shBRD4 lentivirus (scale bars: 100 μm, magnification: 100). (E, F) Cell proliferation ability after 22Rv1 and C4-2 cells transfected by shCYP19A1 or shBRD4 lentivirus. GAPDH as inner control at western blot experiments. * represents *P*<0.05, *** represents *P*<0.001.
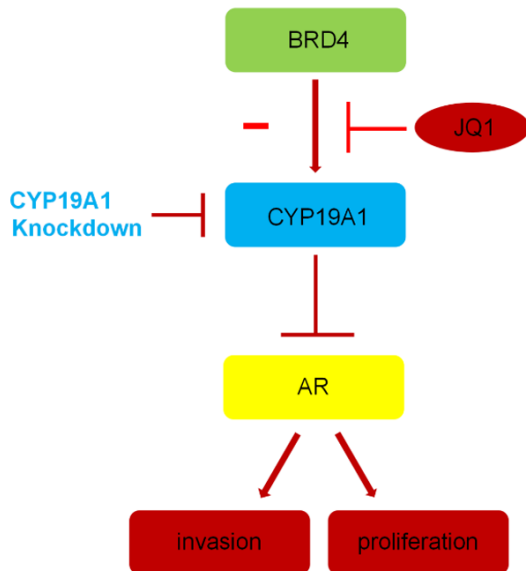
Figure 7. The hypothetical model of the study.

sion and proliferation ability of cells (**Figure 6C-F**). Further, we also found that CYP19A1 enhanced the degressive of cell invasion and proliferation after *BRD4* knockdown (**Figure 6C-F**). These results indicated that CYP19A1 impact the BRD4-induced oncogenesis in CRPC cells.

In summary of the results, we found CYP19A1 could be downregulated by BRD4, and its function could be reversed by JQ1. In addition, CYP19A1 could suppress CRPC cell invasion and proliferation by regulating the AR (**Figure 7**).

Discussion

PCa is one of the most common malignant tumors in elderly men. In the United States, the incidence rate of PCa is more than 20% [1]. It is also the second leading cause of death from cancer in men. In China, PCa is a serious health problem among men. According to the data from the National Cancer Center of China, the incidence and mortality rates of PCa have greatly increased [28]. Androgen plays an important role in the occurrence of PCa and thus is a target in ADT, which is currently the first-line treatment for PCa [3]. However, after a treatment period of 18-24 months, patients with PCa will inevitably relapse into the castration-resistant stage [4]. Patients with CRPC typically have a median survival time of no

more than 2 years [5]. To date, there is no effective method for treating CRPC, and the mechanism of CRPC is still unclear.

The androgen metabolic pathway is important in androgen synthesis and metabolism. It has been demonstrated that the abnormal function of the androgen metabolic pathway plays a key role in the occurrence of CRPC [29]. The androgen metabolic pathway consists of several components, which include CYPs [30]. CYPs are one of the largest and most diverse superfamily of enzymes [6], and are involved in the process of metabolizing various substances. As a terminal oxygenase, they play a role in sterol hormone synthesis in living organisms [7, 8]. The *CYP19A1* gene is a member of the CYPs, which can encode aromatase, a key enzyme that catalyzes the conversion of androgen to estrogen [9, 10]. Therefore, the higher the expression of *CYP19A1*, the lower the androgen level in the serum. In addition, CYP19A1 has been demonstrated to play an important role in the occurrence of breast cancer and even affect the survival of patients [31]. Various drugs that can inhibit the function of CYP19A1, such as letrozole, have been used in clinical settings to treat breast cancer [32]. PCa and breast cancer share many similarities; for example, they are both hormone-dependent neoplasms. In addition, CYP19A1 can metabolize testosterone to estrogen. Therefore, CYP-19A1 may be correlated with the occurrence of CRPC and may be a potential therapeutic target for treating CRPC.

AR binds to androgen and has been found to play an important role in both hormone-dependent PCa and CRPC occurrence and development [33]. AR is a member of the nuclear steroid receptor superfamily of transcription factors. It is located at Xq11-12 and contains eight exons that encode a protein of ~919 amino acids [34-37]. Since 1995, there is a consensus that AR can regulate multiple cellular events such as proliferation, apoptosis, migration, invasion, and differentiation. *AR* mutation and amplification occur in PCa and CRPC. Moreover, *AR* expression changes in PCa with lymph node, visceral, and bone metastases [38, 39]. Apart from primary PCa, *AR* is also highly expressed and transcriptionally active in CRPC [26]. Given its involvement, AR is a target in enzalutamide treatment [40]. A series of *in*

vitro studies have similarly shown increased *AR* expression and the restoration of AR activity in tumors that relapsed after castration, and RNA interference and related approaches established that AR was required for growth in these CRPC models [41-45].

BRD4 has been reported to be overexpressed in various tumors including CRPC [11-13]. As a carcinogenic factor, BRD4 can promote breast cancer cell invasion and migration by targeting the Jagged1/Notch1 pathway [13]. Increased *BRD4* expression is related to lung cancer lymph node metastasis, and the mechanism may involve the binding of BRD4 to RelA, an important component of the NF-κB complex, leading to inflammatory responses [46]. In addition, BRD4 can regulate the transcription of the onco-protein AR and promote PCa progression [47]. JQ1, a BRD4 inhibitor, has been demonstrated to function by targeting BRD4, which is a transcription factor that belongs to the BET family and has been reported to be a novel and important oncogenic protein in human PCa [48, 49]. Moreover, JQ1 disrupts the BRD4-acetylated lysine interaction, further suppressing the transcriptional activity of BRD4 [15]. Here, we found that both BRD4 and JQ1 could affect the expression of *CYP19A1*, indicating that *CYP19A1* may be a potential downstream gene of BRD4.

In this study, the expression of *CYP19A1* was lower in CRPC samples. In addition, CYP19A1 affected CRPC cell invasion and proliferation, which may be attributed to the regulation of AR by CYP19A1. Moreover, as CYP19A1 could metabolize testosterone, it could affect *AR* expression. We found that *CYP19A1* expression could be influenced by BRD4, and it could modulate the effect of JQ1 in CRPC. Taken together, this means that BRD4 can indirectly regulate the expression of *AR*. The results suggest that CYP19A1 may be a potential target for treating CRPC, especially when using BRD4 inhibitors.

Our study is not without limitations. First, although we examined the expression of *CYP-19A1* in public databases, clinical PCa specimens, and PCa cell lines, the sample size was small. In addition, the difference in *CYP19A1* expression between PCa and CRPC was small. Therefore, despite the importance of CYP19A1 in the occurrence of both PCa and CRPC, it may not be important for the development of PCa

into CRPC. Second, although the expression of *CYP19A1* could be affected by BRD4, the specific regulatory mechanisms remain unclear. More studies should be conducted to elucidate the specific mechanisms underlying the effect of BRD4 on the expression of *CYP19A1* in CRPC.

Conclusion

In conclusion, CYP19A1 plays a role in the development of CRPC by decreasing CRPC cell invasion and proliferation. In CRPC cells, CYP19A1 targets the AR by metabolizing testosterone which can be regulated by BRD4. Furthermore, the expression of CYP19A1 influences cell sensitivity to BRD4 inhibitors. Therefore, CYP19A1 may be a potential target for treating CRPC by enhancing the effect of BRD4 inhibitors.

Acknowledgements

Disclosure of conflict of interest

None.

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