Androgen Receptor Variant AR-V9 Is Coexpressed with AR-V7 in Prostate Cancer Metastases and Predicts Abiraterone Resistance

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Purpose: Androgen receptor (AR) variant AR-V7 is a ligand-independent transcription factor that promotes prostate cancer resistance to AR-targeted therapies. Accordingly, efforts are under way to develop strategies for monitoring and inhibiting AR-V7 in castration-resistant prostate cancer (CRPC). The purpose of this study was to understand whether other AR variants may be coexpressed with AR-V7 and promote resistance to AR-targeted therapies.

Experimental Design: We utilized complementary short- and long-read sequencing of intact AR mRNA isoforms to characterize AR expression in CRPC models. Coexpression of AR-V7 and AR-V9 mRNA in CRPC metastases and circulating tumor cells was assessed by RNA-seq and RT-PCR, respectively. Expression of AR-V9 protein in CRPC models was evaluated with polyclonal antibodies. Multivariate analysis was performed to test whether AR variant mRNA expression in metastatic tissues was associated with a 12-week progression-free survival endpoint in a prospective clinical trial of 78 CRPC-stage patients initiating therapy with the androgen synthesis inhibitor, abiraterone acetate.

Results: AR-V9 was frequently coexpressed with AR-V7. Both AR variant species were found to share a common 3′ terminal cryptic exon, which rendered AR-V9 susceptible to experimental manipulations that were previously thought to target AR-V7 uniquely. AR-V9 promoted ligand-independent growth of prostate cancer cells. High AR-V9 mRNA expression in CRPC metastases was predictive of primary resistance to abiraterone acetate (HR = 4.0; 95% confidence interval, 1.31–12.2; P = 0.02).

Conclusions: AR-V9 may be an important component of therapeutic resistance in CRPC. Clin Cancer Res; 23(16); 4704–15. ©2017 AACR.

Introduction

Prostate cancer is the most frequently diagnosed male cancer in the United States (1). Surgery and radiation are curative treatment options for men with localized disease. For metastatic prostate cancer, androgen deprivation therapy (ADT) is the standard of care. ADT inhibits the androgen receptor (AR), a master transcriptional regulator in normal and cancerous prostate cells (2). The major limitation of ADT is the development of castration-resistant prostate cancer (CRPC), which is almost invariably due to transcriptional reactivation of the AR (2, 3). In some patients, AR transcriptional reactivation can be targeted with second-generations of therapies, such as abiraterone acetate and enzalutamide (4, 5). However, resistance is frequent, and CRPC remains the second-leading cause of male cancer deaths (6). Although there is evidence that emergence of AR-null neuroendocrine CRPC accounts for resistance to abiraterone or enzalutamide in a subset of cases (7), AR transcriptional activity persists in the majority of cases (8).

One mechanism of resistance to abiraterone and enzalutamide is expression of truncated AR variants (AR-V). AR-Vs lack the COOH-terminal ligand-binding domain (LBD) of full-length AR due to splicing of alternative 3′ terminal cryptic exons (9–14). Instead of a LBD, these 3′ terminal cryptic exons encode short carboxyl-terminal extensions. One particular AR-V, AR-V7, arises

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that had been assumed to target AR-V7 species, AR-V9 was susceptible to experimental manipulations of discrete regions of transcripts, as is the case for short-read RNA-seq data, quantitative RT-PCR with primers flanking splice junctions, or hybridization of probes to single exons. To address this challenge, we utilized single molecule, real-time (SMRT) isoform sequencing (Iso-Seq; ref. 32) to identify the entire exon composition of AR and AR-V isoforms expressed in CRPC, and estimate their abundance. We report that AR-V9, which was previously reported to arise from contiguous splicing of exons 1, 2, 3, and cryptic exon 3 (CE3; refs. 10, 12), Expression of exon CE3 as the 3′ terminal exon of AR-V7 has been exploited for the development of RT-PCR, in situ hybridization (ISH), and RNA-sequencing (RNA-seq) assays to detect AR-V7 mRNA expression in tissues (10, 12, 15–18), circulating tumor cells (19–25), and blood (26–28) of patients with CRPC. In circulating tumor cells, positivity for AR-V7 expression has been associated with resistance to abiraterone and enzalutamide, but not taxane chemotherapy (19, 20). In line with this, knockdown of AR-V7 using RNAi targeted to AR exon CE3 has indicated that AR-V7 can sustain AR signaling and thereby promote key features of the CRPC phenotype in models of CRPC (10, 29–31). Collectively, these studies have supported the concept that AR-V7 could serve as a predictive biomarker for treatment selection and also an important therapeutic target.

Profilong of clinical CRPC tissues by RNA-seq has indicated that multiple AR-V species are expressed in addition to AR-V7 (16, 17). However, the extent to which AR-Vs may be coexpressed and contribute to resistance is not known. One barrier to understanding these relationships is the challenge in inferring expression levels of complete AR and AR-V isoforms from targeted measurements of discrete regions of transcripts, as is the case for short-read RNA-seq data, quantitative RT-PCR with primers flanking splice junctions, or hybridization of probes to single exons. To address this challenge, we utilized single molecule, real-time (SMRT) isoform sequencing (Iso-Seq; ref. 32) to identify the entire exon composition of AR and AR-V isoforms expressed in CRPC, and estimate their abundance. We report that AR-V9, which was previously reported to arise from contiguous splicing of exons 1, 2, 3, and CE3 (14, 33), is frequently coexpressed with AR-V7 in CRPC. We also reannotate AR exons CE5 and CE3 as a single 3′ terminal exon with two separate splice acceptor sites for synthesis of AR-V9 or AR-V7 mRNA. As predicted by these newly annotated features, AR-V9 was susceptible to experimental manipulations that had been assumed to target AR-V7 specifically. In a biopsy-based clinical trial of metastatic CRPC patients, tumors with high AR-V9 expression displayed an increased risk of progression during treatment with abiraterone. These findings have high significance for design and interpretation of assays interrogating AR-V expression and implicate AR-V9 as a clinically important AR-V in CRPC.

Materials and Methods

Patients and clinical specimens

RNA from a CRPC liver metastasis and the LuCaP 35-CR and LuCaP 147 patient-derived xenografts (PDX) was kindly provided by Drs. Colm Morrissey, Eva Corey, and Robert Vessella (University of Washington) (34, 35). The PROMOTE study (Prostate Cancer Medically-Optimized Genome-Enhanced Therapy, ClinicalTrials.gov identifier NCT01953640) was initiated after obtaining approval from Mayo Clinic Institutional Review Board (IRB), which ensures accordance with Belmont Report ethical guidelines. All patients provided written informed consent. Details of the PROMOTE study and bioinformatics methods for analysis of PROMOTE RNA-seq data are included in the Supplementary Methods section.

Circulating tumor cell analysis

This was a prospective biomarker study evaluating expression of AR in circulating tumor cells from 12 patients with CRPC who were treated with AR-targeted therapies including lyase inhibitors (abiraterone, TAK700, or VT-464) or an AR antagonist (enzalutamide). The study was initiated after obtaining approval by the University of Wisconsin IRB, which ensures accordance with Belmont Report ethical guidelines. All patients supplied written informed consent. Details of circulating tumor cell analysis using the VERSA platform (36) are included in the Supplementary Methods section.

Cell culture

22Rv1 (ATCC, #CRL-2505), LNCaP (ATCC, #CRL-1740), VCaP (ATCC, #CRL-2876), and DU145 (ATCC, #HTB-81) cells were obtained from ATCC. ATCC ensures authenticity of these human cell lines using short tandem repeat analysis. 22Rv1, LNCaP, and DU145 cells were maintained in RPMI1640 (Invitrogen) with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (penicillin/streptomycin) in a 5% CO2 incubator at 37°C. VCaP cells were cultured in DMEM (Invitrogen) with 10% FBS and penicillin/streptomycin in a 5% CO2 incubator at 37°C. CWR-R1 cells (37) were a kind gift from Dr. Elizabeth Wilson (UNC Chapel Hill, Chapel Hill, NC) and cultured in RPMI1640 + 10% FBS and penicillin/streptomycin. Cell line authentication and mycoplasma monitoring are described in the Supplementary Methods section.

Illumina AR RNA-seq

RNA isolated from 22Rv1 cells, CWR-R1 cells, LuCaP 147 PDX tissue, and a CRPC liver metastasis was converted to cDNA using a Clontech Advantage RT Kit using both oligo-dT and random hexamer primers as per the manufacturer’s recommendations. cDNA samples were submitted to the University of Minnesota Genomics Center (Rochester, MN) for RNA-seq library preparation and hybrid capture with a custom AR-based SureSelect (Agilent) bait library (30) using the SureSelect QXT Reagent Kit (Agilent) as per the manufacturer’s recommendations. Postcapture sequencing libraries were pooled and diluted to 10 pmol/L for flow cell clustering and sequenced using Illumina HiSeq 2000 with 2 × 50 bp settings. For metastatic biopsy specimens obtained under the PROMOTE trial, RNA-seq libraries were prepared at
the Mayo Clinic Medical Genome Facility according to the manufacturer's instructions for the TruSeq RNA Sample Prep Kit v2 (Illumina). The concentration and size distribution of the libraries were determined on an Agilent Bioanalyzer DNA 1000 chip and Qubit fluorometry (Invitrogen). Libraries were pooled and diluted to 8 to 10 pmol/L for flow cell clustering and sequenced using an Illumina HiSeq 2000 at 2 × 101 bp settings. Bioinformatics methods for analysis of RNA-seq data are included in the Supplementary Methods section. The RNA-seq data from the PROMOTE study are available through the NCBI database of Genotypes and Phenotypes (dbGaP) under accession phs001141.v1.p1.

3′ Rapid amplification of cDNA ends

RNA extracted from 22Rv1 cells and LuCaP 35-CR PDX tissue was subjected to 3′ rapid amplification of cDNA ends (3′-RACE) using a second-generation 5′/3′ RACE Kit (Roche) according to the manufacturer's instructions. Briefly, 1 μg total RNA was used for first-strand cDNA synthesis using the oligo(dT)-anchor primer provided in the kit. An aliquot of the cDNA reaction was amplified by PCR using Quanta AccuStart II PCR SuperMix (Quanta Biosciences), a forward primer anchored in AR exon 1 (5′-TTACCCGC-CACCTGATGTTG) or AR exon 3 (5′-GCTGAAGGGAAACA-GAAGTACC) paired with a reverse primer provided by the kit (5′-GACCCACCGTATCGATGTCGAC-3′). PCR was performed with an initial melt at 95°C for 2 minutes, followed by 10 cycles of PCR (95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 40 seconds) and then 26 cycles of PCR (95°C for 15 seconds, 60°C for 30 seconds, and 72°C starting at 40 seconds for the first cycle increasing 20 seconds every additional cycle).

PacBio SMRT Iso-Seq

Amplified 3′ RACE products from 22Rv1 cells and LuCaP 35-CR xenografts were converted into SMRTbell sequencing libraries using the SMRTbell Template Prep Kit 1.0 (Pacific Biosciences) per the manufacturer's recommendations. Amplified products were end repaired and ligated to SMRTbell hairpin adapters. SMRTbell libraries were purified using AMPure PB Beads (Pacific Biosciences). Final libraries were sequenced on the Pacific Biosciences (PacBio) RS II using the P6/C4 sequencing chemistry (Pacific Biosciences). Two SMRT cells were sequenced for each sample. Bioinformatics methods for analysis of PacBio Iso-Seq data are included in the Supplementary Methods section.

Statistical analysis of PROMOTE data

Composite progression at 12 weeks was categorized as yes/no by evaluating PSA, RECIST, bone scan, and symptoms as recommended by Prostate Cancer Working Group-2 (38). mRNA expression levels prior to therapy with abiraterone acetate of full-length AR (ARFL), ARV3, ARV7, ARV9, ARV23, ARV45, chromogranin-A (CHGA) together with serum PSA, testosterone, serum chromogranin-A (CHGA) levels, Gleason score at initial diagnosis, high versus low volume disease, and time from starting hormone therapy to metastatic CRPC stage were evaluated using a logistic regression model for predicting resistance at 12 weeks after starting treatment. Because of the relatively high proportion of patients who did not express individual AR-Vs, a dichotomous cut-off point was determined by first splitting levels of each AR-V into quartiles or terciles and choosing the cut-off point that best maximized the difference between responders and nonresponders. Levels of AR and AR-V mRNA expression at baseline were tested for associations with composite progression via the χ² test. To avoid overparameterization during the multivariate modeling process due to a relatively small number of patients and large number of potential covariates, the final multivariate model fitted only factors with an entry threshold of P < 0.2 in univariate analysis. All possible multivariable models fitting these criteria were considered, and the difference in the −2 log likelihoods was determined and tested against the appropriate χ² test.

Transient transfections

Details for construction of AR-V9 plasmid are included in the Supplementary Methods section. 22Rv1 and VCaP cells were transfected with two separate siRNAs targeted to AR exon CE3 or three separate siRNAs targeted to exon CE3 as outlined in the Supplementary Methods. Cells were lysed 48 hours posttransfection for isolation of total RNA and protein. LNCaP and DU1145 cells were transfected with AR-V7 and AR-V9 expression vectors along with AR-responsive luciferase reporter plasmids as described in the Supplementary Methods. Activities of the firefly and Renilla luciferase reporters were assayed using a Dual Luciferase Assay Kit as per the manufacturer’s recommendations. Transfection efficiency was normalized by dividing firefly luciferase activity by Renilla luciferase activity. Data presented represent the mean ± SEM from three independent experiments, each performed in duplicate.

Lentivirus infection and cell proliferation assays

Details for construction and preparation of lentivirus are included in the Supplementary Methods. LNCaP cells were seeded in 6-cm dishes at 4 × 10⁵ cells per dish in RPMI with 10% FBS and penicillin/streptomycin. Cells were transduced the next day by addition of 0.5, 2, 8, 16, or 32 μL of GFP or AR-V9 lentivirus directly to tissue culture medium. Cells were reseeded 120 hours posttransduction on 96-well plates in RPMI + 10% CSS + penicillin/streptomycin and subjected to cell proliferation assays using a BrdU ELISA Kit (Roche) according to the manufacturer’s recommendations.

RT-PCR

Expressions of AR-V7, AR-V9, and full-length AR were assessed by RT-PCR with total RNA extracted from 22Rv1 and VCaP cells as described in the Supplementary Methods. Fold change in mRNA expression levels were calculated by the comparative Cₗ method using the formula 2⁻^ΔΔCₗ and GAPDH as calibrator.

Immunoprecipitation and Western blot analysis

Lysates from siRNA-transfected 22Rv1 and VCaP cells were subjected to immunoprecipitation with rabbit antiserum raised to the unique AR-V9 COOH-terminal peptide (details in the Supplementary Methods), a mouse mAb specific for the unique AR-V7 COOH-terminal peptide (catalog #: AG10008, Precision Antibody), or rabbit/mouse IgG controls. Immunoprecipitated complexes were boiled in 1 × Laemmli buffer and resolved in denaturing gels. Alternatively, transfected cells were harvested in 1 × Laemmli buffer and lysates were resolved in denaturing gels. Gels were transferred to nitrocellulose membranes and subjected to Western blot analysis as described previously. Membranes were incubated with primary antibodies (AR-N20, AR441, ERK2-D2; Santa Cruz Biotechnology) diluted 1:1,000.
Results

RNA-seq reveals frequent coexpression of AR-V9 and AR-V7 in prostate cancer

To investigate AR splicing patterns at high depth in prostate cancer, we used a modified RNA-seq approach that included an AR sequence enrichment step prior to illumina paired-end sequencing. We used this approach to analyze RNA from the 22Rv1 cell line, which is the model in which AR-Vs were first discovered and characterized (9, 10, 12). Visual inspection of RNA-seq reads mapped using the TopHat algorithm (39) confirmed the expected expression of each canonical AR exon 1-8, as well as high expression of a region of the AR gene downstream of exon 3 (Fig. 1A). This is the region of the AR gene where multiple cryptic exons reside, including CE5 and CE3.

Full-length AR is a modular protein encoded by contiguously spliced exons 1–8. Conversely, AR-V9 and AR-V7 are both encoded by AR exons 1–3, with exons CE5 (14, 33) or CE3 (10, 12) as the 3' terminal exons, respectively (Fig. 1B). We quantified split reads, defined as RNA-seq reads spanning discrete splice junctions, to infer expression of discrete AR mRNA species. This approach revealed that AR-V9 and AR-V7 isoforms (reads spanning the exon 3/CE5 splice junction and 3/CE3 splice junction, respectively) were abundant AR-Vs. For example, the number of AR-V9 and AR-V7 split reads were 37% and 63%, respectively, of the number of full-length AR mRNA split reads (reads spanning the exon 3/4 splice junction; Fig. 1A). Half-lives of exon 3/CE5 and 3/CE3 splice junctions were both longer than 6 hours, indicating that neither was a splicing intermediate (Supplementary Fig. S1).

Attenuation of 22Rv1 RNA-seq read coverage was apparent downstream of exon CE3, which is consistent with termination of the AR-V7 transcript at this location. However, attenuation of 22Rv1 RNA-seq read coverage was not observed for exon CE5 (Fig. 1A). In our analyses, we considered previous studies demonstrating that 22Rv1 cells harbor a 35-kb intragenic tandem duplication encompassing AR exon 3, CE5, and CE3, which could confound interpretation of RNA-seq reads mapped to this region (40). We therefore performed similar analysis in CRPC CWR-R1 cells, which do not have a genomic alteration in this exon 3 region of AR (30). Similar to 22Rv1, CWR-R1 cells coexpressed AR-V9 and AR-V7 mRNA. Furthermore, attenuation of RNA-seq read coverage was apparent at the end of exon CE3 but not CE5 (Fig. 1C).

Similar features of AR-V9 and AR-V7 mRNA expression were noted in RNA from the LuCaP147 PDX and a CRPC liver metastasis obtained from rapid autopsy. Split RNA-seq reads spanning the exon 3/CE5 and 3/CE3 splice junctions were quantified to infer expression of AR-V9 and AR-V7, respectively.

Long-read SMRT sequencing of AR isoforms reveals a shared 3' terminal exon for AR-V9 and AR-V7

The short-read fragments yielded by illumina RNA-seq enable quantification of discrete exons as well as discrete splice junctions. However, it is challenging to infer complete mRNAs from 5' end to 3' end with these discrete fragments of information, particularly when multiple RNA isoforms exist for a single gene. To address this challenge, we performed 3' rapid amplification of cDNA ends (RACE) with RNA isolated from 22Rv1 cells using a forward primer anchored in AR exon 1 (Supplementary Fig. S2). 3' RACE
To interpret SMRT Iso-Seq data, we classified exons contained in 3′ RACE products as "canonical exons" (exons 1–8 of full-length AR), "annotated cryptic exons" (cryptic exons reported to be expressed in AR-Vs in previous studies), or "PacBio 22Rv1 exons" (novel exons identified in 22Rv1 mRNAs in this study; Fig. 2A) and developed a visualization scheme to represent the splicing of these exons within each mRNA species. In this visualization scheme, each exon expressed in the context of a specific AR mRNA isoform was denoted by a pixel that was colored based upon whether that exon utilized the exact 5′ and 3′ splice sites annotated for AR exons, the exact 5′ splice site only, the exact 3′ splice site only, or neither of the annotated 5′ or 3′ splice sites (Fig. 2B). To estimate the abundance of each AR mRNA isoform, we counted the number of full-length reads that contained these splicing profiles (Fig. 2B). It should be noted that shorter fragments in a SMRT bell library may be preferentially sequenced by PacBio SMRT cells, which means that comparative levels of full-length AR versus AR-V mRNAs may not be fully quantitative. Nevertheless, an exon 3–duplicated version of full-length AR was the most abundant AR species detected in 22Rv1 cells (Fig. 2B). This finding mitigated concerns about quantitative bias for shorter fragments, as this was the longest PacBio library fragment sequenced, and relative quantification appeared to be generally consistent with Illumina split reads spanning the exon 3/4 splice junction of full-length AR (Fig. 1A).

Similar to Illumina sequencing, this SMRT Iso-Seq approach also indicated that AR-V9 was an abundant isoform, with levels comparable with AR-V7. However, although AR-V9 mRNA displayed correct usage of the exon CE5 5′ splice site, the 3′ terminus of this exon appeared to be located at the end of exon CE3, with no splicing events in between (Fig. 2B). This indicated that the 3′ terminal exon CE5 in AR-V9 is approximately 2.4 kb, which is much longer than annotated (14, 33), encompassing exons CE5, CE3, and the intervening region. High abundance of AR-V9 relative to AR-V7, as well as a long 3′ terminal exon in AR-V9 mRNA consisting of the contiguous CE5–CE3 segment was confirmed when a forward primer anchored in exon 3 was utilized for 3′ RACE/SMRT Iso-Seq with 22Rv1 mRNA (Fig. 2C; Supplementary Fig. S2). Similarly, the 3′ terminal exon in AR-V9 mRNA had the same identity in the LuCaP 35-CRPx PDX, although AR-V7 mRNA was more abundant (Fig. 2D and E). Overall, these 3′-RACE/SMRT Iso-Seq data were in agreement with coverage data from Illumina short-read sequencing (Fig. 1B), supporting the notion that AR-V9 mRNA is abundant and coexpressed with AR-V7 in CRPC cell line models and PDX tissues.

Co-expression of AR-V9 and AR-V7 mRNA in clinical CRPC

To test whether AR-V9 is co-expressed with AR-V7 in clinical CRPC tissues, we interrogated RNA-seq data from biopsies of 56 metastatic tissues available from the AACR-PCF Stand Up To Cancer study of CRPC (17). Quantification of split reads spanning the exon 3/CE5 splice junction and 3/CE3 splice junction revealed a positive correlation between AR-V9 and AR-V7 mRNA expression (Fig. 3A). Next, we used RT-PCR to evaluate expression of AR-V7, AR-V9, and AR target genes in circulating tumor cells from an independent cohort of 12 patients. AR-V9 was expressed in a subset of patients that received therapy with androgen synthesis inhibitors (abiraterone, VT-464, or TAK700; ref. 8) or enzalutamide (Fig. 3B). All AR-positive patient samples exhibited expression of AR-regulated and prostate cancer–specific genes, including NKX3.1 and/or PSMA, TMPRSS2, and KLK2/3. In this small cohort of patients, AR-V9 was frequently coexpressed with AR-V7 in circulating tumor cells.

AR-V9 protein expression is inhibited by RNAi targeted to exon CE3

Previous studies designed to test the functional importance of endogenous AR-V7 in CRPC cell lines utilized siRNA targeted to exon CE3 (10, 29–31). The conclusion that endogenously expressed AR-V7 was driving the CRPC phenotype was based on the assumption that these exon CE3-targeted siRNAs were targeting AR-V7 specifically, and not impacting expression of full-length AR or other AR-Vs. Our finding that AR-V9 and AR-V7 mRNAs both contained the entirety of exon CE3 indicated that both mRNAs may be knocked down by siRNAs targeted to exon CE3 (Fig. 4A). Indeed, transfection of 22Rv1 and VCaP cell lines with two independent siRNAs targeting AR exon CE3 reduced expression of both AR-V7 and AR-V9 mRNA (Fig. 4B). In contrast, two independent siRNAs targeting AR exon CE3 reduced expression of AR-V9 but not AR-V7 (Fig. 4B).

To test whether these findings extended to endogenous AR-V7 and AR-V9 protein, we raised polyclonal antisera to the COOH-terminal amino acid sequence unique to AR-V9 (Fig. 4C). Polyclonal antibodies affinity purified with an AR-V9 COOH-terminal peptide recognized a single approximately 75-kDa species in Western blots with lysates from LNCaP cells transfected with an AR-V9 expression vector (Supplementary Fig. S3). However, purified AR-V9 polyclonal antibodies also displayed binding to non-AR species in 22Rv1 lysates, indicating this reagent did not have adequate specificity to discriminate endogenous AR-V9 in Western blots. To overcome this limitation, we used polyclonal AR-V9 antiserum to immunoprecipitate endogenous AR-V9 protein from 22Rv1 and VCaP cells. In these experiments, AR-V9 antibodies immunoprecipitated an approximately 75-kDa species that was recognized by an mAb specific for the AR NTD (Fig. 4D). Consistent with results from RT-PCR experiments, the level of AR-V9 protein in immunoprecipitates was reduced by siRNAs targeted to both AR exons CE5 and CE3 (Fig. 4D). In contrast, the level of AR-V7 protein immunoprecipitated by an antibody specific for the AR-V7 COOH terminus was only reduced by knockdown with siRNA targeted to AR exon CE3 (Fig. 4D). From this, we concluded that AR-V9 protein is expressed endogenously in 22Rv1 and VCaP cells and can be knocked down with siRNA targeted to AR exon CE3.

High AR-V9 expression is associated with progression during therapy with abiraterone acetate

Given our finding that AR-V9 and AR-V7 are frequently coexpressed in CRPC, and that AR-V7 and AR-V9 mRNA both contain exon CE3 at their 3′ termini, we asked whether AR-V9 expression levels were associated with response to abiraterone in CRPC. For this, we analyzed data from a prospective study wherein biopsies of metastatic CRPC tissues were obtained for genomic analysis before patients initiated therapy with abiraterone acetate plus prednisone. Ninety-two patients were enrolled and followed for outcomes with the primary goal of identifying transcriptomic alterations that were predictive of efficacy of treatment. At 12 weeks posttherapy, patients were assessed for disease progression...
using a 12-week composite progression-free survival (PFS) end-point as per the recommendations of the Prostate Cancer Working Group-2 (38). The clinicopathologic characteristics of the entire cohort are summarized in Supplementary Table S1. There were 78 of 92 patients enrolled in this study for which RNA-seq and composite PFS outcomes were available (Fig. 5A).
and the ratio of AR-V9/full-length AR were significant across CRPC biopsies, but samples negative for AR-V7 and AR-V9 expression (Fig. 5C). Tumor RNA content levels in pretreatment metastatic CRPC biopsies were correlated with 12-week composite PFS. Multivariate analysis determined that AR-V9 levels in the highest quartile predicted primary resistance to therapy (HR = 4.0; 95% confidence interval, 1.31–12.2; P = 0.02). No multivariable model was significantly better than AR-V9 alone due to the high correlation with other AR-Vs.

AR-V9 is constitutively active and promotes androgen-independent growth

AR-V7 has been shown to function as a constitutive effector of the broad androgen/AR signaling program (12, 29, 41). To test whether AR-V9 displays these activities, we transfected cells with an AR-V9 expression vector and AR-responsive promoter–reporter constructs. In AR-positive LNCaP cells (Fig. 6A and B) and AR-negative DU145 cells (Fig. 6C and D), AR-V9 displayed constitutive, ligand-independent transcriptional activity when expressed in isolation, and also when coexpressed with AR-V7. Because abiraterone exerts action at the level of testes, adrenal glands, and tumor cells, this drug is not appropriate for experimental therapy of prostate cancer cells grown in vitro. Therefore, we used enzalutamide as an example of a second-generation AR-targeted therapy to test therapeutic sensitivity of AR-V9 transcriptional activity. Similar to AR-V7, constitutive AR-V9 transcriptional activity was insensitive to treatment with enzalutamide (Fig. 6B and D). Consistent with these findings, infection of LNCaP cells with a lentiviral vector harboring AR-V9 resulted in androgen-independent proliferation at lower virus titers, but suppression of proliferation at higher virus titers (Fig. 6E and F). These results resembled the known biphasic effects of AR-V7 and other AR-Vs on LNCaP cell proliferation due to biphasic regulation of proliferation-associated genes (29, 42). Collectively, these functional data support the concept that AR-V9 may play a significant, yet previously unappreciated, role in promoting androgen-independent growth of CRPC cells.

Discussion

RNA-seq analysis has revealed that multiple AR-Vs are expressed in clinical prostate cancer (16, 17). Among these, AR-V7 is the best characterized due to frequent detection of the AR exon 3/CE3 splice junction by RNA-seq and RT-PCR (12, 13, 15–17), high expression of exon CE3 measured by RNA-ISH (20, 25, 43), and availability of AR-V7–specific antibodies to interrogate protein expression in tissues (19, 44, 45). In this study, we found that AR-V9 is frequently coexpressed with AR-V7 in CRPC cell lines, PDX tissue, circulating tumor cells, and biopsies of metastatic CRPC. Furthermore, our work with 22Rv1 and VCaP cell lines and LuCaP 35-CR PDX tissue revealed that both of these AR-V species contain the entirety of AR exon CE3 nucleotide sequence at their extreme 3′ termini, with AR-V9 mRNA being approximately 1.1 kb longer due to the extended 3′ untranslated region. Analysis of additional samples will be required to conclude whether AR-V9 transcripts always contain this large 3′ untranslated region encompassing CE3.

Our study revealed that high pretherapy AR-V9 mRNA expression in CRPC metastases was correlated with primary resistance to abiraterone acetate. Limitations include the relatively small patient cohort, use of a 12-week composite PFS endpoint, and exploratory nature of using post hoc cutoffs to define low/high AR-V9 expression. Nevertheless, this finding appears to be aligned with previous RNA-seq studies showing that AR-V9 expression in...
prostate cancer tissues is highly enriched in CRPC. For example, in the Cancer Genome Atlas study of localized, hormone-naive localized prostate cancer tissue, AR-V9 was detectable in less than 10% of specimens (16, 17). However, in the AACR-PCF Stand Up To Cancer study of CRPC metastases, AR-V9 was expressed in over 75% of specimens (17). In these same studies, AR-V7 expression was detected in over half of therapy-naive localized prostate cancer and nearly all metastatic CRPC (16, 17).

In circulating tumor cells or plasma, AR-V7 mRNA or protein expression is detectable at frequencies ranging from 3% to 100% depending on treatment history and detection platform (19, 20, 22–24, 26, 27, 46). Moreover, detection of AR-V7 mRNA expression in these blood-based studies was correlated with resistance to therapy with abiraterone or enzalutamide but not taxane chemotherapy (19, 20, 24, 26, 27, 46). Considering these extensive data supporting AR-V7 as a predictive circulating biomarker, it is not clear why high levels of pretherapy AR-V7 expression in CRPC metastases were not significantly correlated with primary resistance to abiraterone in multivariate analysis. It will be important for future studies to address differences in predictive capacity of AR-V7 signals obtained from blood versus tissues (47).

The newly annotated features of AR-V7 and AR-V9 transcripts arising from our study have importance for design and interpretation of biomarker assays. For example, signals from RNA-ISH assays with probes complementary to AR exon CE3 have been utilized to assess AR-V7 mRNA levels in prostate cancer tissues (20, 25, 43). Our data indicate these RNA-ISH signals would represent a composite of AR-V7 and/or AR-V9. Second, our work may provide insight into discordant reports of correlations between AR-V7 mRNA levels in prostatectomy specimens and risk of biochemical recurrence (12, 18). One study supporting a correlation utilized RT-PCR with primers flanking the AR exon 3/CE3 splice junction (12), whereas a study finding no correlation utilized a branched DNA assay with probes targeting a broader region of exon CE3 that may not have discriminated between AR-V7 and AR-V9 (18). It should also be noted that the longer transcript length of AR-V9 relative to AR-V7 could bias reverse transcription reactions utilizing oligo(dT) primers, favoring more efficient detection of AR-V7.
Our findings also have importance for interpreting the functional roles of AR-Vs in prostate cancer. For example, the functional importance of endogenous AR-V7 as a main driver of resistance to AR-targeted therapies has been established with siRNAs targeted to exon CE3 in cell line models such as 22Rv1, CWR-R1, and VCaP (10, 29–31). The main conclusions from these knockdown experiments were that AR-V7 was sufficient to support constitutive transcriptional activation of AR target genes, androgen-independent proliferation, and insensitivity to antiandrogens (10, 29–31). Because our data establish that siRNAs targeted to AR exon CE3 also inhibit expression of AR-V9, it remains unclear whether endogenous AR-V7 is an independent effector of resistance, or requires functional cooperation with AR-V9. Given that AR-Vs require dimerization to support chromatin binding and transcriptional activation of target genes (48–50), it is possible that AR-V7 homodimers, AR-V9 homodimers, and AR-V7:AR-V9 heterodimers are all engaged with chromatin in CRPC cells under conditions of full-length AR inhibition.

In summary, this study used complementary short- and long-read RNA-seq technologies to identify a common shared 3' terminal exon as the molecular basis for frequent AR-V7 and AR-V9 coexpression in CRPC. As AR-V7 and AR-V9 proteins are both constitutively active, the overall levels and functional impact of AR-Vs in prostate cancer may be greater than would be anticipated from analyses of either AR-V alone. Additional studies are warranted to test the predictive capacity of AR-V9 in larger cohorts and investigate whether specific targeting of AR-V9 in addition to AR-V7 may be needed to overcome drug resistance.
Disclosure of Potential Conflicts of Interest

R. Jimenez is an employee of Histowiz. J.M. Lang has ownership interests (including patents) in Salus Discovery, LLC and is a consultant/advisory board member for Sanofi. S.M. Dehm is a consultant/advisory board member for Astellas/Medivation and Janssen Research and Development LLC. No potential conflicts of interest were disclosed by the other authors.

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AR-V9 functions as a constitutively active transcription factor independent of full-length AR. A and B, AR-positive LNCaP prostate cancer cells were transfected with a PSA-driven luciferase reporter and expression vectors encoding AR-Vs as indicated. Cells were treated with DHT, enzalutamide (en), or vehicle controls (ethanol as control for DHT, DMSO as control for enzalutamide) as indicated and subjected to Western blot analysis with antibodies specific for the AR NTD or ERK-2 (A, loading control) or luciferase assay (B). Luciferase activities are expressed relative to the activity of vehicle-treated LNCaP cells transfected with PSA-luciferase and SV40-Renilla only, which was arbitrarily set to 1. Bars, mean; whiskers, SEM from two independent experiments, each of which was performed in triplicate (n = 6). C and D, AR-negative DU145 prostate cancer cells were transfected with an androgen response element (ARE)-driven luciferase reporter and expression vectors encoding AR-Vs as indicated. Cells were treated and subjected to Western blot analysis (C) with antibodies specific for the AR NTD or ERK-2 (loading control) or luciferase assay (D). Luciferase activities are expressed relative to the activity of vehicle-treated LNCaP cells transfected with 4XARE-luciferase and SV40-Renilla only, which was arbitrarily set to 1. Bars, mean; whiskers, SEM from two independent experiments, each of which was performed in triplicate (n = 6). E and F, LNCaP cells were infected with a range of titers of lentivirus encoding GFP (control) or AR-V9 and subjected to Western blot analysis (E) with antibodies specific to the AR NTD or ERK-2 (loading control) or assayed for proliferation by BrdU incorporation assay (F). Data represent mean ± SEM from three biological replicate experiments, each performed in triplicate (n = 9).
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References

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Androgen Receptor Variant AR-V9 Is Coexpressed with AR-V7 in Prostate Cancer Metastases and Predicts Abiraterone Resistance

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