Published OnlineFirst July 20, 2016; DOI: 10.1158/1078-0432.CCR-16-0237

Cancer Therapy: Clinical

Clinical Cancer Research

Branched Chain RNA *In Situ* Hybridization for Androgen Receptor Splice Variant AR-V7 as a Prognostic Biomarker for Metastatic Castration-Sensitive Prostate Cancer

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Abstract

Purpose: The androgen receptor (AR) mRNA splice variant AR-V7 has emerged as a predictive biomarker for response to AR-targeted therapies. There are currently no commercially available assays to detect AR splice variants. The branched chain RNA *in situ* hybridization (ISH) platform enables the highly sensitive detection of RNA transcripts in formalin-fixed, paraffin-embedded (FFPE) tissues.

Experimental design: We designed a branched chain RNA ISH probe to target the unique cryptic exon CE3 of AR-V7 using multiple tiling probes. This automated ISH assay was applied to tumor tissue from two distinct clinical cohorts that we hypothesized would differ in AR-V7 status.

Results: We detected AR-V7 in all tumor samples from men with metastatic castration-resistant prostate cancer with tissue obtained after disease progression despite at least one subsequent line of hormonal therapy (abiraterone, enzalutamide, or bicalutamide; $n = 12$). We detected AR-V7 in just one tumor from men who had undergone prostatectomy for localized adenocarcinoma ($n = 30$; Gleason $4 + 5 = 9$ in the AR-V7–positive sample). Given the apparent distinction between the above groups by AR-V7 signal, we analyzed pretreatment AR-V7 status as a predictive and prognostic biomarker in men with treatment-naïve metastatic disease. Patients with metastases but without detectable AR-V7 RNA at baseline had significantly longer overall survival (log-rank $P = 0.044$) and a trend toward superior progression-free survival (log-rank $P = 0.055$).

Conclusions: Within an institutional cohort, the RNA ISH assay identified AR-V7 within FFPE tissue and may have prognostic value in metastatic castration-sensitive prostate cancer. These preliminary findings warrant further study in larger cohorts.
Clin Cancer Res; 23(2); 363–9. ©2016 AACR.

Introduction

Prostate cancer is common, with rising use of systemic therapy (1) and risk for increasing incidence of advanced disease as screening efforts are discouraged (2, 3). Despite FDA approval of five new systemic therapies for metastatic castration-resistant prostate cancer (mCRPC) since 2010, it remains the second leading cause of cancer death in men. Most of the currently available therapies for advanced prostate cancer target the androgen receptor (AR). This includes standard first-line androgen deprivation therapy (ADT) with a gonadotropin-releasing hormone (GnRH) agonist or antagonist, secondary therapy with androgen biosynthesis inhibitors such as abiraterone (4) or antiandrogens such as enzalutamide (5), and taxane chemotherapies (6, 7). Understanding resistance to AR-targeted therapy is therefore important.

AR splice variants with constitutively active AR function have emerged recently as predictive biomarkers for response to AR-targeted therapies (8–11). AR-V7 is thought to be the most clinically prevalent AR splice variant. It was first described in patients with advanced disease (12–16) and has more recently been detected in localized prostate cancer (17). This variant lacks the ligand-binding domain but retains the transactivating N-terminal domain, and therefore may be resistant to inhibition by AR-targeted hormonal agents.

AR-V7 expression has been associated with resistance to abiraterone and enzalutamide (8) but not taxane chemotherapy (docetaxel or cabazitaxel; ref. 11) in patients with mCRPC. These data suggest that AR-V7 expression could be a predictive biomarker of treatment response for AR-targeted hormonal agents in mCRPC. It remains unknown whether AR splice variant expression may also serve as a prognostic biomarker in castration-sensitive prostate cancer.

Currently, there are no commercially available assays to detect AR splice variants. Assessment of AR-V7 within four tissue samples

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

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doi: 10.1158/1078-0432.CCR-16-0237

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Translational Relevance

Branched chain RNA *in situ* hybridization (ISH) is a technique that facilitates the detection of specific RNA transcripts within formalin-fixed, paraffin-embedded tissues. We used RNA ISH to detect the androgen receptor splice variant AR-V7 within archival prostate cancer tissue. First, we found that this automated AR-V7 RNA ISH assay detected splice variant transcript within samples from men with castration-resistant prostate cancer that were clinically likely to harbor transcript, and seldom detected it in those that were of lower clinical likelihood. Second, we found that the presence of detectable AR-V7 transcript at baseline among men with treatment-naïve metastatic disease showed promise as a negative prognostic biomarker. This methodology can be used to validate the preliminary findings within our cohort and to investigate other clinical questions relevant to the presence of AR-V7 within archival cancer tissue.

has been described with use of the Advanced Cell Diagnostics RNAscope 2.0 Brown kit (8), though this method has not to our knowledge been automated. Reproducible methodology for the assessment of AR splice variant RNA therefore represents an unmet medical need. RNA *in situ* hybridization (ISH) enables the detection of specific RNA transcripts, but the assay has traditionally been limited by poor sensitivity, high background signal, and low-throughput using a manual platform. The recent development of a branched chain RNA ISH platform enables sensitive detection of RNA transcripts in formalin-fixed, paraffin-embedded (FFPE) tissues with little background, based on a technology that uses a branched DNA structure for signal amplification (18). An automated version of this RNA ISH platform is available and can be implemented in a standard clinical laboratory (19, 20). The development of a branched chain RNA ISH assay capable of detecting AR splice variants in FFPE tissues would enable the retrospective analysis of archival tissue banks annotated with clinical outcomes, and potentially serve as a powerful biomarker that is more easily adopted than assays that require frozen tissues or circulating tumor cells (CTCs).

In this pilot study, we developed an AR-V7 branched chain RNA ISH assay and correlated AR-V7 expression in FFPE tissues with clinical characteristics. We hypothesized that AR-V7 would be detectable within archival FFPE prostate cancer tissue using RNA ISH in patients with mCRPC who had experienced progression despite at least one secondary hormonal manipulation, and that AR-V7 would not commonly be detectable among those who had undergone prostatectomy for clinically localized disease not previously exposed to systemic cancer therapy. For men receiving first-line ADT, we hypothesized that detectable baseline AR-V7 would be associated with shorter overall survival (OS). We further hypothesized that detectable baseline AR-V7 would be significantly more common in those who experienced a brief (under 9 months) rather than sustained (>2.5 years) response to first-line ADT.

Materials and Methods

Patients

Patient cohorts were defined from within the Massachusetts General Hospital genitourinary oncology practice. Institutional Review Board approval to evaluate archival tissue was obtained prior to this work (Dana Farber/Harvard Cancer Center Protocol 15-025, Partners Protocol 2005P000774). All evaluations were retrospective and included patients with tissue collected since January 1, 2000.

We defined two clinical cohorts that we hypothesized to represent low clinical likelihood and high clinical likelihood to have detectable AR-V7 transcript using the RNA ISH assay on FFPE tumor tissue. One cohort consisted of patients who had undergone radical prostatectomy at Massachusetts General Hospital (MGH) for clinically localized disease with surgical pathology specimens showing Gleason $3 + 3 = 6$ ($n = 10$), Gleason 7 ($n = 10$), or Gleason ≥ 8 ($n = 10$) adenocarcinoma. Another cohort consisted of patients ($N = 12$) who had developed mCRPC with tumor tissue obtained after the development of progressive disease despite at least one subsequent line of hormonal therapy (abiraterone, enzalutamide, or bicalutamide). Four patients from this cohort had received chemotherapy in addition to at least one secondary hormonal agent (Table 1).

We defined two additional clinical cohorts for whom tissue was available prior to the start of first-line ADT for metastatic prostate cancer. The "sustained response" cohort consisted of patients ($N = 13$) who experienced >2.5 years of response to first-line ADT before the development of biochemical castration resistance (defined as a confirmed rise in PSA from the nadir despite testosterone <50 ng/dL). The "brief response" cohort consisted of patients ($N = 9$) who demonstrated biochemical castration

Table 1. Prior treatments within "resistant disease" cohort

	Tissue	Date	Prior secondary therapies	AR	AR-V7	Housekeeping
1	Bladder	September 30, 2013	Abiraterone	3+	2+	3+
2	Liver	April 27, 2012	Abiraterone	3+	3+	3+
3	Trans-urethral resection of prostate	November 20, 2012	Docetaxel, abiraterone, cabazitaxel, enzalutamide	2+	1+	3+
4	Bladder	November 27, 2014	Abiraterone, enzalutamide	3+	3+	3+
5	Liver	October 31, 2014	Abiraterone	3+	2+	2+
6	Vertebral body decompression	January 10, 2014	Docetaxel, abiraterone, cabazitaxel, enzalutamide	3+	2+	3+
7	Vertebral body decompression	April 30, 2010	Bicalutamide	3+	2+	3+
8	Bladder	September 29, 2010	Abiraterone, docetaxel	3+	1+	1+
9	Vertebral body decompression	January 25, 2014	Bicalutamide	3+	1+	3+
10	Bladder	November 27, 2013	Abiraterone	3+	3+	3+
11	Femur	March 11, 2011	Bicalutamide, docetaxel, cabazitaxel	1+	1+	2+
12	Soft tissue (autopsy)	September 14, 2013	Abiraterone	1+	3+	3+

NOTE: "AR" and "AR-V7" denote androgen receptor transcript scoring by RNA ISH for full-length and AR-V7 splice variant, respectively. "Housekeeping" denotes housekeeping gene transcript scoring. All samples were positive for housekeeping gene staining at 1+ to 3+. The only sample that scored higher for AR-V7 than for AR was the autopsy sample.

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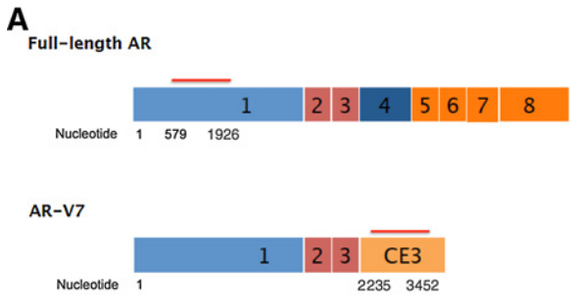

B

RNA ISH Score	Criteria
3+	Signal visible at 2× and 4× magnification
2+	Signal visible at 10× magnification
1+	Signal visible at 20× and 40× magnification
0	Signal not present at 40× magnification

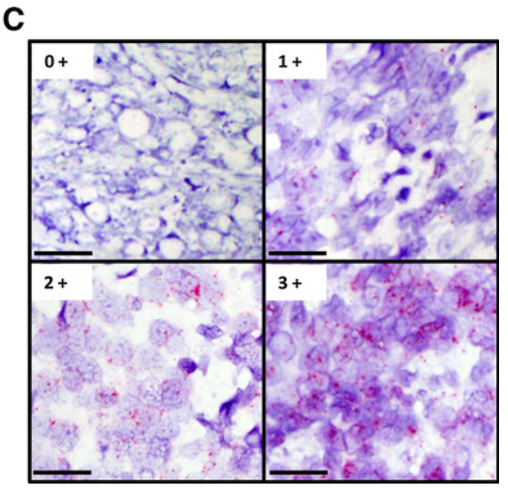

Figure 1.

RNA ISH probes for full-length AR and AR-V7. **A,** Schematic shows target regions for RNA ISH probes within the mRNA for AR and AR-V7. The full-length AR probe was designed to target a region in exon 1 (nucleotides 579–1926), whereas the AR-V7 probe was designed to target the unique cryptic exon CE3 as identified in the NCBI nucleotide database. **B,** RNA ISH scoring system. The RNA ISH signal was scored on a scale of 0 to 3+ according to visual criteria for the presence of transcript at the specified magnifications. **C,** Representative micrographs depicting tumor tissues stained with AR-V7 RNA ISH. When present, AR transcripts stain red. Gleason 6 prostatectomy tumor specimens universally showed no AR-V7 staining at 40X magnification. "Resistant disease" specimens scored 1+ to 3+ for AR-V7 RNA. All micrographs are at 40X magnification. The scale bar indicates 40 μm.

resistance within 9 months of the start of ADT. All patients responded initially to ADT.

Cell lines

Prostate cancer cell lines (VCaP, 22Rv1, PC3, and RWPE-1) were obtained directly from the American Type Culture Collection after authentication by short tandem repeat profiling, and were passaged for fewer than 6 months after receipt or resuscitation. These cell lines were maintained as recommended and used as positive (VCaP and 22Rv1) and negative (RWPE-1 and PC3) controls for the AR-V7 probe. Cell lines were fixed in formalin and incorporated into paraffin blocks, which were then sectioned for testing RNA ISH probes.

Design of RNA ISH probes

Probes are illustrated schematically in Fig. 1. The AR-V7 RNA ISH probe was designed to target the unique cryptic exon CE3 as identified in the NCBI nucleotide database, using multiple tiling probes. The full-length AR probe was designed to target a region in exon 1 (nucleotides 579–1926).

Automated RNA ISH assay

Automated ISH assays for mRNA were performed using the ViewRNA eZl Detection Kit (Affymetrix) on the BOND RX IHC and ISH Staining System with BDZ 6.0 software (Leica Biosystems). FFPE tissue sections on slides were processed automatically from deparaffinization, through ISH staining to hematoxylin counterstaining; sections were coverslipped off-instrument. Briefly, 5-μm sections of FFPE tissue were mounted on Surgipath X-tra glass slides, baked for 1 hour at 60°C, and placed on the BOND RX for processing. The BOND RX user-selectable settings were the ViewRNA ez-l Detection 1-plex (Red) protocol and ViewRNA Dewax1; ViewRNA HIER 10 min, ER1 (95); ViewRNA Enzyme 2 (10); ViewRNA Probe Hybridization. With these settings, the RNA unmasking conditions for the tissue consisted of a 10-minute incubation at 95°C in Bond Epitope Retrieval Solution 1 (Leica Biosystems) followed by 10-minute incubation with Proteinase K from the BOND Enzyme Pretreatment Kit at 1:1,000 dilution (Leica Biosystems). The AR-V7 (Affymetrix; Cat # DVA1-17526) and full-length AR (Affymetrix; Cat # DVA1-17525) mRNA-targeting Probe Sets were diluted 1:40 in ViewRNA Probe Diluent (Affymetrix) for use on the automated platform. mRNA-targeting probe sets composed of a cocktail of GAPDH, PPIB, and ACTB (Affymetrix; Cat # DVA1-16742) were used as positive controls for integrity of RNA. Diluted Probe Set, Diluted Proteinase K, and ViewRNA eZl Detection Kit were loaded onto BOND RX prior to starting the run. After the run, slides were rinsed with water, dried for 30 minutes at room temperature, dipped in xylene, and mounted using Histo-Mount solution (Life Technologies).

Quantification of RNA ISH signal

A semiquantitative scoring method was devised and used to score the RNA ISH signal in FFPE tumor tissues. All the cases were reviewed by three pathologists who were blinded to the clinical background of each sample. Samples with red dots representing signal for AR and AR-V7 seen at 2X and 4X magnification were scored as 3+, signal seen at 10X magnification was scored as 2+, signal seen at 20X and 40X magnification was scored as 1+, and no staining for AR and AR-V7 at 40X magnification was scored as 0 (Fig. 1).

Real-time quantitative reverse transcription PCR

Total RNA was extracted from cell lines using the RNeasy Mini Kit (Qiagen). Note that 1 μg of RNA was used to generate cDNA using SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific). One nanogram of cDNA (equivalent of ~100 cells) was amplified and analyzed in triplicate using the CFX96 Touch

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Real-Time PCR Detection System (Bio-Rad). PrimeTime qPCR assays (IDT) were used. Primer sequences are as follows: AR-V7 (F) 5'-CTTGTCGTCTTCGGAAATGTTATG-3'; AR-V7 (R) 5'-CTTTCTTCAGGGTCTGGTCATT-3'; AR-V7 (probe) 5'-/56-FAM/AAGCAGGGA/ZEN/TGACTCTGGGAGAAA/3IABkFQ/-3'; AR (F) 5'-CAATTACCAGGGTGGGAAGAA-3'; AR (R) 5'-ATGACTTCTG-CAGGTGGATATG-3'; AR (probe) 5'-/56-FAM/AGGCACTAG/ZEN/AACCAGAAACCCTGC-3'.

Statistical analysis

Clinical cohorts were compared with respect to AR-V7 signal using the Fisher exact test for binary variables. OS was measured from the date of the start of ADT to the date of death or last follow-up. Progression-free survival (PFS) was measured from the date of the start of ADT to the date of biochemical disease progression. Survival curves were generated using the Kaplan–Meier method and compared using the log-rank test. Cox proportional hazards regression analyses were used to identify predictors of OS and PFS. Two-sided P values <0.05 were considered statistically significant. Statistical analyses were performed using R, version 3.2.4.

Results

We designed a branched chain RNA ISH assay targeting the unique cryptic exon CE3 (Fig. 1). The automated AR-V7 RNA ISH assay was initially tested on prostate cancer cell lines, demonstrating robust signal in cell lines known to be positive for AR-V7 (VCaP and 22Rv1) and no signal in cell lines negative for AR-V7 (RWPE-1 and PC3; Supplementary Fig. S1). To quantitate the AR-V7 signal, we developed a semiquantitative scoring method based on the magnification required for the visual detection of ISH signal (Fig. 1B and Materials and Methods). Representative images of AR-V7 ISH scoring in patient samples are depicted in Fig. 1C. ISH signal outside of tumor was visualized only in epithelial cell background stroma at a rate of approximately one dot per 50 cells.

We identified cases from within an institutional cohort of men treated for prostate cancer. With four clinical cohorts as described above (see Materials and Methods), a total of 74 archival FFPE prostate cancer patient samples were identified for AR-V7 testing. Among these were 10 samples obtained from targeted needle biopsies of bone lesions. Sample processing of bone specimens was unsuccessful, such that reference positive-control probes for RNA integrity (GAPDH, PPIB, and ACTB) provided inconsistent results (data not shown). We therefore excluded samples obtained from bone biopsy; 64 samples remained for analysis.

"Resistant disease" cohort samples were obtained from patients who had developed mCRPC and progressed despite at least one secondary hormonal manipulation. This cohort demonstrated detectable AR-V7 in all 12 examined samples (Table 1). Prostatectomy cohort samples were obtained from men whose surgical grading demonstrated exclusively Gleason 6, 7, or $\geq$8 adenocarcinoma. This cohort demonstrated detectable AR-V7 in just 1 of the 30 examined samples (Table 2). The sample that did have detectable AR-V7 was Gleason 4 + 5 = 9.

Given the virtually binary distinction of AR-V7 presence by RNA ISH among the above groups, we analyzed pretreatment AR-V7 status as a prognostic biomarker in men with treatment-naïve metastatic disease. Twenty-two men with metastatic prostate cancer had analyzable tissue available prior to the start of ADT. OS was significantly longer among patients who

Table 2. Tissue sample staining by cohort

Brief response				
Sample	**Tissue**	**AR**	**AR-V7**	**Housekeeping**
1	Prostate	1+	1+	3+
2	Urethra	2+	1+	3+
3	Bladder	2+	1+	3+
4	Prostate	1+	1+	3+
5	Prostate	1+	0	2+
6	Prostate	2+	0	3+
7	Prostate	2+	1+	3+
8	Prostate	2+	1+	3+
9	Prostate	1+	0	3+
Sustained response				
Sample	**Tissue**	**AR**	**AR-V7**	**Housekeeping**
1	Prostate	0	0	3+
2	Prostate	1+	1+	3+
3	Lung	0	0	3+
4	Node	2+	1+	3+
5	Node	1+	0	2+
6	Prostate	1+	0	3+
7	Prostate	0	0	2+
8	Pleura	0	0	1+
9	Prostate	1+	0	3+
10	Prostate	1+	0	3+
11	Prostate	3+	1+	3+
12	Node	3+	2+	3+
13	Prostate	1+	0	2+
Gleason 6 prostatectomy				
Sample	**Tissue**	**AR**	**AR-V7**	**Housekeeping**
1	Prostate	0	0	2+
2	Prostate	1+	0	3+
3	Prostate	0	0	3+
4	Prostate	1+	0	3+
5	Prostate	0	0	3+
6	Prostate	0	0	3+
7	Prostate	0	0	2+
8	Prostate	1+	0	3+
9	Prostate	1+	0	2+
10	Prostate	1+	0	2+
Gleason 7 prostatectomy				
Sample	**Tissue**	**AR**	**AR-V7**	**Housekeeping**
1	Prostate	1+	0	3+
2	Prostate	1+	0	3+
3	Prostate	0	0	3+
4	Prostate	1+	0	3+
5	Prostate	0	0	3+
6	Prostate	0	0	3+
7	Prostate	0	0	3+
8	Prostate	0	0	3+
9	Prostate	0	0	3+
10	Prostate	0	0	2+
Gleason >8 prostatectomy				
Sample	**Tissue**	**AR**	**AR-V7**	**Housekeeping**
1	Prostate	0	0	3+
2	Prostate	2+	0	3+
3	Prostate	0	0	0
4	Prostate	0	0	3+
5	Prostate	1+	0	3+
6	Prostate	1+	0	3+
7	Prostate	1+	0	3+
8	Prostate	1+	0	3+
9	Prostate	0	0	0
10	Prostate	2+	1+	3+

NOTE: All samples are baseline biopsy samples prior to systemic therapy (groups 1 and 2) or were prostatectomy specimens. All samples except for two Gleason $\geq$8 prostatectomy samples were positive for housekeeping gene staining at 1+ to 3+.

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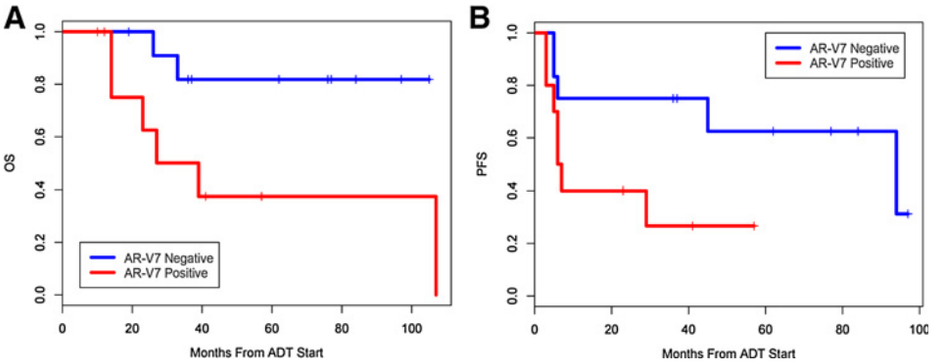

Figure 2.
Clinical outcomes by AR-V7 status within baseline tumor tissue. **A,** Kaplan–Meier curves for OS by AR-V7 status in men with treatment-naïve metastatic disease. OS was significantly longer among patients who did not have detectable AR-V7 (median OS = not reached vs. 33 months; log-rank P = 0.0443). **B,** Kaplan–Meier curves for PFS by AR-V7 status in men with treatment-naïve metastatic disease. There was a trend toward superior PFS among patients who did not have detectable AR-V7 RNA (median PFS = 94 vs. 6.5 months; log-rank P = 0.0549).

did not have detectable AR-V7 (log-rank P = 0.0443; Fig. 2A). There was a trend toward superior PFS in this cohort (log-rank P = 0.0549; Fig. 2B).

We tested several clinically relevant variables in Cox proportional hazards models, although we recognized that the small sample size increased the risk of an overfit model. In a multivariate model for OS that included AR-V7 status, patient age, pretreatment PSA, and sites of disease (lymph node vs. bone vs. visceral), age and PSA were not significant, whereas the HR for AR-V7 remained significant at 18.9 [95% confidence interval (CI), 1.7–216.3; P = 0.018]. In a similar model for PFS, only AR-V7 was significant with an HR of 3.8 (95% CI, 1.02–14.4; P = 0.046). However, these exploratory analyses were based on a limited sample size and a small number of events, and require validation in larger patient cohorts.

We hypothesized that the presence of AR-V7 RNA within pretreatment tumor tissue would be more common among men who exhibited a sustained response to first-line ADT than among men who experienced progression less than 9 months after initiation of ADT. "Sustained response" and "brief response" cohorts were compared for the prevalence of AR-V7 RNA prior to the initiation of therapy. Detectable AR-V7 was more common among "brief response" samples (6 of 9; 67%) than among "sustained response" samples (4 of 13; 31%); this difference was not statistically significant (P = 0.192). Of the pretreatment samples that were positive for AR-V7, the staining score was predominantly 1+ (9 of 10).

All of the tested samples had equal or less splice variant staining compared with full-length AR staining. Within pretreatment samples, AR staining was generally 0+ to 2+, whereas AR-V7 staining was generally 0+ to 1+ (Table 2). Within the heavily pretreated "resistant disease" cohort samples, AR staining was 2+ to 3+ in 11 of 12 samples.

In a single patient who went on to autopsy, AR-V7 was identified within tissue obtained prior to first-line ADT (1+) and in generally greater quantity within tissue that had been exposed to and progressed despite abiraterone/prednisone therapy (1+ to 3+, depending on site; Fig. 3).

Discussion

In order to improve understanding of therapeutic resistance to hormonal therapies for prostate cancer, we developed a branched chain RNA ISH assay to detect the AR-V7 splice variant within archival FFPE prostate cancer. We detected AR-V7 splice variant RNA within all samples from men with mCRPC and progression despite at least one secondary hormonal manipulation. We detected AR-V7 within 1 of 30 samples among men who underwent up-front prostatectomy for clinically localized disease. Finally, patients without detectable AR-V7 RNA at baseline in the presence of metastatic disease had significantly longer OS and a nonsignificant trend toward superior PFS.

Our results demonstrate the feasibility of splice variant RNA assessment in archival tissue dating back to January 2000. Hypothesized and experimental results aligned within the "resistant disease" cohort and prostatectomy cohort samples. These results require broader validation within larger and cross-institutional cohorts. Given that the testing methodology is well-defined and can be carried out on archival tissue, technique reproducibility can be easily examined. Further, the possibility of automated scoring could be investigated.

We generally observed more AR-V7 transcript in resistant samples compared with pretreatment samples. This aligns with the published experience of AR-V7 testing within CTCs as the presence of transcript and quantity become more likely in more heavily treated cancers (8). We observed equal or greater score for AR transcript compared with AR-V7 in all samples. This, too, aligns with the published experience of AR-V7 RNA testing within CTCs.

Patients with detectable AR-V7 prior to starting ADT had significantly shorter OS compared with patients without detectable AR-V7. These data suggest that AR-V7 detection could have utility as a prognostic biomarker in men with a new diagnosis of metastatic prostate cancer. Confirmation of the prognostic utility of AR-V7 for treatment-naïve metastatic prostate cancer patients could identify subgroups that may benefit from novel therapeutic approaches from treatment initiation.

We describe preliminary results of the biomarker characteristics of AR-V7 by RNA ISH in a pilot study of men with newly diagnosed metastatic prostate cancer. Response to first-line ADT is highly variable and can vary from primary resistance to more than one decade of response. Although AR-V7 was detectable more frequently in the "brief response" cohort than the "prolonged response" cohort, there was notable variability. The clinical relevance, for instance, of 2+ AR-V7 staining within pretreatment tissue for a patient who has responded to first-line ADT for greater than 3 years is not known.

The sensitivity of RNA ISH is a potential advantage of the technique, but also presents a technical challenge in evaluating

Published OnlineFirst July 20, 2016; DOI: 10.1158/1078-0432.CCR-16-0237

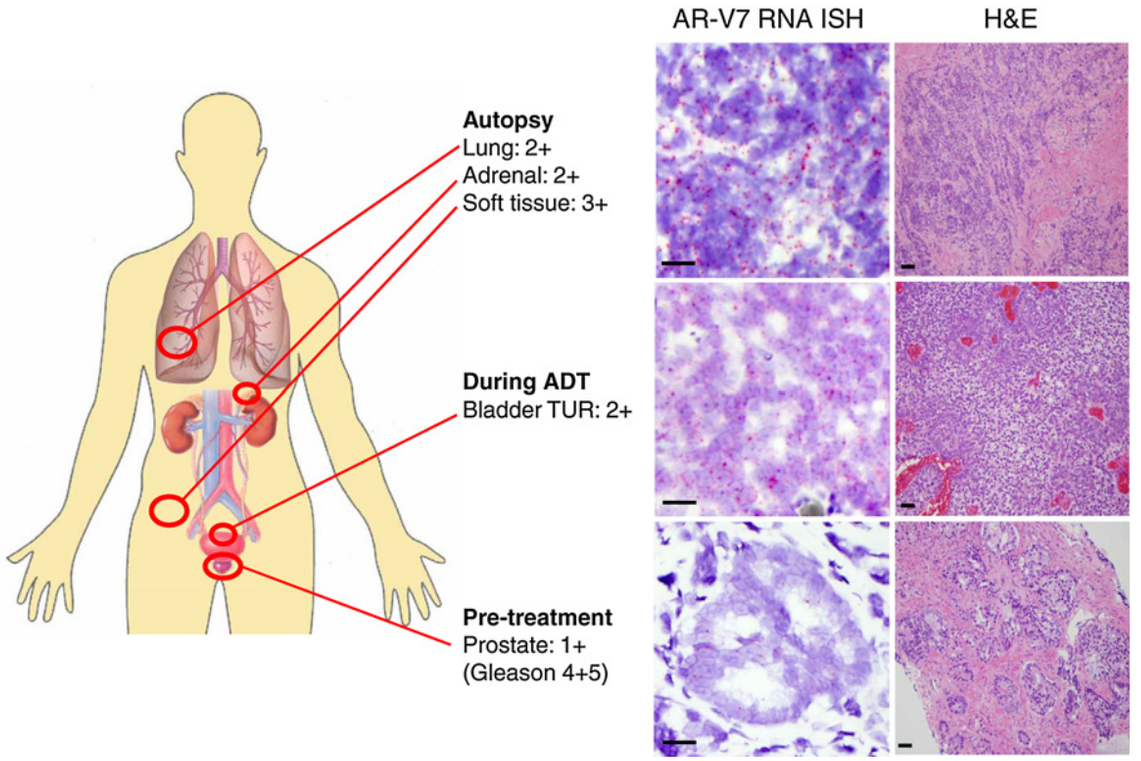

Figure 3.
Serial assessment of AR-V7 expression within an individual patient with metastatic prostate cancer. Serial archival tissue samples within a single patient depict incremental increase in AR-V7 transcript. Right column of images are hematoxylin and eosin (H&E) at 20× magnification; left column of images are RNA ISH at 40× magnification. AR-V7 was detectable with a score of 1+ at time of initial diagnosis when trans-rectal biopsy revealed Gleason 5 + 4 = 9 adenocarcinoma (bottom row). AR-V7 score was 2+ within tumor tissue resected at the bladder neck when the patient presented with hematuria while receiving ADT (middle row). The patient developed biochemical castration resistance 8 months after start of ADT and responded to abiraterone/prednisone for just 2 months. Autopsy revealed prostate cancer tissue staining 2 + and 3 + for AR-V7 within lung, adrenal gland, and soft tissue (top row). The scale bar indicates 40 μm.

its specificity. RNA ISH allows for the detection of RNA transcripts within individual cells in archival FFPE tissues. In contrast, alternative methods such as qRT-PCR require many more intact cells to reliably produce a signal of a given transcript. The quantity and quality of extracted RNA from FFPE tissues are low compared with fresh or frozen tissues, and often insufficient to achieve a reliable qRT-PCR signal. It is therefore difficult to define sensitivity and specificity of RNA ISH in FFPE tissues because of the absence of a sufficiently sensitive gold-standard comparison testing method. We acknowledge this as a limitation.

Our study has additional notable limitations. First, ISH probe design is dependent upon unique sequences for specific splice variants. Probe design generally requires multiple 20mer probes (between 20 and 40) across a given transcript such that 500 to 1,000 base pairs of unique target sequence must be identified. In the case of AR-V567, these requirements limited probe design because the only unique feature of this AR splice variant transcript is the presence of a junction across exons 4 and 8. Second, tumor samples obtained from bone did not produce reliable RNA staining. This is likely a result of necessary decalcification that compromises the integrity of the RNA. Third, our methods for investigating AR-V7 as a biomarker were retrospective and must be validated in a prospective experience. Fourth, although the automated RNA ISH assay is highly reproducible in our hands, it will be necessary to confirm its reproducibility in different laboratories and institutions. Finally, the small number of samples tested is an inherent limitation of our pilot study. Our results are preliminary and require broader validation within larger cross-institutional cohorts.

This methodology for the testing of RNA within FFPE has numerous potential applications. Examination of AR-V7 prevalence and quantity within archival tumor tissue from larger cohorts with long-term clinical follow-up could clarify its role as a predictive biomarker in multiple clinical settings. In addition, probe design could facilitate testing for additional AR splice variants using this same technique. Finally, this technique could be used more broadly to characterize expression patterns of specific transcripts in a variety of cancers.

Disclosure of Potential Conflicts of Interest

V. Deshpande, M.N. Rivera, and D.T. Ting have patent ownership in and report receiving commercial research grants from Affymetrix. No potential conflicts of interest were disclosed by the other authors.

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Acknowledgments

We thank K. Broderick for technical assistance. This work was partially supported by the Department of Defense (D.T. Miyamoto), Prostate Cancer Foundation (P.J. Saylor, R.J. Lee, D.T. Miyamoto), Burroughs Wellcome Trust (D.T. Ting), NIH 4K12CA087723-14 (D.T. Ting), and NIH P01CA120964 (C.-L. Wu).

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Received February 5, 2016; revised June 19, 2016; accepted July 1, 2016; published OnlineFirst July 20, 2016.

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Published OnlineFirst July 20, 2016; DOI: 10.1158/1078-0432.CCR-16-0237

Clinical Cancer Research

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Clin Cancer Res 2017;23:363-369. Published OnlineFirst July 20, 2016.