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

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Abstract

Purpose: Mechanisms mediating androgen receptor (AR) reactivation in prostate cancer 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: Prostate cancer 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 coregulators. In contrast, mRNA encoding full-length AR (AR-FL) and a constitutively active splice variant (AR-V7) were increased compared with xenografts before castration, with an increase in AR-V7 relative to AR-FL. This shift toward AR-V7 was due to a feedback mechanism whereby the androgen-ligated 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, 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 1 to 2 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 prostate cancer 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 to 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...
Translational Relevance

Previous studies have indicated that restoration of androgen receptor (AR) transcriptional activity in prostate cancer that relapses after castration (castration-resistant prostate cancer) 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 (LBD). 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 LBD before the emergence of additional resistance mechanisms.

Androgen synthesis versus alternative mechanisms, including expression of alternatively spliced AR isoforms, in progression to abiraterone resistance.

Materials and Methods

siRNA and transfection analysis

The siRNAs specific for full-length AR (AR-FL; siExon 7, siEX7) and for AR-V7 (siCryptic Exon 3; siCE3) were described previously (17). The siRNA targeting AR exon 1 (siEX1) was described previously (21). Transfection of siRNA was performed using Lipofectamine RNAiMax (Invitrogen) in OptiMEM according to the manufacturer’s protocol. The final siRNA concentration was 20 nmol/L. A scrambled nontargeting control siRNA (Qiagen) was used as a negative control. Sixteen hours later, transfection medium was replaced with medium containing 5% charcoal-dextran stripped serum. Another 24 hours later, transfected cells were stimulated with DHT at 10 nmol/L or vehicle (ethanol) for 16 hours.

Immunoblot and steroid analyses

Whole-cell lysates 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. The antibodies against AR-FL, PSA, FKBP5, TMPRSS2-ERG, PLZF, androgen receptor (AR), androgen receptor ligand binding domain (LBD), were from Precision Antibody. Antibodies against AR-V7 were from Precision Antibody. Antibodies against alternatively spliced AR isoforms, in androgen synthesis versus alternative mechanisms, includ-
we used Affymetrix oligonucleotide microarrays to compare expression of AR-regulated genes in biopsies from CRPC xenografts before 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 before therapy than at relapse, supporting the conclusion that AR transcriptional activity was restored (Fig. 1A).

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 four sets of tumor samples pre-(pre-abi) or posttreatment (Abi-resistant) was analyzed by microarray (Affymetrix HuGene 1.0 ST). Expression of 12 androgen-stimulated genes is shown as heatmap (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 toward lower expression in the relapsed tumors. C, expression of nine genes involved in androgen synthesis is shown as heatmap. D, DHT, testosterone, and androstenedione levels in six sets of abiraterone-relapsed VCaP xenograft tumor samples versus pretreatment levels in tumor biopsies were measured using mass spectrometry. Each sample was measured in duplicate.

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 (Fig. 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 before 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 (Fig. 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, although AR activity may still be dependent on residual androgen synthesis, it seemed 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 pretreatment xenografts. A total of 181 genes were upregulated in abiraterone-resistant tumors and 132 genes were downregulated (Fig. 2A and B, and Supplementary Table S1). 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 (Fig. 2A and B). Gene ontology analysis using the Database for Annotation, Visualization and Integrated Discovery system showed only weak enrichment for genes associated with histone acetylation amongst the genes that were upregulated in the abiraterone-resistant xenografts (Fig. 2C). In contrast, the downregulated genes were most strongly associated with mitosis (Fig. 2D). This latter finding indicated that although the relapsed tumors were growing, the abiraterone was still slowing their proliferative rate relative to the CRPC tumors before 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 (Fig. 2A, in bold and colored...
We reported previously that AR gene transcription is rapidly repressed by the androgen-liganded 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 (Fig. 4A, right). 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; Fig. 4A, left). 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 (Fig. 4B).

Examining a DHT dose response in VCaP cells, we found that AR-V7 was decreased by approximately 80% at 0.1 nmol/L DHT versus approximately 60% for AR-FL, and that AR-V7 was further markedly decreased by >95% at 1 to 10 nmol/L DHT versus approximately 80% for AR-FL (Fig. 4C). To confirm that the effects of DHT were mediated by the AR-FL, we used an siRNA targeting exon 7 (siEX7; which is not present in AR-V7) to selectively deplete the AR-FL. As expected, the siEX7 markedly decreased AR-FL, but not AR-V7 (Fig. 4D). Moreover, depletion of the AR-FL 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 (Fig. 4E, bottom). Significantly, DHT similarly did not increase degradation of the AR-V7 transcript, which instead seemed to be somewhat more stable in the presence of DHT (Fig. 4E, top). 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 approximately 8 hours, and that there was further loss primarily of AR-V7 between 8 to 24 hours (Fig. 4F; DHT+DMSO). Treatment with DHT and cycloheximide (CHX), which blocks new protein synthesis, abrogated the decline in AR-V7 at 24 hours (Fig. 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 prostate cancer 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 seems 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 VCaP xenografts and the matched castration-resistant VCaP xenografts showed only a very minor band migrating at the predicted position of AR-V7, consistent with the low mRNA levels (Fig. 5A). Quantitative analysis of the AR-V7 and AR-FL bands indicated that AR-V7 protein was expressed at approximately 1.0% to 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 (Fig. 5B). Consistent with this protein being AR-V7, it was not detected by an antibody directed against the AR C-terminus, and its expression was markedly decreased by treatment with DHT (Fig. 5B). To confirm VCaP expression of AR-V7 protein, VCaP cells in androgen-depleted medium were treated with siCE3. This markedly decreased the faster migrating AR band without decreasing AR-FL (Fig. 5B). In contrast, both the AR-FL and AR-V7 bands were decreased by siEX1. Finally, immunoblotting with an AR-V7–specific antibody further supported the conclusion that the lower AR band was AR-V7 (Fig. 5B, AR-V7). The AR-V7 and AR-FL bands were quantified and AR-V7 versus AR-FL ratio was approximately 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, although increased after androgen deprivation, is still expressed at low levels.
low levels compared with 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 with 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 with the control siRNA (siCtrl), depletion of AR-V7 decreased mRNA for a series of AR-regulated genes by approximately 30% (Fig. 5C, left). An siRNA targeting AR-FL (siEX7), which is expressed at higher levels than AR-V7, decreased these AR-regulated genes to a similar degree (Fig. 5C, middle). The expression of these genes was not substantially further decreased by transfecting the cells with an siEX1 to knockdown both AR-FL and AR-V7, which may reflect an inability to adequately downregulate AR in a subset of the cells (Fig. 5C, right). 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 (Fig. 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 before 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...
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 phosphoinositide 3-kinase pathway (PIK3IP1) was one of the most upregulated genes in the abiraterone-resistant xenografts. Recent studies show that the 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 LBD (10, 14–20). Indeed, as observed here, previous reports found that AR variants were increased in prostate cancer 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 more 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-ligated 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.

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, although these findings indicate that full restoration of androgen synthesis is not a common mechanisms of abiraterone
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 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 VCaPs 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 LBD, 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. Although these tumors currently seem 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 LBD.

References


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