AD_____

Award Number: W81XWH-12-1-0275

TITLE: Dual-Targeting of AR and Akt Pathways by Berberine in Castration-Resistant Prostate Cancer

PRINCIPAL INVESTIGATOR: Ö¦ÊHaitao Zhang

CONTRACTING ORGANIZATION: Tulane University
ÅÅ¥Á¦Á|^æ•ÊÃŠ¦ÇŒÎÄ€FFGÏÇÎHGÁ

REPORT DATE: Œ*˘•´o´2014

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
                Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
                Distribution Unlimited

REPORT DOCUMENTATION PAGE

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
August 2014	Annual	19 Jul 2013- 18 Jul 2014

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Dual-Targeting of AR and Akt Pathways by Berberine in Castration-Resistant Prostate Cancer	
	5b. GRANT NUMBER
	W81XWH-12-1-0275
	5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)	5d. PROJECT NUMBER
Haitao Zhang	
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
E-Mail: hzhang@tulane.edu	

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
Tulane University New Orleans, LA 70112	

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012	
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

In this grant period, we completed testing the efficacy of berberine in intact Pten-null mice. In addition to inhibiting tumor development, berberine suppresses AR and AKT pathways simultaneously. We found BEZ235 treatment was not effective in this model of prostate cancer, despite its potent inhibition on AKT activation. This is possibly due to a compensatory induction of AR-mediated signaling. We further demonstrated that berberine inhibits the development of castration-resistant prostate cancer in castrated Pten-null mice. In addition, we provided initial evidence of berberine's efficacy against castration-resistance prostate cancer driven by a constitutively active AR splice variant.

15. SUBJECT TERMS

Prostate cancer; Androgen receptor; Berberine; natural compound; Pten knockout mouse model; castration resistant prostate cancer; AR splice variants.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE	UU	36	19b. TELEPHONE NUMBER *(include area code)*
U	U	U			

Table of Contents

1.   Introduction

In the previous report, we investigated the mechanisms underlying the downregulation of full-length and splice variants of AR by the phytochemical berberine (BBR). We concluded that BBR inhibits the transcription of the AR gene, leading to a similar decrease of the full-length AR (AR-FL) and AR-V7 at the transcript level. However, the AR-V7 protein is much less stable than AR-FL; therefore, AR-V7 is depleted at a greater rate than AR-FL under BBR treatment. By using a Pten conditional knockout model, we demonstrated that BBR is effective in blocking prostate cancer development. During the second grant period, we tested the anticancer efficacies of BBR against prostate progression after androgen deprivation and against castration-resistant prostate cancer (CRPC) driven by AR splice variants.
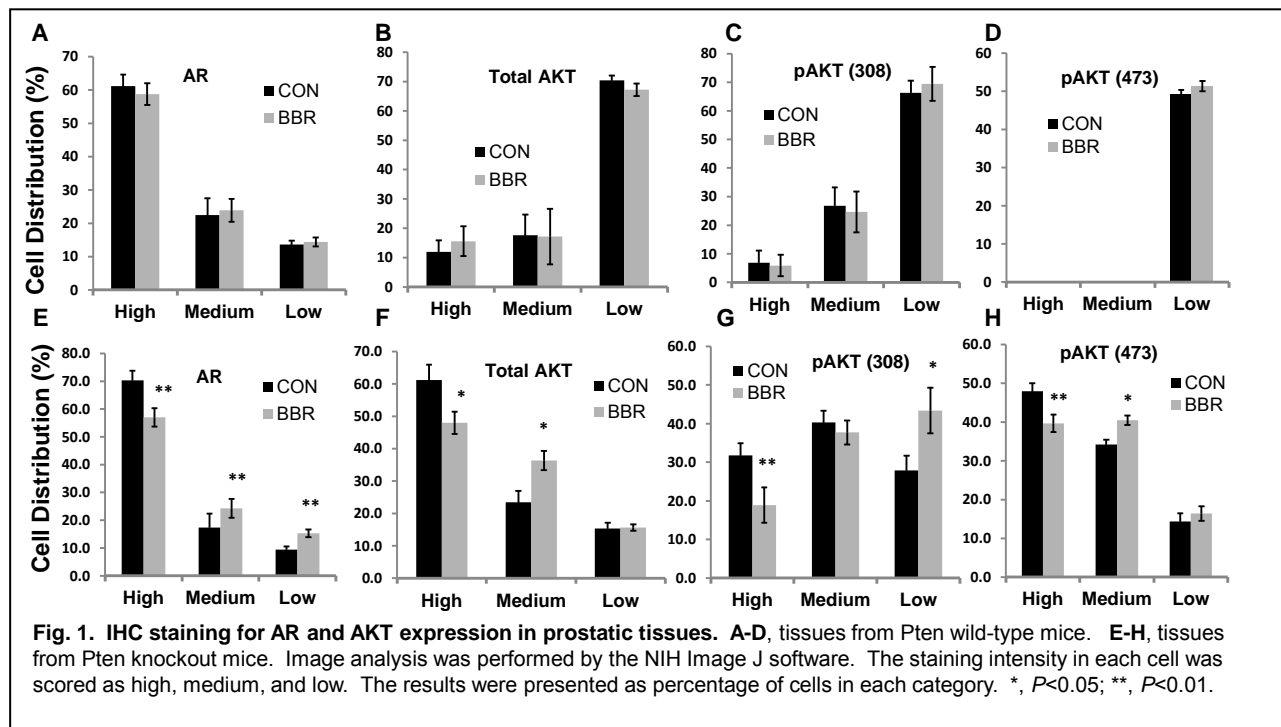
2.   Keywords

Prostate cancer; androgen receptor; berberine; natural compound; Pten knockout mouse model; castration resistant prostate cancer; AR splice variants.
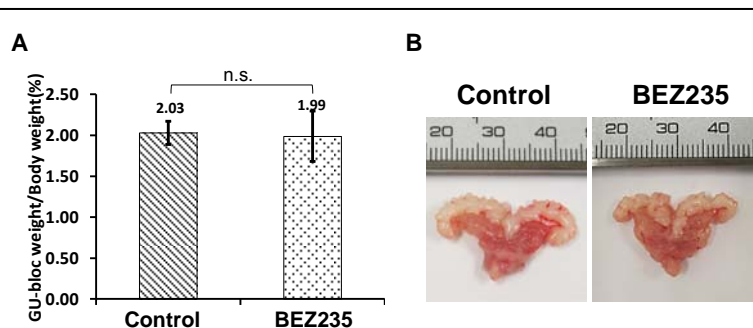
3.   Overall Project Summary

Task 2. To evaluate the *in vivo* efficacy of berberine against prostate cancer growth in Pten knockout mice.

BBR inhibits the expression of AR and AKT in Pten-null prostatic tissues. In the previous report, we presented the data on BBR inhibition of tumor development in intact Pten-null mice. In the period, we analyzed the tissues obtained from this animal experiment by immunohistochemistry (IHC) staining for AR, total and phosphorylated AKT (serine 308 and 473), as previously described (1). All images, excluding areas near the edges or with necrosis, were analyzed using the Image J Software (NIH). The staining intensity was scored as high, medium, and low, and cells in each category were counted. The results were presented as the percentage of cells in each category.   While having no effected on the distribution of cells, BBR treatment significantly lowered the percentage of cells with high staining intensity of AR, and increased the percentages of cells with medium or low staining for AR (Fig. 1, A vs E). Similar observations were made for total AKT (B vs F), pAKT308 (C vs G), and pAKT (D vs H). In summary, the IHC staining data showed BBR suppresses both the AR and the AKT pathways simultaneously. The selectivity of BBR in AR and AKT inhibition in cancerous tissues with regard to is consistent with the tumor growth data.

Fig. 1. IHC staining for AR and AKT expression in prostatic tissues. A-D, tissues from Pten wild-type mice. **E-H**, tissues from Pten knockout mice. Image analysis was performed by the NIH Image J software. The staining intensity in each cell was scored as high, medium, and low. The results were presented as percentage of cells in each category. *, $P<0.05$; **, $P<0.01$.
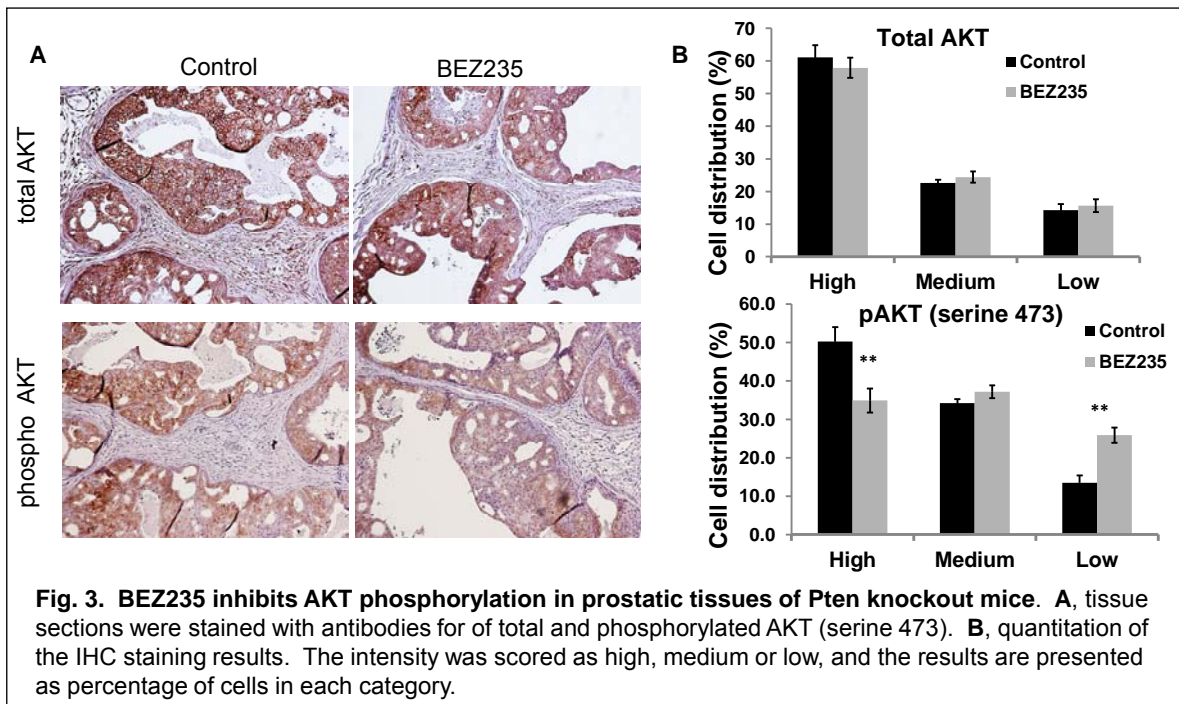
AKT inhibition by BEZ235 is not sufficient to block prostate cancer development in Pten-null mice. To evaluate the benefit of dual-targeting of AR and AKT by BBR, we set out test the efficacy in the Pten-null model with that of BEZ235, a PI3K and mTORC1/2 dual inhibitor which inhibits AKT phosphorylation by PDK1 and mTORC2 (2). The experimental design is similar to that for BBR. Male F2 Pten-/- mice identified by genotyping were randomly assigned to control or BEZ235 treatment group (n=6) at 12 weeks of age and received vehicle (10% 1-methyl-2-pyrrolidone/ 90% PEG 400) or 45 mg/kg/day of BEZ235, respectively, through oral gavage. The treatment continued for 9 weeks and animals were sacrificed by CO_2 inhalation at

Fig. 2. BEZ235 is ineffective in blocking prostate cancer development in Pten KO mice. A, The weight of the GU-bloc is normalized by body weight and presented as mean ± standard deviation, n=6. **B,** Representative photos of the GU bloc in each group.

the end of experiment. The weight of the genitourinary bloc (GU bloc), consisting of the prostate lobes, seminal vesicles, ampullary glands, bladder, proximal ductus deferens, and proximal urethra was excised *en bloc*, was used to represent prostate tumor burden. As shown in Fig. 2, there is little or no difference in GU bloc weight between the control
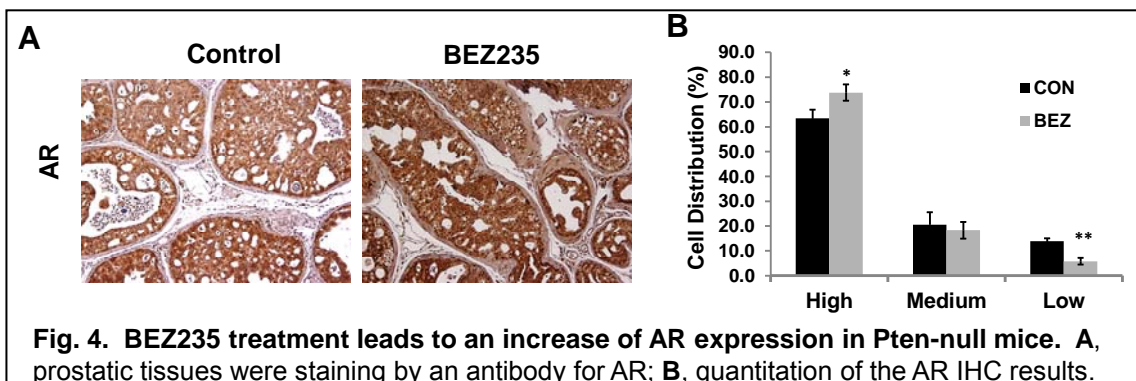
2

and BEZ235 groups. The lack of tumor inhibition is not due to lack of potency of the compound, as immunohistochemistry (IHC) staining analysis showed BEZ235 effectively

Fig. 3. BEZ235 inhibits AKT phosphorylation in prostatic tissues of Pten knockout mice. A, tissue sections were stained with antibodies for of total and phosphorylated AKT (serine 473). B, quantitation of the IHC staining results. The intensity was scored as high, medium or low, and the results are presented as percentage of cells in each category.
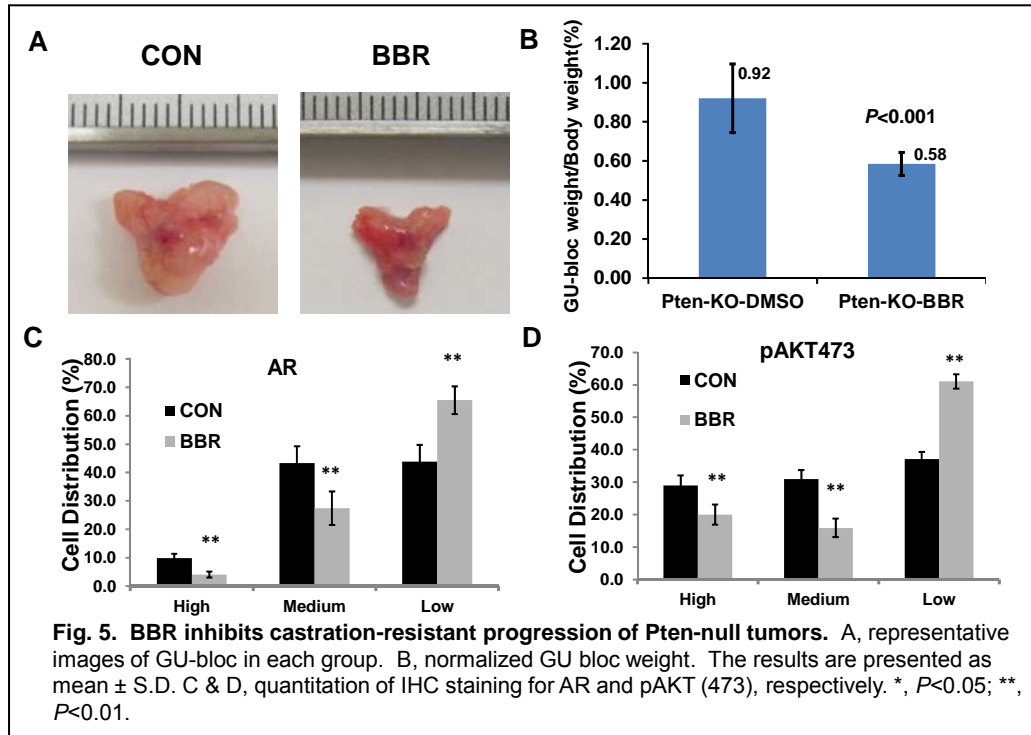
reduced AKT phosphorylation in prostatic tissues from the Pten-null mice (Fig. 3). Based on the reciprocal inhibition between AR and AKT, we hypothesized that BEZ235 treatment led to induction of AR expression. Indeed, IHC analysis showed an increase of AR staining in these tissues (Fig. 4). These results suggest that suppression of the AKT pathway by BEZ235 is negated by a compensatory AR induction in the cancer cells. By comparing the efficacies of BBR and BEZ235, we conclude that it is necessary to inhibit both AR and AKT pathways, at least in this Pten-null model of prostate cancer.

Fig. 4. BEZ235 treatment leads to an increase of AR expression in Pten-null mice. A, prostatic tissues were staining by an antibody for AR; B, quantitation of the AR IHC results.
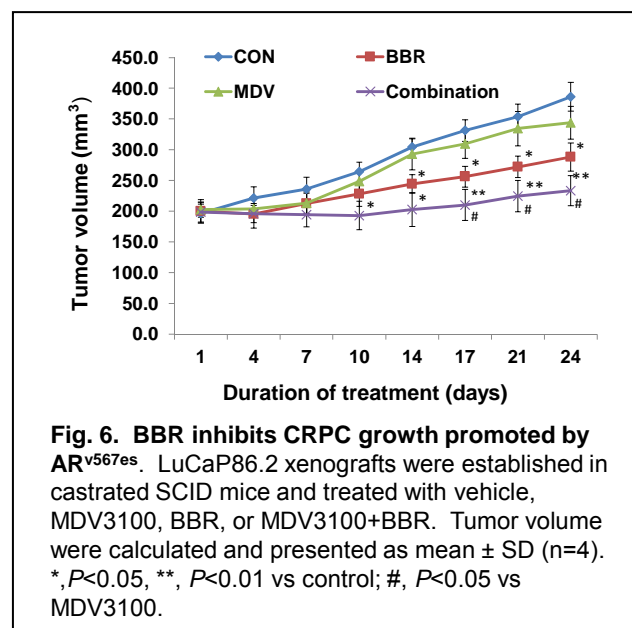
BBR inhibits castration-resistant progression of Pten-null prostate cancer.
Pten-null mice were castrated at 12 weeks of age and randomly assigned to control and

3

treatment groups (n=9) and received vehicle (DMSO) or 5 mg/kg/day of BBR, respectively, through i.p. injection. The treatment continued for 20 weeks and animals were sacrificed at the end of 32 weeks.   As shown in Fig. 5, BBR treatment significantly lowered the weight of the GU bloc. IHC analysis showed BBR reduced the expression of AR and AKT in these tissues. These results suggest BBR is effective in blocking tumor progression following androgen deprivation.

Fig. 5. BBR inhibits castration-resistant progression of Pten-null tumors. A, representative images of GU-bloc in each group. B, normalized GU bloc weight. The results are presented as mean ± S.D. C & D, quantitation of IHC staining for AR and pAKT (473), respectively. *, *P*<0.05; **, *P*<0.01.

Task 3.   To evaluate the *in vivo* efficacy of berberine against AR-fl- or AR-V-promoted CRPC growth.

BBR inhibits CRPC growth promoted by AR[v567es]**.**   In this experiment, we selected the LuCaP86.2 xenograft model, which expresses predominantly the constitutively active AR splice variant AR[v567es], which lacks the ligand-binding domain (3).   LuCap86.2 tumors grow in a castration-resistant manner and were serially passaged in castrated SCID mice. The tumors were cut into 25 mm³ pieces and implanted subcutaneously into castrated SCID mice at 8 weeks of age. Tumor dimensions

Fig. 6. BBR inhibits CRPC growth promoted by AR[v567es]**.** LuCaP86.2 xenografts were established in castrated SCID mice and treated with vehicle, MDV3100, BBR, or MDV3100+BBR. Tumor volume were calculated and presented as mean ± SD (n=4). *,*P*<0.05, **, *P*<0.01 vs control; #, *P*<0.05 vs MDV3100.

4

were measured twice weekly using a Vernier caliper and tumor volume were calculated by the following formula: length x width x height x 0.5236. When tumor size reaches 200 mm^3, mice were randomized to one of four groups and receive vehicle, MDV3100 at 10 mg/kg/day, BBR at 5 mg/kg/day, or the combination of MDV3100 and BBR, respectively, through i.p. injection.   This experiment was still ongoing at the time of this report and an interim analysis was conducted using data collected to this point (Fig. 6).   As expected, the antiandrogen MDV3100 did not alter the growth of LuCaP86.2 tumors during this treatment period (24 days).   In contrast, tumors in the BBR group showed significantly reduced growth rate after 14 days of treatment.   The BBR/MDV3100 combination was most effective in arresting the growth of this tumor model.   This interim analysis suggests that BBR not only is effective by itself, but also enhances the therapeutic efficacy of MDV3100 in CRPC expressing AR splice variants.

4.   Key Research Accomplishments

- We have demonstrated the efficacy of berberine in preventing prostate cancer development and in blocking castration-resistant progression in the Pten-null model of prostate cancer.

- We have shown that inhibition of the PI3K/AKT pathway is ineffective against prostate cancer development in a Pten-null environment, possibly due to a compensatory activation of the AR pathway.

- We have provided the initial evidence that BBR is effective against castration-resistant prostate cancer driven by an AR splice variant lacking the ligand-binding domain, and that it enhances the therapeutic efficacy of MDV3100 in this tumor model.

5.   Conclusion

Through the experiments conducted in this peroid, we concluded that: 1) berberine is effective in preventing prostate cancer development in the Pten-null environment. Given its low toxicity profile and low cost, berberine is an excellent candidate as a preventive agent for prostate cancer. The future plan is to conduct a prevention trial in patients at risk for prostate cancer; 2) berberine is effective in blocking prostate cancer progression following androgen deprivation therapy.   This result is significant, in view of the magnitude of challenge we are facing in the treatment of castration-resistant prostate cancer. This finding provides a rationale for conducting a clinical trial using berberine as an adjuvant therapy to androgen deprivation; 3) berberine is promising in the treatment of castration-resistant prostate cancer expressing constitutively active AR splice variants.   Our immediate plan is to corroborate the

finding in the next grant period. The clinical impact of this finding is significant. Recently studies have shown that the expression of AR splice variants confers resistance to MDV3100 (4,5). This study could lead to the development of a combination strategy using berberine and MDV3100 to improve therapeutic efficacy and to combat the development of resistance to MDV3100.

6.   Publications, Abstracts, and Presentations

Alison Egan, Yan Dong, Haitao Zhang, Yanfeng Qi, Steven P. Balk, Oliver Sartor. (2013). Castration-Resistant Prostate Cancer: Adaptive Responses in the Androgen Axis. Cancer Treatment Reviews 40:426-433.

Haitao Zhang*, Yang Zhan, Xichun Liu, Yanfeng Qi, Guanyi Zhang, Oliver Sartor, Yan Dong. (2013). Splicing variants of androgen receptor in prostate cancer. Am J Clin Exp Urol 2013;1(1):18-24.

Bo Cao, Yanfeng Qi, Guanyi Zhang, Duo Xu, Yang Zhan, Xavier Alvarez, Zhiyong Guo, Xueqi Fu, Stephen R. Plymate, Oliver Sartor, Haitao Zhang*, and Yan Dong. (2014). Androgen receptor splice variants activating the full-length receptor in mediating resistance to androgen-directed therapy. Oncotarget 2014. Mar 30;5(6):1646-56.

   *: corresponding author

7.   Inventions, Patents, and Licenses

Nothing to report.

8.   Reportable Outcomes

Nothing to report.

9.   Other Achievements

Using preliminary data generated with the support of this grant, we have further developed the project into an application and submitted it to the American Cancer Society for Research Scholar Grant award. The proposal, titled "Co-targeting AR and AKT by Berberine in Castration-resistant Prostate Cancer", was awarded in March, 2014.

10.   References

1. Li J, Cao B, Liu X, Fu X, Chen L, Xiong Z, Sartor O, Dong Y, and Zhang H. Berberine suppresses androgen receptor signaling in prostate cancer. Molecular Cancer Therapeutics 2011;10(8):1346-56.

2. Serra V, Markman B, Scaltriti M, Eichhorn PJ, Valero V, Guzman M, et al. NVP-BEZ235, a dual PI3K/mTOR inhibitor, prevents PI3K signaling and inhibits the growth of cancer cells with activating PI3K mutations. Cancer Res 2008;68:8022-30.

3. Sun S, Sprenger CC, Vessella RL, Haugk K, Soriano K, Mostaghel EA, et al. Castration resistance in human prostate cancer is conferred by a frequently occurring androgen receptor splice variant. J Clin Invest 2010;120:2715-30.

4. Li Y, Chan SC, Brand LJ, Hwang TH, Silverstein KA, Dehm SM. Androgen receptor splice variants mediate enzalutamide resistance in castration-resistant prostate cancer cell lines. Cancer Res 2013;15;73(2):483-9.

5. Cao B, Qi Y, Zhang G, Xu D, Zhan Y, Alvarez X, Guo Z, Fu X, Plymate SR, Sartor O, Zhang H, and Dong Y. Androgen receptor splice variants activating the full-length receptor in mediating resistance to androgen-directed therapy. Oncotarget 2014; 30;5(6):1646-56.

11.   Appendices

The 3 papers listed under "Publications, Abstracts, and Presentations" are attached in that order.

Laboratory-Clinic Interface

Castration-resistant prostate cancer: Adaptive responses in the androgen axis

Alison Egan [a], Yan Dong [b,e,f], Haitao Zhang [c,e], Yanfeng Qi [b,e], Steven P. Balk [g], Oliver Sartor [a,d,e,*]

[a] Department of Medicine, Tulane University School of Medicine, New Orleans, LA 70112, USA
[b] Department of Structural and Cellular Biology, Tulane University School of Medicine, New Orleans, LA 70112, USA
[c] Department of Pathology and Laboratory Medicine, Tulane University School of Medicine, New Orleans, LA 70112, USA
[d] Department of Urology, Tulane University School of Medicine, New Orleans, LA 70112, USA
[e] Department of Tulane Cancer Center, Tulane University School of Medicine, New Orleans, LA 70112, USA
[f] National Engineering Laboratory for AIDS Vaccine, Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, 330 Brookline Avenue, Boston, MA 02215, USA
[g] College of Life Sciences, Jilin University, China and Hematology–Oncology Division, Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, 330 Brookline Avenue, Boston, MA 02215, USA

ABSTRACT

The androgen signaling axis in prostate cancer is associated with multiple adaptive mechanisms in response to castration. Herein we review these adaptations with an emphasis on recent molecular insights into the growth and development of castration resistant prostate cancer (CRPC). Alterations include both conventional and novel intracrine androgen synthesis pathways and androgen transport as well as androgen receptor (AR) overexpression, mutation, and splice variation. Each of these underlying mechanisms are potentially linked to post-castration growth, especially after treatment with newer hormonal agents such as abiraterone and enzalutamide. Post-translational AR modifications are well documented and these can affect receptor activity, stability, localization, and interaction with other proteins. Changes in recruitment of androgen receptor associated co-activators/repressors and a distinct AR-induced transcriptional program can dramatically alter proliferation, invasion, and metastasis in a ligand and context-dependent manner. Numerous previously uncharacterized non-coding RNAs, some of which are androgen regulated, may also have important biological function in this disease. Taken together, the view of CRPC has changed dramatically in the last several years. This has occurred not only within the setting of multiple treatment paradigm changes, but also as a multiplicity of potential molecular mechanisms underlying this disease state have been explored and discovered.

© 2013 Elsevier Ltd. All rights reserved.

Introduction

Prostate cancer is by far the most common non-skin cancer and currently the second leading cause of cancer death in men in the United States. Both normal and malignant prostate epithelial cells depend on androgen dependent activation of the androgen receptor (AR) for prostate-specific antigen (PSA) production and survival. Androgen deprivation therapy (ADT) via surgical or medical castration remains the standard form of treatment, and has been so for the last 70 years for clinically advanced prostate cancer [1]. Disease progression after initial ADT, despite castration levels of testosterone, is termed castrate resistant prostate cancer (CRPC). This may either be metastatic or non-metastatic and the natural

history is distinct. For men with metastatic disease, castration resistance as measured by PSA rise develops approximately 16 months after initial ADT [2]. This is markedly distinct from those with no metastases. For patients who start ADT for PSA only progression, time to castration resistance is in part dependent on PSA doubling time, but has been reported to be as long as 10 years [3].

Many studies have found that AR is present in both initially diagnosed prostate cancer cells and in the vast majority of cells in prostates from CRPC patients [4]. PSA, a known AR target gene, will eventually rise in most CRPC patients, serving as a marker that the androgen axis is still functional despite low circulating levels of serum androgens. Multiple mechanisms have been proposed for the continued activation of AR and the development of CRPC. Molecular studies dissecting the androgen signaling pathways in CRPC are ongoing with multiple new insights in the last several years. This review covers a broad review of these potential mechanisms (see Table 1).

Table 1
Proposed mechanisms for continued AR signaling in CRPC.

Intracrine synthesis of androgens
Amplification and/or overexpression of AR
Overexpression and/or polymorphism of steroid transporters
Mutation of the AR gene
Constitutively-active AR splice variants
Alteration in AR co-regulators
Crosstalk between AR and other signaling pathways
Post-translational modifications of AR
Distinct AR mediated transcriptional programs

Androgen receptor: overview

AR is a member of the steroid receptor superfamily that acts predominately as a ligand-dependent transcription factor after binding to various DNA binding sites. The AR gene is located on the X-chromosome (Xq12), made up of 8 exons [5]. AR consists of an N-terminal domain (NTD) which contains a transactivation domain (AF1) that serves as a primary transcription regulatory region (see Fig. 1). The central DNA binding domain (DBD) contains two zinc fingers that connect to the hinge region allowing DNA recognition, dimerization, and stabilization. The DBD is highly homologous with the DBD of the human glucocorticoid receptor and the human progesterone receptor. The hinge region contains a canonical nuclear localization signal that regulates the nuclear import of the receptor. The hinge region is also a target site for acetylation, ubiquitination, and methylation [6]. The C-terminal domain (CTD) contains the ligand binding domain (LBD) and the AF2 domain, a second transcriptional regulation domain. The NTD and CTD both contain transactivation domains (AF1/AF2), but AF1 is considered dominant in most AR signaling studies conducted under normal physiological conditions. This is particularly relevant in the study of AR splice variants (vide infra).

After synthesis of AR protein, a variety of conformational changes are required to generate a receptor with high-ligand-binding affinity. This requires a complex cascade of events initiated by a "foldosome" that includes complex interactions of a variety of chaperone proteins including HSP40, HSP90, and HSP23 [7]. Upon ligand binding, further conformational changes of AR occur, leading to intra-receptor NTD/CTD interaction followed by translocation of the ligand-bound receptor to the nucleus and homodimerization. Various studies now distinguish nuclear and cytoplasmic AR in both clinical specimens and pharmacological responses, with the nuclear AR contributing to androgen-axis signaling via transcriptional regulation [8].

In the nucleus, the ligand-bound AR homodimers recruit various co-activators and co-repressors, bind to androgen-response elements (ARE), and lead to a broad program of transcriptional activation in AR target genes such as PSA and TMPRSS2. AR-regulated target genes can be both up- or down-regulated and can vary according to ligand concentration. AR target genes vary in cells derived from hormone sensitive cancer as compared to cells derived from CRPC.

Intracrine synthesis of androgens

CRPC tissue exhibits persistent levels of androgens, despite ADT, albeit some androgen levels are lower compared to hormone-naïve tissue [9,10]. Studies have shown the upregulation of steroidogenic enzymes in both model CRPC systems and in tissue from CRPC patients suggesting increased intratumoral synthesis of androgens [11]. In metastases of CRPC patients, relative to primary tumors, there is increased expression of a number of genes involved in androgen metabolism including HSD3B2 (3 beta-hydroxysteroid dehydrogenase), AKR1C3 (also known as 17 hydroxysteroid dehydrogenase type 5 or hydroxysteroid 17-beta-dehydrogenase 5), AKR1C2 (3α-hydroxysteroid dehydrogenase), AKR1C1 (20 alpha-hydroxysteroid dehydrogenase), SRD5A1 (5-alpha reductase type 1), and UGT2B15 (UDP-glucuronosyltransferase 2B15). Of note, AKR1C3 is involved in the conversion of androstenedione to testosterone; SRD5A1/2 converts testosterone to DHT (see Fig. 2). Other investigators have reported variations on this theme [12–14] but taken together, from a functional perspective, these changes are compatible with the over-arching hypothesis that CRPC cells can synthesize potent androgens from various steroidal precursors.

The conventional mechanisms of androgen synthesis are emphasized by many but alternative mechanisms of testosterone and DHT synthesis are also demonstrated in CRPC (see Fig. 2). Androstenedione may be 17-keto reduced to testosterone, and/or
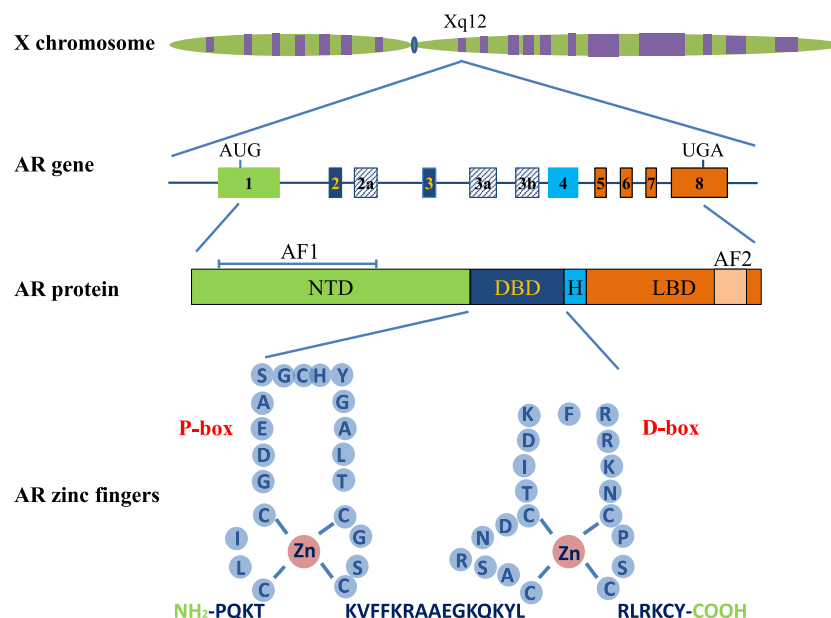
Fig. 1. Schematic representation of the structure of the AR gene, protein, and the two zinc fingers in AR DBD. H, hinge region; P-box mediates DNA recognition; D-box mediates AR DBD-dependent dimerization.
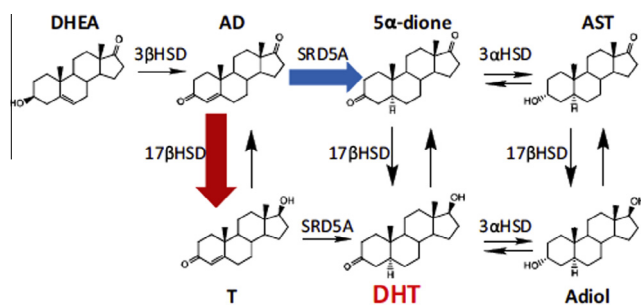
Fig. 2. Androstenedione (AD) can be converted to testosterone directly or it can be 5-alpha reduced to 5-alpha-dione. DHT (dihydrotestosterone) can then be synthesized directly (via 17-beta HSD) or indirectly as shown above. Other abbreviations include T (testosterone), DHT (dihydrotestosterone), DHEA (dehydroepiandrosterone), AD (androstenedione), AST (androsterone), 5α-dione (5α-androstenedione), and Adiol (5α-androstane-3α,17β-diol). Enzymes include 3αHSD or 3βHSD or 17βHSD (3α or 3β or 17β-hydroxysteroid dehydrogenases, respectively), or SRD5A (steroid-5α-reductase). Figure from K.H. Chang et al. [15].

5α reduced by to 5α-dione. In cell lines derived from CRPC patients, the reduction of androstenedione to 5α-dione occurs earlier and more rapidly than the 17-keto reduction to testosterone suggesting primary utilization of the pathway that synthesizes DHT from precursors other than testosterone. Assays in fresh tumor samples confirm the cell line findings [15]. The SRD5A1 enzyme is dominant over the SRD5A2 enzyme in this process [11,15]. 5α-Dione can be converted into DHT via two different mechanisms, either via the direct 17-keto reduction of 5α-dione by AKR1C3, or via conversion of 5α-dione to androsterone, and then conversion of 5α-androstane-3α,17β-diol to DHT. Interestingly in these experiments using CRPC cells, the dominant form of DHT synthesis does not appear to involve testosterone as precursor. "Back door" synthesis of DHT is also possible via 5α-reduction of either progesterone or 17α-hydroxy-progesterone [15]. These reductions are followed by a series of enzymatic conversions similar to the classic DHT steroidogenesis pathway except that conversion of testosterone to DHT is bypassed [15]. Whether or not the "back door" pathway is critical for CRPC development is not yet clear.

Steroid transporters

The efficiency of androgen transport has been shown to affect both efficacy of ADT and transition rate to CRPC. The solute carriers (SLCOs) are a complex family of genes that are involved in transport of organic molecules across cell membranes. The proteins derived from these genes are termed organic acid transporters (OATs). Several of these transporters, including those derived from the SLCO2B1 and SLCO1B3 genes, have significantly increased expression in metastatic CRPC tissues compared to primary cancers [16]. Certain single nucleotide polymorphism (SNP) variants of SLCO2B1 are associated with shorter responses to ADT (hence more rapid CRPC development). One of these SLCO2B1 risk SNPs is exonic, and two are intronic. There also appears to be a gene–gene interaction between the SLCO 2B1 SNPS in terms of time to progression post-ADT. Increasing the number of risk SNPs to 2 or more is associated with a shortened time to progression post-ADT compared to those with zero to one risk genotype [17]. There was an 18 month survival difference if 3 risk genotypes were present compared to none/one and a 12 month difference if 2 risk genotypes were present compared to none/one [17]. In a separate study of CRPC patients, individuals with SLCO1B3 334GG/699AA haplotype showed longer median survival and improved survival probability at 10 years than patients carrying TT/AA and TG/GA haplotypes [18].

The SLCO transporters are involved in various steroid hormonal uptakes in a complex fashion. SLCO2B1 is found in multiple tissues and is involved in transport of compounds including atorvastatin, DHEAS, and estrone-3 sulfate. One of the variants of SCLO2B1 associated with more rapid post-ADT progression is also associated with enhanced transport of dehydroepiandrosterone sulfate (DHEAS), increased AR expression, and increased PSA expression using an in vitro prostate cancer model [17]. SLCO1B3 is primarily found in liver and cancer cells and transports testosterone across membranes in a SNP dependent manner. One of the provocative aspects of the OATs is that they are potentially "druggable" targets. We note that atorvastatin strongly interacts with SLCO2B1 and that (hypothetically) this observation could have therapeutic implications.

AR overexpression

CRPC has been shown to express more AR than benign tissue and hormone naïve cancers [4,19]. Increased AR expression may sensitize the receptor to low levels of androgen [20]. Donovan et al. demonstrated that an increase in nuclear AR expression in advanced disease in either diagnostic biopsy or radical prostatectomy samples was associated with reduced time to prostate-cancer specific mortality [21].

One mechanism for AR protein overexpression is via AR genetic amplification, which has been reported in approximately 30% of CRPC cases [22]. An increase in AR mRNA levels without genomic amplification has also been well described [12]. A number of potential links between AR expression and other signaling pathways are postulated. AR signaling in normal prostate decreases AR gene transcription via lysine specific demethylase I recruitment and H3K4me1,2 demethylation of a specific enhancer in intron 2 of the AR gene [23,24]. Considerable data now suggest that "relief" from AR mediated repression of AR expression can increase AR mRNA in CRPC [23,24]. A link between retinoblastoma protein (RB1) loss and AR expression may be important as well. RB1 loss is frequently seen in transition to CRPC and is associated with poor clinical outcomes. By losing RB1, the transcription factor E2F1 is increased, driving AR overexpression via an increased transcription rate [25]. Micro-RNAs such as miR-let-7c may also serve as important regulator of AR expression. Down-regulation of Let-7c is inversely correlated with AR expression, whereas the expression of Lin28 (a repressor of let-7) is positively correlated [26]. These types of studies suggest potential new drug targets to decrease AR expression in selected patients.

AR mutations in CRPC

Loss-of-function, germline mutations of AR are frequently associated with androgen insensitivity syndrome but AR mutations in prostate cancer are almost exclusively somatic and often associated with gain-of-function [27]. In general, the frequency of AR mutation is lower in untreated, hormone-sensitive prostate cancer than in CRPC [28]. Employing exome sequencing, Grasso et al. [29] reported somatic AR mutations in 5 of 50 cases of lethal, heavily pre-treated CRPC, but none of 11 cases of untreated, localized prostate cancer.

The higher incidence of AR mutations in CRPC suggests an adaptive response and a "Darwinian" mutant-AR clonal selection in some cases [30,31]. This hypothesis is further supported by the observation that AR mutations are more frequent in patients treated with an anti-androgen/castration combination as compared to castration alone [31]. Mutations of the AR gene most frequently localize to exons that encode the LBD (∼49%), followed by the N-terminal domain (∼40%), the DBD (∼7%), and the hinge domain

(2%). Mutations are rarely found in untranslated regions [27]. Mutations in the LBD could potentially affect the ligand specificity of AR, allowing it to be activated by non-androgenic steroids, or anti-androgens, in a promiscuous manner.

The T877A mutation, which has been described by multiple investigators, expands AR ligand binding to estrogen, progestin, selected corticosteroids, and selected anti-androgens [32]. Another mutation in the ligand-binding pocket, H874Y, was identified in CRPC patients treated with flutamide. This mutation also increases ligand promiscuity, allowing DHEA, estradiol, progesterone, and hydroxyflutamide to activate transcription in various model systems [33]. Mutations outside of the LBD could cause gain-of-function or loss-of-function of the receptor by influencing on nuclear localization, co-regulator binding, protein stability, and promoter selectivity [34]. Constitutively active mutants have been described in the regulatory NTD (G142V, M523V, G524D, and M537V) [35].

When certain steroid or steroid binding treatments are withdrawn (flutamide, bicalutamide, nilutamide, megestrol, cyproterone acetate, prednisone, or estramustine), there is potential for improvement in PSA and/or other parameters of disease progression [36]. Whether or not mutated AR is responsible for these withdrawal responses is not clear but laboratory-based experiments clearly uphold the feasibility of such a hypothesis.

Taken together, AR mutations in CRPC potentially allow for continued ligand dependent activation of AR by creating promiscuous ligand binding, altered binding of co-regulators, and/or alterations in genomic regulatory element binding. More studies are needed to assess the clinical impact of these mutations on disease progression. Better categorization of these mutants in patients may provide a greater degree of personalization of therapeutic selection.

AR splice variants

A large number of AR splice variants (AR-Vs) have been recently identified and characterized in CRPC patients (see Fig. 3). These variants have insertions of cryptic exons downstream of the sequences encoding the DBD or deletions of the exons encoding the AR-LBD, resulting in a disrupted AR open reading frame and the expression of truncated AR-V proteins devoid of the functional LBD [37–42]. The majority of the AR-Vs identified to date displays constitutive activity. Two major AR-Vs, AR-V7 (also named as AR3) and ARv567es, have been shown to be capable of regulating target gene expression in the absence of the full-length AR (AR-FL) signaling. Profiling of gene expression changes after knockdown or ectopic expression of AR-V7 or ARv567es suggests that AR-Vs and AR-FL regulate an overlapping yet distinctive set of target genes [43–45]. These studies are rapidly evolving and significant differences in the AR-V transcriptome have been identified in different studies, possibly due to the use of different model systems.

AR-Vs are prevalently upregulated in CRPC compared to hormone-naïve cancers, and can emerge as an adaptive response to therapies targeting the androgen signaling axis, especially new potent drugs such as abiraterone and enzalutamide [46,47]. It is important to recognize the existence of discrepancy between the abundance of AR-V mRNAs and that of AR-V proteins reported in clinical specimens. Although the levels of AR-V mRNAs have been reported to be relatively low, Western analyses of 13 CRPC bone metastases demonstrate that the levels of AR-V proteins could constitute a median of 32% of the AR-FL protein level [39]. In 38% of these CRPC bone metastases, the AR-V proteins are expressed at a level comparable to that of the AR-FL protein [39].

There is now intriguing evidence supporting the important contribution of the constitutively-active AR-Vs to the development of castration resistance. Ectopic expression of AR-V7 or ARv567es confers castration-resistant growth of LNCaP xenograft tumors [42], whereas specific knockdown of AR-V7 attenuates the growth of castration-resistant 22Rv1 xenograft tumors in castrated host [38]. In addition, AR-V7 or ARv567es expression level has been shown to be associated with adverse clinical outcomes. Higher expression of AR-V7 in hormone-naïve prostate tumors predicts increased risk of biochemical recurrence following radical prostatectomy [38,40]. Patients with high AR-V7 or detectable ARv567es expression have significantly shorter cancer-specific survival than other CRPC patients [39]. Thus, the extensive in vitro and xenograft literature on AR-V expression translates into clinically relevant observations.

In addition to the role of the constitutively-active AR-Vs in promoting castration-resistant progression after first-line ADT, their
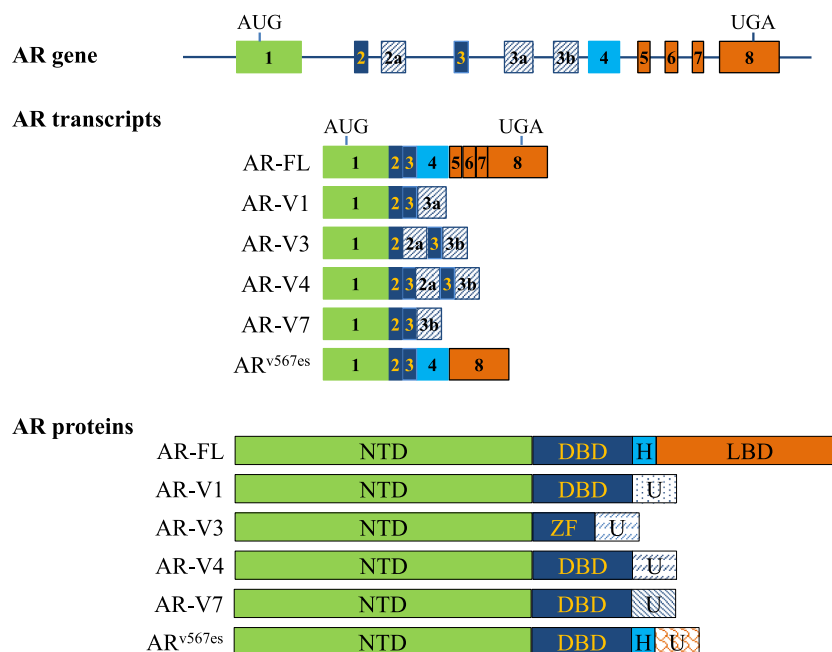

Fig. 3. Schematic representation of the structure of AR-FL and AR-V transcripts and proteins. H, hinge region; U, untranslated region; ZF, zinc finger.

lack of the functional LBD also predicts resistance to the new androgen-axis-targeting drugs, such as abiraterone and enzalutamide [46,47]. Several natural or synthetic compounds have been shown pre-clinically to inhibit AR-V actions [48–52]. Targeting the AR-Vs with these novel agents is an important concept in the therapeutic advances against CRPC, but all of these agents will require clinical trials for proof of action.

Alterations in AR co-regulators

Over 170 potential co-regulators of AR have been identified that bind to AR, stabilize the protein, and lead to either increased or decreased transcriptional activity by altering ligand specificity or allowing transactivation of AR in low levels of androgen [53]. This deregulated co-regulator expression affects AR activity and is repeatedly hypothesized to contribute to the development of CRPC [54,55].

Members of the steroid receptor co-activator family including SRC-1, SRC-2/TIF-2, and SRC-3, have been found to bind the AR NTD and activate AR transactivation via histone acetyltransferase activity as well as by recruiting additional co-activators including CBP/p300. Members of the SRC family are increased in prostate cancer; even higher levels are seen in CRPC [53,55]. SRC-2 has been frequently noted to be amplified as well [54]. These higher levels may lead to increased sensitivity of AR to weak agonists such as androstenedione and DHEA [55]. When phosphorylated by mitogen-activated kinase (MAPK) signaling pathways, SRC1 can activate AR in the absence of androgens to the same magnitude as potent androgens [56]. p300 can also acetylate AR [57], and this post-translational change can enhance co-activator and inhibit co-repressor binding to AR [58].

Co-activators ARA70 and ARA55 also affect ligand specificity of the receptor. ARA70 overexpression enhances AR activation in response to normal weak agonists or to enable antagonists to act as agonists [59]. ARA55 binds to AR leading to an increase in AR activity and altered specificity to binding ligands [58]. Some studies have shown increased expression is associated with shorter recurrence-free survival and overall survival in CRPC patients [60].

Recently, a member of the Snail family of transcription factors (Slug or SNAI2), has been implicated as an AR target gene that can both help upregulate AR and enhance AR-mediated signaling in both wild type, and splice variant, expressing systems [61]. Slug may be particularly important given it acts as a potential oncogene in other cancers and can trigger epithelial–mesenchymal transition (EMT), a frequently described attribute of advanced CRPC [62].

Research on AR co-regulators has focused on both co-activators and co-repressors [63]. SMRT and NCoR are particularly important co-repressors, and their recruitment may play a role in mediating the inhibition of androgen-axis signaling by anti-androgens. Thus alteration in the relative ratios of co-repressors/co-activators recruitment may play a role in the development of CRPC. The scope of this article prevents a full discussion of this area.

Crosstalk with growth factor and cytokine signaling pathways and post-translational AR modifications

Extracellular peptides, including cytokines and growth factors, along with their downstream intracellular signaling cascades, have been implicated in prostate cancer. In CRPC, the crosstalk between these signaling pathways and AR provides a ligand-independent mechanism to sustain AR activation and promote cell growth [6,53,56,64–66].

Post-translational modifications of nuclear receptors including AR effect receptor function, stability, localization, and interactions with other proteins. Phosphorylation, sumoylation, acetylation, and ubiquitination are potential reversible mechanisms behind these alterations.

AR phosphorylations have been described both at serine/threonine residues and various tyrosine residues. MAPK, AKT, protein kinase A, protein kinase C, src-family kinases and Ack1 kinases have all been implicated. In some cases these kinase pathways are thought to be driven by various cytokines and growth factors. Phosphorylation of the AR can localize the receptor to the nucleus and alter AR-dependent transcriptional activity [64–66].

Acetylation occurs at highly conserved lysine residues in AR due to a physiologic stimulus including DHT and the recruitment of co-activators such as p300, which contain intrinsic histone acetyltransferase activity [6,66,67]. Ligand induced AR function is enhanced by this acetyltransferase activity, and augments AR activity at promoter sites of cell cycle genes leading to increased cell proliferation. Mutations in the hinge region can lead to loss of acetylation and decreased DHT binding and signaling [6,66].

SUMOylation typically results in repression of AR. This process results in the attachment of small ubiquitin like modifiers (SUMO) to AR with reversal via SUMO specific proteases including SENP1. AR is modified by SUMO-1 in an androgen dependent manner leading to rapid reversal of AR function. Mutations at the site of SUMOylation within the NTD lead to decreased SUMO-1 binding and enhanced transcriptional activity of AR [6,66,68,69].

The ubiquitin E3 ligase, RNF6, induces AR ubiquitination and promotes AR transcriptional activity. RNF6 is overexpressed in CRPC tissues and has been linked to prostate cancer cell growth after androgen-depletion. Data suggest that RNF6-induced ubiquitination regulates both AR transcriptional activity and specificity by altering cofactor recruitment [66,68,70].

These mechanisms illustrate the effects of post-translational modifications and their impacts on AR activity. Further in vivo studies are needed to address their clinical significance and influence on disease progression, as well as their potential for developing novel prostate cancer specific therapies.

Altered gene expression in CRPC

Recent studies have shed considerable insight into how AR signaling assists cancer cells in adapting to the decline in androgen levels and how AR transcriptional networks are regulated in CRPC, as compared to androgen-dependent prostate cancer. A variety of studies reveal that AR can alter the transcription of a significant number of genes mediating androgen synthesis, DNA synthesis, and cell cycle progression. Chromatin immunoprecipitation coupled with sequencing (ChIP-seq) of DNA adjacent to histone marks H3K4me1 and H3K4me3 in CRPC tissue reveal significant overlapping binding sites with AR (26% of AR binding sites overlap with these H3K4 peaks in CRPC tissue) [71]. These and other studies emphasize the critical importance of histone methylation in regulating AR mediated transcription.

Urbanucci et al. demonstrate that, when AR-transfected LNCaP cells are exposed to low concentrations of androgens, even a modest overexpression of AR could lead to a significant increase in the number of binding sites in the AR cistrome, as well as the strength of AR binding [72]. Moreover, in these cells, receptor binding to chromatin can take place at a concentration of androgen that is 100-fold lower than that in control cells. The canonical androgen-response elements (AREs) and ARE half-sites are among the most significantly overrepresented motifs in this setting [73]. Additional data suggest that AR may be preferentially recruited to chromatin sites that lack the canonical AREs or ARE half-sites by tethering proteins [74].

Via gene expression profiling, a distinct set of genes are clearly upregulated in CRPC compared to the androgen-dependent state. Many AR driven promoters in CRPC tissue demonstrate preferential

co-occurrence of AR binding sites and histone mark H3K4me3. AR recruitment to chromatin associated with the H3K4me1 histone marks is more pronounced at enhancers rather than promoters [71,72]. A complex but distinct transcription program has been identified in CRPC resulting in cellular growth rather than differentiation [72]. In normal prostate cells, AR signaling primarily promotes a differentiation of cells. In CRPC, unlike androgen-dependent cells, AR directly promotes transcription of M-phase cell cycle regulatory genes such as CDC20, UBE2C, CDK1, and these alterations increase cellular proliferation in combination with various collaborating transcription factors (such as FoxA1) and co-activators (such as MED1).

As an example of re-directed gene expression in CRPC, the ubiquitin-conjugating enzyme E2C (UBE2C) may be particularly important as expression is critical for inactivation of the cell cycle M-phase checkpoint [72]. In CRPC models, two UBE2C gene enhancer sites contain H3K4me1 and H3K4me2. These epigenetic marks allow FoxA1 and MED1 binding, which then directs AR binding to the enhancer, thereby activating AR-dependent UBE2C transcription. Both the H3K4 marks and the "pioneer factor" FoxA1 are required for AR binding to occur. The critical role of UBE2C is suggested by silencing of UBE2C, which stops proliferation in CRPC models. Interestingly, though silencing of FOXA1 abolishes AR mediated UBE2C transcription in CRPC models, it has no effect on traditional AR target gene expression (PSA and TMPRSS2) in androgen dependent models, suggesting the critical importance of FoxA1 in unique CRPC transcription programs [72].

It is not appropriate to view AR-mediated CRPC gene reprogramming solely in terms of gene expression. Liganded AR has distinct effects on AR mediated transcription that are dependent on both ligand concentration and cellular context. One hypothesis suggests that androgen levels in CRPC cells are adequate to stimulate selected enhancer elements, but are not adequate to effectively recruit AR to suppressor elements that can negatively regulate cellular proliferation. Under these circumstances, both the concentration of ligand and the CRPC context are keys to understanding AR regulated gene expression [24].

Enhancer of zeste homolog 2 (EZH2), which is over-expressed in CRPC (and many other advanced cancers), can functionally switch from transcriptional repression in normal cells to gene activation in CRPC. EZH2 phosphorylation at Ser21, potentially an Akt mediated event in CRPC, promotes association with an AR-containing complex and dramatically alters expression of a large number of transcripts involved in cellular proliferation. Suppression of EZH2 decreases growth rates in various CRPC model systems suggesting potential as a drug target [75].

Another example of dramatic alterations in the AR cistrome involves the AR-regulated TMPRSS2:ERG fusion gene. Expression of the TMPRSS2:ERG fusion gene is restored in CRPC, in concert with activation of AR transcriptional activity, and a continued important role for ERG in CRPC has been postulated [23]. In CRPC cells, ERG over-expression redirects AR to a set of genes (including the potentially important SOX9) that are not normally androgen stimulated [76].

Taken together, AR gene expression signatures in CRPC are dynamic, context-dependent, and involve hundreds of transcripts (both coding and non-coding) that promote cell proliferation, motility, and invasion. Significant variations are noted between cell cultures and human samples [71], thus caution is advised in over-interpreting cell culture studies. Developing gene expression signatures with clinical relevance is an ongoing goal [71].

Non-AR mutations in CRPC

In a study of metastatic CRPC tissues derived from autopsy specimens, mutations were recurrently noted in a variety of exomes including those derived from p53, ZFHX3, RB1, PTEN, APC, MLL2, OR5L1 and CDK12 [29]. These appear to constitute adaptive responses that arise in CRPC but are distinct from those seen in the AR-axis. Next-generation sequencing promises to further elucidate further genomic alterations/mutations in both coding and non-coding regions of the genome. This area will rapidly progress in the near future. Whether clinically actionable mutations will arise from these analyses is unclear. Additional focus on this important arena is beyond the scope of this manuscript.

Non-coding RNAs

This complex arena is only now beginning to be explored. Recent studies of the human transcriptome indicate that the number of non-coding RNAs is in far excess of the number of protein coding genes. Non-coding RNAs vary tremendously in size. Thus far, microRNAs are among those best studied given their stability and annotation. Androgens can up- or down-regulate a number of microRNAs, with exact results being dependent on the model studied [77]. Androgen represses the miR-99a/let7c/125b-2 cluster [26]. Serum samples from metastatic CRPC patients exhibit distinct circulating microRNA signatures. miR-375, miR-378, and miR-141 are over-expressed in serum from CRPC patients compared with serum from low-risk localized patients, while miR-409-3p is under-expressed [78]. Among longer non-coding RNAs, approximately 120 are transcriptionally dysregulated in prostate cancer [79]. One of these, PCAT-1 has been potentially implicated in advanced disease [80]. Considerable evolution in this area is expected in the next several years.

Conclusion

Multiple adaptive mechanisms involve the androgen signaling axis in CRPC. Alterations in the AR-axis include altered ligand/receptor interactions, and/or altered transcriptional response to AR. Both AR hypersensitivity due to AR overexpression and intratumoral androgen synthesis have been shown to continue androgen dependent growth despite a relatively hormone-depleted environment. AR mutations are noted, especially in tumors treated with selected anti-androgens. AR splice variants can create a constitutively active receptor, alter recruitment of co-activators/repressors, and alter gene expression leading to sustained CRPC growth. Changes in co-regulator expression and various polymorphisms in androgen transporters have also been identified. Ligand independent activation of AR has been seen with cross talk between extracellular peptides, intracellular kinase pathways, co-regulatory proteins, and intrinsic activation of AR. Post-translational modifications can affect AR activity, stability, localization, and interaction with other proteins. A distinct AR-induced transcriptional program leading to proliferation, invasion, and metastasis provides an additional important mechanism for the development of CRPC. The exploration of numerous uncharacterized non-coding RNAs, some of which are androgen-regulated, may also have important biological functions in this disease. Non-AR-axis mutations are also increasingly being documented and their relevance to the growth of CRPC is currently being explored. In summary, CRPC development and progression is centered, in part, around adaptations of the androgen signaling, opening doors for continued research and potential development of novel therapeutic concepts.

Funding source

Consortium Fund (Y.D. and H.Z.), and the Laborde endowed professorship (O.S.). The study sponsors had no involvement in the collection, analysis and interpretation of data, the writing of the manuscript, or the decision to submit the manuscript for publication.

Conflict of interest statement

There are no conflicts to declare.

References

[1] Huggins C, Hodges CV. Studies on prostatic cancer. I. The effect of castration, of estrogen and androgen injection on serum phosphatases in metastatic carcinoma of the prostate. Cancer Res 1941;1(4):293–7.

[2] Ross RW, Xie W, Regan MM, et al. Efficacy of androgen deprivation therapy (ADT) in patients with advanced prostate cancer: association between Gleason score, prostate-specific antigen level, and prior ADT exposure with duration of ADT effect. Cancer 2008;112(6):1247–53.

[3] Crook JM, O'Callaghan CJ, Duncan G, et al. Intermittent androgen suppression for rising PSA level after radiotherapy. N Engl J Med 2012;367(10):895–903.

[4] Ford III OH, Gregory CW, Kim D, Smitherman AB, Mohler JL. Androgen receptor gene amplification and protein expression in recurrent prostate cancer. J Urol 2003;170(5):1817–21.

[5] Dehm SM, Tindall DJ. Androgen receptor structural and functional elements: role and regulation in prostate cancer. Mol Endocrinol 2007;21(12):2855–63.

[6] Clinckemalie L, Vanderschueren D, Boonen S, Claessens F. The hinge region in androgen receptor control. Mol Cell Endocrinol 2012;358(1):1–8.

[7] Cano LQ, Lavery DN, Bevan CL. Mini-review: foldosome regulation of androgen receptor action in prostate cancer. Mol Cell Endocrinol 2013;369(1–2):52–62.

[8] Watson PA, Chen YF, Balbas MD, et al. Constitutively active androgen receptor splice variants expressed in castration-resistant prostate cancer require full-length androgen receptor. Proc Natl Acad Sci USA 2010;107(39):16759–65.

[9] Geller J, Albert JD, Nachtsheim DA, Loza D. Comparison of prostatic cancer tissue dihydrotestosterone levels at the time of relapse following orchiectomy or estrogen therapy. J Urol 1984;132(4):693–6.

[10] Mohler JL, Gregory CW, Ford III OH, et al. The androgen axis in recurrent prostate cancer. Clin Cancer Res 2004;10(2):440–8.

[11] Stanbrough M, Bubley GJ, Ross K, et al. Increased expression of genes converting adrenal androgens to testosterone in androgen-independent prostate cancer. Cancer Res 2006;66(5):2815–25.

[12] Holzbeierlein J, Lal P, LaTulippe E, et al. Gene expression analysis of human prostate carcinoma during hormonal therapy identifies androgen-responsive genes and mechanisms of therapy resistance. Am J Pathol 2004;164(1):217–27.

[13] Locke JA, Guns ES, Lubik AA, et al. Androgen levels increase by intratumoral de novo steroidogenesis during progression of castration-resistant prostate cancer. Cancer Res 2008;68(15):6407–15.

[14] Montgomery RB, Mostaghel EA, Vessella R, et al. Maintenance of intratumoral androgens in metastatic prostate cancer: a mechanism for castration-resistant tumor growth. Cancer Res 2008;68(11):4447–54.

[15] Chang KH, Li R, Papari-Zareei M, et al. Dihydrotestosterone synthesis bypasses testosterone to drive castration-resistant prostate cancer. Proc Natl Acad Sci USA 2011;108(33):13728–33.

[16] Wright JL, Kwon EM, Ostrander EA, et al. Expression of SLCO transport genes in castration-resistant prostate cancer and impact of genetic variation in SLCO1B3 and SLCO2B1 on prostate cancer outcomes. Cancer Epidemiol Biomarkers Prev 2011;20(4):619–27.

[17] Yang M, Xie W, Mostaghel E, et al. SLCO2B1 and SLCO1B3 may determine time to progression for patients receiving androgen deprivation therapy for prostate cancer. J Clin Oncol 2011;29(18):2565–73.

[18] Hamada A, Sissung T, Price DK, et al. Effect of SLCO1B3 haplotype on testosterone transport and clinical outcome in caucasian patients with androgen-independent prostatic cancer. Clin Cancer Res 2008;14(11):3312–8.

[19] Linja MJ, Savinainen KJ, Saramaki OR, Tammela TL, Vessella RL, Visakorpi T. Amplification and overexpression of androgen receptor gene in hormone-refractory prostate cancer. Cancer Res 2001;61(9):3550–5.

[20] Waltering KK, Helenius MA, Sahu B, et al. Increased expression of androgen receptor sensitizes prostate cancer cells to low levels of androgens. Cancer Res 2009;69(20):8141–9.

[21] Donovan MJ, Osman I, Khan FM, et al. Androgen receptor expression is associated with prostate cancer-specific survival in castrate patients with metastatic disease. BJU Int 2010;105(4):462–7.

[22] Visakorpi T, Hyytinen E, Koivisto P, et al. In vivo amplification of the androgen receptor gene and progression of human prostate cancer. Nat Genet 1995;9(4):401–6.

[23] Cai C, Wang H, Xu Y, Chen S, Balk SP. Reactivation of androgen receptor-regulated TMPRSS2: ERG gene expression in castration-resistant prostate cancer. Cancer Res 2009;69(15):6027–32.

[24] Cai C, He HH, Chen S, et al. Androgen receptor gene expression in prostate cancer is directly suppressed by the androgen receptor through recruitment of lysine-specific demethylase 1. Cancer Cell 2011;20(4):457–71.

[25] Sharma A, Yeow WS, Ertel A, et al. The retinoblastoma tumor suppressor controls androgen signaling and human prostate cancer progression. J Clin Invest 2010;120(12):4478–92.

[26] Sun D, Layer R, Mueller AC, et al. Regulation of several androgen-induced genes through the repression of the miR-99a/let-7c/miR-125b-2 miRNA cluster in prostate cancer cells. Oncogene 2013. http://dx.doi.org/10.1038/onc.2013.77 (Epub ahead of print)..

[27] Gottlieb B, Beitel LK, Nadarajah A, Paliouras M, Trifiro M. The androgen receptor gene mutations database: 2012 update. Hum Mutat 2012;33(5):887–94.

[28] Koochekpour S. Androgen receptor signaling and mutations in prostate cancer. Asian J Androl 2010;12(5):639–57.

[29] Grasso CS, Wu YM, Robinson DR, et al. The mutational landscape of lethal castration-resistant prostate cancer. Nature 2012;487(7406):239–43.

[30] Taplin ME, Bubley GJ, Shuster TD, et al. Mutation of the androgen-receptor gene in metastatic androgen-independent prostate cancer. N Engl J Med 1995;332(21):1393–8.

[31] Taplin ME, Bubley GJ, Ko YJ, et al. Selection for androgen receptor mutations in prostate cancers treated with androgen antagonist. Cancer Res 1999;59(11):2511–5.

[32] Veldscholte J, Voorhorst-Ogink MM, Bolt-de VJ, van Rooij HC, Trapman J, Mulder E. Unusual specificity of the androgen receptor in the human prostate tumor cell line LNCaP: high affinity for progestogenic and estrogenic steroids. Biochim Biophys Acta 1990;1052(1):187–94.

[33] Steketee K, Timmerman L, Ziel-van der Made AC, Doesburg P, Brinkmann AO, Trapman J. Broadened ligand responsiveness of androgen receptor mutants obtained by random amino acid substitution of H874 and mutation hot spot T877 in prostate cancer. Int J Cancer 2002;100(3):309–17.

[34] Steinkamp MP, O'Mahony OA, Brogley M, et al. Treatment-dependent androgen receptor mutations in prostate cancer exploit multiple mechanisms to evade therapy. Cancer Res 2009;69(10):4434–42.

[35] Hay CW, McEwan IJ. The impact of point mutations in the human androgen receptor: classification of mutations on the basis of transcriptional activity. PLoS One 2012;7(3):e32514.

[36] Kelly WK, Slovin S, Scher HI. Steroid hormone withdrawal syndromes. Pathophysiology and clinical significance. Urol Clin North Am 1997;24(2):421–31.

[37] 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(13):5469–77.

[38] Guo Z, Yang X, Sun F, et al. A novel androgen receptor splice variant is up-regulated during prostate cancer progression and promotes androgen depletion-resistant growth. Cancer Res 2009;69(6):2305–13.

[39] Hornberg E, Ylitalo EB, Crnalic S, et al. Expression of androgen receptor splice variants in prostate cancer bone metastases is associated with castration-resistance and short survival. PLoS One 2011;6(4):e19059.

[40] Hu R, Dunn TA, Wei S, et al. Ligand-independent androgen receptor variants derived from splicing of cryptic exons signify hormone-refractory prostate cancer. Cancer Res 2009;69(1):16–22.

[41] Sun S, Sprenger CC, Vessella RL, et al. Castration resistance in human prostate cancer is conferred by a frequently occurring androgen receptor splice variant. J Clin Invest 2010;120(8):2715–30.

[42] Zhang X, Morrissey C, Sun S, et al. Androgen receptor variants occur frequently in castration resistant prostate cancer metastases. PLoS One 2011;6(11):e27970.

[43] Chan SC, Li Y, Dehm SM. Androgen receptor splice variants activate androgen receptor target genes and support aberrant prostate cancer cell growth independent of canonical androgen receptor nuclear localization signal. J Biol Chem 2012;287(23):19736–49.

[44] Hu R, Isaacs WB, Luo J. A snapshot of the expression signature of androgen receptor splicing variants and their distinctive transcriptional activities. Prostate 2011;71(15):1656–67.

[45] Hu R, Lu C, Mostaghel EA, et al. Distinct transcriptional programs mediated by the ligand-dependent full-length androgen receptor and its splice variants in castration-resistant prostate cancer. Cancer Res 2012;72(14):3457–62.

[46] Li Y, Chan SC, Brand LJ, Hwang TH, Silverstein KA, Dehm SM. Androgen receptor splice variants mediate enzalutamide resistance in castration-resistant prostate cancer cell lines. Cancer Res 2013;73(2):483–9.

[47] Mostaghel EA, Marck BT, Plymate SR, et al. Resistance to CYP17A1 inhibition with abiraterone in castration-resistant prostate cancer: induction of steroidogenesis and androgen receptor splice variants. Clin Cancer Res 2011;17(18):5913–25.

[48] Andersen RJ, Mawji NR, Wang J, et al. Regression of castrate-recurrent prostate cancer by a small-molecule inhibitor of the amino-terminus domain of the androgen receptor. Cancer Cell 2010;17(6):535–46.

[49] Cao B, Liu X, Li J, et al. 20(S)-protopanaxadiol-aglycone downregulation of the full-length and splice variants of androgen receptor. Int J Cancer 2013;132(6):1277–87.

[50] Li J, Cao B, Liu X, et al. Berberine suppresses androgen receptor signaling in prostate cancer. Mol Cancer Ther 2011;10(8):1346–56.

[51] Li X, Liu Z, Xu X, et al. Kava components down-regulate expression of AR and AR splice variants and reduce growth in patient-derived prostate cancer xenografts in mice. PLoS One 2012;7(2):e31213.
[52] Mashima T, Okabe S, Seimiya H. Pharmacological targeting of constitutively active truncated androgen receptor by nigericin and suppression of hormone-refractory prostate cancer cell growth. Mol Pharmacol 2010;78(5):846–54.
[53] Heemers HV, Tindall DJ. Androgen receptor (AR) coregulators: a diversity of functions converging on and regulating the AR transcriptional complex. Endocr Rev 2007;28(7):778–808.
[54] Comuzzi B, Nemes C, Schmidt S, et al. The androgen receptor co-activator CBP is up-regulated following androgen withdrawal and is highly expressed in advanced prostate cancer. J Pathol 2004;204(2):159–66.
[55] Taylor BS, Schultz N, Hieronymus H, et al. Integrative genomic profiling of human prostate cancer. Cancer Cell 2010;18(1):11–22.
[56] Ueda T, Bruchovsky N, Sadar MD. Activation of the androgen receptor N-terminal domain by interleukin-6 via MAPK and STAT3 signal transduction pathways. J Biol Chem 2002;277(9):7076–85.
[57] Fu M, Wang C, Reutens AT, et al. P300 and p300/cAMP-response element-binding protein-associated factor acetylate the androgen receptor at sites governing hormone-dependent transactivation. J Biol Chem 2000;275(27):20853–60.
[58] Fu M, Rao M, Wang C, et al. Acetylation of androgen receptor enhances coactivator binding and promotes prostate cancer cell growth. Mol Cell Biol 2003;23(23):8563–75.
[59] Yeh S, Chang HC, Miyamoto H, et al. Differential induction of the androgen receptor transcriptional activity by selective androgen receptor coactivators. Keio J Med 1999;48(2):87–92.
[60] Miyoshi Y, Ishiguro H, Uemura H, et al. Expression of AR associated protein 55 (ARA55) and androgen receptor in prostate cancer. Prostate 2003;56(4):280–6.
[61] Wu K, Gore C, Yang L, et al. Slug, a unique androgen-regulated transcription factor, coordinates androgen receptor to facilitate castration resistance in prostate cancer. Mol Endocrinol 2012;26(9):1496–507.
[62] Alves CC, Carneiro F, Hoefler H, Becker KF. Role of the epithelial–mesenchymal transition regulator Slug in primary human cancers. Front Biosci 2009;14:3035–50.
[63] Godoy AS, Sotomayor PC, Villagran M, et al. Altered corepressor SMRT expression and recruitment to target genes as a mechanism that change the response to androgens in prostate cancer progression. Biochem Biophys Res Commun 2012;423(3):564–70.
[64] Chen S, Kesler CT, Paschal BM, Balk SP. Androgen receptor phosphorylation and activity are regulated by an association with protein phosphatase 1. J Biol Chem 2009;284(38):25576–84.
[65] Chen S, Gulla S, Cai C, Balk SP. Androgen receptor serine 81 phosphorylation mediates chromatin binding and transcriptional activation. J Biol Chem 2012;287(11):8571–83.
[66] Gioeli D, Paschal BM. Post-translational modification of the androgen receptor. Mol Cell Endocrinol 2012;352(1–2):70–8.
[67] Gong J, Zhu J, Goodman Jr OB, et al. Activation of p300 histone acetyltransferase activity and acetylation of the androgen receptor by bombesin in prostate cancer cells. Oncogene 2006;25(14):2011–21.
[68] Coffey K, Robson CN. Regulation of the androgen receptor by post-translational modifications. J Endocrinol 2012;215(2):221–37.
[69] Poukka H, Karvonen U, Janne OA, Palvimo JJ. Covalent modification of the androgen receptor by small ubiquitin-like modifier 1 (SUMO-1). Proc Natl Acad Sci USA 2000;97(26):14145–50.
[70] Xu K, Shimelis H, Linn DE, et al. Regulation of androgen receptor transcriptional activity and specificity by RNF6-induced ubiquitination. Cancer Cell 2009;15(4):270–82.
[71] Sharma NL, Massie CE, Ramos-Montoya A, et al. The androgen receptor induces a distinct transcriptional program in castration-resistant prostate cancer in man. Cancer Cell 2013;23(1):35–47.
[72] Wang Q, Li W, Zhang Y, et al. Androgen receptor regulates a distinct transcription program in androgen-independent prostate cancer. Cell 2009;138(2):245–56.
[73] Urbanucci A, Marttila S, Janne OA, Visakorpi T. Androgen receptor overexpression alters binding dynamics of the receptor to chromatin and chromatin structure. Prostate 2012;72(11):1223–32.
[74] Gonit M, Zhang J, Salazar M, et al. Hormone depletion-insensitivity of prostate cancer cells is supported by the AR without binding to classical response elements. Mol Endocrinol 2011;25(4):621–34.
[75] Xu K, Wu ZJ, Groner AC, et al. EZH2 oncogenic activity in castration-resistant prostate cancer cells is polycomb-independent. Science 2012;338(6113):1465–9.
[76] Cai C, Wang H, He HH, et al. ERG induces androgen receptor-mediated regulation of SOX9 in prostate cancer. J Clin Invest 2013;123(3):1109–22.
[77] Waltering KK, Porkka KP, Jalava SE, et al. Androgen regulation of micro-RNAs in prostate cancer. Prostate 2011;71(6):604–14.
[78] Nguyen HC, Xie W, Yang M, et al. Expression differences of circulating microRNAs in metastatic castration resistant prostate cancer and low-risk, localized prostate cancer. Prostate 2013;73(4):346–54.
[79] Prensner JR, Chinnaiyan AM. The emergence of lncRNAs in cancer biology. Cancer Discov 2011;1(5):391–407.
[80] Prensner JR, Iyer MK, Balbin OA, et al. Transcriptome sequencing across a prostate cancer cohort identifies PCAT-1, an unannotated lincRNA implicated in disease progression. Nat Biotechnol 2011;29(8):742–9.

Review Article
Splicing variants of androgen receptor in prostate cancer

Haitao Zhang[1,5], Yang Zhan[2], Xichun Liu[1], Yanfeng Qi[2], Guanyi Zhang[1], Oliver Sartor[3,4,5], Yan Dong[2,5,6]

Departments of [1]Pathology and Laboratory Medicine, [2]Structural and Cellular Biology, [3]Urology, [4]Medicine, Tulane University School of Medicine, [5]Tulane Cancer Center, New Orleans, LA; [6]National Engineering Laboratory for AIDS Vaccine, College of Life Sciences, Jilin University, China

Abstract: Significant advances in our understanding of continued androgen receptor (AR) signaling in castration-resistant prostate cancer have led to the development and FDA approval of two next-generation androgen-directed therapies, abiraterone and enzalutamide. These new therapies heralded a new era of prostate cancer therapy. However, disease progression during androgen-directed therapies remains the most critical challenge in the clinical management of prostate cancer. Accumulating evidence points to an important contribution of constitutively-active AR splice variants to AR-driven tumor progression during androgen-directed therapies. In this review, we will focus on the structure, activity, detection, clinical relevance, and mechanisms of production of AR splice variants.

Keywords: Androgen receptor, AR splice variants, castration resistance, prostate cancer
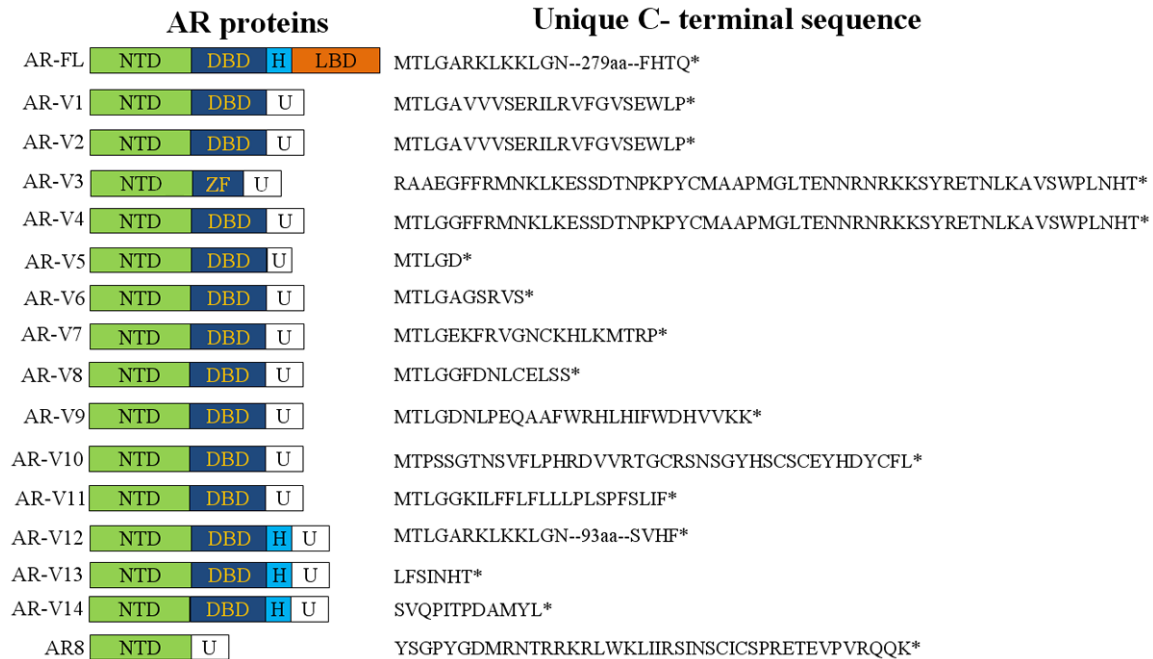
Introduction

Prostate cancer is the most common non-skin cancer and the second leading cause of cancer mortality in men in the United States. Androgen deprivation therapy, which disrupts androgen receptor (AR) signaling by reducing androgen levels through surgical or chemical castration, or by administration of anti-androgens that compete with androgens for binding to AR [2], is the first-line treatment for metastatic and locally advanced prostate cancer. While this regimen is effective initially, progression to the presently incurable and lethal stage, termed castration-resistant prostate cancer (CRPC), invariably occurs [1, 2]. With a median survival of ~16-18 months [3], CRPC accounts for the majority of disease-related mortality. Mounting evidence suggests that resurgent AR drives therapeutic failure and castration-resistant progression [1, 2]. A number of ligand-dependent and -independent mechanisms have been proposed to underlie AR reactivation during androgen deprivation therapy [1, 2]. While these mechanisms are thoroughly reviewed by many, this review is focused on the discussion of the role of constitutively-active AR splice variants that lack the functional ligand-binding domain (LBD) in AR-signaling reactivation.

Structure and activity of the AR splice variants

The canonical AR protein is 919 amino acids long, encoded by 8 exons on the X-chromosome (Xq12) [4]. Structurally, the full-length AR (AR-FL) resembles other members of the steroid receptor family, consisting of 4 domains (**Figure 1**). The N-terminal domain (NTD) contains an activation function 1 (AF1) domain that functions as a ligand-independent transcriptional activation domain. Another important function of NTD is recruitment of coregulators. The DNA-binding domain (DBD) contains two zinc fingers that are involved in DNA recognition, dimerization, and stabilization. The hinge domain (H) provides flexibility to the protein and regulates the nuclear translocation of the receptor through a canonical nuclear localization signal. The C-terminal LBD contains a ligand-binding pocket that mediates high affinity ligand-binding. A second activation function domain (AF2) is located in the LBD and regulates transcriptional activation in a ligand-dependent manner. All hormonal therapies currently accepted in

AR proteins   **Unique C- terminal sequence**

AR-FL	NTD DBD H LBD	MTLGARKLKKLGN--279aa--FHTQ*
AR-V1	NTD DBD U	MTLGAVVVSERILRVFGVSEWLP*
AR-V2	NTD DBD U	MTLGAVVVSERILRVFGVSEWLP*
AR-V3	NTD ZF U	RAAEGFFRMNKLKESSDTNPKPYCMAAPMGLTENNRNRKKSYRETNLKAVSWPLNHT*
AR-V4	NTD DBD U	MTLGGFFRMNKLKESSDTNPKPYCMAAPMGLTENNRNRKKSYRETNLKAVSWPLNHT*
AR-V5	NTD DBD U	MTLGD*
AR-V6	NTD DBD U	MTLGAGSRVS*
AR-V7	NTD DBD U	MTLGEKFRVGNCKHLKMTRP*
AR-V8	NTD DBD U	MTLGGFDNLCELSS*
AR-V9	NTD DBD U	MTLGDNLPEQAAFWRHLHIFWDHVVKK*
AR-V10	NTD DBD U	MTPSSGTNSVFLPHRDVVRTGCRSNSGYHSCSCEYHDYCFL*
AR-V11	NTD DBD U	MTLGGKILFFLFLLLPLSPFSLIF*
AR-V12	NTD DBD H U	MTLGARKLKKLGN--93aa--SVHF*
AR-V13	NTD DBD H U	LFSINHT*
AR-V14	NTD DBD H U	SVQPITPDAMYL*
AR8	NTD U	YSGPYGDMRNTRRKRLWKLIIRSINSCICSPRETEVPVRQQK*

Figure 1. Schematic representation of the structure of AR-FL and AR-V proteins. U, unique sequence; ZF, zinc finger.

the clinic for treatment of prostate cancer, including the recent FDA-approved abiraterone [5] and enzalutamide [6], target the LBD *de facto.*

Recently, a cadre of AR splice variants (AR-Vs) that are devoid of a functional LBD have been identified (**Figure 1**). Structurally, these variants either have insertions of cryptic exons immediately downstream of the exons encoding the DBD or have deletions of the exons encoding the LBD, resulting in a disrupted AR open reading frame and the expression of truncated proteins [7-12]. Since the NTD and DBD remain intact in the majority of the AR-Vs identified to date, many variants display constitutive activity [7-12]. Others are considered conditionally active because these variants display ligand-independent activity only in certain cell models [12]. Two major AR-Vs, AR-V7 (also known as AR3) and AR[v567es] (a.k.a. AR-V12), have been shown to be capable of regulating target gene expression in the absence of the AR-FL signaling [8-10, 13]. Gene expression profiling showed that AR-V7 and AR[v567es] regulate the expression of both canonical androgen-responsive genes and a distinct set of targets enriched for cell-cycle function [9, 10, 13]. However, there exists significant difference in the AR-V transcriptome identified by different studies [8-10, 13], possibly due to the use of different model systems.

Notably, not all AR-Vs function as a transcription factor. For example, AR8 lacks a functional DBD, and has been shown to localize mainly to the plasma membrane [14]. AR8 may play a role in mediating Src kinase activation as well as AR-FL tyrosine phosphorylation and subsequent nuclear translocation in response to EGF treatment, possibly by forming a membrane-associated signaling complex that includes AR8, AR-FL, Src kinase, and EGFR [14]. Depletion of AR8 by RNA interference compromised EGF-induced Src activation and AR phosphorylation, as well as inhibited cell proliferation and induced cell death [14]. Thus, AR8 activates the AR signaling pathway and promotes cell survival via a nongenomic mechanism.

Potentiation of AR-FL activity is not limited to the transcriptionally inactive AR8. In cells co-expressing AR[v567es] and AR-FL, AR[v567es] could bind to AR-FL and facilitate the nuclear translocation of AR-FL in the absence of ligand [10]. Similarly, AR-V7 could also facilitate the nuclear translocation of AR-FL in an androgen depleted condition or in the presence of the potent AR antagonist enzalutamide (Cao *et al.*, manuscript under review).

Table 1. Isoform-specific PCR primers for detecting AR-Vs

AR-Vs	Alias	PCR Primers	Primer locations	Product size (bp)	Ref.
AR-V1	AR4	F: 5'-CCATCTTGTCGTCTTCGGAAATGTTATGAAGC-3' R: 5'-CTGTTGTGGATGAGCAGCTGAGAGTCT-3'	Exon 3 CE1	149	[8]
		F: 5'-CTACTCCGGACCTTACGGGGACATGCG-3' R: 5'-GATTCTTTCAGAAACAACAACAGCTGCT-3'	Exon 1 Exon 3/CE1	322	[9]
AR-V2		N/A			[8]
AR-V3	AR1/2/2b	N/A			[7, 8]
AR-V4	AR1/2/3/2b, AR5	F: 5'-CTACTCCGGACCTTACGGGGACATGCG-3' R: 5'-CTTTTAATTTGTTCATTCTGAAAAATCCTC-3'	Exon 1 CE2	323	[9]
AR-V5		N/A			[8]
AR-V6		N/A			[8]
AR-V7	AR3	F: 5'-CCATCTTGTCGTCTTCGGAAATGTTATGAAGC-3' F: 5'-TTTGAATGAGGCAAGTCAGCCTTTCT-3'	Exon 3 CE3	125	[8]
		F: 5'-CTACTCCGGACCTTACGGGGACATGCG-3' R: 5'-TGCCAACCCGGAATTTTTCTCCC-3'	Exon 1 Exon 3/CE3	314	[9]
AR-V8		N/A			[11]
AR-V9		F: 5'-CCATCTTGTCGTCTTCGGAAATGTTATGAAGC-3' R: 5'-TTAGTTCTACTTCTTAACAACGTGATCCCA-3'	Exon 3 CE5	128	[12]
AR-V10		N/A			[11]
AR-V11		N/A			[11]
AR-V12	AR^{v567es}	F: 5-GCCTTGCCTGATTGCGAG R: 5'-CATGTGTGACTTGATTAGCAGGTCAAA	Exons 4/8 Exon 8	64	[12]
		F: 5'-CCAAGGCCTTGCCTGATTGC-3' R: 5'-TTGGGCACTTGCACAGAGAT-3'	Exons 4/8 Exon 8	124	[10]
AR-V13		N/A			[12]
AR-V14		N/A			[12]
AR8		F: 5'-CGACTTCACCGCACCTGATG-3' R: 5'-CTCTTTCTTCGGGTATTTCGCATG-3'	Exon 1 Exons 1/3'	150	[14]

Detection of AR-V expression

The majority of AR-V transcripts can be detected by reverse transcription polymerase chain reaction (RT-PCR), taking advantage of their unique exon compositions and exon-exon junctions. A collation of published PCR primers for AR-Vs is presented in **Table 1**. Although RT-PCR provides a sensitive and specific assay for the mRNA, the results do not always correlate with protein expression. For example, the transcript, but not the protein product, of AR-V7 has been detected in the LNCaP cell line [15]. To date, isoform-specific antibodies have only been reported for AR-V7 [8, 9] and AR8 [14], and the only commercially available antibody is for AR-V7 (Precision Antibodies, Columbia, MD). To overcome this limitation, Zhang *et al.* developed an immunohistochemical assay to detect the expression of AR-Vs by using two antibodies recognizing the N- and C-terminus of AR, respectively [16]. A decrease of AR nuclear staining by the C-terminal antibody when compared to that by the N-terminal antibody was used to indicate the presence of LBD-truncated, transcriptionally active AR-Vs. The authors

reported a significant loss in AR C-terminal nuclear immunoreactivity in CRPC specimens, but not in tissues from primary cancers, suggesting the prevalence of AR-Vs in CRPC. The results were further corroborated by RT-PCR [16]. Despite the demonstrated success, this approach requires significant efforts in assay optimization and standardization. There is a great demand for the development of additional isoform-specific antibodies for AR-Vs. The unique C-terminal peptide sequences of AR-Vs are presented in **Figure 1**.

Clinical relevance of AR-Vs

AR-Vs are prevalently upregulated in CRPC compared to hormone-naïve cancers, and may emerge as an adaptive response to therapies targeting the androgen signaling axis [8-11, 16, 17]. It is important to recognize the existence of discrepancy between the relative abundance of AR-V mRNAs and that of AR-V proteins in clinical specimens. Although the levels of AR-V mRNAs in metastatic CRPCs have been shown to constitute at most 7% of the AR-FL mRNA level [11, 17], Western analyses of 13 CRPC

bone metastases demonstrated that the levels of AR-V proteins could constitute a median of 32% of the AR-FL protein level (ranging from 0 to 95%) [17]. In fact, in 38% of these CRPC bone metastases, the AR-V proteins are expressed at a level comparable to that of the AR-FL protein [17]. The relative high abundance of AR-V proteins is also supported by data from immunohistochemistry analysis. With the use of an antibody specific to AR-V7, several groups show that AR-V7 is readily detectable in prostate cancer specimens [9, 13, 18]. Using the two-antibodies approach described above, Zhang and colleagues analyzed 50 primary prostate cancer and 162 metastatic CRPC tissues and found that 24% of these CRPC tissues display a staining pattern similar to that of the LuCaP86.2 xenograft, which predominantly expresses AR-V [16].

It is also important to recognize that the absolute levels of AR-Vs may not be as important as that of AR-FL for their respective activity. This is because AR-FL is located in the cytoplasm in the absence of ligand and translocates to the nucleus and activates target-gene expression upon ligand binding, whereas constitutively-active AR-Vs localize to the nucleus and activate target-gene expression in the absence of ligand [8-12, 19]. Strikingly, higher expression of AR-V7 in hormone-naïve prostate tumors predicts increased risk of biochemical recurrence following radical prostatectomy [8, 9], and patients with high levels of expression of AR-V7 or detectable expression of AR[v567es] have a significantly shorter survival than other CRPC patients [17], indicating an association between AR-V expression and a more lethal form of prostate cancer. Collectively, the existing data support that AR-V proteins are expressed at a significant level in clinical specimens and should not be trivialized simply based on their relative low mRNA abundance.

Preclinical studies have pointed to an important role of AR-Vs in mediating castration resistance. Ectopic expression of AR-V7 or AR[v567es] confers castration-resistant growth of LNCaP xenograft tumors [9-11]. Conversely, knockdown of AR-V7 attenuates the growth of castration-resistant 22Rv1 xenograft tumors [9]. Targeted expression of AR[v567es] in prostate epithelium induces *de novo* prostate cancer development and promotes castration-resistant progression of the tumors in transgenic mice [20].

Although only prostatic intraepithelial neoplasia lesions are observed in AR-V7 transgenic mice, the majority of AR-V7-positive cells in castrated AR-V7 transgenic mice are ck5+/ck8+ intermediate cells, indicating a role of AR-V7 in maintaining or expanding prostate progenitor cell population during androgen deprivation [21].

AR-Vs have also been indicated to confer resistance to abiraterone and enzalutamide in preclinical studies. AR-Vs are increased in CRPC xenografts that recurred after abiraterone [22] or enzalutamide treatment (Cao *et al.*, manuscript under review). Knockdown of AR-Vs sensitizes 22Rv1 cells and NFκB p52-transfected LNCaP cells to enzalutamide inhibition of growth [23, 24]. Reducing AR-V levels with small-molecule drugs improves enzalutamide efficacy against the growth of 22Rv1 cells and xenografts [25]. Intriguingly, ectopic expression of AR-V7 in AR-FL-overexpressing LNCaP xenografts does not affect the growth inhibitory efficacy of enzalutamide [11]. A plausible explanation for the discrepancy is that, in the context of AR overexpression, the growth of LNCaP tumors may be driven mainly by the AR-FL signaling, making enzalutamide highly effective irrespective of AR-V expression. Nonetheless, our data showed that, when the ectopically-expressed AR-FL is lost in these xenografts, they can become resistant to enzalutamide, and the resistance is accompanied by increased expression of AR-Vs (Cao *et al.*, manuscript under review). Thus, prostate cancers may evade all androgen-directed therapies through shifting towards AR-V-mediated signaling.

Mechanisms of AR-V production

Two mechanisms have been proposed for AR-V production, AR gene rearrangement [26, 27] and increased pre-mRNA splicing [35]. Modeling gene rearrangement in prostate cancer cells showed expression of AR[v567es] without AR-FL in clonally selected cells [27]. While AR gene rearrangement could contribute to AR-V production in a subset of prostate cancers, AR-V production at the expense of AR-FL appears to be inconsistent with the tight correlation between AR-V and AR-FL mRNAs observed in individual clinical specimens and in xenograft [11, 17] or co-expression of AR-FL and AR-V7 in CRPC specimens, as indicated by overlapping AR-FL and AR-V7 immunohistochemistry staining of

adjacent tumor sections [13]. Moreover, while a clonal selection process is required for gene-rearrangement-mediated AR-V production to be manifested at the level of tumor tissues, change in AR-V levels in response to androgen deprivation was rather rapid in xenograft tumors [10, 11]. Further, different AR-Vs can be expressed in the same tissues. Clonal expansion of cells with one type of gene arrangement could lead to expression of one specific AR-V but may not be able to account for the expression of different AR-Vs. Compared to gene arrangement, increased splicing appears to be more generalizable. RNA splicing is closely coupled with gene transcription [36]. Androgen deprivation was shown to enhance the rate of AR-gene transcription and thereby indirectly contribute to increased AR pre-mRNA splicing to produce both AR-FL and AR-V7 [35]. A comprehension of the mechanisms of AR-V production is paramount for developing effective means to suppress AR-V expression.

Conclusion

AR signaling is active in CRPC although the cancer is no longer responsive to androgen deprivation therapy. LBD-truncated AR splice variants not only may play a role in maintaining the canonical AR transcriptome in a genuine ligand-independent manner, but may also regulate a unique subset of target genes. Accumulating clinical and preclinical data suggest that AR-Vs are critically involved in the treatment failure of first- and second-line hormonal therapies. Therefore, targeting the AR-Vs appears to an important concept and a fruitful direction of therapeutic development. To this end, several natural or synthetic compounds have been shown pre-clinically to inhibit AR-V actions [18, 29-34], and proof of efficacy in clinical trials is keenly awaited. Furthermore, the expression of constitutively active AR-Vs could serve as a prognostic and response biomarker to guide treatment decisions.

Acknowledgements

Address correspondence to: Haitao Zhang, 1430 Tulane Avenue SL-79, New Orleans, LA 70112. Tel: 504-988-7696; Fax: 504-988-7389; E-mail: hzhang@tulane.edu; Yan Dong, 1430 Tulane Avenue SL-49, New Orleans, LA 70112. Tel: 504-988-4761; Fax: 504-988-0468; E-mail: ydong@tulane.edu

References

[1] Egan A, Dong Y, Zhang H, Qi Y, Balk SP, Sartor O. Castration-resistant prostate cancer: Adaptive responses in the androgen axis. Cancer Treat Rev 2013 Sep 14; [Epub ahead of print]. Available at: http://www.sciencedirect.com/science/article/pii/S0305737213002004.

[2] Knudsen KE, Scher HI. Starving the addiction: new opportunities for durable suppression of AR signaling in prostate cancer. Clin Cancer Res 2009; 15: 4792-8.

[3] Pienta KJ, Bradley D. Mechanisms underlying the development of androgen-independent prostate cancer. Clin Cancer Res 2006; 12: 1665-71.

[4] Bennett NC, Gardiner RA, Hooper JD, Johnson DW, Gobe GC. Molecular cell biology of androgen receptor signalling. Int J Biochem Cell Biol 2010; 42: 813-27.

[5] de Bono JS, Logothetis CJ, Molina A, Fizazi K, North S, Chu L, Chi KN, Jones RJ, Goodman OB Jr, Saad F, Staffurth JN, Mainwaring P, Harland S, Flaig TW, Hutson TE, Cheng T, Patterson H, Hainsworth JD, Ryan CJ, Sternberg CN, Ellard SL, Fléchon A, Saleh M, Scholz M, Efstathiou E, Zivi A, Bianchini D, Loriot Y, Chieffo N, Kheoh T, Haqq CM, Scher HI; COU-AA-301 Investigators. Abiraterone and increased survival in metastatic prostate cancer. N Engl J Med 2011; 364: 1995-2005.

[6] Scher HI, Fizazi K, Saad F, Taplin ME, Sternberg CN, Miller K, de Wit R, Mulders P, Chi KN, Shore ND, Armstrong AJ, Flaig TW, Fléchon A, Mainwaring P, Fleming M, Hainsworth JD, Hirmand M, Selby B, Seely L, de Bono JS; AFFIRM Investigators. Increased survival with enzalutamide in prostate cancer after chemotherapy. N Engl J Med 2012; 367: 1187-97.

[7] 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.

[8] Hu R, Dunn TA, Wei S, Isharwal S, Veltri RW, Humphreys E, Han M, Partin AW, Vessella RL,

Isaacs WB, Bova GS, Luo J. Ligand-independent androgen receptor variants derived from splicing of cryptic exons signify hormone-refractory prostate cancer. Cancer Res 2009; 69: 16-22.

[9] Guo Z, Yang X, Sun F, Jiang R, Linn DE, Chen H, Chen H, Kong X, Melamed J, Tepper CG, Kung HJ, Brodie AM, Edwards J, Qiu Y. A novel androgen receptor splice variant is up-regulated during prostate cancer progression and promotes androgen depletion-resistant growth. Cancer Res 2009; 69: 2305-13.

[10] Sun S, Sprenger CC, Vessella RL, Haugk K, Soriano K, Mostaghel EA, Page ST, Coleman IM, Nguyen HM, Sun H, Nelson PS, Plymate SR. Castration resistance in human prostate cancer is conferred by a frequently occurring androgen receptor splice variant. J Clin Invest 2010; 120: 2715-30.

[11] Watson PA, Chen YF, Balbas MD, Wongvipat J, Socci ND, Viale A, Kim K, Sawyers CL. Constitutively active androgen receptor splice variants expressed in castration-resistant prostate cancer require full-length androgen receptor. Proc Natl Acad Sci U S A 2010; 107: 16759-65.

[12] Hu R, Isaacs WB, Luo J. A snapshot of the expression signature of androgen receptor splicing variants and their distinctive transcriptional activities. Prostate 2011; 71: 1656-67.

[13] Hu R, Lu C, Mostaghel EA, Yegnasubramanian S, Gurel M, Tannahill C, Edwards J, Isaacs WB, Nelson PS, Bluemn E, Plymate SR, Luo J. Distinct transcriptional programs mediated by the ligand-dependent full-length androgen receptor and its splice variants in castration-resistant prostate cancer. Cancer Res 2012; 72: 3457-62.

[14] Yang X, Guo Z, Sun F, Li W, Alfano A, Shimelis H, Chen M, Brodie AM, Chen H, Xiao Z, Veenstra TD, Qiu Y. Novel membrane-associated androgen receptor splice variant potentiates proliferative and survival responses in prostate cancer cells. J Biol Chem 2011; 286: 36152-60.

[15] Haile S, Sadar MD. Androgen receptor and its splice variants in prostate cancer. Cell Mol Life Sci 2011; 68: 3971-81.

[16] Zhang X, Morrissey C, Sun S, Ketchandji M, Nelson PS, True LD, Vakar-Lopez F, Vessella RL, Plymate SR. Androgen Receptor Variants Occur Frequently in Castration Resistant Prostate Cancer Metastases. PLoS One 2011; 6: e27970.

[17] Hörnberg E, Ylitalo EB, Crnalic S, Antti H, Stattin P, Widmark A, Bergh A, Wikström P. Expression of androgen receptor splice variants in prostate cancer bone metastases is associated with castration-resistance and short survival. PLoS One 2011; 6: e19059.

[18] Yamashita S, Lai KP, Chuang KL, Xu D, Miyamoto H, Tochigi T, Pang ST, Li L, Arai Y, Kung HJ, Yeh S, Chang C. ASC-J9 Suppresses Castration-Resistant Prostate Cancer Growth through Degradation of Full-length and Splice Variant Androgen Receptors. Neoplasia 2012; 14: 74-83.

[19] Chan SC, Li Y, Dehm SM. Androgen Receptor Splice Variants Activate Androgen Receptor Target Genes and Support Aberrant Prostate Cancer Cell Growth Independent of Canonical Androgen Receptor Nuclear Localization Signal. J Biol Chem 2012; 287: 19736-49.

[20] Liu G, Sprenger C, Sun S, Epilepsia KS, Haugk K, Zhang X, Coleman I, Nelson PS, Plymate S. AR variant ARv567es induces carcinogenesis in a novel transgenic mouse model of prostate cancer. Neoplasia 2013; 15: 1009-17.

[21] Sun F, Chen HG, Li W, Yang X, Wang X, Jiang R, Guo Z, Chen H, Huang J, Borowsky AD, Qiu Y. Androgen receptor splice variant AR3 promotes prostate cancer via modulating expression of autocrine/paracrine factors. J Biol Chem 2013 Dec 2; [Epub ahead of print].

[22] Mostaghel EA, Marck BT, Plymate SR, Vessella RL, Balk S, Matsumoto AM, Nelson PS, Montgomery RB. Resistance to CYP17A1 inhibition with abiraterone in castration resistant prostate cancer: Induction of steroidogenesis and androgen receptor splice variants. Clin Cancer Res 2011; 17: 5913-25.

[23] Li Y, Chan SC, Brand LJ, Hwang TH, Silverstein KAT, Dehm SM. Androgen Receptor Splice Variants Mediate Enzalutamide Resistance in Castration-Resistant Prostate Cancer Cell Lines. Cancer Res 2013; 73: 483-9.

[24] Nadiminty N, Tummala R, Liu C, Yang J, Lou W, Evans CP, Gao AC. NF-κB2/p52 Induces Resistance to Enzalutamide in Prostate Cancer: Role of Androgen Receptor and Its Variants. Mol Cancer Ther 2013; 12: 1629-37.

[25] Zhan Y, Cao B, Qi Y, Liu S, Zhang Q, Zhou W, Xu D, Lu H, Sartor O, Kong W, Zhang H, Dong Y. Methylselenol prodrug enhances MDV3100 efficacy for treatment of castration-resistant prostate cancer. Int J Cancer 2013; 133: 2225-33.

[26] Li Y, Hwang TH, Oseth LA, Hauge A, Vessella RL, Schmechel SC, Hirsch B, Beckman KB, Silverstein KA, Dehm SM. AR intragenic deletions linked to androgen receptor splice variant expression and activity in models of prostate cancer progression. Oncogene 2012; 31: 4759-67.

[27] Nyquist MD, Li Y, Hwang TH, Manlove LS, Vessella RL, Silverstein KA, Voytas DF, Dehm SM. TALEN-engineered AR gene rearrangements reveal endocrine uncoupling of androgen receptor in prostate cancer. Proc Natl Acad Sci U S A 2013; 110: 17492-7.

[28] Edwards J, Krishna NS, Grigor KM, Bartlett JMS. Androgen receptor gene amplification and protein expression in hormone refractory prostate cancer. Br J Cancer 2003; 89: 552-6.

[29] Andersen RJ, Mawji NR, Wang J, Wang G, Haile S, Myung JK, Watt K, Tam T, Yang YC, Bañuelos CA, Williams DE, McEwan IJ, Wang Y, Sadar MD. Regression of castrate-recurrent prostate cancer by a small-molecule inhibitor of the amino-terminus domain of the androgen receptor. Cancer Cell 2010; 17: 535-46.

[30] Li J, Cao B, Liu X, Fu X, Xiong Z, Chen L, Sartor O, Dong Y, Zhang H. Berberine suppresses androgen receptor signaling in prostate cancer. Mol Cancer Ther 2011; 10: 1346-56.

[31] Cao B, Liu X, Li J, Liu S, Qi Y, Xiong Z, Zhang A, Wiese T, Fu X, Gu J, Rennie PS, Sartor O, Lee BR, Ip C, Zhao L, Zhang H, Dong Y. 20(S)-protopanaxadiol-aglycone downregulation of the full-length and splice variants of androgen receptor. J Int Cancer 2013; 132: 1277-87.

[32] Li X, Liu Z, Xu X, Blair CA, Sun Z, Xie J, Lilly MB, Zi X. Kava components down-regulate expression of AR and AR splice variants and reduce growth in patient-derived prostate cancer xenografts in mice. PLoS One 2012; 7: e31213.

[33] Mashima T, Okabe S, Seimiya H. Pharmacological targeting of constitutively active truncated androgen receptor by nigericin and suppression of hormone-refractory prostate cancer cell growth. Mol Pharmacol 2010; 78: 46-54.

[34] Narizhneva NV, Tararova ND, Ryabokon P, Shyshynova I, Prokvolit A, Komarov PG, Purmal AA, Gudkov AV, Gurova KV. Small molecule screening reveals a transcription-independent prosurvival function of androgen receptor in castration-resistant prostate cancer. Cell Cycle 2009; 8: 4155-67.

[35] Liu LL, Xie N, Sun S, Plymate S, Mostaghel E and Dong X. Mechanisms of the androgen receptor splicing in prostate cancer cells. Oncogene 2013; [Epub ahead of print].

[36] Kornblihtt AR. Coupling transcription and alternative splicing. Adv Exp Med Biol 2007; 623: 175-89.

Androgen receptor splice variants activating the full-length receptor in mediating resistance to androgen-directed therapy

Bo Cao[1,2,*], Yanfeng Qi[1,*], Guanyi Zhang[2,3], Duo Xu[1,2], Yang Zhan[1], Xavier Alvarez[4], Zhiyong Guo[5], Xueqi Fu[2], Stephen R. Plymate[6,7], Oliver Sartor[8,9], Haitao Zhang[2,3], and Yan Dong[1,10]

[1] Department of Structural and Cellular Biology, Tulane University School of Medicine, Tulane Cancer Center, New Orleans, LA

[2] College of Life Sciences, Jilin University, China

[3] Department of Pathology and Laboratory Medicine, Tulane University School of Medicine, Tulane Cancer Center, New Orleans, LA

[4] Tulane National Primate Research Center, Covington, LA

[5] Department of Pharmacology, University of Maryland School of Medicine, Baltimore, MD

[6] Departments of Medicine, University of Washington School of Medicine, Seattle, WA

[7] Department of Urology, University of Washington School of Medicine, Seattle, WA

[8] Department of Urology, Tulane University School of Medicine, Tulane Cancer Center, New Orleans, LA

[9] Department of Medicine, Tulane University School of Medicine, Tulane Cancer Center, New Orleans, LA

[10] National Engineering Laboratory for AIDS Vaccine, College of Life Sciences, Jilin University, China

[*] These authors contributed equally; in alphabetical order

Correspondence to: Yan Dong, *email*: ydong@tulane.edu; Haitao Zhang, *email*: hzhang@tulane.edu

ABSTRACT:

Upregulation of constitutively-active androgen receptor splice variants (AR-Vs) has been implicated in AR-driven tumor progression in castration-resistant prostate cancer. To date, functional studies of AR-Vs have been focused mainly on their ability to regulate gene expression independent of the full-length AR (AR-FL). Here, we showed that AR-V7 and AR^{v567es}, two major AR-Vs, both facilitated AR-FL nuclear localization in the absence of androgen and mitigated the ability of the antiandrogen enzalutamide to inhibit AR-FL nuclear trafficking. AR-V bound to the promoter of its specific target without AR-FL, but co-occupied the promoter of canonical AR target with AR-FL in a mutually-dependent manner. AR-V expression attenuated both androgen and enzalutamide modulation of AR-FL activity/cell growth, and mitigated the *in vivo* antitumor efficacy of enzalutamide. Furthermore, AR^{v567es} levels were upregulated in xenograft tumors that had acquired enzalutamide resistance. Collectively, this study highlights a dual function of AR-Vs in mediating castration resistance. In addition to trans-activating target genes independent of AR-FL, AR-Vs can serve as a "rheostat" to control the degree of response of AR-FL to androgen-directed therapy via activating AR-FL in an androgen-independent manner. The findings shed new insights into the mechanisms of AR-V-mediated castration resistance and have significant therapeutic implications.

INTRODUCTION

Androgen deprivation therapy, which disrupts androgen receptor (AR) signaling through androgen ablation or AR antagonists, is the first-line treatment for disseminated prostate cancer. While this regimen is effective initially, progression to the presently incurable and lethal stage, termed castration-resistant prostate

cancer (CRPC), invariably occurs [1,2]. Resurgent AR activity is an established driver of therapeutic failure and castration-resistant progression [1,2]. A number of ligand-dependent and –independent mechanisms have been proposed to underlie AR reactivation after androgen-directed therapies [1,2]. For example, overexpression of the full-length AR (AR-FL) was shown to convert prostate cancer growth from a castration-sensitive to a castration-resistant stage [3]. In addition, CRPC tissues were shown to exhibit persistent levels of androgens despite androgen deprivation [1,2]. These led to the development of the potent AR antagonist enzalutamide (MDV3100) and the androgen biosynthesis inhibitor abiraterone for treatment of metastatic CRPC [4,5]. They heralded a new era of prostate cancer therapy. However, many patients presented with therapy-resistant disease, and most initial responders developed acquired resistance within months of therapy initiation, again accompanied by increased prostate-specific antigen (PSA), indicating reactivated AR signaling [4,5]. Emerging evidences indicate that prostate tumors can adapt to these androgen-directed therapies, including the new agents, by signaling through constitutively-active AR splice variants (AR-Vs) that lack the functional ligand-binding domain [6-16].

AR-Vs are upregulated in most CRPCs compared to hormone-naïve cancers [6,7,13-17]. Intriguingly, there is a significant discrepancy between the relative abundance of AR-V mRNAs and that of AR-V proteins in clinical specimens. While the level of AR-V mRNAs is low relative to that of the AR-FL, the AR-V proteins are expressed at a level comparable to that of AR-FL in a considerable portion of metastatic CRPC tissues [6,16]. In addition, the absolute levels of AR-Vs may not be as important as that of AR-FL for their respective activity. This is because AR-FL is located in the cytoplasm in the absence of ligand and translocates to the nucleus and activates target-gene expression upon ligand binding, whereas constitutively-active AR-Vs localize to the nucleus and activate target-gene expression in the absence of ligand [13-15,18-20]. AR-V7 (aka AR3) and AR[v567es] are two major AR-Vs expressed in clinical specimens [6,7,13-15]. Strikingly, patients with high levels of expression of AR-V7 or detectable expression of AR[v567es] have a significantly shorter survival than other CRPC patients [6], indicating an association between AR-V expression and a more lethal form of prostate cancer.

Preclinical studies have pointed to an important role of AR-Vs in mediating castration resistance. Ectopic expression of AR-V7 or AR[v567es] confers castration-resistant growth of LNCaP xenograft tumors [13,15,20]. Conversely, knockdown of AR-V7 attenuates the growth of castration-resistant 22Rv1 xenograft tumors [13]. AR-Vs have also been shown to confer resistance to enzalutamide in preclinical studies. Knockdown of AR-Vs sensitizes 22Rv1 cells and NFκB p52-transfected LNCaP cells to enzalutamide inhibition of growth [8,11]. Reducing AR-V levels with small-molecule drugs improves enzalutamide efficacy against the growth of 22Rv1 cells and xenografts [21]. Thus, AR-V upregulation appears to be a mechanism for prostate cancer cells to evade androgen-directed therapies. A comprehension of mechanisms of AR-V action is paramount for developing effective means to suppress AR-V signaling.

Gene expression profiling showed that AR-Vs regulate the expression of both canonical androgen-responsive genes and a distinct set of targets enriched for cell-cycle function [7,13,15]. The ability of AR-Vs to regulate target-gene expression has been attributed largely to their AR-FL-independent activity [7,8,12-15,19]. However, AR-FL and AR-V7 immunohistochemistry staining of adjacent sections of CRPC specimens showed that AR-V is often co-expressed with AR-FL [7]. We reason that, in addition to binding to chromatin sites and regulating gene expression independent of AR-FL, AR-Vs may bind to chromatin as a complex with AR-FL. Combined, these two activities may account for the expanded AR-V transcriptome. In fact, AR[v567es] has been shown to coimmunoprecipitate with AR-FL and facilitate AR-FL nuclear localization in the absence of androgen [15]. In the present study, we dissected the interplay between AR-Vs and AR-FL in regulating gene expression and mediating resistance to androgen-directed therapies.

RESULTS

AR-V mitigates enzalutamide inhibition of AR-FL nuclear localization

Both AR[v567es] and AR-V7 can reside constitutively in the nucleus [14,15,18], and AR[v567es] has been shown to facilitate AR-FL nuclear localization in the absence of androgen [15]. Enzalutamide is known to attenuate androgen-induced AR-FL nuclear localization in cells expressing AR-FL alone [22]. To assess the effect of AR-V7 on AR-FL subcellular localization and the impact of AR-Vs on enzalutamide modulation of AR-FL localization, we expressed AR-FL-green-fluorescent-protein (AR-FL-GFP) with or without AR-V7-turbo-red-fluorescent-protein (AR-V7-TurboFP) or AR[v567es]-TurboFP in the AR-null COS-7 cells. Consistent with previous reports [14,15,18], as shown in Figure 1A, both AR-Vs were found primarily in the nucleus, whereas AR-FL localized predominantly in the cytoplasm in androgen-deprived conditions. Enzalutamide caused ~50% reduction of androgen-induced AR-FL nuclear localization, but had no effect on AR-V localization or AR-FL localization in the absence of androgen.

When co-expressed with AR-V7 or AR[v567es] (Figure 1B), AR-FL could localize to the nucleus in the absence of androgen. The nuclear localization was unaffected by

enzalutamide. Strikingly, although addition of androgen further induced AR-FL nuclear localization, enzalutamide could not retain AR-FL in the cytoplasm when AR-V was present. Moreover, AR-V localization was not affected by androgen or enzalutamide even when co-expressed with AR-FL. A similar result was obtained in the PC-3 prostate cancer cells (Supplementary Figure 1). Taken together, the data suggest that AR-Vs facilitate AR-FL nuclear localization in the absence of androgen and mitigate the ability of enzalutamide to inhibit androgen-induced AR-FL nuclear localization.

AR-V and AR-FL co-occupy the target-gene promoter

Although AR-V-mediated AR-FL nuclear localization may not necessarily entail a physical interaction between AR-V and AR-FL, AR^{v567es} has been shown to coimmunoprecipitate with AR-FL, indicating AR-V can form a complex with AR-FL [15]. To find out whether they bind to target promoters as a complex, we performed sequential chromatin immunoprecipitation (Re-ChIP) analysis with an AR-V7 antibody followed by an AR-FL antibody in 22Rv1 cells, which express endogenous AR-V7 and are in part driven by AR-V7 [23]. We had to limit the analysis to AR-V7 because it is the only AR-V to which a specific antibody has been developed. As shown in Figure 2A, we detected co-occupancy of AR-V7 and AR-FL on the promoter of the PSA gene, and the co-occupancy was unaffected by androgen or enzalutamide treatment. In contrast, the promoter of ubiquitin-conjugating enzyme E2C (UBE2C) is only bound by AR-V7 (Figure 2A and 2B), and ChIP assay showed that AR-FL knockdown (shFL) did not significantly affect the binding (Figure 2B). This is consistent with UBE2C as an AR-V-specific target [6,7]. We then conducted a ChIP assay on the PSA promoter in 22Rv1 cells with or without specific knockdown of AR-FL or AR-V7 in androgen-deprived condition. As shown in Figure 2C, AR-FL knockdown diminished AR-V7 binding to the PSA promoter. Similarly, AR-V7 knockdown (shV7) reduced androgen-independent AR-FL binding to the promoter (Figure 2D). Collectively, the data indicate that,
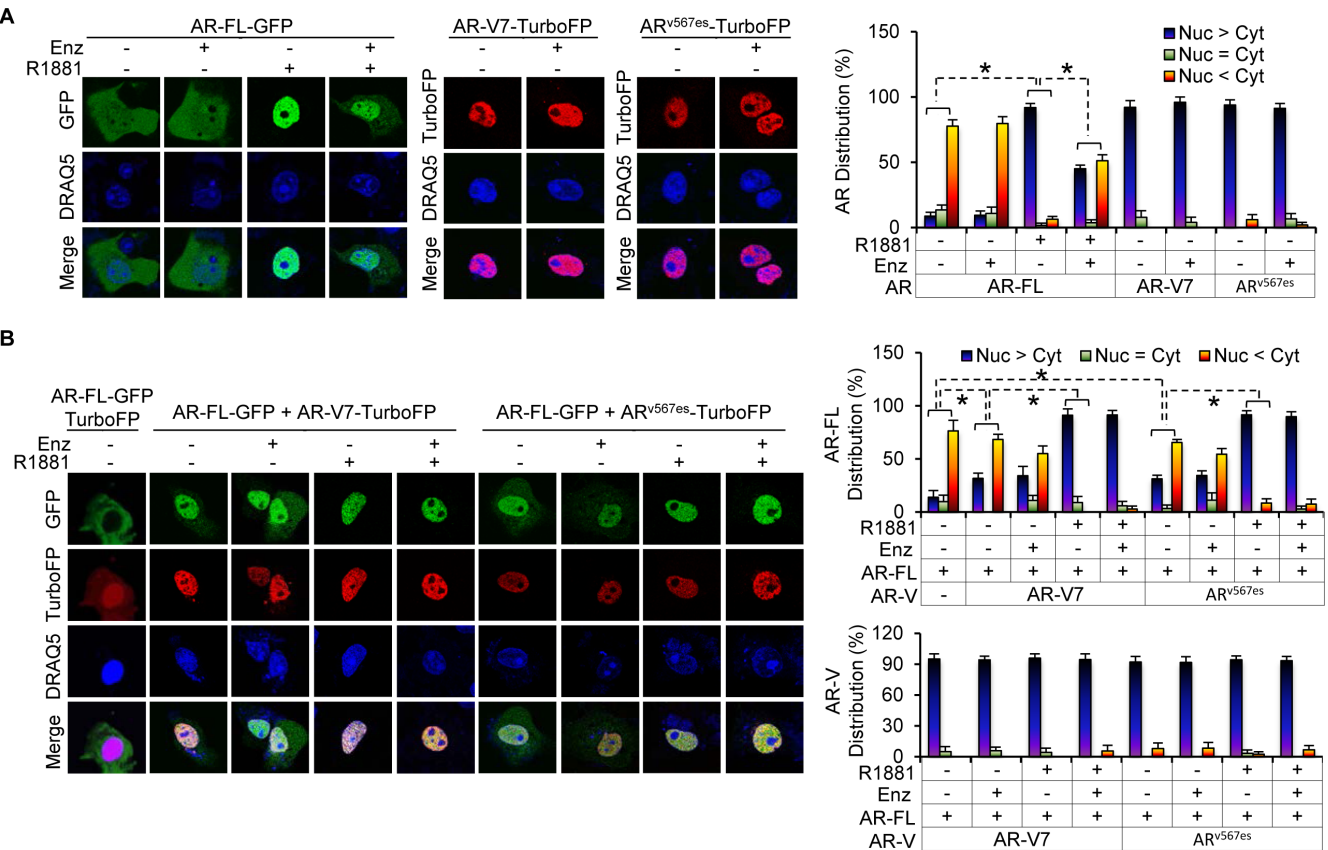
Figure 1: AR-V facilitates AR-FL nuclear localization in the absence of androgen and mitigates enzalutamide inhibition of androgen-induced AR-FL nuclear localization. A & B. Confocal fluorescence microscopy of AR-FL and AR-V subcellular localization when expressed alone (A) or when co-expressed with AR-V (B) in COS-7 cells. Right panels, quantitation of % of cells with predominantly nuclear, equally nuclear and cytoplasmic, or predominantly cytoplasmic expression. DRAQ5, nuclear stain. Cells cultured in androgen-deprived condition were pre-treated with 10 μM enzalutamide (Enz) for 2 hr, followed by treatment with or without 1 nM R1881 for 3 hr. *, $P < 0.05$.

in the absence of androgen, AR-V and AR-FL co-occupy the promoter of canonical androgen-responsive gene, but not AR-V-specific target, in a mutually-dependent manner.

AR-V attenuates androgen-induced AR-FL transactivation

To determine the impact of promoter co-occupancy on target gene expression, we measured the mRNA levels of both canonical androgen-responsive genes (PSA and TMPRSS2) and AR-V-specific targets (CCNA2 and UBE2C) in 22Rv1 cells in response to AR-FL or AR-V7 knockdown (Figure 3A). While knockdown of AR-FL and AR-V7 both reduced androgen-independent expression of PSA and TMPRSS2, only AR-V7 knockdown downregulated CCNA2 and UBE2C. Notably, although AR-V7 knockdown diminished basal PSA and TMPRSS2 levels, the levels after androgen stimulation were essentially the same in control and AR-V7-knockdown cells. AR-V7 knockdown thus led to a higher magnitude of androgen induction of PSA (2.7-fold vs. 1.7-fold) and TMPRSS2 (2.6-fold vs. 1.4-fold), and enzalutamide was very effective in blocking the induction. Conversely, ectopic expression of AR-V7 or AR^{v567es} in LNCaP cells dose-dependently induced basal PSA and TMPRSS2

expression and diminished the degree of response of PSA and TMPRSS2 to androgen (Figure 3B and Supplementary Figure 2). Taken together, the data indicate that, in addition to *trans*-activating a distinct set of genes, AR-Vs activate AR-FL in an androgen-independent manner to induce the expression of their shared targets. In doing so, AR-Vs could serve as "rheostats" to control the degree of response of AR-FL to androgen and to androgen-directed therapy. Interestingly, while ectopic co-expression of AR-V7 or AR^{v567es} rendered enzalutamide ineffective against androgen-induced AR-FL nuclear localization (Figure 1B), the presence of AR-V7 did not affect the ability of enzalutamide to inhibit androgen-dependent expression of PSA and TMPRSS2 (Figure 3A and Supplementary Figure 2). Collectively, these results suggest that AR-Vs could facilitate the nuclear localization of AR-FL in the presence of enzalutamide, but are unable to overcome the suppression of ligand-activated AR-FL transactivation by enzalutamide.

AR-V mitigates androgen and enzalutamide modulation of cell growth

We proceeded to characterize the effect of AR-V7 knockdown on androgen and enzalutamide modulation
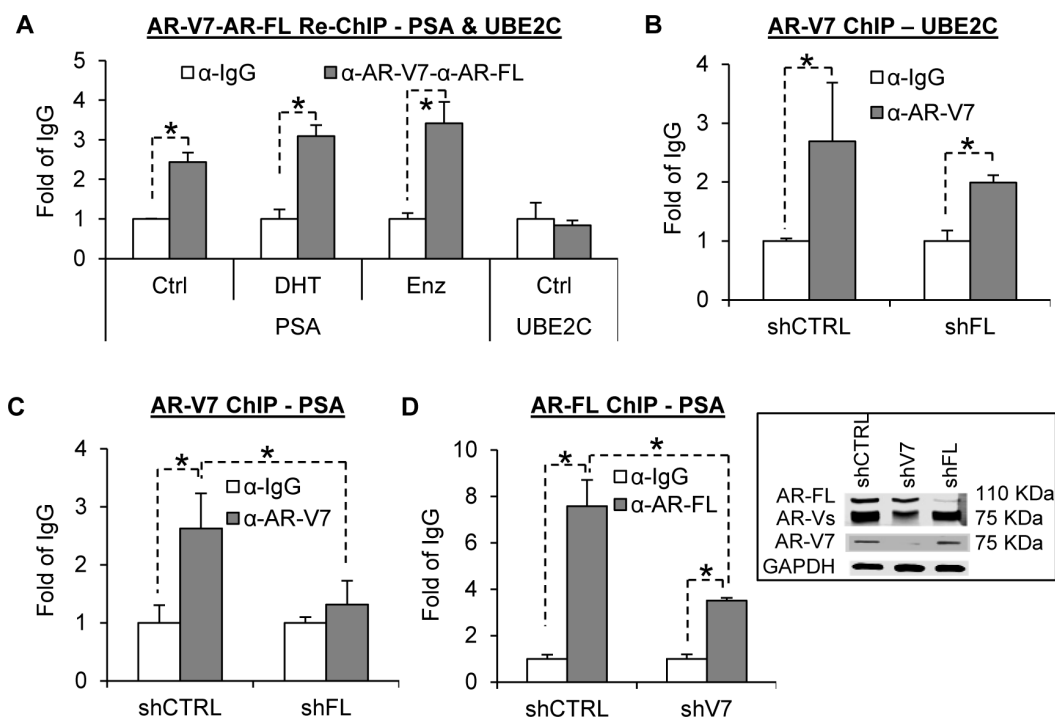
Figure 2: AR-V7 and AR-FL co-occupy the PSA, but not UBE2C, promoter in a mutually dependent manner. A. Sequential ChIP analysis in 22Rv1 cells with an AR-V7 antibody followed by an AR-FL antibody showing co-occupancy of the PSA, but not UBE2C, promoter by AR-V7 and AR-FL. Enzalutamide (Enz), 10 μM. DHT, 1 nM. B. AR-V7 ChIP analysis in 22Rv1 cells showing AR-V7 binding to the UBE2C promoter. C. AR-V7 ChIP analysis in 22Rv1 cells showing AR-FL knockdown diminishes AR-V7 binding to the PSA promoter. D. AR-FL ChIP analysis in 22Rv1 cells showing AR-V7 knockdown reduces AR-FL binding to the PSA promoter. The values of the IgG samples are set as 1, and the ChIP results are presented as relative fold of IgG. *, $P < 0.05$. Western blots showed the knockdown efficacy of AR-FL and AR-V7.

of the growth of 22Rv1 cells. Congruent with the mRNA data, after AR-V7 knockdown, the cells became more sensitive to DHT induction of growth (Figure 4A; ~2-fold in AR-V7-knockdown cells *vs.* 1.3-fold in control cells). Consequently, the knockdown cells were more responsive to enzalutamide growth inhibition than the control cells. We next inoculated AR-V7-knockdown cells or control cells in nude mice, and characterized the response of the ensuing tumors to enzalutamide. As shown in Figure 4B, growth inhibition by enzalutamide was more pronounced after AR-V7 knockdown (the tumor growth curves are presented in Supplementary Figure 3). Collectively, the data suggest that AR-V may contribute to enzalutamide resistance by dampening the response of the cells to androgen induction of growth.

Increased AR-Vs in tumors that had developed acquired resistance to enzalutamide

Enzalutamide has been demonstrated to be very effective against the growth of castration-resistant AR-FL-overexpressing LNCaP xenografts [22]. As shown in Figure 5A, we observed the same phenomenon in xenografts established by inoculating LNCaP cells

that were transduced with wild-type-AR-FL-encoding lentivirus into castrated nude mice. Some tumors resumed growth with prolonged treatment (after 7-17 weeks) (Figure 5B). We serially passaged the relapsed Tumor #1 and #2 (Figure 5B) in castrated mice treated with enzalutamide, and considered tumors from the second to fourth passages as enzalutamide resistant. RNA-seq analysis of four enzalutamide-sensitive tumors and six enzalutamide-resistant tumors showed that none of the tumors carried the AR F876L missense mutation (Figure 5C), which was identified in enzalutamide-resistant LNCaP cells and shown to confer agonist activity to enzalutamide [24-26]. Instead, the transcripts of AR^{v567es} and AR-V7 (trending toward significance) were upregulated in enzalutamide-resistant tumors, while the levels of AR-V4 or AR-FL transcript did not differ (Figure 6A-D). The upregulation of AR-V was also reflected at the protein level (Figure 6E). Interestingly, all the enzalutamide-resistant tumors that showed higher AR-V protein expression also express increased levels of glucocorticoid receptor (Supplementary Figure 4), the upregulation of which has been shown to be a mechanism of acquired resistance to enzalutamide [27]. The data indicate that these tumors may use multiple mechanisms to evade enzalutamide treatment.
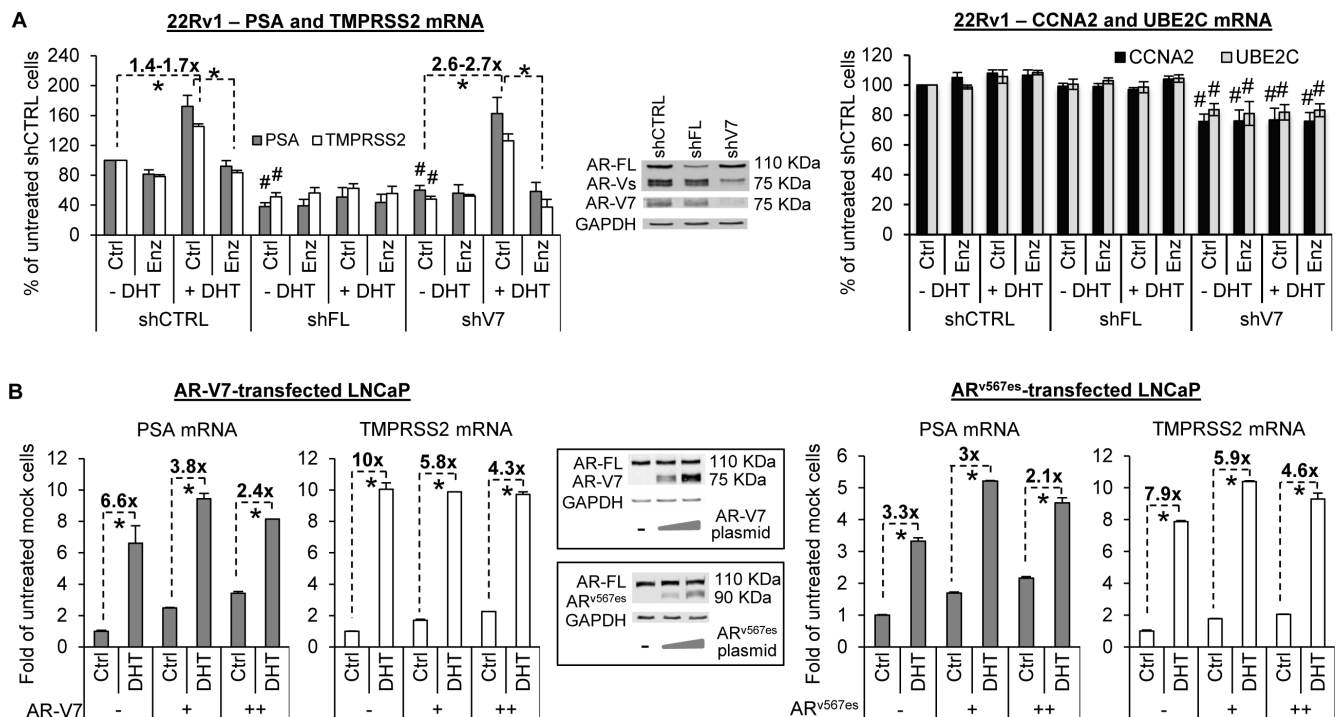
Figure 3: AR-V attenuates androgen and enzalutamide modulation of AR-target expression. A. qRT-PCR analysis showing reduced androgen-independent expression of PSA and TMPRSS2 after knockdown of either AR-FL or AR-V7 (left panel) and reduced expression of CCNA2 and UBE2C only after AR-V7 knockdown (right panel). AR-V7 knockdown also renders 22Rv1 cells more sensitive to DHT and enzalutamide modulation of PSA and TMPRSS2 expression. B. qRT-PCR analysis showing that AR-V transfection dose-dependently attenuates DHT induction of PSA and TMPRSS2 in LNCaP cells. Treatment duration, 8 hr (A); 4 hr (B). Enzalutamide (Enz), 10 μM. DHT, 1 nM. *, $P < 0.05$. #, $P < 0.05$ from untreated control-shRNA cells.
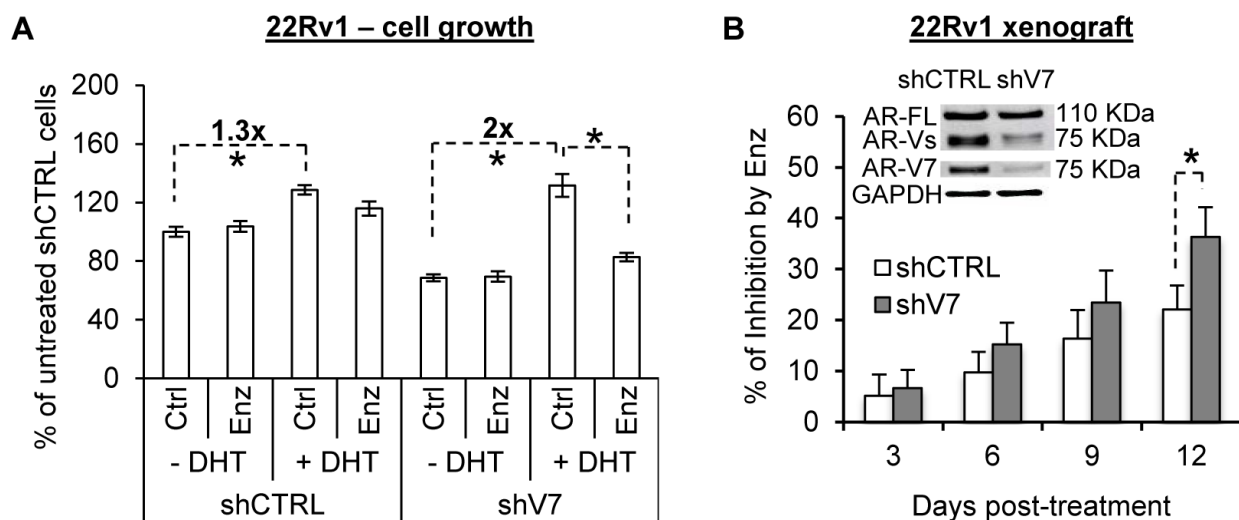
Figure 4: AR-V attenuates androgen and enzalutamide modulation of cell growth. A. AR-V7 knockdown enhances the response of 22Rv1 cells to androgen and enzalutamide modulation of cell growth. B. Enzalutamide inhibition of 22Rv1 tumor growth becomes more pronounced after AR-V7 knockdown. Data are expressed as % of inhibition by enzalutamide. *, $P < 0.05$. Enzalutamide (Enz), 10 mg/kg/day. n = 8.
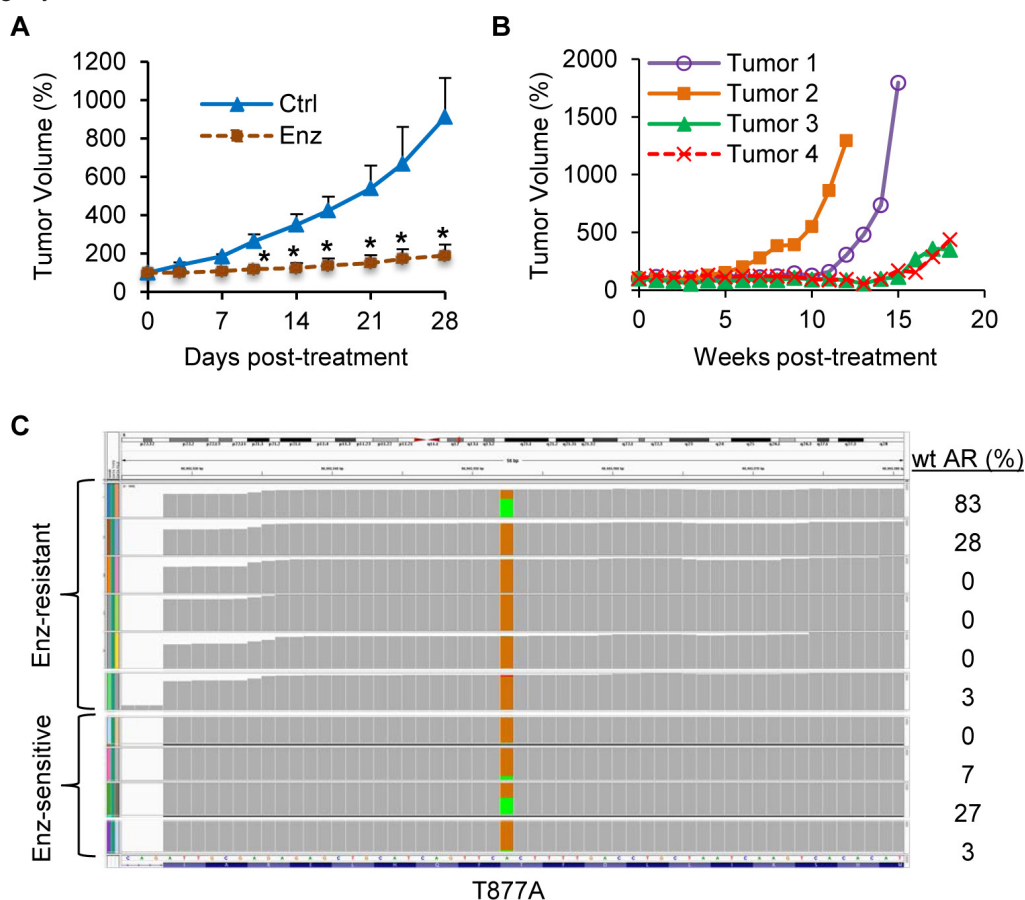

Figure 5: Absence of AR F876L mutation in LNCaP tumors that have developed acquired resistance to enzalutamide. A. Enzalutamide (Enz) inhibits the growth of castration-resistant LNCaP tumors initially. LNCaP cells were transduced with lentivirus encoding wild-type (wt) AR-FL before inoculated into castrated mice. *, $P < 0.05$ from the control group. n = 5. B. LNCaP tumors resume growth after 7-17 weeks of enzalutamide treatment. The mean tumor volumes were presented as % of original tumor size at Day 0 of treatment. C. Integrative Genomics Viewer (IGV) plot of RNA-seq data showing no detection of F876L mutation in the AR gene in enzalutamide-sensitive and –resistant LNCaP tumors. The brown boxes represent the relative frequencies of T877A-mutated AR that is present in the LNCaP tumors. The relative frequencies of the transduced wt AR remained in the tumors are denoted by the green boxes and tabled on the right. Allele frequency threshold was set at 0.01.

DISCUSSION

To date, the ability of AR-Vs to contribute to castration resistance has been attributed largely to their AR-FL-independent constitutive activity in regulating gene expression. Here, we identified what we believe to be a novel mechanism of AR-V action. We showed that AR-V7 and AR^{v567es}, two major AR-Vs, not only facilitate AR-FL nuclear localization in the absence of androgen but also mitigate the ability of the antiandrogen enzalutamide to inhibit androgen-induced AR-FL nuclear localization. In the nucleus, AR-V7 binds to the promoter of its specific target without AR-FL, but co-occupies the promoter of canonical androgen-responsive gene with AR-FL in a mutually-dependent manner. The co-occupancy is not affected by androgen or enzalutamide. Concordantly, knockdown of AR-FL and AR-V7 both result in reduced androgen-independent expression of canonical androgen-responsive genes, but only AR-V7 knockdown downregulates AR-V-specific targets. Notably, although basal levels of canonical androgen-responsive genes are diminished after AR-V7 knockdown, or elevated after AR-V7 or AR^{v567es} overexpression, the levels after androgen stimulation are unaffected. Thus, AR-Vs appear to repress the degree of response of AR-

FL to androgen by activating AR-FL to induce target expression in an androgen-independent manner. This is further supported by the improved sensitivity of the cells to androgen induction of cell growth and enzalutamide inhibition of cell growth after AR-V7 knockdown. These collective findings suggest that, in addition to AR-FL-independent constitutive transactivation, AR-Vs may serve as "rheostats" to control the degree of response of AR-FL to androgen and to androgen-directed therapy.

In the present study, we also showed that enzalutamide becomes more potent in thwarting the growth of 22Rv1 xenograft tumors after AR-V7 knockdown, indicating that targeting both AR-Vs and AR-FL is needed to achieve complete AR blockade. While corroborating the *in vitro* observations from Li *et al*. [8] and Nadiminty *et al*. [11], the data contrast the finding from Watson *et al*. that ectopic expression of AR-V7 in AR-FL-overexpressing LNCaP xenograft tumors does not affect the growth inhibitory efficacy of enzalutamide [20]. A plausible explanation for the discrepancy is that, in the context of AR overexpression, the growth of LNCaP tumors may be driven mainly by the AR-FL signaling, making enzalutamide highly effective irrespective of AR-V expression. Nonetheless, we showed that, when the ectopically-expressed AR-FL is lost in these tumors, they can become resistant to enzalutamide. The resistance is
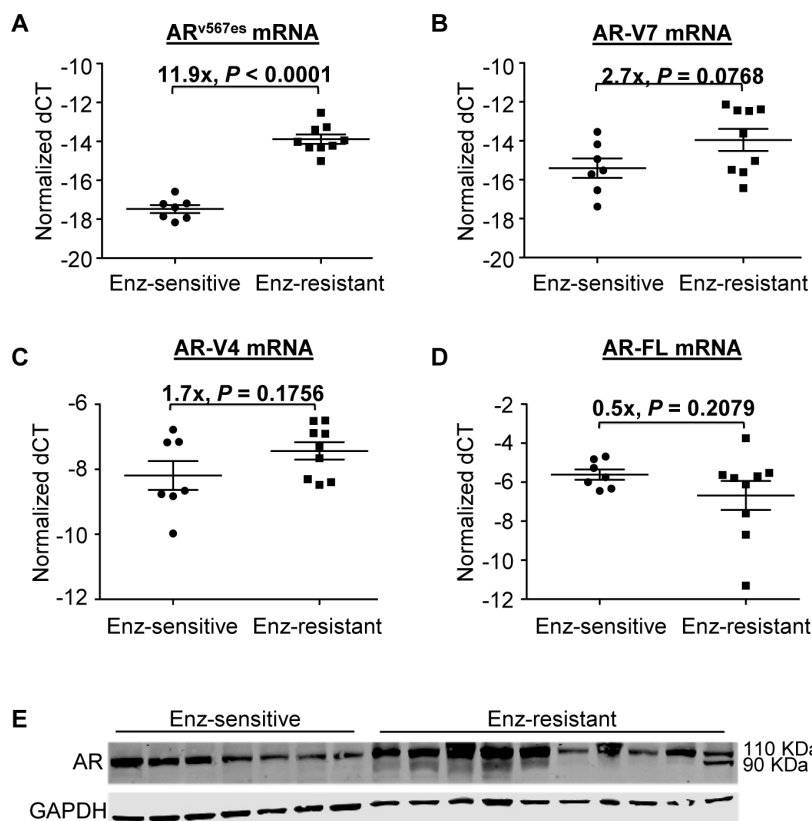
Figure 6: Increased AR-V expression in LNCaP tumors that have developed acquired resistance to enzalutamide.
A-D. qRT-PCR analysis of the levels of AR-V transcripts. Fold changes are calculated from the difference in mean ΔC_T between the enzalutamide-sensitive and enzalutamide-resistant groups ($2^{\Delta\Delta CT}$). E. Western blot analysis of the levels of AR-FL and AR-V proteins.

accompanied by increased expression of AR^{v567es}. Thus, these tumors may also evade enzalutamide treatment through shifting towards AR-V-mediated signaling.

The significance of our finding that AR-Vs activate AR-FL to induce target-gene expression in an androgen-independent manner is based on the premise that AR-Vs and AR-FL are often co-expressed in biological contexts. This is supported by overlapping AR-FL and AR-V7 immunohistochemistry staining of adjacent sections of CRPC specimens [7]. This is also supported by the finding that androgen deprivation coordinately increases AR-FL and AR-V mRNAs by inducing the transcription of the AR gene and thereby increasing the recruitment of splicing factors to AR pre-mRNA to splice both AR-FL and AR-V mRNAs [9]. AR-V expression may also be a result of AR gene rearrangements [28,29], and gene-arrangement-caused AR-V production appears to occur at the expense of AR-FL [29]. However, a clonal selection process is required for gene-rearrangement-mediated AR-V production to be manifested at the level of tumor tissues. This appears to be in contrast to the rather rapid change of AR-V levels observed in xenograft tumors after androgen ablation or androgen replacement [15,20]. Further, different AR-Vs can be expressed in the same tissues. Clonal expansion of cells with one type of gene arrangement could lead to expression of one specific AR-V but may not be able to account for the expression of different AR-Vs. Finally, our data showing co-occupancy of AR-V7 and AR-FL on the PSA promoter in a mutually-dependent manner and increased response of AR-FL to androgen after AR-V7 knockdown provided further support to the co-expression of AR-FL and AR-V in the same cells. Thus, the ability of AR-Vs to activate AR-FL in an androgen-independent manner could be as important as their AR-FL-independent *trans*-activating activity in mediating castration resistance.

Our finding of AR-V and AR-FL co-regulating the expression of canonical androgen-responsive genes in androgen-deprived condition is reminiscent of the transcriptome data from Hu *et al*. that knockout of AR-FL in AR-V-transfected LNCaP cells almost completely abolishes the expression of at least a subset of canonical androgen-responsive genes [7]. In addition to regulating canonical androgen-responsive genes, AR-Vs have also been shown to regulate a distinct set of targets enriched for cell-cycle function [6,7,13]. This is further corroborated by our ChIP data showing the promoter of UBE2C is bound by AR-V7 but not AR-FL. Receptor dimerization is a crucial step of AR-FL activation [30]. AR^{v567es} has been shown to co-immunoprecipitate with AR-FL [15]. Here, we showed that AR-V7 and AR-FL co-reside on the promoter of their shared target. AR-V7 and AR^{v567es} can localize constitutively to the nucleus, and facilitate AR-FL nuclear localization in the absence of androgen. It is therefore possible that AR-V7 and AR^{v567es} dimerize with AR-FL in the cytoplasm in an androgen-independent

manner, and the heterodimer translocates to the nucleus and binds to regulatory elements of their shared targets to regulate the transcription of these targets. It remains unknown as to whether dimerization is required for AR-Vs to regulate their specific targets. Future studies are needed to define the dimeric nature of AR-Vs in regulating gene expression.

In summary, our study provides further evidence to support AR-V upregulation as a means for prostate cancer cells to evade all androgen-directed therapies currently accepted in the clinic. Mechanistically, we identified a novel mechanism by which AR-Vs mediate castration-resistant progression. We showed that AR-Vs can activate AR-FL to induce target expression in an androgen-independent manner. By doing so, AR-Vs may serve as "rheostats" to control the degree of response of AR-FL to androgen and to androgen-directed therapy. Since AR-Vs are often co-expressed with AR-FL in biological contexts, this mechanism of AR-V action may be equally important as its AR-FL-independent activity to castration resistance. These findings underscore a critical need to develop effective means to target both AR-Vs and AR-FL to achieve complete AR blockade for more effective combat of these clinically challenging tumors. Several natural or synthetic compounds have been shown pre-clinically to inhibit AR-V and AR-FL actions [17,21,31-35]. Proof of efficacy in clinical trials is keenly awaited.

METHODS

Cell Lines and Reagents

LNCaP, 22Rv1, COS-7, and PC-3 cells were obtained from American Type Culture Collection at Passage 4. Cells used in this study were within 20 passages (~3 months of non-continuous culturing). All cell lines were tested and authenticated by the method of short tandem repeat profiling. Enzalutamide was purchased from Selleck Chemicals (Houston, TX), and the purity of >99% was confirmed by Nuclear Magnetic Resonance. The following antibodies were used in Western blot analysis: anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Millipore), anti-AR (N-terminus-directed; PG-21, Millipore), and anti-AR-V7 (Precision Antibody). Cell growth was determined by the Sulforhodamine assay.

Subcellular Localization

AR subcellular localization is detected by confocal fluorescence microscopy. The pTurboFP-AR-V7 and pTurboFP-AR^{v567es} plasmids were generated by cloning the cDNA fragments for AR-V7 and AR^{v567es}, respectively, into the pCMV-TurboFP635 vector. COS-7 or PC-3 cells were transfected with indicated plasmids and cultured in phenol

red-free RPMI-1640 supplemented with 10% charcoal-stripped fetal bovine serum. At 40 hr after transfection, cells were pre-treated with or without 10 μM enzalutamide for 2 hr, followed by treatment with or without 1 nM R1881 for 3 hr. The COS-7 cells were then fixed with 2% paraformaldehyde, and the nuclei stained with 2.5 μM DRAQ5 (Cell Signaling). The PC-3 cells were then fixed with 70% ethanol, and the nuclei stained with DAPI. Confocal images were obtained by using a Leica TCS SP2 system with a 63X oil-immersion objective on a Z-stage, and an average of 6 fields with ~10 cells per field was captured for each group. Data quantitation was performed as described [18].

qRT-PCR

qRT-PCR was performed as described [36]. The qPCR primer-probe sets for PSA, transmembrane protease, serine 2 (TMPRSS2), cyclin A2 (CCNA2), and UBE2C were from IDT. The primer sequences for AR isoforms were as described [13].

ChIP and Re-ChIP

ChIP and Re-ChIP were performed as described [37]. The following antibodies were used: mouse IgG2a (ab18413, abcam), rabbit IgG (ab46540, abcam), AR-FL-specific antibody (C-terminus-directed; C-19, sc-815 x, Santa Cruz Biotech), AR-V7-specific antibody (AG10008, Precision Antibody). The PSA promoter P2-ARE primers described by Guo *et al*. [13] and the UBE2C promoter primers described by Wang *et al*. [38] were used for qPCR analysis of ChIP or re-ChIP DNA. The RPL30 exon 3 control region (Cell Signaling) was used as a negative control.

Tumor Xenografts

Xenograft studies were conducted essentially as described [22,32]. LNCaP cells (4×10^6) infected with lentivirus encoding AR-FL or 22Rv1 cells infected with lentivirus encoding control shRNA or AR-V7 shRNA were inoculated into castrated or intact nude mice (Charles River), respectively. The cells were mixed with 50% Matrigel and inoculated subcutaneously on the right dorsal flank. Tumor volume was calculated as *0.524 x width² x length* [39]. When the tumor size reached ~100 mm³, the mice were randomized to daily treatment with vehicle or 10 mg/kg/day enzalutamide through oral gavage as described [22].

For the development of enzalutamide-resistant tumors, two LNCaP tumors that relapsed after enzalutamide treatment were resected, and ~20 mm³ pieces of the tumors were transplanted into castrated nude mice.

When the tumor bits grew to 100~200 mm³, the mice started to receive 10 mg/kg/day enzalutamide through oral gavage. The tumors were harvested when they reached ~800 mm³ and serially passaged in castrated nude mice following the same protocol. The second to fourth passages of tumors were considered as enzalutamide-resistant. All animal procedures were approved by the Tulane University Institutional Animal Care and Use Committee.

Statistical Analysis

The *Student's* two-tailed t test was used to determine the mean differences between two groups. $P < 0.05$ is considered significant. Data are presented as mean ± SEM.

Editorial note:

This paper has been accepted based in part on peer-review conducted by another journal and the authors' response and revisions as well as expedited peer-review in Oncotarget

REFERENCES

1. Egan A, Dong Y, Zhang H, Qi Y, Balk SP, Sartor O. Castration-resistant prostate cancer: Adaptive responses in the androgen axis. Cancer Treat.Rev. 2014; 40: 426-33.

2. Knudsen KE, Scher HI. Starving the addiction: new opportunities for durable suppression of AR signaling in prostate cancer. Clin.Cancer Res. 2009; 15: 4792-8.

3. Chen CD, Welsbie DS, Tran C, Baek SH, Chen R, Vessella R, Rosenfeld MG, Sawyers CL. Molecular determinants of resistance to antiandrogen therapy. Nat.Med. 2004; 10: 33-9.

4. Fizazi K, Scher HI, Molina A, Logothetis CJ, Chi KN, Jones RJ, Staffurth JN, North S, Vogelzang NJ, Saad F, Mainwaring P, Harland S, Goodman OB, Jr., Sternberg CN, Li JH, Kheoh T et al. Abiraterone acetate for treatment of

metastatic castration-resistant prostate cancer: final overall survival analysis of the COU-AA-301 randomised, double-blind, placebo-controlled phase 3 study. Lancet Oncol. 2012; 13: 983-92.

5. Scher HI, Fizazi K, Saad F, Taplin ME, Sternberg CN, Miller K, de WR, Mulders P, Chi KN, Shore ND, Armstrong AJ, Flaig TW, Flechon A, Mainwaring P, Fleming M, Hainsworth JD et al. Increased survival with enzalutamide in prostate cancer after chemotherapy. N.Engl.J.Med. 2012; 367: 1187-97.

6. Hornberg E, Ylitalo EB, Crnalic S, Antti H, Stattin P, Widmark A, Bergh A, Wikstrom P. Expression of androgen receptor splice variants in prostate cancer bone metastases is associated with castration-resistance and short survival. PLoS One. 2011; 6: e19059.

7. Hu R, Lu C, Mostaghel EA, Yegnasubramanian S, Gurel M, Tannahill C, Edwards J, Isaacs WB, Nelson PS, Bluemn E, Plymate SR, Luo J. Distinct transcriptional programs mediated by the ligand-dependent full-length androgen receptor and its splice variants in castration-resistant prostate cancer. Cancer Res. 2012; 72: 3457-62.

8. Li Y, Chan SC, Brand LJ, Hwang TH, Silverstein KA, Dehm SM. Androgen receptor splice variants mediate enzalutamide resistance in castration-resistant prostate cancer cell lines. Cancer Res. 2012; 73: 483-9.

9. Liu LL, Xie N, Sun S, Plymate S, Mostaghel E, Dong X. Mechanisms of the androgen receptor splicing in prostate cancer cells. Oncogene. 2013 Jul 15; [Epub ahead of print].

10. Mostaghel EA, Marck BT, Plymate SR, Vessella RL, Balk S, Matsumoto AM, Nelson PS, Montgomery RB. Resistance to CYP17A1 Inhibition with Abiraterone in Castration-Resistant Prostate Cancer: Induction of Steroidogenesis and Androgen Receptor Splice Variants. Clinical Cancer Research. 2011; 17: 5913-25.

11. Nadiminty N, Tummala R, Liu C, Yang J, Lou W, Evans CP, Gao AC. NF-kappaB2/p52 Induces Resistance to Enzalutamide in Prostate Cancer: Role of Androgen Receptor and Its Variants. Mol.Cancer Ther. 2013; 12: 1629-37.

12. 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.

13. Guo Z, Yang X, Sun F, Jiang R, Linn DE, Chen H, Chen H, Kong X, Melamed J, Tepper CG, Kung HJ, Brodie AM, Edwards J, Qiu Y. A novel androgen receptor splice variant is up-regulated during prostate cancer progression and promotes androgen depletion-resistant growth. Cancer Res. 2009; 69: 2305-13.

14. Hu R, Dunn TA, Wei S, Isharwal S, Veltri RW, Humphreys E, Han M, Partin AW, Vessella RL, Isaacs WB, Bova GS, Luo J. Ligand-independent androgen receptor variants derived from splicing of cryptic exons signify hormone-refractory prostate cancer. Cancer Res. 2009; 69: 16-22.

15. Sun S, Sprenger CC, Vessella RL, Haugk K, Soriano K, Mostaghel EA, Page ST, Coleman IM, Nguyen HM, Sun H, Nelson PS, Plymate SR. Castration resistance in human prostate cancer is conferred by a frequently occurring androgen receptor splice variant. J.Clin.Invest. 2010; 120: 2715-30.

16. Zhang X, Morrissey C, Sun S, Ketchandji M, Nelson PS, True LD, Vakar-Lopez F, Vessella RL, Plymate SR. Androgen receptor variants occur frequently in castration resistant prostate cancer metastases. PLoS One. 2011; 6: e27970.

17. Yamashita S, Lai KP, Chuang KL, Xu D, Miyamoto H, Tochigi T, Pang ST, Li L, Arai Y, Kung HJ, Yeh S, Chang C. ASC-J9 suppresses castration-resistant prostate cancer growth through degradation of full-length and splice variant androgen receptors. Neoplasia. 2012; 14: 74-83.

18. Chan SC, Li Y, Dehm SM. Androgen receptor splice variants activate androgen receptor target genes and support aberrant prostate cancer cell growth independent of canonical androgen receptor nuclear localization signal. J.Biol.Chem. 2012; 287: 19736-49.

19. Hu R, Isaacs WB, Luo J. A snapshot of the expression signature of androgen receptor splicing variants and their distinctive transcriptional activities. Prostate. 2011; 71: 1656-67.

20. Watson PA, Chen YF, Balbas MD, Wongvipat J, Socci ND, Viale A, Kim K, Sawyers CL. Constitutively active androgen receptor splice variants expressed in castration-resistant prostate cancer require full-length androgen receptor. Proc.Natl.Acad.Sci.U.S.A. 2010; 107: 16759-65.

21. Zhan Y, Cao B, Qi Y, Liu S, Zhang Q, Zhou W, Xu D, Lu H, Sartor O, Kong W, Zhang H, Dong Y. Methylselenol prodrug enhances MDV3100 efficacy for treatment of castration-resistant prostate cancer. Int.J.Cancer. 2013; 133: 2225-33.

22. Tran C, Ouk S, Clegg NJ, Chen Y, Watson PA, Arora V, Wongvipat J, Smith-Jones PM, Yoo D, Kwon A, Wasielewska T, Welsbie D, Chen CD, Higano CS, Beer TM, Hung DT et al. Development of a second-generation antiandrogen for treatment of advanced prostate cancer. Science. 2009; 324: 787-90.

23. Guo Z, Qiu Y. A new trick of an old molecule: androgen receptor splice variants taking the stage?! Int.J.Biol.Sci. 2011; 7: 815-22.

24. Balbas MD, Evans MJ, Hosfield DJ, Wongvipat J, Arora VK, Watson PA, Chen Y, Greene GL, Shen Y, Sawyers CL. Overcoming mutation-based resistance to antiandrogens with rational drug design. Elife. 2013; 2: e00499.

25. Korpal M, Korn JM, Gao X, Rakiec DP, Ruddy DA, Doshi S, Yuan J, Kovats SG, Kim S, Cooke VG, Monahan JE, Stegmeier F, Roberts TM, Sellers WR, Zhou W, Zhu P. An F876L Mutation in Androgen Receptor Confers Genetic and Phenotypic Resistance to MDV3100 (Enzalutamide). Cancer Discov. 2013; 3: 1030-43.

26. Joseph JD, Lu N, Qian J, Sensintaffar J, Shao G, Brigham D, Moon M, Maneval EC, Chen I, Darimont B, Hager JH. A Clinically Relevant Androgen Receptor Mutation Confers Resistance to Second-Generation Antiandrogens Enzalutamide and ARN-509. Cancer Discov. 2013; 3: 1020-9.

27. Arora VK, Schenkein E, Murali R, Subudhi SK, Wongvipat J, Balbas MD, Shah N, Cai L, Efstathiou E, Logothetis C, Zheng D, Sawyers CL. Glucocorticoid receptor confers resistance to antiandrogens by bypassing androgen receptor blockade. Cell. 2013; 155: 1309-22.

28. Li Y, Hwang TH, Oseth LA, Hauge A, Vessella RL, Schmechel SC, Hirsch B, Beckman KB, Silverstein KA, Dehm SM. AR intragenic deletions linked to androgen receptor splice variant expression and activity in models of prostate cancer progression. Oncogene. 2012; 31: 4759-67.

29. Nyquist MD, Li Y, Hwang TH, Manlove LS, Vessella RL, Silverstein KA, Voytas DF, Dehm SM. TALEN-engineered AR gene rearrangements reveal endocrine uncoupling of androgen receptor in prostate cancer. Proc.Natl.Acad. Sci.U.S.A. 2013; 110: 17492-7.

30. Centenera MM, Harris JM, Tilley WD, Butler LM. Minireview: The Contribution of Different Androgen Receptor Domains to Receptor Dimerization and Signaling. Mol Endocrinol. 2008; 22: 2373-82.

31. Andersen RJ, Mawji NR, Wang J, Wang G, Haile S, Myung JK, Watt K, Tam T, Yang YC, Banuelos CA, Williams DE, McEwan IJ, Wang Y, Sadar MD. Regression of Castrate-Recurrent Prostate Cancer by a Small-Molecule Inhibitor of the Amino-Terminus Domain of the Androgen Receptor. Cancer Cell. 2010; 17: 535-46.

32. Cao B, Liu X, Li J, Liu S, Qi Y, Xiong Z, Zhang A, Wiese T, Fu X, Gu J, Rennie PS, Sartor O, Lee BR, Ip C, Zhao L, Zhang H et al. 20(S)-protopanaxadiol-aglycone downregulation of the full-length and splice variants of androgen receptor. Int.J.Cancer. 2013; 132: 1277-87.

33. Li J, Cao B, Liu X, Fu X, Xiong Z, Chen L, Sartor O, Dong Y, Zhang H. Berberine Suppresses Androgen Receptor Signaling in Prostate Cancer. Molecular Cancer Therapeutics. 2011; 10: 1346-56.

34. Li X, Liu Z, Xu X, Blair CA, Sun Z, Xie J, Lilly MB, Zi X. Kava components down-regulate expression of AR and AR splice variants and reduce growth in patient-derived prostate cancer xenografts in mice. PLoS One. 2012; 7: e31213.

35. Mashima T, Okabe S, Seimiya H. Pharmacological targeting of constitutively active truncated androgen receptor by nigericin and suppression of hormone-refractory prostate cancer cell growth. Mol.Pharmacol. 2010; 78: 846-54.

36. Dong Y, Lee SO, Zhang H, Marshall J, Gao AC, Ip C. Prostate specific antigen expression is down-regulated by selenium through disruption of androgen receptor signaling. Cancer Res. 2004; 64: 19-22.

37. Furlan-Magaril M, Rincón-Arano Hc, Recillas-Targa F. Sequential Chromatin Immunoprecipitation Protocol: ChIP-reChIP. DNA-Protein Interactions (543 edition) 2008: 253-66.

38. Wang Q, Li W, Zhang Y, Yuan X, Xu K, Yu J, Chen Z, Beroukhim R, Wang H, Lupien M, Wu T, Regan MM, Meyer CA, Carroll JS, Manrai AK, Janne OA et al. Androgen receptor regulates a distinct transcription program in androgen-independent prostate cancer. Cell. 2009; 138: 245-56.

39. Gleave ME, Hsieh JT, Wu HC, von Eschenbach AC, Chung LW. Serum prostate specific antigen levels in mice bearing human prostate LNCaP tumors are determined by tumor volume and endocrine and growth factors. Cancer Res. 1992; 52: 1598-605.