Rapid Induction of Androgen Receptor Splice Variants by Androgen Deprivation in Prostate Cancer

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Running Title: Androgen repression of AR splice variant
Statement of Translational Relevance

Previous studies have indicated that restoration of androgen receptor (AR) transcriptional activity in prostate cancer (PCa) that relapses after castration (castration-resistant prostate cancer, CRPC) or after subsequent therapy with abiraterone, a CYP17A1 inhibitor that further suppresses androgen synthesis, may be mediated by abiraterone-resistant intratumoral androgen synthesis or by constitutively active AR splice variants lacking the ligand binding domain. We show that AR reactivation in abiraterone-resistant VCaP xenografts is not associated with restoration of intratumoral androgens. Moreover, we find that increases in the major AR splice variant (AR-V7) occur rapidly through a feedback mechanism and can mediate low basal AR activity immediately after androgen-deprivation, but cannot mediate the high-level AR activity in relapsed tumors. These results indicate that agents targeting AR splice variants may be most effective when used very early in conjunction with therapies targeting the AR ligand binding domain, prior to the emergence of additional resistance mechanisms.
Abstract

**Purpose:** Mechanisms mediating androgen receptor (AR) reactivation in prostate cancer (PCa) that progresses after castration (castration-resistant prostate cancer, CRPC) and subsequent treatment with abiraterone (CYP17A1 inhibitor that further suppresses androgen synthesis) remain unclear.

**Experimental Design:** PCa xenografts were examined to identify mechanism of progression after castration and abiraterone.

**Results:** AR reactivation in abiraterone-resistant VCaP xenografts was not associated with restoration of intratumoral androgens or alterations in AR co-regulators. In contrast, mRNA encoding full length AR (AR-FL) and a constitutively active splice variant (AR-V7) were increased compared to xenografts prior to castration, with an increase in AR-V7 relative to AR-FL. This shift towards AR-V7 was due to a feedback mechanism whereby the androgen-liganded AR stimulates expression of proteins that suppress generation of AR-V7 relative to AR-FL transcripts. However, despite the increases in AR-V7 mRNA, it remained a minor transcript (<1%) relative to AR-FL in resistant VCaP xenografts and CRPC clinical samples. AR-V7 protein expression was similarly low relative to AR-FL in castration-resistant VCaP xenografts and androgen-deprived VCaP cells, but the weak basal AR activity in these latter cells was further repressed by AR-V7 siRNA.

**Conclusions:** AR-V7 at these low levels is not adequate to restore AR activity, but its rapid induction after androgen deprivation allows tumors to retain basal AR activity that may be needed for survival until more potent mechanisms emerge to activate AR. Agents targeting AR splice variants may be most effective when used very early in conjunction with therapies targeting the AR ligand binding domain.
Introduction

Blockade of testicular androgen production by surgical or medical castration (androgen deprivation therapy) is a standard treatment for metastatic prostate cancer (PCa), but tumors invariably relapse and progress into a stage termed castration-resistant prostate cancer (CRPC). One mechanism driving these resistant tumors is intratumoral synthesis of androgens (testosterone and dihydrotestosterone, DHT) from precursor steroids produced by the adrenal glands or de novo from cholesterol (1-6). Synthesis of these precursor steroids is dependent on the enzyme CYP17A1, and a specific inhibitor of this enzyme (abiraterone) was recently approved for treatment of CRPC, but most men who initially respond will relapse within one to two years (6-9). These relapses are generally associated with increases in serum prostate-specific antigen (PSA), suggesting that androgen receptor (AR) activity has again been restored. However, the mechanisms mediating this AR activity and the role of AR in resistance to CYP17A1 inhibitor therapy remain unclear (1, 10, 11).

The human VCaP PCa cell xenograft expresses the androgen regulated TMPRSS2:ERG fusion gene and has been used as a model for progression to CRPC after castration (12, 13). We recently reported that castration-resistant VCaP xenografts initially respond to abiraterone, but relapse within 1-2 months (2). Consistent with findings in patients, these abiraterone-relapsed xenografts expressed high levels of several AR regulated genes, indicating restoration of AR transcriptional activity. These relapsed tumors also had increased expression of CYP17A1 mRNA, suggesting restoration of androgen synthesis as a possible resistance mechanism (2). Recent findings in other xenograft models have similarly suggested that androgen synthesis may mediate resistance in some cases (10), and have identified expression of alternatively spliced AR isoforms as another potential resistance mechanism (10, 14-20). In this study we assess the contribution of intratumoral androgen synthesis versus alternative mechanisms, including expression of alternatively spliced AR isoforms, in progression to abiraterone-resistance.

Materials and Methods

Small interfering RNA (siRNA) and transfection analysis

The siRNAs specific for full-length AR (siExon 7, siEX7) and for AR-V7 (siCryptic Exon 3, siCE3) were
described previously (17). The siRNA targeting AR Exon 1 was described previously (21). Transfection of siRNA was performed using Lipofectamine RNAiMax (Invitrogen, Carlsbad, CA) in OptiMEM according to the manufacturer’s protocol. The final siRNA concentration was 20 nM. A scrambled non-targeting control siRNA (Qiagen, Valencia, CA) was employed as a negative control. Sixteen hours later, transfection medium was replaced with medium containing 5% charcoal-dextran stripped serum (CSS). Another 24 hours later, transfected cells were stimulated with dihydrotestosterone (DHT) at 10 nM or vehicle (ethanol) for 16 h.

**Immunoblot and steroid analyses**

Whole cell lysates (WCL) were prepared using lysis buffer containing 2% SDS and subjected to immunoblotting. The antibodies against human AR (N20 and C19) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies against AR-V7 were from Precision Antibody (Columbia, MD). Antibodies against β-actin (AC-15) and β-tubulin were from Millipore (Billerica, MA). The results from a minimum of three experiments were subjected to densitometry and normalized to β-actin or β-tubulin loading control and the mean values relative to control empty vector (EV) cells (set to 1.0) given. AR immunoblots were further quantified by comparison with blots containing serial dilutions of AR protein. Steroid extractions from xenografts and mass spectrometry were performed as described previously (5).

**RNA Sequencing (RNA-Seq)**

Total cellular RNA was extracted and purified from tissues using the RNeasy Mini Kit (Qiagen). 1 µg of RNA was treated with DNase in-solution (Qiagen) and purified with the RNeasy MinElute Cleanup Kit (Qiagen). DNA-free RNA was then depleted of ribosomal RNA using the Ribo-Zero rRNA Removal Kit (Epicentre). The remaining fraction of RNA was prepared into an indexed, strand-specific library using the Script-Seq v2 RNA-Seq Library Preparation Kit (Epicentre), pooled, and then clustered and sequenced on a Hi-Seq 2000 (Illumina) with 100-base paired-end reads (100×100) and 7 indexing cycles. Demultiplexed FASTQ files were aligned to the human genome and genetic features were quantified with the RNA-seq Unified Mapper.
Data were visualized using the Integrative Genome Viewer (22). Data are submitted (SRP019503).

**Reverse Transcriptase (RT)-PCR analysis**

RNA was isolated using RNeasy Mini Kit (Invitrogen). Superscript III reverse transcriptase (Invitrogen) was used for reverse transcription with 500 ng RNA in the presence of 100 ng of random primers (Invitrogen). For conventional PCR, the primers for *AR-V7* were described previously (17), *GAPDH* primers were as follows: Forward: 5’-tcaccatcttcaggag-3’, Reverse: 5’-gcctcaccaccttcttg-3’. For real-time quantitative PCR (qRT-PCR), the *AR-V7* TaqMan primers and probe were as follows: Forward: 5’-cggaatgttatgaaggagtga-3’, reverse: 5’-ctggctattttgagatgcttgcaat-3’, probe: 5’-FAM-ggagaaaaattccgggt-3’. The specific TaqMan primer-probe sets for *AR-FL*, *PSA*, *FKBP5*, *TMPRSS2-ERG*, *PLZF*, and *GAPDH* were as described previously (2, 21). *KLK2* and *NKX3.1* primer and probe sets were purchased from Applied Biosystems. qRT-PCR was performed in an ABI7900 thermal cycler.

**Results**

**Expression of AR-stimulated genes in abiraterone-resistant VCaP xenografts**

We previously reported a castration-resistant VCaP xenograft model that responds initially to abiraterone and then relapses after approximately 6 weeks of abiraterone treatment (2, 12). It should be noted that the abiraterone response in these castration-resistant xenografts primarily reflects blockade of de novo androgen synthesis by intratumoral CYP17A1, as the murine adrenal gland does not synthesize the substantial levels of androgen precursors that are produced in humans and are hence a major target of CYP17A1 inhibitors in men with CRPC. AR activity, based on expression of a small panel of AR regulated genes, was initially markedly repressed by abiraterone and appeared to be restored in these abiraterone-resistant xenografts. To more comprehensively assess AR activity in these xenografts, we used Affymetrix oligonucleotide microarrays to compare expression of AR regulated genes in biopsies from CRPC xenografts prior to starting abiraterone and at relapse. This analysis showed that expression of multiple well-recognized
AR stimulated genes, including ERG from the TMPRSS2:ERG fusion gene and the recently reported AR and ERG dependent oncogene SOX9 (23), was not significantly higher in the tumors prior to therapy and at relapse, supporting the conclusion that AR transcriptional activity was restored (Figure 1A).

To more systematically identify alterations in the spectrum of AR regulated genes, we also examined expression of all genes shown previously to be induced at least 2-fold by androgen in VCaP cells (21). Figure 1B shows a plot of their fold induction by DHT in VCaP cells versus the ratio of their expression in the xenografts prior to abiraterone and at relapse. These results show that expression of androgen-stimulated genes, whether they are weakly or strongly androgen-induced, was broadly restored in the abiraterone-relapsed tumors, with no significant trend towards lower expression in the relapsed tumors.

**Androgen synthesis in abiraterone-resistant VCaP xenografts**

Consistent with our previous report, expression of CYP17A1 and AKR1C3 were increased in three of the four abiraterone-relapsed xenografts, and there were variable changes in other androgen synthetic enzymes (Figure 1C). To determine whether restoration of intratumoral androgen synthesis may be mediating relapses, we performed mass spectrometry to examine intratumoral androgens in biopsies from these xenografts prior to starting abiraterone and at relapse. Significantly, we could readily detect DHT, testosterone and androstenedione in the relapsed tumors. However, in all cases their levels were markedly lower than those in biopsies from the matched tumors before starting abiraterone treatment (Figure 1D). These findings are consistent with recent clinical studies showing sustained suppression of testosterone in both blood and bone marrow in patients relapsing after abiraterone treatment (24). Therefore, while AR activity may still be dependent on residual androgen synthesis, it appeared that additional mechanisms must also be driving AR activity at low androgen levels.

**Genes and pathways altered in abiraterone-relapsed xenografts**

We next did an unbiased analysis and identified all genes with significant (p<0.05) changes in expression in the abiraterone-resistant xenografts versus the matched pre-treatment xenografts. A total of 181
genes were upregulated in abiraterone-resistant tumors and 132 genes were downregulated (Figure 2A, B, and Supplementary Table 1). Amongst the 30 most upregulated and downregulated genes, only GATA2 has been shown to associate with AR and may contribute to enhancing AR activity, and we did not observe consistent increases in any established AR coactivator proteins or decreases in AR corepressors (Figure 2A, B). Gene ontology analysis using the Database for Annotation, Visualization and Integrated Discovery (DAVID) system showed only weak enrichment for genes associated with histone acetylation amongst the genes that were upregulated in the abiraterone-resistant xenografts (Figure 2C). In contrast, the downregulated genes were most strongly associated with mitosis (Figure 2D). This latter finding indicated that while the relapsed tumors were growing, the abiraterone was still slowing their proliferative rate relative to the CRPC tumors prior to starting abiraterone.

We reported previously that AR functions directly as a transcriptional repressor on a subset of genes, including the AR gene itself and multiple genes involved in nucleotide and DNA synthesis (21). Interestingly, 10 of the 30 most upregulated genes in the abiraterone-resistant xenografts were genes we found previously to be AR-repressed in VCaP or VCaP derived CRPC cells (Figure 2A, in bold and colored red). Microarray analyses also showed increases in many additional AR-repressed genes in the abiraterone-resistant xenografts (Figure 2E). Taken together, these findings indicate that the transcriptional activation functions of AR were largely restored in these abiraterone-resistant xenografts, but that its function as a transcriptional repressor (which require somewhat higher androgen levels) were diminished. As the predominant functions of AR-repressed genes are related to nucleotide and DNA synthesis (21), their increased expression may be contributing to proliferation in the abiraterone-relapsed xenografts.

**Structural alterations in AR in abiraterone-resistant xenografts**

VCaP cells have an amplified AR that is wild-type (25), and Sanger sequencing of AR from the abiraterone-relapsed xenografts did not reveal any AR mutations (not shown). To identify mutations that may be present in a subset of resistant cells or in only one of the amplified AR genes, and therefore present in a minority of AR transcripts, we also performed RNA-seq on three abiraterone resistant xenografts. However,
this analysis only identified AR mutations at low frequencies that were not shared amongst the xenografts and were of unclear functional significance (Supplementary Table 3; RNA-seq data is available in the NCBI Sequence Real Archive: SRP019503).

Previous studies have shown that AR can undergo alternative splicing from exon 3 in the DNA binding domain to cryptic exons in intron 3 or to exon 8 (14-20). These AR isoforms lack the carboxyl-terminal (C-terminal) ligand binding domain (LBD) and can have ligand-independent constitutive activity that may mediate resistance to androgen-deprivation therapy and to AR antagonists that target the LBD. Using RT-PCR with primer sets specific for AR-V1 to V7 and V12 in the VCaP xenografts, the only variant we could consistently detect was AR-V7 (not shown). Consistent with this result, using paired-end RNA-seq in 3 abiraterone-resistant VCaP xenografts, the only AR variant we detected was AR-V7. Relative to transcripts containing exon 3 spliced to exon 4, the abundance of transcripts containing exon 3 spliced to the V7 cryptic exon was 1.0% (1/99), 0.9% (48/5323), and 0.2% (5/2223).

As shown in Figure 3A, AR-V7 was readily detectable and consistently increased in the abiraterone-resistant xenografts relative to levels in biopsies from the matched CRPC xenografts prior to starting abiraterone. AR-FL was also increased, but the increase in AR-V7 expression appeared to be greater. This was confirmed by qRT-PCR for AR-V7 versus full length AR (AR-FL) in the pre- and post-abiraterone treated tumors (Figure 3B). We further assessed expression of AR-V7 and AR-FL in a series of previously described VCaP xenografts that were biopsied prior to castration (androgen-dependent, AD), at 4 days post-castration (CS), or at relapse after castration (CR) (12). AR-V7 expression was increased at 4 days, was further increased in the relapsed castration-resistant xenografts, and these fold-increases in AR-V7 were greater than those of AR-FL (Figure 3C). As summarized in Figure 3D, the mean increase of AR-V7 expression was 53-fold during the development of castration-resistance, while AR-FL was increased to a lesser extent (10-fold). Similarly, AR-V7 was increased ~3-fold in abiraterone-resistant xenografts relative to levels in castration-resistant xenografts prior to abiraterone, while AR-FL was less increased (1.4-fold).

Androgen preferentially suppresses expression of AR-V7 versus AR-FL
We reported previously that AR gene transcription is rapidly repressed by the androgen-ligated AR through AR binding to a site in intron 2 of the AR gene (21). Therefore, we next addressed whether the increase in AR-V7 may reflect this feedback mechanism, versus selection for subpopulations of cells expressing higher AR-V7. Consistent with our previous results, VCaP cells cultured in steroid-depleted medium expressed high levels of AR-FL mRNA that were substantially decreased after 24 hours treatment with DHT (Figure 4A, right panel). AR-FL mRNA was similarly decreased by DHT in VCS2 cells, which were derived from a castration-resistant VCaP xenograft (2). Significantly, DHT treatment caused an even greater decrease in the levels of AR-V7 in both the VCaP and VCS2 cells (~20 and ~80-fold in VCaP and VCS2 cells, respectively) (Figure 4A, left panel). AR-V7 is also expressed in high passage LNCaP cells (LN-HP) and in the LNCaP derived C4-2 line. Although its levels are lower than in VCaP, DHT in both of these lines similarly decreased AR-V7 expression to a greater extent than AR-FL (Figure 4B).

Examining a DHT dose response in VCaP cells, we found that AR-V7 was decreased by ~80% at 0.1 nM DHT versus ~60% for AR-FL, and that AR-V7 was further markedly decreased by >95% at 1-10 nM DHT versus ~80% for AR-FL (Figure 4C). To confirm that the effects of DHT were mediated by the AR-FL, we used an siRNA targeting exon 7 (which is not present in AR-V7) to selectively deplete the full length AR. As expected, the siEX7 markedly decreased AR-FL, but not AR-V7 (Figure 4D). Moreover, depletion of the full length AR by siEX7 prevented the DHT-mediated decrease in AR-V7. To determine whether DHT may be preferentially enhancing degradation of the AR-V7 transcript, we assessed AR-V7 and AR-FL mRNA levels after treatment with actinomycin D to block new mRNA synthesis. Consistent with our previous results (21), DHT did not increase degradation of AR-FL mRNA (Figure 4E, lower panel). Significantly, DHT similarly did not increase degradation of the AR-V7 transcript, which instead appeared to be somewhat more stable in the presence of DHT (Figure 4E, upper panel). These results indicate that increased mRNA degradation does not account for the relative decrease in AR-V7 versus AR-FL in response to DHT.

Interestingly, examination of AR-FL versus AR-V7 transcripts over a 24 hour time-course showed that both declined similarly in response to DHT for ~8 hours, and that there was further loss primarily of AR-V7 between 8-24 hours (Figure 4F, DHT+DMSO). Treatment with DHT and cycloheximide (CHX), which blocks
new protein synthesis, abrogated the decline in AR-V7 at 24 hours (Figure 4F, DHT+CHX), indicating that an androgen-stimulated increased in the synthesis of one or more proteins mediates the preferential decline in AR-V7 versus AR-FL mRNAs. These may be splicing factors, but also may be proteins that enhance RNA II polymerase elongation and thereby prevent stalling and premature chain termination in intron 3. In any case, our overall conclusion from these data is that the increased expression of AR-V7 in the castration-resistant and abiraterone-resistant xenografts reflects a feedback mechanism that rapidly increases AR-V7 relative to AR-FL at low androgen levels, rather than selective pressure for subsets of cells expressing higher AR-V7.

AR-V7 contribution to AR activity after androgen-deprivation

AR-V7 has been detected in PCa cell lines as well as in clinical samples, and higher AR-V7 staining has been associated with progression to CRPC (16-19, 26-28). Functional analyses based on ectopic expression demonstrated that AR-V7 is constitutively active and can induce CRPC growth (16, 29). However, the contribution of endogenous AR-V7, which appears to be expressed at low levels relative to AR-FL, to AR activity remains to be clarified. As noted above, despite the marked increases in AR-V7 mRNA in the castration-resistant VCaP xenografts, and further increases in the abiraterone-resistant xenografts, RNA-seq indicated that AR-V7 mRNA in the abiraterone-resistant xenografts was still only a small fraction (<1%) of total AR mRNA. However, as AR-V7 protein could be higher than suggested by the mRNA levels, we next assessed AR-V7 protein. Immunoblotting of proteins extracted from biopsies of androgen-dependent (AD) VCaP xenografts and the matched castration-resistant (CR) VCaP xenografts showed only a very minor band migrating at the predicted position of AR-V7, consistent with the low mRNA levels (Figure 5A). Quantitative analysis of the AR-V7 and AR-FL bands indicated that AR-V7 protein was expressed at ~1.0 – 1.5% of the AR-FL levels in castration-resistant xenografts, which was at least 10-fold higher than the AR-V7 to AR-FL ratio in the androgen-dependent xenografts.

Immunoblotting of VCaP cells cultured in vitro in androgen depleted medium similarly showed low levels of a protein that migrated at the predicted position of AR-V7 (Figure 5B). Consistent with this protein being AR-V7, it was not detected by an antibody directed against the AR C-terminus (AR-C), and its...
expression was markedly decreased by treatment with DHT (Figure 5B). To confirm VCaP expression of AR-V7 protein, VCaP cells in androgen depleted medium were treated with siRNA targeting the cryptic exon 3 (siCE3). This markedly decreased the faster migrating AR band without decreasing full length AR (Figure 5B). In contrast, both the AR-FL and AR-V7 bands were decreased by siRNA targeting exon 1 (siEX1). Finally, immunoblotting with an AR-V7 specific antibody further supported the conclusion that the lower AR band was AR-V7 (Figure 5B, AR-V7). The AR-V7 and AR-FL bands were quantified and AR-V7 versus AR-FL ratio was ~1.6% in the negative control siRNA (siCtrl), and was markedly decreased by addition of DHT (siCtrl+DHT).

Overall these results indicate that AR-V7 protein, while increased after androgen deprivation, is still expressed at low levels compared to AR-FL, and that this is consistent with the low levels of AR-V7 mRNA.

Culturing VCaP cells in steroid depleted medium markedly reduces their AR transcriptional activity compared to androgen stimulated VCaP cells, but they still retain basal AR activity that can be further reduced by blocking de novo androgen synthesis with abiraterone or other agents (2). To assess whether AR-V7 contributes to this basal AR transcriptional activity, we transfected VCaP cells in steroid depleted with siRNA targeting AR-V7 (siCE3). Compared to the control siRNA (siCtrl), depletion of AR-V7 decreased mRNA for a series of AR regulated genes by ~30% (Figure 5C, left panel). An siRNA targeting full length AR (siEX7) decreased these AR regulated genes to a similar degree (Figure 5C, middle panel). Expression of these genes was not substantially further decreased by transfecting the cells with an siRNA targeting exon 1 (siEX1) to knockdown both AR-FL and AR-V7, which may reflect an inability to adequately downregulate AR in a subset of the cells Figure 5C, right panel). In contrast, when we used enzalutamide to block activation of the AR-FL by residual androgens, we found that expression of AR regulated genes could be further reduced by knockdown of AR-V7 (Figure 5D). Taken together these findings indicate that the rapid induction of AR-V7 protein, although still expressed at low levels, can contribute to maintaining a low basal level of AR transcriptional activity immediately after androgen deprivation therapy. However, these findings further indicate that AR-V7 expressed at these levels is not a major contributor to the high-level AR activity observed in castration-resistant or abiraterone-relapsed tumors.
**AR-V7 mRNA expression in CRPC clinical samples**

We previously analyzed RNA from a series of CRPC bone marrow metastases and showed that AR regulated genes were highly expressed, although their levels were not fully restored to those in primary prostate cancers prior to castration (6). To determine the potential contribution of AR-V7 to this AR activity, we used qRT-PCR to assess expression of AR-V7 and AR-FL in these clinical samples relative to VCaP cells. Significantly, AR-V7 mRNA in some clinical samples was markedly higher than in androgen-deprived VCaP cells, suggesting that it could be making a more substantial contribution to AR activity in some tumors (Figure 6, upper panel, all levels are normalized to the level in androgen-deprived VCaP cells). However, these samples also had corresponding increases in AR-FL, indicating that the increase in AR-V7 may reflect primarily an increase in overall AR gene transcription rather than a marked shift in splicing towards AR-V7 (Figure 6, lower panel). However, it should be noted that the therapies in these patients did not include abiraterone, suggesting substantial intratumoral androgens may be suppressing generation of AR-V7, and that ongoing studies of patients relapsing on abiraterone or enzalutamide may reveal higher levels of AR-V7 or other AR splice variants relative to AR-FL.

**Discussion**

Intratumoral androgen synthesis is now well established as a mechanism that contributes to AR reactivation after castration, but its role in resistance to CYP17A1 inhibitors or AR antagonists is not clear. Results in this study show that intratumoral levels of androstenedione, testosterone, and DHT are not restored in abiraterone-resistant VCaP xenografts. A recent study similarly found that androgen levels remained low in bone marrow aspirates from patients with abiraterone-resistant tumors (24). Importantly, while these findings indicate that full restoration of androgen synthesis is not a common mechanisms of abiraterone resistance, the AR may remain dependent on the low levels of androgen that are still being produced. Indeed, previous studies have shown that AR may become sensitized to low levels of androgen through a variety of mechanisms (30). Amongst the genes that were increased in the abiraterone-resistant VCaP xenografts, GATA2 was previously shown to cooperate with AR in the activation of multiple androgen-regulated genes (31).
However, we did not observe increases in other well-characterized AR coactivators or decreases in AR corepressors. Interestingly, a negative regulator of the PI3 kinase pathway (PIK3IP1) was one of the most upregulated genes in the abiraterone-resistant xenografts. Recent studies show that PI3 kinase pathway inhibition may enhance AR signaling (32, 33), but the basis for this effect seen in PTEN deficient mouse models is not clear and studies in other models have yielded conflicting results (34-36). We also observed increased expression of BMX, a nonreceptor tyrosine kinase shown previously to enhance AR activity and to be increased in CRPC (37, 38). We recently reported that BMX enhances the activity of multiple receptor tyrosine kinases by phosphorylating a regulatory site in their kinase domains, but its role in CRPC remains to be established (39). Finally, expression of the mineralocorticoid receptor (NR3C2) was also increased, but its potential contribution to AR signaling remains to be determined.

A further mechanism implicated in resistance to androgen deprivation therapies is increased expression of constitutively active AR splice variants that have deleted the ligand binding domain (10, 14-20). Indeed, as observed here, previous reports found that AR variants were increased in PCa xenografts after treatment with abiraterone or enzalutamide (10, 40). Analyses of our abiraterone-resistant VCaP xenografts showed that AR-V7 was the only consistently expressed AR variant. Expression of both AR-FL and AR-V7 transcripts were increased in the castration-resistant VCaP xenografts, and were further increased in the abiraterone-resistant xenografts. Significantly, the fold-increase in AR-V7 was markedly greater than for AR-FL, which suggested that there may be positive selection for cells with increased AR-V7. However, further studies showed that the increase in AR-V7 reflected rapid feedback mechanisms rather than selection for subclones with increased AR-V7. Consistent with our report showing that AR gene transcription is negatively regulated by agonist-liganded AR (21), we found that DHT rapidly decreased expression of both AR-FL and AR-V7. Moreover, we found that generation of AR-V7 was further suppressed by DHT through an additional mechanism that was dependent on AR-FL and required new protein synthesis. Significantly, a report that came out while this study was under review found that androgen deprivation could increase recruitment of certain splicing factors that enhanced splicing of AR pre-mRNA to AR-V7 (41). Further studies are needed to determine whether new
proteins synthesized in response to DHT impair the recruitment of these splicing factors or suppress AR-V7 by other mechanisms, and whether they have broader roles in AR function.

Despite the marked fold-increase in AR-V7 mRNA during the development of castration- and abiraterone-resistance, AR-V7 mRNA remained at low levels compared with that of AR-FL (<1%). Moreover, although AR-V7 protein may be somewhat more stable than AR-FL under some conditions (29, 42), we found that AR-V7 protein was similarly present at very low levels relative to AR-FL in castration-resistant VCaP xenografts and in androgen starved VCaP cells in vitro. As AR activity in the androgen starved VCaP cells in vitro is markedly decreased, these observations indicate that AR-V7 expressed at these low levels is unlikely to be the major factor driving high-level AR activity in the castration- or abiraterone-resistant xenografts. Nonetheless, the functional analysis of AR-V7 in androgen starved VCaP cells showed that it could make a substantial contribution to the residual basal AR activity under conditions where AR-FL is impaired. Therefore, we hypothesize that the rapid induction of AR-V7 immediately after androgen deprivation, mediated by both an increase in AR gene transcription and a decrease in androgen regulated factors that suppress generation of the AR-V7 splice variant, represents a mechanism by which tumor cells retain low levels of AR activity needed for survival until more potent mechanisms emerge. Importantly, this would suggest that agents targeting AR splice variants may be most effective when used early in conjunction with androgen deprivation or antagonists that target the ligand binding domain, and that their efficacy may be markedly diminished if used later at relapse when additional mechanisms are driving AR activity. An exception may be in tumors expressing very high levels of AR splice variants relative to AR-FL. While these tumors currently appear to be infrequent, and may occur primarily due to structural alterations in the AR gene (14, 18, 27, 43), they may become frequent as patients become resistant to more potent agents targeting the AR ligand binding domain.

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References


42. Shafi AA, Cox MB, Weigel NL. Androgen receptor splice variants are resistant to inhibitors of Hsp90 and FKBP52, which alter androgen receptor activity and expression. Steroids. 2013;78:548-54.
Figure Legends

Figure 1. Expression of AR-stimulated genes and androgen synthesis in abiraterone-resistant VCaP xenografts. A, mice bearing recurrent VCaP xenografts were treated with abiraterone until relapse (0.5 mg/ml in drinking water for 4-6 weeks). RNA extracted from 4 sets of tumor samples pre- (pre-abi) or post-treatment (Abi-resistant) was analyzed by microarray (Affymetrix HuGene 1.0 ST). Expression of 12 androgen-stimulated genes is shown as heat map (red, high expression; green, low expression). B, the Log2 ratio for expression of androgen stimulated genes (>2-fold) in abiraterone-relapsed versus pretreatment xenograft is plotted versus their fold androgen induction. R² is presented as an indication of the correlation between androgen-induction and change induced by abiraterone treatment, and showed no trend towards lower expression in the relapsed tumors. C, expression of 9 genes involved in androgen synthesis is shown as heat map. D, DHT, testosterone, and androstenedione levels in 6 sets of abiraterone-relapsed VCaP xenograft tumor samples versus pre-treatment levels in tumor biopsies were measured using mass spectrometry. Each sample was measured in duplicate.

Figure 2. Genes and pathways altered in abiraterone-relapsed xenografts. A and B, heat map presentations of expression of top 30 most consistently (A) upregulated genes or (B) downregulated genes in abiraterone resistant tumors. Genes shown in bold and red in (A) were shown previously to be repressed by DHT in VCaP cells. C and D, gene ontology analysis on (C) 181 upregulated genes or (D) 132 downregulated genes (p<0.05). E, expression of 13 androgen-suppressed genes shown as heat map.

Figure 3. Structural alterations in AR in abiraterone-resistant xenografts. A and B, expression of AR-V7 transcripts versus AR-FL in a series of VCaP xenografts (1, 2, 3, 4, 7, 8) were examined using (A) semi-quantitative RT-PCR (-1, pre-abiraterone; -2, post-abiraterone) or (B) real-time RT-PCR. C, VCaP xenografts were established and biopsied at three stages: androgen-dependent tumor (AD), 4d post-castration (CS), and castration-resistant relapsed tumor (CR). Expression of AR-V7 and AR-FL were examined in 4 sets of these
tumor samples. D, fold change in expression of AR-V7 and AR-FL in different stages of xenograft tumors are summarized.

Figure 4. Androgen preferentially suppresses expression of AR-V7 versus AR-FL. A, VCaP or VCS2 cells were treated with (A) 10 nM DHT or vehicle (ethanol) for 24 h and mRNA for AR-V7 or AR-FL were measured using qRT-PCR (GAPDH as internal normalization control). B, C4-2 and high-passage LNCaP (LN-HP) cells were androgen deprived for 3 or 10 days, respectively, before being treated with 10 nM DHT for overnight. RNA samples were subjected to qRT-PCR for AR-V7 and AR-FL. C, VCaP cells were treated with increasing doses of DHT (0-10 nM) for 24 h. RNA samples were subjected to qRT-PCR for AR-V7 and AR-FL expression, and the levels shown are normalized to the levels in the absence of added DHT. D, VCaP cells were transfected with siRNA against exon 7 of AR-FL (siEX7) for 48 h and then treated with 0-10 nM DHT for 24 h. RNA samples were subjected to qRT-PCR. E and F, VCaP cells were pretreated with/out DHT for 2 h followed by addition of (E) actinomycin D (10 μM) for 0-6 h or (F) cycloheximide (CHX, 10 μg/ml) for 0-24 h. Note: all cells were androgen starved by culturing in steroid-depleted medium prior to treatments.

Figure 5. AR-V7 contributes for AR activity in hormone depleted conditions. A, AR protein in androgen dependent (AD) or castration resistant (CR) VCaP xenograft tumors was immunoblotted, with β-tubulin as loading control. The AR-V7 and AR-FL bands were quantified using NIH Image J software in comparison to band intensity on blots with serial dilutions of AR, and values were further normalized to β-tubulin. Ratios of AR-V7 versus AR-FL expression are presented. B, VCaP cells were transfected with siRNA against non-target control (siCtrl), cryptic exon 3 (siCE3), or exon 1 (siEX1) followed with treatment of DHT or vehicle for 24 h, and lysates were then immunoblotted with AR N- or C- terminal antibodies or an AR-V7 specific antibody. β-actin and β-tubulin were used as loading control. The AR-V7 and AR-FL bands were quantified as in A and presented as ratios of AR-V7 versus AR-FL. C, effects of siCE3, siEX7 (siRNA against exon 7) and siEX1 (siRNA targeting exon 1 of both AR-FL and AR-V7) on a panel of androgen stimulated genes in androgen
starved VCaP cells. D, Androgen-deprived VCaP cells were transfected with siCtrl or siCE3 for 24 h and then treated with enzalutamide (enza, 10 μM) or vehicle (DMSO) for overnight. SiCtrl-transfected VCaP cells treated with DHT were included as a positive control for AR activation. RNA samples were subjected to qRT-PCR for PSA, PLZF, KLK2 and GAPDH (as internal control).

Figure 6. AR-V7 expression in CRPC patient samples. Expression of AR-V7 and AR-FL were assessed by qRT-PCR in 20 CRPC bone marrow biopsy tumor samples, with GAPDH co-amplified as an internal control. The levels for each are normalized to those in androgen starved VCaP cells (VCaP cells treated with ethanol vehicle control). The bottom panel shows the ratios of AR-V7 versus AR-FL, which are similarly normalized to the ratio in androgen starved VCaP cells. *AR-FL in Sample 28 was 17.0-fold relative to androgen-deprived VCaP cells; **AR-V7 and AR-FL in Sample 49 were 14.0-fold and 860-fold, respectively, relative to those in the androgen-deprived VCaP cells.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Rapid Induction of Androgen Receptor Splice Variants by Androgen Deprivation in Prostate Cancer


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