A Novel Androgen Receptor Splice Variant Is Up-regulated during Prostate Cancer Progression and Promotes Androgen Depletion–Resistant Growth

Zhiyong Guo,[1] Xi Yang,[1] Feng Sun,[1] Richeng Jiang,[1] Douglas E. Linn,[1] Hege Chen,[1] Hegang Chen,[2] Xiangtian Kong,[3] Jonathan Melamed,[3] Clifford G. Tepper,[4] Hsing-Jien Kung,[4] Angela M.H. Brodie,[1] Joanne Edwards,[5] and Yun Qiu[1]

[1]Department of Pharmacology and Experimental Therapeutics and The Greenebaum Cancer Center and [2]Department of Epidemiology and Preventive Medicine, University of Maryland School of Medicine, Baltimore, Maryland; [3]Department of Pathology, New York University School of Medicine, New York, New York; [4]Division of Basic Sciences, University of California Davis Cancer Center, Sacramento, California; and [5]Division of Cancer Sciences and Molecular Pathology, University of Glasgow, Glasgow Royal Infirmary, Glasgow, United Kingdom

Abstract

The androgen receptor (AR) plays a key role in progression to incurable androgen ablation–resistant prostate cancer (PCA). We have identified three novel AR splice variants lacking the ligand-binding domain (designated as AR3, AR4, and AR5) in hormone-insensitive PCA cells. AR3, one of the major splice variants expressed in human prostate tissues, is constitutively active, and its transcriptional activity is not regulated by androgens or antiandrogens. Immunohistochemistry analysis on tissue microarrays containing 429 human prostate tissue samples shows that AR3 is significantly up-regulated during PCA progression and AR3 expression level is correlated with the risk of tumor recurrence after radical prostatectomy. Overexpression of AR3 confers ablation-independent growth of PCA cells, whereas specific knockdown of AR3 expression (without altering AR level) in hormone-resistant PCA cells attenuates their growth under androgen-depleted conditions in both cell culture and xenograft models, suggesting an indispensable role of AR3 in ablation-independent growth of PCA cells. Furthermore, AR3 may play a distinct, yet essential, role in ablation-independent growth through the regulation of a unique set of genes, including *AKT1*, which are not regulated by the prototype AR. Our data suggest that aberrant expression of AR splice variants may be a novel mechanism underlying ablation independence during PCA progression, and AR3 may serve as a prognostic marker to predict patient outcome in response to hormonal therapy. Given that these novel AR splice variants are not inhibited by currently available antiandrogen drugs, development of new drugs targeting these AR isoforms may potentially be effective for treatment of ablation-resistant PCA. [Cancer Res 2009;69(6):2305–13]

Introduction

Androgen ablation therapy is one of the most common treatments for patients with advanced prostate cancer (PCA). However, the majority of PCA patients will eventually develop androgen depletion–independent recurrent tumors that are resistant to currently available treatments (1). It has, therefore, become a focus of intensive study to understand the mechanisms underlying the transition to ablation-resistant PCA (2–4). The androgen receptor (AR) is primarily responsible for mediating the physiologic effects of androgens by binding to specific DNA sequences, known as androgen-responsive elements (ARE; refs. 5, 6). Upon ligand binding, AR undergoes a conformational change and translocates into the nucleus, where it binds to specific AREs in the androgen-responsive genes and thereby modulates their expression (7, 8). Human *AR* gene is structurally composed of eight exons and encodes a multidomain protein, including an NH_2 terminal transactivation domain (NTD), a central DNA-binding domain (DBD), a hinge region, and a COOH terminal ligand-binding domain (LBD; refs. 9, 10). The LBD seems to be dispensable for AR transcriptional activity as its deletion leads to constitutive activation of its transcriptional capability in reporter assays (11–14). However, it remains elusive whether such constitutively active AR isoform(s) are naturally expressed in the prostate gland. Several studies have been carried out to identify potential AR-regulated genes by microarray and chromatin immunoprecipitation (ChIP)–chip analyses and revealed a cell-specific and gene-specific transcriptional regulation by AR in prostate cells (15, 16).

A majority of ablation-resistant PCAs express AR and androgen-responsive genes, indicating that AR signaling pathway is still functional under androgen-depleted conditions (17, 18). Several studies established an essential role for AR in both hormone-sensitive and ablation-resistant PCA (19, 20). The mechanisms underlying ablation-resistant AR-mediated signaling have yet to be fully elucidated. Mutation and amplification of *AR* gene, alterations in protein kinases, growth factors, nuclear receptor coactivators, and steroid metabolism enzymes have all been proposed to modulate AR signaling and may, therefore, contribute to androgen ablation resistance of PCA (3, 18, 21–29). Another plausible hypothesis for activation of AR in the absence of hormones was proposed by Tepper and colleagues (30). In their study, a mutant AR was identified in hormone-insensitive PCA cell line CWR22Rv1 that contains an in-frame tandem duplication of the exon 3 encoding the second zinc finger of the DBD, and this insertional mutation renders AR susceptible to the protease cleavage and generates a constitutively active form around 80 kDa. A recent report showed that two AR splice variants may possibly be involved in ablation resistance in 22Rv1 (31). However, the clinical significance of these AR isoforms remains elusive.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Yun Qiu, Department of Pharmacology and Experimental Therapeutics, University of Maryland School of Medicine, 655 West Baltimore Street, BRB Room 4-002, Baltimore, MD 21201. Phone: 410-706-4535; Fax: 410-706-0032; E-mail: yqiu@som.umaryland.edu.

doi:10.1158/0008-5472.CAN-08-3795

In this study, we have identified three novel AR splice variants (designated as AR3, AR4, and AR5) in androgen-insensitive PCA cells. Our data suggest that aberrant expression of AR alternative splicing variants may be a novel mechanism underlying androgen ablation independence during PCA progression.

Materials and Methods

Cell culture. LNCaP, PC-3, 22Rv1, and COS-1 cells were purchased from American Type Culture Collection. PCA cell lines C-81, C4-2, C4-2B, and CWR-R1 were kindly provided by Drs. MF Lin (32), D. Tindall, and E. Wilson (33), respectively. The cells were transfected with FuGENE 6 (Roche) or Lipofectamine 2000 (Invitrogen) following the manufacturer's instruction.

Cloning and constructs. The primer corresponding to the shARc (5′-CAGAGTCGCGACTACTACAACTTTCCA-3′) was used to amplify the 3′ end of the AR transcripts using the 5′/3′ rapid amplification of cDNA ends (RACE) kit (Roche) according to the manufacturer's instructions.

The short hairpin RNAs (shRNA) specific for human *AR* and *AKT1* were purchased from Sigma. The shRNAs specific for AR3 (shAR3) were constructed as described previously (26). The oligo sequences used were as follows: shAR3-1 5′-TGTAATAGTGGTTACCACTCTTCAAGAGAGAGTGGTAACCACTATTACTTTTTTTTC-3′, 5′-TCGAGAAAAAAAAAGTAATAGTGGTTACCACTCTCTCTTGAAGAGTGGTAACCACTATTACA-3′; shAR3-2 5′-TAGGCTAATGAGGTTTATTTCTCAAGAGAAAATAAACCTCATTAGCCTTTTTTTTTC-3′, 5′-TCGAGaaAAAAAAAGGCTAATGAGGTTTATTTTCTCTTGAGAAATAAACCTCATTAGCCTA-3′; shAR3 scrambled control 5′-TAAGAAACAGTCCGACTCAATTCAAGAGATTGAGTCGGACTGTTTCTTTCTTTTTTC-3′, 5′-TCGAGAAAAAAGAAAGAAACAGTCCGACTCAATCTCTTGAATTGAGTCGGACTGTTTCTTA-3′.

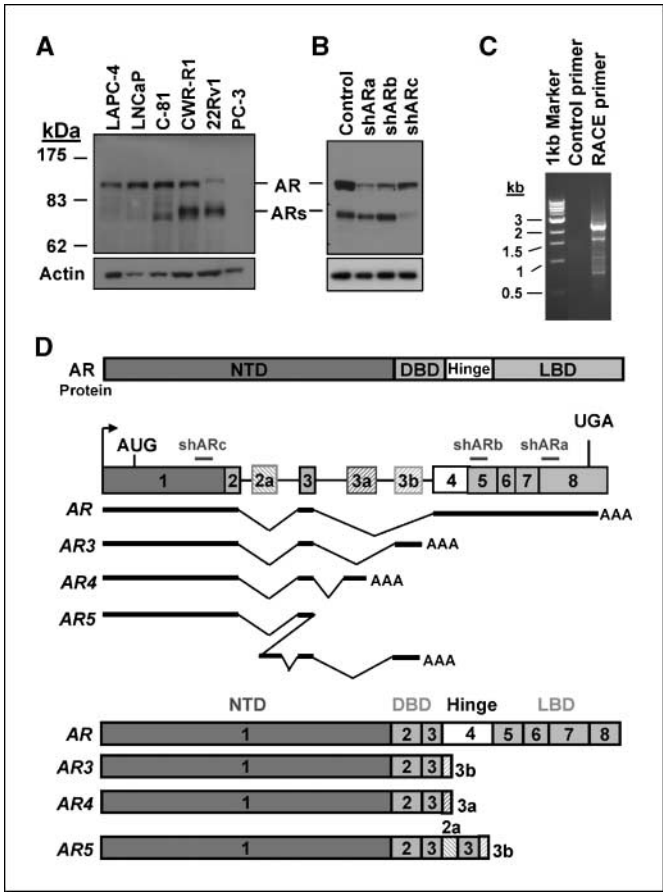

Figure 1. Cloning of novel alternative splice AR isoforms. *A,* cell lysates of different PCA cells were blotted with anti-AR (*top*) and anti-actin (*bottom*). *B,* CWR-R1 were infected with the lentivirus encoding the GFPshRNA (*Control*), ARshRNAa (*shARa*), ARshRNAb (*shARb*), and ARshRNAc (*shARc*) targeting different exons of *AR* as indicated in *D*. At 48 h postinfection, cell lysates were subjected to Western blot with anti-AR and anti-Actin, respectively. *C,* total RNA was isolated from CWR-R1 cells and reverse transcribed. The primer derived from AR shRNAc sequence or a control primer was used to perform 3′ RACE. *D,* schematic structure of the human AR splice variants. *Hatched cassettes,* cryptic exons; *solid thick lines,* transcribed sequences.

Quantitative real-time PCR. Quantitative real-time PCR was performed as described previously (34). The primer sequences used for AR isoforms are AR sense 5′-ctactccggaccttacggggacatgcg-3′, antisense 5′-gggctgacattcatagccttcaatgtgtgac-3′; AR3 sense 5′-ctactccggaccttacggggacatgcg-3′, antisense 5′-TGCCAACCCGGAATTTTTCTCCC-3′; AR4 sense 5′-ctactccggaccttacggggacatgcg-3′, antisense 5′-gattctttcagaaacaacaacagctgct-3′; AR5 sense 5′-ctactccggaccttacggggacatgcg-3′, antisense 5′-cttttaatttgttcattctgaaaaatcctc-3′; and 18S sense 5′-TTGACGGAAGGGCA CCACCAG-3′, antisense 5′-GCACCACCACCCACGGAATCG-3′. The relative abundance of each AR isoform transcript was quantified by using the comparative $\Delta\Delta C_t$ with 18S as an internal control. The primer sequences used for human *AKT1* were sense 5-TCTATGGCGCTGAGATTGTG-3 and antisense 5-CTTAATGTGCCCGTCCTTGT-3. Human *actin* and *PSA* primers are described previously (26). The relative expression levels of AKT1 and PSA transcript were quantified by using the comparative $\Delta\Delta C_t$ with actin as an internal control.

Antibodies. The antibodies used in this study include mouse monoclonal anti-Akt1 (2H10; Cell Signaling), mouse monoclonal anti-AR (441), anti-actin (C2), and rabbit polyclonal anti-AR (H-280) and anti-AR (C-19; Santa Cruz). The anti-AR3 was developed by immunizing the rabbits with a synthetic peptide corresponding to the COOH terminal 16 unique amino acids of AR3 (EKFRVGNCKHLKMTRP), and the antisera were affinity purified against the immobilized immunogen.

Luciferase reporter assay. Luciferase assay was carried out as described previously (26). Briefly, at 24 h after transfection, cells were incubated with phenol red–free medium containing 5% charcoal–stripped fetal bovine serum. Dual-luciferase assays were performed according to the manufacturer's instructions (Promega). The results are presented as the relative changes of luciferase activity to the untreated control.

Immunohistochemical analysis. Immunohistochemical staining was carried out with anti-AR3 or anti-AR after a procedure as described previously (26). Immunoreactivity of prostatic epithelial cells was evaluated manually by pathologists (J.M. and X.K.) and graded using a two-score system based on intensity score and proportion score, as described previously (26, 35). Intensity was scored on a scale of 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). Distribution of immunopositive tumor cells was scored on a scale of 0 (0%), 1 (0.1–1%), 2 (2–10%), 3 (11–33%), 4 (34–66%), and 5 (67–100%). The immunoreactivity score was the sum of intensity score and proportion score. Additional information on tissue microarrays (TMA) is available in the supplementary material.

Chromatin immunoprecipitation. Chromatin immunoprecipitation was performed as described previously (26). The PCR primers were as follows: P1-ARE sense 5′-CCACAGAGCACCTCAGCAGTCC-3′, antisense 5′-GAGCAGGGCACCCTCTCATGG-3′; P2-ARE sense 5′-GCTCCTCACTGACGGACTTGTCTG-3′, antisense, 5′-CCCCTGGTGACAGATGGCC-3′; and P3-ARE sense 5′-GTGCATTTGAGAGAAGCCACGCTG-3′, antisense, 5′-CACATTGCGCATAGCTGCAGAAG-3′. The *PSA* enhancer ARE (PSA-E) and promoter ARE (PSA-P) detection primers were used as described previously (26).

***In vitro* cell growth assay and *in vivo* tumor growth in xenograft models.** The tumor growth of LNCaP, 22Rv1, and CWR-R1 in the xenograft models was carried out, as described previously (26). Briefly, at 48-h postinfection, 10^6 cells were mixed with 100 μL of Matrigel and then s.c. injected in the flanks of the castrated SCID/nude mice. Tumor volumes ($0.5236 \times r_1^2 \times r_2$, wherein $r_1 < r_2$) were measured weekly and calculated. The differences in tumor sizes formed on both flanks were compared by the paired *t* test.

Results

Cloning of AR splice variants. Expression of AR in a panel of PCA cell lines was examined using an antibody recognizing the

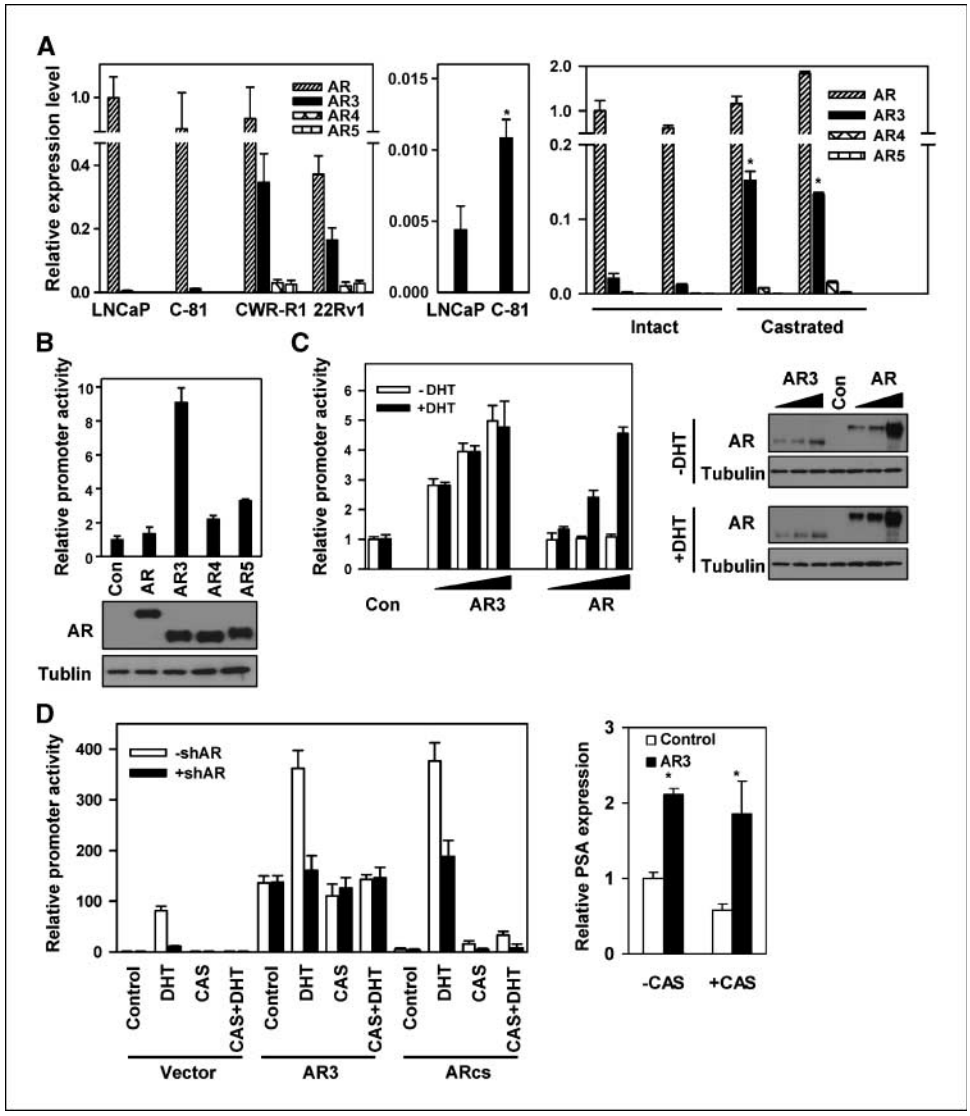

Figure 2. Expression of AR isoforms in PCA cells. *A,* the relative expression levels of AR, AR3, AR4, and AR5 were quantified using real-time PCR (*left*). The AR level in LNCaP was arbitrarily set as 1. AR3 expression in LNCaP and C-81 were further plotted with a higher resolution. *, $P < 0.05$ (*middle*). Their expression in two pairs of CW22R xenograft tumors derived from the intact and castrated male mice were also quantified (*right*). *, $P < 0.05$. *B,* transcriptional activity of AR isoforms. COS-1 were transfected with ARR2-luciferase reporter, together with the indicated expression vector. At 24 h posttransfection, the luciferase activity was measured. Cell lysates were blotted with anti-AR and anti-tubulin, respectively (*bottom*). *C,* COS-1 were transfected with ARR2-luciferase reporter, along with increasing doses of AR3- or AR-expressing vector. At 24 h posttransfection, cells were treated with or without 10 nmol/L DHT for 24 h and luciferase activities were measured. Cell lysates were blotted with anti-AR and anti-tubulin, respectively (*right*). *D,* LNCaP were infected with (+) or without (-) the lentivirus encoding ARshRNA (shAR) as described previously (26). At 6 h postinfection, cells were transfected with ARR2-luciferase reporter along with AR3 or the ARcs. At 24 h posttransfection, cells were treated with DHT and casodex (*CAS*), as indicated for 24 h before luciferase activity was measured (*left*). Cell lysates were blotted with anti-AR and anti-tubulin (Supplementary Fig. S15). *Right,* LNCaP were transfected with AR3 vector or control. At 24 h posttransfection, cells were treated with or without Casodex (+*CAS* or −*CAS*) and the relative PSA levels were quantified using real-time PCR. The PSA level in the control LNCaP was arbitrarily set as 1. *, $P < 0.05$.

NH_2 terminus of AR. In addition to the well-characterized 110-kDa AR protein, we detected one 80-kDa band in the LNCaP derivative C-81, CWR-R1, and 22Rv1 cells, which are known to grow in the androgen-depleted medium (Fig. 1*A*). This short-form AR (ARs) seemed to correspond to the truncated AR previously reported in CWR-R1 (36) and 22Rv1 cells (11, 30). On the other hand, ARs was barely detectable in the androgen-dependent LAPC4 and LNCaP cells. These data implied an inverse correlation between ARs expression and androgen dependency of these cell lines. To confirm that ARs was indeed derived from the *AR* gene, we treated CWR-R1 with a panel of shRNAs targeting distinct regions of the *AR* gene. These shRNAs seemed to differentially knockdown AR and ARs (Fig. 1*B*), suggesting that AR and ARs may be translated from more than one transcript. Similar effects were also observed in 22Rv1 cells (Supplementary Fig. S17*A*). These findings prompted us to clone possible alternative splice variants of AR by 3′ RACE using a primer corresponding to the shARc target sequence. As shown in Fig. 1*C*, multiple PCR products resulted from the 3′ RACE were detected. Subsequent cloning and sequencing analysis revealed that the major band around 2 kb turned out to be the 110-kDa prototype AR. The other bands were found to be resulted from alternative splicing through various mechanisms, including exon skipping, cryptic splicing donor or acceptor usage, cryptic exon inclusion, etc. More than 20 splicing variants have been identified thus far. Among them, three variants (designated as AR3, AR4, and AR5) were predicted to encode a protein around 80 kDa (Supplementary Figs. S1–S3). The schematic structures of these AR variants are shown in Fig. 1*D*. They contain the intact NTD and DBD but lack the hinge region and LBD. Instead, they contain 16 to 53 unique amino acids at their COOH termini, respectively.

AR3 is one of the major AR splice variants in PCA. To determine the relative abundance of these AR splice variants, we designed the isoform-specific primers, recognizing the unique junction sequence present in each isoform. These isoforms were detected in a panel of human prostate tissues by reverse transcription–PCR (Supplementary Fig. S5). Figure 2*A* shows that AR3 seemed to be one of the most frequently and abundantly expressed isoforms detected in all three hormone-insensitive cell lines. Consistent with the Western blot data in Fig. 1*A*, AR3 expression is significantly increased in the high-passage LNCaP androgen–insensitive derivative C-81 compared with the parental androgen–sensitive LNCaP cells (Fig. 2*A, middle*). We also detected

a dramatic increase of AR3 expression in castration-resistant CWR22 xenografts compared with their hormone-naive counterparts (Fig. 2*A, right*), suggesting a role of AR3 in developing androgen ablation resistance. All three AR isoforms were able to induce androgen-independent activation of the ARR2 reporter in COS-1 cells, and AR3 seemed to be more active compared with the other two splice variants (Fig. 2*B*). In addition, the AR3 activity was increased in a dose-dependent manner; however, unlike the prototype AR whose activity was dramatically stimulated by DHT, AR3 activity is independent of androgen (Fig. 2*C*). We also overexpressed AR3 in LNCaP cells and examined whether its activity could be modulated by AR, androgen, or antiandrogen. Figure 2*D* (*left*) shows

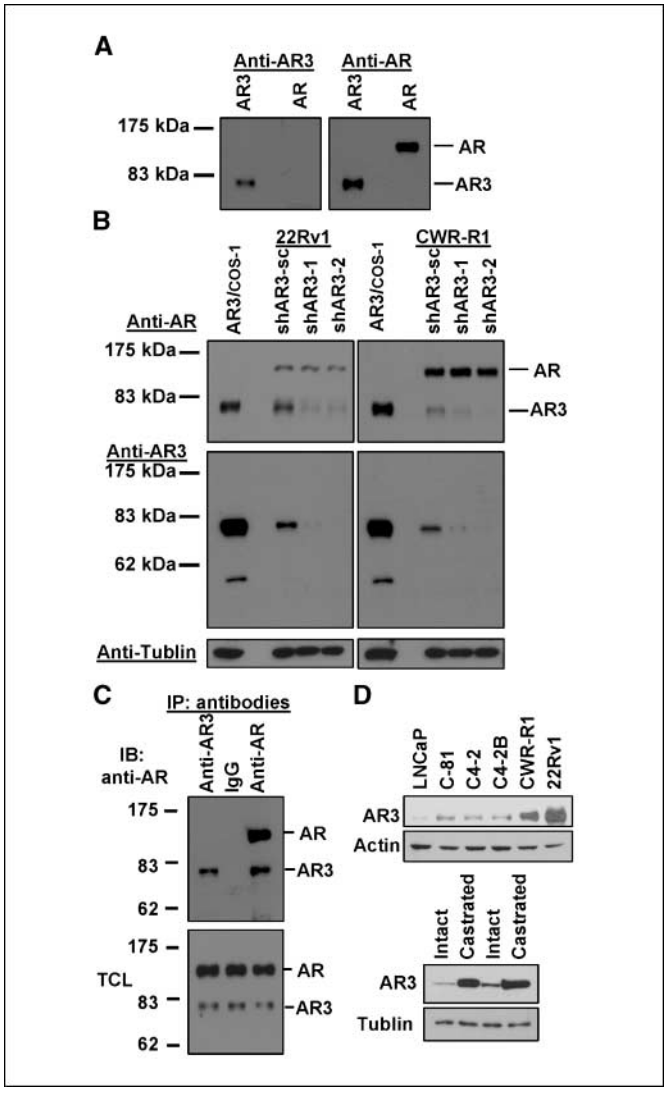

Figure 3. Detection of AR3 in hormone-insensitive PCA cells. *A,* COS-1 were transfected with AR3 or AR vector. Total protein lysates were immunoblotted with anti-AR3 and anti-AR, respectively. *B,* CWR-R1 and 22Rv1 were infected with lentivirus encoding AR3shRNAs (shAR3-1, shAR3-2) or the scrambled control (*shAR3-sc*). At 48 h postinfection, cell lysates were subjected to immunoblotting with anti-AR and anti-AR3, respectively. COS-1 overexpressing AR3 was used as a positive control (*first lane*). *C,* CWR-R1 lysates were split into three equal aliquots and immnuoprecipitated with anti-AR3, control IgG, and anti-AR, respectively. The resultant immunoprecipitates and the input total cell lysates were immunoblotted with anti-AR. *D,* total cell lysates of a panel of PCA cells were blotted with anti-AR3 and anti-actin, respectively (*top*). *Bottom,* extracts of two pairs of CW22R tumor xenografts derived from the intact and castrated male mice were blotted with anti-AR3 and anti-tubulin, respectively.

that inhibition of AR, either by the specific shRNA or casodex, did not affect AR3 activity regardless of DHT treatment, the activity of endogenous AR or exogenous codon-switched wild-type AR (ARcs), as described previously (26), was induced by DHT and blocked by casodex, as expected. Furthermore, overexpression of AR3 in LNCaP cultured in androgen-depleted medium induced a moderate increase of endogenous PSA expression, and such change was not inhibited by casodex (Fig. 2*D, right*). Thus, AR3 activity is not controlled by DHT, casodex, or AR, suggesting that AR3 may be a true androgen-independent transcription factor.

AR3 expression is increased in androgen depletion–insensitive PCA cells and predicts PCA recurrence. To further characterize the endogenous AR3, we developed a polyclonal antibody specific for AR3 and two AR3 shRNAs, specifically targeting the unique exon 3b of AR3. Figure 3*A* shows that the anti-AR3 antibody only detected the overexpressed AR3, but not AR. Knockdown of AR3 in 22Rv1 and CWR-R1 cells diminished the immunoreactivity of anti-AR3 and anti-AR with the 80-kDa ARs, whereas it had little effect on anti-AR reactivity with the 110-kDa AR (Fig. 3*B*). The anti-AR3 antibody could efficiently and selectively immunoprecipitate the endogenous AR3 but, not AR (Fig. 3*C*). The specificity of anti-AR3 in immunohistochemical staining was further validated, as shown in Supplementary Fig. S6. Immunofluorescence staining revealed that AR3 was present in both nucleus and cytoplasm in CWR-R1 and 22Rv1 cells (Supplementary Fig. S7). Western blot analysis showed that AR3 is expressed in all tested AR-positive PCA lines. It is noteworthy that the level of AR3 in the androgen-insensitive LNCaP derivatives C81, C4-2, and C4-2B was significantly higher than that in the androgen-sensitive LNCaP (Fig. 3*D, top*). In addition, AR3 protein level was also dramatically increased in CWR22 xenograft tumor derived from the castrated mice compared with that from the intact animals (Fig. 3*D, bottom*). Immunohistochemistry analysis on human prostate tissue microarrays revealed a marked change in AR3 expression level and pattern in malignant prostate tissues compared with the benign counterparts (Fig. 4*A*). In benign tissues, anti-AR3 mainly stained basal and stromal cells, but most of luminal epithelial cells were barely stained (mean epithelial cytoplasmic staining score, = 1.52 $\pm$ 0.34; Fig. 4*B* and Supplementary Fig. S9). On the other hand, the majority of luminal cells in malignant glands showed stronger cytoplasmic AR3 staining (mean score = 4.74 $\pm$ 0.13). In addition, a significant redistribution of AR3 protein to the nucleus was observed in hormone-resistant tumor samples (44% nuclear positive) compared with hormone-naive counterparts (9% nuclear positive). Thus, nuclear translocation of AR3 is significantly increased in hormone-resistant tumors. To assess whether AR3 could be used as a potential prognostic marker, clinical outcome analysis was performed on 224 PCA patient samples with clinicopathological information. Patients with elevated PSA levels after radical prostatectomy are at a high risk to develop distant metastases and die of PCA. Clinical failure was defined as a PSA elevation of >0.2 ng/mL after radical prostatectomy with successive increasing PSA values. Kaplan-Meier analysis indicated that PCA patients who have higher cytoplasmic staining of AR3 (staining score $\geq$ 6) have a greater risk for PSA recurrence after radical prostatectomy (Fig. 4*C*; log-rank test, $P < 0.0001$). Furthermore, multivariable Cox regression analysis showed that AR3 is a significant predictor of PCA recurrence after adjusting for other important clinicopathologic variables, including Gleason sum score, preoperative PSA, size of largest individual nodule of invasive cancer, and surgical margin status (Table 1). As indicated in the

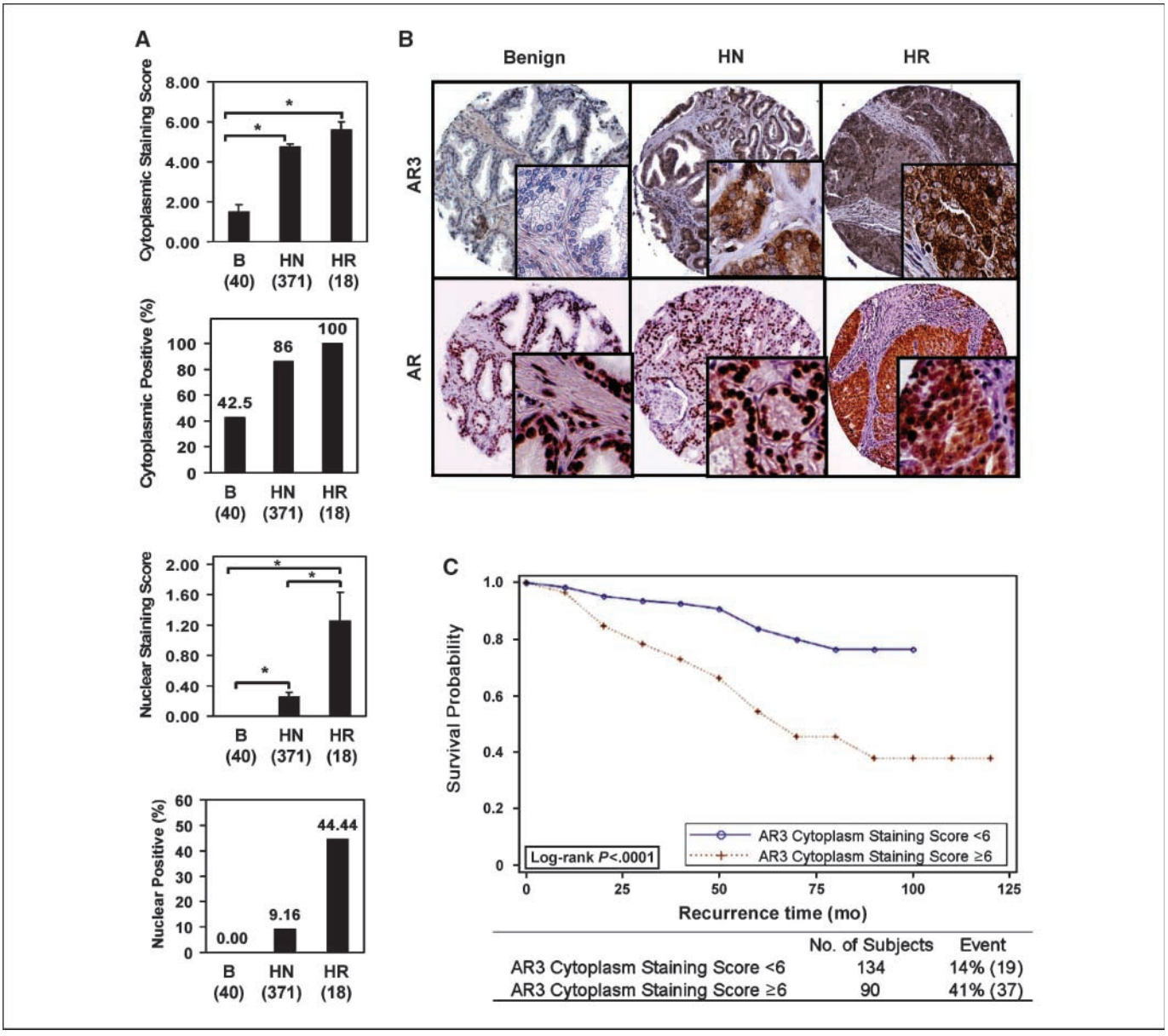

Figure 4. Increased AR3 expression in androgen depletion–resistant PCA cells. *A,* human prostate TMAs were stained with anti-AR3. *Columns,* the mean scores of cytoplasmic and nuclear staining of the luminal cell and the positive rates for cytoplasmic and nuclear staining; *bars,* SE. *, $P < 0.01$. *B,* benign; *HN,* hormone-naive; *HR,* hormone-resistant. *B,* the representative fields of TMAs stained with anti-AR3. The anti-AR–stained arrays were included as control. *C,* correlation of AR3 cytoplasmic staining with PSA recurrence after prostatectomy.

model, AR3 was the strongest predictor with a hazard ratio of 2.5 [95% confidence interval (95% CI), 1.3–4.6; $P = 0.004$].

AR3 promotes androgen depletion–independent growth. Overexpression of AR3 in LNCaP cell promoted growth in androgen-depleted medium (Fig. 5*A*). This was accompanied by the increase of DNA synthesis measured by EdU incorporation (Supplementary Fig. S14*A*), suggesting that overexpression of AR3 stimulates LNCaP proliferation in androgen-depleted medium. Such growth enhancement was also observed in the castrated SCID mice xenograft models (Fig. 5*B*). We further investigated whether expression of AR3 in hormone-insensitive PCA cells is required for their growth by specifically knocking down AR3. As shown in Fig. 5*C* and *D*, treatment of both 22Rv1 and CWR-R1 cells with the shRNA specific for AR3 attenuated their growth in the androgen-depleted medium, as well as in the castrated nude mice, suggesting that AR3 activity is required for PCA cell growth under androgen-depleted conditions. We also examined the effects of AR3 knockdown on DNA synthesis and apoptosis in CWR-R1 cells in parallel with AR knockdown. We found that AR3 knockdown significantly reduced EdU incorporation (Supplementary Fig. S14*B*) but had little effects on apoptosis (Supplementary Fig. S12). Meanwhile, AR knockdown seemed to induce a marked increase in the number of apoptotic cells and reduce the number of proliferating cells. It should be noted that knockdown of AR3 in these cells did not alter the expression of AR; therefore, AR3 may play an indispensable role in promoting PCA cell proliferation, possibly through regulating a different set of target genes.

AR3 regulates AKT1 expression in PCA cells. To identify potential AR3-regulated genes, we selectively knocked down AR3 or AR by the specific shRNAs in CWR-R1 and 22Rv1. The differential

Table 1. Multivariable Cox regression analysis of association of AR3 and clinicopathologic variables with PCA recurrence

Variables	Hazard ratio (95% CI)	*P*
AR3 (≥6 vs. <6)	2.486 (1.331–4.643)	0.0043
Gleason sum score (≥7 vs. <7)	1.451 (1.034–2.035)	0.0311
Preoperative PSA (>7, 4–7, ≤4)	1.018 (0.645–1.605)	0.9391
Tumor size (≥2 cm vs. <2 cm)	0.719 (0.355–1.454)	0.3580
Surgical margin (positive vs. negative)	2.055 (1.120–3.772)	0.0201

gene expression resulted from AR3 or AR knockdown were determined by microarray analysis. The differential expression of a set of 188 genes was consistently detected in both cell lines when AR3 was specifically knocked down, whereas the expression of 412 genes was altered in both cell lines when AR was specifically inhibited (Fig. 6*A*). Among them, 71 genes are commonly regulated by both AR and AR3. A partial list of these genes is summarized in Supplementary Table S1. Several known AR-regulated genes, such as *IGFBP3* and *FKBP5*, are also regulated by AR3. However, many classic AR-regulated genes, such as *CLU, TMEPAI, KLK3* (*PSA*), and *CLDN4* were not affected by AR3 knockdown under our experimental conditions (Supplementary Table S2). Among the 117 genes that are preferentially regulated by AR3 (Supplementary Table S3), a number of genes, such as *MAP4K4, HOXB7*, and *ELK1*, have been found to be up-regulated in hormone-resistant or metastatic PCAs in previous gene profiling studies (19, 37, 38). Interestingly, the serine/threonine kinase *AKT1*, which has been implicated in PCA development and progression, seemed to be preferentially regulated by AR3 as well. As shown in Fig. 6*B* (*left*), the level of *AKT1* transcript in AR3 knockdown cells is significantly less than that in cells treated with the scrambled shRNA or the shRNA specific for AR. The protein level of AKT1 was also reduced accordingly in AR3 knockdown CWR-R1 cells (Fig. 6*B, middle*). In addition, we examined AKT1 protein level in some of the xenograft tumors described in Fig. 4. Consistent with the results in cell lines, AKT1 protein level is increased in the xenograft tumor of LNCaP overexpressing AR3 and decreased in the xenograft tumor of 22Rv1 with AR3 knockdown (Fig. 6*B, right*). Concurrent with the change of AKT1 expression level in these cells, the phosphorylation status of the AKT substrate GSK3β was also altered accordingly. To test whether AKT1 is essential for PCA growth under androgen-depleted conditions, AKT1 in CWR-R1 cells was knocked down by the specific shRNAs. Consistent with the previous report on the mouse model (39), even a 50% reduction of AKT1 expression diminished PCA cell growth (Fig. 6*C*). Furthermore, we identified at least two putative ARE sites in the

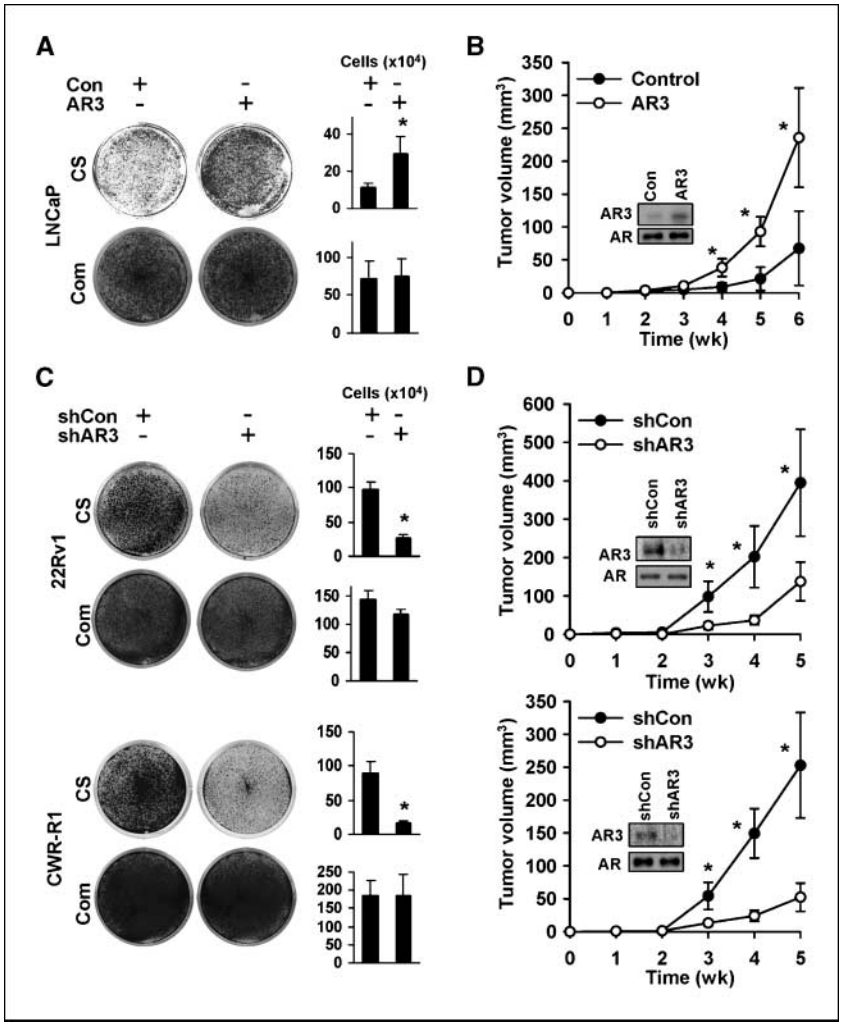

Figure 5. AR3 promotes PCA cell growth under androgen-depleted conditions. *A*, LNCaP were infected with lentivirus encoding AR3 or the control vector. After a 2-wk culture in the androgen-depleted (*CS*) or the complete (*Com*) medium, cells were visualized by Coomassie Blue staining. Under the same experimental conditions, cell numbers were quantified and plotted as a bar graph (*right;* *, $P < 0.05$). *B*, at 48 h postinfection, cells were injected into the castrated SCID mice and tumor growth were monitored weekly. *Points,* mean tumor volume $n = 5$ mice/group; *bars,* SE. *, $P < 0.05$. *Inset,* Western blots of anti-AR3 and anti-AR of LNCaP xenograft tumor lysates. *C*, CWR-R1 and 22Rv1 cells were infected with lentivirus encoding AR3shRNA-1 (*shAR3*) or control shRNA (*shCon*). Cell growth was visualized and quantified as in Fig. 5*A*. *D,* tumor growth was monitored as in Fig. 5*B*. *Inset,* Western blots of anti-AR3 and anti-AR of the CWR-R1 and 22Rv1 xenograft tumor lysates.

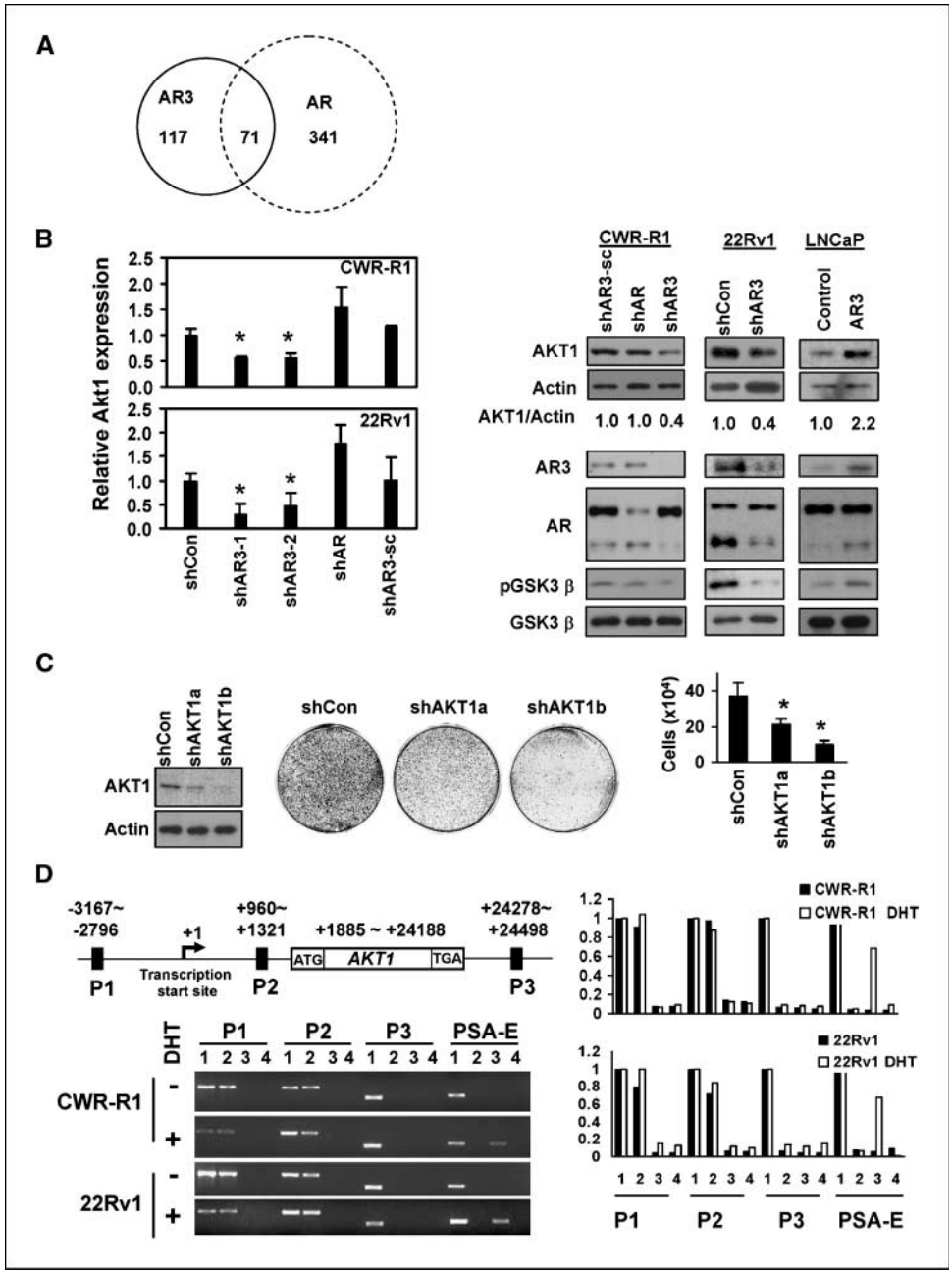

Figure 6. *AKT1* is a target gene regulated by AR3. *A,* schematic representation of AR3 and AR target genes. *B,* the effects of AR3 on *AKT1* transcription. CWR-R1 and 22Rv1 were infected with lentivirus encoding the control shRNA (*shCon*), AR3shRNAs (*shAR3-1* or *shAR3-2*), ARshRNA (*shAR*), or the scrambled control (*shAR3-sc*). At 48 h postinfection, the relative expression levels of *AKT1* transcripts compared with the shCon was quantified by real-time PCR; *, $P < 0.05$ (*left*). The protein levels of AKT1, actin, AR3, AR, pGSK3β, and GSK3β were also detected by immunoblotting. The levels of AKT1 from the immunoblots were normalized by calculating the ratios of AKT1/actin. Changes in fold compared with the control (*bottom*). *Right,* the lysates of LNCaP and 22Rv1 xenograft tumors from Fig. 5*A* and *B* were subjected to immunoblotting, as described above. *C,* the effects of AKT1 knockdown on PCA cell growth. CWR-R1 cells were infected with the lentivirus encoding two independent AKT1 shRNAs (shAKT1a and shAKT1b), respectively. After 2-wk culture in androgen-depleted medium, cells were visualized and quantified, as described in Fig. 5*A*. *D,* CWR-R1 and 22Rv1 were treated with or without DHT (10 nmol/L) for 1 h. Binding of AR3 or AR to the putative ARE sites (*P1, P2*, and *P3*) of human *AKT1* gene was analyzed by ChIP assays. The ARE at the *PSA* enhancer region (*PSA-E*) was used as a positive control for AR. PCR products from input (*1*), immunoprecipitation with anti-AR3 (*2*), anti-AR (*3*), or the control antibody (*4*) were resolved on agarose gels (*left*). The PCR products were quantified by using the software Quantity One (*right*).

AKT1 regulatory region and showed that AR3, but not AR, was able to bind to these ARE sites determined by ChIP assays (Fig. 6*D*), suggesting that AR3 may directly regulate *AKT1* transcription. Meanwhile, AR3 failed to bind to the ARE site located at the enhancer region of *PSA* gene. Taken together, our data suggest that AR3 and AR may play an overlapping yet distinct role in prostate cells by regulating their respective target genes.

Discussion

Mechanisms underlying PCA progression to androgen ablation resistance are complicated, and many factors may be involved. In this report, we showed that alternative splicing of human *AR* gene may be one of the means to diversify its signaling and confer androgen-independent activation of AR in prostate cells. We showed that several AR splice variants are constitutively active in transcription, and their activity is not expected to be affected by either androgens or antiandrogen drugs. Among these variants, AR3 seems to be one of the most abundantly and ubiquitously expressed isoforms in our screening of a panel of human PCA cell lines and tissues. In normal prostate tissues, AR3 seems to be mainly expressed in the basal and stromal cells, but virtually no or only weak AR3 expression was detected in the luminal epithelial cells. This is consistent with the previous reports that the basal and stromal compartments are insensitive to androgen ablation (40–43). Our data suggested that AR3 may play a role in androgen-insensitive regulation of normal prostate gland homeostasis. However, in malignant glands, a marked increase of cytoplasmic AR3 expression is detected in carcinoma cells in 86% of the cases examined in this study. Although the cytoplasmic AR3 may not be transcriptionally

active before hormonal therapy, AR3 may translocate into the nucleus later on during disease progression (e.g., when Src activity is up-regulated upon androgen ablation) and exerts its transcriptional activity under androgen-depleted conditions. This is consistent with our observation that PCA patients with higher cytoplasmic AR3 protein level have a greater risk for tumor recurrence after prostatectomy. Therefore, the expression of AR3 may potentially be used to predict patient outcome in response to hormonal therapy. It is possible that AR3 transcriptional activity is tightly regulated by its subcellular localization, like the prototype AR. However, the underlying mechanisms have yet to be determined. We previously showed that Src kinase may regulate AR nuclear translocation under androgen-depleted conditions, possibly through phosphorylating Y^{534} in the NTD. Most of Src-induced phosphorylation sites are present in AR3; therefore, Src family kinases may likely be involved.

It is possible that tumor cells hijack the active AR splice variants lacking the LBD to escape from the hormonal therapy, and aberrant expression of the constitutively active AR3 may contribute to ablation-independent growth. This is supported by our observations that overexpression of AR3 in LNCaP promoted tumor growth and knockdown of AR3 in CWR-R1 and 22Rv1 attenuated their growth under androgen-depleted conditions. Our gene expression profiling in CWR-R1 and 22Rv1 cells revealed that AR3 shares some overlapping target genes with AR despite of its lack of the AF2 domain (LBD). However, a large subset of classic androgen-responsive genes, including *KLK3* (PSA), is preferentially regulated by AR under our experimental conditions. It should be noted that these cells were maintained in normal growth medium, and under such conditions, both AR and AR3 are believed to be active. Although AR3 knockdown did not alter PSA expression under this condition as the active AR may be the main driver for controlling PSA expression, overexpression of AR3 in LNCaP cultured in the androgen-depleted medium did induce a moderate increase in PSA transcription, suggesting that AR3 is able to compensate, at least in part, for AR activity under androgen-depleted conditions. This is supported by our observation that AR3 was able to bind to the proximal ARE site, although it did not bind to the distal ARE site of the *PSA* gene (Fig. 6*D* and Supplementary Fig. S11).

Most importantly, microarray analysis allowed us to uncover a subset of genes that are preferentially regulated by AR3. These genes are involved in the regulation of diverse biological processes, including signal transduction, posttranslational modifications, transcription, chromatin remodeling, ion transportation, and metabolism, suggesting that AR3 may play a critical role in homeostasis maintenance of its target cells although AR3 is relatively less abundant compared with the prototype AR. This is supported by our observation that knockdown of AR3 attenuated PCA cell growth. We have confirmed that *AKT1* is one of AR3 preferred target genes in PTEN-positive CWR-R1 and 22Rv1 cells by real-time PCR and Western blot. *AKT1* was reported to be overexpressed in the primary epithelial cultures derived from human prostate tumors (44), suggesting that AKT signaling may be elevated in the epithelial compartment through a transcriptional mechanism. An increase of AKT1 expression in PCA patients is associated with PSA relapse (45). Thus, AR3 may contribute to the up-regulation of AKT1 signaling at the transcriptional level during PCA progression. Although the magnitude of changes of AKT1 is moderate ($\sim$2-fold to 3-fold), such change may have a substantial effect on prostate cell growth, as shown in Fig. 6*C*. This is supported by a previous study, showing that haplodeficiency of *Akt1* dramatically inhibits prostate tumor development in $Pten^{+/-}$ mice (39). Therefore, the increase of AR3 in luminal epithelial cells may be sufficient to confer a growth advantage, at least in part, through increasing AKT1 expression. Taken together, our data suggest that AR3 may have a distinct biological activity despite a partial overlapping biological function with AR. AR3 may primarily play a role in the regulation of androgen-independent biological processes and maintain homeostasis of the prostate gland in concert with AR.

Although we showed that AR3 can function as a transcriptional factor independent of AR, it is still possible that AR3 may bind to a subset of ARE sites (e.g., the proximal PSA-P site) in complex with AR. However, thus far, we have not yet been able to detect such complex using our AR3-specific antibody. Future study should be carried out to examine whether they may synergistically function together to regulate a subset of ARE-containing promoters.

We also identified another splice variant AR6 lacking the second zinc finger in the DBD (Supplementary Fig. S4). AR6 did not display detectable transcriptional activity on the PSA and ARR2 reporters in COS-1 cells and, therefore, was not characterized in this study. During the preparation of this manuscript, two AR variants expressed in 22Rv1 cells were reported recently (31). Notably, they seem to be different from the ones identified in the present study. We propose that technical approaches (cDNA preparation, PCR amplification, cloning, etc.) might account for our differing observations. This possibility is supported by that the sequences of our splice variants have longer 3′ untranslated regions and contain the conserved AATAAA polyadenylation signals. Although their variants seem to share some sequence homology with AR5 and AR6, the unique coding sequences at the COOH termini are quite different. Future study on human tissue samples will be necessary to resolve these discrepancies.

Nevertheless, these studies suggest that aberrant expression of AR splicing variants may be a mechanism underlying PCA progression. Given that these AR isoforms are not inhibited by currently available antiandrogens, development of new drugs targeting these AR variants may potentially be effective for ablation-resistant PCA.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 9/30/2008; revised 12/11/2008; accepted 12/31/2008; published OnlineFirst 2/24/2009.

Grant support: NIH grant CA106504, DOD grants W81XWH-06-1-0199 and W81XWH-08-1-0126 (Y. Qiu), NIH grant NCI UO1-CA86772 (J. Melamed), DOD Postdoctoral Fellowship grant W81XWH-04-1-0015 (Z. Guo), and DOD Predoctoral Fellowship grant W81XWH-08-1-0068 (D.E. Linn).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

1. Denmeade SR, Isaacs JT. A history of prostate cancer treatment. Nat Rev Cancer 2002;2:389–96.
2. Litvinov IV, Antony L, Isaacs JT. Molecular characterization of an improved vector for evaluation of the tumor suppressor versus oncogene abilities of the androgen receptor. Prostate 2004;61:299–304.
3. Feldman BJ, Feldman D. The development of androgen-independent prostate cancer. Nat Rev Cancer 2001;1:34–45.
4. Debes JD, Tindall DJ. Mechanisms of androgen-refractory prostate cancer. N Engl J Med 2004;351:1488–90.

5. Chang CS, Kokontis J, Liao ST. Molecular cloning of human and rat complementary DNA encoding androgen receptors. Science 1988;240:324–6.
6. Lubahn DB, Joseph DR, Sar M, et al. The human androgen receptor: complementary deoxyribonucleic acid cloning, sequence analysis and gene expression in prostate. Mol Endocrinol 1988;2:1265–75.
7. McPhaul MJ. Factors that mediate and modulate androgen action. J Investig Dermatol Symp Proc 2003;8:1–5.
8. Heinlein CA, Chang C. Androgen receptor in prostate cancer. Endocr Rev 2004;25:276–308.
9. Kuiper GG, Faber PW, van Rooij HC, et al. Structural organization of the human androgen receptor gene. J Mol Endocrinol 1989;2:R1–4.
10. Lubahn DB, Brown TR, Simental JA, et al. Sequence of the intron/exon junctions of the coding region of the human androgen receptor gene and identification of a point mutation in a family with complete androgen insensitivity. Proc Natl Acad Sci U S A 1989;86:9534–8.
11. Libertini SJ, Tepper CG, Rodriguez V, Asmuth DM, Kung HJ, Mudryj M. Evidence for calpain-mediated androgen receptor cleavage as a mechanism for androgen independence. Cancer Res 2007;67:9001–5.
12. Simental JA, Sar M, Lane MV, French FS, Wilson EM. Transcriptional activation and nuclear targeting signals of the human androgen receptor. J Biol Chem 1991;266:510–8.
13. Jenster G, van der Korput HA, van Vroonhoven C, van der Kwast TH, Trapman J, Brinkmann AO. Domains of the human androgen receptor involved in steroid binding, transcriptional activation, and subcellular localization. Mol Endocrinol 1991;5:1396–404.
14. Rundlett SE, Wu XP, Miesfeld RL. Functional characterizations of the androgen receptor confirm that the molecular basis of androgen action is transcriptional regulation. Mol Endocrinol 1990;4:708–14.
15. Bolton EC, So AY, Chaivorapol C, Haqq CM, Li H, Yamamoto KR. Cell- and gene-specific regulation of primary target genes by the androgen receptor. Genes Dev 2007;21:2005–17.
16. Wang Q, Li W, Liu XS, et al. A hierarchical network of transcription factors governs androgen receptor-dependent prostate cancer growth. Mol Cell 2007;27:380–92.
17. Marcelli M, Cunningham GR. Hormonal signaling in prostatic hyperplasia and neoplasia. J Clin Endocrinol Metab 1999;84:3463–8.
18. Culig Z, Klocker H, Bartsch G, Hobisch A. Androgen receptors in prostate cancer. Endocr Relat Cancer 2002;9:155–70.
19. Chen CD, Welsbie DS, Tran C, et al. Molecular determinants of resistance to antiandrogen therapy. Nat Med 2004;10:33–9.
20. Zegarra-Moro OL, Schmidt LJ, Huang H, Tindall DJ. Disruption of androgen receptor function inhibits proliferation of androgen-refractory prostate cancer cells. Cancer Res 2002;62:1008–13.
21. Yeh S, Lin HK, Kang HY, Thin TH, Lin MF, Chang C. From HER2/Neu signal cascade to androgen receptor and its coactivators: a novel pathway by induction of androgen target genes through MAP kinase in prostate cancer cells. Proc Natl Acad Sci U S A 1999;96:5458–63.
22. Ling MT, Chan KW, Choo CK. Androgen induces differentiation of a human papillomavirus 16 E6/E7 immortalized prostate epithelial cell line. J Endocrinol 2001;170:287–96.
23. Lin HK, Hu YC, Yang L, et al. Suppression versus induction of androgen receptor functions by the phosphatidylinositol 3-kinase/Akt pathway in prostate cancer LNCaP cells with different passage numbers. J Biol Chem 2003;278:50902–7.
24. Gregory CW, Fei X, Ponguta LA, et al. Epidermal growth factor increases coactivation of the androgen receptor in recurrent prostate cancer. J Biol Chem 2004;279:7119–30.
25. Ueda T, Mawji NR, Bruchovsky N, Sadar MD. Ligand-independent activation of the androgen receptor by interleukin-6 and the role of steroid receptor coactivator-1 in prostate cancer cells. J Biol Chem 2002;277:38087–94.
26. Guo Z, Dai B, Jiang T, et al. Regulation of androgen receptor activity by tyrosine phosphorylation. Cancer Cell 2006;10:309–19.
27. Mahajan NP, Liu Y, Majumder S, et al. Activated Cdc42-associated kinase Ack1 promotes prostate cancer progression via androgen receptor tyrosine phosphorylation. Proc Natl Acad Sci U S A 2007;104:8438–43.
28. Gelmann EP. Molecular biology of the androgen receptor. J Clin Oncol 2002;20:3001–15.
29. Titus MA, Schell MJ, Lih FB, Tomer KB, Mohler JL. Testosterone and dihydrotestosterone tissue levels in recurrent prostate cancer. Clin Cancer Res 2005;11:4653–7.
30. Tepper CG, Boucher DL, Ryan PE, et al. Characterization of a novel androgen receptor mutation in a relapsed CWR22 prostate cancer xenograft and cell line. Cancer Res 2002;62:6606–14.
31. Dehm SM, Schmidt LJ, Heemers HV, Vessella RL, Tindall DJ. Splicing of a Novel Androgen Receptor Exon Generates a Constitutively Active Androgen Receptor that Mediates Prostate Cancer Therapy Resistance. Cancer Res 2008;68:5469–77.
32. Igawa T, Lin FF, Lee MS, Karan D, Batra SK, Lin MF. Establishment and characterization of androgen-independent human prostate cancer LNCaP cell model. Prostate 2002;50:222–35.
33. Gregory CW, Johnson RT, Jr., Mohler JL, French FS, Wilson EM. Androgen receptor stabilization in recurrent prostate cancer is associated with hypersensitivity to low androgen. Cancer Res 2001;61:2892–8.
34. Zhang Y, Wang XW, Jelovac D, et al. The ErbB3-binding protein Ebp1 suppresses androgen receptor-mediated gene transcription and tumorigenesis of prostate cancer cells. Proc Natl Acad Sci U S A 2005;102:9890–5.
35. Harvey JM, Clark GM, Osborne CK, Allred DC. Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. J Clin Oncol 1999;17:1474–81.
36. Gregory CW, He B, Wilson EM. The putative androgen receptor-A form results from *in vitro* proteolysis. J Mol Endocrinol 2001;27:309–19.
37. Best CJ, Gillespie JW, Yi Y, et al. Molecular alterations in primary prostate cancer after androgen ablation therapy. Clin Cancer Res 2005;11:6823–34.
38. Varambally S, Yu J, Laxman B, et al. Integrative genomic and proteomic analysis of prostate cancer reveals signatures of metastatic progression. Cancer Cell 2005;8:393–406.
39. Chen ML, Xu PZ, Peng XD, et al. The deficiency of Akt1 is sufficient to suppress tumor development in Pten+/- mice. Genes Dev 2006;20:1569–74.
40. Johansson A, Jones J, Pietras K, et al. A stroma targeted therapy enhances castration effects in a transplantable rat prostate cancer model. Prostate 2007;67:1664–76.
41. Cunha GR, Cooke PS, Kurita T. Role of stromal-epithelial interactions in hormonal responses. Arch Histol Cytol 2004;67:417–34.
42. Kurita T, Wang YZ, Donjacour AA, et al. Paracrine regulation of apoptosis by steroid hormones in the male and female reproductive system. Cell Death Differ 2001;8:192–200.
43. English HF, Santen RJ, Isaacs JT. Response of glandular versus basal rat ventral prostatic epithelial cells to androgen withdrawal and replacement. Prostate 1987;11:229–42.
44. Nanni S, Priolo C, Grasselli A, et al. Epithelial-restricted gene profile of primary cultures from human prostate tumors: a molecular approach to predict clinical behavior of prostate cancer. Mol Cancer Res 2006;4:79–92.
45. Le Page C, Koumakpayi IH, Alam-Fahmy M, Mes-Masson AM, Saad F. Expression and localisation of Akt-1, Akt-2 and Akt-3 correlate with clinical outcome of prostate cancer patients. Br J Cancer 2006;94:1906–12.