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TITLE: Noninvasive Detection of AR-FL/AR-V7 as a Predictive Biomarker for
Therapeutic Resistance in Men with Metastatic Castration-Resistant Prostate Cancer

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14. ABSTRACT Understanding primary and acquired resistance to abiraterone and enzalutamide, and developing analytically validated and clinically qualified predictive biomarkers, remains a critically important unmet medical need. We propose non-invasive detection of full-length androgen receptor (AR-FL) and the androgen receptor splice variant 7 (AR-V7) (AR-FL/AR-V7) as a predictive biomarker for therapeutic resistance in men with metastatic castration-resistant prostate cancer.					
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- 1. INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Understanding primary and acquired resistance to abiraterone and enzalutamide, and developing analytically validated and clinically qualified predictive biomarkers, remains a critically important unmet medical need. We propose non-invasive detection of full-length androgen receptor (AR-FL) and the androgen receptor splice variant 7 (AR-V7) (AR-FL/AR-V7) as a predictive biomarker for therapeutic resistance in men with metastatic castration-resistant prostate cancer. Using a laboratory-developed, RNA-based assay modified from a commercially available circulating tumor cell (CTC) detection platform, we have developed standard operating procedures and performed extensive internal validation and quality control studies to determine its feasibility for detection of AR-FL/AR-V7 in blood samples. Although our recent studies show data supporting this predictive biomarker, analytical validation is required prior to clinical use, and a large-scale, multi-institutional study is needed to further establish clinical utility. The overall objective of the project is to enable precision therapy of metastatic castration-resistant prostate cancer by developing non-invasive tests for the AR-FL/AR-V7.

- 2. KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Prostate cancer, CRPC, AR-V7, liquid biopsy, resistance, abiraterone, enzalutamide

- 3. ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.

What were the major goals of the project

Major Task 1: Development of robust and standardized SOPs pertaining to the accurate and reliable detection of AR-FL/ARV7.

Subtask 1: To conduct essential study planning activities including IRB and HRPO approval, ordering of a common set of reagents, equipment readiness, protocol review, distribution of SOPs, personnel assignment, and review of documentation requirements (Months 1-6).

Subtask 2: Testing SOPs pertaining to the accurate and reliable detection of AR-FL/AR-V7.

Subtask 3: Development of robust SOPs for sample collection, processing, and transfer (Months 7-12).

Major Task 2: Correlation between CTC AR expression with contemporaneously acquired fresh CRPC biopsy expression, and with expression detected in cell-free exosome RNA.

Subtask 1: Correlation between CTC AR expression with contemporaneously acquired fresh CRPC biopsy expression. (Months 7-24).

Subtask 2: Correlation between CTC AR expression with expression detected in cell-free exosome RNA. (Months 7-24).

Major Task 3: Development of new CTC selection and molecular detection platforms

Subtask 1: Evaluation of new CTC selection platform for the purpose of detection of AR-FL/AR-V7 (Months 12-24).

Subtask 2: Evaluation of new molecular detection platforms (Months 12-24).

Major Task 4: Clinical validation of the AR-FL/AR-V7 test

Subtask 1: Prospective recruitment of 300 patients with mCRPC initiating standard-of-care treatment with abiraterone, enzalutamide, or chemotherapy consenting for blood draw (baseline, 2nd at the time of response if any, and 3rd time at the time of progression), and optional biopsy (~n=50) (Months 12-30)

Subtask 2: Biomarker implementation in certified labs (Months 12-30).

Subtask 3: Data analysis (Months 30-36).

Major Task 5: Biomarker-embedded trial of enzalutamide and AKT inhibitor

Subtask 1:

• Recruit, consent, and enroll 140 patients/human subjects to Phase I/II trial.

• Evaluation of the association between CTC counts, ARFL/ AR-V7 expression, and PTEN status, and all these parameters to response to treatment (Months 6-30).

Subtask 2: Collection and documentation of 20 pre and post-treatment biopsies from men enrolled in the trial for collaborative studies with Dr. Luo (Months 6-12).

Major Task 6: Alternative approaches

Subtask 1: Formulation of additional biomarker-driven clinical trials (Months 24-36).

Subtask 2: Additional studies according to FDA/EMA guidance (Months 24-36).

What was accomplished under these goals?

Task 1: We have completed this task. All regulatory documents are in place and all required collaborative agreements have been signed. We have distributed SOPs and compared the data across different institutions. The test has been analytically validated at Johns Hopkins University, leading to a publication focusing on analytical performance of the test.

Task 2: We have completed the experimental part of this task. A manuscript evaluating the correlation between CRPC biopsy and CTC marker status is under preparation by the three principle investigators.

Task 3: Subtask 1 will be reported by one of the principle PIs, Dr. Stephen Plymate. Subtask 2 has been completed by Drs. Luo, de Bono, and Plymate, leading to publication currently in press.

Task 4: On-going. JHU has recruited 130 patients. Biomarker has been implemented in JHU CLIA lab. A total of ~500 patients have been tested in the clinic. A separate cohort of 135 patients have been tested by both the CLIA lab and the Luo research lab. A manuscript on clinical utility of the test has been accepted for publication.

Task 5: Samples are being obtained routinely from patients and are being processed to the cDNA stage. JHU has tested 63 samples shipped from the de Bono group. Data is being unblinded and analyzed.

Task 6: Future work

What opportunities for training and professional development has the project provided?

The laboratory of Dr. Luo hosted a Scientific Officer from the Prof. de Bono group to train in the Adnatest to ensure good technical practice. A postdoc research fellow from Dr. Luo group (Dr. Yezi Zhu) and a clinical fellow from Dr. de Bono group (Dr. Adam Sharp) have collaborated and co-authored a manuscript to be published in European Urology.

How were the results disseminated to communities of interest?

Results from this project were disseminated to communities of interest through peer-reviewed publications.

What do you plan to do during the next reporting period to accomplish the goals?

We will continue to recruit patients into this study by coordinating with collaborating sites. We will continue to advocate the utility of the test in clinical trials. In addition, we are expanding our cohort for more robust clinical utility studies. We expect to complete all Tasks 3 during year 3 of the project period. We will continue to disseminate study results to communities of interest.

4. IMPACT: Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?

Following analytical validation, we have realized patient benefit by making a clinical grade test available to patients at the Johns Hopkins University. Since the implementation of the test, more than 500 patients have been tested. A small cohort of the patients were evaluated for patient benefit. A manuscript describing our experience in analytical validation of the test was published, and a manuscript focusing on clinical utility and patient benefit is currently in press.

What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Nothing to Report.

What was the impact on society beyond science and technology?

We believe men with metastatic CRPC will benefit from the availability of the test. A manuscript evaluating how the test results are utilized by providers and patients and whether the availability of the test resulted in better patient outcome is currently in press. This information will provide guidance to providers, patients, and insurers.

- 5. CHANGES/PROBLEMS:** The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Nothing to Report.

Actual or anticipated problems or delays and actions or plans to resolve them

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Nothing to Report.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to Report.

Significant changes in use or care of vertebrate animals

N/A

Significant changes in use of biohazards and/or select agents

Nothing to Report.

6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state “Nothing to Report.”

i Publications, conference papers, and presentations

Report only the major publication(s) resulting from the work under this award.

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

1. Markowski MC, Silberstein JL, Eshleman JR, Eisenberger MA, Luo J, Antonarakis ES. Clinical Utility of CLIA-Grade AR-V7 Testing in Patients With Metastatic Castration-Resistant Prostate Cancer. *JCO Precis Oncol.* 2017;2017. Epub 2017/11/25. doi: 10.1200/PO.17.00127. PubMed PMID: 29170762. (In press, acknowledged federal support)
2. Zhu Y, Sharp A, Anderson CM, Silberstein JL, Taylor M, Lu C, Zhao P, De Marzo AM, Antonarakis ES, Wang M, Wu X, Luo Y, Su N, Nava Rodrigues D, Figueiredo I, Welti J, Park E, Ma XJ, Coleman I, Morrissey C, Plymate SR, Nelson PS, de Bono JS, Luo J. Novel Junction-specific and Quantifiable In Situ Detection of AR-V7 and its Clinical Correlates in Metastatic Castration-resistant Prostate Cancer. *European urology.* 2017. Epub 2017/09/04. doi: 10.1016/j.eururo.2017.08.009. PubMed PMID: 28866255. (In press, acknowledged federal support)
3. Antonarakis ES, Lu C, Luber B, Wang H, Chen Y, Zhu Y, Silberstein JL, Taylor MN, Maughan BL, Denmeade SR, Pienta KJ, Paller CJ, Carducci MA, Eisenberger MA, Luo J. Clinical Significance of Androgen Receptor Splice Variant-7 mRNA Detection in Circulating Tumor Cells of Men With Metastatic Castration-Resistant Prostate Cancer Treated With First- and Second-Line Abiraterone and Enzalutamide. *J Clin Oncol.* 2017;35(19):2149-56. Epub 2017/04/07. doi: 10.1200/JCO.2016.70.1961. PubMed PMID: 28384066; PMCID: PMC5493048. (Published, acknowledged federal support)
4. Lokhandwala PM, Riel SL, Haley L, Lu C, Chen Y, Silberstein J, Zhu Y, Zheng G, Lin MT, Gocke CD, Partin AW, Antonarakis ES, Luo J, Eshleman JR. Analytical Validation of Androgen Receptor Splice Variant 7 Detection in a Clinical Laboratory Improvement Amendments (CLIA) Laboratory Setting. *J Mol Diagn.* 2017;19(1):115-25. Epub 2016/12/06. doi: 10.1016/j.jmoldx.2016.08.003. PubMed PMID: 27916435. (Published, acknowledged federal support)
5. Antonarakis ES, Armstrong AJ, Dehm SM, Luo J. Androgen receptor variant-driven prostate cancer: clinical implications and therapeutic targeting. *Prostate cancer and prostatic diseases.* 2016;19(3):231-41. Epub 2016/05/18. doi: 10.1038/pcan.2016.17. PubMed PMID: 27184811; PMCID: PMC5493501. (Published, acknowledged federal support)

Books or other non-periodical, on(1)e-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to Report.

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to Report.

ï Website(s) or other Internet site(s)

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to Report.

ï Technologies or techniques

Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to Report.

i Inventions, patent applications, and/or licenses

Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to Report.

i Other Products

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name	Role	Percent Effort
Sokoll , Lori	Logistical and regulatory consult, Co-Investigator	5
Luo , Jun	Principle Investigator, overall management	30
Demarzo , Angelo	Tissue-based studies, Co-Investigator	3.99
Eshleman , James	CLIA lab activities, Co-Investigator	4.02
Paller , Channing	Oncology planning, Co-Investigator	3.67
Isaacs , William	Scientific guidance, Co-Investigator	7.83
Antonarakis , Emmanuel	Oncology lead, Co-Investigator	8.40
Wang , Hao	Statistician, Co-Investigator	15
Lu , Changxue	quality control, protocol development	50
Zhu, Yezi	technological development	60
Riel, Stacy	CLIA coordination, lab management	50

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not

necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to Report.

What other organizations were involved as partners?

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to <https://ers.amedd.army.mil> for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on <https://www.usamraa.army.mil>) should be updated and submitted with attachments.

- 9. APPENDICES:** Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

Five journal articles are attached.

Clinical Utility of CLIA-Grade AR-V7 Testing in Patients With Metastatic Castration-Resistant Prostate Cancer

Purpose A splice variant of the androgen receptor, AR-V7, confers resistance to AR-targeted therapies (ATTs) but not taxane chemotherapies in patients with metastatic castration-resistant prostate cancer. Since August 2015, a clinical-grade assay to detect AR-V7 messenger RNA expression in circulating tumors cells (CTCs) has been available to providers through a Clinical Laboratory Improvement Amendments–certified laboratory at Johns Hopkins University.

Methods We contacted ordering providers of the first 150 consecutive tests by using a questionnaire-based survey to determine how the results of AR-V7 testing were used to influence clinical practice.

Results In all, 142 (95%) of 150 questionnaires were completed by 38 providers from 29 sites across the United States and Canada. AR-V7 test results were reported either as CTC– (28%), CTC+/AR-V7– (30%), or CTC+/AR-V7+ (42%). Prevalence of AR-V7 detection increased with prior exposure to ATTs (abiraterone and enzalutamide naïve, 22%; after abiraterone or enzalutamide, 35%; after abiraterone and enzalutamide, 43%). Overall, management was affected by AR-V7 testing in 53% of the patients and even more often with CTC+/AR-V7+ results. AR-V7+ patients were commonly switched from ATT to taxane chemotherapy (43%) or were offered a clinical trial (43%); management remained unchanged in only 14% of these patients. Overall, patients who had a change in management on the basis of AR-V7 testing were significantly more likely to achieve a physician-reported 50% decline in prostate-specific antigen response on next-line therapy than those who did not change treatment (54% v 31%; $P = .015$).

Conclusion Providers used AR-V7 testing to influence clinical decision making more often than not. Physicians reported that men with AR-V7+ results had the most treatment changes, and such men were preferentially managed with taxane therapy or offered a clinical trial, which may have improved outcomes.

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INTRODUCTION

Metastatic castration-resistant prostate cancer (mCRPC) remains dependent on androgen receptor (AR) signaling for survival in the presence of low testosterone levels.¹ Two novel AR-targeted therapies (ATTs), abiraterone and enzalutamide, induced high objective response rates and improved overall survival in patients with mCRPC.^{2,3} Treatment with abiraterone or enzalutamide in the prechemotherapy setting led to a 50% decline in prostate-specific antigen (PSA₅₀) response in 62% and 78% of these patients,

respectively.^{2,3} However, sequential use of abiraterone and enzalutamide has resulted in much lower PSA response rates with the use of the second ATT agent (ie, abiraterone after enzalutamide, 10% to 15%; enzalutamide after abiraterone, 20% to 30%).⁴⁻⁶ These data underscore the importance of identifying a predictive biomarker of resistance to ATT to prevent the use of subsequent futile therapy.

Potential mechanisms of resistance to ATT include AR amplification and mutation, as well as the expression of AR splice variants.⁷ A well-characterized

Mark C. Markowski
John L. Silberstein
James R. Eshleman
Mario A. Eisenberger
Jun Luo
Emmanuel S.
Antonarakis

Author affiliations and support information (if applicable) appear at the end of this article.
The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Cancer Institute or the National Institutes of Health.

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splice variant, AR-V7, is a truncated form of full-length AR (AR-FL) that lacks the ligand-binding domain but retains both the transactivation and DNA-binding domains, allowing for constitutive AR signaling in the absence of androgen.⁸⁻¹⁰ In patients with mCRPC, detection of AR-V7 in circulating tumors cells (CTCs) was shown to predict resistance to novel ATTs.¹¹⁻¹³ Moreover, AR-V7+ patients had a significantly shorter overall survival, suggesting a prognostic value of AR-V7 in addition to its use as a predictive biomarker.^{11,13}

Chemotherapy is an alternative to ATT for AR-V7+ patients with mCRPC. Detection of AR-V7 has been shown not to preclude response to taxane-based chemotherapy.^{14,15} Further prospective investigation found a significant survival benefit with the use of taxanes versus ATTs in patients with AR-V7+ disease.¹⁶ Interestingly, the presence of this splice variant is a dynamic feature with possible conversion from AR-V7+ to AR-V7- status after chemotherapy with taxanes.^{14,17} These data suggest that serial AR-V7 testing may guide the clinical treatment of patients with mCRPC. Those patients with AR-V7- prostate cancer may continue to benefit from ATT, whereas chemotherapy may be more effective in patients with detectable AR-V7 transcript.¹⁸

If the clinical utility of AR-V7 testing can be confirmed, it may also have an economic benefit. In a recent study, we modeled the cost of treating all patients with mCRPC with abiraterone or enzalutamide versus using AR-V7 testing to direct treatment.¹⁹ By using a clinical scenario in which AR-V7+ patients were changed from treatment with abiraterone and/or enzalutamide to chemotherapy thus avoiding the cost of futile ATT therapy, AR-V7 testing resulted in a theoretical cost savings to the health care system of \$150 million per year. To this end, since August 2015, clinical-grade AR-V7 testing performed in a Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory at Johns Hopkins University has been available to health care providers for clinical use.²⁰ However, despite the commercial availability of this AR-V7 test, its clinical utility is unknown. Here, we have retrospectively compiled questionnaire-based data on how ordering providers are applying the results of AR-V7 testing in their clinical practice to influence decision making.

METHODS

The analytical validation and test characteristics of our CLIA-grade AR-V7 assay have been described previously.²⁰ Our molecular pathology database was queried for all AR-V7 tests ordered by internal and external providers for clinical

purposes. We identified patients by name, date of birth, date of testing, AR-V7 status, and the ordering provider of each test. Clinical results of this test were reported as CTC-, CTC+/AR-V7-, or CTC+/AR-V7+, because each of these categories is associated with different outcomes.¹² A clinical utility questionnaire (Data Supplement) was generated for each AR-V7 test ordered and was mailed or e-mailed to each ordering provider. We defined a biomarker-based change in treatment as a confirmation of treatment choice or a change from one therapy to another after AR-V7 testing. The institutional review board at Johns Hopkins University approved this study and granted a waiver of consent to contact the provider of each AR-V7 test ordered because that was considered a clinical audit. The ordering provider was then contacted for participation and asked to complete a questionnaire pertaining to treatment decisions that were made on the basis of results of that specific AR-V7 test. Participation in this study was voluntary. If a provider did not wish to participate or the questionnaire was not returned after two attempts to contact the provider, data for that patient were not included in the analysis.

One hundred fifty consecutive AR-V7 clinical test results were obtained between August 31, 2015, and August 31, 2016, representing the first 150 tests ordered. From these, 142 questionnaires (95%) were completed and returned by 38 providers across 29 sites (28 in the United States [in 22 states] and one in Canada). Statistical analyses for this project were largely descriptive. In specified cases, a two-tailed Fisher's exact test was used to compare proportions between two or more groups. The significance level was set at $P < .05$, and corrections were not performed for multiple comparisons.

RESULTS

Information from 142 of 150 questionnaires sent (95% participant response rate) were included in this analysis. All 142 AR-V7 tests were ordered for patients with mCRPC. The number of lines of additional systemic therapies for mCRPC among these patients was reported as follows: 24% ($n = 33$) had received no other lines, 27% ($n = 39$) had received one line, 27% ($n = 39$) had received two lines, 13% ($n = 18$) had received three lines, and 9% ($n = 13$) had received four or more lines of systemic therapy before testing. Eighteen percent of men ($n = 26$) had previously received bicalutamide, 46% ($n = 66$) had received abiraterone, 49% ($n = 70$) had received enzalutamide, 48% ($n = 69$) had received docetaxel, and 13% ($n = 18$) had received cabazitaxel.

Table 1. Summary of AR-V7 Test Results According to the Number of Novel ATTs Previously Received

Test Result	Total No. of Tests		Abiraterone and Enzalutamide		Abiraterone or Enzalutamide		Abiraterone and Enzalutamide Naïve	
	n/N	%	n/N	%	n/N	%	n/N	%
CTC–	40/142	28.17	8/40	20.0	11/40	27.5	21/40	52.5
CTC+/AR-V7–	42/142	29.58	8/42	19.0	20/42	47.62	14/42	33.33
CTC+/AR-V7+	60/142	42.25	26/60	43.33	21/60	35.0	13/60	21.66

Abbreviations: AR-V7, androgen receptor splice variant 7; ATT, AR-targeted therapy; CTC, circulating tumor cell; n/N, number of patients in that category divided by total number of patients.

Overall, the prevalence of a CTC– result was 28%, the prevalence of a CTC+/AR-V7– result was 30%, and the prevalence of a CTC+/AR-V7+ result was 42%. We then subdivided test results according to physician-reported prior treatment with a novel ATT (Table 1). The majority of patients without detectable CTCs were naïve to both abiraterone and enzalutamide (53%). Patients who were treated with abiraterone and/or enzalutamide resulted in a higher prevalence of AR-V7 detection compared with patients who were not treated with an ATT: 22% of treatment-naïve patients were AR-V7+; after treatment with abiraterone or enzalutamide, 35% of patients were AR-V7+; and after treatment with abiraterone and enzalutamide, 43% of patients were AR-V7+.

To assess the clinical utility of AR-V7 testing, providers were asked whether the AR-V7 status influenced their decision making. The majority of AR-V7– tests (CTC– or CTC+/AR-V7–) did not change the clinical practice of the providers (Table 2). However, almost two thirds (62%) of AR-V7+ tests resulted in a change in management. In patients for whom treatment was changed, providers were then asked to specify the type of therapy selected on the basis of the test result. We

also stratified those responses by AR-V7 test result (Table 3). Patients with an AR-V7– result (CTC– or CTC+/AR-V7–) were preferentially treated with an ATT agent (confirmed AR treatment, or changed from taxane to AR therapy). A smaller subset of AR-V7– patients were treated on a clinical trial or changed to chemotherapy. Conversely, after an AR-V7+ result, most patients were changed from an ATT agent to taxane chemotherapy (43%) or were enrolled in a clinical trial (43%). A list of these AR-V7–directed clinical trials is provided in Appendix Table A1. Providers were next asked to self-report whether each patient achieved a PSA₅₀ response on next-line systemic therapy (ie, the subsequent therapy selected after the AR-V7 test result (Table 4). The physician-reported PSA₅₀ response rate (PSA₅₀ RR) was significantly higher among patients in whom management was changed on the basis of AR-V7 testing compared with those in whom treatment was not altered (54% *v* 31%; *P* = .015). PSA₅₀ RR data were missing from 16% (*n* = 12) and 22% (*n* = 15) of questionnaires in which management was changed or not changed, respectively.

We also investigated which systemic therapy was used in patients with mCRPC after progression on both abiraterone and enzalutamide, according to AR-V7 status (Table 5). For patients with an AR-V7– result, most (44%) were offered standard taxane-based chemotherapy, and 19% enrolled on a clinical trial. By contrast, AR-V7+ patients who had already received abiraterone and enzalutamide were more often treated on a clinical trial (54%) compared with treatment using chemotherapy (19%). For patients who had received abiraterone and enzalutamide, we investigated the prevalence of AR-V7 positivity after physician-reported treatment with docetaxel (Appendix Table A2). No significant difference in prior docetaxel treatment was observed between AR-V7– (56% [nine of 16]) and AR-V7+ (73% [19 of 26]; *P* = .32) patients. In the chemotherapy-naïve group, no numerical difference was noted in the reported clinical trial enrollment based on AR-V7 status. In the patients who were treated with docetaxel, those who were AR-V7– were commonly treated with standard chemotherapy (ie, cabazitaxel; 67% [six of nine]) whereas AR-V7+ patients were more frequently placed on a clinical trial (58% [11 of 19]).

Finally, we examined provider treatment preferences for AR-V7+ patients irrespective of the prior therapies received (Table 6). These patients were most commonly treated on either a clinical trial (35%) or with taxane chemotherapy (32%), similar to those patients who had previously exhausted

Table 2. Clinical Utility of AR-V7 Testing in Patients With mCRPC

Test Result	Did the AR-V7 Assay Result in a Change in Management for This Patient?			
	Yes		No	
	n/N	%	n/N	%
CTC–	18/40	45.0	22/40	55.0
CTC+/AR-V7–	20/42	47.62	22/42	52.38
CTC+/AR-V7+	37/60	61.67	23/60	38.33

Abbreviations: AR-V7, androgen receptor splice variant 7; CTC, circulating tumor; mCRPC, metastatic castration-resistant prostate cancer; n/N, number of patients in that category divided by total number of patients.

Table 3. Change in Management on the Basis of AR-V7 Testing in Patients with mCRPC

Test Result	How Did the AR-V7 Assay Change Your Management?											
	AR → Taxane		Taxane → AR		Confirmed AR		Confirmed Taxane		Clinical Trial		No Response	
	n/N	%	n/N	%	n/N	%	n/N	%	n/N	%	n/N	%
CTC–	0		7/18	38.89	6/18	33.33	1/18	5.56	4/18	22.22	0	
CTC+/AR-V7–	0		6/20	30.0	10/20	50.0	2/20	10.0	1/20	5.0	1/20	5.0
CTC+/AR-V7+	16/37	43.24	0		1/37	2.70	3/37	8.11	16/37	43.24	1/37	2.70

Abbreviations: AR, androgen receptor; AR-V7, androgen receptor splice variant 7; CTC, circulating tumor; mCRPC, metastatic castration-resistant prostate cancer; n/N, number of patients in that category divided by total number of patients.

all ATT options. A minority of patients (7%) received either enzalutamide or abiraterone despite an AR-V7+ result, whereas all AR-V7+ patients managed with observation (10%) enrolled in hospice shortly thereafter.

To summarize the data compiled from providers' real-world experience with AR-V7 testing, we propose a hypothetical treatment algorithm for making decisions regarding patients with mCRPC using AR-V7 as a potential treatment-selection biomarker (Fig 1). After first-line systemic therapy with abiraterone or enzalutamide, AR-V7+ patients would preferentially cross over to taxane-based therapy, whereas those who are AR-V7– may continue on a second ATT. Because of occasional conversions from AR-V7+ to AR-V7– status, men progressing on taxane treatment can be retested and could potentially consider treatment with an ATT if the AR-V7 status reverts to negative. Finally, even patients progressing after treatment with abiraterone and enzalutamide may be considered for AR-V7 testing if an AR-V7–directed clinical trial is available.

DISCUSSION

The optimal sequencing of therapeutic agents in patients with mCRPC is unknown and remains a major challenge. The recent discovery of CTC-based AR-V7 detection as a potential predictive biomarker of ATT resistance (but not taxane resistance) may aid in such treatment decisions. To this end, the National Comprehensive Cancer Network prostate cancer guidelines now suggest that AR-V7 testing can be considered and may play a role in guiding therapy selection in mCRPC, but at this time, these guidelines have not gone as far as recommending testing to determine treatment choice. In another recent consensus report, the majority of prostate cancer specialists polled (59%) stated that AR-V7 testing would be useful for some (majority or minority of) patients with mCRPC.²¹ Although further prospective validation of the predictive ability of

AR-V7 is currently ongoing, here we investigated the clinical utility of AR-V7 testing in a real-world setting.

We asked providers whether the result of the AR-V7 test influenced their clinical practice for that specific patient. Overall, more than 50% of providers stated that the AR-V7 test changed their treatment decision. Providers were also asked to self-report whether patients achieved a PSA₅₀ response on their next-line therapy. Importantly, we observed a significantly higher PSA₅₀ RR in patients whose providers used the AR-V7 result to change their therapy. Although our findings are retrospective, they suggest that AR-V7 testing may possibly lead to improved clinical responses to treatment, at least in terms of PSA₅₀ RR. We did not assess for radiographic progression-free or overall survival, which may not have correlated with PSA₅₀ RR.

The statistically significant PSA₅₀ RR difference between the change and no-change groups is largely driven by the AR-V7+ subgroup (described in Appendix Table A3). AR-V7+ patients for whom management did not change had a PSA₅₀ RR of 5% (*v* 39% in AR-V7+ patients for whom treatment was changed). A clear limitation to our study is that we did not explicitly ask providers to list the specific next-line therapy for those patients

Table 4. PSA₅₀ Response Rate to Next-Line Therapy Based on Change in Clinical Practice After AR-V7 Testing

Management	Did the Patient Achieve a PSA ₅₀ Response on Next-Line Therapy?			
	Yes		No	
	n/N	%	n/N	%
Changed	34/63	53.97	29/63	46.03
Did not change	16/52	30.77	36/52	69.23

NOTE. Fisher's exact test *P* = .015.

Abbreviations: AR-V7, androgen receptor splice variant 7; n/N, number of patients in that category divided by total number of patients; PSA₅₀, 50% decline in prostate-specific antigen.

Table 5. Next-Line Systemic Therapy in Patients After Treatment With Abiraterone and Enzalutamide on the Basis of AR-V7 Status

Status	Next-Line Therapy After Abiraterone and Enzalutamide							
	Taxane		Clinical Trial		Observation		Unknown	
	n/N	%	n/N	%	n/N	%	n/N	%
AR-V7–	7/16	43.75	3/16	18.75	1/16	6.25	5/16	31.25
AR-V7+	5/26	19.23	14/26	53.84	3/26	11.54	4/26	15.38

Abbreviation: AR-V7, androgen receptor splice variant 7; n/N, number of patients in that category divided by total number of patients.

who did not have a treatment change based on the AR-V7 test. Therefore we cannot directly compare between these groups. We hypothesize that AR-V7+ patients in the change group were more commonly treated with chemotherapy compared with the no-change group, resulting in improved outcomes. The no-change group may also have been less clinically fit for chemotherapy or did not meet eligibility criteria for enrolling on a clinical trial. We also acknowledge a high PSA₅₀ RR in AR-V7+ patients treated with chemotherapy and AR-V7– patients treated with ATT (both in the change group). Another potential weakness is the subjective definition of changing clinical practice and including patients whose treatment choice was confirmed on the basis of AR-V7 testing. This may have inflated the perceived clinical utility of the biomarker. Nonetheless, these data suggest that further prospective investigation is warranted to study biomarker-driven clinical outcomes.

By using the treatment history captured by our questionnaires, we were able to observe an increasing prevalence of AR-V7 detection with prior exposure to ATTs, as expected. This finding is consistent with previously published data^{12,13,16} and suggests that the clinical data obtained in this study are representative. In addition, we observed interesting nonsignificant trends in our physician-reported data regarding prior treatment. For instance, in ATT-naïve patients, prior treatment with docetaxel increased the incidence of AR-V7 to 40% (*v* 21% in the chemotherapy-naïve

group). This finding is corroborated by the recent biomarker data from the ARMOR3-SV (A Study of Galeterone Compared with Enzalutamide in Men Expressing Androgen Receptor Splice Variant-7 mRNA [AR-V7] Metastatic CRPC) trial, in which first-line patients with mCRPC who had previously received docetaxel for metastatic hormone-sensitive disease had a higher prevalence of AR-V7 detection compared with chemotherapy-naïve patients.²² Moreover, 79% of men with newly developed mCRPC had prior exposure to bicalutamide. Interestingly, the incidence of AR-V7 was 27% in patients who had received bicalutamide compared with 0% in patients with no prior bicalutamide treatment. The higher trend of AR-V7+ tests after treatment with docetaxel or bicalutamide, in the absence of a novel ATT, may suggest that total number of therapies (ie, more advanced disease) contributes to AR-V7 expression in addition to the known relationship with prior ATT exposure. Another provocative hypothesis might be that docetaxel works as an AR-modulating therapy in prostate cancer, inhibiting microtubule-dependent nuclear transport of wild-type AR but not AR-V7, thereby selecting for the emergence of AR-V7–expressing clones during or after chemotherapy treatment.

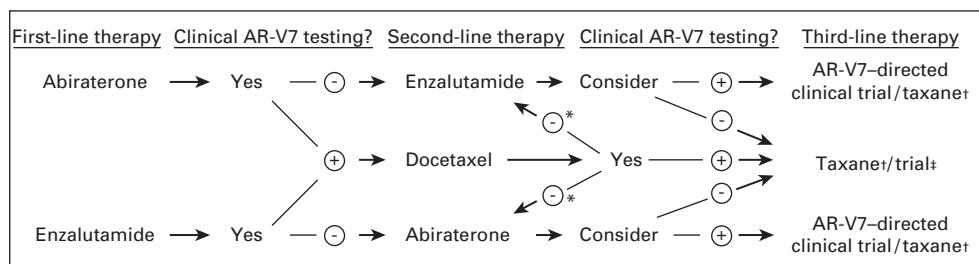
This study had some additional limitations. First, the prevalence of AR-V7 was probably overestimated compared with earlier reports because providers were more likely to order a test if the clinical scenario suggested that the test might be positive. Other significant weaknesses of this analysis were its retrospective nature and reliance on self-reporting by providers. To this end, we did not review the medical records of all patients to confirm their prior treatment history or their PSA₅₀ response. An audit of 14 randomly selected questionnaires determined that providers gave accurate responses to the questionnaire in most instances (see Appendix), suggesting that our data represent the true clinical course for each patient. Finally, we concede that many patients will receive all ATTs approved by the US Food and Drug Administration, regardless of AR-V7 status, because clinical responses to ATTs are sometimes observed in AR-V7+ patients.¹³ However, the clinical utility of AR-V7 testing may potentially be clearest in AR-V7+ patients who have additional ATTs still available for treatment. By identifying high-risk patients (ie, AR-V7+) who have adequate performance status earlier in their mCRPC treatment, this would allow them to be treated with taxane therapy before the chemotherapy window closes. AR-V7– tests are probably less clinically useful because patients could

Table 6. Next-Line Systemic Therapy Selected by Treating Physicians for Patients With AR-V7+ mCRPC

Next-Line Therapy for AR-V7+ Patients									
AR Targeted		Taxane		Clinical Trial		Observation		Unknown	
n/N	%	n/N	%	n/N	%	n/N	%	n/N	%
4/60	6.67	19/60	31.67	21/60	35.0	6/60	10.0	10/60	16.67

Abbreviations: AR, androgen receptor; AR-V7, androgen receptor splice variant 7; mCRPC, metastatic castration-resistant prostate cancer; n/N, number of patients in that category divided by total number of patients.

Fig 1. Potential decision algorithm based on serial androgen receptor splice variant 7 (AR-V7) testing across the castration-resistant prostate cancer landscape. After each line of therapy, we propose an algorithm for consideration of AR-V7 testing. Patients with a positive AR-V7 test could be changed from an AR-targeted therapy (ATT) agent to taxane-based chemotherapy. After taxane treatment, repeat AR-V7 testing may be clinically helpful, and patients with a negative AR-V7 test can be considered for additional ATT. After abiraterone and enzalutamide treatment, patients should be subject to AR-V7 testing only if an AR-V7-directed clinical trial is available. (*) Indicates limited clinical data supporting the use of ATTs in AR-V7+ patients that subsequently convert to AR-V7-. (†) Denotes either docetaxel (first-line) or cabazitaxel (second-line), depending on prior treatment. (§) Denotes a clinical trial that does not require a positive AR-V7 test for AR-V7- patients, whereas AR-V7+ patients should consider an AR-V7-directed trial, if available.



receive either chemotherapy or ATTs at their physician's discretion.

We propose a decision algorithm using serial AR-V7 testing across the mCRPC landscape. This algorithm is based largely on published data suggesting that the presence of AR-V7 confers resistance to ATTs but does not influence response to taxane chemotherapy.^{11,13,14,16} We note that at this time, there is no clinical trial evidence to support the use of ATTs in AR-V7+ patients who convert to AR-V7- status after chemotherapy. In the setting of patients who have received abiraterone and enzalutamide, AR-V7 testing may be considered if AR-V7-selected clinical trials are available. In our study, 17 of 22 AR-V7+ patients were enrolled on a clinical trial that mandated AR-V7 detection as an entry criterion, suggesting that many of the tests in this study were ordered for the purpose of screening for a clinical trial. In these instances, the clinical utility of AR-V7 testing is primarily to allow enrollment on an AR-V7-directed trial. Finally, there is ongoing debate in the prostate cancer

community about whether AR-V7 detection is merely a proxy for AR amplification or overexpression and not an independent predictor of response to therapy or clinical outcomes.²¹ Although several studies have shown that AR-V7 detection is indeed correlated with AR-FL expression, AR-V7 still remains independently prognostic in multivariable analysis after controlling for AR-FL levels.^{11,13,23}

In conclusion, to our knowledge, this is the first study to examine the preliminary real-world clinical utility of CLIA-grade AR-V7 testing in patients with mCRPC. We show that AR-V7 testing influenced clinical decision making overall (regardless of test results) but that its utility was greatest in the setting of AR-V7+ results. Additional prospective studies are needed and are ongoing (eg, NCT02269982; Prospective Circulating Prostate Cancer Predictors in Higher Risk mCRPC Study [PROPHET]).

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APPENDIX

Analysis of PSA₅₀ Response Rate Stratified by AR-V7 Status and Change/No Change Designation

We further queried our database to investigate the difference in the 50% decline in prostate-specific antigen (PSA₅₀) response rate (RR) between the change and no-change groups on the basis of androgen receptor splice variant 7 (AR-V7) status. For patients with AR-V7+ disease, the PSA₅₀ RR in those who did not change therapy was 5.3% (one of 19) compared with 38.7% (12 of 31) in the change group (Table A3). In the AR-V7- patients, the PSA₅₀ RR was 45.45% (15 of 33) in the no-change group versus 68.88% (22 of 32) in the change group. We asked whether there was a difference in the number of lines of therapy (ie, more advanced disease) between the change and no-change groups. Each group was subdivided by AR-V7 status (Table A3). Within both the AR-V7+ and AR-V7- groups, there was no discernible difference in number of lines of therapy between the change and no-change group. We also examined PSA₅₀ RR by treatment choice in those patients who had a change in treatment (these data were not solicited for patients who did not have a treatment change in the questionnaire). In AR-V7+ patients, the PSA₅₀ RR to chemotherapy (either changed or confirmed) was 73.33% (11 of 15). The PSA₅₀ RR in clinical trials for AR-V7+ patients was one (6.7%) of 15 in the change group. In AR-V7- patients who had treatment changed (or confirmed), the PSA₅₀ RR was 70.8% (17 of 24) on AR-targeted therapy (chemotherapy, 100% [three of three]; clinical trial, 0% [zero of three]; observation, 100% [one of one]; unknown, 100% [one of one]).

Audit of Randomly Selected Questionnaires Completed by Johns Hopkins University Providers

We (M.C.M.) performed a medical record audit of 14 randomly selected questionnaires completed by Johns Hopkins University providers to assess for accuracy. Clinical data from other sites were not accessible for audit. We investigated only objective questionnaire responses: clinical stage, prior therapies, most recent treatment, PSA₅₀ response on next-line therapy, and clinical trial eligibility. In the 14 questionnaires audited, we found two minor discrepancies: (1) bicalutamide was listed in error as a prior therapy (one time), and (2) the correct clinical trial was misidentified for a patient (one time). Subjective responses (ie, clinical helpfulness and change in management) were not assessed. Although this audit reflects a small sample size (approximately 10% of the entire population included in this study), it suggests that providers documented accurate responses to the questionnaire in almost all instances.

Table A1. AR-V7–Directed Clinical Trials for Patients With mCRPC

Identifier	Title	Selection
NCT02438007	A Study of Galeterone Compared with Enzalutamide in Men Expressing Androgen Receptor Splice Variant-7 mRNA (AR-V7) Metastatic CRPC (ARMOR3-SV)	AR-V7 specific
NCT02532114	Niclosamide and Enzalutamide in Treating Patients With Castration-Resistant, Metastatic Prostate Cancer	
NCT02601014	Biomarker-Driven Therapy With Nivolumab and Ipilimumab in Treating Patients With Metastatic Hormone-Resistant Prostate Cancer Expressing AR-V7 (STARVE-PC)	AR-V7 specific
NCT03050866	Cabazitaxel in mCRPC Patients With AR-V7 Positive Circulating Tumor Cells (CTCs) (CABA-V7)	AR-V7 specific

Abbreviations: AR-V7, androgen receptor splice variant 7; mCRPC, metastatic castration-resistant prostate cancer.

Table A2. Next-Line Systemic Therapy in Patients After Abiraterone and Enzalutamide on the Basis of AR-V7 Status and Prior Treatment With Docetaxel

Next-Line Therapy in Patients After Abiraterone and Enzalutamide								
Docetaxel Status	Taxane		Clinical Trial		Observation		Unknown	
	n/N	%	n/N	%	n/N	%	n/N	%
Docetaxel naïve								
AR-V7–	1/7	14.2	3/7	42.86	0		3/7	42.86
AR-V7+	2/7	28.57	3/7	42.86	2/7	28.57	0	
After docetaxel								
AR-V7–	6/9	66.67	0		1/9	11.11	2/9	22.22
AR-V7+	3/19	15.79	11/19	57.89	1/19	5.26	4/19	21.05

Abbreviation: AR-V7, androgen receptor splice variant 7; n/N, number of patients in that category divided by total number of patients.

Table A3. PSA₅₀ RR and Lines of Therapy Subgrouped by AR-V7 Status in Patients With mCRPC Who Had a Change or No Change in Management

Management	Lines of Therapy											
	PSA ₅₀ RR		0		1		2		3		4+	
	n/N	%	n/N	%	n/N	%	n/N	%	n/N	%	n/N	%
Change												
AR-V7–	22/32	68.75	11/32	34.4	9/32	28.1	8/32	25	2/32	6.25	2/32	6.25
AR-V7+	12/31	38.7	4/31	12.9	8/31	25.8	8/31	25.8	8/31	25.8	3/31	9.7
No change												
AR-V7–	15/33	45.5	10/33	30.3	14/33	42.4	5/33	15.2	2/33	6	2/33	6
AR-V7+	1/19	5.3	2/19	10.5	4/19	21.0	5/19	26.3	5/19	26.3	3/19	15.8

Abbreviations: AR-V7, androgen receptor splice variant 7; mCRPC, metastatic castration-resistant prostate cancer; n/N, number of patients in that category divided by total number of patients; PSA₅₀, 50% decline in prostate-specific antigen; RR, response rate.

Clinical Significance of Androgen Receptor Splice Variant-7 mRNA Detection in Circulating Tumor Cells of Men With Metastatic Castration-Resistant Prostate Cancer Treated With First- and Second-Line Abiraterone and Enzalutamide

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ABSTRACT

Purpose

We reported previously that the detection of androgen receptor splice variant-7 (AR-V7) mRNA in circulating tumor cells (CTCs) correlated with poor outcomes from the use of abiraterone and enzalutamide in patients with castration-resistant prostate cancer (CRPC). Here, we expanded our cohort size to better characterize the prognostic significance of AR-V7 in this setting.

Methods

We prospectively enrolled 202 patients with CRPC starting abiraterone or enzalutamide and investigated the prognostic value of CTC detection (+ v -) and AR-V7 detection (+ v -) using a CTC-based AR-V7 mRNA assay. We examined $\geq 50\%$ prostate-specific antigen (PSA) responses, PSA progression-free survival, clinical and radiologic progression-free survival, and overall survival. We constructed multivariable models adjusting for PSA, Gleason sum, number of prior hormone therapies, prior abiraterone or enzalutamide use, prior taxane use, presence of visceral metastases, and Eastern Cooperative Oncology Group score. We also separately examined the first-line and second-line novel hormonal therapy (NHT) settings.

Results

Median follow-up times were 15.0, 21.7, and 14.6 months for CTC-, CTC+/AR-V7-, and CTC+/AR-V7+ patients, respectively. CTC+/AR-V7+ patients were more likely to have Gleason scores ≥ 8 ($P = .05$), metastatic disease at diagnosis ($P = .01$), higher PSA ($P < .01$), prior abiraterone or enzalutamide use ($P = .03$), prior taxane use ($P = .02$), and Eastern Cooperative Oncology Group ≥ 1 ($P = .01$). Outcomes for the overall cohort (and separately for the first-line and second-line NHT cohorts) were best for CTC- patients, intermediate for CTC+/AR-V7- patients, and worse for CTC+/AR-V7+ patients. These correlations remained significant in multivariable models.

Conclusion

This expanded analysis further characterizes the importance of CTC-based AR-V7 mRNA detection in predicting outcomes in patients with CRPC receiving first- and second-line NHT and, to the best of our knowledge, is the first to suggest that this assay be interpreted using three separate prognostic categories: CTC-, CTC+/AR-V7-, and CTC+/AR-V7+.

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INTRODUCTION

Treatment-specific biomarkers (eg, markers that help select or exclude a particular therapy) for metastatic castration-resistant prostate cancer (CRPC) are currently lacking. It has emerged recently that detection of androgen receptor splice variant-7 (AR-V7) in circulating tumor cells (CTCs) may represent one such treatment-

selection marker in men with metastatic CRPC.¹ AR-V7 is an abnormally spliced mRNA isoform of the androgen receptor, producing a protein product lacking the C-terminal ligand-binding domain but retaining the transcriptionally active N-terminal domain. Despite its inability to bind ligand (eg, dihydrotestosterone), AR-V7 remains constitutively active in a ligand-independent manner and is capable of driving CRPC growth.¹

ASSOCIATED CONTENT



See accompanying Editorial on page 2103



Data Supplement
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Accumulating evidence suggests that CTC-based detection of AR-V7 may be associated with a lack of benefit of novel hormonal therapies (NHT) including abiraterone² and enzalutamide.³ An initial pilot study conducted by our group (n = 62) suggested that AR-V7 mRNA detection was associated with resistance to abiraterone and enzalutamide.⁴ This was confirmed subsequently by additional studies showing a similar lack of benefit of NHT in patients with detectable AR-V7 mRNA or protein.^{5,6} In addition, it has been suggested that CTC-based AR-V7 detection is compatible with sensitivity to taxane chemotherapies such as docetaxel and cabazitaxel.^{7,8} Furthermore, the relative benefit of taxane chemotherapy over NHT may be greater in AR-V7+ patients than in AR-V7- patients where chemotherapy and NHT seem to have comparable efficacy.^{6,7}

These prior studies suffered from two significant limitations. First, because of small sample sizes, these studies were generally not able to explore the prognostic value of AR-V7 separately in the first-line and second-line NHT settings (and typically included a mix of patients, some of whom had received prior NHT). Indeed, the usefulness of the AR-V7 biomarker could be different in the first-line and second-line settings. Second, none of the above-mentioned studies included data on patients without detectable CTCs. This is problematic because some patients with CRPC may not harbor CTCs and therefore cannot be evaluated for AR-V7. The purpose of our analysis was to expand on our study examining AR-V7 in men receiving abiraterone or enzalutamide (currently, n = 202). By doing so, we aimed to better understand the clinical significance of AR-V7 in both the first-line and second-line NHT settings and to explore the prognostic value of CTC- results compared with CTC+/AR-V7- and CTC+/AR-V7+ results.

METHODS

Patients

We prospectively enrolled men with metastatic CRPC who were beginning treatment with enzalutamide or abiraterone. Patients had to have histologically confirmed prostate adenocarcinoma, progressive disease despite castration levels of serum testosterone (< 50 ng/dL), and radiographic metastases on computed tomography (CT) or technetium-99 bone scans. Patients had to have three or more rising serum prostate-specific antigen (PSA) values taken $\geq$ 2 weeks apart, consistent with Prostate Cancer Working Group guidelines.⁹ Patients were excluded if they planned to receive additional concurrent anticancer therapies. Prior taxane chemotherapy was permitted, as was previous treatment with the alternative NHT (ie, prior abiraterone in enzalutamide-treated patients, and vice versa). This study was approved by the Johns Hopkins University institutional review board, and patients provided written informed consent.

Study Design

This was a prospective study evaluating the ability of baseline CTC status (+ v -) and AR-V7 status (+ v -) to predict clinical benefit from NHT. Patients were asked to provide peripheral blood CTC samples at baseline (before beginning NHT) and at the time of progression. Enzalutamide was administered at 160 mg once daily, and abiraterone was administered at 1,000 mg once daily (with prednisone 5 mg twice a day). Follow-up was prospectively defined: patients had PSA measurements every 1 to 2 months, as well as CT (chest, abdomen, and pelvis) and technetium-99 bone scans every 2 to 4 months. Therapy with enzalutamide

or abiraterone was continued until PSA progression, clinical or radiographic progression, or unmanageable drug-related toxicity.

CTC Assay and AR-V7 Detection

CTC analyses were conducted using a modified AdnaTest platform (QIAGEN, Hannover, Germany), as described previously.⁴ Capture of CTCs was performed using the EpCAM-based ProstateCancerSelect kit, and mRNA expression analyses were performed using the ProstateCancerDetect kit with multiplexed reverse-transcription polymerase chain reaction primers to establish the presence or absence of CTCs. Custom primers were designed to detect full-length androgen receptor (AR-FL) mRNA and AR-V7 mRNA, as described previously.⁴ The relative abundance of AR-V7 was determined by calculating the ratio of AR-V7 transcript to AR-FL transcript.

In our previous reports,^{4,7} we presented data only on CTC+ patients, whereas CTC- patients were excluded from analysis (because their AR-V7 status could not be determined). Here, we aimed to consider our biomarker readout in three separate categories: CTC- (AR-V7 agnostic), CTC+/AR-V7-, and CTC+/AR-V7+. In this way, all enrolled patients, even those without detectable CTCs, would contribute data to our study.

Outcome Measures

The primary end point was clinical and radiographic progression-free survival (PFS); progression was defined as symptomatic progression (worsening disease-related symptoms or new cancer-related complications) or radiologic progression (on CT scan: $\geq$ 20% enlargement in sum diameter of target lesions [Response Evaluation Criteria in Solid Tumors⁹]; on bone scan: two or more new bone lesions not caused by flare), or death, whichever occurred first.¹⁰ Secondary end points included PSA response rate, PSA progression-free survival (PSA-PFS), and overall survival (OS). PSA response was defined as the proportion of patients with a $\geq$ 50% PSA decline from baseline at any time point after therapy (and maintained for $\geq$ 3 weeks); best PSA response (maximal percentage decrease from baseline) was also determined. PSA progression was defined as a $\geq$ 25% increase in PSA from nadir (and by $\geq$ 2 ng/mL), requiring confirmation $\geq$ 3 weeks later (Prostate Cancer Working Group criteria).¹⁰ OS was defined as the interval from enrolment to death from any cause.

Statistical Analyses

Clinical outcomes were analyzed separately in the three biomarker groups: CTC-, CTC+/AR-V7-, and CTC+/AR-V7+. PSA response rates were compared using Fisher's exact test. Time-to-event outcomes (PFS, PSA-PFS, and OS) were evaluated using Kaplan-Meier analysis, and survival-time differences were compared using the log-rank test. Univariable and multivariable logistic regressions (for PSA response) and Cox regressions (for time-to-event end points) were used to assess the independent effect of biomarker status on clinical outcomes. Covariates included in the multivariable models were baseline PSA, number of prior hormonal therapies, presence of visceral metastases, Gleason sum, Eastern Cooperative Oncology Group score, prior taxane chemotherapy, and prior abiraterone or enzalutamide. These variables were strongly associated with clinical outcomes in our prior AR-V7 studies.^{4,7} Because of fewer events in the multivariable models for OS, only three covariates were included in these models (baseline PSA, prior chemotherapy, and prior abiraterone or enzalutamide).

Statistical analyses were performed for the cohort as a whole (n = 202; primary analysis) and also separately for the first-line NHT (n = 124) and second-line NHT (n = 78) cohorts. Sample size was determined on the basis of the primary comparison of PFS between CTC+/AR-V7- and CTC+/AR-V7+ patients in the overall cohort. Assuming a 30% prevalence of AR-V7 detection among CTC+ men, 148 CTC+ patients provided 90% power to detect a hazard ratio of 2.0 when median PFS in CTC+/AR-V7- men was 8.0 months, using a two-sided log-rank test at a significance level of .05. This calculation assumed 36 months of accrual time and a 2% dropout rate. Fifty-three of the enrolled patients were CTC- when the

study reached the required sample size of 148 men with CTC+, and these patients were included in the analysis as a separate group. The first-line NHT cohort included patients beginning abiraterone or enzalutamide who had not previously received the alternative agent. The second-line NHT cohort included men beginning abiraterone or enzalutamide who had previously received the alternative drug. All statistical tests were two sided, and *P* values were not corrected for multiple comparisons. Statistical analyses were performed using *R* (version 2.15.1).

RESULTS

Patients

Between December 2012 and November 2015, 202 men (95 starting abiraterone, 107 starting enzalutamide) were prospectively enrolled. As of February 2016, median follow-up times for the at-risk population, calculated using the reverse Kaplan-Meier method, were 15.0, 21.7, and 14.6 months for CTC-, CTC+/AR-V7-, and CTC+/AR-V7+ patients, respectively. Baseline patient characteristics are summarized in Table 1. Overall, 53 of the 202 men (26.2%) were CTC-, 113 of the 202 men (56.0%) were CTC+/AR-V7-, and 36 of the 202 men (17.8%) were CTC+/AR-V7+. CTC+/AR-V7+ patients were more likely to have Gleason scores ≥ 8 ($P = .05$), metastatic disease at diagnosis ($P = .01$), higher PSA levels ($P < .01$), higher alkaline phosphatase levels ($P < .01$), prior abiraterone or enzalutamide use ($P = .03$), prior taxane use ($P = .02$), presence of pain ($P < .01$), and Eastern Cooperative Oncology Group status ≥ 1 ($P = .01$).

One hundred twenty-four patients had not received abiraterone or enzalutamide previously (first-line NHT cohort). Of these, 36 (29.0%) were CTC-, 73 (58.9%) were CTC+/AR-V7-, and 15 (12.1%) were CTC+/AR-V7+. Seventy-eight men had received abiraterone or enzalutamide previously (second-line NHT cohort). Of these, 17 (21.8%) were CTC-, 40 (51.3%) were CTC+/AR-V7-, and 21 (26.9%) were CTC+/AR-V7+.

The prevalence of CTC- patients was lower and the prevalence of CTC+/AR-V7+ patients was higher in the second-line compared with the first-line NHT cohorts.

PSA Responses

Overall ($n = 202$), PSA response rates to enzalutamide or abiraterone were 75.5% (40 of 53) in CTC- patients, 52.2% (59 of 113) in CTC+/AR-V7- patients, and 13.9% (5 of 36) in CTC+/AR-V7+ patients ($P < .001$; Fig 1A). In multivariable logistic regression analysis (Data Supplement), biomarker status remained an independent predictor of PSA response. To understand the clinical characteristics that may permit PSA response to abiraterone or enzalutamide despite detection of AR-V7, we compared baseline characteristics among all CTC+/AR-V7+ patients stratified by whether they achieved a PSA response (Data Supplement). PSA responders had more favorable clinical characteristics (less prior abiraterone or enzalutamide use, less prior docetaxel use, less visceral metastases, less bone pain, lower PSAs, lower AR-FL levels, and lower AR-V7/AR-FL ratios) than did PSA nonresponders. We also discovered that two of these PSA responders received concurrent palliative radiotherapy to an osseous metastatic site, which may have influenced PSA trends.

Among first-line NHT patients ($n = 124$), PSA response rates in CTC- patients, CTC+/AR-V7- patients, and CTC+/AR-V7+ patients were 86.1% (31 of 36), 65.8% (48 of 73), and 26.7% (four of 15), respectively ($P < .001$; Fig 2A). In multivariable logistic regression analysis (Data Supplement), biomarker status remained an independent predictor of PSA response. Among second-line NHT patients ($n = 78$), PSA response rates in CTC- men, CTC+/AR-V7- men, and CTC+/AR-V7+ men were 52.9% (nine of 17), 27.5% (11 of 40), and 4.8% (one of 21), respectively ($P = .003$; Fig 3A). In multivariable logistic regression analysis (Data Supplement), biomarker status generally remained an independent predictor of PSA response.

Table 1. Baseline Characteristics of Patients With CRPC Starting Treatment With Abiraterone or Enzalutamide ($N = 202$)

Baseline Characteristic	CTC- ($n = 53$ [26.2%])	CTC+/AR-V7- ($n = 113$ [56.0%])	CTC+/AR-V7+ ($n = 36$ [17.8%])	<i>P</i> *
Median age, years	70	71	70	.888
Nonwhite ethnicity, %	9	14	17	.575
Years since diagnosis, mean	7.9	7.3	5.6	.098
Gleason sum ≥ 8 at diagnosis, %	68	60	83	.052
Type of primary local therapy, %				.011
Surgery only	12	24	25	
Radiation only	31	30	19	
Both	35	20	8	
None	23	26	47	
M1 disease at diagnosis, %	16	22	47	
No. prior hormonal therapies, median	2	2	3	.158
Time to castration resistance, median no. months	23.0	20.5	14.0	.148
Prior use of abiraterone or enzalutamide, %	32	35	58	.025
Prior use of docetaxel, %	19	25	44	.022
Presence of bone metastases, %	76	83	100	.010
Presence of visceral metastases, %	23	27	34	.559
ECOG performance status ≥ 1 , %	41	24	53	.006
Presence of pain, %	32	40	72	.002
Baseline PSA, ng/mL, median	13.7	31.4	92.0	< .001
Baseline alkaline phosphatase, U/L, median	80	96	120	< .001

Abbreviations: AR-V7, androgen receptor splice variant-7; CRPC, castration-resistant prostate cancer; CTC, circulating tumor cell; ECOG, Eastern Cooperative Oncology Group; PSA, prostate-specific antigen.

**P* values are based on Fisher's exact test and the Mann-Whitney *U* test for categorical and continuous variables, respectively.

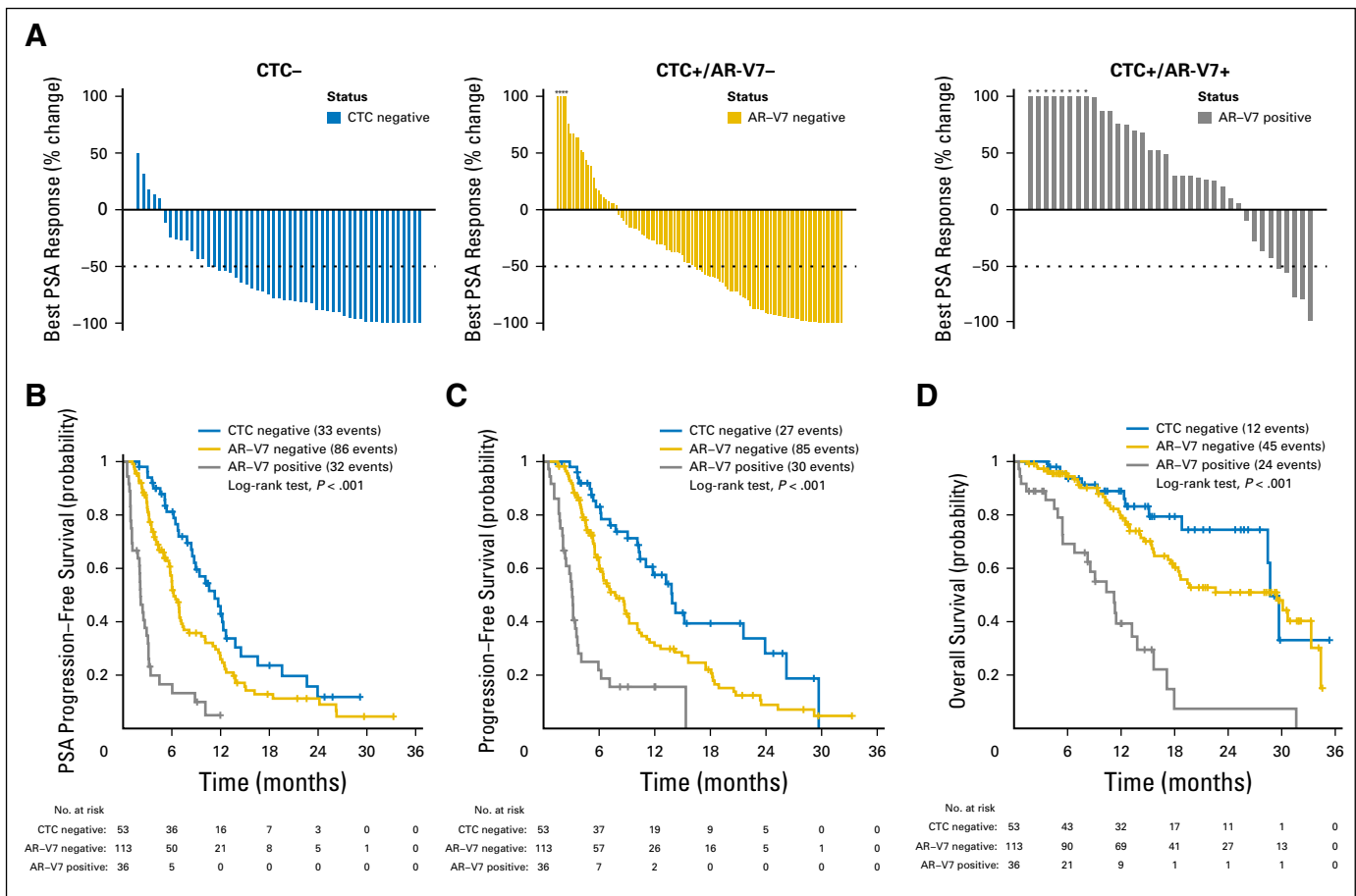


Fig 1. Clinical outcomes in the overall cohort of men starting treatment with abiraterone or enzalutamide (N = 202), according to CTC status and AR-V7 status. (A) Waterfall plots depicting best PSA responses according to CTC status and AR-V7 status, expressed in three categories: CTC-, CTC+/AR-V7-, and CTC+/AR-V7+. The dotted line illustrates the threshold for defining a PSA response ($\geq 50\%$ PSA reduction from baseline). PSA response rates in CTC- patients, CTC+/AR-V7- patients, and CTC+/AR-V7+ patients were 75.5% (40 of 53), 52.2% (59 of 113), and 13.9% (5 of 36), respectively ($P < .001$). All three groups were significantly different from each other (CTC- v CTC+/AR-V7-, $P = .005$; CTC- v CTC+/AR-V7+, $P < .001$; CTC+/AR-V7- v CTC+/AR-V7+, $P < .001$). (B) Kaplan-Meier curves indicating PSA progression-free survival according to CTC status and AR-V7 status. Median PSA progression-free survival in CTC- patients, CTC+/AR-V7- patients, and CTC+/AR-V7+ patients was 11.3 months (95% CI, 8.7 to 13.8), 6.2 months (95% CI, 5.8 to 7.3), and 2.1 months (95% CI, 1.9 to 3.1), respectively ($P < .001$). (C) Kaplan-Meier curves indicating clinical and radiographic progression-free survival according to CTC status and AR-V7 status. Median progression-free survival in CTC- patients, CTC+/AR-V7- patients, and CTC+/AR-V7+ patients was 13.9 months (95% CI, 11.0 to not reached), 7.7 months (95% CI, 6.2 to 10.1), and 3.1 months (95% CI, 2.3 to 3.7), respectively ($P < .001$). (D) Kaplan-Meier curves indicating overall survival according to CTC status and AR-V7 status. Median overall survival in CTC- patients, CTC+/AR-V7- patients, and CTC+/AR-V7+ patients was 28.7 months (95% CI, 28.4 to not reached), 29.5 months (95% CI, 18.4 to not reached), and 11.2 months (95% CI, 8.3 to 17.1), respectively ($P < .001$). AR-V7, androgen receptor splice variant-7; CTC, circulating tumor cell; PSA, prostate-specific antigen.

PSA-PFS

In the overall cohort, median PSA-PFS to enzalutamide or abiraterone was 11.3 months (95% CI, 8.7 to 13.8) in CTC- patients, 6.2 months (95% CI, 5.8 to 7.3) in CTC+/AR-V7- patients, and 2.1 months (95% CI, 1.9 to 3.1) in CTC+/AR-V7+ patients ($P < .001$; Fig 1B). In multivariable Cox regression analysis (Data Supplement), biomarker status remained independently prognostic for PSA-PFS.

In the first-line NHT cohort, median PSA-PFS in CTC- patients, CTC+/AR-V7- patients, and CTC+/AR-V7+ patients was 12.7 months (95% CI, 11.7 to 23.9), 7.3 months (95% CI, 6.2 to 12.0), and 2.9 months (95% CI, 2.0 to not reached), respectively ($P < .001$; Fig 2B). In multivariable Cox regression analysis (Data Supplement), biomarker status remained independently prognostic for PSA-PFS. In the second-line NHT cohort, median PSA-PFS in CTC- patients, CTC+/AR-V7- patients, and CTC+/AR-V7+ patients

was 6.4 months (95% CI, 5.1 to not reached), 4.4 months (95% CI, 3.2 to 6.0), and 1.1 months (95% CI, 1.0 to 3.1), respectively ($P < .001$; Fig 3B). In multivariable Cox regression analysis (Data Supplement), biomarker status remained independently prognostic for PSA-PFS.

PFS

In the overall cohort, median PFS was 13.9 months (95% CI, 11.0 to not reached) in CTC- patients, 7.7 months (95% CI, 6.2 to 10.1) in CTC+/AR-V7- patients, and 3.1 months (95% CI, 2.3 to 3.7) in CTC+/AR-V7+ patients ($P < .001$; Fig 1C). In multivariable Cox regression analysis (Data Supplement), biomarker status remained independently prognostic for PFS.

In the first-line NHT cohort, median PFS in CTC- patients, CTC+/AR-V7- patients, and CTC+/AR-V7+ patients was 21.6 months (95% CI, 13.9 to not reached), 10.1 months

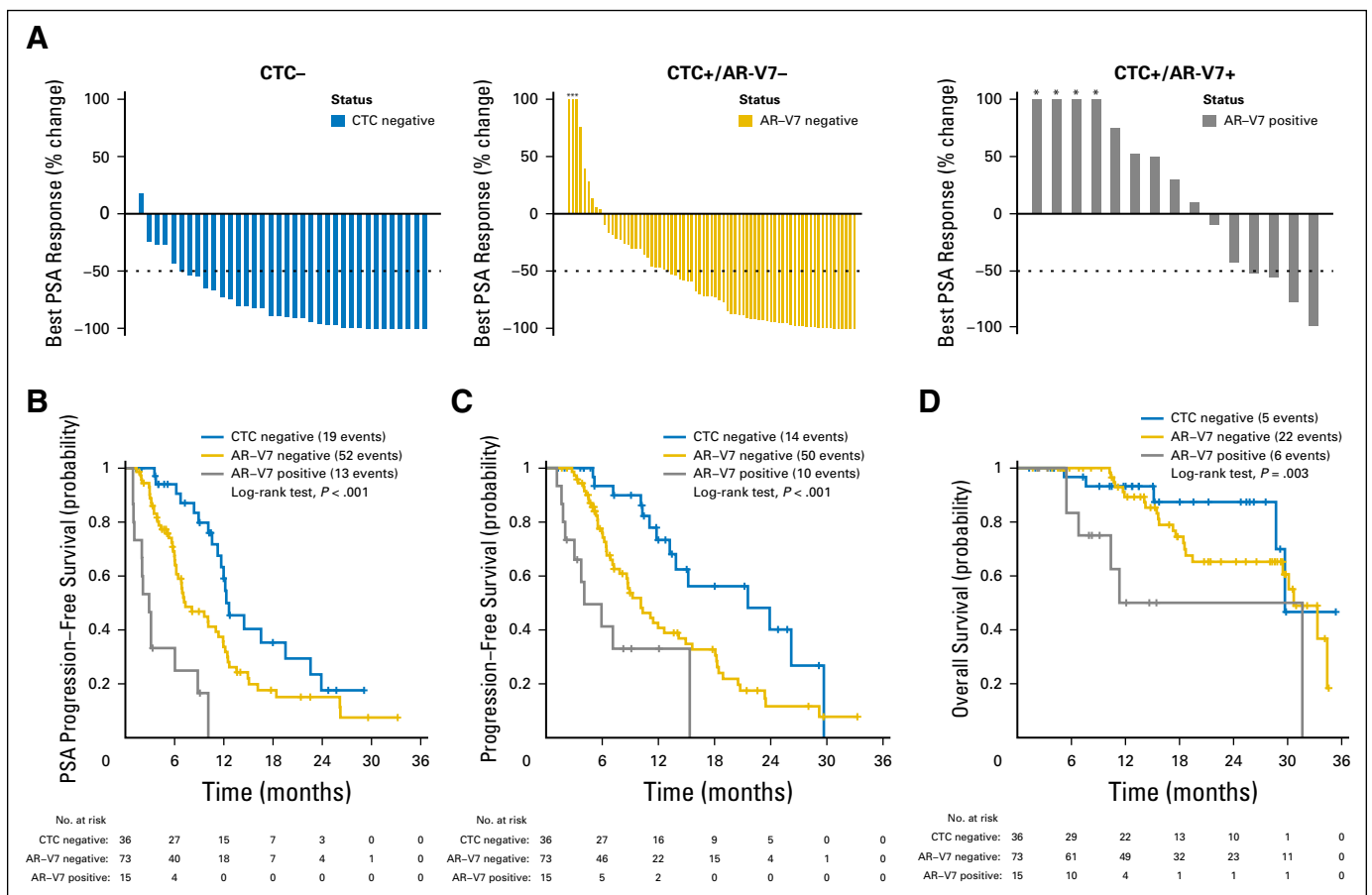


Fig 2. Clinical outcomes for men starting abiraterone or enzalutamide in the first-line novel hormonal therapy setting (N = 124), according to CTC status and AR-V7 status. (A) Waterfall plots depicting best PSA responses according to CTC status and AR-V7 status, expressed in three categories: CTC-, CTC+/AR-V7-, and CTC+/AR-V7+. The dotted line illustrates the threshold for defining a PSA response ($\geq 50\%$ PSA reduction from baseline). PSA response rates in CTC- patients, CTC+/AR-V7- patients, and CTC+/AR-V7+ patients were 86.1% (31 of 36), 65.8% (48 of 73), and 26.7% (4 of 15), respectively ($P < .001$). All three groups were significantly different from each other (CTC- v CTC+/AR-V7-, $P = .03$; CTC- v CTC+/AR-V7+, $P < .001$; CTC+/AR-V7- v CTC+/AR-V7+, $P = .009$). (B) Kaplan-Meier curves indicating PSA progression-free survival according to CTC status and AR-V7 status. Median PSA progression-free survival in CTC- patients, CTC+/AR-V7- patients, and CTC+/AR-V7+ patients was 12.7 months (95% CI, 11.7 to 23.9), 7.3 months (95% CI, 6.2 to 12.0), and 2.9 months (95% CI, 2.0 to not reached), respectively ($P < .001$). (C) Kaplan-Meier curves indicating clinical and radiographic progression-free survival according to CTC status and AR-V7 status. Median progression-free survival in CTC- patients, CTC+/AR-V7- patients, and CTC+/AR-V7+ patients was 10.1 months (95% CI, 13.9 to not reached), 10.1 months (95% CI, 7.9 to 14.9), and 4.1 months (95% CI, 3.0 to not reached), respectively ($P < .001$). (D) Kaplan-Meier curves indicating overall survival according to CTC status and AR-V7 status. Median overall survival in CTC- patients, CTC+/AR-V7- patients, and CTC+/AR-V7+ patients was 29.7 months (95% CI, 28.7 to not reached), 30.7 months (95% CI, 29.5 to not reached), and 21.5 months (95% CI, 10.4 to not reached), respectively ($P = .003$). AR-V7, androgen receptor splice variant-7; CTC, circulating tumor cell; PSA, prostate-specific antigen.

(95% CI, 7.9 to 14.9), and 4.1 months (95% CI, 3.0 to not reached), respectively ($P < .001$; Fig 2C). In multivariable Cox regression analysis (Data Supplement), biomarker status generally remained independently prognostic for PFS. In the second-line NHT cohort, median PFS in CTC- patients, CTC+/AR-V7- patients, and CTC+/AR-V7+ patients was 6.2 months (95% CI, 5.4 to not reached), 5.3 months (95% CI, 4.1 to 7.7), and 2.8 months (95% CI, 2.1 to 3.4), respectively ($P < .001$; Fig 3C). In multivariable Cox regression analysis (Data Supplement), biomarker status generally remained independently prognostic for PFS.

OS

In the overall cohort, median OS was 28.7 months (95% CI, 28.4 to not reached) in CTC- patients, 29.5 months (95% CI, 18.4 to not reached) in CTC+/AR-V7- patients, and 11.2 months

(95% CI, 8.3 to 17.1) in CTC+/AR-V7+ patients ($P < .001$; Fig 1D). In multivariable Cox regression analysis (Data Supplement), biomarker status generally remained independently prognostic for OS.

In the first-line NHT cohort, median OS in CTC- patients, CTC+/AR-V7- patients, and CTC+/AR-V7+ patients was 29.7 months (95% CI, 28.7 to not reached), 30.7 months (95% CI, 29.5 to not reached), and 21.5 months (95% CI, 10.4 to not reached), respectively ($P = .003$; Fig 2D). In multivariable Cox regression analysis (Data Supplement), biomarker status generally remained independently prognostic for OS. In the second-line NHT cohort, median OS in CTC- patients, CTC+/AR-V7- patients, and CTC+/AR-V7+ patients was 18.8 months (95% CI, 12.5 to not reached), 13.0 months (95% CI, 10.0 to 22.6), and 8.5 months (95% CI, 4.9 to 15.6), respectively ($P < .001$; Fig 3D). In multivariable Cox regression analysis (Data Supplement), biomarker status generally remained independently prognostic for OS.

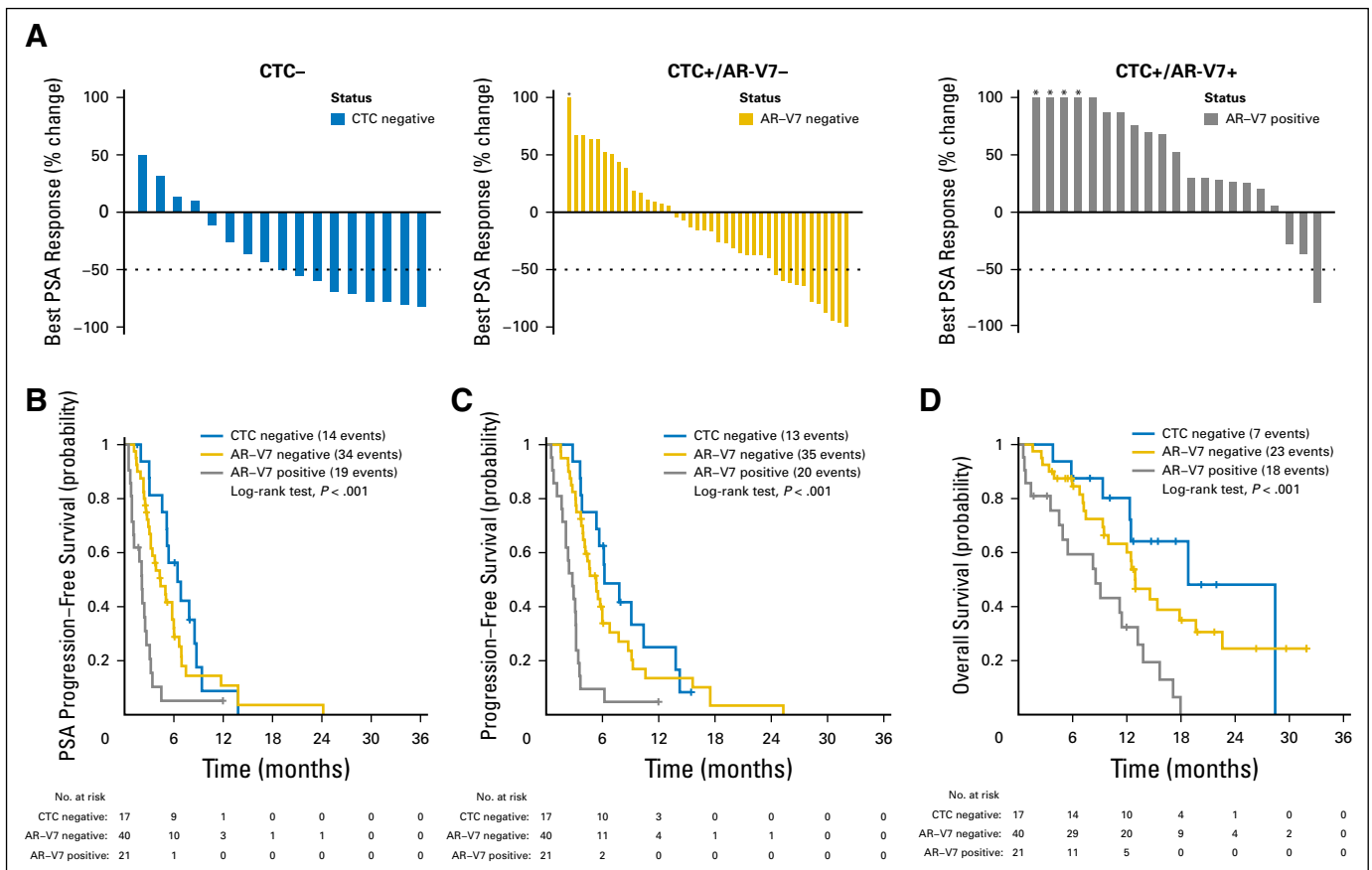


Fig 3. Clinical outcomes for men starting abiraterone or enzalutamide in the second-line novel hormonal therapy setting (N = 78), according to CTC status and AR-V7 status. (A) Waterfall plots depicting best PSA responses according to CTC status and AR-V7 status, expressed in three categories: CTC-, CTC+/AR-V7-, and CTC+/AR-V7+. The dotted line illustrates the threshold for defining a PSA response ($\geq 50\%$ PSA reduction from baseline). PSA response rates in CTC- patients, CTC+/AR-V7- patients, and CTC+/AR-V7+ patients were 52.9% (nine of 17), 27.5% (11 of 40), and 4.8% (one of 21), respectively ($P = .003$). All three groups were significantly different from each other (CTC- vs CTC+/AR-V7-, $P = .078$; CTC- vs CTC+/AR-V7+, $P = .002$; CTC+/AR-V7- vs CTC+/AR-V7+, $P = .044$). (B) Kaplan-Meier curves indicating PSA progression-free survival according to CTC status and AR-V7 status. Median PSA progression-free survival in CTC- patients, CTC+/AR-V7- patients, and CTC+/AR-V7+ patients was 6.4 months (95% CI, 5.1 to not reached), 4.4 months (95% CI, 3.2 to 6.0), and 1.1 months (95% CI, 1.0 to 3.1), respectively ($P < .001$). (C) Kaplan-Meier curves indicating clinical and radiographic progression-free survival according to CTC status and AR-V7 status. Median progression-free survival in CTC- patients, CTC+/AR-V7- patients, and CTC+/AR-V7+ patients was 6.2 months (95% CI 5.4–not reached), 5.3 months (95% CI 4.1–7.7), and 2.8 months (95% CI 2.1–3.4), respectively ($P < .001$). (D) Kaplan-Meier curves indicating overall survival according to CTC status and AR-V7 status. Median overall survival in CTC- patients, CTC+/AR-V7- patients, and CTC+/AR-V7+ patients was 18.8 months (95% CI 12.5–not reached), 13.0 months (95% CI 10.0–22.6), and 8.5 months (95% CI 4.9–15.6), respectively ($P < .001$).

Conversions

Fifty-nine patients had evaluable paired blood samples from baseline and progression. Of the 14 patients with baseline CTC- samples, six (43%) remained CTC-, and the remainder converted to CTC+/AR-V7- (n = 6, 43%) or CTC+/AR-V7+ (n = 2, 14%). Of the 35 men with baseline CTC+/AR-V7- samples, 20 (57%) remained CTC+/AR-V7-, and the remainder converted to CTC- (n = 5, 14%) or CTC+/AR-V7+ (n = 10, 29%). Of the 10 men with baseline CTC+/AR-V7+ samples, nine (90%) remained CTC+/AR-V7+, and one patient (10%) converted to CTC+/AR-V7- (none of these patients converted to CTC-).

DISCUSSION

We report, to the best of our knowledge, the largest prospective study to date examining the prognostic significance of CTC-based AR-V7 testing in patients with CRPC receiving NHT with

abiraterone or enzalutamide. The current results confirm our previous pilot data that CTC+/AR-V7+ patients have inferior clinical outcomes compared with CTC+/AR-V7- individuals, with respect to PSA responses, PSA-PFS, PFS, and OS. Furthermore, we report, we believe for the first time, the prognostic value of CTC- results using the AdnaTest platform. As expected, CTC- patients demonstrated clinical outcomes that were superior even to CTC+/AR-V7- individuals. To this end, CTC- patients seem to have the best outcomes with NHT, CTC+/AR-V7- patients have intermediate outcomes, and CTC+/AR-V7+ patients have the worst outcomes. Furthermore, because of the increased sample size of this study, we were able to evaluate the clinical significance of biomarker status separately in patients receiving first-line NHT and second-line NHT. To this end, biomarker status remained prognostic for all clinical outcomes in both the first-line and the second-line NHT settings, although there was no statistical difference in survival between CTC- and CTC+/AR-V7- subgroups.

The fact that a small proportion (13.9%) of CTC+/AR-V7+ patients achieved PSA responses with NHT was an important observation of our study (Data Supplement). Interestingly, responding patients generally had lower AR-FL transcript levels (median 21 v 36 copies) and lower AR-V7/AR-FL ratios (median, 8.8% v 21.2%), perhaps suggesting that a higher abundance of AR-FL and higher AR-V7 ratios may be associated with worse prognosis. It is also possible that some CTCs in a given blood sample expressed detectable levels of AR-V7, whereas others did not, reflecting individual tumor cell heterogeneity as demonstrated recently by RNA sequencing.¹¹ Moreover, our assay, which detects AR-V7 mRNA, does not document the presence or nuclear localization of AR-V7 protein,⁶ and it is possible that untranslated mRNA was detected, which would not be expected to be pathogenic in the absence of nuclear-localized protein. Finally, it should be highlighted that despite the possibility of a PSA response in some CTC+/AR-V7+ patients, PSA responses in the CTC+/AR-V7- and CTC- populations were much higher (52.2% and 75.5%, respectively). Nevertheless, this observation highlights the notion that not all CTC+/AR-V7+ patients may have an absolute primary resistance to abiraterone and enzalutamide. Rare PSA responses in CTC+/AR-V7+ patients have also been observed in other series,^{5,12} although PSA reductions alone do not constitute a clinical benefit.

The current prostate cancer clinical states model¹³ recommends considering metastatic CRPC as a series of states defined by the number of prior systemic therapies received. Accordingly, it was important to examine our biomarker outcomes separately in the first-line NHT and second-line NHT settings, representing two distinct contexts of use. Interestingly, but not surprisingly, the prevalence of CTC- patients was lower and the prevalence of CTC+/AR-V7+ patients was higher in the second-line compared with the first-line NHT setting. Similarly, in another study using a CTC-based assay relying on immunofluorescence staining of nuclear AR-V7 protein,⁶ AR-V7 prevalence in that analysis also increased with subsequent lines of CRPC therapy. Importantly, our current data show that our biomarker assay retains its prognostic value in both the first-line and the second-line NHT settings, with clinical outcomes remaining distinct for each of the three biomarker categories in each setting. Our data also underscore the value of considering CTC- patients as a distinct category from CTC+/AR-V7- patients. Other CTC-based AR-V7 platforms should also evaluate the prognostic implications of CTC- results.

This study has some limitations, the most significant of which was that there was some variability in the timing of PSA assessments and imaging assessments (which may have influenced our PSA-PFS and PFS estimates). In addition, this study as designed

allowed us to draw conclusions only on the prognostic usefulness of our biomarker in the context of androgen-directed therapy, because all patients exclusively received NHT and we did not include chemotherapy-treated patients. Therefore, the predictive usefulness of this biomarker and the interaction between biomarker status and treatment type could not be evaluated and will form the basis of future work. Finally, this study was not powered to assess OS, and we have not yet observed enough death events to make conclusive statements about biomarker status and survival. In addition, because of the exploratory nature of this study, *P* values were not corrected for multiple comparisons.

In conclusion, this expanded analysis confirms the negative prognostic impact of CTC-based AR-V7 detection in patients with CRPC undergoing therapy with abiraterone and enzalutamide and suggests that this biomarker panel may be useful in the prediction of response to AR-targeted treatment applied in the first- and second-line NHT settings. Furthermore, we believe our study is the first to suggest that the modified-AdnaTest CTC-based AR-V7 mRNA assay should be interpreted using three separate prognostic categories: CTC-, CTC+/AR-V7-, and CTC+/AR-V7+. Prospective studies ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT02269982) identifier NCT02269982) are currently underway to validate these findings.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Disclosures provided by the authors are available with this article at jco.org.

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Clinical Significance of Androgen Receptor Splice Variant-7 mRNA Detection in Circulating Tumor Cells of Men With Metastatic Castration-Resistant Prostate Cancer Treated With First- and Second-Line Abiraterone and Enzalutamide

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Analytical Validation of Androgen Receptor Splice Variant 7 Detection in a Clinical Laboratory Improvement Amendments (CLIA) Laboratory Setting



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Patients with castration-resistant prostate cancer (CRPC) often are treated with drugs that target the androgen receptor (AR) ligand-binding domain. Constitutively active AR splice variant 7 (AR-V7) lacks the ligand-binding domain and, if detected in circulating tumor cells, may be associated with resistance to these agents. We validated an AR-V7 assay in a Clinical Laboratory Improvement Amendments (CLIA)—certified laboratory. Circulating tumor cells were isolated, and mRNA was reverse-transcribed into cDNA. Real-time quantitative PCR amplification of reference transcripts (beta-actin and glyceraldehyde-3-phosphate dehydrogenase), prostate-specific transcripts (prostate-specific membrane antigen, prostate-specific antigen, and AR-full length), and AR-V7 was performed. Specimens for validation included an AR-V7 expressing prostate cancer (LNCaP95), 38 peripheral blood controls, and 21 blood samples from CRPC patients. The assay detected as few as five LNCaP95 cells spiked into peripheral blood, showing high analytical sensitivity. Multiple inter-run and intrarun replicates of LNCaP95 cell line experiments yielded similar cycle threshold values for all genes, showing high analytical precision (AR-V7 cycle threshold CV of 0.67%). All 38 healthy control samples were negative for AR-V7, showing high diagnostic specificity (100%). The diagnostic accuracy was confirmed by concurrent testing of 21 CRPC samples in the research laboratory and the clinical diagnostic laboratory: concordance in AR-V7 status was achieved in all cases (positive in 4, negative in 17) (100% accuracy). This first validated clinical assay detects the AR-V7 with high analytical sensitivity, precision, specificity, and accuracy. (*J Mol Diagn* 2017, 19: 115–125; <http://dx.doi.org/10.1016/j.jmoldx.2016.08.003>)

Alterations of the androgen pathway are critical to prostate cancer progression. Suppression of androgen receptor (AR) signaling is the therapeutic goal of many prostate cancer drugs. Patients with residual or recurrent disease after surgery and radiotherapy are treated with chemical castration (gonadotropin-releasing hormone agonists or antagonists). After showing an initial response, most patients develop progressive disease, referred to as castration-resistant prostate cancer (CRPC). Intriguingly, CRPC is not androgen-independent and several drugs designed to further suppress the AR pathway have shown improved survival, including abiraterone and enzalutamide.^{1–4} Abiraterone is a CYP17A1 inhibitor that impairs synthesis of dihydrotestosterone (AR ligand) precursors in the adrenal glands

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and in the prostate. Alternatively, enzalutamide suppresses signaling by antagonistic binding to the ligand-binding domain of AR.

However, not all patients respond equally to these newer AR-targeting drugs. Approximately 20% to 40% of CRPC patients have poor clinical response to these agents, and nearly all patients who initially respond acquire secondary resistance. Several mechanisms of resistance to these agents have been proposed.⁵ One mechanism of resistance is alternate splicing of the AR transcript that results in the truncated AR splice variant 7 (AR-V7) protein.^{6,7}

The AR protein has four domains—the N-terminal domain, the DNA binding domain, the hinge-region, and the androgen-binding C-terminal ligand-binding domain. Binding of the ligand (dihydrotestosterone) to the AR ligand-binding domain results in nuclear localization of the AR. In the nucleus, the AR N-terminal domain acts as a transcriptional activator of proliferative signals and androgen-regulated genes (eg, *PSA*). Discovered in 2008, the splice variants of the AR arise through splicing of the intronic cryptic exons to the upstream exons encoding the DNA-binding domain.^{6,8} Of the 22 known variants, AR-V7 is the most widely studied variant and carries the most prognostic information. In addition, AR-V7 is the only variant that produces a protein that can be detected in clinical samples.⁹ AR-V7 contains only the first three of the eight exons seen in AR—full length (FL), followed by cryptic exon 3 encoding a novel, variant-specific peptide of 16 amino acids. AR-V7 lacks the ligand-binding domain of the protein but retains transcriptional activity. Therefore, AR-V7 acts as a constitutively active AR protein, independently of its binding by dihydrotestosterone. AR-targeting drugs disrupt the dihydrotestosterone-dependent AR signaling. Therefore, these drugs can inhibit signaling by AR-FL protein, but should not inhibit signaling by AR-V7. In CRPC patients, multiple preclinical studies have correlated the disease progression and mortality to the presence and abundance of AR splice variants.^{10–14}

Clinical detection of AR-V7 in circulating tumor cells (CTCs) of CRPC patients has been shown by four independent groups to be associated with resistance to AR-targeting agents (*Discussion*).^{15–18} However, expression of AR-V7 does not appear to affect the response to taxane chemotherapy adversely.^{18–21} Taxanes affect the microtubule network resulting in cytoplasmic sequestration of AR, thereby disrupting the AR nuclear signaling. AR-V7 detection in the CTCs of CRPC patients therefore could be incorporated into therapeutic decision making if a clinically validated assay were available.^{22,23}

Despite the many published studies supporting the clinical significance of AR-V7 to date, the analytical performance of the test has not been reported. AR-V7 testing now is being used for several ongoing clinical trials. Seven registered clinical trials (1 phase III trial, 5 phase II trials, and 1 pilot trial) were identified from ClinicalTrials.gov (<https://clinicaltrials.gov>, last accessed January 3, 2016) using the

terms “AR-V7” or “ARV7” (summarized by Maughan and Antonarakis²⁴). Analytical validation of the AR-V7 test is critically important given the collective data supporting AR-V7 as a predictive marker and the need for further clinical validation through clinical trials. In this report, we describe the analytical validation and the performance characteristics of the first laboratory-developed AR-V7 test in a Clinical Laboratory Improvement Amendments (CLIA)—certified laboratory (live date: August 13, 2015).

Materials and Methods

This study was performed under institutional review board approval, with informed consent.

Cell Lines and Specimens

The prostate cancer cell lines LNCaP and LNCaP95, generously provided by Dr. Luo's laboratory (Baltimore, Maryland), were cultured as described.¹⁵ Validation included DNA fingerprinting, RT-PCR, and Sanger sequencing of the AR-FL transcript from LNCaP cells, and the AR-V7 transcript from LNCaP95 cells. After confirming that LNCaP95 expressed AR-V7, RNA was used as a positive control,⁹ and human spleen mRNA (category number: 636525; Clontech, Mountain View, CA) was used as a negative control. As a positive control for prostate cancer cell isolation, LNCaP95 cells were expanded, subcultured, counted, and cryopreserved in single-use aliquots (10,000 cells) in liquid nitrogen. Empirically, larger numbers of cells were required in contrast to the analyte spike in experiments. Cells were diluted into 5 mL of RPMI and processed in parallel to whole blood samples.

Deidentified discarded peripheral blood specimens obtained from 38 healthy bone marrow donors were used to characterize the expression of AR and AR-V7 (14 males, age, 20 to 40 years; 24 females, age, 20 to 69 years).

Blood samples (minimum, 10 mL) from men with metastatic CRPC were collected in two or more acid citrate dextrose (yellow-top) vacutainer tubes (BD, Franklin Lakes, NJ) and sent to the clinical laboratory cold. Two tubes of blood were used for duplicate routine clinical testing. When a third tube was available, it was analyzed independently in the research laboratory. The CTCs were enriched and RNA was harvested within 24 hours of sample collection (generally within 4 hours).

CTC Enrichment

CTCs were enriched using the AdnaTest Prostate-CancerSelect kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Briefly, this method uses immunomagnetic enrichment of tumor cells via epithelial and tumor-associated antigens. Magnetic beads with proprietary antibodies were used to bind to the tumor cells in peripheral blood, and captured using a magnet (LSKMAGS15;

MilliPore, Darnsted, Germany). mRNA from cell lysates was isolated using the AdnaTest ProstateCancerDetect kit (Qiagen), which contains oligo-(dT)₂₅-coated beads, according to the manufacturer's protocol. All mRNA isolated was reverse-transcribed to cDNA by the SensiScript RT kit (catalog number: 205213; Qiagen) in a 20-μL reaction using the following thermocycler settings: 37°C for 60 minutes, 93°C for 5 minutes, and 4°C hold. Negative and positive control cDNA were produced in single master lots and stored as single-use aliquots at -20°C.

cDNA was used as a template in a multiplex PCR reaction using HotStar Taq Master Mix (Qiagen) and a pool of primers (provided with the kit) that amplify three tumor-associated antigen mRNAs [epidermal growth factor receptor, prostate-specific antigen (PSA), and prostate-specific membrane antigen (PSMA)] and one reference mRNA beta-actin (ACTB). The following PCR conditions were used: 95°C for 15 minutes; 94°C for 30 seconds, 61°C for 30 seconds, and 72°C for 30 seconds × 42 cycles; 72°C for 10 minutes; and 4°C hold. The amplified PCR products were detected using an Agilent TapeStation 2200 (Agilent, Santa Clara, CA) to confirm cDNA synthesis.

qPCR

Each of the two cDNA syntheses were tested independently as replicates. The cDNA (1/10 of each cell lysate yield) was used as a template for six independent real-time quantitative PCRs (qPCRs) (20 μL reactions) using TaqMan Universal MasterMix II without UNG (Life Technologies, Carlsbad, CA) and primer-probe sets (Table 1) to amplify three prostate-associated antigen mRNAs (PSA, PSMA, and AR-FL), two reference transcripts [glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ACTB], and the AR-V7 transcript.

Table 1 Custom-Ordered qPCR and Sequencing Oligonucleotides

AR-FL qPCR	
Forward primer	5'-CCTGCTCAAGACGCTTCTAC-3'
Reverse primer	5'-GAAGTATGTCAGCTCTCTCG-3'
Hydrolysis probe	5'-CTCCTGGACTCCGTGCAGCCTA-3'
AR-V7 qPCR	
Forward primer	5'-TGAAGCAGGGATGACTCTGG-3'
Reverse primer	5'-TCAGCCTTTCTTCAGGGTCTG-3'
Hydrolysis probe	5'-CCGGGTGGCAATTGCAAGCA-TCT-3'
Sequencing	
PSMA forward primer	5'-CCACCTTTTCAGTGCTTTCTC-3'
PSMA reverse primer	5'-TTTTTCATGTCCCGTTCCAAT-3'
PSA forward primer	5'-GTCCCGGTTGTCTTCTCTAC-3'
PSA reverse primer	5'-GGGAATGCTTCTCGCACT-3'
AR-FL forward primer	5'-CCTGCTCAAGACGCTTCTAC-3'
AR-FL reverse primer	5'-GAAGTATGTCAGCTCTCTCG-3'
AR-V7 reverse primer	5'-TTTGAATGAGGCAAGTCAGCCT-TTCT-3'

AR, androgen receptor; FL, full length; PSA, prostate-specific antigen; PSMA, prostate specific membrane antigen; qPCR, real-time quantitative PCR; V7, splice variant 7.

PCR conditions were as follows: 95°C for 10 minutes; and 95°C for 15 seconds and 60°C for 1 minute × 50 cycles.

The qPCR assay was performed using hydrolysis probes containing fluorescein amidite and a minor groove binding nonfluorescent quencher and the ABI 7900 HT qPCR instrument (Applied Biosystems, Waltham, MA). The cycle threshold (Ct) value was determined visually as the cycle number at which the fluorescence signal crosses 50% of maximal intensity. Commercially available sets of primers and probes (ThermoFisher Scientific, Waltham, MA) were used for GAPDH (Hs02758991_g1), ACTB (Hs01060665_g1), PSMA (Hs00379515_m1), and PSA (Hs02576345_m1) transcripts. Custom-designed primers and probes (Table 1) spanning the exon 7—exon 8 junction for AR-FL (NM_000044.3) and the exon 3—cryptic exon 3 junction for AR-V7 (transcript described by Hu et al,¹¹) were used to amplify 77 and 84 base pair amplicons, respectively. Primer specificity was confirmed by the Basic Local Alignment Search Tool (BLAST; NCBI, <https://blast.ncbi.nlm.nih.gov/Blast.cgi>, last accessed November 3, 2016) against the human transcriptome. Primers and probes were from single master lots in single-use aliquots.

Sanger Sequencing

qPCR amplification products from PSMA, PSA, AR-FL, and AR-V7 were sequenced using primers in Table 1. Products were cycle-sequenced using BigDye v3.1 (Applied Biosystems) per the manufacturer's instructions on an Applied Biosystems 9700 thermocycler and products were resolved on an Applied Biosystems 3730XL DNA sequencer.

Results

We developed an assay to detect AR-V7 mRNA from the CTCs from metastatic CRPC patients (Figure 1). Briefly, CTCs were isolated, and their presence or absence initially was assessed by using cDNA in a multiplex PCR (Adna ProstateCancerDetect kit) for amplification of three tumor-associated antigens (epidermal growth factor receptor, PSA, and PSMA) using the Agilent TapeStation 2200. The mRNA from enriched cells was isolated using Oligo-d(T)₂₅-coated beads and reverse-transcribed into cDNA. Separate qPCR reactions for six target mRNAs were performed in duplicate, including two reference gene transcripts (ACTB and GAPDH), three prostate-specific gene transcripts (PSMA, PSA, and AR-FL), and AR-V7 mRNA. Each analyte was interpreted qualitatively and the test results were reported as follows: i) no mRNA detected: this category is used when all RT-PCR reactions have failed; ii) no CTCs detected: used when housekeeping genes are positive and prostate-specific markers are negative; iii) CTCs detected/AR-V7 not detected: used when housekeeping and prostate-specific markers both are positive; and iv) CTCs detected/AR-V7 detected: used when

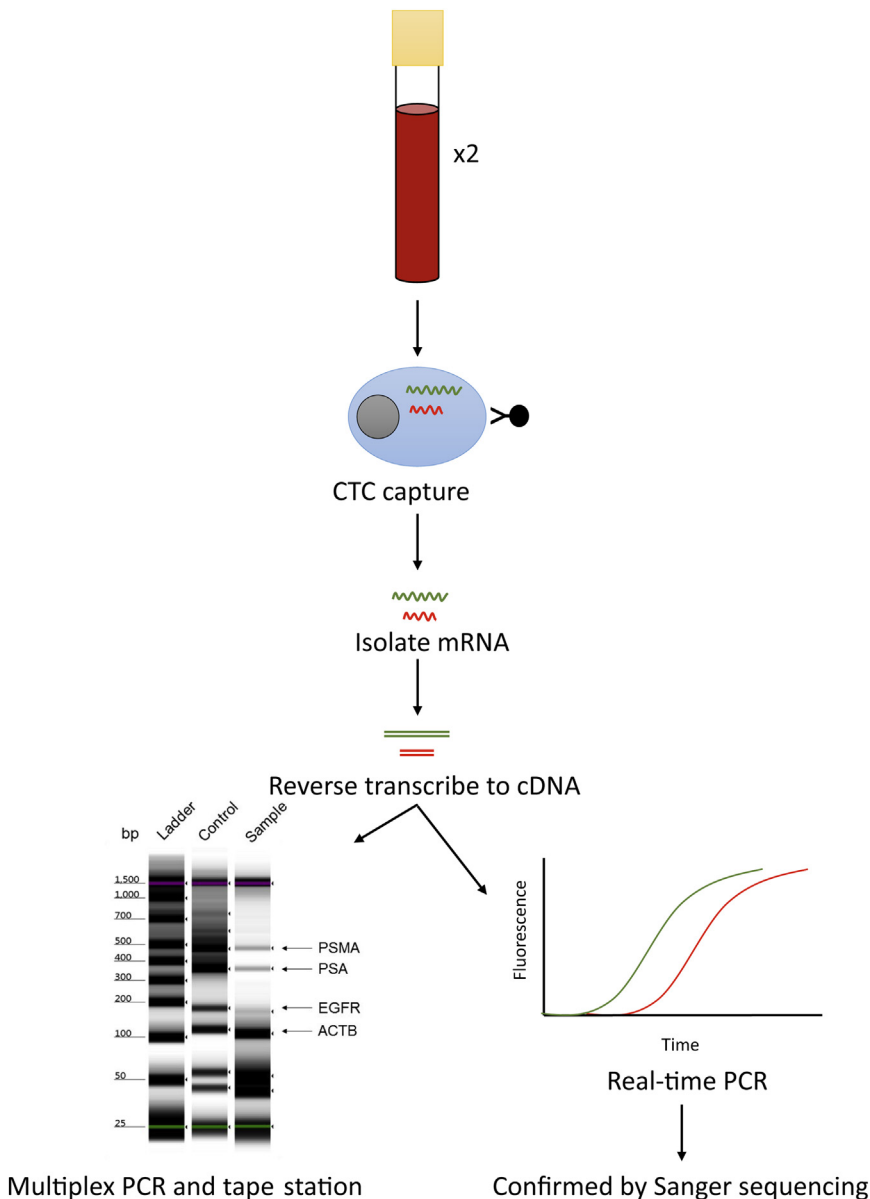


Figure 1 Androgen receptor splice variant 7 (AR-V7) test workflow. Patient blood samples in a minimum of two (preferably three) yellow-top tubes are obtained. Two tubes are used to test the patient in duplicate using our assay as follows. Circulating tumor cells (CTCs) are enriched from the blood samples and mRNA is isolated. **Green lines** and **red lines** designate AR-FL and AR-V7 mRNA, respectively. The RNA is reverse-transcribed to cDNA. cDNA is used in a multiplexed PCR reaction followed by electrophoresis on TapeStation to detect the presence of CTCs. cDNA also is used as a template for real-time quantitative PCR to amplify six target regions to investigate the presence of CTCs and AR-V7. Any amplified prostate-specific antigen (PSA), prostate-specific membrane antigen (PSMA), AR-FL, or AR-V7 products were confirmed by Sanger sequencing. **Purple horizontal bar** shows 1500-bp size standard; **green horizontal bar**, 25-bp size standard. ACTB, beta-actin; EGFR, epidermal growth factor receptor.

prostate-specific markers and AR-V7 reactions both are positive. Amplification of either PSA or PSMA was evidence for CTC detected. Text from the reports is provided in [Supplemental Appendix S1](#). Positive control (LNCaP95 mRNA), negative control (human splenic mRNA), and no-template control (water) were included in each qPCR run.

Validation Approach

The approach to assay validation included confirming the identity of the cell line and the presence of the AR-V7 transcript, validation of the individual steps of the assay including CTC isolation, confirming the identity of qPCR amplification products by Sanger sequencing, and testing the performance characteristics of the assay.

Characterization of LNCaP95 Cell Lines

The LNCaP95 cell line is a derivative of LNCaP, and was characterized by amplifying DNA with the Identifier kit ([Figure 2B](#)). The microsatellite profile of LNCaP95 matched the parental LNCaP (not shown), and the profile listed by ATCC (Manassas, VA). We confirmed AR-V7 expression using RT-PCR and Sanger sequencing (data not shown).

Validation of CTC Enrichment

We showed that the AdnaTest ProstateCancerSelect kit was capable of isolating CTCs from blood by spiking 10,000 and 50,000 prostate cancer (LNCaP95) cells into 4 mL of a donor blood specimen. The large number of cells was required because we used microsatellite fingerprinting as the

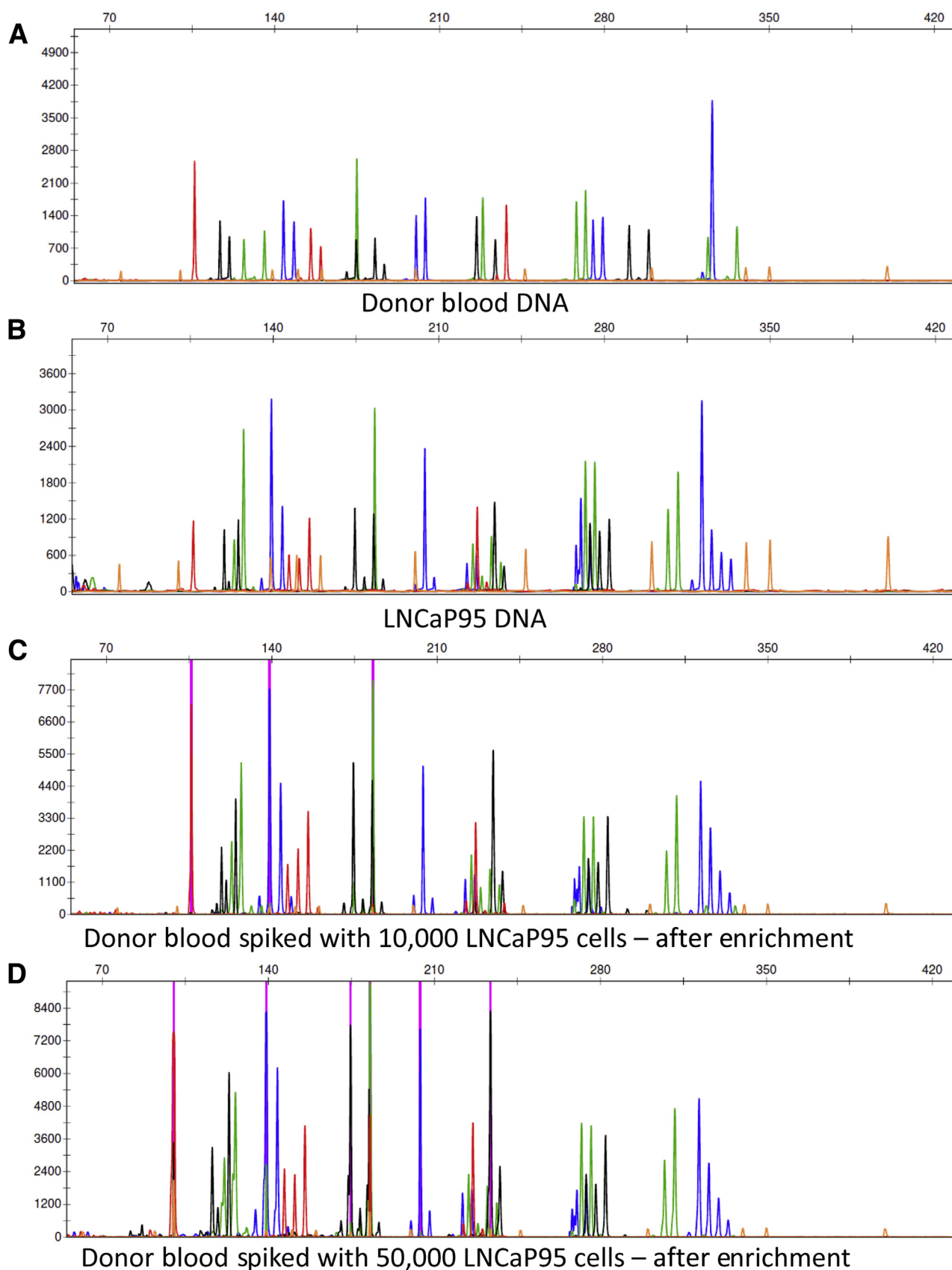


Figure 2 **A:** Microsatellite profile of unspiked donor blood DNA. **B:** Microsatellite profile of LNCaP95 DNA. **C:** Microsatellite profile of donor blood spiked with 10,000 LNCaP95 cells after enrichment. **D:** Microsatellite profile of donor blood spiked with 50,000 LNCaP95 cells after enrichment. After CTC-enrichment the majority of the DNA ($\geq 90\%$) is derived from the prostate cancer cells, showing effective enrichment.

Table 2 Validation of CTC Enrichment

LNCaP95 cells spiked, <i>n</i>	Total donor cells	LNCaP95 cells in sample, %	Pre-enrichment		After enrichment		Fold enrichment
			Donor DNA, %	LNCaP95 DNA, %	Donor DNA, %	LNCaP95 DNA, %	
0	1.5×10^7	0.00	100	0			N/A
10,000	1.5×10^7	0.07	100	0	10	90	1350
50,000	1.5×10^7	0.33	100	0	2	98	294

CTC, circulating tumor cell; N/A, not applicable.

end point. The percentages of the LNCaP95 DNA and donor DNA were quantified (Figure 2, A and B). No LNCaP95 DNA was detected in any of the pre-enriched specimens (Table 2). After enrichment, the samples with 10,000 (Figure 2C) and 50,000 (Figure 2D) spiked cells consisted of approximately 90% and 98% of LNCaP95, respectively. This represents an approximate 1350-fold and 294-fold enrichment, respectively (Table 2).

Validation of qPCR

We tested the cDNA that was reverse-transcribed from 1 ng of harvested LNCaP95 mRNA for the presence of six-target transcripts (AR-V7, AR-FL, PSA, PSMA, GAPDH, and ACTB) using qPCR. As expected LNCaP95 showed expression of all six-target regions, including AR-V7 (Supplemental Figure S1). The identity of the amplified PCR products from the four nonreference targets (PSMA, PSA, AR-FL, and AR-V7) was confirmed using Sanger sequencing (Supplemental Figure S2). In contrast, by using human splenic mRNA, GAPDH, ACTB, PSMA, and AR-FL were detected, consistent with the documented expression of some prostate-specific antigens in other organs (<http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Hs.654487>, last accessed May 9, 2016), but did not show expression of PSA or AR-V7 (Supplemental Figure S1).

Limit of Detection of the CTC

We spiked 5 mL of healthy blood samples with different numbers of freshly harvested LNCaP95 cells, in duplicate. After enrichment, we consistently detected as few as five LNCaP95 cells using both the CTC detection multiplex kit (Figure 3A), as well as by qPCR of the four prostate-specific transcripts: AR-V7, AR-FL, PSA, and PSMA (Supplemental Table S1). The average Ct value for qPCR correlated inversely with the number of spiked LNCaP95 cells as expected (Figure 3B). The average RNA concentration obtained after CTC enrichment correlated with the number of spiked LNCaP95 cells (Supplemental Figure S3). We were unable to detect CTCs reliably from samples spiked with fewer than 5 cells (data not shown). This compares favorably with data from Bitting et al²⁵ who showed a median number of 16 CTCs per 7.5 mL of blood.

Analytical Sensitivity

To determine the analytical sensitivity, we generated serial dilutions of harvested LNCaP95 RNA, converted to cDNA, and tested for the presence of all six target transcripts. Our assay consistently detected all analytes, except PSA, from an estimated 1 pg of input LNCaP95 RNA (Figure 4 and Supplemental Table S2). The limit of detection for PSA transcripts was 10 pg of input RNA.

Analytical Precision

We compared the intrarun and inter-run Ct values for each of the amplified targets when using 1 ng input RNA from LNCaP95 to measure the analytical precision of our assay. There was good intrarun reproducibility of the Ct values (means ± SD) for each of the amplified targets from 12 separate reactions (Table 3 and Supplemental Table S3). The average ΔCt (Ct of AR-V7 – Ct of AR-FL) for LNCaP95 was 3.69 ± 0.23 (CV, 6.2%), implying an AR-FL to AR-V7 transcript ratio of between 8- and 16-fold (Supplemental Table S3). Similar reproducibility was seen in two replicates from an independent run. The Ct (and ΔCt) values of the positive control (LNCaP95 RNA) were determined for each run and plotted on a Levey–Jennings chart as quality control.²⁶

Diagnostic Specificity

We de-identified and tested the peripheral blood from 38 healthy controls (14 males, age, 20 to 40 years; 24 females, age, 20 to 69 years) for the six transcripts. All control samples were negative for AR-V7, but expressed both reference genes, showing high (100%) diagnostic specificity (Supplemental Table S4). All healthy control samples were negative for PSA and PSMA; however, AR-FL transcripts were detected at very low levels (Ct value, >39) in 3 healthy donors: 2 older women (age, >50 years) and 1 man (age, <40 years) (Supplemental Table S4), consistent with the documented AR-FL expression from nonprostatic tissue (<http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Hs.76704>, last accessed May 9, 2016).

Diagnostic Accuracy

Before clinical implementation, we tested 21 CRPC patient blood specimens concurrently with Dr. Luo’s laboratory.¹⁵

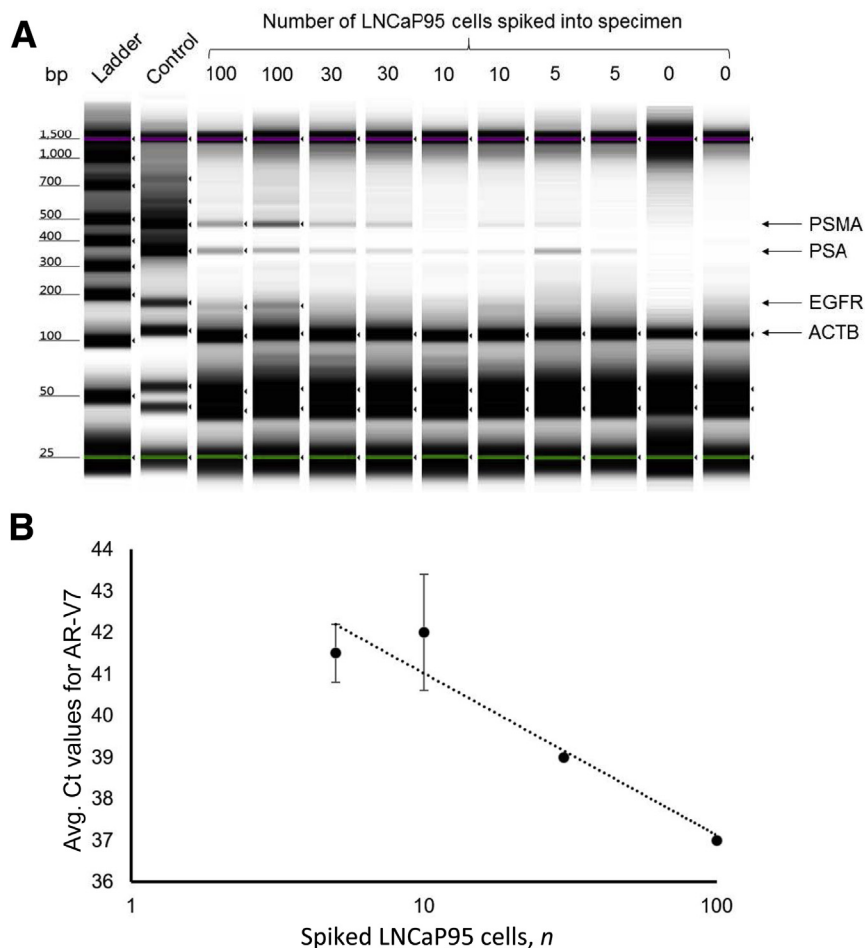


Figure 3 Different estimated numbers of LNCaP95 cells in duplicate were spiked into a healthy donor blood sample and detected using the Adna ProstateCancerDetect kit, as well as our assay. **A:** TapeStation image after ProstateCancerDetect kit showing amplification of prostate-associated antigens [prostate-specific antigen (PSA), prostate-specific membrane antigen (PSMA), epidermal growth factor receptor (EGFR), beta-actin (ACTB)] from samples spiked with 5 or more cells. **B:** The average cycle threshold (Ct) value of androgen receptor splice variant 7 (AR-V7) real-time quantitative PCR amplification correlates inversely with the number of spiked LNCaP95 cells. **Purple horizontal bar** shows 1500-bp size standard; **green horizontal bar**, 25-bp size standard. Experiments were performed in duplicate and error bars are ± 1 SD. Avg, average.

Results were concordant for AR-V7 for all 21 cases (positive in 4 cases, negative in 17 cases) (Table 4 and Supplemental Table S5). In addition, the ratio of AR-FL to AR-V7 transcripts, as estimated by Δ Ct (Ct of AR-FL – Ct of AR-V7), was comparable for all four positive cases

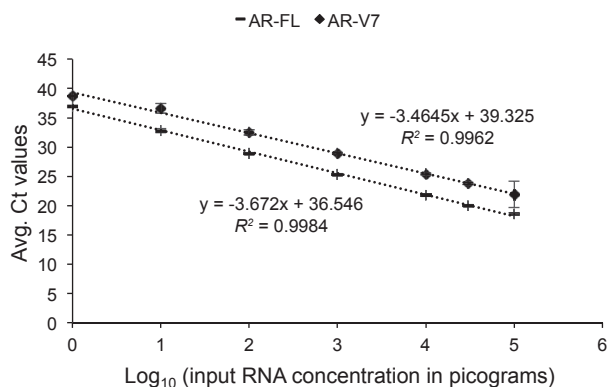


Figure 4 Real-time quantitative PCR amplification using diluting concentrations of input LNCaP95 RNA was performed. The graph of average cycle threshold (Ct) value versus $\log_{10}$ (input RNA concentration) shows amplification of both AR-FL and AR-V7 from up to 1 pg (0 on $\log_{10}$ scale) of input LNCaP95 RNA. Experiments were performed in duplicate and error bars are ± 1 SD. AR, androgen receptor; Avg, average; FL, full length; V7, splice variant 7.

between the laboratories (Supplemental Table S5). The difference in Δ Ct between the laboratories averaged 0.725 (range, -0.77 to 1.825). There were occasional discrepancies between the laboratories regarding the presence or absence of individual prostate-specific transcripts (PSA, PSMA, and AR-FL). However, overall, 19 of the 21 CRPC cases were categorized identically by both laboratories (Table 4). The laboratories differed in two cases regarding the presence of CTCs. Our assay detected low levels of PSMA transcripts in these two patients at high Ct values of 38.82 and 38.86, respectively, but not PSA or AR-FL (Supplemental Table S5). The research laboratory categorized these cases as no CTCs detected. In this regard, the presence or absence of CTCs was not clinically actionable because the indication for testing was limited to men with known metastatic disease.

Proposed Indications for Testing and Clinical Implementation

AR-V7 testing may be indicated in men with metastatic CRPC, to be integrated into therapeutic decision making about the potential benefit of AR-directed agents (enzalutamide and abiraterone) versus taxanes (docetaxel and

Table 3 Precision

mRNA	Mean Ct	SD	CV
GAPDH	25.0	0.06	0.24%
ACTB	23.55	0.11	0.47%
PSA	31.76	0.13	0.41%
PSMA	27.92	0.08	0.29%
AR-FL	27.64	0.17	0.62%
AR-V7	31.34	0.21	0.67%

ACTB, beta-actin; AR, androgen receptor; Ct, cycle threshold; FL, full length; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PSA, prostate-specific antigen; PSMA, prostate-specific membrane antigen; V7, splice variant 7.

cabazitaxel) based on the correlation between the presence of AR-V7 transcripts and the lack of therapeutic response to AR-targeting agents.^{15–17}

Clinical testing was implemented as follows. Each clinical sample was tested in duplicate. The parental LNCaP cell line spiked into RPMI was used as a positive control with each CTC isolation. The cDNA was tested in duplicate in a multiplex PCR using the proprietary kit and detected using electrophoresis (TapeStation) to initially confirm the presence/absence of CTCs. Stored cDNA was tested by qPCR, with a positive control (LNCaP95 RNA), a negative control (human splenic RNA), and a no-template control in each run. The identity of amplified prostate-specific targets (PSA, PSMA, AR-FL, and AR-V7) was confirmed by Sanger sequencing. Residual blood samples subsequently were tested by the research laboratory independently to determine concordance as quality control.

Subsequent Testing After Validation

After launching the clinical test, patient results were released based on testing in the molecular diagnostic laboratory only, and without knowledge of the research testing laboratory results. Seventeen patient samples subsequently were tested by the research laboratory. Overall, 16 of 17 patients were

categorized identically between the two laboratories (94.11% concurrency): 4 as no CTCs detected; 6 as CTCs detected, no AR-V7 detected; and 6 as CTCs detected, AR-V7 detected (Table 4 and Supplemental Table S6). All prostate-associated targets amplified by our laboratory from the first 13 clinical samples (PSMA, PSA, AR-FL, and AR-V7) were confirmed by sequencing. From sample 14 onward, only AR-FL– and AR-V7–positive amplicons were sequenced. There was one discrepancy (sample 17) between the laboratories, the clinical laboratory detected the AR-V7 transcript at a very low level (Ct, 38.10) in one of the two duplicates, but this was not detected by the research laboratory. The residual cDNA for this sample was tested and confirmed the presence of AR-V7, and was confirmed further by Sanger sequencing. To date, no sample has failed testing (ie, no mRNA detected). Since implementation, all AR-V7–positive samples have been confirmed by Sanger sequencing.

Discussion

Detection of AR-V7 in patients with metastatic CRPC has been shown to correlate with a lack of response to AR-targeting agents by four independent groups.^{15–18} The first report by Antonarakis et al¹⁵ from Johns Hopkins reported 62 patients treated with either enzalutamide or abiraterone. Eighteen (29%) patients expressed AR-V7 at baseline, showed a lack of PSA response, and showed significantly reduced progression-free and overall survival compared with patients who lacked AR-V7 expression. The second study from Houston, TX, followed up 60 patients with bone metastatic CRPC treated with enzalutamide. The presence of AR-V7 in bone marrow specimens (detected at the protein level using immunohistochemistry) in 26% of patients was associated with primary resistance to enzalutamide treatment.¹⁷ The third study from Germany looked at the clinical response of 37 patients treated with hormonal therapy (enzalutamide or abiraterone). Only 1 of 15 (7%) AR-V7–positive patients showed a PSA response to therapy in

Table 4 Diagnostic Accuracy

Results	Dr. Luo's Laboratory			
	No mRNA detected	No CTCs detected	CTCs (+), AR-V7 (–)	CTCs (+), AR-V7 (+)
Results of concurrent testing during validation				
CLIA laboratory	0	0	0	0
No mRNA detected				
No CTCs detected	0	6	0	0
CTCs (+), AR-V7 (–)	0	2	9	0
CTCs (+), AR-V7 (+)	0	0	0	4
Results of subsequent testing during live testing				
CLIA laboratory				
No mRNA detected	0	0	0	0
No CTCs detected	0	4	0	0
CTCs (+), AR-V7 (–)	0	0	6	0
CTCs (+), AR-V7 (+)	0	0	1	6

AR, androgen receptor; CLIA, Clinical Laboratory Improvement Amendments; CTC, circulating tumor cell; V7, splice variant 7.

contrast to 71% of AR-V7–negative patients.¹⁶ Most recently, a fourth study by Scher et al¹⁸ from Memorial Sloan Kettering Cancer Center, using independent methodology, confirmed that the expression of AR-V7 predicted a lack of response to AR targeting agents. Although additional confirmation is desirable, we consider the AR-V7 test to be sufficiently clinically validated.

In this study, we present analytical validation of the first qualitative AR-V7 test with high analytical sensitivity, precision, diagnostic specificity, and accuracy. The clinical assay is similar to that described previously by Dr. Luo's research laboratory¹⁵ with the following differences. We used sequence-specific hydrolysis probes instead of the intercalating dye (SYBR green), and we Sanger sequenced the amplified products to confirm their identity.

In our assay, detection of the prostate-associated antigen transcripts (PSA or PSMA) from the patient's blood was interpreted as CTCs detected. Because the AR-FL transcript was detected in 3 healthy control patients' blood, we did not use AR-FL by itself as evidence of CTC positivity. AR-FL positivity is used as additional evidence of the presence of CTCs when another transcript (PSA or PSMA) is positive. Furthermore, none of the patients had detectable AR-FL transcript in the absence of PSA and PSMA.

Our current assay is qualitative. Low levels of expression of AR-V7 are reported as AR-V7 positive. Whether quantitative AR-V7 differences are associated with a differential response to therapy remains to be determined. In theory, low-level AR-V7 positivity by our assay may reflect either capture of scant CTCs that are all AR-V7 positive, or may reflect a small subset of captured CTCs that express AR-V7. Whether tumor heterogeneity exists in the CTCs, and if that is representative of the *in situ* tumor, and how that affects response to therapy, is not currently known.

Because our laboratory is the first CLIA–certified laboratory performing AR-V7 testing, interlaboratory proficiency currently is not possible. In the interim, we plan to perform repeat testing of stored cDNA. Currently, residual samples subsequently are tested by Dr. Luo's laboratory and results are compared. Detection of AR-V7 and other transcripts could be adapted to next-generation sequencing–based testing, possibly using RNA annealing, selection, and ligation sequencing technology.²⁷

CRPC patients resistant to the novel hormonal therapy often are treated with taxane chemotherapy. Taxanes disrupt the intracellular microtubule trafficking and thus disrupt androgen signaling by sequestering the AR protein in the cytoplasm. Thus, they should be effective against both the AR-FL and AR-V7 CRPCs. However, data from *in vitro* and xenograft model systems have shown that when treated with taxanes (docetaxel), AR-V7 can traffic into the nucleus in a microtubule-independent mechanism, and the AR-V7–expressing cells can escape growth inhibition.^{28–30} In contrast, clinical data has documented better outcomes for AR-V7–expressing CRPC patients when treated with taxanes in comparison with novel hormonal therapy.¹⁹ Another

group has shown that response to cabazitaxel in CRPC patients is independent of the AR-V7 status of the patient.²¹ Prospective clinical trials (eg, NCT02379390, <https://clinicaltrials.gov/ct2/show/NCT02379390>, last accessed November 18, 2016) are ongoing to confirm the clinical response to taxanes in AR-V7–expressing patients.

In addition to taxanes, several newer therapeutic agents have shown promising results in preclinical studies against AR splice variants. These include galeterone (Tokai Pharmaceuticals, Boston, MA), an AR-targeting agent that potentially can inhibit both AR-FL– and AR-V7–mediated signaling^{31–33}; the ESSA Pharmaceuticals Inc. (EPI, Vancouver, CA) family of small molecules (including EPI-506) that target the AR N-terminal domain^{34–36}; agents targeting the bromodomain extraterminal family of proteins that bind with AR to facilitate its transcriptional activation^{37,38}; and niclosamide, which can inhibit AR-FL– and AR-V7–mediated transcriptional activity in preclinical models.^{39,40}

In addition to the AR splice variants, several less common possible mechanisms of resistance to androgen-deprivation therapy have been documented in CRPC patients.⁴¹ These include AR gene amplification and overexpression, conversion of weak androgens to potent androgens, intratumoral androgen production, somatic point mutations in the N-terminal domain of AR, and stimulation of AR via androgen-independent pathways (reviewed by Maughan and Antonarakis²²). Our assay does not detect these possible alternative mechanisms of resistance to androgen-deprivation therapy. Data that AR-V7–positive CRPCs respond to taxanes potentially allows AR-V7 to serve as a clinically actionable treatment-selection biomarker in this setting.

In summary, AR-V7 testing in metastatic CRPC patients may serve as a clinically relevant biomarker to guide prognosis as well as therapy decisions. Several ongoing clinical trials are likely to provide information regarding the best therapeutic choice in this high-risk population. The development of a robust AR-V7 assay performed in a CLIA–certified laboratory documented in this report is the crucial first step in its further clinical validation.

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.jmoldx.2016.08.003>.

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REVIEW

Androgen receptor variant-driven prostate cancer: clinical implications and therapeutic targeting

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While there are myriad mechanisms of primary and acquired resistance to conventional and next-generation hormonal therapies in prostate cancer, the potential role of androgen receptor splice variants (AR-Vs) has recently gained momentum. AR-Vs are abnormally truncated isoforms of the androgen receptor (AR) protein that lack the COOH-terminal domain but retain the NH₂-terminal domain and DNA-binding domain and are thus constitutively active even in the absence of ligands. Although multiple preclinical studies have previously implicated AR-Vs in the development of castration resistance as well as resistance to abiraterone and enzalutamide, recent technological advances have made it possible to reliably detect and quantify AR-Vs from human clinical tumor specimens including blood samples. Initial clinical studies have now shown that certain AR-Vs, in particular AR-V7, may be associated with resistance to abiraterone and enzalutamide but not taxane chemotherapies when detected in circulating tumor cells. Efforts are now underway to clinically validate AR-V7 as a relevant treatment-selection biomarker in the context of other key genomic aberrations in men with metastatic castration-resistant prostate cancer. Additional efforts are underway to therapeutically target both AR and AR-Vs either directly or indirectly. Whether AR-Vs represent drivers of castration-resistant prostate cancer, or whether they are simply passenger events associated with aggressive disease or clonal heterogeneity, will ultimately be answered only through these types of clinical trials.

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INTRODUCTION

In 1941, Huggins and Hodges¹ first demonstrated the clinical efficacy of hormonal manipulation for the treatment of metastatic prostate cancer. Androgen deprivation therapy (ADT), involving surgical or chemical castration, remains the standard first-line option for men with metastatic prostate cancer, and suppression of androgen receptor (AR) signaling has been the therapeutic goal in prostate cancer drug development for seven decades. It is well known, however, that ADT only provides temporary clinical benefit and progression to castration-resistant prostate cancer (CRPC) almost always occurs after a variable period of time. In general, prostate cancer progression upon first-line ADT continues to rely on AR signaling sustained by adrenal and intratumoral androgens as well as upregulation of AR protein expression in tumor cells. The established concept that sustained AR signaling is a key molecular determinant of CRPC has directly contributed to the successful clinical development of abiraterone and enzalutamide,^{2,3} both of which have been approved by the US Food and Drug Administration (FDA) to treat metastatic CRPC on the basis of survival improvements.^{4–7} However, a significant subset of CRPC patients demonstrates primary resistance to the two agents, and nearly all patients that are treated eventually develop acquired resistance during the course of treatment. Therefore, understanding and managing primary and acquired resistance to abiraterone and enzalutamide has become a critical

unmet need.⁸ One potential explanation for this resistance is the generation of AR splice variants (AR-Vs). In this review, we will discuss evolving insights into AR-V expression in prostate cancers and their implications in contemporary prostate cancer clinical care, as well as current efforts in therapeutic targeting of AR-Vs aiming to overcome resistance to novel hormonal therapies.

MECHANISMS OF ANDROGEN/AR RESISTANCE

A significant fraction of prostate tumors treated with androgen/AR-directed therapies, including abiraterone and enzalutamide, will demonstrate a molecular signature consistent with continued 'addiction' to AR signaling. General mechanisms of androgen/AR resistance focusing on the AR pathway have been covered in several recent reviews.^{8–11} Tumors treated by therapies designed to suppress AR signaling are expected to acquire molecular alterations in this axis to maintain their addiction. Indeed, the AR gene is frequently amplified or mutated (less common than AR amplification) in CRPC.^{12,13} In a recent study involving 150 metastatic CRPC cases, AR amplifications or mutations were found in ~62% of these cases.¹³ In contrast, focal amplification of the AR gene was detected in <1% of hormone-naïve prostate cancers ($n=596$).¹² In addition, overexpression of both the canonical full-length AR (AR-FL) and AR-Vs are frequently observed in CRPC. However, a wider spectrum of molecular aberrations may be

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responsible for sustained AR signaling, requiring carefully designed studies to dissect key drivers and determinants of resistance.^{11,14} For example, characterization of the relative frequency of previously reported molecular aberrations (including CYP17A1, AKR1C3, HSD3B1, GR and PR) in the context of aforementioned AR aberrations may help to further clarify their importance and clinical relevance. As prostate cancer is now being managed by increasingly more potent androgen/AR-directed therapies, it is reasonable to anticipate a rise in tumors in which AR expression may be low or even absent. These tumors may demonstrate histological and molecular features of neuroendocrine differentiation and/or small cell carcinoma, in which loss and/or mutations of the *RB1*, *TP53* and *PTEN* genes are often observed. A recent report suggested that up to a quarter of prostate tumors resistant to abiraterone or enzalutamide may demonstrate distinct morphological and molecular features intermediate between typical acinar adenocarcinoma and neuroendocrine differentiation/small cell carcinoma.¹⁵ It is currently unknown whether AR-Vs may be present in some of these tumors, and indeed in many of these cases serum PSA can be quite elevated suggesting ongoing AR activation. The various resistance mechanisms also portend increasingly complex patterns of intra- and inter-tumor heterogeneity that will need to be accounted for in the clinical setting and relevant study designs.

AR SPLICED VARIANTS

The availability of enzalutamide and abiraterone has facilitated studies aimed at understanding the role of AR-Vs in the presence of potent inhibitors of AR signaling. AR-Vs^{10,16} are alternatively spliced isoforms of the AR mRNA usually resulting in premature termination of the AR protein product. Most AR-Vs retain the NH₂-terminal transactivating domain (NTD) but are missing variable portions of the COOH-terminal domain including the ligand-binding domain (LBD) (Figure 1). In particular, AR-V7, the most frequently expressed AR-V,¹⁶ has been implicated in resistance to enzalutamide and abiraterone in both preclinical experiments and a few recent clinical studies (see later sections). Conceptually, AR-V7 along with a number of other AR-Vs, is a biologically plausible

mechanism of resistance to abiraterone and enzalutamide. AR-Vs lack the LBD, which is the intended therapeutic target of all existing androgen/AR-directed therapies (Figure 1). Preclinical studies have confirmed that AR-Vs are capable of mediating constitutively active AR signaling (that is, in the absence of androgens or AR-FL),^{17–21} that expression levels of AR-Vs are typically elevated in CRPC and in response to inhibition of AR-FL signaling,^{19–24} and that AR-V expression is associated with disease progression in retrospective studies.^{18,19,25} In clinical CRPC specimens, individual AR-Vs are often co-expressed with AR-FL and are usually less abundant than the wild-type transcripts.^{13,19,21,26} For example, quantitative PCR with reverse transcription (RT-PCR) analysis of circulating tumor cells from men with CRPC has revealed the median mRNA ratio of AR-V7/AR-FL is 21% (range, 1.8–208%).²⁷ RNA-seq analysis of CRPC autopsy and biopsy tissue has similarly revealed a broad range of AR-V7 expression ratios,^{13,27} with the median level of AR-V7 expression as a function of overall AR expression being ~5% in metastatic biopsies.¹³ However, because AR-FL is a very abundant transcript (increased by ~10-fold in CRPC compared with hormone-sensitive prostate cancer),¹⁹ a ratio of AR-V7/AR-FL at 5–20% would bring the levels of AR-V7 in CRPC specimens on par with the levels of AR-FL in an untreated hormone-naïve tumor. Presently the levels of nuclear AR-Vs required to drive an androgen-independent transcriptome remains unclear. However, even a low level of AR-V expression may be sufficient in the setting of castration or potent full-length AR blockade. Another AR-V, designated ARv567^{es}, is also expressed at levels equivalent to AR-FL in certain contexts.²⁸ These quantitative data provide the context to fathom a clinically relevant role of AR-Vs as ligand-independent transcription factors that may or may not require AR-FL.

STRUCTURE AND FUNCTION OF AR VARIANTS

Detailed structures of various AR-Vs have been described in recent reviews.^{10,16} The key domains shared among wild-type AR-FL and all AR-Vs are the NTD and DNA-binding domain (DBD). AR-Vs lack the LBD and instead have divergent COOH-terminal extensions encoded by mRNAs derived from exon-skipping events or

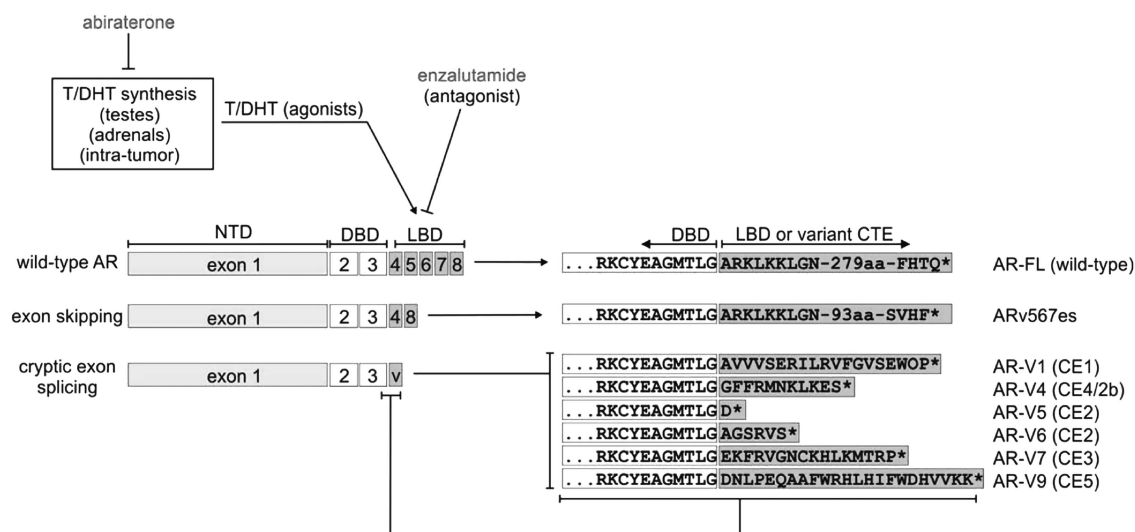


Figure 1. Domain structures of full-length androgen receptor and androgen receptor splice variants. Full-length androgen receptor and androgen receptor splice variants share a core structure composed of the transcriptionally active AR NH₂-terminal domain encoded by exon 1 and the DNA-binding domain encoded by exons 2 and 3. Androgen receptor splice variants (AR-Vs) lack the AR ligand-binding domain (LBD), which is the binding site for agonists testosterone (T) and DHT and competitive antagonists such as enzalutamide. Instead of a ligand-binding domain, androgen receptor splice variants contain CTE of variable length and sequence, which arise from exon skipping or splicing of various CE. Amino-acid sequences of CTEs from selected androgen receptor splice variants are shown. AR, androgen receptor; CE, cryptic exons; CTE, COOH-terminal extensions; DHT, dihydrotestosterone.

incorporation of alternative 3'-terminal cryptic exons (Figure 1). The AR-NTD is an intrinsically disordered protein domain, and is responsible for the majority of AR transcriptional activity. The AR-DBD is composed of two zinc fingers that mediate AR/DNA interactions and also AR/AR dimerization. There have been multiple review articles written on various aspects of molecular structure and function of AR-FL.^{29,30} Importantly, several lines of evidence support the notion that the function and regulation of the AR-NTD and DBD may proceed similarly between AR-FL and AR-Vs. For example, the AR transactivation unit-5 (TAU5) domain in the AR-NTD has been shown to function as a key transactivation domain for AR-FL under conditions of no/low androgen.³¹ The transcription factor FOXO1 has been shown to bind the AR TAU5 domain and thereby repress transcriptional activity of AR-FL and AR-Vs.³² This indicates that TAU5 has similar roles in mediating ligand-independent transcriptional activation in the context of AR-FL and AR-Vs. In addition, the highest-affinity genome-wide binding sites for both AR-FL and AR-FL vs AR-Vs are canonical inverted repeat androgen response elements (AREs), which indicates that AR-FL and AR-Vs display similar regulation of the DBD.³³ In line with this, dimerization between AR-V monomers requires the D-box dimerization interface in the second zinc finger of the AR-DBD,^{33,34} which is the same interface required for dimerization of AR-FL.³⁵

An outstanding question regarding AR-V structure/function is the identity of the precise domain(s) that promote nuclear localization of AR-Vs. The hinge region located between the AR DBD and LBD harbors the canonical AR nuclear localization signal, which is required for nuclear localization of AR-FL following ligand-binding.³⁶ However, only certain AR-Vs encoded by mRNAs that retain AR exon 4 (such as ARv567^{es}) retain this hinge region. Some AR-Vs encoded by mRNAs lacking exon 4 (such as AR-V7) do not harbor this hinge region, yet are still able to localize efficiently to the nucleus in a constitutive manner, possibly due to a NLS-like signal in their unique COOH-terminal extensions,^{19,26} although mutation of these residues has only a modest impact on the ability of AR-V7 to localize to the nucleus and does not impact on transcriptional activity.³⁷ One factor contributing to this efficient nuclear localization may be that most AR-Vs lack a nuclear export signal (NES) encoded by AR exon 6, which is required for nuclear exclusion of AR-FL in the absence of ligand.³⁸ In other studies however, some AR-Vs (including AR-V1 and AR-V9) did not demonstrate efficient nuclear localization although they lack NES, suggesting that some of the differential nuclear localization activities could be mediated by the short variant-specific COOH-terminal extensions.²⁶

DIMERIZATION AND CONSTITUTIVE ACTIVATION

Because AR-Vs often co-exist with AR-FL in CRPC, the role of AR-FL in mediating the function of AR-Vs remains unclear and warrants further studies. It is possible that AR-Vs may form heterodimers with AR-FL.^{20,21} If so, therapeutic targeting of AR-FL alone may theoretically disrupt AR-V-mediated functions.²¹ Xu *et al.*³⁴ characterized protein-protein interactions between AR-Vs (AR-V7 and ARv567^{es}) and AR-FL using bimolecular fluorescence complementation and bioluminescence resonance energy transfer assays. In this study, AR-Vs were found to form heterodimers with AR-FL and also to form homodimers with themselves in the absence of androgens, possibly through NTD-to-CTD interactions (NTD of AR-V and CTD of AR-FL) as well as DBD-to-DBD interactions (present in both AR-Vs and AR-FL). These data suggest potential interdependency between AR-FL and AR-Vs. However, the focus on overexpression models (as opposed to cells expressing endogenous AR-FL and AR-Vs) may limit the generalizability and clinical relevance of the findings.³⁹ Indeed, overexpression experiments using diverse readouts (co-immunoprecipitation, bimolecular fluorescence complementation) have

consistently been successful in detecting AR-FL/AR-V heterodimers.^{20,34,40} However, it remains plausible that the detected interactions may represent transient, DNA-dependent interactions given that AR-FL and AR-Vs have overlapping DNA-binding sites.³³ In line with this, reports testing heterodimerization between endogenously expressed AR-FL and AR-Vs have had mixed results. For example, an endogenous interaction between AR-FL and AR-Vs was not detectable in the 22Rv1 or CWR-R1 cell lines^{18,41,42} and in only one instance were interactions between AR-FL and ARv567^{es} detected in lysates from patient-derived xenografts.²⁰ Because of these mixed findings, it remains unclear whether AR-FL and AR-Vs directly interact or in what contexts, particularly in patients. Studies designed to detect dimer formation *in situ* may help to shed additional light on this important topic.

MOLECULAR ORIGINS OF AR VARIANTS

The mRNA and protein expression levels of AR-Vs relative to AR-FL varies within normal and malignant hormone-naïve and castration-resistant prostate tissues,^{13,19,22,26,43} circulating tumor cells^{27,44} and prostate cancer cell lines.^{17–19} This suggests that AR-V synthesis is not simply a byproduct of AR splicing. One mechanism that can dramatically alter the ratio of AR-V expression to AR-FL is rearrangement of the *AR* gene. For example, the 22Rv1 and CWR-R1 cell lines, which express high levels of AR-V7 and display AR-V-driven resistance to AR-targeted therapies, harbor large intragenic structural rearrangements in *AR*.^{45–47} Interestingly, the CWR-R1 cell line is heterogeneous, and the cell sub-population harboring a rearranged *AR* allele was shown to be the cell sub-population with AR-V7-driven antiandrogen resistance. Conversely, the CWR-R1 cell sub-population without this rearranged *AR* gene expressed very low levels of AR-V7 and displayed sensitivity to antiandrogens.⁴⁶ In addition, intragenic *AR* rearrangements have also been shown to dramatically impact expression of ARv567^{es}. For example, the LuCaP86.2 and LuCaP136 patient-derived xenografts harbor intragenic deletions and inversions, respectively, of a segment of the *AR* gene containing AR exons 5, 6 and 7.^{28,47} In the case of these alleles, AR-FL expression is abolished, and ARv567^{es} is the only AR species synthesized.²⁸

Additional mechanisms have been demonstrated to impact AR-V mRNA and protein levels in prostate cancer cells, which may function independently from or synergistically with structural alterations in the *AR* gene. An early observation in several prostate cancer cell lines was that specific inhibition of the AR-FL protein (via castration, antiandrogen treatment or siRNA) led to increased expression of AR-V7.^{22,23} However, concomitant increases in AR-FL expression have also been observed with these manipulations, which is likely the result of negative feedback inhibition through AR transcriptional autoregulation.⁴⁸ Similarly, overexpression of components of the canonical and non-canonical NF-κB signaling pathways in LNCaP cells (including IKK2, p65/RelA and p52) led to increased expression of AR-FL and AR-Vs, and inhibition of components of these pathways via siRNA or chemical inhibitors led to decreased expression of AR-FL and AR-Vs.^{49–51} Further, knockdown of hnRNPA1 (encoded by the *HNRNPA1* gene) and hnRNP2 (encoded by the *HNRNPA2B1* gene), which are both regulated by NF-κB, decreased AR-FL and AR-V7 protein expression in 22Rv1 and VCaP prostate cancer cells.⁵⁰ Additional hnRNPs, including hnRNP1 (encoded by the *PTBP1* gene) and hnRNP1 (encoded by the *HNRNPH1* gene), have also been shown to physically associate with the *AR* gene locus and positively affect expression of AR-FL and AR-Vs in various cell lines.⁵² Interestingly, splicing factors SF2 (encoded by the *SRSF1* gene) and U2AF65 (encoded by the *U2AF2* gene) have been shown to associate with the AR pre-RNA near the exon CE3/3b (encoding the 3'-terminal exon of AR-V7) splice acceptor. Further, knockdown of these factors impaired splicing of exon 3 to CE3 but not exons 3 to 4 in LNCaP95, a cell line derived from the parental LNCaP line with

high levels of AR-V7.⁵³ Overall, these studies have provided a diverse set of factors that can affect AR-V synthesis, but it should be noted that most of these factors also have similar effects on AR-FL synthesis, indicating that in these cell line models, production of AR-Vs may be influenced by aberrant AR transcription and perhaps a consequential change in splicing dynamics, rather than aberrant AR splicing that specifically affect the expression of AR-Vs.

Contemporary prostate cancer genome-sequencing studies have not been able to confirm an early report of a Q640X stop point-mutation in prostate cancer that can give rise to an AR-V-like truncated AR protein species.^{13,43,54} This raises the question: if truncation of the AR LBD is an effective resistance mechanism in prostate cancer, why are not truncating mutations observed? One possibility may be that mRNAs harboring premature termination codons in exons 4–7 of the *AR* gene would have subsequent exons splice downstream of this mutant termination codon, which is the classical signal for degradation by nonsense-mediated mRNA decay.⁵⁵ Therefore, rearrangement of the *AR* gene and/or changes in splicing dynamics may be the only mechanism(s) available to achieve expression of AR-V proteins in prostate cancer cells.

AR AND AR-V TRANSCRIPTOMES

AR-Vs were initially found to function as constitutively active transcription factors that can activate transcription of AR-regulated target genes, such as *KLK3* (*PSA*), *KLK2* (*HK2*), *TMPPRSS2* and *NKX3-1*.¹⁷ Subsequent studies indicated that AR-Vs can not only support the broad androgen/AR transcriptional program^{22,46} but may also uniquely transcriptionally activate alternative targets such as *AKT1*,¹⁸ genes associated with M-phase regulation of the cell cycle including *UBE2C* and *CCNA2* [ref. 22] and the *FOXA1*-repressed target genes *EDN2* and *ETS2*.⁵⁶ In contrast, a ChIP-seq study found that ARv567^{es} displayed the same genome-wide binding preference as AR-FL, but engaged chromatin sites with weaker affinity.³³ Chromatin sites found to be engaged by both ARv567^{es} and AR-FL included *UBE2C*, *CCNA2*, *EDN2* and *ETS2*, which would not be expected if these genes were unique transcriptional targets of AR-Vs.³³ One explanation for the apparent discrepancy, among many others,⁵⁷ may be the known biphasic nature of androgen signaling.⁴⁶ This biphasic signaling profile, wherein prostate cancer cells proliferate maximally when exposed to androgens in the 0.1–1.0 nM range, but display paradoxical proliferative inhibition when androgens are 10 nM and above, is one of the justifications for trials of high-dose testosterone therapy for men with castration-resistant prostate cancer.⁵⁸ Accordingly, many of the gene targets that have been proposed to be unique to AR-Vs, including *UBE2C* and *CCNA2*, may be induced by AR-FL in the low androgen environment but repressed under high suppressive androgen levels.⁴⁶ Similarly, AR-V7 has been shown to activate (while AR-FL has been shown to repress) the tricarboxylic acid cycle-related genes *MDH1* and *OGDH*. This differential gene expression has been linked to differences in metabolism noted for LNCaP cells expressing AR-V7 versus LNCaP cells treated with androgen.⁵⁹ Overall, these studies have documented several differences when AR-FL and AR-V transcriptional targets have been evaluated head-to-head. Further investigation is required to determine whether these and other AR-V gene targets are truly unique, or instead gene targets common to AR-FL and AR-Vs that display threshold and/or biphasic responses to varying levels of AR transcriptional output.

PRECLINICAL DATA ON AR-V INHIBITION

Despite many studies documenting expression of AR-Vs in prostate cancer cell lines, patient-derived xenografts, patient tissues and circulating tumor cells, relatively few studies have

evaluated the potential therapeutic efficacy of directly inhibiting AR-Vs. This is because many of the manipulations or treatments that have been applied to AR-V-expressing prostate cancer cells can inhibit both AR-FL and AR-Vs. Therefore, it has been difficult to discern the relative contributions of these two AR species to key biological parameters. For example, development of enzalutamide resistance in the LNCaP cell line model has been shown to be associated with increased expression of AR-FL as well as AR-V7. Antisense oligonucleotides (ASOs) that inhibited AR-FL expression inhibited growth and induced apoptosis in these enzalutamide-resistant cells. However, these effects were equivalent for various AR-targeted ASOs, even those that blocked AR-FL but not AR-V7. Alternatively, in 22Rv1 cells, ASOs that blocked expression of AR-FL and AR-V7 were more effective in inhibiting growth and inducing apoptosis than ASOs that only blocked AR-FL.⁶⁰ Similarly, a separate study showed that LNCaP cells stably expressing AR-Vs remained sensitive to inhibition of AR-FL via siRNA-mediated knockdown or treatment with enzalutamide,²¹ indicating that mere expression of AR-Vs in this cell line model may not be sufficient to drive all parameters of resistance. In contrast, in cases where AR-V synthesis is due to underlying rearrangements in the *AR* gene, selective ablation of AR-V expression can impair proliferation of prostate cancer cells and restore responses to androgens and antiandrogens.^{28,46}

AR VARIANTS AND EPITHELIAL PLASTICITY

Emerging data suggest a complex relationship between AR biology and epithelial plasticity, defined as the ability of cells to reversibly undergo phenotypic changes.^{61,62} These phenotypic changes range from alterations in gene expression to protein translation, changes in invasion, proliferation and metastasis, and changes in morphology, such as the mesenchymal or neuroendocrine transition. In transgenic mice engineered to overexpress either AR-V7 or AR-V567^{es}, increases in invasion and stemness/plasticity biomarkers were observed during castration-resistant outgrowth.^{63,64} This was accompanied by a promotion of paracrine signaling in the tumor microenvironment, which then contributed further into treatment resistance and growth/invasion. It has long been appreciated that castration itself can induce mesenchymal biomarker expression in cell lines, xenografts and patient samples, including induction of N- and OB-cadherin expression, SNAIL and ZEB1, and loss of cytokeratin and PSA expression.^{65,66} Loss of cytokeratin expression has been associated with activation of stemness pathways, such as NOTCH or GLI, as well as chemotherapy resistance in prostate cancer.⁶⁷ Growth of prostate cancer cells in charcoal-stripped media can also induce higher levels of AR-FL and AR-Vs.^{22,23} Finally, circulating tumor cells from men with metastatic CRPC frequently express both epithelial and mesenchymal markers, suggesting that this plasticity is important in lethal disease.⁶⁸ A key question that emerges from these observations is: what is the relationship between such plasticity and AR biology?

Overexpression of AR-V7 has been demonstrated to result in higher levels of SNAIL, TWIST, N-cadherin and ZEB1, despite a lack of impact on cytokeratin or E-cadherin downregulation.^{69,70} These data suggest that overexpression of AR-Vs may induce a partial EMT in some contexts. These data have also been observed with androgens and overexpression of AR-FL, which may also drive an EMT program in some contexts. Induced cellular plasticity and invasion has also been observed with exogenous androgens, which may also drive an EMT program in some contexts of low AR activity and TGF- β signaling dependence.⁷¹ These authors observed suppression of epithelial plasticity by overexpression of AR-FL, but did not measure AR-Vs in their system. A relationship between SNAIL overexpression and induction of both AR-FL and AR-V7 has recently been demonstrated, which promotes resistance to enzalutamide.⁷² SNAIL has been demonstrated to be

overexpressed in patients with metastatic prostate cancer and high-grade disease, and is commonly found in neuroendocrine prostate cancer metastases.^{73–76} Overexpression of SNAIL was accompanied by increased migration and invasion in the context of these alterations in AR biology. Although in some contexts, SNAIL may lead to loss of AR activity and plasticity or neuroendocrine prostate cancer transformation, in other contexts it appears that SNAIL may lead to alternative splicing of AR and/or increased AR expression, which may promote enzalutamide resistance. Further work will clarify the mechanisms of this relationship, including the impact of epithelial plasticity proteins on alternative splicing and epigenetic regulation of AR. However, these data suggest that AR biology may be directly impacted by cellular differentiation programs normally operative during embryology, and which are re-awakened during metastasis. In addition, AR inhibition may lead to generation of AR-Vs which can then activate these latent programs as part of a reciprocal relationship.

CLINICAL SIGNIFICANCE OF AR-V7

Although at least 22 AR-Vs have been discovered to date in tumor samples from patients with metastatic CRPC,¹³ AR-V7 is the most clinically relevant variant. This is because it is the most frequently observed and the most abundant AR-V in clinical specimens, and is the only variant that can be detected reproducibly at both the mRNA and protein levels. In addition, a number of retrospective studies (assessing AR-V7 using a variety of methods) have suggested that this variant is associated with more rapid disease progression and shorter survival in men with metastatic CRPC.^{18,19,25,77,78}

The first prospective study to evaluate the prognostic impact of AR-V7 was conducted by Efstathiou *et al.*⁷⁹ In that study, 60 men with bone-metastatic CRPC were treated with enzalutamide and underwent bone marrow biopsies at baseline and after 8 weeks. The presence of AR-V7 (on > 1% of tumor cells, detected at the protein level using immunohistochemistry on formalin-fixed paraffin-embedded specimens) was associated with primary resistance to enzalutamide. More specifically, AR-V7 protein was detected in 57% of men who developed disease progression within 4 months of starting enzalutamide, but was not detected in any patient who responded to enzalutamide for longer than 6 months. These findings were inline with an earlier study by the same investigators⁸⁰ evaluating the combination of abiraterone and enzalutamide in 60 men with bone-metastatic CRPC. In that study, AR-V7 protein was detected in bone marrow biopsies from 66% of patients who developed progression within 4 months, but in none of the men who responded to therapy for more than 6 months.

More recently, Antonarakis *et al.*²⁷ have developed an assay to serially evaluate AR-V7 at the mRNA level from circulating tumor cells (CTCs), using a RT-PCR detection method (positivity is defined as detection of AR-V7 cDNA at ≤ 36 PCR cycles). These authors conducted a prospective study assessing the prognostic role of AR-V7 in 31 CRPC patients receiving abiraterone and 31 patients receiving enzalutamide. In the abiraterone-treated cohort, AR-V7 was detected at baseline in 19% of patients. AR-V7-positive men had lower PSA response rates (0 vs 68%), shorter progression-free survival (hazard ratio (HR) 16.5) and shorter overall survival (HR 9.9) to abiraterone than AR-V7-negative men. The baseline prevalence of AR-V7 in the enzalutamide-treated cohort was 39%. Again, AR-V7-positive men had lower PSA response rates (0 vs 53%), shorter progression-free survival (HR 8.5) and shorter overall survival (HR 4.3) to enzalutamide than AR-V7-negative men. Notably, the prevalence of AR-V7 was higher in enzalutamide-treated men who had previously received abiraterone and in abiraterone-treated men who had previously received enzalutamide; AR-V7 prevalence was lowest in men who did not

receive either agent. In addition, when assessing serial CTC samples over time, the authors reported that all men with baseline detection of AR-V7 remained AR-V7-positive during the course of therapy with abiraterone and enzalutamide, while 14% of men with negative AR-V7 status at baseline converted to AR-V7-positive during treatment; these patients had intermediate clinical outcomes.

These findings were supported by an independent study recently published by Steinestel *et al.*⁸¹ In this prospective study, the authors used a CTC-based RT-PCR assay to detect AR-V7 in the context of various therapies for CRPC, including 22 patients receiving abiraterone (*n* = 10) or enzalutamide (*n* = 12). To this end, the PSA response rate to abiraterone or enzalutamide was 7% among AR-V7-positive patients and 63% among AR-V7-negative patients. Notably, one AR-V7-positive patient did have a PSA response to abiraterone, suggesting that a small proportion of AR-V7-positive men may derive some benefit from abiraterone or enzalutamide. In addition, this study confirmed that the prevalence of AR-V7 was higher in patients who had previously received abiraterone or enzalutamide compared with those who had not.

Another important question is whether the presence of AR-V7 is relevant in the setting of taxane chemotherapy, especially because two preclinical studies had previously produced conflicting results in this regard.^{82,83} To answer this question, Antonarakis *et al.*⁸⁴ performed a second prospective study using their CTC-based AR-V7 RT-PCR assay on 37 patients beginning treatment with docetaxel (*n* = 30) or cabazitaxel (*n* = 7). The prevalence of AR-V7 in these patients, most of which had previously received abiraterone and/or enzalutamide, was 46%. Encouragingly, PSA responses were observed in both AR-V7-positive and AR-V7-negative men (41 vs 65%). Similarly, progression-free survival was not statistically different in AR-V7-positive and negative patients. As a hypothesis-generating exercise, the authors then incorporated data from their prior study of 62 abiraterone- and enzalutamide-treated patients, and showed that clinical outcomes appeared to be better with taxanes compared with enzalutamide or abiraterone in AR-V7-positive men, while outcomes did not appear to differ by treatment type in AR-V7-negative men. More specifically, in AR-V7-positive patients, PSA responses were higher in taxane-treated vs enzalutamide- or abiraterone-treated men (41 vs 0%), and progression-free survival was longer in taxane-treated men (HR 0.21). A very interesting observation from this study was that a significant proportion of patients (58%) with baseline positive AR-V7 converted to AR-V7-negative during treatment with docetaxel or cabazitaxel. Whether or not such transitions in AR-V7 status may re-sensitize such patients to further AR-directed therapies is unknown.⁸⁵

In an independent prospective clinical trial, Onstenk *et al.*⁸⁶ developed a CTC assay to evaluate AR-V7 mRNA in 29 CRPC patients starting therapy with cabazitaxel. The prevalence of AR-V7 at baseline in these patients, who had all received prior docetaxel as well as abiraterone in most cases, was 55%. In support of the previous study, the authors showed no significant differences between AR-V7-negative and AR-V7-positive patients with respect to PSA responses (18 vs 8%), progression-free survival (HR 0.8) or overall survival (HR 1.6). Therefore, taken together with the findings from the previous study, the preliminary evidence to date suggests that presence of AR-V7 may not be a marker of resistance to taxane chemotherapy and may therefore represent a treatment-selection biomarker in CRPC.

CLINICAL QUALIFICATION OF AR-V7: A TREATMENT-SELECTION BIOMARKER?

Despite the intriguing clinical correlations discussed above, these findings remain preliminary and will require systematic prospective validation and clinical qualification. As such, it remains

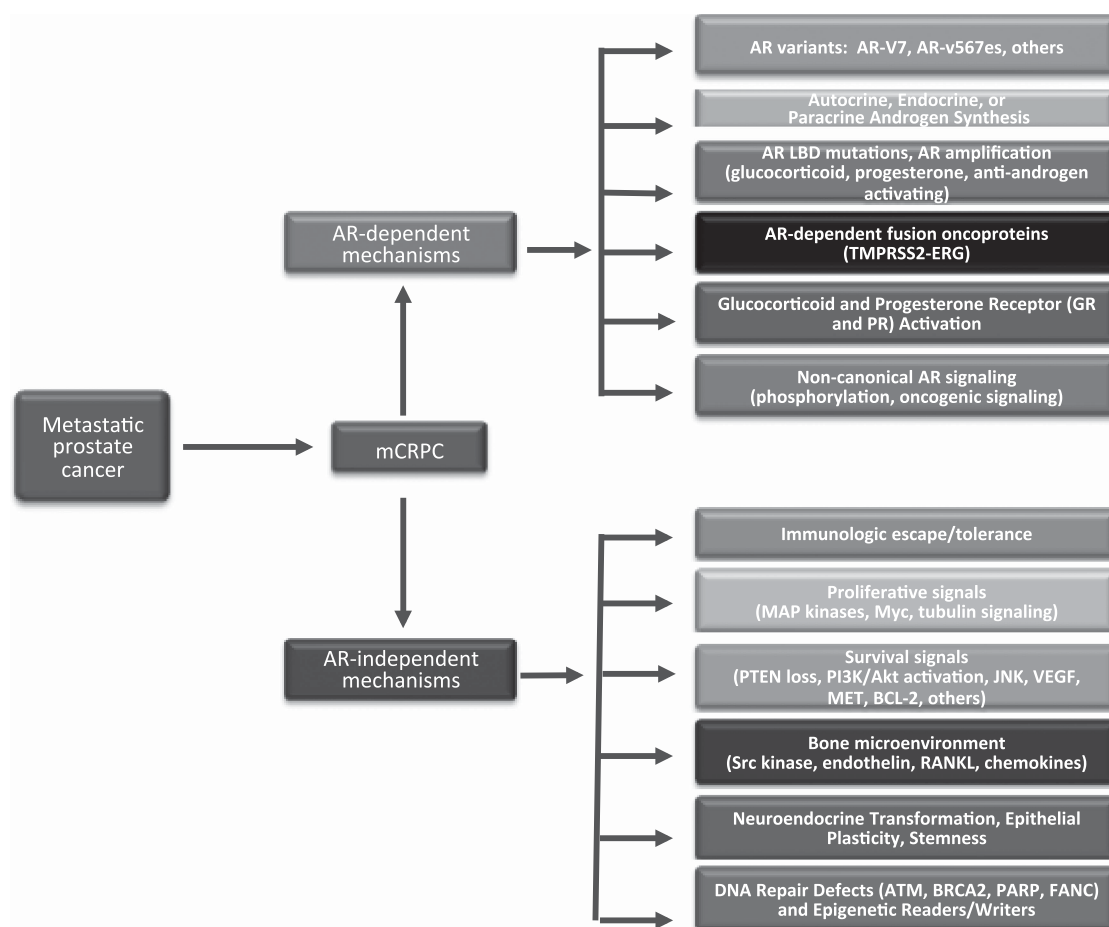


Figure 2. Mechanisms of castration-resistant progression, reflecting various molecular disease states. AR, androgen receptor; LBD, ligand-binding domain; mCRPC, metastatic castration-resistant prostate cancer.

premature at this time to use CTC-based AR-V7 testing in routing clinical practice to inform treatment decisions. AR-V detection and presence in men with metastatic CRPC must be understood in the context of a number of additional known resistance and progression-related genomic and epigenomic alterations (Figure 2). These include both AR-dependent and AR-independent mechanisms, immune tolerance, DNA repair defects and aberrations in key oncogenes or tumor suppressors implicated in CRPC progression. Although there are a number of AR-V7 validation studies currently being conducted, this review will highlight two examples. Other studies aiming to confirm (or refute) the clinical relevance of AR-V7 in CRPC patients are summarized in Table 1.

The first study is the Sanofi-sponsored PRIMCAB trial (NCT02379390), whose target population is men with metastatic CRPC who have developed clinical disease progression within 6 months of starting abiraterone or enzalutamide. Such patients ($n = 274$) will be randomized equally to receive either cabazitaxel chemotherapy or the alternative AR-directed therapy. The primary end point of this trial is radiographic progression-free survival. As a secondary end point, the trial will prospectively evaluate baseline AR-V7 mRNA status from CTCs as a putative predictive biomarker in this setting, where the prevalence of AR-V7 mRNA is expected to be ~33%. Johns Hopkins will serve as the central laboratory for AR-V7 testing in this trial. Exploratory analyses will also evaluate transitions in AR-V7 status at the time of progression.

The second study is a Prostate Cancer Foundation (PCF)-sponsored prospective biomarker trial (NCT02269982) evaluating three different CTC-based AR-V7 assays in 120 men with metastatic CRPC who have not received taxane chemotherapy

for CRPC. In an attempt to enrich for patients with evaluable CTCs, eligible subjects must have at least two of the following high-risk features: radiographic progression, hemoglobin $< 10 \text{ g dl}^{-1}$, alkaline phosphatase above normal, lactate dehydrogenase above normal, PSA doubling time < 3 months, prior abiraterone or enzalutamide use, presence of visceral metastases, presence of pain requiring narcotics or detectable CTC using the CellSearch platform. In this non-interventional trial, patients will receive standard-of-care abiraterone or enzalutamide and then may also chose to receive standard-of-care taxane at progression. AR-V7 testing will be performed before AR-directed therapy, at progression on AR-directed therapy, and also at progression on chemotherapy (for those patients subsequently receiving taxane treatment). Each patient will undergo AR-V7 testing with three clinical assays at each time point: the Johns Hopkins mRNA-based assay, the Weill-Cornell mRNA-based assay (which also evaluates other AR-Vs), and the EPIC Sciences protein-based AR assay (San Diego, CA, USA). The coordinating center for this trial is the Duke Cancer Institute. In this study, the relationship of AR-Vs with outcome will be analyzed in the context of CTC enumeration, clinical phenotypes and other genomic aberrations detected in CTCs and cell-free DNA through copy number analysis and whole-exome sequencing, including AR amplification and other pathways implicated in CRPC (Figure 2).

THERAPEUTIC TARGETING OF AR-V7

While there are currently no agents in clinical use that can specifically target AR-V7 or other AR-Vs in prostate cancer, a

Table 1. Selected biomarker trials evaluating the clinical utility of AR-V7 in CRPC patients

Therapeutic agents	Trial phase	Description	Key outcomes	Biomarker platform	NCT number
Cabazitaxel vs abiraterone/enzalutamide [PRIMCAB]	Phase 2	Randomized open-label trial of cabazitaxel vs abiraterone or enzalutamide in mCRPC patients refractory to enzalutamide or abiraterone, with prospective validation of AR-V7 biomarker	rPFS	From CTCs; mRNA-based, AdnaTest (Qiagen)	NCT02379390
Cabazitaxel vs abiraterone/enzalutamide [OZM-054]	Phase 2	Randomized open-label trial of cabazitaxel vs abiraterone or enzalutamide in mCRPC patients with poor-prognosis features who have not previously received abiraterone or enzalutamide	PSA response rate and/or radiographic response	From whole-blood RNA; mRNA-based, PAXgene (PreAnalytiX, Hombrechtikon, Switzerland)	NCT02254785
Abiraterone, enzalutamide, taxanes [PCF 00056936]	Phase 2	Prospective observational study in mCRPC patients starting standard-of-care abiraterone or enzalutamide, with potential switch to taxane chemotherapy upon progression, evaluating mechanisms of resistance related to AR-V7 and other biomarkers. Three AR-V7 assays will be compared.	PFS, OS	(1) From CTCs; mRNA-based, AdnaTest (Qiagen) (2) From CTCs; mRNA-based, RosetteSep (StemCell Technologies, Vancouver, BC, Canada) (3) From CTCs; protein-based, Epic Sciences	NCT02269982
Abiraterone, enzalutamide [GUTG-001]	Phase 2	Randomized open-label sequencing study of abiraterone → enzalutamide vs enzalutamide → abiraterone in mCRPC	PSA response rate	From whole-blood RNA; mRNA-based, PAXgene (PreAnalytiX)	NCT02125357
Abiraterone, enzalutamide [BARRIER-P]	Phase 2	Open-label trial of standard-of-care enzalutamide or abiraterone for mCRPC, evaluating biomarkers of response and resistance including AR-V7	PSA response rate, PSA progression	From whole-blood RNA; mRNA-based, PAXgene (PreAnalytiX)	NCT02429193

Abbreviations: AR-V7, androgen receptor splice variant 7; CTC, circulating tumor cell; mCRPC, metastatic castration-resistant prostate cancer; OS, overall survival; rPFS, radiographic progression-free survival.

number of interesting compounds are now in clinical development that may have AR-V-directed activities. Here we will highlight three of these agents, while others are summarized in Table 2.

The first drug is galeterone, manufactured by Tokai Pharmaceuticals, Boston, MA, USA. Galeterone is an oral AR signaling inhibitor that possesses three mechanisms of AR-directed action: it inhibits CYP17 lyase, it antagonizes the AR ligand-binding domain, and it destabilizes AR protein via an unknown, proteasome-dependent mechanism.⁸⁷ Interestingly, treatment of AR-V-expressing prostate cancer cells with galeterone also leads to reduced AR-V expression (including AR-V7), presumably through this same proteasome-dependent mechanism.⁸⁸ In a *post hoc* analysis of the phase 2 ARMOR2 trial, six out of seven men with reduced/lost expression of the AR COOH-terminal domain (as determined by immunohistochemistry on CTCs using an AR COOH-terminal domain-specific antibody) achieved a >50% PSA reduction with galeterone. Based on these preliminary data, a registrational phase-3 trial, ARMOR3-SV, was launched in Q4 2015 (NCT02438007). Eligible patients are those with AR-V7-positive metastatic CRPC without prior treatment with abiraterone, enzalutamide, or taxane chemotherapies. AR-V7 testing will be conducted using a CLIA-certified assay developed by Qiagen (Hilden, Germany). Patients with CTCs positive for AR-V7 mRNA ($n = 148$) will be equally randomized to receive either galeterone 2550 mg daily or enzalutamide 160 mg daily. The primary end point is radiographic progression-free survival, with the key secondary end point being overall survival. Notably, ARMOR3-SV is the first registrational trial in prostate cancer to use a biomarker-selection precision-medicine trial design, and will test the efficacy of a multi-targeted AR-directed agent in men with detectable CTCs who would not be predicted to benefit substantially from a pure AR-FL inhibitor.

The second agent is EPI-506 (an oral prodrug of EPI-002, manufactured by ESSA), which is the first drug capable of targeting the AR-NTD.⁸⁹ Specifically, EPI-002 is one out of four stereoisomers of racemic EPI-001 which is a chlorinated bisphenol compound that can bind covalently to the AR-NTD. Because the NTD is common to both in the AR-FL and in all of the AR-Vs (including AR-V7), treatment with EPI-506 would be expected to extinguish all forms of AR signaling. Indeed, preclinical studies with EPI-002 have shown that this compound has activity in several AR-V expressing cell lines and xenograft models, including LNCaP95 and VCaP.^{90,91} However, EPI-001 has also been shown to have anti-proliferative activity at higher doses in AR-null prostate cancer cells, and demonstrates effects that are independent of the AR-NTD, including inhibition of AR expression, selective PPAR γ agonist activity, and a general pH-dependent alkylating activity.⁹² Based on these encouraging preclinical data, a phase 1 trial (including a subsequent phase 2 expansion) was initiated in Q1 2016 (NCT02606123). Eligible patients will be those with metastatic CRPC who have previously received either abiraterone or enzalutamide; one prior taxane therapy is also permitted but not required.⁹³ Exploratory analyses of AR-V7 and AR mutations will also be conducted in this trial, but this information will not be used for patient selection or stratification.

The third agent with potential activity against AR-V-expressing prostate cancer is the anti-helminthic drug niclosamide. In a drug library screen aimed at identifying FDA-approved drugs capable of targeting AR-V7, niclosamide emerged as an unexpected hit.^{94,95} Further mechanistic studies suggested that this agent functioned by promoting degradation of AR-FL and AR-V7 through a proteasome-dependent pathway. Interestingly, this study noted that AR-V7 degradation occurred more rapidly than AR-FL degradation in niclosamide-treated cells. Remarkably, niclosamide demonstrated significant antitumor activity in a number of AR-V-expressing CRPC cell lines (C4-2 and CWR22Rv1), as well as in a CWR22Rv1 xenograft model. Notably, 22Rv1 was

Table 2. Selected therapeutic trials using agents with potential activity against AR-V7-expressing CRPC

Investigational agents	Trial phase	Description	Key outcomes	Biomarker platform	NCT number
Galeterone vs enzalutamide [ARMOR3-SV]	Phase 3	Randomized open-label trial of enzalutamide vs galeterone in treatment-naïve AR-V7-positive mCRPC patients	rPFS, OS	From CTCs; mRNA-based, AdnaTest (Qiagen)	NCT02438007
EPI-506 (AR-NTD inhibitor)	Phase 1/2	Single-arm trial in men with mCRPC after progression on enzalutamide or abiraterone (one prior taxane also permitted)	Safety, PSA response rate	From CTCs; protein-based, Epic Sciences	NCT02606123
Niclosamide+enzalutamide [NCT-2015-01246]	Phase 1	Open-label trial of niclosamide plus enzalutamide in AR-V7-positive abiraterone-refractory mCRPC	Safety, PSA response rate	From CTCs; mRNA-based, AdnaTest (Qiagen)	NCT02532114
High-dose testosterone [RESTORE]	Phase 2	Single-arm trial of high-dose testosterone for abiraterone- or enzalutamide-refractory mCRPC	PSA response rate, safety	From CTCs; mRNA-based, AdnaTest (Qiagen)	NCT02090114
High-dose testosterone vs enzalutamide [TRANSFORMER]	Phase 3	Randomized study of high-dose testosterone vs enzalutamide for abiraterone-refractory mCRPC	rPFS	From CTCs; mRNA-based, AdnaTest (Qiagen)	NCT02286921
Cabazitaxel [CARVE]	Phase 2	Single-arm open-label trial of cabazitaxel in mCRPC patients with AR-V7-positive CTCs who have previously received docetaxel	PSA response rate	From CTCs; mRNA-based, CellSearch (Janssen, Horsham, PA, USA)	NCT02621190
GSK525762 (BET inhibitor) [GSK-115521]	Phase 1	Open-label trial of GSK525762 in solid tumors, including CRPC	Safety, response rate	No AR-V7 testing	NCT01587703
GS-5829 (BET inhibitor) [GS-US-350-1604]	Phase 1/2	Open-label trial of GS-5829 specifically for mCRPC, used alone (phase 1) and in combination with enzalutamide (phase 2)	Safety (phase 1); PFS at 24 weeks (phase 2)	From CTCs; protein-based, Epic Sciences	NCT02607228
Ipilimumab+nivolumab [STARVE-PC]	Phase 2	Single-arm trial of ipilimumab plus nivolumab in AR-V7-positive mCRPC	PSA response rate, safety	From CTCs; mRNA-based, AdnaTest (Qiagen)	NCT02601014

Abbreviations: AR-NTD, androgen receptor N-terminal domain; BET, bromodomain extra-terminal motif; CTC, circulating tumor cell; mCRPC, metastatic castration-resistant prostate cancer; OS, overall survival; rPFS, radiographic progression-free survival.

found to be completely resistant to enzalutamide, but niclosamide resulted in tumor growth restriction in this model, while the combination of niclosamide and enzalutamide produced maximal tumor inhibition. Based on these preclinical data, a phase 1 clinical trial was launched in Q4 2015 (NCT02532114) for men with abiraterone-pretreated CRPC who test positive for AR-V7 using a CTC-based AR-V7 assay developed at the University of Washington, Seattle. In this trial, patients will receive enzalutamide 160 mg plus escalating doses of oral niclosamide (500 mg three times daily, 1000 mg three times daily and 1500 mg three times daily). Exploratory analyses will evaluate changes in AR-V7 status during the course of niclosamide treatment and at the time of progression.

UNRESOLVED QUESTIONS AND FUTURE RESEARCH

The principal question that arises from the data presented is whether AR-Vs, particularly AR-V7, are drivers of malignant progression and treatment resistance in the clinic, or whether AR-Vs are passenger markers of aggressive disease. For example, high levels of AR copy number amplification may be associated with altered splicing which may lead to the detection of these variants in CTCs or tissues, but it may be that AR-FL gains rather than AR-V expression is important given their relative abundance. Recent data suggest a strong relationship between AR copy gains detectable in plasma cell-free DNA and poor outcomes with abiraterone in men with metastatic CRPC.⁹⁶ The relationship between AR copy gains and altered splicing of AR in patients is unclear, and which biomarker is most associated with poor response remains undetermined. In addition, heterogeneity of CTCs clearly exists in men with mCRPC, and while AR-Vs may be detectable, they may co-exist with other aggressive disease genotypes and phenotypes, such as neuroendocrine transformation, de-differentiation and stem-like phenotypes, as well as AR-null CTCs (Figure 2,^{13,97}). The dissemination of CTCs, which allows AR-Vs to be detectable, may itself be a marker of highly aggressive/invasive disease and epithelial plasticity which may or may not be causally related to AR-Vs.⁹⁸ In addition, targeting of AR-Vs in preclinical models appears to be context-dependent. In some contexts, reduction of AR-Vs may restore sensitivity to AR antagonists, while in other contexts AR-V action appears expendable while AR-FL activity appears dominant. Some of this model dependence may be related to the presence of agonistic mutations that prevent AR-Vs from emerging in the presence of drugs such as enzalutamide, while in other contexts, AR-Vs may be found at low levels or unlinked from tumor cell growth.⁶⁰

Men with metastatic CRPC present with a large number of genomic alterations that impact on DNA repair pathways, PI3K pathway signaling, cell cycle pathways, stemness/differentiation pathways (WNT and NOTCH signaling), epigenetic signaling and p53 loss among many others.¹³ In addition, epigenetic divergent evolution toward a neuroendocrine phenotype may lead to a loss of AR dependence,⁹⁹ which may co-exist and eventually overtake AR-dependent clones under the selection pressures of hormonal therapy. These genomic lesions may take on a more important role when AR-FL is suppressed, and while AR-Vs may become detectable due to AR-FL suppression, their persistence may be transient, and may be expendable in the context of these additional mutations. Furthermore, the metastatic process in prostate cancer is accompanied by widespread alterations in splicing decisions, which may impact many genes other than AR. Silencing of AR and a movement away from AR dependency is clearly operative in several cases after progression on enzalutamide/abiraterone, and in this context it is unlikely that further AR inhibition (even with inhibitors that target the AR-NTD or DBD, or anti-AR immunotherapy) would have therapeutic efficacy.

To discern the driver vs. passenger role of AR-Vs in the clinical setting, trials in which agents that selectively inhibit AR-Vs (or both

AR-FL and AR-Vs) are needed. Such trials are listed in Table 2, as agents such as galeterone, EPI-506, and perhaps others may have activity in AR degradation or inhibition of AR more broadly. Demonstrating reversal of resistance with these agents would provide proof-of-concept that at least some of the resistance to AR-LBD inhibitors is mediated by AR-Vs. However, if efficacy is modest or short-lived, it would imply that AR-Vs are merely markers of aggressive disease, and that other approaches beyond AR targeting are needed, such as immunotherapy or combinations with poly (ADP-ribose) polymerase inhibitors or chemotherapy.¹⁰⁰ However, the fact that AR-V protein expression appears to increase during hormonal therapy indicates strong clonal selection or plasticity induced by drug treatment, and suggests that further targeting of the AR is likely to provide clinical benefit. The key question is whether the root/trunk cells in CRPC remain AR-negative and de-differentiated, even while spawning more differentiated progeny. If this is the case, potent AR inhibition with NTD inhibitors may only select for more aggressive AR-null clones such as the neuroendocrine prostate cancer transformation over time.

Given this, ongoing and future trials of novel AR inhibitors should measure and account for this heterogeneity in both genotype and phenotype, and track it longitudinally. One such ongoing study (NCT02269982) is doing exactly this, using cell-free RNA/DNA, CTC-derived RNA/DNA, and measures of tumor heterogeneity in the context of AR-V detection. Given that data suggest that AR-Vs may promote plasticity itself in a feed-forward loop, which may eventually lead to escape from AR dependency, measures of the relationship between plasticity, stemness, neuroendocrine prostate cancer transformation and AR biology in CRPC patients receiving standard-of-care therapies are needed over time. Only through such pharmacodynamic and mechanistic studies can we develop broader therapeutic approaches, such as immunotherapies or combinations of targeted agents, to address this heterogeneity within patients and between patients. Finally, such predictive biomarker-driven studies may permit the optimization of care delivery to those men with CRPC who are most likely to benefit, saving patients and society from the burdens of cost and excess toxicity from ineffective agents.

CONCLUSIONS

We have reviewed the origin, structure, and biology of AR variants, and have demonstrated the strong clinical associations of measurements of AR-Vs in CTCs with clinical outcomes in CRPC patients receiving novel AR-targeted therapies and taxane chemotherapies. AR variants are likely an important contributor to CRPC progression and AR therapy resistance, and emerging biomarkers of AR variant expression in patients should help to select men with prostate cancer most likely to benefit from AR-targeted therapies or to select men who are appropriate for other systemic approaches. Much of these data suggest that in some patients, targeting of the AR-NTD or DBD may provide greater therapeutic benefit than targeting the AR LBD alone, or that combination approaches with AR LBD inhibitors may be beneficial. A number of biomarker-based predictive trials are ongoing to examine AR variants in the contexts of standard enzalutamide or abiraterone therapy. In addition, AR variant expression is being utilized in several trials of novel hormonal or immunologic agents in order to demonstrate clinical benefit in AR variant-driven tumors. As most of oncology moves toward using precision biomarkers, we anticipate that measures of AR biology, including AR-Vs, in the context of a broad genomic characterization of patients, will help to select patients for AR-directed therapies vs chemotherapies, to monitor more closely those men who appear to have only a modest AR dependence due to tumor heterogeneity or plasticity, and to direct patients who appear to have AR-independent disease to other therapies (including radium-223,

immunotherapy approaches or clinical trials). The ultimate goal driving these trials is a personalized medicine approach to optimizing care based on the underlying and treatment-induced genotype and phenotype of men with mCRPC.

CONFLICT OF INTEREST

ESA has served as a paid consultant/advisor for Janssen, Astellas, Sanofi, Dendreon, Essa, and Medivation; has received research funding to his institution from Janssen, Johnson & Johnson, Sanofi, Dendreon, Exelixis, Genentech, Novartis and Tokai; and is a co-inventor of a technology that has been licensed to Tokai. AJA has served as a paid consultant for Sanofi-aventis, Dendreon, Janssen, Eisai, Bayer and Medivation/Astellas; is on the speaker's bureau for Sanofi-aventis and Dendreon; and receives research funding to his institution from Janssen, Medivation/Astellas, Sanofi-aventis, Active Biotech, Bayer, Dendreon, Novartis and Pfizer. SMD has served as a paid consultant/advisor for Medivation/Astellas. JL has served as a paid consultant/advisor for Astellas, Gilead and Sanofi; has received research funding to his institution from Orion, Mirati, Astellas, Sanofi and Gilead; and is a co-inventor of a technology that has been licensed to A&G and Tokai.

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Platinum Priority – Prostate Cancer
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Novel Junction-specific and Quantifiable In Situ Detection of AR-V7 and its Clinical Correlates in Metastatic Castration-resistant Prostate Cancer

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Abstract

Background: Androgen receptor splice variant 7 (AR-V7) has been implicated in resistance to abiraterone and enzalutamide treatment in men with metastatic castration-resistant prostate cancer (mCRPC). Tissue- or cell-based in situ detection of AR-V7, however, has been limited by lack of specificity.

Objective: To address current limitations in precision measurement of AR-V7 by developing a novel junction-specific AR-V7 RNA in situ hybridization (RISH) assay compatible with automated quantification.

Design, setting, and participants: We designed a RISH method to visualize single splice junctions in cells and tissue. Using the validated assay for junction-specific detection of the full-length AR (AR-FL) and AR-V7, we generated quantitative data, blinded to clinical data, for 63 prostate tumor biopsies.

Outcome measurements and statistical analysis: We evaluated clinical correlates of AR-FL/AR-V7 measurements, including association with prostate-specific antigen progression-free survival (PSA-PFS) and clinical and radiographic progression-free survival (PFS), in a subset of patients starting treatment with abiraterone or enzalutamide following biopsy.

Results and limitations: Quantitative AR-FL/AR-V7 data were generated from 56 of the 63 (88.9%) biopsy specimens examined, of which 44 were mCRPC biopsies. Positive AR-V7 signals were detected in 34.1% (15/44) mCRPC specimens, all of which also co-expressed AR-FL. The median AR-V7/AR-FL ratio was 11.9% (range 2.7–30.3%). Positive detection of AR-V7 was correlated with indicators of high disease burden at baseline. Among the 25 CRPC biopsies collected before treatment with abiraterone or enzalutamide, positive AR-V7 detection, but not higher AR-FL, was significantly associated with shorter PSA-PFS (hazard ratio 2.789, 95% confidence interval 1.12–6.95; $p = 0.0081$).

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Conclusions: We report for the first time a RISH method for highly specific and quantifiable detection of splice junctions, allowing further characterization of AR-V7 and its clinical significance.

Patient summary: Higher AR-V7 levels detected and quantified using a novel method were associated with poorer response to abiraterone or enzalutamide in prostate cancer.

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1. Introduction

Androgen receptor splice variant 7 (AR-V7) is one of the AR aberrations implicated in the development of castration-resistant prostate cancer (CRPC) [1,2]. AR-V7 originates from contiguous splicing of AR exons 1, 2, and 3 and the cryptic exon 3 (CE3) within the canonical intron 3 of the AR gene [1]. Specific detection of AR-V7 can be achieved by targeting the exon 3/CE3 splice junction via reverse transcription polymerase chain reaction (RT-PCR) [3]. A number of previous studies have demonstrated the prognostic value of AR-V7 detection by RT-PCR in men with metastatic CRPC (mCRPC) treated with abiraterone and/or enzalutamide. These studies used biological substrates such as prostate cancer tissues [4–8] and liquid biopsy samples, including circulating tumor cells (CTCs) [9–11], plasma exosomes [12], peripheral blood mononuclear cells (PBMCs) [13], and even whole blood samples [14,15]. While these approaches generally allow sensitive and specific detection of AR-V7, they are limited by a number of analytical and preanalytical challenges mainly attributable to low amounts of AR-V7 mRNA in liquid biopsy samples [16]. Critically, determination of AR-V7 status and its quantification were not possible in a significant proportion of mCRPC patients who were CTC-negative, even though the CTC-based AR-V7 test has been analytically validated and implemented in a clinical laboratory [17].

An alternative and potentially complementary approach to RT-PCR-based detection is RNA in situ hybridization (RISH). In contrast to the RT-PCR approach, RISH allows visualization of gene expression with spatial and morphological context [18]. Traditional RISH methods have been hampered by low sensitivity and a low signal-to-noise ratio, as well as the time-consuming effort required to develop experimental protocols for each detection target [19]. The RNAscope method is a recently developed RISH technique that uses an integrated probe design and signal amplification strategy to amplify target-specific signals by thousands fold without amplifying the background noise [20]. Importantly, this technique is compatible with routine formalin-fixed paraffin-embedded (FFPE) tissues. Following an initial report on AR-V7 RISH by RNAscope [10], two recent reports showed that AR-V7 detected in FFPE tissue specimens by two different RISH methods was associated with CRPC and prognostic in those treated with AR-targeting therapies [21,22]. However, these RISH methods, while revolutionary in RNA detection, require multiple tiling probes covering a target sequence of ~1 kb, and therefore lack the resolution for detecting a variant-specific splice junction. For AR-V7 detection, the published methods [10,21,22] targeted the

1.3-kb CE3 sequence. Because the CE3 sequence is also present in AR genomic DNA and AR pre-mRNA that are retained in the nucleus before being spliced and exported to the cytoplasm, detection of the CE3 sequence described in these previous studies should not be equated to detection of AR-V7. Indeed, detection of pre-mRNA was reported in a previous study [21] and detection of AR genomic DNA cannot be ruled out, particularly in mCRPC specimens with AR amplification. In addition, specificity for AR-V7 detection that targets the CE3 sequence may be further compromised by simultaneous detection of AR-V9, another androgen receptor variant that shares the same 3' CE3 sequence [23]. Therefore, accurate detection and quantification of AR-V7 mRNA in intact cells would not be possible given the lack of resolution and detection specificity of existing RISH methods.

In the present study, we developed a novel RISH detection method targeting a single splice junction using probes straddling the targeted junction. We applied this novel method to detect and quantify AR-V7, by targeting the exon 3/CE3 junction, and full-length AR (AR-FL), by targeting the exon 7/exon 8 junction. Following validation of junction-specific detection of the AR transcripts in cell lines and in FFPE specimens from mCRPC patients, we applied the prototype technology and quantified AR-V7/AR-FL levels in biopsies from mCRPC patients. We then conducted exploratory clinical correlative analysis for men treated with abiraterone or enzalutamide. We present the first example of visualization of splice junctions in morphologically intact cells, and demonstrate for the first time a highly specific and quantifiable AR-V7 RISH test for detection of clinically significant levels of AR-V7 mRNA in mCRPC patients.

2. Patients and methods

2.1. Patients

Two biopsy cohorts, one from the Johns Hopkins University School of Medicine (JHU cohort) and one from the Institute of Cancer Research and Royal Marsden NHS Foundation Trust (UK cohort), were used in this study. For the JHU cohort, 35 patients with metastatic prostate cancer gave informed consent to undergo the biopsy procedure under a study protocol approved by the institutional review board. Within this unselected and diverse cohort (Supplementary Table 1), nine patients with mCRPC underwent treatment with abiraterone or enzalutamide immediately following the biopsy procedure. For the UK cohort, 28 retrospective biopsies, including mainly bone marrow and prostate biopsies (Supplementary Table 1) were selected from patients treated with first-line abiraterone or enzalutamide (mainly abiraterone) following the biopsies. All study participants had given written informed

consent and were enrolled in institutional protocols approved by a multicenter research ethics committee (Chelsea Research Ethics Committee, reference 04/Q0801/60). There were no other sample selection criteria; all samples tested are included in Supplementary Table 1. All experimental processes were performed while blinded to the sample type and related data.

2.2. RISH by BaseScope

The BaseScope assays (Advanced Cell Diagnostics, Inc., Hayward, CA) for AR-FL/AR-V7 were developed to achieve junction-specific detection of the AR transcripts. The BaseScope assay is based on the RNAscope technology [20] but uses an additional signal amplification step and requires only one “double Z” (1 ZZ) probe pair for single-molecule detection. The 1-ZZ probe for AR-V7 was designed to target the AR-V7-specific junction of exon 3 and CE3 (AR-E3/CE3) (ZZ probe target sequence GAC TCT GGG AGA AAA ATT CCG GGT TGG CAA TTG CAA GCA TCT C), and the 1-ZZ probe for AR-FL was designed to target the splice junction of exon 7 and exon 8 (AR-E7/E8) (ZZ probe target sequence GCT CAC CAA GCT CCT GGA CTC CGT GCA GCC TAT TGC GAG A), as illustrated schematically in Figure 1A. For each sample,

four probes were used in four adjacent sections: AR-E7/E8, AR-E3/CE3, 1-ZZ Hs-POLR2A as a positive control, and 1-ZZ DapB as a negative control. Slides with negative POLR2A staining ($n = 4$ in the JHU cohort and $n = 3$ in the UK cohort), indicative of poor tissue quality, were excluded from analysis. Automated quantification of AR transcripts was performed using RNAscope Spot Studio software (Supplementary material).

2.3. Statistical analysis

The baseline clinical characteristics in the JHU cohort ($n = 28$, excluding 4 disqualified samples and 3 samples diagnosed with small cell carcinoma/neuroendocrine [SC/NE]), and UK cohort ($n = 16$, including all those collected before abiraterone or enzalutamide treatment) were separately compared according to AR-V7 status (positive vs negative). Categorical and continuous variables were compared using Fisher's exact test and a Mann-Whitney test, respectively.

Exploratory evaluations of an association between AR status and treatment outcome were conducted among the combined cohort of all patients treated with abiraterone or enzalutamide ($n = 25$) following the biopsy procedure. Outcome measures included

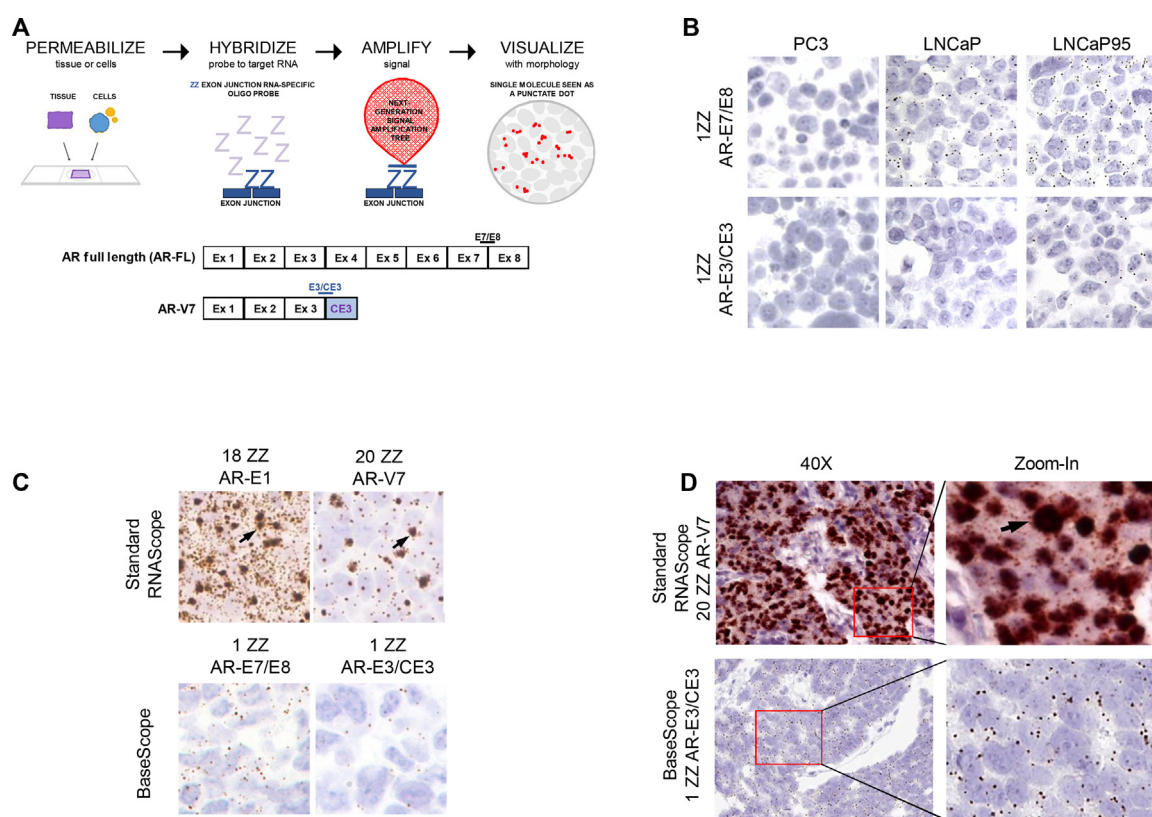


Fig. 1 – Development of the BaseScope RNA in situ hybridization assay for detection of splice junctions specific to AR-FL and AR-V7. (A) Schematic illustration of the BaseScope assay and the splice junctions targeted for probe design. **Top:** Overview of the BaseScope assay workflow. Sections containing fixed tissues or cells were permeabilized, and exposed mRNA were hybridized with a single pair of BaseScope probes (ZZ pair) that straddle the exon/exon junction of interest. Following amplification by an advanced, next-generation signal amplification system, junction-specific signals can be visualized as punctate dots under a standard bright-field microscope. **Bottom:** AR splice junctions targeted for BaseScope probe design. The splice junction between AR exons 7 and 8 (E7/E8) was targeted for specific detection of the full-length AR (AR-FL), while the splice junction between exon 3 and cryptic exon 3 (CE3) (E3/CE3) was targeted for specific detection of AR-V7. **(B)** Specificity of the BaseScope AR probes as demonstrated by signals detected in prostate cancer cell lines with known AR-FL/AR-V7 profiles. The cell lines PC3 (AR-FL-negative, AR-V7-negative), LNCaP (AR-FL-positive, AR-V7-negative), and LNCaP95 (AR-FL-positive, AR-V7-positive) were stained using the following 1 ZZ BaseScope probes: AR-E7/E8 for AR-FL and AR-E3/CE3 for AR-V7. **(C)** The BaseScope assay detects mature mRNA exclusively in cytoplasm. LNCaP95 cells (AR-FL-positive, AR-V7-positive) were stained with standard RNAscope (top) and BaseScope (bottom) assays. Both cytoplasmic and intranuclear (arrows) signals were detected with 18 ZZ AR-E1 and 20 ZZ AR-V7 probes used in the standard RNAscope (top) assay, while the 1 ZZ probes used in the BaseScope (bottom) assays detected punctate signals representing mature mRNA exclusively in the cytoplasm. **(D)** Comparison of AR-V7 signals detected by the standard RNAscope and BaseScope assays in a metastatic castration-resistant prostate cancer (mCRPC) biopsy specimen. The same mCRPC biopsy core was processed and stained for AR-V7 using 20 ZZ AR-V7 probes in the standard RNAscope assay (top) and the BaseScope assay (bottom) using the 1 ZZ AR-E3/CE3 probe. Note the intense intranuclear AR-V7 signal (arrow) with the RNAscope assay.

prostate-specific antigen progression-free survival (PSA-PFS) and clinical/radiographic progression-free survival (PFS). Survival time differences were analyzed using a log-rank test. In all tests, $p \leq 0.05$ was considered statistically significant. Statistical analyses were performed using GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Junction specific AR RISH assay development

The splice junction between AR exon 3 and CE3 (E3/CE3) is specific to AR-V7 mRNA. Detection of this junction (ie, specific detection of AR-V7) has not been possible in morphologically intact cells and the native tissue environment because of technical constraints of the existing RNAscope RISH assay requiring 20 ZZ probes targeting the 1.3-kb CE3 sequence [10,21,22]. We designed and optimized a novel AR-V7 RISH probe consisting of a 1-ZZ pair of oligonucleotide sequences straddling the AR E3/CE3 junction, in parallel with a novel 1-ZZ probe for the AR-FL that straddles the splice junction between AR exon 7 and exon 8 (E7/E8; Fig. 1A). To validate the specificity of these novel junction-specific AR probes, we first performed RISH in human prostate cancer cell lines with known AR-FL/AR-V7 expression profiles. As shown in Figure 1B, probes each consisting of 1-ZZ pairs (termed BaseScope probes) detected punctate cytoplasmic signals consistent with the known AR-FL/AR-V7 status of the cell lines. The improvement in specificity of the BaseScope assay over the RNAscope assay was shown by comparison of two RISH assays in LNCaP95 cells (positive for both AR-FL and AR-V7). Consistent with previous findings [21], the RNAscope probes (20 ZZ over ~1 kb) designed to target the entire CE3 sequence detected both cytoplasmic dots from mature AR-V7 mRNA and nonspecific intranuclear signals from AR-V7 pre-mRNA (Fig. 1C), precluding accurate quantification. By contrast, the junction-specific AR-V7 probe (<50 bases) detected signals for mature AR-V7 mRNA exclusively in the cytoplasm (Fig. 1C). Parallel comparison of these two AR-V7 RISH assays in a metastatic CRPC biopsy specimen further confirmed this distinction (Fig. 1D).

Although the novel prototype AR-V7 RISH assay appeared to detect fewer transcripts than the RNAscope assay owing to significantly fewer ZZ pairs for detection (Fig. 1C,D), the junction-specific detection made it possible to conduct automated quantification of AR-V7-specific signals (Supplementary Fig. 1). As shown in Supplementary Figure 2, quantitative measurements of AR-V7, AR-FL, and AR-V7/AR-FL ratios from the novel assay were consistent with values derived from RT-PCR in a set of metastatic biopsies from CRPC patients ($n = 13$) with matching FFPE and frozen specimens. AR-V7 can also be detected in a tissue microarray containing autopsy specimens from CRPC patients (Supplementary Fig. 3), although no statistically significant correlation between RISH and RNA-Seq was found ($n = 7$; Supplementary Fig. 3). Therefore, we have demonstrated the validity and feasibility of AR-V7 quantification by the novel RISH assay.

3.2. AR-V7/AR-FL quantification in biopsy specimens and correlation with baseline clinical characteristics

Having established the novel junction-specific AR RISH method, we generated quantitative AR-V7 and AR-FL RISH data from two independent biopsy cohorts while blinded to the sample identity. The first cohort consisted of 35 biopsies from patients with metastatic prostate cancer collected at JHU (Supplementary Table 1). After excluding four samples that did not meet the quality control criteria (no signal with the POLR2A-positive control probe), samples were grouped into SC/NE ($n = 3$), castration-sensitive prostate cancer (CSPC; $n = 3$), and CRPC ($n = 25$) on the basis of pathology reports and clinical notes. Representative images showing AR-V7/AR-FL measurements were shown in Figure 2A, and quantitative values for all 31 samples were shown in Figure 2B. Notably, samples with AR-V7 signals were always concurrently positive for AR-FL and, without exception, AR-FL measurement values were higher than those for AR-V7 (Fig. 2B and Supplementary Table 1).

Because AR-V7 values exhibited a continuous range (Supplementary Table 1), it was necessary to define AR-V7 “positivity” before clinical correlative analysis. We used a cutoff value of 0.4 to define AR-V7 “positivity” (Supplementary material). Using this cutoff, six of the 12 samples (50%) that had an AR-V7 RISH value above zero were AR-V7-positive (Fig. 2B and Supplementary Table 1). AR-V7 positivity was associated with prior treatment with ketoconazole, abiraterone, or enzalutamide, but not with any other baseline variable in this set of 28 biopsies (Supplementary Table 2). After defining the cutoff, a second cohort of 28 biopsies (UK cohort) was evaluated (Supplementary Table 1) using the same RISH method, among which nine biopsies were AR-V7-positive according to the predefined cutoff (Fig. 2C). In this cohort, 16 samples had baseline data available at the sampling time before treatment with abiraterone or enzalutamide (Supplementary Table 2). AR-V7 positivity was associated with serum PSA, but not with any other baseline variables in this cohort (Supplementary Table 2). Quantitative AR-V7/AR-FL RISH values from the combined 56 biopsy samples are presented in Supplementary Figure 4. Notably, all CSPC specimens ($n = 9$) and SC/NE samples ($n = 3$) were negative for AR-V7 according to this novel detection method (Supplementary Fig. 4). Among the CRPC specimens ($n = 44$), the AR-V7-positive rate was 34.1% (15/44), and the median AR-V7/AR-FL ratio was ~11.9% among AR-V7-positive samples (Supplementary Fig. 4).

3.3. Comparison of AR-V7 RISH and AR-V7 immunohistochemistry (IHC)

Detection of clinically significant AR-V7 can also be achieved by IHC using antibodies raised against the AR-V7-specific peptide [8,24]. However, detection of nonspecific, unidentified protein targets in AR/AR-V7-negative cells has been reported [8]. To allow comparison of AR-V7 RISH and IHC results, we developed an optimized AR-V7 IHC method (Supplementary material) that uses a new AR-V7

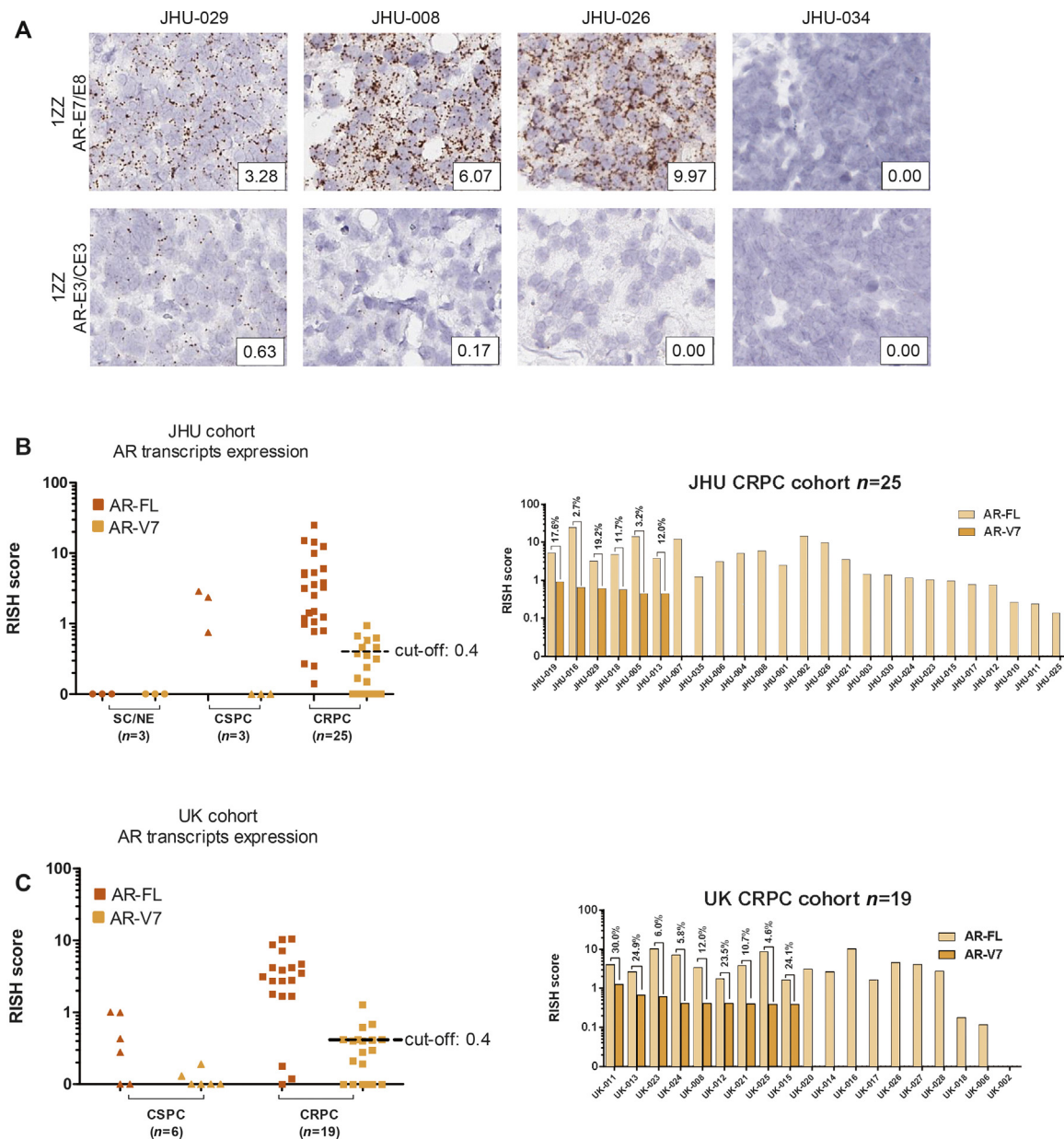


Fig. 2 – Detection and quantification of AR-FL and AR-V7 in two independent biopsy cohorts. (A) Representative images and quantified RNA in situ hybridization (RISH) scores for AR-FL (probe AR-E7/E8, top) and AR-V7 (probe AR-E3/CE3, bottom) mRNA detection in tissue biopsies from patients with metastatic prostate cancer. (B) AR quantification by junction-specific RISH in the JHU cohort. Left panel: Quantified AR-FL and AR-V7 mRNA expression in three small cell carcinoma/neuroendocrine (SC/NE) biopsies, three castration-sensitive prostate cancer (CSPEC) biopsies, and 25 castration-resistant prostate cancer (CRPC) biopsies from the JHU cohort. The line indicates the value for the AR-V7 cutoff (0.4). Right panel: Relative AR-V7/AR-FL values and ratios (for those that were AR-V7-positive defined by the cutoff) in each of the 25 CRPC specimens. (C) AR quantification by junction-specific RISH in the UK cohort. Left panel: Quantified AR-FL and AR-V7 mRNA expression in six CSPEC biopsies and 19 CRPC biopsies from the UK cohort. The line denoted the AR-V7 cutoff (0.4). Right panel: Relative AR-V7/AR-FL values and ratios (for those that were AR-V7-positive defined by the cutoff) in each of the 19 CRPC specimens.

antibody that specifically detected AR-V7 protein in cells with known AR-V7 status (Fig. 3A). In addition, areas of positive IHC staining corresponded to positive RISH staining in a sample with mixed SC/NE and adenocarcinoma histology (Fig. 3B). To further characterize the novel AR-V7 RISH test, we compared AR-V7 measurements obtained with RISH and IHC methods (Supplementary material) in matched sections from 36 mCRPC biopsies (mainly from the UK cohort). The IHC results robustly correlated with the RISH results (Fig. 3C,D, Supplementary Table 3).

3.4. Association with treatment outcome

We conducted exploratory treatment outcome analyses after combining biopsies collected from patients treated with abiraterone or enzalutamide in the two cohorts. A total of 25 patients ($n = 9$ in the JHU cohort and $n = 16$ in the UK cohort) were biopsied before treatment with abiraterone or enzalutamide. PSA response rates were not significantly different by AR-V7 status, although a numerically better PSA response rate was observed in subjects with AR-V7 scores

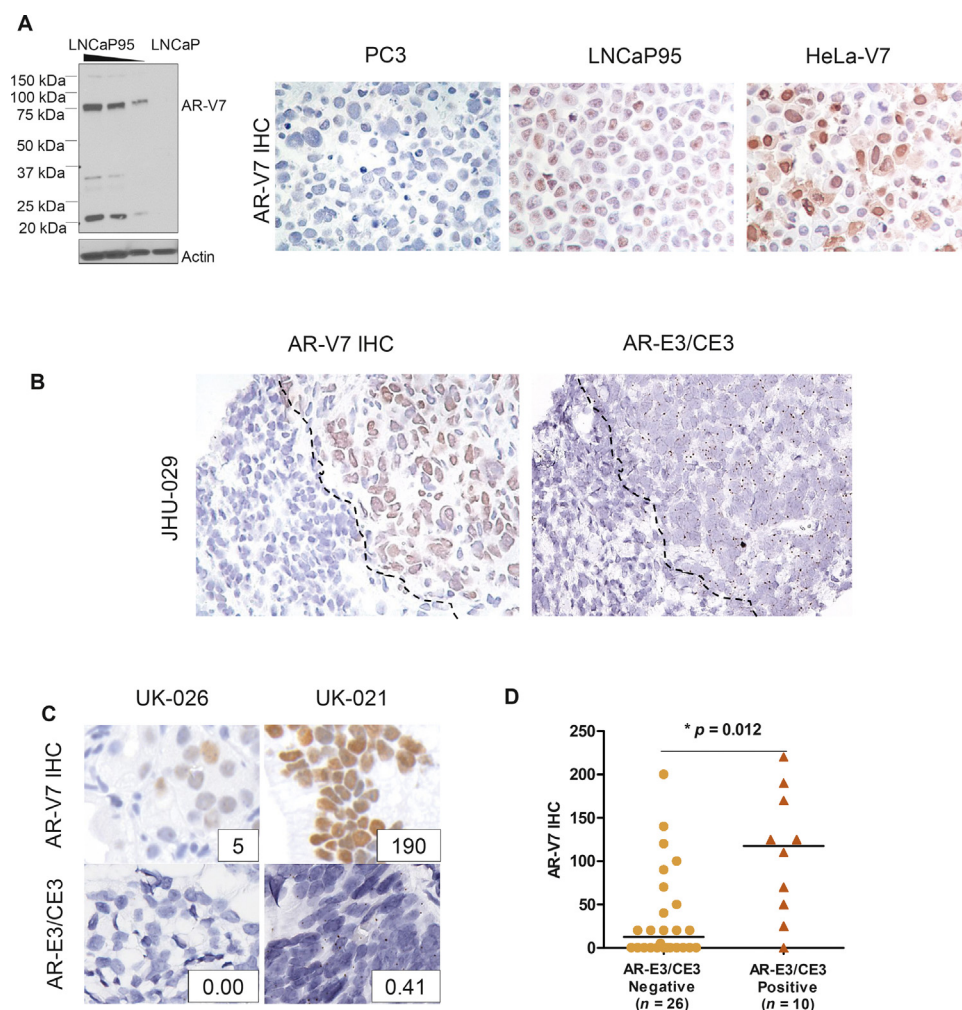


Fig. 3 – Comparison of AR-FL/AR-V7 levels quantified by RNA in situ hybridization (RISH) and immunohistochemistry (IHC). (A) Western blot and IHC using the RevMab-RM7 AR-V7 antibody in prostate cancer cells with known AR profiles. Western blot showed the ~80-kDa AR-V7 band consistent with known AR-V7 status in LNCaP (AR-V7-negative) and LNCaP95 (AR-V7-positive) cells. Different doses of LNCaP95 protein lysates were loaded. Non-specific staining was shown at approximately 30 and 23 kDa. β -Actin was blotted as a loading control. In IHC experiments, PC3 cells showed negative AR-V7 IHC staining, LNCaP95 cells showed moderate AR-V7 staining, and HeLa cells transiently transfected with AR-V7 showed the highest level of AR-V7 IHC staining (heterogeneity reflected the transfection efficiency). (B) AR-V7 IHC staining was compared with the AR-E3/CE3 BaseScope assay in a metastatic castration-resistant prostate cancer CRPC biopsy with mixed SC/NE and adenocarcinoma histology. (C) Representative images and quantified scores comparing IHC and RISH results in biopsies from the UK cohort. (D) Comparison of AR-V7 IHC values in AR-V7-positive ($n = 10$) and AR-V7-negative biopsies ($n = 26$) defined by junction-specific RISH. The p value was determined using an unpaired t test.

below the cutoff (Supplementary Fig. 5). AR-V7 status was significantly associated with shorter PSA-PFS ($p = 0.0081$; Fig. 4A) and showed a trend towards an association with PFS ($p = 0.054$; Fig. 4B). However, AR-FL status was not associated with either PSA-PFS or PFS in this combined cohort (Fig. 4C,D).

4. Discussion

Here we present the first example of visualization of splice junctions in morphologically intact cells using a novel RISH assay, and quantitative analysis of AR-FL/AR-V7 mRNA levels in FFPE biopsies obtained from mCRPC patients. Although the study was limited by cohort size, AR-V7 status was correlated with clinical characteristics and clinical outcomes after treatment with abiraterone or enzalutamide. This novel

AR-V7 RISH test may help to address some of the limitations of the RT-PCR-based test, for which clinical development may be limited by preanalytical and analytical challenges because of reliance on detection of CTCs and low levels of the analytes in liquid biopsy samples [16]. For example, the CTC-based test requires relatively fresh blood samples delivered and processed within 24 h of collection. In addition, reporting of AR-V7 status would not be possible for patients with no detectable CTCs, although they usually present with lower disease burden and favorable treatment outcome [25]. For AR-V7 tests using biological substrates other than CTCs (exosomes, PBMCs, and whole blood), full analytical performance data have not been reported [12–15]. Although tissue-based tests require an invasive sampling procedure and may be further compromised by tissue heterogeneity, the role of molecular aberrations detected in tissue biopsies

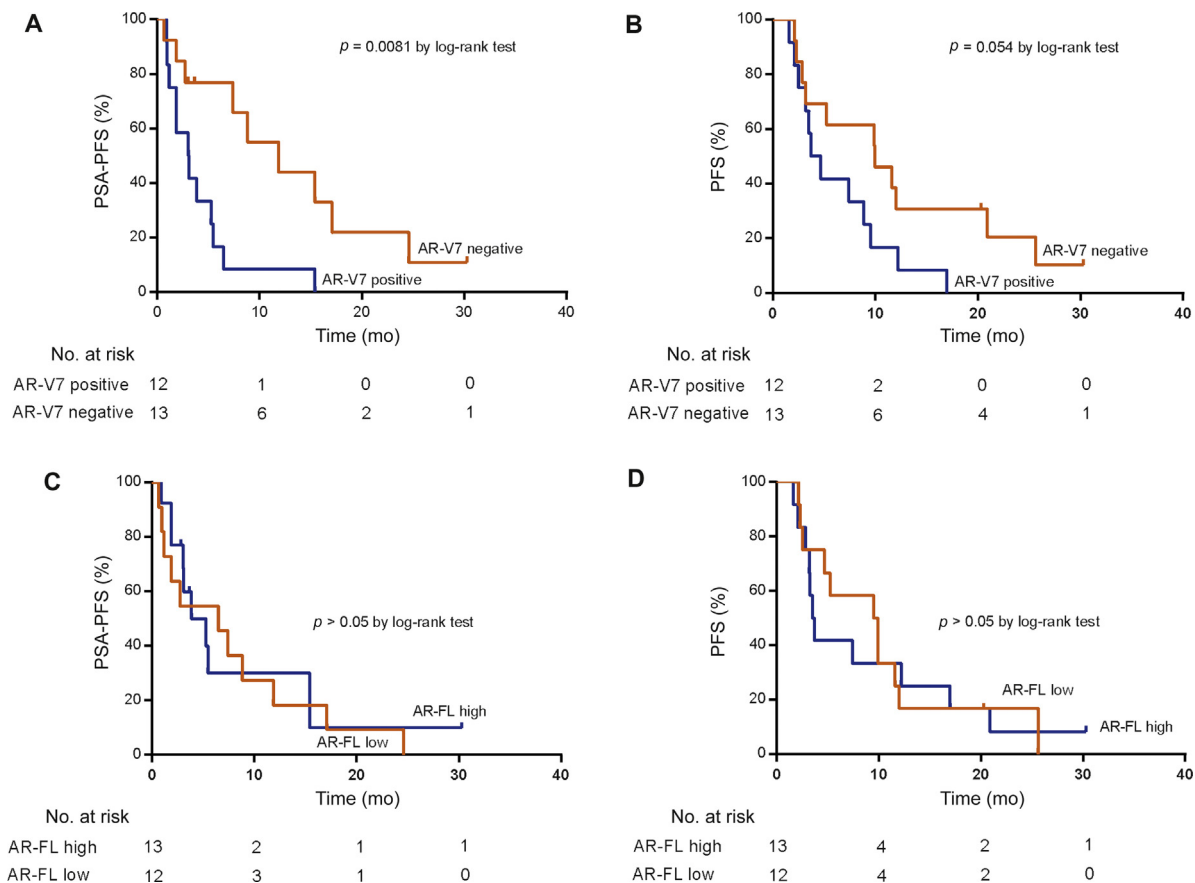


Fig. 4 – Exploratory clinical outcome analysis of AR-V7 and AR-FL status determined by BaseScope assay in patients treated with abiraterone or enzalutamide ($n = 25$). (A) Kaplan-Meier analysis of prostate-specific antigen progression-free survival (PSA-PFS) by AR-V7 status. The median PSA-PFS was 3.1 mo in AR-V7-positive patients and 11.8 mo in AR-V7-negative patients (AR-V7 positivity hazard ratio [HR] for PSA-PFS 2.789, 95% confidence interval [CI] 1.12–6.95; $p = 0.0081$ by log-rank test). (B) Kaplan-Meier analysis of clinical or radiographic progression-free survival (PFS) by AR-V7 status. The median clinical or radiographic PFS was 4.2 mo in AR-V7-positive patients and 9.9 mo in AR-V7-negative patients (AR-V7 positivity HR for PFS 2.118, 95% CI 0.89–5.02; $p = 0.054$ by log-rank test). (C) Kaplan-Meier analysis of PSA-PFS by AR-FL status. The median PSA-PFS was 3.9 mo in AR-FL high patients and 6.5 mo in AR-FL low patients (AR-FL high status HR for PSA-PFS 1.167, 95% CI 0.4957–2.745; $p = 0.7145$ by log-rank test). (D) Kaplan-Meier analysis of clinical or radiographic PFS by AR-FL status. The median clinical or radiographic PFS was 3.6 mo in AR-FL high patients and 9.8 mo in AR-FL low patients (AR-FL high status HR for PFS 1.073, 95% CI 0.464–2.475; $p = 0.8675$ by log-rank test).

remains important [26]. It may be possible to develop treatment or patient selection markers on the basis of a biopsy, as indicated in a recent article suggesting the feasibility of obtaining molecular information representative of the patient by sampling a single metastasis [27]. Therefore, the newly developed capability for detection and quantification of a critical AR aberration in biopsy specimens, upon further work, may address a significant hurdle in measurement science for treatment and patient selection.

In situ detection of AR-V7 can also be achieved by IHC. Two recent studies demonstrated the prognostic value of AR-V7 detection by IHC in tissue specimens or CTCs immobilized on glass slides [8,24]. However, nonspecific signals from this antibody were acknowledged [8]. While antibody-based tests have a number of advantages, development of an optimized antibody is technically challenging and time-consuming. In our comparison of RISH and IHC (Fig. 3), we used a new AR-V7 antibody that was determined to be more specific than those evaluated in previous studies [8,24]. Although the measurements were generally concordant (Fig. 3), discrepancies were found

(Supplementary Table 3), potentially reflecting measurement variations that may be related to nonspecific detection by IHC or different regulation of translation from mRNA to protein, as well as protein degradation among cases. Nevertheless, there is merit in further developing IHC-based detection methods for AR-V7, particularly since AR splice variant protein may have a longer half-life than its parent mRNA transcript [28]. Importantly, however, the RISH method described here can also be adapted for application in the CTC platforms described earlier [24] to allow further comparison of RISH and IHC.

Owing to the small sample size limited by difficulty in obtaining an adequate number of pretreatment biopsies, our clinical correlative analysis is exploratory and we did not conduct multivariable analysis adjusting for other prognostic factors. The small sample size also limited our ability to further optimize and validate the cutoff used to define AR-V7 status. As a result of these limitations, the potential clinical utility of the tissue-based RISH test (eg, in CTC-negative patients) remains to be determined. The main goal of the present study was to develop and

validate a novel in situ AR-V7 test for detection of clinically significant levels of AR-V7, using a novel prototype method that had recently undergone substantial improvement with respect to detection sensitivity (personal communication between J.L. and X.M.). The present study achieved this goal with the clinical resources currently available to the study investigators. Full clinical validation may be conducted in tissue or immobilized CTC specimens collected from ongoing clinical trials, and prospective studies can be designed to evaluate the potential utility of this novel test in drug development and patient management.

5. Conclusions

We demonstrated for the first time a highly specific and quantifiable AR-V7 RISH test for detection of clinically significant levels of AR-V7 mRNA in prostate tissue specimens. Our data lend further credence to the clinical importance of AR splice variants and describe a novel assay that merits further clinical qualification in both tissue and CTCs in future clinical trials.

Author contributions: Jun Luo had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Zhu, Sharp, Anderson, Lu, Ma, Y. Luo, Plymate, Nelson, de Bono, J. Luo.

Acquisition of data: Zhu, Sharp, Anderson, Silberstein, Taylor, Lu, Zhao, Demarzo, Antonarakis, Wang, Wu, Su, Nava Rodrigues, Figueiredo, Coleman, Morrissey.

Analysis and interpretation of data: Zhu, Sharp, Anderson, Lu, Demarzo, Antonarakis, Figueiredo, Welti, Park, Ma, Coleman, Morrissey, de Bono, J. Luo.

Drafting of the manuscript: Zhu, Sharp, de Bono, J. Luo.

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Statistical analysis: Zhu, Sharp, Nava Rodrigues, Figueiredo, Welti, Coleman, Morrissey, de Bono, J. Luo.

Obtaining funding: J. Luo, de Bono, Ma, Y. Luo, Plymate, Nelson.

Administrative, technical, or material support: Anderson, Silberstein, Taylor, Demarzo, Antonarakis, Wang, Wu, Y. Luo, Su, Nava Rodrigues, Figueiredo, Park, Ma, Coleman, Morrissey, Plymate, Nelson.

Supervision: J. Luo, de Bono, Plymate, Nelson.

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Orion Pharma, Pfizer, Tesaro, and Sanofi. Peter S. Nelson has served as a consultant/advisor for Janssen, Astellas, and Genentech. CA, Courtney Anderson Mindy Wang, Xingyong Wu, Yuling Luo, Nan Su, Emily Park, and Xiao-Jun Ma are employees of Advanced Cell Diagnostics. The remaining authors have nothing to disclose.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.eururo.2017.08.009>.

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