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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. 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.

15. SUBJECT TERMS

None listed

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1. INTRODUCTION:

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:

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:

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

ï 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 this task. A manuscript evaluating the correlation between CRPC biopsy and CTC marker status is under review.

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 a recent publication in European Urology.

Task 4: We have completed this task. A manuscript on clinical utility of the test has been published. Beyond the scope of our original SOW, additional analysis is being conducted.

Task 5: Will be mainly reported by the de Bono group from the ICR site. 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 was unblinded and analyzed. A manuscript is under review.

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. Both Dr. Zhu and Sharp presented their most recent work at the annual Prostate Cancer Foundation retreat.

How were the results disseminated to communities of interest?

Results from this project were disseminated to communities of interest through peer-reviewed publications as well as poster and podium presentations at national meetings.

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

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 600 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 was also published.

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 has been published(1). This information will provide guidance to providers, patients, and insurers.

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.

5. 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."

ï **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. Boudadi K, Suzman DL, Anagnostou V, Fu W, Luber B, Wang H, Niknafs N, White JR, Silberstein JL, Sullivan R, Dowling D, Harb R, Nirschl TR, Veeneman BA, Tomlins SA, Wang Y, Jendrisak A, Graf RP, Dittamore R, Carducci MA, Eisenberger MA, Haffner MC, Meeker AK, Eshleman JR, Luo J, Velculescu VE, Drake CG, Antonarakis ES. Ipilimumab plus nivolumab and DNA-repair defects in AR-V7-expressing metastatic prostate cancer. Oncotarget. 2018;9(47):28561-71. Epub 2018/07/10. doi: 10.18632/oncotarget.25564. PubMed PMID: 29983880; PMCID: PMC6033362.
2. Luo J, Attard G, Balk SP, Bevan C, Burnstein K, Cato L, Cherkasov A, De Bono JS, Dong Y, Gao AC, Gleave M, Heemers H, Kanayama M, Kittler R, Lang JM, Lee RJ, Logothetis CJ, Matusik R, Plymate S, Sawyers CL, Selth LA, Soule H, Tilley W, Weigel NL, Zoubeidi A, Dehm SM, Raj GV. Role of Androgen Receptor Variants in Prostate Cancer: Report from the 2017 Mission Androgen Receptor Variants Meeting. Eur Urol. 2018;73(5):715-23. Epub 2017/12/21. doi: 10.1016/j.eururo.2017.11.038. PubMed PMID: 29258679; PMCID: PMC5929166.
3. Paschalis A, Sharp A, Welti JC, Neeb A, Raj GV, Luo J, Plymate SR, de Bono JS. Alternative splicing in prostate cancer. Nat Rev Clin Oncol. 2018;15(11):663-75. Epub 2018/08/24. doi: 10.1038/s41571-018-0085-0. PubMed PMID: 30135575.
4. 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. Eur Urol. 2018;73(5):727-35. Epub 2017/09/04. doi: 10.1016/j.eururo.2017.08.009. PubMed PMID: 28866255.
5. Androgen receptor splice variant-7 expression emerges with castration resistance in prostate cancer.Sharp A, Coleman I, Yuan W, Sprenger C, Dolling D, Nava Rodrigues D, Russo JW, Figueiredo I, Bertan C, Seed G, Riisnaes R, Uo T, Neeb A, Welti J, Morrissey C, Carreira S, Luo J, Nelson PS, Balk SP, True LD, De Bono J, Plymate SR.J Clin Invest. 2018 Oct 18 [Epub ahead of print]PMID:30334814

Books or other non-periodical, on(5)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.

ï **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.

ï **Other Products**

Nothing to Report.

6. 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

7. 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.

8. 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.

available at www.sciencedirect.com
journal homepage: www.europeanurology.com

European Association of Urology

Platinum Priority – Prostate Cancer
Editorial by XXX on pp. x–y of this issue

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

Yezi Zhu [a,1], Adam Sharp [b,c,1], Courtney M. Anderson [d], John L. Silberstein [a], Maritza Taylor [a], Changxue Lu [a], Pei Zhao [a], Angelo M. De Marzo [a,e,f], Emmanuel S. Antonarakis [f], Mindy Wang [d], Xingyong Wu [d], Yuling Luo [d], Nan Su [d], Daniel Nava Rodrigues [b], Ines Figueiredo [b], Jonathan Welti [b], Emily Park [d], Xiao-Jun Ma [d], Ilsa Coleman [g], Colm Morrissey [h], Stephen R. Plymate [h], Peter S. Nelson [g,h], Johann S. de Bono [b,c,], Jun Luo [a,*]*

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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; $p = 0.0081$).

[1] These authors contributed equally to this work.

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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.

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

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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

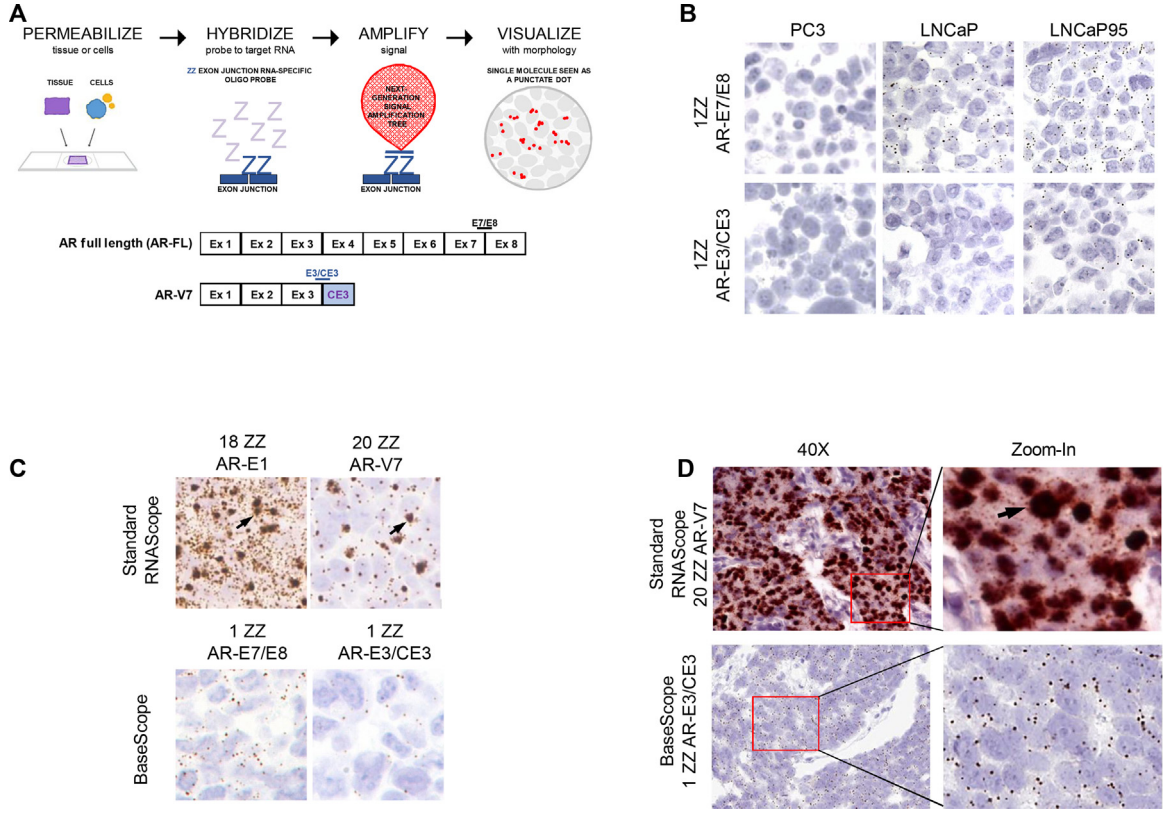

Fig. 1 – Development of the BaseScope RNA in situ hybridization assay for detection of splice junctions specific to AR-FL and AR-V7. (A) Schematic illustration of the BaseScope assay and the splice junctions targeted for probe design. Top: Overview of the BaseScope assay workflow. Sections containing 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.

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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

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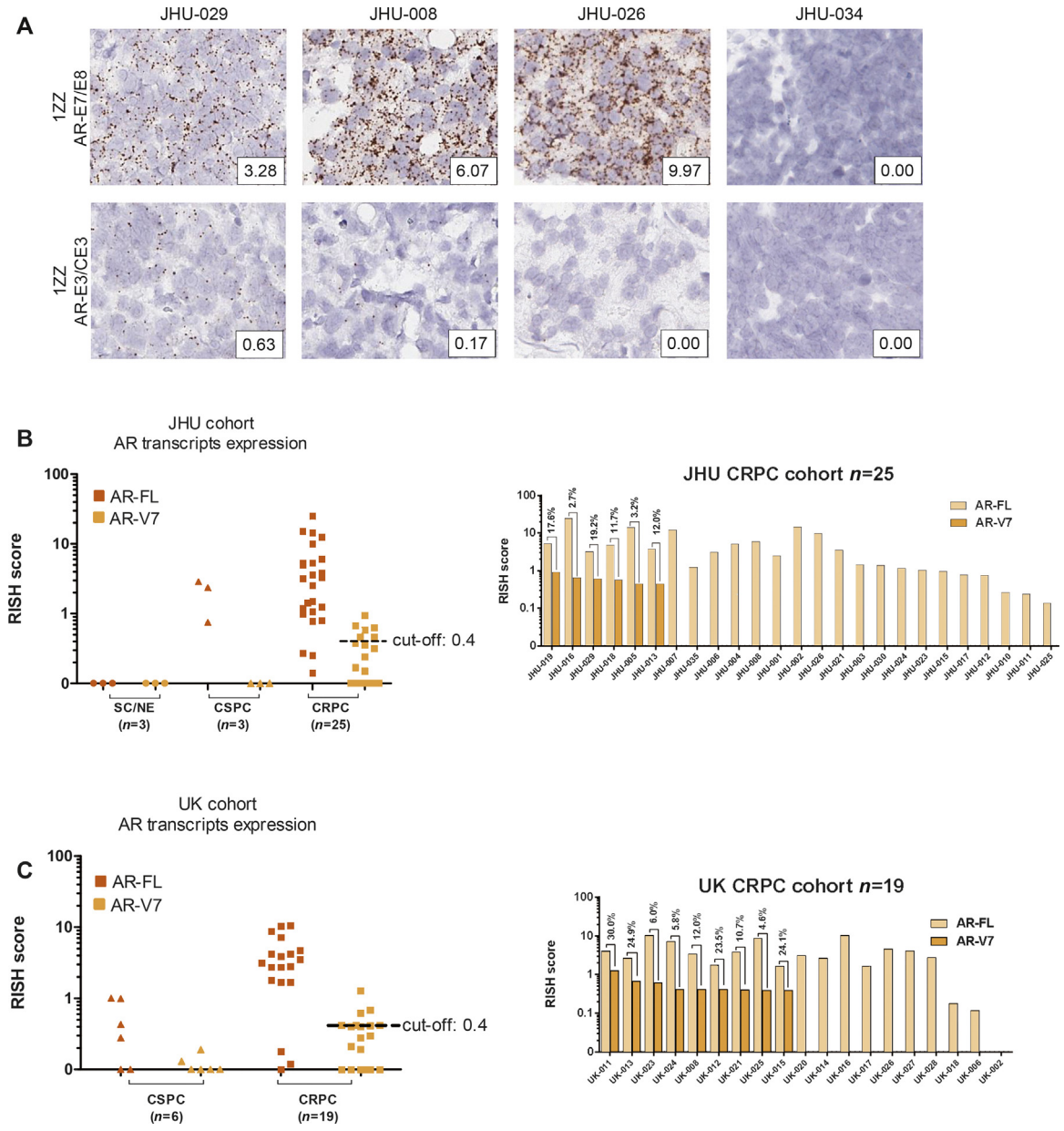

Fig. 2 – Detection and quantification of AR-FL and AR-V7 in two independent biopsy cohorts. (A) Representative images and quantified RNA in situ hybridization (RISH) scores for AR-FL (probe AR-E7/E8, top) and AR-V7 (probe AR-E3/CE3, bottom) mRNA detection in tissue biopsies from patients with metastatic 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 (CSPC) 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 CSPC 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

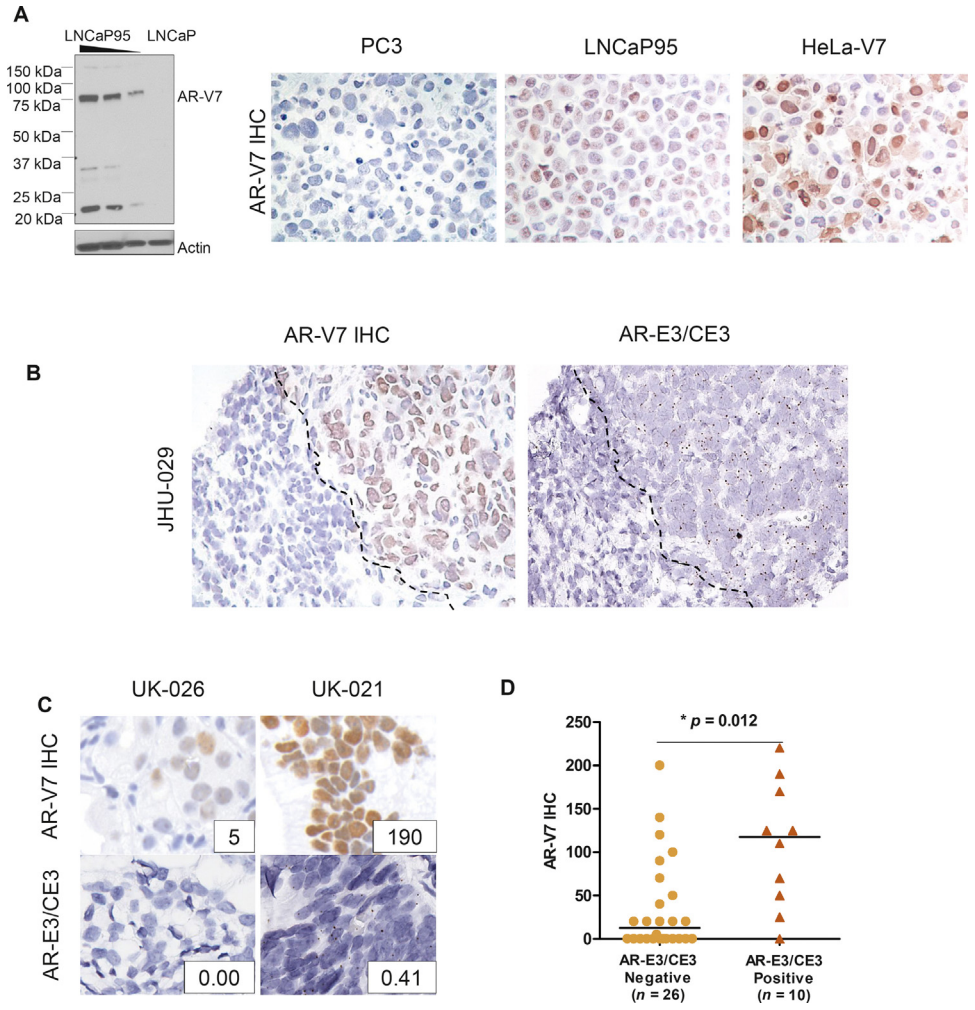

Fig. 3 – Comparison of AR-FL/AR-V7 levels quantified by RNA in situ hybridization (RISH) and immunohistochemistry (IHC). (A) Western blot and IHC using the RevMab-RM7 AR-V7 antibody in prostate cancer cells with known AR profiles. Western blot showed the ~80-kDa AR-V7 band consistent with known AR-V7 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

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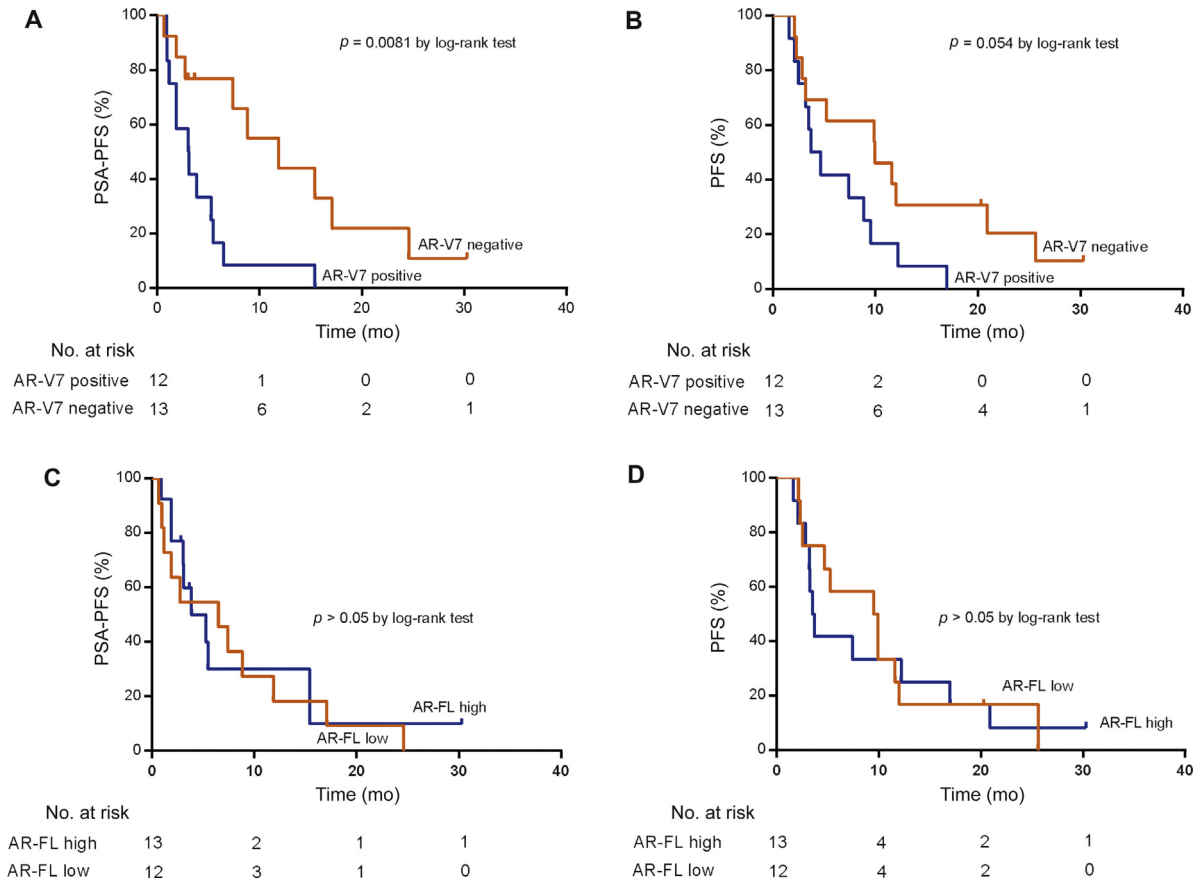

Fig. 4 – Exploratory clinical outcome analysis of AR-V7 and AR-FL status determined by BaseScope assay in patients treated with abiraterone or enzalutamide ($n = 25$). (A) Kaplan-Meier analysis of prostate-specific antigen progression-free survival (PSA-PFS) by AR-V7 status. The median PSA-PFS was 3.1 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

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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.
Critical revision of the manuscript for important intellectual content: Anderson, Ma, Coleman, Morrissey, Plymate, Nelson.
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.
Other: None.

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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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Role of Androgen Receptor Variants in Prostate Cancer: Report from the 2017 Mission Androgen Receptor Variants Meeting

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Abstract

Context: Although a number of studies have demonstrated the importance of constitutively active androgen receptor variants (AR-Vs) in prostate cancer, questions still remain about the precise role of AR-Vs in the progression of castration-resistant prostate cancer (CRPC).
Objective: Key stakeholders and opinion leaders in prostate cancer convened on May 11, 2017 in Boston to establish the current state of the field of AR-Vs.
Evidence acquisition: The meeting "Mission Androgen Receptor Variants" was the second of its kind sponsored by the Prostate Cancer Foundation (PCF). This invitation-only event was attended by international leaders in the field and representatives from sponsoring organizations (PCF and industry sponsors). Eighteen faculty members gave short presentations, which were followed by in-depth discussions. Discussions focused on three thematic topics: (1) potential of AR-Vs as biomarkers of therapeutic resistance; (2) role of AR-Vs as functionally active CRPC progression drivers; and (3) utility of AR-Vs as therapeutic targets in CRPC.
Evidence synthesis: The three meeting organizers synthesized this meeting report, which is intended to summarize major data discussed at the meeting and identify key questions as well as strategies for addressing these questions. There was a critical consensus that further study of the AR-Vs is an important research focus in CRPC. Contrasting views and emphasis, each supported by data, were presented at the meeting, discussed among the participants, and synthesized in this report.
Conclusions: This article highlights the state of knowledge and outlines the most pressing questions that need to be addressed to advance the AR-V field.
Patient summary: Although further investigation is needed to delineate the role of androgen receptor (AR) variants in metastatic castration-resistant prostate cancer, advances in measurement science have enabled development of blood-based tests for treatment selection. Detection of AR variants (eg, AR-V7) identified a patient population with poor outcomes to existing AR-targeting therapies, highlighting the need for novel therapeutic agents currently under development.

1. Introduction

As prostate cancer is an androgen-dependent disease, the androgen receptor (AR) is the primary molecular target for systemic prostate cancer therapy. Despite initial robust responses to first-line androgen deprivation therapies (ADTs), nearly all patients with advanced prostate cancer progress to lethal castration-resistant prostate cancer (CRPC). Importantly, in CRPC, the AR continues to be the primary molecular driver, as evidenced by efficacy of novel hormonal therapies, abiraterone and enzalutamide, in CRPC patients [1–4]. While effective, therapies targeting AR are not curative, due to intrinsic and acquired resistance to first-line ADTs and novel hormonal therapies. Molecular mechanisms of resistance are largely driven by AR aberrations including AR protein overexpression, AR gene amplification, AR gene mutations, and AR variants (AR-Vs) [5].

AR-Vs are truncated AR proteins lacking the AR ligand-binding domain (AR-LBD) [6]. While AR-Vs have frequently been detected in CRPC, their expression and functional role in benign prostate tissues and primary prostate cancers is not readily apparent. Structural rearrangements in the AR gene and alternative AR mRNA splicing are at least two mechanisms for expression of AR-Vs in CRPC [6]. Multiple AR-Vs arising from AR gene rearrangements and/or alternative splicing have been characterized. To date, AR splice variant-7 (AR-V7) has been studied in greatest detail owing to its relative abundance and frequency of detection in CRPC [7,8], as well as its potential clinical utility as a marker for treatment selection in men with metastatic CRPC (mCRPC) [9]. However, in-depth studies have also been conducted on other AR-Vs, including AR-V1, AR-V3, AR-V7, AR-V9, and ARv567es [10–12]. Structural differences of these AR-Vs are illustrated in Figure 1. Since AR-Vs contain the AR DNA-binding domain (DBD) and the AR transcriptional activation domain, they are capable of transcriptional regulation, in spite of the loss of the AR-LBD. Further, since the AR-Vs lack the AR-LBD, they are not regulated by either first-line or novel hormonal therapies currently used in the clinic. At the Mission Androgen Receptor Variants (MARS) 2 meeting, our efforts were streamlined to evaluate the role of AR-Vs as biomarkers, molecular drivers, and therapeutic targets. The authors identified key consensus, discussion points, and critical future work needed to advance the field.

2. Evidence acquisition

The MARS meeting was the second of its kind sponsored by the Prostate Cancer Foundation (PCF). This invitation-only event was attended by international leaders in the field and representatives from sponsoring organizations (PCF and industry sponsors). Eighteen faculty members gave short presentations, which were followed by in-depth discussions. Discussions focused on three thematic topics: (1) potential of AR-Vs as biomarkers of therapeutic resistance;

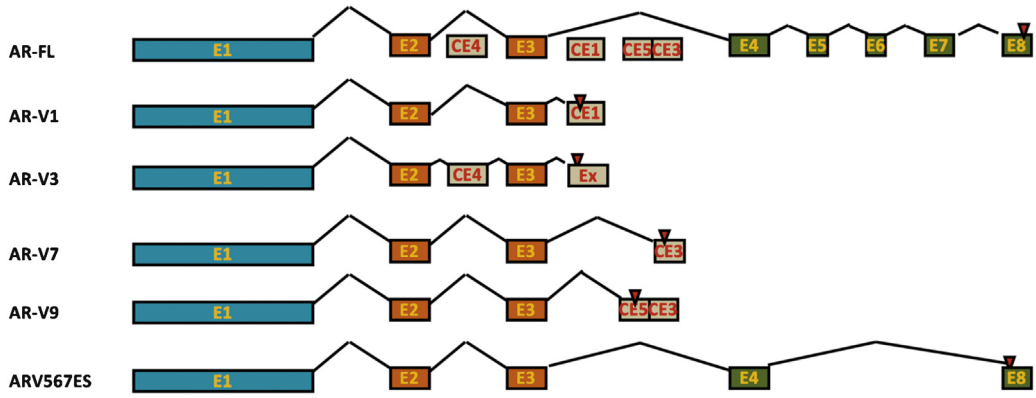

Fig. 1 – Transcript structure for representative AR-Vs. Stop codon positions are marked for each AR transcript. AR = androgen receptor; AR-FL = full length androgen receptor; AR-V = androgen receptor variant; E1–E8 = canonical androgen receptor exons 1–8; CE1–5 = cryptic exons 1–5; Ex = unknown exon in AR-V3.

(2) role of AR-Vs as functionally active CRPC progression drivers; and (3) utility of AR-Vs as therapeutic targets in CRPC.

3. Evidence synthesis

The three meeting organizers (J.L., S.M.D., G.V.R.) conceived the idea of an invitation-only meeting focusing on AR-Vs. Three thematic topics were predefined prior to the meeting: (1) potential of AR-Vs as biomarkers of therapeutic resistance; (2) role of AR-Vs as functionally active CRPC progression drivers; and (3) utility of AR-Vs as therapeutic targets in CRPC. The meeting was sponsored by the PCF, Sanofi, Astellas, Janssen Research and Development LLC, and Sun Pharma, and held in Boston, MA, prior to the American Urological Association annual meeting. Academic physicians and scientists from the USA, the UK, Canada, Australia, and Japan, as well as representatives from four sponsoring pharmaceutical companies attended this meeting. Eighteen faculty members gave short presentations, which were followed by in-depth discussions. The three meeting organizers summarized major data discussed at the meeting, identified 26 key questions in the field, and synthesized this meeting report. The 26 key questions were included in an online survey sent to all nonindustry participants after the meeting. Detailed voting results (percent and number of approval votes) are included in the three boxes of the Supplementary material, summarizing general consensus reached at the meeting.

3.1. Session 1: Measurement science pertaining to AR-Vs and other AR aberrations

3.1.1. Tissue-based testing of AR-Vs

Prior studies had established that AR-Vs can be detected at the RNA and protein levels in CRPC samples. Expression of several AR-Vs including AR-V7 has been reported in benign prostate tissue and primary prostate cancers [9]. Although AR-Vs were detected in untreated prostate tumors and benign prostate tissues; their levels were substantially lower and likely represented background splice events detectable by reverse transcription polymerase chain reaction and RNA-seq that may not lead to robust detection by RNA in situ hybridization (RISH) and immunohistochemistry (IHC). However, expression of AR-V7 is higher in CRPC, potentially due to AR gene amplification and/or induction of AR-V7 by ADTs [13–15], which occurs in CRPC cell lines and xenografts. Therefore, amplification of the AR gene, a frequent event in CRPC, is likely to underlie the increased expression of AR-Vs at this disease stage. Whether the selective advantage for AR gene amplification is overexpression of AR or AR-Vs (or both) remains to be established.

Dr. Richard Lee reported the development of a branched chain RNA in situ histochemistry (RNA ISH) assay for the detection of AR-V7 mRNA within archival prostate cancer tissue. This branched chain RNA ISH assays and other similar assays reported in the literature utilized 20-mer probes tiled across 500–1000 base pairs of the unique cryptic exon 3 (CE3) at the 3′ terminus of AR-V7 mRNA [16–18]. Using an automated ISH system, AR-V7 was detected in formalin-fixed paraffin-embedded (FFPE) samples from radical prostatectomy (n = 30, detection rate 1/30), metastatic, castration-sensitive prostate cancer (n = 22, detection rate 10/22), and mCRPC (n = 12, detection rate 12/12), suggesting that AR-V7 expression dramatically increases with disease progression [18]. In this pilot study, AR-V7 mRNA detection was also associated with the duration of response to first-line ADTs. Dr. Lee presented an individual case with serial tissues collected before, during, and after ADT showing progressively higher AR-V7 expression. Thus, automated RNA ISH assay is feasible for AR-V7 evaluation in archival FFPE prostate cancer tissue, and this small-cohort study suggests that baseline AR-V7 by this method is a negative predictive marker for treatment with ADTs.

Dr. Johann de Bono reported on advances in tissue-based measurements of AR-V7 mRNA and protein. The primary drawback of RNA ISH– and IHC-based detection of AR-V7 mRNA and protein has been the lack of specificity [19,20]. Dr. de Bono presented data on a novel RNA ISH

method, as well as a new AR-V7 antibody developed by RevMab shown to be analytically valid with a single band on Western blot. The novel RNA ISH method detected a single splice junction specific to AR-V7 [20], and the new AR-V7 antibody did not detect false-positive signals reported in a previous study with the Abcam antibody [19]. These validated in situ detection methods enabled precision measurement of AR-V7 in morphologically intact clinical tissue specimens. A recent study reported a novel RISH detection method and compared the RISH results with the IHC results by the RevMab antibody [20]. The findings further confirmed the improved specificity of the new antibody. Detection of AR-V7 by the novel RNA ISH method in a cohort of CRPC biopsies was generally associated with poorer response to abiraterone and enzalutamide [20]. Dr. de Bono suggested that AR-V7 mRNA detection alone may not correlate with AR-V7 protein levels, due to altered kinetics of AR-V7 protein degradation [21].

3.1.1.1. Synthesis. AR-V7 mRNA and protein can be detected in morphologically intact tissues. Tissue-based studies support the feasibility of measuring AR-V7 in metastatic tissue biopsies. As AR-V7 is infrequently detected in untreated patients by RISH or IHC, elevated AR-V7 expression may be an acquired event after hormonal therapies, although detection in untreated cases may theoretically have prognostic or predictive value. Both RISH and IHC detection values are continuous variables. Thus, differences in detection rate between studies could reflect the different cutoff values. In addition, difference in techniques, reliance on RNA versus protein, and sampling criteria may result in different detection rates.

3.1.2. Blood-based detection of AR-Vs and other AR aberrations

Previous studies have utilized circulating tumor cells (CTCs) [16,22–25], plasma exosomes [26], peripheral blood mononucleated cells [27], and even whole blood samples [28–31] for the detection of AR-Vs (mainly AR-V7) in men with mCRPC. Among these, the CTC-based AR-V7 test has been analytically validated and implemented in Clinical Laboratory Improvement Amendments (CLIA)-certified laboratories on the basis of clinical correlative findings [25,32]. However, CTC-based AR-V7 tests may be limited. Critically, determination of the AR-V7 status and its quantification were not possible in a significant proportion of mCRPC patients who were CTC negative. In addition, many other relevant molecular targets are compatible with blood-based measurements, including AR amplification/mutation, AR-Vs other than AR-V7, as well as non-AR genomic alterations implicated in CRPC progression.

Novel CTC platforms may partially address this limitation. Dr. Joshua Lang demonstrated the feasibility of the VERSA system to detect mRNAs of multiple AR-Vs using CTCs captured using antibodies targeted to cell surface proteins EpCAM and TROP2. The overall AR-V7 detection rate was 6/26 (26%) in this study, in line with the literature. In addition to AR-V7, AR-V9 was also detected at a high frequency [11]. Using this platform, ARv567es was detected at a lower frequency (~1% of patients). The system allowed analysis of additional genes involved in epithelial–mesenchymal transition and neuroendocrine differentiation.

Dr. Mayuko Kanayama reported the Juntendo University pilot study, where they evaluated the detection of AR-V7–positive CTCs along with examination of prostate-specific membrane antigen (PSMA) in 19 Japanese mCRPC patients treated with different CRPC therapies. The overall AR-V7 detection rate was 26% (5/19). In addition to AR-V7, Dr. Kanayama discussed the potential negative predictive value of PSMA detection (positive rate 47%, 9/19) in this small cohort.

Drs. Martin Gleave and Gerhard Attard presented data on blood-based measurements through the isolation of circulating tumor DNA (CtDNA). Published data have established the feasibility of detecting genomic alterations in CtDNA samples in patients with CRPC and evolution in these genomic alterations over time under selective pressures of treatment [33–35]. A wide spectrum of genomic alterations (AR, DNA repair genes, TP53, PIK3CA, and RB1) detected in CtDNA samples are also detected in matched metastatic tissues. In addition, CtDNA assays may reveal greater heterogeneity of alterations in some patients than is possible through biopsy of a single metastatic site, suggesting that metastatic tissue biopsy may not be required to determine the somatic status of clinically actionable prostate cancer driver genes [36]. CtDNA assays, therefore, may be utilized for the development of prognostic and predictive biomarkers. There is a growing need to optimize panels and develop CLIA-certified assay for prospective validation of CtDNA markers.

3.1.3. Synthesis

It is feasible to measure AR aberrations (AR-V7, AR-V9, AR amplification, and AR mutation) as well as other disease drivers using blood-based assays. However, no studies have integrated these measurements. The challenge ahead is to perform analytical as well as clinical validation of individual and integrated assays with concerted efforts.

3.2. Session 2: AR-V functional role and regulation

3.2.1. Regulation of gene expression by AR-Vs

At the functional level, ADTs cause increased expression of AR and AR-Vs due to relief of androgen/AR-mediated suppression of AR gene transcription [37]. Thus, AR-Vs (and full-length AW [AR-FL]) induced by ADTs may regulate the same genes regulated by androgen-activated AR-FL. An alternative model is that elevated AR-Vs may confer a distinct transcriptional program and cellular phenotype. Dr. Nancy L. Weigel used an inducible AR-V7 system to show that while AR-V7 induces canonical AR genes such as *FKBP5* and *KLK3*, RNA-seq analysis revealed expression of distinct genes that were associated with AR-V7 induction. For example, EDN2, ETS2, SRD5A1, ORM1, BIRC3, HSP27, and HES1 were specifically induced following induction of AR-V7 expression, while SGK1 was specifically induced by AR-FL activation [38]. Interestingly, the AR-V7–specific target genes, *EDN2*, and *ETS2* are genes that can be regulated by AR if the pioneer transcription factor FOXA1 is depleted.

Conversely, AR can induce RASSF3 only when FOXA1 is present, but AR-V7 does not induce RASSF3 under either condition. When ARv567es was investigated, there also appeared to be significant overlap with AR-V7 and AR-FL targets. However, there may also be ARv567es-specific target genes owing to the retention of the AR hinge region in ARv567es as opposed to AR-V7 (Fig. 1).

Dr. Laura Cato showed LNCaP95 cells (LNCaP cells derived from long-term passage under castrate conditions) may depend on both AR-FL and AR-V7 for short-term proliferation, as evidenced by slowed LNCaP95 cell growth after dox-inducible knockdown of either AR-FL or AR-V7 [14]. Using the AR-V7 RevMab and an AR C-terminal antibody for ChIP-seq, they demonstrate that AR-V7 and AR-FL bind to the same genomic location in this dox-inducible model. AR-V7 chromatin binding was reduced in response to AR-FL knockdown and vice versa; knockdown of AR-V7 reduced AR-FL chromatin binding. Further work is ongoing to identify similarities and differences in the transcriptional activities of the two receptors.

3.2.1.1. Synthesis. Laboratory research using cell-line model systems may be used to dissect gene expression and chromatin-binding programs directed by AR-FL and AR-Vs. The majority of chromatin-binding events and transcriptional targets of AR-Vs and androgen-activated AR-FL display significant overlap. However, genome-wide analyses have suggested that an AR-V–specific transcriptional program may exist. The interplay between AR-FL, AR-V7, and other AR-Vs deserves further in-depth investigation, particularly in the setting of the native molecular context of clinical specimens.

3.2.2. Role of AR dimerization and AR coregulators

An important question in the AR-V field is whether AR-V functions as a dimer, and if so, how AR-V/AR-FL homo- and heterodimers influence therapeutic targeting [39]. Studies published from Dr. Yan Dong's laboratory [40–42] focused on interactions between AR-FL and various AR-Vs. Using tagged forms of AR-FL and AR-Vs, Dr. Dong suggested that AR-Vs form dimers through DBD/DBD interactions, and that this dimerization is required for function but not nuclear localization. AR-Vs can modulate the function and localization of AR-FL through formation of AR-FL/AR-V heterodimers, which are mediated by the DBD/DBD interactions, but also binding of the AR-V N terminus with the AR-FL C terminus. Importantly, this AR-V/AR-FL heterodimer was not inhibited by enzalutamide. The critical function of the dimer was supported by mutually dependent co-occupancy of genomic sites by AR-FL and AR-V7.

AR coregulators are important for AR-FL transcriptional activity [43] and may drive context-specific AR functions. The AR-V cistrome consists of canonical androgen response elements (AREs) and overlaps with AR-FL, and AR-V–specific genes may reflect the biphasic nature of AR transcriptional activation of certain target genes [10,44], indicating that the same or a similar set of coregulators may be involved in AR-FL and AR-V function. However, AR-Vs such as ARv567es and AR-V7 have reduced affinity for AREs compared with AR-FL [10], and altered kinetics of DNA binding may require different sets of coregulators. Dr. Luke Selth presented his work on dissecting the sets of coregulators bound by AR and ARv567es using Rapid Immunoprecipitation Mass-spectrometry of Endogenous proteins (RIME) [45]. This work showed a high degree of overlap between the AR-FL and ARv567es interactomes, but also yielded differences that may arise from coregulators specific to the AR-LBD and/or unique binding surfaces on the variant protein. One new example of an AR-FL and ARv567es-shared coregulator recently identified by Dr. Selth's group is GRHL2 [45], which was shown to participate in a feed-forward transcriptional loop with active AR-FL and AR-Vs that likely drives their activity in CRPC. Dr. Selth also noted that there are likely to be differences in regulation of AR/AR-V by the ubiquitin–proteasome system [46], as AR-Vs lose interaction and dependency on HSP90 [47].

3.2.2.1. Synthesis. Current data indicate that chromatin binding and transcriptional activation mediated by AR-Vs require homodimerization. While evidence also exists to support a role for heterodimerization between AR-Vs and AR-FL, the relative contributions of AR-V homodimers and AR-FL/AR-V heterodimers to the activation of AR-FL/AR-V target genes remain to be characterized. RIME represents an effective approach to study complexes nucleated by these homo- and heterodimers, and will be useful for characterizing AR-FL and AR-V interactomes in clinical specimens.

3.2.3. AR-V as a potential disease driver

Whether AR-Vs drive therapeutic resistance in CRPC remains an unresolved topic. Studies in favor of AR-Vs functioning as drivers of resistance have come from models where AR-Vs are endogenously expressed at high levels, and their knockdown restores sensitivity to castration and/or antiandrogens [44,48]. In contrast, studies arguing against AR-Vs functioning as drivers of resistance have come from models where AR-Vs are introduced ectopically or are expressed at extremely low levels relative to AR-FL. For instance, Dr. Charles Sawyers discussed his early published work indicating that AR-Vs lack the key properties of drug resistance drivers. In these studies, overexpression of AR-V7 in LNCaP cells was able to confer gain of function in terms of ligand-independent growth but did not impart resistance to enzalutamide, suggesting that AR-Vs may require AR-FL for gain of function [49]. In this context, Dr. Sawyers indicated that rapid induction of AR-V7 by ADTs may be a by-product of the increased transcription of the AR gene and simply reflects a mechanism for rapid induction of AR-FL expression by ADTs [14,49]. Another argument against a driver role for AR-Vs came from his observations with a prostate cancer cell line (Myc-Cap) derived from the Hi-Myc mouse, which expressed AR-Vs but remained sensitive to castration and enzalutamide. However, he also noted the Myc transgene in this model was under the control of an androgen-responsive promoter, which clouds interpretation. The murine AR-V, although structurally different from the human AR-V, demonstrated in vitro functional activities similar to human AR-V. Additionally, the 3′ terminal exons

in these murine AR-Vs were not located within the AR gene locus, as is the case for human AR-V7. Instead, these 3′ terminal exons were located hundreds of kb upstream or downstream of the AR locus, indicating that underlying AR gene rearrangements were responsible for their splicing into AR mRNA.

Dr. Steven Balk presented data on progressive increases of AR-V7 expression in VCaP cells treated with enzalutamide in vitro and combination of abiraterone/enzalutamide in vivo. Using these cells with high AR-V7 expression, he showed that AR activity was inhibited by knockdown with an siRNA targeted to AR exon 1 (which encodes the AR amino terminus [NTD]) or the 3′ terminal exon CE3 of AR-V7. Interestingly, AR activity in these cells was not inhibited by siRNA that selectively knocked down AR-FL. In contrast to this adaptive model, VCaP cells treated acutely with enzalutamide and displaying rapid induction of AR-V7 displayed very low AR activity, suggesting that additional cofactors may be important for AR-V function in settings of acute versus adapted AR-V7 expression.

3.2.3.1. Synthesis. Discrepancies have been noted in the contribution of AR-Vs to the phenotype of therapeutic resistance in CRPC. It is possible that these discrepancies may be due to whether knockdown or overexpression approaches were used to interrogate AR-V function. Additionally, several of the models used for AR-V knockdown experiments and displaying AR-V–driven resistance phenotypes also harbor structural rearrangements in the AR gene and/or were adapted to long-term treatment with enzalutamide. This suggests that alterations in AR gene structure or adaptive changes cofactor milieu may be important determinants of AR-V function. Additionally, it has recently been shown that AR-V9, AR-V1, and additional AR-Vs utilizing CEs in AR intron 3 are coordinately expressed in CRPC and susceptible to knockdown with siRNAs/shRNAs that had previously been thought to target AR-V7 exclusively [11,50]. Thus, AR-V7 knockdown studies reported in the literature were actually inhibiting expression of multiple AR-Vs simultaneously. Further work is required to elucidate the impact of these parameters on AR-V function as drivers of resistance in CRPC.

3.3. Session 3: Therapeutic targets and strategies

3.3.1. Utility of targeting AR amino terminus

The AR-NTD (amino acids 1–538) is structurally unique among steroid receptors: it is much longer, has a stronger transactivation domain, and is critical for the transactivation and function of the AR. Experimental and bioinformatic analyses reveal that the AR-NTD is intrinsically disordered in solution and exists as an ensemble of interconverting conformations. In response to environmental stresses, the AR-NTD may rapidly and reversibly fluctuate between conformations. These alterations in domain structure may enable transient interactions between AR-NTD and protein coregulators that may allosterically regulate AR function. Analyses by circular dichroism, Fourier transform infrared spectroscopy, secondary structure prediction, and mutagenesis have revealed variations in the degree of intrinsic disorder of different regions of the AR-NTD: some domains may adopt more stable secondary structure than others. While the flexibility and intrinsic disorder of the AR-NTD in solution and the lack of a crystal structure hamper rational design of drugs using virtual docking approaches, the relative stability of some domains makes them potentially targetable. In addition, the lack of sequence homology between the NTDs of AR and other members of the steroid hormone receptor family suggests that drugs targeting the AR-NTD will be more selective for the AR, yielding fewer side effects.

The primary advantage of drugs targeting the AR-NTD is the potential to fundamentally target all forms of the AR, including those that drive resistance to AR-LBD–targeting therapies. Since the AR-NTD is retained in all forms of biologically active AR, including amplified AR-FL, AR-LBD mutations (eg, W741C for bicalutamide and F876L for enzalutamide), and AR-Vs, drugs targeting AR-NTD should be effective against all these AR forms. The addition of such drugs to the CRPC armamentarium is likely to have significant clinical utility to prevent and overcome drug resistance.

A bisphenol A derivative EPI-001 has been shown to bind the AF-1 region of the AR-NTD and inhibit AR function. EPI-001 has been shown to inhibit AR-NTD transactivation, inhibit proliferation of cell lines expressing various forms of AR, and selectively block AR–protein interactions and recruitment of the AR to DNA response elements [51]. A derivative of EPI-001 is currently in phase I clinical trials. Importantly, EPI-001 provided proof of principle of the translatability of drugs targeting the AR-NTD; however, its utility as a therapeutic agent remains to be proved.

3.3.2. Development of novel agents

Dr. Artem Cherkasov performed in silico screening of 150 million compounds to identify drugs targeting the AR DBD [52]. As the AR DBD is shared between AR-FL, AR-LBD mutations, and AR-Vs, such an approach is likely to target all forms of the AR. Indeed, their lead compound VPC-14449 inhibits binding of AR-FL as well as AR-V7 and ARv567es to chromatin, and consequently blocks transcriptional activity driven by AR [53]. Dr. Cherkasov and colleagues also developed VPC-17005 to block AR dimerization, and this compound resulted in the abrogation of AR function. Importantly, VPC-17005 selectively inhibited AR DNA binding without affecting other steroid hormone receptors, including progesterone receptor, estrogen receptor, and glucocorticoid receptor. Further development of these drugs could pave the way forward for rational design of drugs targeting the undruggable transcription factors.

Dr. Allen Gao showed that niclosamide, a Food and Drug Administration–approved drug to treat tapeworm infections, displayed activity against AR-V7 function [54–56]. A gene expression signature associated with treatment with niclosamide overlapped significantly with AR knockdown. This activity against AR-V7 may be driven by AR degradation as evidenced by MG132, a 26S proteasome inhibitor, inhibiting niclosamide-mediated AR-V7 protein degrada-

tion. Niclosamide inhibited growth of enzalutamide-resistant C4-2B cells, and synergized with treatment with abiraterone and enzalutamide [55]. Phase Ib/II clinical trials combining niclosamide and abiraterone/enzalutamide in mCRPC patients are currently ongoing.

Additional indirect strategies to interfere with AR-V function are to target their interactions with either protein coregulators or downstream transcriptional targets. Dr. Kerry Burnstein proposed indirectly targeting AR-V activity by disrupting interactions with key AR-V coactivators such as VAV3 and other AR amino terminal–interacting regulators [57]. She showed that a coactivator-enhanced AR-V transcriptional target could be exploited therapeutically in CRPC xenograft models. Similarly, another indirect approach to targeting AR-Vs rose out of an observation that certain kinase inhibitors inhibited the growth of AR-positive cells but not of AR-negative cells. Dr. Stephen Plymate presented data showing that bumped kinase inhibitors (BKIs) could inhibit prostate cancer cells that are driven by the constitutively active AR-V7. One proposed mechanism of action was inhibition of serine 81 phosphorylation on both AR-FL and AR-V7. Of note, in the absence of androgen, serine 81 is phosphorylated in cells expressing AR-FL and AR-V7. They also demonstrated that their candidate BKIs inhibited the growth of the AR-FL–driven LuCaP35 human PDX model in noncastrate mice as well as AR-V7–driven LNCaP95 xenografts in castrate mice. Their current BKI PK data demonstrate that they can reach effective EC50 levels in mice with a single daily oral dose, and there was no observable toxicity after 6 wk of treatment. Importantly, the BKIs have a narrow kinase target range. They will continue to modify the BKIs to achieve increased potency.

Dr. Robert Matusik showed that nuclear factor kB (NFkB) induces expression of AR-V7, and inhibition of NFkB using bortezomib reduced AR-V7 levels and restored CRPC responsiveness to antiandrogens in cell line and xenograft models using CRW22RV1 and C4-2B cells. Further, neuropeptides released by neuroendocrine prostate cancer activate the gastrin-releasing peptide receptor to induce NFkB and AR-V7 expression resulting in CRPC [58,59]. Other inhibitors of NFkB, such as methotrexate or LC-1 (dimethylaminoparthenolide), would reduce AR-V7 expression in CRPC cell lines and restore responsiveness to antiandrogens.

Dr. Amina Zoubeidi suggested that a significant fraction (9/35) of AR-positive enzalutamide-resistant xenografts are potentially AR indifferent, as indicated by a lack of prostate-specific antigen (PSA) expression [60], and have biologic similarity to pluripotent and neuroendocrine tumors. Stem-cell factors likely emerge early during therapeutic inhibition of the AR pathway, which can then be followed later by the expression of neuroendocrine markers. Inhibition of EZH2 can reverse the process back to an AR-driven state. Dr. Zoubeidi discussed BRN2, which was found to be overexpressed in CRPC tumors associated with low serum PSA. She further demonstrated that BRN2 was a transcription factor that likely functioned as a master regulator of enzalutamide-induced neuroendocrine transdifferentiation required for the expression of neuroendocrine markers. Mechanistically, AR suppressed BRN2, and this negative feedback can be relieved by enzalutamide. Thus, BRN2 is a potential target in advanced CRPC, and inhibition of BRN2 in combination with enzalutamide is being explored.

3.3.2.1. Synthesis. The unresolved question of whether AR-Vs function as the primary molecular drivers in CRPC has led to the question of whether AR-Vs are viable therapeutic targets for CRPC. Since AR-Vs lack the AR-LBD, novel drugs would be required to target AR-Vs. Since the amino terminus of AR-Vs is identical to the amino terminus of AR-FL, therapeutic strategies developed against AR-Vs are likely to have effects against all forms of the AR. This rationale has spawned significant interest from multiple investigators to target the AR-NTD or downregulate the expression of AR-Vs. However, approaches to target the N terminus of the AR are limited by the intrinsic disorder and lack of a crystal structure of this domain.

4. Conclusions

To realize the potential of translating laboratory discoveries to patient benefit, it is important to understand the biology, measurement science, and relevant experimental therapeutic approaches from multiple perspectives. In the 9 yr since the first report of AR-Vs, critical advances have been made. Importantly, testing platforms have been developed to facilitate AR-V measurements for biomarker-driven or biomarker-stratified clinical trials, and AR-Vs are being explored as a therapeutic target. In spite of these advances, there remains a need to conduct prospective trials to further assess the clinical utility of AR-Vs in mCRPC, and future efforts are also needed to improve blood-based testing platforms beyond AR-V7 by integrating multiple AR aberrations to enable robust treatment selection and patient selection. In addition, how AR-Vs mediate genomic function as a transcription factor, particularly in homo- versus heterodimer contexts, remains incompletely characterized. Additionally, an unresolved question that remains is whether AR-Vs drive therapeutic resistance in CRPC and, if so, under which specific contexts. Blood-based detection of AR-V7 identified a patient population with poor outcomes to existing therapeutic agents, highlighting the need to develop novel therapeutic approaches for mCRPC. Finally, there is a pressing need to develop markers and therapeutic approaches targeting AR-indifferent prostate cancers.

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Study concept and design: Luo, Dehm, Raj.
Acquisition of data: Luo, Attard, Balk, Bevan, Burnstein, Cato, Cherkasov, De Bono, Dong, Gao, Gleave, Heemers, Kanayama, Kittler, Lang, Lee, Logothetis, Matusik, Plymate, Sawyers, Selth, Soule, Tilley, Weigel, Zoubeidi, Dehm, Raj.
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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.eururo.2017.11.038.

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Alternative splicing in prostate cancer

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Abstract | Androgen receptor (AR) splice variants (AR-Vs) have been implicated in the development and progression of metastatic prostate cancer. AR-Vs are truncated isoforms of the AR, a subset of which lack a ligand-binding domain and remain constitutively active in the absence of circulating androgens, thus promoting cancer cell proliferation. Consequently, AR-Vs have been proposed to contribute not only to resistance to anti-androgen therapies but also to resistance to radiotherapy in patients receiving combination therapy by promoting DNA repair. AR-Vs, such as AR-V7, have been associated with unfavourable clinical outcomes in patients; however, attempts to specifically inhibit or prevent the formation of AR-Vs have, to date, been unsuccessful. Thus, novel therapeutic strategies are desperately needed to address the oncogenic effects of AR-Vs, which can drive lethal forms of prostate cancer. Disruption of alternative splicing through modulation of the spliceosome is one such potential therapeutic avenue; however, our understanding of the biology of the spliceosome and how it contributes to prostate cancer remains incomplete, as reflected in the dearth of spliceosome-targeted therapeutic agents. In this Review, the authors outline the current understanding of the role of the spliceosome in the progression of prostate cancer and explore the therapeutic utility of manipulating alternative splicing to improve patient care.

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Prostate cancer is the second most frequently diagnosed cancer among men worldwide[1], with one man dying of prostate cancer every 45 minutes in the United Kingdom alone[2]. Since the pioneering work of Charles Huggins and Clarence Hodges, who first demonstrated the benefits of androgen deprivation therapy (ADT) in patients with metastatic prostate cancer in 1941 (REF.[3]), our understanding of the pathogenesis has increased substantially, particularly with regards to the fundamental importance of the androgen receptor (AR) in all stages of disease from tumorigenesis to progression and ultimately treatment resistance and death[4].

The AR and prostate cancer

The AR is a ligand-activated transcription factor that has a central role in male sexual development. This receptor is a member of the steroid and nuclear hormone receptor superfamily and is encoded by the *AR* gene located on chromosome Xq12 (REF.[5]), the transcriptional activity of which is modulated by its interactions with potentially >200 different transcriptional co-regulators[6]. In prostate cancer, in addition to these regulators, genomic aberrations such as *AR* copy number gains, mutations, and gene rearrangements are also thought to have a major role in *AR* gene expression, with *AR* overexpression, in particular, being key to the development and progression of castration-resistant prostate cancer (CRPC)[7].

The structure of the full-length product of *AR* transcription was first reported in 1988 (REFS[8,9]). The AR comprises four discrete functional domains (FIG. 1), namely, an amino-terminal transcriptional domain (NTD), the sequence of which is highly variable and inherently disordered[5]; a DNA-binding domain (DBD), which consists of a highly conserved 66-residue core made up of 2 zinc-nucleated modules[10]; a hinge region; and a carboxy-terminal ligand-binding domain (LBD)[11]. Of note, while the carboxy terminus and the DBD have been crystalized, the crystal structure of the amino terminus remains elusive, thus hindering the development of amino-terminal-targeted agents.

In the absence of activating ligands, the AR is sequestered within the cytoplasm by a complex of heat shock protein (HSP) chaperones[12] and their co-chaperones, such as BAG family molecular chaperone regulator 1 (BAG-1). In the presence of circulating androgens, namely, dihydrotestosterone (DHT) and, to a lesser degree, testosterone, the AR undergoes conformational changes[11] and dimerizes with other ligand-bound AR subunits to form homodimers. Nuclear localization of the AR is dependent on the AR bipartite nuclear localization sequence (NLS), which is highly conserved between many nuclear receptors and contains two clusters of basic amino acids[13]. The NLS is recognized by the transport adaptor proteins importin-α and importin-β,

Key points

- Persistent androgen receptor (AR) signalling is fundamental for the development of treatment resistance in prostate cancer and for its progression to lethal castration-resistant prostate cancer (CRPC).
- Truncated AR splice variants that lack the AR ligand-binding domain (LBD) and remain constitutively active in the absence of androgen ligands are a biologically credible mechanism of treatment resistance in CRPC. These splice variants occur through the process of alternative splicing, which is regulated by the spliceosome.
- AR splice variant 7 (AR-V7) is the most widely studied AR splice variant and has been associated with both an increased risk of biochemical relapse and inferior overall survival outcomes.
- Efforts to directly target AR splice variants have proved challenging owing to the loss of the LBD (the target of the currently approved anti-androgen therapies) and the intrinsically disordered nature of the AR amino-terminal domain.
- Targeting spliceosomal activity to inhibit the generation of AR splice variants represents an attractive alternative therapeutic strategy; however, the complexity of the spliceosome, and a lack of understanding of its biology, has resulted in a paucity of such agents being developed.
- Further research is urgently required to improve understanding of the splicing abnormalities that contribute to the progression of CRPC, as well as the consequences of inhibiting these factors, before the true utility of these therapies can be realized.

which regulate the shuttling of the AR homodimers into the cell nucleus. The NLS is also recognized and bound by dynein, a motor protein that interacts with cellular microtubules to enhance nuclear translocation of the AR via a cytoskeletal transport network[14]. Once in the nucleus, the AR complex binds with DNA at specific sites known as androgen-response elements through its DBD. In this way, the AR can upregulate or downregulate the transcription and activation of various genes, many of which are involved in the regulation of crucial cellular functions such as growth and proliferation. As a consequence of this ability to regulate cellular survival, persistent activation of the AR has been shown to be a pivotal driving force in the development and progression of prostate cancer. Furthermore, inhibition of AR signalling with ADT (as achieved, for example, with luteinizing-hormone-releasing hormone (LHRH) agonists such as goserelin and leuprorelin acetate) remains the standard of care in the treatment of prostate cancer to this day[15,16]. However, while nearly all patients initially respond to ADT, the duration of response varies from months to years, and, ultimately, all patients will eventually acquire resistance to ADT and progress to CRPC, which is often lethal[17].

CRPC was long thought of as being an androgen-independent entity; however, over the past decade, in particular, the continuing importance of the AR in the progression of advanced-stage prostate cancer has become better appreciated, culminating in the introduction of abiraterone and enzalutamide into routine clinical practice, which have both provided additional improvements in survival benefit for patients with CRPC[18,19]. Despite the success of these second-generation AR-targeted therapies, treatment resistance continues to be a major challenge, leaving patients with only a limited number of meaningful treatment options following disease progression. These options include taxane chemotherapy, which is limited by the risk of severe adverse events such as cytopenia and neurotoxicities[20,21], and targeted therapies that are only effective in a subgroup of patients, such as poly(ADP-ribose) polymerase (PARP) inhibitors or carboplatin in homologous recombination-deficient prostate cancers (as yet unapproved) and anti-programmed cell death protein 1 (PD-1) antibodies for patients with mismatch repair defective or microsatellite unstable disease[22]. In addition, with clinical evidence emerging that use of abiraterone at diagnosis of castration-sensitive prostate cancer improves outcomes[23,24], it is foreseeable that, in the future, these agents will be used much earlier in the disease trajectory. Such a change could result in resistance to anti-androgens occurring at the time of progression from first-line therapy rather than as a later event, creating the possibility of new clinical dilemmas.

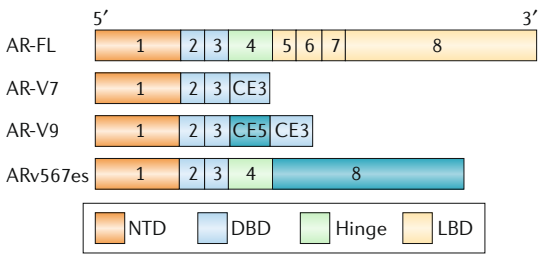


Fig. 1 | **AR splice variants.** A schematic diagram depicting the full-length androgen receptor (AR-FL) alongside a selection of its truncated protein isoforms, the androgen receptor (AR) splice variants (AR-Vs) AR-V7, AR-V9, and ARv567es. These proteins share identical amino-terminal domains (NTDs) and DNA-binding domains (DBDs) but have unique carboxy-terminal extensions. AR-V7 and AR-V9 have a common 3′-terminal cryptic exon (CE), while ARv567es has a complete hinge region and nuclear localization signal, similar to that of the full-length protein, but lacks a ligand-binding domain (LBD).

The many faces of the AR

The full-length AR (AR-FL) has been well described in the literature[11,25]; however, over the past 10 years, a variety of alternate versions of the AR have been shown to exist. Evidence for these variants first emerged through the work of Dehm and colleagues[26], who identified two truncated AR isoforms lacking the carboxy-terminal domain in 22Rv1 prostate cancer cell lines, which were encoded by mRNAs with a novel exon 2b located at their 3′ end. These AR isoforms were found to remain constitutively active and maintained the proliferation of 22Rv1 cells in the absence of exposure to androgens[26]. Subsequently, with the development of more-advanced sequencing techniques, numerous other truncated forms of the AR have been reported[25,27,28].

Expression of AR protein results from the transcription and translation of the *AR* gene. However, owing to the discontinuous nature of eukaryotic genes, typically featuring regions of non-coding DNA (introns) interspersed between stretches of coding DNA (exons), the resultant precursor mRNA (pre-mRNA)

transcript typically contains both sequences when initially transcribed. Therefore, before translation, nascent pre-mRNA transcripts are edited through a process known as splicing, which removes introns and produces mature mRNAs that can be translated into functional proteins.

RNA splicing is performed by complex cellular machinery referred to generally as the spliceosome. The importance of this complex gained increased recognition with the discovery that, through the alternative inclusion and exclusion of exons and introns termed alternative splicing, a single gene can encode multiple different proteins[29]. Alternative splicing enables eukaryotic cells to transform a genome that contains only 20,000 genes into a substantially larger and more diverse proteome of approximately 95,000 unique proteins[30]. As such, awareness of the role of the spliceosome in numerous diseases, including in cancer, is growing. However, our understanding of its underlying biological mechanisms remains incomplete, making it an important area of clinical research.

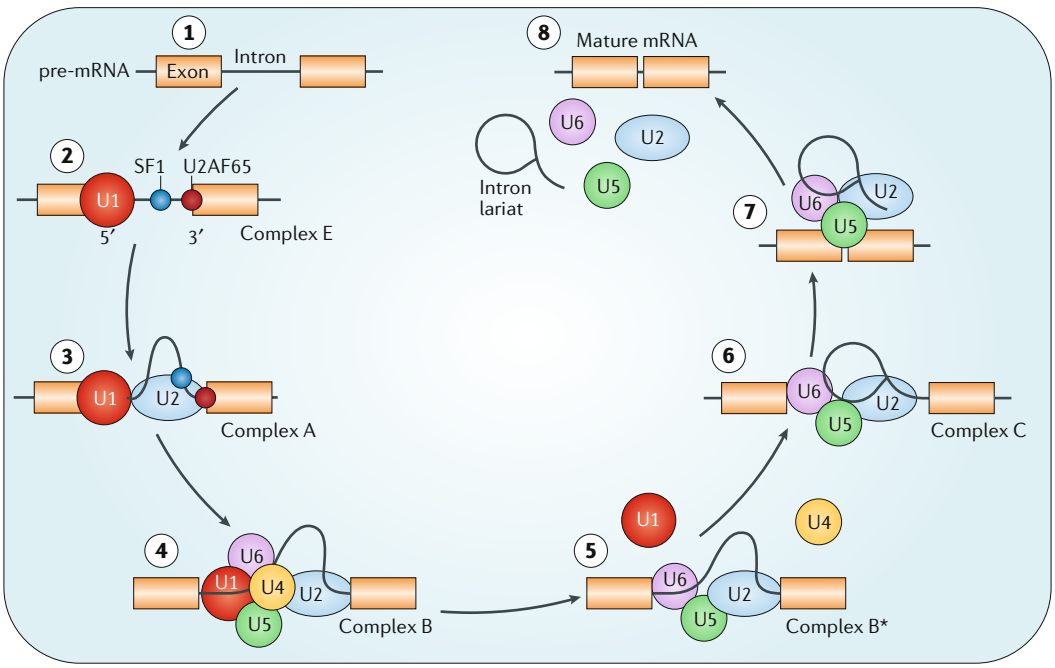
The spliceosome

The spliceosome is a dynamic cellular machine composed of small nuclear ribonucleoproteins (snRNPs) and their associated protein cofactors (BOX 1). Importantly, all major steps in spliceosome formation are reversible, suggesting that a proofreading mechanism is in operation during splicing, with data from in vitro studies showing that partially assembled spliceosomes are able to disassemble and reassemble at alternative splicing sites[31]. This effect is particularly apparent during the early stages of spliceosome assembly because commitment to splicing increases as spliceosome assembly progresses[31].

Spliceosome regulation. The core constituents of the spliceosome complex, such as the snRNPs U1 and U2, are able to define exon–intron boundaries; however, splicing sequences within nascent mRNA precursors often contain too little information to unambiguously define specific splice sites[32]. In addition, human introns often contain sequences that are not canonical splice sites but have a high degree of similarity to authentic

Box 1 | **Spliceosome assembly and alternative splicing**

Splicing occurs in a stepwise manner beginning with coupling of the small nuclear ribonucleoprotein (snRNP) U1 with the intron 5′ splice site (step 1). This reaction is ATP-independent and results in a weak interaction, which is then stabilized by the binding of splicing factor 1 (SF1) and splicing factor U2AF 65 kDa subunit (U2AF65) to the 3′ splice site (step 2). Together these structures form the early complex (complex E) and trigger the ATP-dependent recruitment of the snRNP U2 to the intron branch point, thus forming the pre-spliceosome (complex A) and defining the end of one exon and the beginning of the next, a process referred to as exon definition (step 3). This also brings the 5′ splice site, branch point, and 3′ splice site, known as the intron definition complex, into closer proximity (step 4). Next, the pre-assembled U4–U6–U5 tri-snRNP is recruited to the pre-spliceosome to form complex B, which then undergoes a series of compositional and conformational changes including the release of U1 and U4, to form the catalytically active complex B (complex B*), which hosts the first catalytic step of splicing (step 5). The resultant complex, complex C, which contains the free end of the first exon and the remaining intron–exon lariat intermediate (step 6), then undergoes further ATP-dependent rearrangements before performing the second catalytic step of splicing to form the post-spliceosomal complex that contains the mature mRNA product, as well as the entire looped intron lariat (step 7). Finally, the U2, U5, and U6 snRNPs are released and recycled for subsequent splicing reactions (step 8).



pre-mRNA, precursor mRNA.

splice sites. As such, additional *cis* and *trans* regulatory factors are required to accurately define exon–intron junctions and maintain splicing fidelity. *Cis*-regulatory RNA elements are nucleotide sequences within pre-mRNA transcripts that can modify the splicing of the same pre-mRNA transcript in which they are located. As such, these sequences are referred to as splicing regulatory elements (SREs) and contribute to splicing in a context-dependent manner, whereby they can serve as either splicing enhancers or silencers depending on their position within the pre-mRNA transcript[33]. SREs exert their effects by recruiting *trans*-acting splicing factors, auxiliary proteins of the spliceosome such as serine-rich and/or arginine-rich (SR) proteins, and heterogeneous nuclear ribonuclear proteins (hnRNPs). These proteins interact with core components of the spliceosome, often the snRNPs U1 and U2, to either activate or suppress the splicing reaction during the early steps of spliceosome assembly. In addition, as with SREs, *trans*-acting splicing factors modify splicing in a context-dependent manner. For example, SR proteins can promote splicing when bound to SREs located within exons but can also inhibit splicing when associated with SREs located in introns[34].

Other factors contributing to the regulation of splicing include tissue-restricted splicing factors (such as members of the neuro-oncological ventral antigen (NOVA) protein family[35] and RNA-binding protein fox-1 homologue 1)[36], the rate of transcription elongation[37], tissue hypoxia[38,39], heat stress[40,41], genotoxic stress[42], chromatin structure, and nucleosome positioning[43]. Knowledge of this complexity has been furthered by the findings that not only can most splicing factors recognize multiple SREs, but each SRE is also often bound by multiple different factors. This observation suggests the presence of a complex network of protein–RNA interactions working alongside the spliceosome and regulating splicing to not only protect the proteome from error but also provide a level of cellular plasticity[44,45].

Alternative splicing. Splice site selection is reported to depend on the strength of a splice site. Sites that bear a close resemblance to recognizable consensus sequences, such as CAG/GUAAGU at the 5′ splice site and NYAG/G at the 3′ splice site, and that form stable interactions with core constituents of the spliceosome, such as snRNP U1, are referred to as strong splice sites. Strong splice sites are more efficiently recognized by the spliceosome and are selected for over weaker sites, with splicing consequently occurring more consistently at strong sites. However, the spliceosome regulatory network can modify the strength of these competing sites by silencing stronger splice sites and enhancing weaker ones, predominantly through *trans*-acting splicing factors. In this way, the interplay between these competing spliceosomal homing signals within a nascent pre-mRNA can lead to the preferential selection of non-canonical splice sites and result in alternative splicing[46].

The findings of high-throughput RNA sequencing studies have shown that alternative splicing is a routine biological process, with 90–95% of human multi-exon gene transcripts demonstrating evidence of alternative splicing events, thereby generating a more diverse proteome[47]. Patterns of alternative splicing range from alternative 3′ or 5′ splice site recognition to retained introns and mutually exclusive exons; however, cassette exon skipping is the most commonly observed event in humans[48] (FIG. 2).

Despite the abundance of alternative splicing events, the functional roles of the many isoforms generated by alternative splicing remain largely uncertain. While this has led some authors to speculate that alternative splicing is a fundamental factor in the development of biodiversity and thus evolution[49], others have implicated alternative splicing in the pathogenesis of a number of diseases, including cancer[48,50,51].

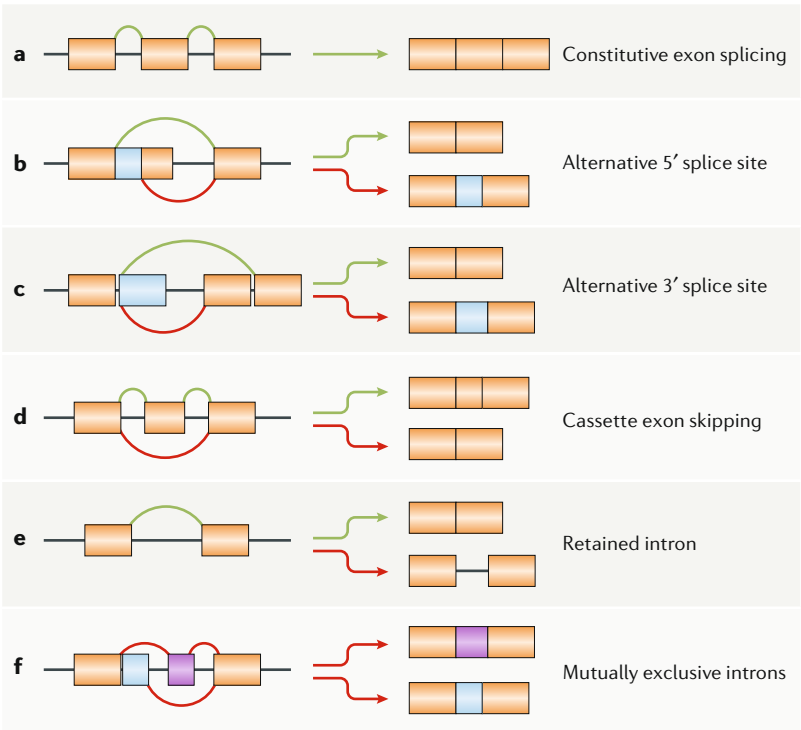


Fig. 2 | **Summary of constitutive and alternative splicing events. a** | Graphic depiction of constitutive splicing where introns are removed and sequential exons are ligated to produce mature mRNA. **b**,**c** | Alternative splicing, in which changes in 5' and 3' splice site selection can result in the generation of alternatively spliced protein variants such as androgen receptor (AR) splice variant 7 (AR-V7), which possesses a 3'-terminal cryptic exon. **d** | Exon skipping, in which a cassette exon is spliced out of the nascent mRNA transcript altogether, along with its adjacent introns. **e** | Intron retention; an intron that does not form part of the canonical mRNA transcript is not removed and remains within the mature mRNA. **f** | Splicing, in which complex events that give rise to mutually exclusive alternative splicing events, in which only one of a set of two or more exons in a gene is included in the final transcript can also occur. Orange exons indicate those that are part of the canonical mRNA sequence; blue or purple exons indicate alternative sequences that might or might not be included in the mature mRNA. Black lines indicate introns, green lines indicate constitutive splicing patterns, and red lines indicate alternative splicing events.

The spliceosome in prostate cancer

The role of the spliceosome in prostate cancer is currently a major area of clinical research. Alternatively spliced variants of the AR that remain constitutively active in the absence of circulating androgens are currently the best-described splicing aberrations in patients with prostate cancer. However, the spliceosome has been

implicated in the pathogenesis of prostate cancer in a number of other ways (FIG. 3).

Mutations of spliceosome regulators. Recurrent somatic mutations in genes encoding splicing factors have been identified in a variety of different cancers such as uveal melanoma[52], pancreatic ductal adenocarcinoma[53], lung adenocarcinoma[54], breast cancer[55], and prostate cancer[56]. Despite this diversity in terms of tumour origin, most reported mutations in splicing factors occur in one of four genes, namely, those encoding splicing factor 3B subunit 1 (SF3B1), SR splicing factor 2 (SRSF2), splicing factor U2AF 35 kDa subunit (U2AF1), and CCCH-type zinc-finger RNA-binding motif and serine/arginine-rich protein 2 (ZRSR2)[57]. Of these, mutations in *SF3B1* are the most common and have been observed in patients with both haematological and solid malignancies, reportedly occurring in 15% of chronic lymphocytic leukaemias, 15–20% of uveal melanomas, and 4% of pancreatic cancers[57]. The product of this gene, SF3B1, is a core spliceosomal protein that binds upstream of the pre-mRNA branch site and is thought to be required for the recognition of most 3′ splice sites[29]. As such, *SF3B1* mutations have been associated with improved recognition of cryptic 3′ splice sites and the formation of alternatively spliced protein isoforms[58]. However, while alternatively spliced versions of the AR spliced at cryptic exon 3 have been implicated in the development of treatment resistance and disease progression in patients with CRPC, with the reported incidence of *SF3B1* mutations in patients with prostate cancer being in the region of 1%[56,59], the contribution of *SF3B1* mutations to treatment resistance through this mechanism could prove to be limited.

Alterations in spliceosome regulator activity. Changes in the activity of splicing factors have been reported to have direct implications for tumorigenesis and disease progression in patients with prostate cancer. For example, KH domain-containing, RNA-binding, signal transduction-associated protein 1 (KHDRBS1) is a nuclear splicing factor involved in the regulation of G1–S-specific cyclin D1 (CCND1) splicing[60], which is a central component of cell cycle control. However, KHDRBS1 is activated through ERK-mediated phosphorylation[61], which is dysregulated in approximately a third of human cancers[62], including prostate cancer. As such, KHDRBS1 has been found to be frequently upregulated in prostate cancer and consequently has been associated with the increased expression of the truncated CCND1b isoform, rather than the canonical CCND1a protein, which promotes the proliferation and survival of prostate cancer cells in vitro[63].

Splicing factor upregulation has also been linked with epithelial–mesenchymal transition in the prostate and thus disease progression in CRPC. Following androgen deprivation, upregulation of the splicing factor serine/arginine repetitive matrix protein 4 (SRRM4) has been shown to cause the alternative splicing of RE1-silencing transcription factor (REST)[64], a neuronal master regulator that, in the absence of alternative splicing, prevents the expression of neuronal genes such as synaptophysin in non-neuronal cells[65]. Consequently, SRRM4 upregulation results in the expression of a truncated form of REST that lacks its canonical transcriptional repressor domain and gives rise to a more AR-independent, neuroendocrine phenotype, which confers a poorer prognosis[66].

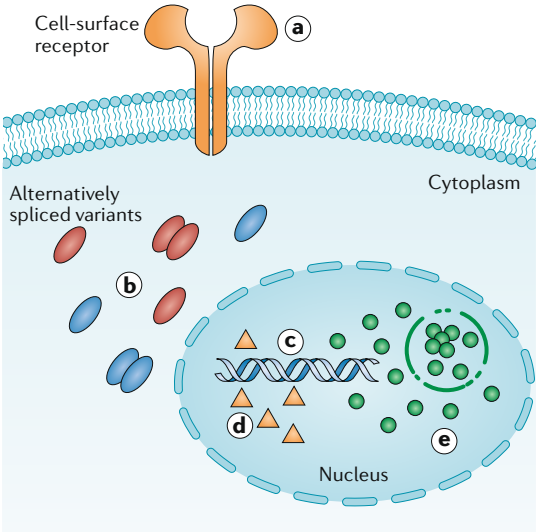


Fig. 3 | **Mechanisms through which the spliceosome contributes to tumorigenesis and disease progression in prostate cancer. a** | Alternative splicing of cell-surface receptors such as the FGFR have been reported to cause aberrant activation of key survival pathways in the absence of circulating androgens. **b** | Constitutively active splice variants of intracellular transcription factors such as the androgen receptor (AR; red ovals) have been linked with disease progression in patients with castration-resistant prostate cancer and are correlated with inferior overall survival outcomes. **c** | Gain-of-function mutations in *cis*-regulatory elements have been proposed to increase AR transcription in the absence of circulating androgens. **d** | Alternative splicing of key cellular regulatory proteins (orange triangles) such as G1–S-specific cyclin D1 (CCND1), a central component of cell cycle control, can promote the proliferation and survival of prostate cancer cells. **e** | Upregulation, as well as alternative splicing, of nuclear splicing factors (green circles) such as Kruppel-like factor 6 (KLF6) is able to increase cellular proliferation, colony formation, and invasion, as well as epithelial–mesenchymal transition, which contributes to AR-independent treatment resistance.

As well as directly contributing to disease progression, the upregulation of canonical splicing factors has also been shown to be pivotal in the activation of other drivers of prostate cancer, such as oncogenes. The proto-oncogene MYC is reported to be overexpressed in up to 90% of all primary human prostate cancer lesions[67]. MYC hyperactivation amplifies pre-mRNA production, leading to stress on the spliceosome[68]. As such, these cancers are as equally dependent on the availability of splicing factors to sustain proliferation and survival as they are on MYC[68], as demonstrated by the upregulation of a number of splicing factors, such as serine/arginine-rich splicing factor 1 (SRSF1), hnRNP A1 and hnRNPs A2/B1 in MYC-overexpressing tumours, and the disruption of many vital cellular processes, which occurs when they are inhibited[68–71].

Alternative splicing of cellular signal transduction pathways. The spliceosome and its associated proteins are involved in the routine operation of a wide range of cellular processes including DNA repair, transcription, and nonsense-mediated RNA decay. For example, the findings of chromatin immunoprecipitation (ChIP) studies demonstrate that SF3B1 and U2AF1 have been shown to interact with breast cancer type 1 susceptibility protein (*BRCA1*) following DNA damage[72].

KLF6 (which encodes Kruppel-like factor 6) is a key tumour suppressor gene that is often mutated in prostate cancer. This gene encodes a member of the Kruppel-like family of transcription factors, which bind with DNA and regulate growth-related signal transduction pathways, cellular proliferation, apoptosis, and angiogenesis[73]. Wild-type KLF6 has inhibitory effects on cell growth, although a common germline single-nucleotide polymorphism in *KLF6* (IVS1−27 G > A/IVSΔA) results in the production of an alternatively spliced isoform, KLF6 splice variant 1 (KLF6–SV1), which increases the level of cellular proliferation, colony formation, and invasion. Furthermore, upregulation of KLF6-SV1 in prostate cancer is associated with an inferior prognosis[74,75]. To our knowledge, attempts to target this splice variant have not been made yet.

As well as affecting the function of several important protein signal transducers, the alternative splicing of cell-surface receptors, leading to aberrant activation of key survival pathways, is an equally important aspect of the contribution of the spliceosome to prostate cancer progression. For example, FGFR2 is a tyrosine kinase receptor, which, when activated by FGF, is involved in the regulation of numerous key cellular processes such as proliferation and differentiation that contribute to cell survival[76]. Under nonmalignant physiological conditions, FGFR2 exists as a number of alternatively spliced isoforms, which tend to be cell type-specific, with isoform IIIb predominantly expressed in epithelial cells and isoform IIIc predominantly expressed in mesenchymal cells. However, in prostate cancer, this distribution has been found to change, with isoform IIIc becoming more prevalent[77]. This increase in isoform IIIc expression favours the binding of FGF8b[77], which is the major FGF isoform expressed in prostate cancer and is thought to have an important role in disease progression, as evidenced by the association of this isoform with higher tumour Gleason grade and clinical stage[78].

In summary, splicing influences prostate cancer carcinogenesis in a multitude of ways, and the breadth of these alterations suggests that endocrine therapy resistance is a multifactorial process. However, the most clinically relevant role of the spliceosome in the progression of prostate cancer is currently considered to be the generation of alternatively spliced AR isoforms.

AR splice variants

To date, a number of AR splice variants (AR-Vs) have been identified and examined in metastases from patients with CRPC[27,79,80] (FIG. 1); however, of these, the role of AR splice variant 7 (AR-V7) is the most widely studied and has been associated with both an increased risk of biochemical relapse[81] and inferior overall survival outcomes[79,82–84]. In 2017, *AR-V9* was shown not only to be co-expressed with *AR-V7* but also to share a common 3′ terminal cryptic exon[85]. Furthermore, AR-V9 might also lead to the ligand-independent growth of prostate cancer cells; high levels of *AR-V9* mRNA are reported to be predictive of primary resistance to abiraterone in cellular models and in a small cohort of patients[85]; however, the clinical significance of this observation remains uncertain.

AR-V7 is a truncated isoform of the canonical AR-FL protein that lacks the LBD but retains both the DBD, which mediates AR dimerization and DNA interactions, and the NTD, which is responsible for the majority of AR transcriptional activity[86]. Crucially, this confirmatory change has been shown to maintain AR-V7 in a constitutively active state in the absence of a ligand, resulting in persistent AR activation and survival signalling in tumour cells[5]. Furthermore, this structural difference might also enable AR-V7 to induce a distinctly different set of transcriptional programmes compared with those induced by AR-FL activation. For example, expression of AR-V7 but not AR-FL is positively correlated with the expression of *UBE2C*, which encodes ubiquitin-conjugating enzyme E2C, a protein required for the degradation of mitotic cyclins and for cell cycle progression in prostate cancer cells and in CRPC xenografts[87]. This observation suggests a shift towards AR-V-mediated signalling following anti-androgen therapy in a subset of patients with CRPC, although attempts to disentangle the functional role of AR-V7 from that of AR-FL have been challenging, and this area of investigation remains an active one. Further evidence is required before firm conclusions can be drawn on this possibility.

AR-V7 is to date considered the most commonly expressed AR-V[27,86], and the prevalence of this splice variant increases substantially as patients progress to CRPC[28,88,89]. This increased expression can, in part, be explained as a consequence of treatment with ADT. AR-V7 expression is intimately linked with *AR* transcription[81], which is increased by approximately tenfold in response to ADT[86], and, as such, AR-V7 expression is consequently also increased. In addition, as activation of AR signalling decreases transcription of AR-V7, inhibition of AR signalling with ADT results in the loss of this negative feedback and leads to further upregulation of AR-V7 (REFS[5,86]). Ultimately, however, the processes determining AR-V7 expression, as opposed to those determining expression of the canonical AR-FL, remain unclear, although an increasing appreciation of the importance of the spliceosome in this process is beginning to emerge.

AR-V7 and the spliceosome. The AR-V7 protein arises from alternative splicing of *AR* mRNA at cryptic exon 3 as opposed to the 3′ splice site of the canonical AR-FL (FIG. 1). *AR* gene copy number gain is considered an important determinant of *AR-V7* mRNA levels in patients with CRPC metastases[90], although this observation alone does not explain why a proportion of encoded *AR* mRNAs become alternatively spliced. For example, in LNCap95 cells, which are not reported to possess this *AR* copy number gain, *AR-V7* RNA is still expressed at

levels comparable to those of VCaP cells in which AR expression is amplified[81], whereas the parent cell line, LNCaP, does not express AR-V7. Therefore, rather than alternative splicing of *AR* mRNA occurring through random splicing error as a consequence of increased substrate concentration, these variations instead suggest the existence of a regulatory mechanism that is responsible for splice site selection.

In preclinical models of prostate cancer, Liu and colleagues reported that androgen deprivation leads to increased recruitment of the spliceosome to the *AR* transcript, thus facilitating both conventional and alternative splicing[81]. Furthermore, treatment with enzalutamide specifically enhances the recruitment of a number of splicing factors to the P2 region of the *AR* mRNA, which contains the 3′ splice site of *AR-V7*. This research group further demonstrated that the splicing proteins splicing factor U2AF 65 kDa subunit (U2AF65) and SRSF1 acted as 'pioneer' factors, directing the recruitment of the spliceosome to SREs located adjacent to the 3′ splice site of *AR-V7*, thus increasing the expression of *AR-V7* mRNA[81]. Interestingly, while knockdown of these splicing factors resulted in a reduction in the levels of *AR-V7* mRNA in both VCaP and LnCap95 cell lines, levels of *AR-FL* mRNA remained unaffected[81], suggesting that these splicing factors play an important role in AR-V7 splicing. Polypyrimidine tract-binding protein 1 (PTB) has also been proposed as a regulator of AR-V7 splicing; however, the evidence for this is currently less conclusive than for U2AF65. Work by Nadiminty et al[91]. has shown that overexpression of PTB results in AR-V7 upregulation, while downregulation of this protein both reduces AR-V7 expression and re-sensitizes CRPC cell lines to enzalutamide. However, PTB knockdown has also been shown to reduce the level of AR-FL expression[81], suggesting that PTB is a general regulator of AR mRNA splicing rather than a specific regulator of AR-V7.

Importantly, and in keeping with the concept of a proofreading process within the spliceosomal network, AR-V7 splicing seems to be both a dynamic and a plastic process. For example, the re-introduction of androgens to androgen-deprived cell lines has been shown to repress *AR-V7* RNA levels, and this effect occurs within 24 hours of re-exposure in VCaP cells. Similarly, in primary cultures from enzalutamide-resistant VCaP xenograft models, both *AR* and *AR-V7* mRNA levels decreased significantly upon exposure to DHT[81]. As an interesting aside, the rapidity of this plasticity might contribute to the encouraging levels of efficacy demonstrated using bipolar androgen therapy, in which patients receive monthly doses of high-dose testosterone while remaining on ADT, as demonstrated in a phase II clinical trial with results published in 2017. In this trial, 52% of patients with metastatic CRPC and previous disease progression on enzalutamide had a 50% reduction in serum prostate-specific antigen (PSA) level on enzalutamide re-challenge following bipolar androgen therapy[92]. This observation suggests that re-sensitization of treatment-resistant prostate cancer to enzalutamide through manipulation of AR-FL and AR-V expression by modulating an individual's exposure to testosterone is feasible. However, definitive conclusions regarding this possibility are difficult to elucidate from this cohort alone given that patient's AR-V7 status in this study was determined through analysis of circulating tumour cells (CTCs) rather than tissue-based assessments. More than half of the patients included in this study were found to lack detectable CTCs, and, therefore, a large proportion of patients in this cohort could not be assessed for AR-V7 expression, and so a number of patients expressing AR-V7 could have been omitted from the analysis. Furthermore, preclinical evidence supporting the efficacy of this possible treatment approach remains inconclusive[93].

Alternative splicing and resistance

Over the past 5–10 years, appreciation of the role of alternative splicing in the development of resistance to anticancer therapies has greatly increased. For example, alternative splicing of survivin, a member of the inhibitor of apoptosis protein family, has been reported to confer resistance to taxanes in preclinical models of ovarian cancer[94], while the alternative splicing of B lymphocyte antigen CD19 might promote resistance to immunotherapy involving adoptive T cells expressing anti-CD19 chimeric antigen receptors in preclinical models of B cell acute lymphoblastic leukaemia[95].

Similarly, even though the development and improvements in genome sequencing have heralded the arrival of various new targeted anticancer therapies, evidence is emerging that patients receiving these agents are similarly vulnerable to the development of resistance as a consequence of alternative splicing. For example, a subset of *BRAF*-mutant melanomas have been reported to acquire resistance to vemurafenib through the expression of a variant $BRAF^{V600E}$ isoform, $p61BRAF^{V600E}$, that lacks exons 4–8, a region that encompasses the RAS-binding domain[96]. Furthermore, and perhaps more pertinently with regards to prostate cancer, alternative splicing has been suggested to contribute to acquired resistance to PARP inhibition[97].

The PARP inhibitor olaparib has a therapeutic effect in cancers harbouring DNA repair defects by inhibiting PARP, a protein that is important for repairing DNA single-strand breaks, resulting in synthetic lethality. Inhibiting the repair of single-strand breaks in this way results in the generation of double-strand breaks during cell division, leading to the death of cells harbouring loss-of-function mutations in *BRCA1* and/or *BRCA2*. Olaparib has been shown to improve overall survival in patients with DNA repair-deficient metastatic prostate cancer, with a response rate of 88% reported in biomarker-positive patients (defined as those with homozygous deletions of *BRCA1* and/or *BRCA2*, *ATM*, Fanconi anaemia-related genes, or *CHEK2* (REF.[22])), thus marking a major step forward in the management of this patient group. PARP inhibition has also demonstrated efficacy in patients with other forms of cancer such as breast[98] and ovarian[99] cancers; however, evidence is emerging from these cancer types suggesting that alternative splicing contributes to resistance to olaparib. Wang et al. report that a proportion of patients possessing PARP-sensitizing *BRCA1* germline

mutations either do not respond or eventually develop resistance to PARP inhibition as a result of frameshift mutations in exon 11, leading to nonsense-mediated RNA decay of full-length *BRCA1* mRNA transcripts and increased expression of an alternatively spliced BRCA1 isoform, BRCA1-Δ11q. The authors suggest that *BRCA1*-deficient cancer cells remove deleterious germline *BRCA1* mutations by producing alternatively spliced protein isoforms that retain residual DNA repair activity and contribute to treatment resistance[97]. Notably, *BRCA2* mutations are much more common than *BRCA1* mutations in patients with prostate cancer[100], although whether or not mechanisms of resistance similar to those seen in other cancers will emerge in patients with prostate cancer will be determined by clinical trials involving novel targeted therapies such as PARP inhibitors. However, these examples do serve to highlight the clinical implications of alternative splicing and add weight to the rationale of harnessing the spliceosome as a novel therapeutic target. Overall, and notwithstanding a growing body of literature in this area, AR-Vs are currently the most widely understood and clinically important mechanism through which alternative splicing is thought to contribute to treatment resistance in patients with CRPC.

AR splice variants and treatment resistance. The emergence of AR splice variants is proposed as a biologically credible mechanism of treatment resistance through the restoration of AR signalling. Data from preclinical studies have shown that inhibition of AR-V7 can re-sensitize enzalutamide-resistant prostate cancer cell lines to anti-androgen treatment[101–103]. AR-Vs have also been implicated in treatment failure in patients receiving combined ADT and radiotherapy, with aberrant AR-V signalling bolstering the DNA damage response and increasing the clonogenic survival of prostate cancer cells following irradiation[104].

However, evidence supporting the role of AR-Vs in treatment resistance currently remains inconclusive. Despite the advantageous characteristics conferred by their structural properties, which hypothetically enable AR-Vs to remain constitutively active in the absence of androgens, only a minority of AR splice variant isoforms have demonstrated this ability in AR transactivation reporter assays[4], raising questions regarding the clinical significance of the majority of AR-Vs. A proposed explanation for this observation is that most AR-Vs are truncated after exon 3 and thus lack a complete NLS and therefore are expected to be predominantly sequestered within the cytoplasm[105]. AR-V7 is, however, an exception to this rule and despite having an incomplete NLS has been shown to reside in the nucleus for prolonged periods of time[5], where it has also been shown to be transcriptionally active[87].

An alternative theory exists that AR-Vs are a consequence of the physiological response to androgen deprivation. Support for this hypothesis is provided by the rapidity of increased AR-V7 expression following ADT. In xenograft models, expression of both AR-FL and AR-V7 has been shown to increase just 2 days following castration and to reach peak levels at 2 weeks, with *AR-V7* mRNA being only a fraction of total AR-FL levels[105]. In addition, the re-introduction of androgens in these models restores the expression of both variants to baseline levels in only 8 days[105]. Thus, if AR-Vs were to cause treatment resistance, one would expect this resistance to occur much sooner than is typically seen in clinical scenarios[17,18]. In support of this argument, while data from a number of clinical studies corroborate reports that AR-V7 expression confers a worse prognosis and contributes to treatment resistance[79,82–84], some research groups have failed to validate this relationship. For example, overexpression of AR-V7 in LNCaP cell lines, which do not innately express AR-V7, did not confer resistance to enzalutamide both in vitro and in in vivo mouse xenograft models of CRPC[105]. Furthermore, in a retrospective analysis of patient records, authors identified 6 out of 21 patients with detectable AR-V7 who derived benefit from treatment with abiraterone or enzalutamide, suggesting that a subgroup of AR-V7-positive patients obtains benefit from novel anti-androgen therapies despite detection of AR-V7 in their CTCs[106]. Similarly, in a prospective study, investigators found no significant difference in either serum PSA response or median serum PSA-defined progression-free survival durations between patients with AR-V7-positive, AR-V9-positive or AR-V7-negative disease treated with abiraterone or enzalutamide, as defined using CTCs. The investigators concluded that AR-V expression did not predict outcomes in patients with metastatic CRPC receiving either agent[107].

Recognizing that nearly all studies with results currently reported rely on the determination of AR-V7 status using CTCs is an important point. Therefore, both positive and negative associations between AR-V7 expression and clinical outcomes of patients with CRPC have to be interpreted with careful consideration of the validity of the assays that were used, with multiple lines of evidence clearly indicating the limitations of these binary assays[79,86–93,106,107]. First, the ability of each assay to determine AR-V7 status (either mRNA or protein) only in patients with detectable CTCs needs to be considered; patients with detectable CTCs who lack AR-V7 expression are not the same as those with undetectable CTCs, in whom AR-V7 status cannot be determined, although patients with undetectable CTCs have been shown to have the best prognosis, relative to those with detectable CTCs and either the presence or absence of AR-V7, after treatment with abiraterone or enzalutamide[108]. Second, although assays designed to measure AR-V7 protein expression overcome concerns regarding the stability of *AR-V7* mRNA, such assays remain susceptible to off-target liabilities, specifically false positive results, as associated with use of the Abcam–Epitomics antibody previously described in the EPIC AR-V7 assay[109]. Moreover, consideration needs to be given to the possibility that despite detectable AR-V7 expression, large numbers of AR-V7-negative cells might also be present, which means that these patients could benefit from abiraterone or enzalutamide. Finally, these molecular association studies will need to be supported by further understanding of AR-V7 biology and the development of novel therapies that

abrogate AR-V7 signalling and induce robust responses in patients with CRPC. Only then will the biological and clinical significance of AR-V7 be truly confirmed; this remains a priority for the field and an unmet urgent clinical need.

Overcoming treatment resistance

Targeting the core spliceosome complex. Several bacterial fermentation products with potent anticancer activity, owing to an ability to modulate the core spliceosome complex, have been identified using large-scale drug screens. The molecules can be broadly categorized into three classes, namely, pladienolides, herboxidienes, and spliceostatins (TABLE 1). These compounds are structurally distinct, although they also share a common mechanism of action whereby they bind with and inhibit SF3B1 (REF.[110]). Under nonmalignant conditions, SF3B1 interacts with U2AF65 to recruit the snRNP U2 to the 3′ splice site of the intron. However, by binding to SF3B1, these compounds interfere with the early stages of spliceosome assembly and therefore destabilize the interactions between U2 and its pre-mRNA target, thus modifying splice site selection[111]. This perturbation of U2 also causes an accumulation on unspliced pre-mRNA in the nucleus, of which a small proportion has been shown to 'leak out' into the cytoplasm and undergo translation, generating aberrant protein products, which themselves can be cytotoxic[112,113]. In addition, several of these compounds have also been shown to decrease the expression of VEGF, thus inhibiting angiogenesis in chick chorioallantoic membrane assays[114].

The potential clinical utility of bacterial fermentation products has been adequately demonstrated in preclinical studies, as observed, for example, in the dose-dependent inhibition of growth seen in experiments involving prostate cancer xenografts following treatment with pladienolide B[115]. However, the findings of early phase clinical trials have been more mixed. In two phase I, open-label, single-arm, dose-escalation studies, investigators assessed the safety and efficacy of pladienolide E7107 in patients with locally advanced or metastatic solid tumours. Data from both trials showed that E7107 was generally well tolerated and produced both dose-dependent and reversible inhibition of pre-mRNA processing in target genes in vivo[116], although both trials

Table 1 | **Small molecules reported to target the process of splicing**

Agents	Stage of development	Effects and/or mechanism of action	Refs
Targeting the core spliceosome complex			
Pladienolides A–G	Preclinical	Binds with and inhibits SF3B1, thus destabilizing the recruitment of snRNP U2; decreases levels of circulating VEGF and thus inhibits tumour angiogenesis; cell cycle arrest at G1 and G2–M; disrupts spliceosome assembly; generates a truncated form of the cell cycle inhibitor cyclin-dependent kinase inhibitor p27 that remains functional; and reduces the number of nuclear speckles	131,132
E7107	Phase I		114–116
Herboxidiene (GEX1A)	Preclinical		133
FR901463, FR901464, and FR901465	Preclinical		134
Meayamycin B	Preclinical		135
Spliceostatin A	Preclinical		112
H3B-8800	Phase I (NCT02841540)	Small-molecule modulator of SF3B1 that displays preferential lethality towards spliceosome-mutant cancer cells owing to retention of short, GC-rich introns	118
Targeting spliceosomal regulatory proteins			
TG003	Preclinical	Competitive inhibitor of CLK1, CLK2, and CLK4 binding with ATP that inhibits CLK enzymatic phosphorylation, activates splicing factors such as SR proteins and leads to dissociation of nuclear speckles	120
SRPIN340	Preclinical	Competitive antagonist of SRPK1 and SRPK2 binding of ATP and a nicotinamide inhibitor, which inhibits SRPK phosphorylation and activation of splicing factors such as SR proteins and modulates splicing of VEGF	136
Cpd-1, Cpd-2, and Cpd-3	Preclinical	Inhibits both CLKs (CLK1 and CLK2) and SRPKs (SPRK1 and SPRK2), which are components of the splicing machinery that are crucial for exon selection, reduces phosphorylation of SR proteins, causes enlargement of nuclear speckles, and causes widespread splicing alterations	137
GSK525762	Phase I (NCT03150056)	Inhibits the BET family proteins BRD2, BRD3, BRD4, and BRDT, downregulates expression of splicing factors, and decreases alternative splicing events in preclinical models	138
ZEN003694	Phase I/II (NCT02711956)		139
OTX105/MK-8628	Phase I (NCT02259114)		122
Other small-molecule inhibitors			
Isoginkgetin	Preclinical	Biflavonoid natural plant product that interferes with the recruitment of the snRNPs U4, U5, and U6 and prevents transition from spliceosomal complex A to complex B	125
NB-506	Preclinical	Inhibits SRFS1 phosphorylation by topoisomerase I, disrupts early spliceosome assembly in vitro, and produces a cytotoxic effect	124

BET, bromodomain and extraterminal domain; BRD, bromodomain-containing protein; BRDT, bromodomain testis-specific protein; CLK, dual specificity protein kinase CLK; SF3B1, splicing factor 3B subunit 1; snRNP, small nuclear ribonucleoprotein; SR, serine-rich and/or arginine-rich; SRPK, serine and arginine protein kinase; SRPIN340, *N*-(2-(piperidin-1-yl)-5-(trifluoromethyl)phenyl); SRSF1, SR splicing factor 1.

were suspended owing to unexpected incidences of bilateral optic neuritis[116,117].

H3B-8800, a small-molecule modulator of SF3B1 (REF.[118]), has also entered a phase I clinical trial (NCT02841540). This trial aims to determine the safety and recommended phase II dose in patients with myelodysplastic syndromes, acute myeloid leukaemia, or chronic myelomonocytic leukaemia, in which recurrent heterozygous mutations of SF3B1 are thought to have a pathological role. If found to be efficacious in subsequent phase II and phase III trials, H3B-8800 could provide proof of principle that targeting the spliceosome is a valid treatment strategy, and this could also open a variety of new therapeutic avenues. However, the toxicity and tolerability of these agents will equally prove to be important factors that will dictate whether or not these agents ever enter routine clinical use.

Targeting spliceosomal regulatory proteins. Rather than targeting the core spliceosome complex, an alternative approach is to modulate splicing by targeting one or more of the regulatory proteins. For example, a variety of compounds have been identified that can inhibit SR protein phosphorylation, and these have been shown in preclinical models to inhibit splicing[119]. TG-003, a benzothiazole, is one such agent and functions as an inhibitor of CLK1, CLK2, and CLK4, all of which are members of the CDC2-like (or LAMMER) family of dual-specificity protein kinases. These kinases are typically involved in the phosphorylation of SR proteins in the nucleus[120], the inhibition of which results in inhibition of splicing and dissociation of spliceosomal nuclear speckles[120].

In the past 5 years, bromodomain and extraterminal domain (BET) inhibition, a promising therapeutic approach that is currently undergoing clinical evaluation in patients with CRPC (NCT03150056 and NCT02711956), has also been shown to affect alternative splicing by modulating spliceosomal regulators[121]. In a study by Asangani et al.[122], the BET inhibitor JQ1 was found to decrease the expression of AR-V7 in preclinical models of CRPC by downregulating the activity of the splicing factors SRSF1 and U2AF65, and in doing so, re-sensitized enzalutamide-resistant prostate cancer cells to AR-targeted therapy. However, as with therapeutic agents targeting the core spliceosomal complex, the long-term success of BET inhibition as a clinically useful therapeutic modality will hinge on the toxicity profile of BET inhibitors, as determined in the ongoing clinical trials.

Other small-molecule inhibitors of the spliceosome. Several other small molecules have also been identified as being capable of modulating the spliceosome, some of which have been reported to have efficacy in preclinical cancer models. However, these studies have generally been limited by their use of cell-free and non-mammalian models[123], and, as such, the therapeutic application of many of these agents is currently considered limited. Despite this lack of clinical implementation thus far, some interesting results have been seen with a number of these agents. For example, NB-506, a glycosylated indolocarbazole derivative that inhibits the capacity of topoisomerase I to phosphorylate SRFS1, has been shown to disrupt early spliceosome assembly and produces a cytotoxic effect in murine P388 leukaemia cells[124]. In addition, preclinical antitumour activity of the biflavonoid natural plant product isoginkgetin has also been demonstrated, which occurs, at least in part, through the ability of this agent to interfere with the recruitment of the snRNPs U4, U5, and U6 and to inhibit splicing by precluding the transition from spliceosomal complex A to complex B[125].

Targeting the spliceosome in oncogene-driven cancers. As described previously, *MYC* overexpression places considerable oncogenic stress on the spliceosome, resulting in cells becoming equally dependent on the spliceosome for survival as they are on MYC. This observation has led to the hypothesis that, in these tumours, inhibition of the spliceosome might have an anticancer effect. In support of this view, spliceosome dysregulation through inhibition of SF3B1 using sudemycin D has been reported to increase survival and limit the formation of metastases in xenograft models of MYC-dependent breast cancer[69]. Ultimately, although intriguing, whether this principle will be applicable to other similarly important genomic aberrations or whether the clinical utility of this approach will be limited to a subset of MYC-dependent cancers remains to be seen.

Targeting alternatively spliced variants. When devising therapeutic strategies to target pathological alternatively spliced variants, in addition to considering those generated through the action of the spliceosome, taking into account protein variants generated through alternative alterations such as genomic fusions or rearrangements, including proteins that are altered in many cancers such as programmed cell death 1 ligand 1 (PD-L1), is equally important[90]. As such, while targeting the spliceosome remains a key consideration in this process, given the multiple routes through which alternatively spliced variants can arise, the concept of directly targeting these protein variants, rather than their mechanism of origin, seems logical.

Efforts to target alternatively spliced proteins remain attractive, but doing so directly with small-molecule inhibitors has to date proved challenging, often owing to the inherent nature of these alternatively spliced variants. For example, because truncated alternatively spliced AR-Vs generally lack an LBD, alternative target sites are required to facilitate their inhibition. However, the disordered nature of the AR NTD renders a consistent target site difficult to ascertain and has to date hindered drug discovery efforts, thus necessitating the development of novel therapeutic strategies. One such proposed approach involves the use of monoclonal antibodies such as GP369, which specifically blocks the IIIb splice variant of FGFR2 (REF.[126]). GP369 showed efficacy as an inhibitor of tumour growth in preclinical studies involving human cancer cell lines and tumour xenografts driven by activated FGFR2 signalling[127]. A phase I trial involving patients with advanced-stage

solid tumours known to express FGFR2 was opened (NCT02368951) on this basis, although the trial was terminated early owing to safety concerns regarding the development of nephrotic syndrome in two participants during dose-escalation, preventing the attainment of a therapeutic dose. Despite this setback, the ability to target alternatively spliced protein isoforms using monoclonal antibodies could yet help to circumvent the difficulties associated with directly inhibiting splice variants, which have hampered drug discovery efforts in this area to date.

Oligonucleotide therapy. Oligonucleotide-based therapies involve the use of engineered oligonucleotides designed to hybridize with RNA sequences that are known to be responsible for specific splicing events in order to prevent their alternative splicing and the production of erroneous protein products with pathological consequences. The potential of these therapeutic agents has so far been best realized in patients with neurodegenerative conditions, including those with Duchenne muscular dystrophy[128] or spinal muscular atrophy[129], in which late-stage clinical trials are underway. However, an important question remains as to whether oligonucleotide therapy is a viable treatment approach in cancer and particularly in cancers with more diverse splicing events. Evidence supporting the use of oligonucleotide therapy in patients with cancer stems from work by Smith et al.[130], who developed a novel RNA splice-switching oligonucleotide designed to induce skipping of exon 11 in *BRCA1*, which is crucial to the DNA damage repair functions of the protein. In doing so the authors successfully rendered wild-type *BRCA1*-expressing cell lines more susceptible to PARP inhibition[130]. This approach provides a fascinating potential therapeutic strategy for targeting cancers with wild-type *BRCA1*, although the challenge in this setting is to maintain BRCA1 function in nonmalignant cells and thus minimize the potentially widespread risks of toxicity.

Conclusions

Splicing events are a plausible mechanism of treatment resistance and disease progression in patients with CRPC and have been proposed as a potential therapeutic target. Drug discovery efforts to date have, however, been challenging; thus, modulation of the spliceosome as a therapeutic tool represents an attractive alternative option, although, as yet, spliceosome inhibitors have not entered clinical practice in patients with prostate cancer, largely owing to the complexity of the spliceosome and a lack of understanding of its biology. Further research is required in order to identify the exact mechanisms underpinning the splicing abnormalities that are thought to contribute to the progression of CRPC, as well as the consequences of inhibiting these factors, before the true utility of these therapies can be realized.

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Competing interests
A.P., A.S., J.C.W., A.N., and J.S.d.B. are employees of The Institute of Cancer Research, a not-for-profit research organization and independent college of The University of London, which has a commercial interest in abiraterone. A.P., A.S., J.C.W., A.N., and J.S.d.-B. have no personal financial interests in abiraterone. G.V.R. has acted as a consultant of Astellas, Bayer, Pfizer, and Sanofi; receives research funding from Bayer; and has ownership interest in C-diagnostics EtiraRx and GaudiumRx. J.L. has served as a paid consultant and/or adviser of Janssen, Sanofi, and Sun Pharma; has received research funding from Astellas, Constellation, Gilead, Orion, and Sanofi; and is the lead inventor of a technology that has been licensed to A&G, Qiagen, and Tokai. J.S.d.B. has served as an advisory board member for Astellas, AstraZeneca, Bayer, Genentech, Genmab, GlaxoSmithKline, Janssen, Medivation, Merck MSD, Menarini, Orion Pharma, Pfizer, Sanofi–Aventis, and Serono. S.R.P. declares no competing interests.

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Androgen receptor splice variant-7 expression emerges with castration resistance in prostate cancer

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BACKGROUND. Liquid biopsies have demonstrated that the constitutively active androgen receptor splice variant-7 (AR-V7) associates with reduced response and overall survival (OS) from endocrine therapies in castration resistant prostate cancer (CRPC). However, these studies provide little information pertaining to AR-V7 expression in prostate cancer (PC) tissue.

METHODS. Following generation and validation of a novel AR-V7 antibody for immunohistochemistry, AR-V7 protein expression was determined for 358 primary prostate samples and 293 metastatic biopsies. Associations with disease progression, full length AR (AR-FL) expression, response to therapy, and gene expression was determined.

RESULTS. We demonstrated that AR-V7 protein is rarely expressed (<1%) in primary PC but is frequently detected (75% of cases) following androgen deprivation therapy, with further significant ($P = 0.020$) increase in expression following abiraterone acetate or enzalutamide therapy. In CRPC, AR-V7 expression is predominantly (94% of cases) nuclear and correlates with AR-FL expression ($P \leq 0.001$) and AR copy number ($P = 0.026$). However, dissociation of expression was observed suggesting mRNA splicing remains crucial for AR-V7 generation. AR-V7 expression was heterogeneous between different metastases from a patient although AR-V7 expression was similar within a metastasis. Moreover, AR-V7 expression correlated with […]

Androgen receptor splice variant-7 expression emerges with castration resistance in prostate cancer

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Abstract

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Conclusion: This study provides impetus to develop therapies that abrogate AR-V7 signaling to improve our understanding of AR-V7 biology, and to confirm its clinical significance.

1. Introduction

Prostate cancer (PC) is the most commonly diagnosed non-cutaneous cancer and second leading cause of male cancer-related death in the Western world [1]. Androgen receptor (AR) signaling is critical for PC development and progression [2-5]. PC patients with advanced disease after primary therapy respond robustly to androgen deprivation therapy (ADT) but nearly all will progress to fatal castration resistant PC (CRPC). There is now mounting evidence that progression to CRPC remains dependent on persistent AR signaling driven by increased androgen synthesis, overexpression of AR co-activators, AR amplification and AR activating point mutations [3, 5-9]. These molecular findings have driven the development of new anti-androgen therapies, such as abiraterone acetate (AA), enzalutamide (E) and apalutamide (AP), that target the AR axis in patients with castration sensitive PC (CSPC) and CRPC. These therapies have led to improved patient outcome and health related quality of life [10-18].

Despite this significant progress, resistance to AA and E is common and on average occurs within a year of starting therapy; this is due, at least in part, to the emergence of constitutively active AR splice variants, of which AR variant-7 (AR-V7) is regarded as the most significant and most extensively characterized [19-32]. AR-V7 is thought to arise from aberrant mRNA splicing of AR exons 1, 2, 3, loss of exons 4-8, and inclusion of the cryptic exon 3 (CE3) into the transcribed AR gene [22, 32]. The resultant protein product is constitutively active in the absence of androgens and drives growth of PC cell lines and patient derived xenografts in the presence of AR directed therapies such as AA or E [23, 24, 32, 33]. AR-V7 forms homodimers with itself and heterodimers with full length AR (AR-FL) and in the absence of androgens binds to AR response elements, facilitating the generation of a pro-tumorigenic transcriptome [34]. In addition, transgenic mice with forced expression of AR-V7 display a pro-tumorigenic phenotype [35]. These pre-clinical studies confirm that AR-V7 may facilitate ligand independent AR signaling to drive resistance to established endocrine therapies.

Insufficient data have been available on AR-V7 mRNA and protein expression in primary PC, although some studies suggest expression [36-39]. AR-V7 protein expression increases as patients develop CRPC and resistance to AA or E [30-32, 40]. A plethora of clinical studies have confirmed AR-V7 expression to be correlated with resistance to AA and E therapy in CRPC; the majority of these measuring AR-V7 mRNA or protein from liquid biopsies (i.e. circulating tumor cells, exosomes, or whole blood) as opposed to utilizing metastatic tumor biopsies [19, 20, 26-31, 41-43]. All clinically licensed therapies modulate AR activity through its ligand-binding domain and therefore conceptually have no activity against AR-V7 mediated oncogenic signaling. Pharmacological inhibitors of bromodomain and extra-terminal proteins and HSP90 suppress AR-V7 generation through inhibition of mRNA splicing and inhibit the growth of CRPC models [33, 44, 45]. However, these therapies target multiple cellular pathways and therefore concerns with regard to clinical utility and safety remain. The development of novel therapies that overcome AR-V7 signaling in CRPC remains an area of urgent unmet clinical need.

In this work, we have performed an extensive cross-institutional study to determine nuclear AR-V7 protein expression in tissue biopsies and autopsies from primary and metastatic PC tumors using a novel AR-V7 antibody. We establish that expression of AR-V7 protein is rare in primary PC. In addition, nuclear AR-V7 expression emerges in response to primary ADT alone in most patients, and further increases in response to AA or E therapy, with nuclear AR-V7 being an important marker of response to these endocrine therapies in CRPC. Furthermore, AR-V7 expression associates with AR-FL expression and AR copy number in CRPC, although many cases with high AR-FL protein expression have undetectable/low AR-V7 protein expression. Moreover, nuclear AR-V7 expression is heterogeneous in different CRPC metastases in the same patient. Finally, nuclear AR-V7 expression is associated with a unique gene signature in CRPC patients. These data support a critical role for AR-V7 in CRPC biology and resistance to established endocrine therapies, providing further impetus for the development of therapeutic strategies that overcome AR-V7 mediated signaling to improve the outcome for patients with this lethal disease.

2. Results

2.1 Validation and optimization of a novel AR-V7 antibody (Clone RM7) for immunohistochemistry.

A novel recombinant rabbit monoclonal antibody (Clone RM7) was developed, in collaboration with RevMAb Biosciences, to CE3 of AR-V7. Antibody validation was performed at The Institute of Cancer Research/Royal Marsden (ICR/RMH) and University of Washington (UW), western blot analysis of AR-V7 positive cell lines (LNCaP95, 22Rv1 and VCaP) demonstrated a strong AR-V7 band at 80KDa (**Figure 1A and Supplementary Figure S1A**). In contrast, no band was seen in AR-V7 negative cell lines (LNCaP, PC3 and DU145) at 80KDa (**Figure 1A and Supplementary Figure S1A**). We next compared RM7 to EPR15656, an AR-V7 antibody that has been studied in PC tissue and circulating tumor cells (CTCs), and utilized for biomarker studies of treatment stratification in CRPC [27, 28, 30]. EPR15656 primarily recognizes AR-V7 but may also bind other proteins demonstrating staining in PC3 cells, colorectal (liver) metastasis, normal lung epithelium and cytoplasmic compartment [27, 28, 30]. EPR15656 demonstrated a strong AR-V7 band at 80KDa in LNCaP95, 22Rv1 and VCaP (**Figure 1A and Supplementary Figure S1A**). However, consistent with reports of positive staining in PC3 cells, EPR15656 demonstrated a strong band at 70KDa in PC3 [30]. Following initial validation, specificity and increased affinity (compared to EPR15656) of RM7 for AR-V7 was confirmed by immunoprecipitation using M12-cumate inducible AR-V7 cells demonstrating a single band at 80KDa (**Figure 1B**). RM7 detected additional bands at approximately 150KDa and 32KDa. However, both the strong 80KDa AR-V7 band and faint 32KDa band disappeared upon shRNA induction by doxycycline suggesting that the 32KDa band is a degradation product of AR-V7 (**Figure 1C**). In addition, the 150KDa band was not seen by Western blot analysis using an alternative extraction method at UW (**Figure 1C**). Furthermore, when RM7 was optimized for immunohistochemistry (IHC); AR-V7 expressing cell lines (22Rv1, LNCaP95 and VCaP) were positive for AR-V7 by IHC and AR-V7 negative cell lines (LNCaP, DU145 and PC3) were negative for AR-V7; confirming that the 150KDa band (present in LNCaP) was not recognized by IHC and RM7 does not stain PC3 cells (**Figure 1D**). In addition, RM7 stained neither a colorectal cancer (liver) metastasis nor normal lung epithelium, which stained positive with EPR15656 previously (**Supplementary Figure S1B**) [30]. Having confirmed that RM7 recognizes AR-V7, we performed IHC on formalin-fixed, paraffin-embedded PC patient tissue biopsies within our study cohorts demonstrating strong, almost exclusively, nuclear staining (**Figure 2 and Figure 3A**). In contrast, EPR15656 demonstrated cytoplasmic staining in PC tissue and CTCs [27, 28, 30]. Taken together, these data demonstrate that RM7 specifically recognizes AR-V7 protein in tissue biopsies from PC patients, with reduced off target liabilities compared to EPR15656.

2.2 Primary prostate cancers rarely express AR-V7 protein.

Previous studies have demonstrated AR-V7 mRNA and protein expression in primary PC [36-39]. Having validated and optimized RM7 for IHC on PC patient samples we

investigated nuclear AR-V7 protein expression in early PC specimens. We utilized the ICR/RMH and UW CSPC cohorts (**Figure 2**). A single biopsy (1.6%) of 63 CSPC biopsies (ICR/RMH CSPC cohort) expressed nuclear AR-V7 (**Figure 3B and Supplementary Table 1**). Similarly, in 295 primary PC specimens (UW CSPC cohort) from men who were treated with a radical prostatectomy, and had not received AR directed therapy, there were no (0%) nuclear AR-V7 positive cases (**Figure 3C**). Clinical data (for PSA progression free survival) was available on 128 patients from the UW CSPC cohort (**Supplementary Table 2**). Of the 128 patients with 5-year follow-up 64 had biochemical recurrence; none of these 64 patients had detectable AR-V7 protein in their initial prostatectomy tissue. These data confirm that AR-V7 is rarely (0.3%; 1 of 358) expressed and therefore nuclear AR-V7 protein expression cannot predict disease recurrence in radically treated primary PC.

2.3 AR-V7 protein emerges as prostate cancer patients and mouse xenografts progress to castration resistant disease and develop resistance to abiraterone acetate or enzalutamide therapy.

Having demonstrated that nuclear AR-V7 protein is infrequently expressed in CSPC we next explored nuclear AR-V7 expression in same patient, matched biopsies, as 63 patients progressed from CSPC to CRPC (**Figure 2**). Nuclear AR-V7 protein significantly ($p<0.001$) increased from CSPC (median H-score, IQR; 0, 0-0) to CRPC (70, 5-130) (**Figure 3B**). Next, we expanded this cohort to 160 CRPC biopsies (**Figure 2**). Median nuclear AR-V7 expression was 75 (5-130) (**Figure 3D and Supplementary Table 3**); 3 (1.9%) mCRPC biopsies of 160 cases had neuroendocrine-like features and were negative for both nuclear AR-FL and nuclear AR-V7 expression. We next determined whether nuclear AR-V7 expression altered as patients progressed through standard of care AR targeting therapies for CRPC (**Figure 3D**). Interestingly, 15 (75%) of 20 biopsies taken after progression on primary ADT (with or without bicalutamide) prior to starting standard systemic therapy for CRPC had detectable nuclear AR-V7 protein expression (40, 1.25-92.5) (**Figure 3D**). Furthermore, nuclear AR-V7 expression was significantly lower ($p=0.020$) in 40 biopsies prior to AA or E therapy (40, 1-107.5) than in 120 biopsies post AA or E therapy (90, 20-150) (**Figure 3D**). There was no clear association between nuclear AR-V7 expression and time of biopsy after starting AA or E therapy ($r=-0.11$ [-0.31-0.09]; $p=0.27$) (**Supplementary Figure S2**). Next we determined whether nuclear AR-V7 expression differed between site of CRPC biopsy. Nuclear AR-V7 expression was higher ($p=0.013$) in lymph node (120, 60-170) metastases compared to bone (50, 1-110), liver (70, 3.75-132.5), prostate (50, 0-70) and other (90, 0-150) sites of metastases (**Figure 3E**). Finally, we investigated whether the same pattern of nuclear AR-V7 expression was observed as VCaP (androgen dependent) mouse xenograft models developed therapeutic resistance. Consistent with our tissue studies, nuclear AR-V7 expression increased in VCaP mouse xenografts as they progressed from the castration sensitive (0, 0-0) to the castration resistance state (155, 102.5-175) to AA/E resistance (180, 160-190) (**Supplementary Figure S3 and S4**). Although nuclear AR-V7 protein is rarely expressed in primary PC, AR-V7 protein expression emerges as patients and mouse xenografts progress to castration resistant disease, and levels increase further as resistance to AA or E therapy develops.

2.4 AR-V7 protein expression associates with response to abiraterone acetate and enzalutamide, but not docetaxel, in castration resistant prostate cancer.

Studies have shown AR-V7 protein and mRNA to be a marker of next generation AR targeted therapy (AA and E) resistance [19, 20, 26-31]. To investigate this further, we determined the response of the ICR/RMH CRPC cohort to AR targeted therapies pre- and post-chemotherapy in AR-V7 negative (nuclear H-score ≤ 10) and AR-V7 positive (nuclear H-score >10) patients. Thirty-six patients received AA or E for CRPC prior to chemotherapy and had fully evaluable response data (**Figure 2**). Patients negative for AR-V7 expression were younger (p=0.04) at the time of starting AR targeting therapy pre-chemotherapy, but no other differences in baseline characteristics were observed (**Table 1**). Patients negative for AR-V7 (n=8) had a greater prostate specific antigen (PSA) 50% nadir (100 vs 68%, p=0.16) and PSA 50% response rate (100 vs 54%, p=0.03) than AR-V7 positive patients (n=28) (**Figure 4A-B**). Patients achieving a 50% PSA fall had significantly lower (p=0.012) nuclear AR-V7 expression (40, 0-100) than those that did not (120, 55-180) (**Supplementary Figure S5**). Furthermore; AR-V7 negative patients had a longer time to PSA progression (11.5 vs 4.8 months (mo), hazard ratio (HR) 0.33 [0.14-0.81], p=0.02), longer time to clinical/radiological progression (13.9 vs 7.2 mo, HR 0.47 [0.20-1.10], p=0.08) and improved overall survival (74.3 vs 25.2mo, HR 0.23 [0.07-0.79], p=0.02) (**Figure 4C-E**). Fifty-four patients received AA or E for CRPC after chemotherapy and had fully evaluable response data (**Figure 2**). There were no differences in the baseline characteristics by AR-V7 status at the time of starting AR targeting therapy post-chemotherapy (**Supplementary Table 4**). Patients negative for AR-V7 (n=17) had a significantly greater PSA 50% nadir (71 vs 24%; p=0.002) and PSA 50% response rate (59 vs 22%; p=0.012) than those positive for AR-V7 (n=37) (**Supplementary Figure S6A-B**). Patients achieving a 50% PSA fall had significantly lower (p=0.011) nuclear AR-V7 expression (3, 0-80) that those that did not (95, 25-125) (**Supplementary Figure S6C**). Interestingly, despite these significant differences in response rates, there was no significant difference in time to PSA progression (2.8 vs 2.3 mo, HR 0.96 [0.54-1.73], p=0.90), time to clinical/radiological progression (4.9 vs 5.1 mo, HR 0.92 [0.51-1.66], p=0.77) or overall survival (14.0 vs 15.7 mo, HR 1.01 [0.56-1.82], p=0.98) (**Supplementary Figure S6D-F**). Having explored response to AR targeted therapy, we next investigated response to docetaxel chemotherapy. Fifty-five patients were treated with docetaxel chemotherapy for CRPC and had fully evaluable response data (**Figure 2**). There was no evidence of a difference in baseline characteristics at the time of starting docetaxel chemotherapy (**Supplementary Table 5**). In contrast to AR targeting therapies, there was no difference in PSA 50% nadir (56 vs 46%, p=0.57) and PSA 50% response rate (39 vs 27%, p=0.54) between AR-V7 negative (n=18) and positive patients (n=37) (**Supplementary Figure S7A-B**). Nuclear AR-V7 expression was not significantly (p=0.14) different in patients achieving a 50% PSA fall with docetaxel (20, 0-85) compared to those that did not (70, 5-132.5) (**Supplementary Figure S7C**). Consistent with this, there was no significant difference in time to PSA progression (4.8 vs 4.7 mo, HR 1.04 [0.57-1.92], p=0.90) and time to clinical/radiological progression (6.9 vs 7.5 mo, HR 1.31 [0.73-2.34], p=0.36) (**Supplementary Figure S7D-E**). However, AR-V7

negative patients had improved overall survival (26.3 vs 18.5 mo, HR 0.50 [0.27-0.95], p=0.03) compared to AR-V7 positive patients (**Supplementary Figure S7F**). Taken together; these data confirm that AR-V7 is a robust prognostic biomarker and an important indicator of sensitivity to AR targeted therapies but not docetaxel treatment in CRPC.

2.5 AR-FL and AR-V7 mRNA and protein expression associate in a high proportion of, but not all, castration resistant prostate cancers.

We and others have shown that AR-FL and AR-V7 mRNA and protein are induced upon castration, and that therapies suppressing mRNA splicing prevent AR-V7 mRNA and protein generation in CRPC [33, 44-47]. Therefore, we next investigated the association between AR-FL and AR-V7 mRNA and protein expression (**Figure 2**). Analysis of RNA-sequencing (RNA-seq) data obtained from 122 CRPC biopsies demonstrated that AR-FL mRNA expression significantly correlated with AR-V7 mRNA expression (r=0.69 [0.58-0.77]; p=<0.001) (**Figure 5A**). In light of RNA quantification not discriminating against cellular localization, we next quantified total (nuclear and cytoplasmic) AR-FL and AR-V7 protein expression in 144 CRPC biopsies (ICR/RMH CRPC cohort) (**Figure 5B and Supplementary Figure S8**). Unlike AR-FL, of which 124 of 144 (86%) biopsies had both cytoplasmic and nuclear AR-FL protein expression, AR-V7 protein was almost exclusively (136/144; 94% of biopsies) nuclear in localization (**Figure 5B)**. There was a significant correlation between total AR-FL and AR-V7 protein expression in 144 CRPC biopsies from the ICR/RMH CRPC cohort (r=0.28 [0.11-0.42], p=<0.001) (**Figure 5C**). However, it is important to recognize that a substantial number of patients with high AR-FL mRNA and protein expression had low or undetectable levels of AR-V7 mRNA and protein. Furthermore, both total AR-FL (r=0.46 [0.28-0.61], p=<0.001) and total AR-V7 (r=0.23 (0.02 to 0.42); p=0.026) protein significantly correlated with AR copy number in 95 CRPC biopsies from the ICR/RMH CRPC cohort (**Figure 5D-E**). Finally, consistent with the demonstration that AR-FL and AR-V7 are differentially localized, there was no significant correlation between nuclear AR-FL and nuclear AR-V7 protein expression in 144 CRPC biopsies from the ICR/RMH CRPC cohort (r=0.11 [-0.06-0.27], p=0.20) (**Figure 5F**). These data demonstrate that taken together, total AR-FL and AR-V7 mRNA and protein expression correlate in CRPC biopsies, although importantly many patients have tumors expressing high levels of AR-FL mRNA and protein but that have low or undetectable AR-V7 mRNA and protein expression. This suggests that the presence of AR-V7 mRNA and protein is not simply a consequence of higher AR-FL levels in all cases.

2.6 AR-V7 protein expression is largely homogenous within metastasis but heterogeneous between metastases from patients with castration resistant prostate cancer.

We have previously shown that in a patient with a genomic rearrangement resulting in the constitutively active AR^{v567es} variant that each of 5 metastases expressed the variant AR in a homogenous fashion [48]. Since AR-V7 is usually not generated from a structural rearrangement of the AR gene but rather from aberrant mRNA splicing, we

next quantitated expression in 133 metastases from 34 CRPC patients that were collected as part of the University of Washington Medical Center Prostate Cancer Donor Rapid Autopsy Program (UW CRPC cohort) (**Figure 2, Figure 6A and Supplementary Table 6**). Automated digital scoring reported as optical density (OD) correlated significantly (r=0.86 [0.83-0.89]; p=<0.001) with manual scoring and was used to determine nuclear AR-V7 expression in the UW CRPC cohort (**Supplementary Figure S9A-C**). Three tissue microarray spots were stained from each metastasis and AR-V7 levels quantified. We found that expression of AR-V7 was largely consistent within each metastasis from a patient and that the variance was not statistically significant (Fligner-Killeen p=0.9999; Levene's p=0.9972) (**Figure 6B and C)**. However, expression of AR-V7 in different metastases in an individual patient differed widely and the variance was statistically significant (Fligner-Killeen p=3.73 x 10^{-06}; Levene's p=3.25 X 10^{-07}) (**Figure 6B and C**). These data suggest that within an individual patient the degree to which AR-V7 may be driving different metastases varies and may result in mixed response to endocrine therapies.

2.7 AR-V7 expression is associated with a unique gene signature in castration resistant prostate cancer.

Having demonstrated inter-patient and intra-patient heterogeneity in nuclear AR-V7 expression we next investigated whether nuclear AR-V7 expression was associated with a specific gene signature in CRPC patients. Forty-one metastatic biopsies from 24 men within the UW CRPC cohort had mRNA expression (RNA-seq) and AR-V7 protein expression (IHC) available [31, 54]. The correlation between AR-V7 protein expression (optical density) and gene mRNA expression (log_2 counts per million) was determined and corrected for multiple testing. We identified 487 genes that correlated (q<0.05) with AR-V7 protein expression; of these 407 positively correlated and 80 negatively correlated (**Figure 7A**). Pathway analysis of the 407 genes that positively correlated with AR-V7 protein expression identified an enrichment for pathways involved in transcription and the androgen response (**Supplementary Figure S10 and Supplementary Table 7**) [49, 50]. We confirmed that AR-V7 mRNA expression and AR-V7 protein expression correlated significantly in 41 UW (p<0.001) and 21 ICR/RMH (p=0.004) CRPC biopsies (data not shown). Next we independently tested the positively correlated 407-gene signature in 21 CRPC metastasis from an ICR/RMH CRPC cohort and 122 CRPC tumor transcriptomes (SU2C/PCF cohort) (**Supplementary Table 8**) [51]. Of the genes identified, 59 were found to be significantly correlated with nuclear AR-V7 protein expression in the ICR/RMH cohort or with AR-V7 mRNA expression in the SU2C/PCF cohort (**Figure 7B-C and Supplementary Table 9**). Following this, pathway analysis of the 59 independently validated genes confirmed a role in transcriptional activity (**Figure 7D and Supplementary Table 10**). Consistent with this finding, 33% were zinc finger containing (ZNF) genes correlated with chromatin binding including HOXB13, ELL2, STEAP2 and BAZ2A. Furthermore, a large number of the genes identified have been associated with PC progression (**Supplementary Table 9**) [52-64]. In addition, genome wide analyses demonstrated that AR-V7 protein expression associates with AR gene expression (**Figure 7C**). Although an association between AR-FL mRNA and AR-V7 mRNA using junction specific reads was confirmed,

there was further confirmation that a substantial number of cases that express high AR-FL mRNA levels have low or undetectable AR-V7 mRNA (**Supplementary Figure S11**). These data suggest that AR-V7 expression is associated with a specific gene signature in a large patient population that may play a key role in transcriptional activity and prostate cancer progression in patients with CRPC.

3. Discussion

Since the pioneering studies of Huggins and Hodges in 1941, the androgen receptor has remained the focus of therapeutic targeting in CRPC. Inhibition of the AR axis with AA and E has improved both overall survival and quality of life for patients with CRPC [10-17, 65]. Although these modalities are initially effective, resistance develops with ongoing AR activity and tumor progression. This is, at least in part, due to the expression of constitutively active AR splice variants of which AR-V7 appears to be most common [19-32]. AR-V7 mRNA and protein expression has been detected at low levels in primary, treatment-naïve PC, and studies have reported a potential association with worse outcome [36-39]. Surprisingly, in two separate patient cohorts we found that nuclear AR-V7 protein was expressed in <1% of PC tumors at diagnosis and therefore it cannot be predictive of outcome after primary therapy. The difference in prevalence of AR-V7 protein expression reported in different studies is likely attributable to the different AR-V7 antibodies used, and in particular, the observation that one antibody used has off-target liabilities as we have previously reported [30]. Consistent with this, we demonstrate AR-V7 protein expression to be almost exclusively nuclear, whereas previous studies have demonstrated cytoplasmic positivity [27, 28, 30]. These data suggest that AR-V7 testing is unlikely to be of use for treatment stratification at time of diagnosis and maybe better utilized beyond first-line treatment [11, 18].

In contrast to primary PC, 75% of CRPC patients who had progressed on primary ADT alone (with or without bicalutamide) expressed nuclear AR-V7 prior to receiving AA or E. Despite AR-V7 expression after primary ADT, subsequent AA or E treatment has significant antitumor activity with response rates ranging from 57-78% [12, 14, 66]. These data indicate that AR-V7 protein expression in biopsies cannot indicate absolute refractoriness to treatment. Although, consistent with previous reports, nuclear AR-V7 levels increased further in response to AA or E; these data suggest that AR-V7 expression is a factor at the initial phase of castration resistance following primary ADT in advanced PC [30, 40]. These data were further confirmed in VCaP mouse xenograft models as they developed resistance to castration and AA/E therapy. Recent studies have shown AA therapy at diagnosis to improve overall survival in PC patients with *de novo* metastatic disease [11, 18]. The demonstration that nuclear AR-V7 expression is rare in primary PC, but emerges with primary therapy, may provide insight into the greater efficacy of AA in CSPC. Importantly, these data show that increased AR-V7 expression is an early event in resistance and if targeted agents to AR splice variants become clinically available, therapy may need to be combined at time of initial ADT.

Critically, we found that AR-V7 protein expression is more prevalent in CRPC biopsies than previously reported from AR-V7 mRNA and protein expression studies in liquid biopsies (11-46%) [19, 20, 27-29]. This important observation is likely due to the differences in sensitivities of the assays used. In addition, CRPC biopsies demonstrate lymph node metastases to express higher levels of AR-V7 than other sites of disease, which may (depending on the source of CTCs) account for the lower incidence of AR-V7 detected in liquid biopsies. Furthermore, we demonstrate intra-patient heterogeneity of nuclear AR-V7 expression between multiple metastases indicating that this is a

further potential source of variation in CTC based AR-V7 assessment. Finally, depending on the biomarkers used to select CTCs, assessment of AR-V7 may be underestimated if not all CTCs are identified. These findings suggest that the detection of AR-V7 in CTCs may not be representative of all metastases, and that while sites of disease expressing AR-V7 may be resistant to current endocrine therapies, those expressing low/no AR-V7 may still respond, within the same subject. Additional studies focusing on the prognostic value of tissue-based AR-V7 detection in CTC-negative patients may be warranted.

The majority of clinical studies have demonstrated that AR-V7 positivity confers resistance and poorer outcome to AR targeting therapies in patients with CRPC [19, 20, 26-31]. We confirmed that AR-V7 positive patients treated with AA or E had a worse PSA response rate in the pre- and post-chemotherapy setting; and those patients who responded had lower levels of AR-V7 expression. Interestingly, despite poorer response rates, only AR-V7 positive patients treated with AA or E prior to chemotherapy had shorter progression free and overall survival. This observation could be multifactorial. Firstly, not all patients had tissue biopsies prior to starting treatment and therefore AR-V7 status may have changed prior to therapy. Secondly, patients without CTCs and therefore no AR-V7 result may be underrepresented in previous studies. In contrast to endocrine therapies, AR-V7 status did not associate with PSA response or progression free survival in patients treated with docetaxel, as previously described [27, 28, 67]. Interestingly, AR-V7 positive patients had shorter overall survival suggesting that AR-V7 positivity may be associated with more aggressive disease, or this group of patients may have derived less benefit from treatment with further novel endocrine therapies. Taken together, these studies will be important to understand, as the landscape of CRPC changes as patients with *de novo* metastatic CSPC receive AA, and as we explore the potential use of AR-V7 to stratify patients to further AR targeted therapies as they progress to CRPC.

The mechanisms by which AR splice variants are generated include genomic rearrangements and/or aberrant alternative mRNA splicing [22, 24, 32, 48, 68-71]. AR-V7 generation has generally been attributed to aberrant splicing of AR pre-mRNA [72]. This does not negate the fact that AR-FL increases under conditions such as castration, and that this leads to the generation of AR-V7 [33, 46, 47]. However, these data indicate that the mechanisms driving increased AR-FL expression and AR signaling in CRPC differ from those required for AR-V7 generation [72-76]. Consistent with this, we demonstrate that although AR-FL and AR-V7 expression associate in CRPC, a substantial number of patients with high levels of AR-FL demonstrate undetectable or low levels of AR-V7 expression. In keeping with this also is evidence emerging that therapies that suppress mRNA splicing decrease AR-V7 generation in CRPC models [33, 45]. JMJD1A has recently been reported to be critical for mRNA splicing and AR-V7 generation but does not impact on AR-FL levels [77]. These data suggest that mRNA splicing is important for AR-V7 generation and is not simply a consequence of increased AR-FL expression. Further understanding of the mechanisms underpinning AR-V7 generation are now required to support the development of therapeutic strategies to suppress splice variant generation in CRPC.

Previous studies examining AR-V7 cistromes and transcriptomes demonstrated proliferative cistomes/transcriptomes which are likely not specific to AR-V7 but a marker of rapidly progressive disease [23, 78-80]. Although a recent study suggested that CRPC transcriptomes are diverse, we identified 59-genes using three independent patient cohorts that associate with AR-V7 expression [79]. One important consideration is that AR-V7 protein expression associates with AR gene expression in genome-wide analysis. However, as both AR-V7 and AR-FL mRNA would be represented in such studies, further junction-specific quantification was performed. Despite this approach confirming a correlation between AR-FL and AR-V7 mRNA, there was further evidence of dissociation in many cases. In addition, a gene signature was derived from AR-V7 protein expression; unlike AR-FL protein, AR-V7 is almost exclusively nuclear and therefore, unlike mRNA analysis, this takes into consideration its likely functional importance. Interestingly, BAZ2A, SRC, STEAP1, STEAP2, DCAF6, TMBIM6, HOXB13, GALANT7, WWC1, SPATS2 and GSTP1 expression associated with AR-V7 expression and all of these have been previously linked to PC progression [52-64]. In addition, HOXB13 has recently been shown to be critical for AR-V7 chromatin binding [79]. Furthermore, we found a number of ZNF contigs to be associated with AR-V7 expression, providing evidence of increased transcriptional activity. These data suggest that AR-V7 protein expression is associated with a unique gene signature important for prostate cancer progression and transcriptional activity. It is important to stress that the 59-gene set derived does not represent the AR-V7 cistrome but is a set of genes associated with AR-V7 expression between cohorts and may identify common characteristics of AR-V7 associated disease. Components of this gene signature, including HOXB13 that has recently been shown to be critical for AR-V7 function, may provide insight into therapeutic targets for novel treatment strategies in patients with high levels of AR-V7 expression [79].

In conclusion, our results show that AR-V7 protein expression, using a validated, highly specific antibody, is not seen in primary CSPC and does not appear until initial resistance to standard ADT occurs, and increases further with AA and E therapy. In addition, AR-V7 protein expression associates with resistance to AR targeted therapies but not taxane treatment in patients with CRPC. Furthermore, although AR-V7 and AR-FL expression levels associate in CRPC, there are many cases in which expression levels are uncoupled suggesting that AR-V7 protein expression is not simply a function of AR-FL protein expression. Moreover, AR-V7 protein is heterogeneously expressed, especially between metastases from the same patient, indicating multiple resistance mechanisms in the same subject. These data suggest that multiple therapeutic modalities may be needed simultaneously to adequately reverse endocrine resistance in AR-V7 positive PC. Finally, AR-V7 protein expression associates with a unique gene signature that may drive transcriptional activity and PC progression. These results further confirm the importance of AR-V7 in CRPC biology and provide impetus for the development of novel therapeutic strategies that abrogate AR-V7 expression at the time of initial ADT in CSPC in order to prevent or delay development of CRPC and improve the outcome for patients with lethal PC.

4. Methods

Cell lines

LNCaP95 cells were kindly provided by Drs. Alan K Meeker and Jun Luo (Johns Hopkins University). 22Rv1 (CRL-2505), VCaP (CRL-2876), DU145 (HTB-81), M12 (a gift from Joy Ware, Medical College of Virginia) and PC3 (CRL-1345) cells were obtained from American Type Culture Collection. Doxycycline inducible cell lines were created using lentiviral vectors in pLKO-Tet-On backbones targeting either GFP (shGFP; 5'-GCAAGCTGACCCTGAAGTTCA-3'), AR-FL exon 8 (shAR-FL; 5'-CCTGCTAATCAAGTCACACAT-3') or AR-V7 cryptic exon 3 (shAR-V7; 5'-GTAGTTGTGAGTATCATGA-3') and lentiviral particles were produced as previously described [81, 82]. Cells were infected with virus and selected with 1 μg/ml puromycin. shRNA expression was induced by treating cells with 1 μg/ml doxycycline for 72 h. All cell lines were grown in recommended media at 37 °C in 5 % CO_2. Cell lines were tested for mycoplasma using the VenorGem One Step PCR Kit (Cambio) and STR-profiled.

Immunoblotting

ICR/RMH antibody validation: Cells were lysed with RIPA buffer (Pierce) supplemented with protease inhibitor cocktail (Roche). Protein extracts (20 μg) were separated on 7% NuPAGE® Tris-Acetate gel (Invitrogen) by electrophoresis and subsequently transferred onto Immobilon-P™ PVDF membranes of 0.45 μm pore size (Millipore). Primary antibodies used were rabbit monoclonal anti-AR-V7 antibody (1 in 1000; RM7; RevMAb biosciences), rabbit monoclonal anti-AR-V7 antibody (1 in 1000; EPR15656; abcam) and mouse monoclonal anti-vinculin antibody (1 in 200000; V9131; Sigma-Aldrich) with species specific secondary antibodies conjugated to horseradish peroxidase. Chemiluminescence was detected on the Chemidoc Touch imaging system (Bio-Rad).

UW antibody validation: Cells were lysed with M-PER™ Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) supplemented with Halt™ Protease Inhibitor and Halt™ Phosphatase Inhibitor Cocktail. Protein extracts (30 μg) were separated on 4–15% Mini-PROTEAN® TGX™ Precast Protein Gel (Bio rad) by electrophoresis and subsequently transferred to a nitrocellulose membrane with an iBlot system. Primary antibodies used were rabbit monoclonal anti-AR-V7 (1 in 2000; RM7; RevMAb biosciences), mouse monoclonal anti-AR N-terminus antibody (1 in 2000; AR441; Santa Cruz Biotechnology) and rabbit monoclonal anti-GAPDH antibody (1 in 10000; #2118; Cell Signaling Technology). The specific signals were visualized on the Blue Ultra Autorad Film (GeneMate) with species specific secondary antibodies conjugated to horseradish peroxidase by chemiluminescence.

AR-V7 immunoprecipitation

Cellular extracts were prepared from cumate-treated M12 cells expressing cumate-inducible AR-V7 lentivirus were prepared using the SparQcumate switch lentivector

system (Systems Bioscience) as previously described [30]. Precleared cell lysate was incubated with rabbit monoclonal anti-AR-V7 antibodies (EPR15656; abcam or RM7; RevMAb biosciences). Rabbit IgG were used as a negative control. Immune complexes were collected using protein A/G Plus agarose beads and analyzed by immunoblotting as described above.

VCaP mouse xenograft models

All animal studies performed in accordance with Beth Israel Deaconess Medical Center IACUC regulations (protocol #086-2016). VCaP mouse xenograft models have been previously described [83]. Briefly, 5 million VCaP cells in 100% Matrigel were injected subcutaneously into 6-week old ICR scid mice (Taconic Biosciences). Xenografts were grown until 1000mm^3, then mice were castrated. For AA and E resistant xenograft model, when castrate tumors exceeded 150% nadir volume they were treated with AA (30mg/kg) and E (50mg/kg). Tumors were biopsied prior to castration resistance, at castration resistance and when resistant to AA and E therapy.

Institute of Cancer Research and Royal Marsden Hospital (ICR/RMH) and University of Washington (UW) tissue samples

The ICR/RMH CSPC and CRPC cohort was identified from men with CRPC treated at the Royal Marsden NHS Foundation Trust. The ICR/RMH CSPC cohort contained 63 patients with sufficient formalin-fixed, paraffin embedded (FFPE) diagnostic (archival) CSPC biopsies; all biopsies demonstrated adenocarcinoma and were from either prostate needle biopsies (47), transurethral resection of the prostate (TURP; 5), transurethral resection of the bladder (TURBT; 1), prostatectomy (8), bone (1) and rectal (1). The ICR/RMH CRPC cohort contained 160 patients (which included all 63 patients in the CSPC cohort) with sufficient FFPE CRPC biopsies from metastatic biopsies of bone (81), lymph node (51), soft tissue (8), liver (10) and TURP (7), TURBT (1) or prostate (2). All tissue blocks were freshly sectioned and only considered for IHC analyses if adequate material was present (author DNR.). Demographic and clinical data for each patient were retrospectively collected from the hospital electronic patient record system.

The UW CSPC cohort was identified from men who received radical prostatectomy without neoadjuvant therapy. Tissue microarrays (TMAs) of FFPE tissue from primary prostate acinar adenocarcinomas was generated. The tissue came from the radical prostatectomy samples of 295 patients, none of whom had received neoadjuvant therapy. The TMAs consisted of single cores of 12 carcinomas, duplicate cores of 167 carcinomas, triplicate cores of 44 carcinomas and quadruplicate cores of 72 carcinomas. The UW CRPC cohort was identified from men who died from their prostate cancer and were part of the University of Washington GU Cancer Rapid autopsy program [5]. The cohort consisted of TMA 83 generated from biopsies of 133 metastases from 34 patients. Triplicate cores of the 133 metastases were placed on the TMA.

Immunohistochemistry (IHC)

ICR/RMH CSPC and CRPC cohort: AR-V7 and AR-FL IHC was performed as previously described [30, 31]. Briefly, AR-V7 IHC was performed using recombinant rabbit monoclonal anti-AR-V7 antibody (Clone RM7; RevMAb biosciences). Biopsies were first deparaffinized prior to antigen retrieval by microwaving (in Tris/EDTA buffer, pH 8.1) for 18 minutes at 800W and anti-AR-V7 antibody (1:500 dilution in Dako REAL diluent, Agilent Technologies) was incubated with tissue for 1 hour at room temperature. After washes, bound antibody was visualized using Dako EnVision Detection System (Agilent Technologies). Sections were counterstained with hematoxylin. Cell pellets from 22Rv1 (positive) and PC3 (negative) were used as controls. Rabbit IgGs were used as a further negative control. AR-FL IHC was performed using mouse monoclonal anti-AR antibody (AR441, Agilent Technologies). Biopsies were first deparaffinized prior to antigen retrieval using pH 8.1 Tris/EDTA solution heated in a water bath and anti-AR antibody (1:1000 dilution in Dako REAL diluent, Agilent Technologies) was incubated with tissue for 1 hour at room temperature. After washes, bound antibody was visualized using Dako EnVision Detection System (Agilent Technologies). Sections were counterstained with hematoxylin. Cell pellets from VCaP (positive) and PC3 (negative) were used as controls. Mouse IgGs were used as a further negative control.

UW CSPC and CRPC cohort: Briefly, AR-V7 IHC was performed using recombinant rabbit monoclonal anti-AR-V7 antibody (Clone RM7; RevMAb biosciences). Deparaffinization, antigen retrieval (Cell conditioner 1; Ventana Medical Systems) and immunostaining were performed on the Ventana Benchmark automated stainer (Ventana Medical Systems). Sections were incubated for 2 hours at 37^{o}C with anti-AR-V7 antibody (1:50 in antibody diluent, Ventana Medical Systems). After washes, bound antibody was visualized using Ventana Optiview DAB detection kit (Ventana Medical Systems). Sections were counterstained with hematoxylin. Controls were sections of a TMA made of cell lines known to express AR-FL and/or AR-V7 (LNCaP, 22Rv1, VCaP), to not express AR-FL or AR-V7 (DU145, PC3, M12) and cells engineered to stably express both AR-FL (M12 AR-FL) and AR-V7 (M12 AR-V7) by transfection.

IHC quantification

ICR/RMH CSPC and CRPC cohort: AR-V7 and AR-FL protein expression was determined for each case by a pathologist (author DNR) blinded to clinical data using the modified H score (HS) method; a semi-quantitative assessment of staining intensity that reflects antigen concentration. HS was determined according to the formula: [(% of weak staining) × 1] + [(% of moderate staining) × 2] + [(% of strong staining) × 3], yielding a range from 0 to 300 [84].

UW CSPC and CRPC cohort: AR-V7 protein expression in the UW CSPC cohort was determined for each case by a pathologist (author LDT) as described above. AR-V7 protein expression in the UW CRPC cohort was determined using automated digital scoring as follows: TMA slides were scanned with an Aperio ScanScope (Leica Biosystems) at 40x (0.25 microns/pixel). Using Aperio ImageScope software, the AR-V7

stained TMA slides were annotated to create regions of interest (ROI) for analysis. Quantitative image analysis of the annotated ROIs was performed using Aperio Brightfield Image Analysis Toolbox software (Leica Biosystems). The analysis data for each TMA spot was extracted into Microsoft Excel for further analysis. The quantitative analysis data for each TMA spot included total numbers and percentages of nuclei (positive and negative), average positive intensity, average positive optical density, and area of analysis. The intensity is a measurement of the light transmission, or brightness, of the positive staining in the nuclei and is logarithmically related to the optical density. The optical density is a measurement of absorbance and is linearly related to the amount of staining present. Automated scores for AR-V7 protein expression were reviewed and confirmed by a pathologist (author LDT); and have been shown to correlate highly with manual scoring (**Supplementary Figure S9**) [84-86].

RNA-seq analysis

UW CRPC cohort: A set of 41 metastatic tumors from 24 men with CRPC were obtained through the University of Washington Prostate Cancer Donor Autopsy Program and used for transcript profiling by RNA-seq, as described using frozen tissues [31, 87]. RNA sequencing data are deposited in the Gene Expression Omnibus database under the accession number GSE118435. These tissues were from metastases where we had tissue available from the same block that had been used to spot the tissue microarray. Tissue microarray AR-V7 IHC scores were matched to mRNA samples by block ID. We then computed the Pearson correlation between AR-V7 expression (automated digital scoring) and gene mRNA expression (log_2 counts per million), controlling for multiple testing using the cor.test and qvalue functions in R. There were 487 genes with q-value <0.05, 407 of which correlated with higher expression in AR-V7 expressing tumors. This 407-gene signature was independently tested in a set of 21 CRPC biopsies (RMH/ICR cohort; see below) and 122 CRPC transcriptomes (SU2C/PCF cohort; see below). AR-FL and AR-V7 mRNA expression in spliced reads per million mapped reads (SRPM) for 41 CRPC transcriptomes from the UW cohort were calculated as previously described [88]. Junction reads spanning the AR exon 3 to exon 4 junction were used to estimate AR-FL specific expression, while reads spanning the AR exon 3 to cryptic exon 3 junction were used to estimate AR-V7 specific reads, normalized by total spliced reads (genome-wide) to correct for sequencing depth.

ICR/RMH cohort: Twenty-one patients with AR-V7 protein expression by IHC and RNA-seq analysis (from the SU2C/PCF consortium) from same biopsy were used. Data from 21 transcriptomes generated by the International Stand Up To Cancer/Prostate Cancer Foundation (SU2C/PCF) Prostate Cancer Dream Team were downloaded and re-analyzed [3]. Paired-end transcriptome sequencing reads were aligned to the human reference genome (GRCh37/hg19) using Tophat2 (v2.0.7). Gene expression, Fragments Per Kilobase of transcript per Million mapped reads (FPKM), was calculated using Cufflinks [89]. For those genes identified in the UW CRPC cohort; association between nuclear AR-V7 protein expression (IHC) and each gene mRNA expression (RNA-seq) from the same biopsy was determined using Pearson correlation coefficient.

SU2C/PCF cohort: Data from 122 CRPC transcriptomes generated by the International Stand Up To Cancer/Prostate Cancer Foundation (SU2C/PCF) Prostate Cancer Dream Team were downloaded and re-analyzed as described above. For those genes identified in the UW CRPC cohort; association between AR-V7 mRNA expression and each gene mRNA expression was determined using Pearson correlation coefficient.

Pathway enrichment analysis

Out of the 407 genes positively associated with higher AR-V7 levels with a q-value <0.05 in the UW CRPC cohort (described above), 59 were found to be positively associated and significant with p-value <0.05 in either the ICR/RMH cohort or the SU2C/PCF cohort. The UpSetR R package was used to plot overlap between cohorts. Pathway over-representation analysis of the 407 and 59 gene sets were conducted using the compute overlaps tool with MSigDBv6.2 (H – Hallmark, CP – Canonical Pathways, C4 – Computational Gene Sets, C5 – GO and C6 – Oncogenic Pathways) [49, 50, 90].

Statistical analysis

All statistical analyses were conducted using Stata v13.1 or GraphPad Prism v6 and are indicated within all figures and tables. H-scores (HS) were reported as median values with interquartile range (IQR). For ICR/RMH CSPC and CRPC cohort, Mann-Whitney test was used to compare differences in nuclear AR-V7 protein expression levels. Spearman's rank correlation coefficient was used to determine the association between nuclear AR-V7 protein expression and timing of CRPC biopsy after starting AA or E therapy. Nonparametric equality-of-medians test was used to determine the difference in nuclear AR-V7 protein expression between metastatic sites. Wilcoxon matched-pairs signed rank test was used to determine the difference between nuclear AR-V7 protein expression as VCaP mouse xenografts progressed from castration sensitive through castration resistant to AA/E resistance. Spearman's rank correlation coefficient was used to determine associations between AR-FL and AR-V7 mRNA expression, total AR-V7 and total AR-FL protein expression, total AR-V7 protein expression and AR copy number, total AR-FL protein expression and AR copy number, nuclear AR-V7 and nuclear AR-FL protein expression, and optical density and HS quantification for nuclear AR-V7 expression. Fligner-Killeen and Levene's tests for homogeneity of variances between-tumors and within-tumors were performed in R using the fligner.test and leveneTest functions. Patients response to AR targeted therapy (AA or E) pre- and post-chemotherapy, and docetaxel were determined. For each therapy, PSA nadir was calculated as the lowest PSA level on therapy and 12-week PSA response was calculated as the % change in PSA between the start of therapy (baseline) and 12-weeks treatment (or closest available PSA reading). Time to PSA progression was defined as time from start of therapy to first PSA increase that is ≥ 25% and ≥ 2µg/L above the PSA nadir. Time to clinical/radiological progression was defined as time from start of therapy to documented radiological progression or clinical progression (including change of therapy, addition of investigational medicinal product or stopping treatment). Overall survival was defined as time from start of therapy to date of death or last follow

up/contact. Patients baseline characteristics and clinical outcomes were compared by positive (nuclear AR-V7 HS > 10) or negative (nuclear AR-V7 HS ≤ 10) AR-V7 status. Patient's baseline characteristics were compared using Fisher's exact test, Student's t-tests (2 tailed) and Wilcoxon rank-sum test as indicated. 50% PSA nadir and 12 week 50% PSA response rates were compared using Fisher's exact test. The difference between nuclear AR-V7 expression by 50% PSA response rate was compared using Mann-Whitney test. Median time to PSA progression, time to clinical/radiological progression and overall survival were estimated using the Kaplan-Meier method. Association with AR-V7 status (positive vs negative) was tested using univariable Cox proportional hazards models. For all statistical analysis, a p-value of less than 0.05 was considered to be statistically significant.

Study approvals

All animal studies were performed in accordance with Beth Israel Deaconess Medical Center IACUC regulations (protocol #086-2016). All patients treated at the Royal Marsden NHS Foundation Trust had provided written informed consent and were enrolled in institutional protocols approved by the Royal Marsden NHS Foundation Trust Hospital (London, UK) ethics review committee (reference no. 04/Q0801/60). All procedures involving human subjects at the University of Washington (Seattle, Washington) and Fred Hutchinson Cancer Research Center (Seattle, Washington) were approved by the Institutional Review Board at those institutions.

5. Author contributions

AS, IC, WY, CS, DNR, JWR, IF, CB, DD, GS, RR, TU, AN, JW, CM, SC, PSN, SPB, LDT, JSdB and SRP designed the research studies. AS, IL, WY, CS, DNR, JWR, IF, CB, RR, TU, AN, JW, SC and LDT conducted experiments and acquired data. AS, IC, WY, CS, DD, DNR, JWR, IF, CB, GS, TU, AN, JW, SC, PSN, SPB, LDT, JSdB and SRP analyzed the data. AS, IC, WY, CS, DD, DNR, JWR, IF, CB, GS, RR, TU, AN, JW, CM, SC, JL, PSN, SPB, LDT, JSdB and SRP wrote and critically reviewed the manuscript.

6. Competing interests

AS, WY, DD, DNR, IF, CB, GS, RR, AN, JW, SC and JSdB are employees of The Institute of Cancer Research, which has a commercial interest in abiraterone. AS has served as an advisory member for Sanofi. JL is an inventor of a relevant technology that has been licensed to A&G, Tokai, and Qiagen. PSN has served as an advisory member for Janssen, Astellas and Roche. SPB has served as an advisory member for Janssen, Sanofi and Astellas. JSdB has served as an advisory member for Astellas, Janssen, Taiho, Vertex, AstraZeneca, Bayer, Genmab, Genentech, GlaxoSmithKline, Merck, Pfizer, Roche and Sanofi-Aventis. IC, CS, JWR, TU, CM, LDT and SRP have no competing interests.

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9. Figures and figure legends

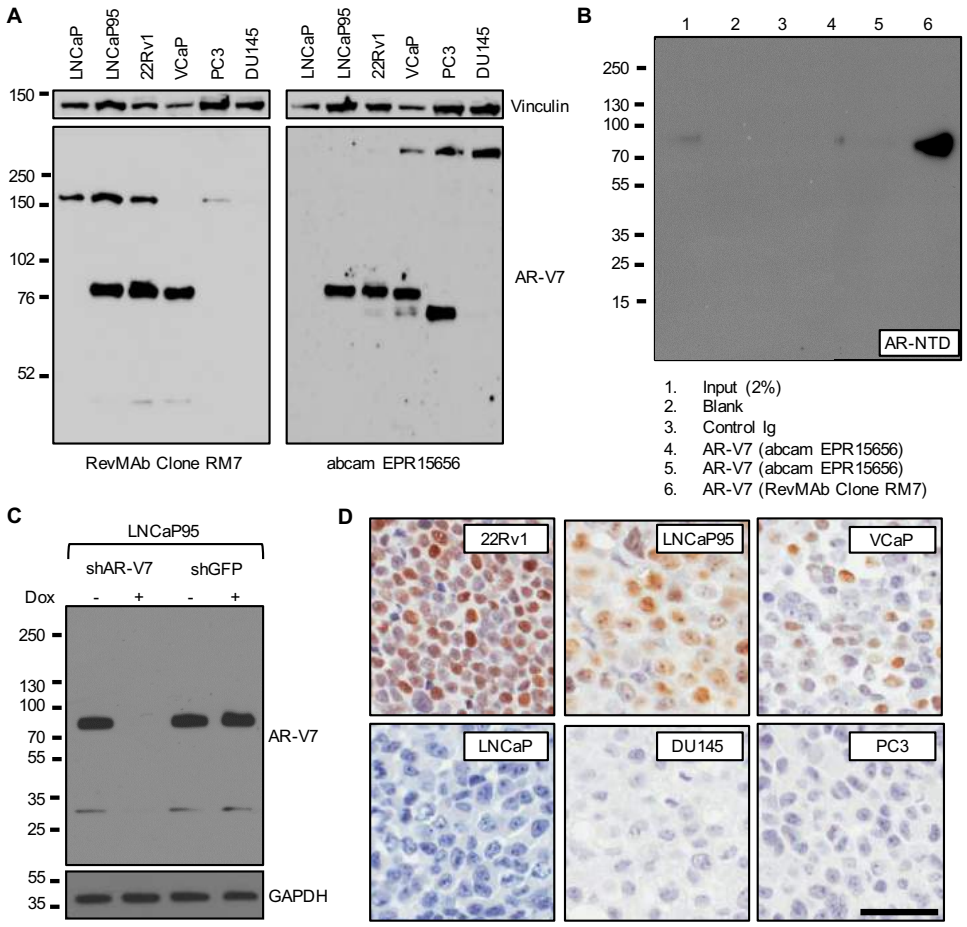

Figure 1: Validation and optimization of a novel AR-V7 antibody (Clone RM7) for immunohistochemistry.

(A) Western blot (long exposure) of AR-V7 positive (LNCaP95, 22Rv1 and VCaP) and negative (LNCaP, PC3 and DU145) PC cell lines using a novel recombinant rabbit monoclonal anti-AR-V7 antibody (Clone RM7) and a previously reported anti-AR-V7 antibody (EPR15656). All cell lines except for LNCaP95 (10% charcoal stripped serum) were grown in 10% fetal bovine serum. **(B)** Immunoprecipitation of AR-V7 from M12-cumate inducible AR-V7 cells using same concentration of AR-V7 antibodies and western blot performed with AR N-terminal domain (AR-NTD) antibody. **(C)** LNCaP95 cells with (doxycycline) inducible shRNA to AR-V7 were treated with (or without) doxycycline and western blot performed with AR-V7 antibody (RM7). **(D)** Micrographs of AR-V7 detection by IHC using AR-V7 antibody (RM7) in cell line pellets positive (22Rv1, LNCaP95 and VCaP) and negative (LNCaP, DU145 and PC3) for AR-V7 (magnification 200x; scale bar 50 μm).

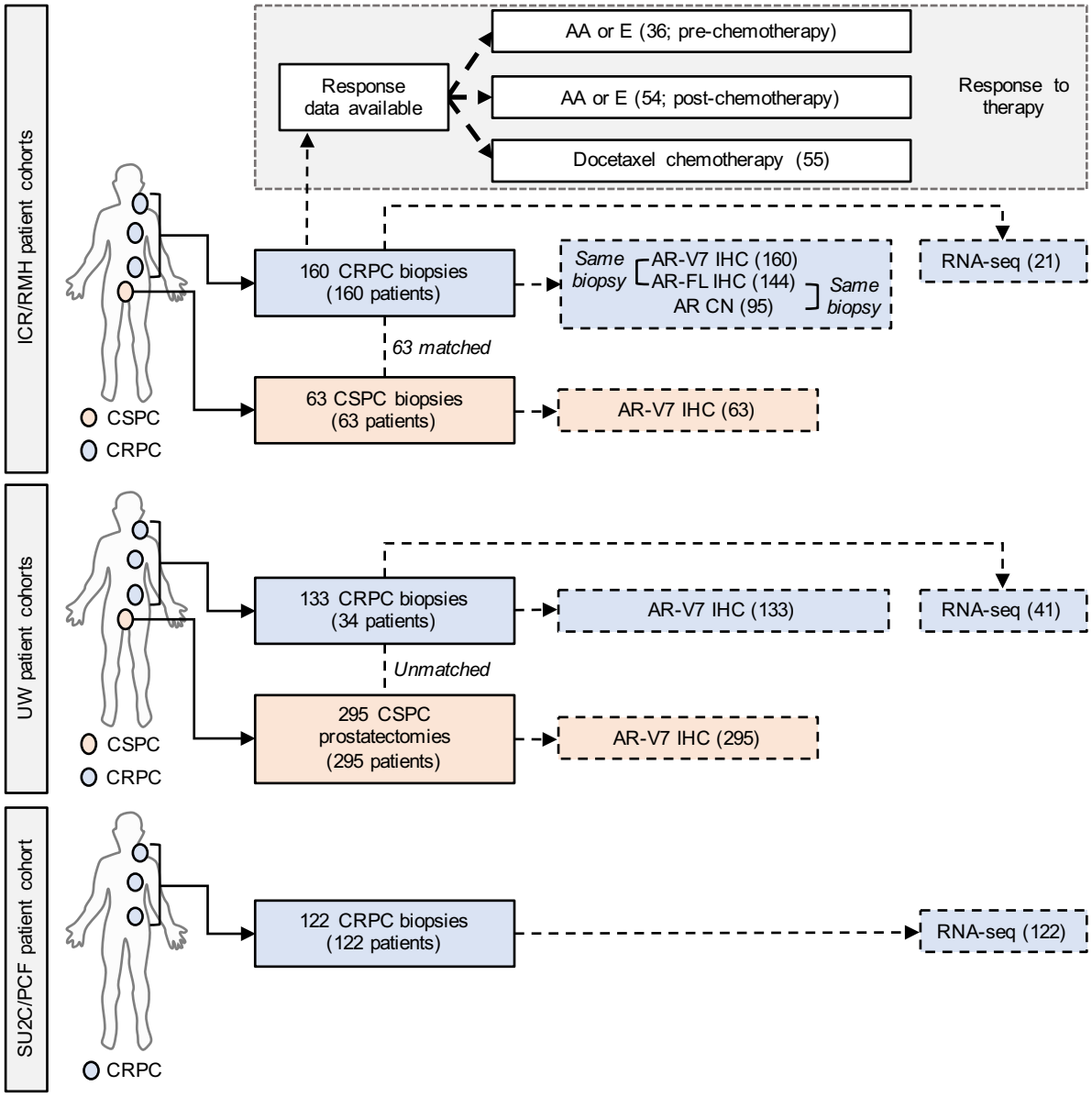

Figure 2: Summary of clinical samples analyzed.

Overview of the ICR/RMH, UW and SU2C/PCF patient cohorts utilized for this study. ICR/RMH patient cohort included 63 CSPC biopsies and 160 CRPC biopsies stained for nuclear AR-V7 expression. Of the 160 biopsies with AR-V7 expression; AR-FL expression (144 biopsies), AR copy number (95 biopsies) and RNA-seq (21 biopsies) was available. Response data was available for abiraterone acetate (AA) or enzalutamide (E) pre-chemotherapy (36 patients), AA or E post-chemotherapy (54 patients) and docetaxel chemotherapy (55 patients). UW patient cohort included 295 CSPC tissues (from 295 patients) who had radical prostatectomy as primary therapy and 133 CRPC biopsies of metastases (from 34 patients). Of 133 CRPC biopsies from 34 patients with AR-V7 expression; RNA-seq (41 biopsies) was available. SU2C/PCF patient cohort included 122 CRPC biopsies with RNA-seq analysis.

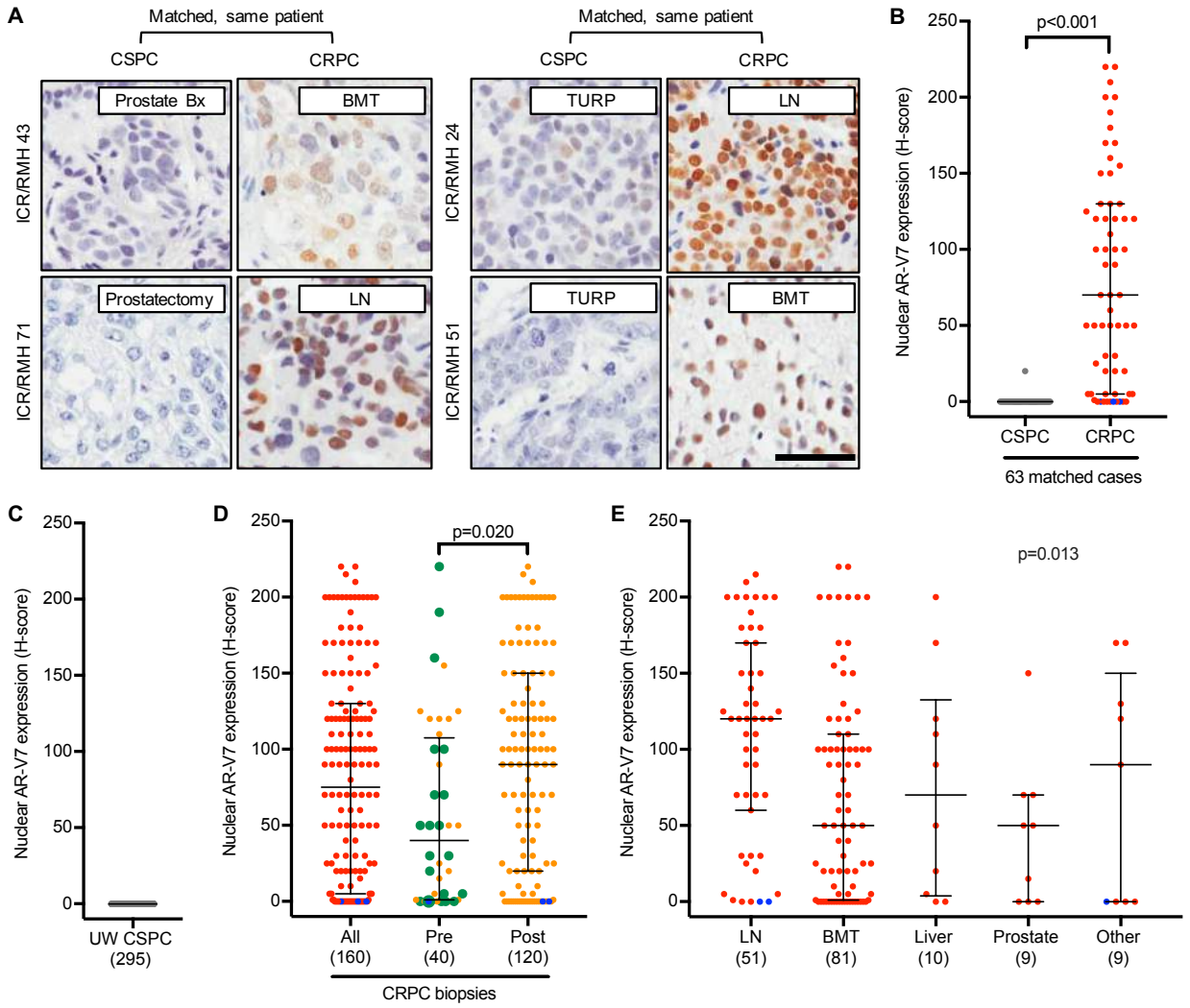

Figure 3: AR-V7 protein expression in prostate cancer.

(A) Representative micrographs of AR-V7 detection by immunohistochemistry (IHC) in four ICR/RMH patients with matched castration sensitive prostate cancer (CSPC) and castration resistant prostate cancer (CRPC) biopsies (magnification 200x; scale bar 50 µm). Prostate (Prostate Bx), prostatectomy, transurethral resection of the prostate (TURP), lymph node (LN) and bone marrow trephine (BMT) biopsies are shown. **(B)** Expression (H-score; HS) of nuclear AR-V7 expression in 63 same patient matched CSPC (grey) and CRPC (red) biopsies from ICR/RMH cohort. Three AR null CRPC cases with neuroendocrine features are shown (blue). Median HS and interquartile range is shown. p-value was calculated using Wilcoxon signed-rank test. **(C)** Expression (HS) of nuclear AR-V7 expression in 295 prostatectomy samples prior to any AR targeted therapy. Median HS and interquartile range is shown. **(D)** Expression (HS) of nuclear AR-V7 expression in 160 CRPC biopsies (red) and dichotomized (orange) by pre (40 biopsies) and post (120 biopsies) abiraterone acetate (AA) or enzalutamide (E) treatment. Three AR null CRPC cases with neuroendocrine features are shown (blue). Twenty biopsies taken after progression on primary ADT (with or without bicalutamide)

and prior to standard therapy for CRPC are shown (green). Median HS and interquartile range is shown. p-value was calculated using Mann-Whitney test. **(E)** Expression (HS) of nuclear AR-V7 expression in 160 CRPC biopsies (red) from lymph node (LN), bone (BMT), liver, prostate and other sites of metastases. Three AR null CRPC cases with neuroendocrine features are shown (blue). Median HS and interquartile range is shown. p-value was calculated using Nonparametric equality-of-medians test.

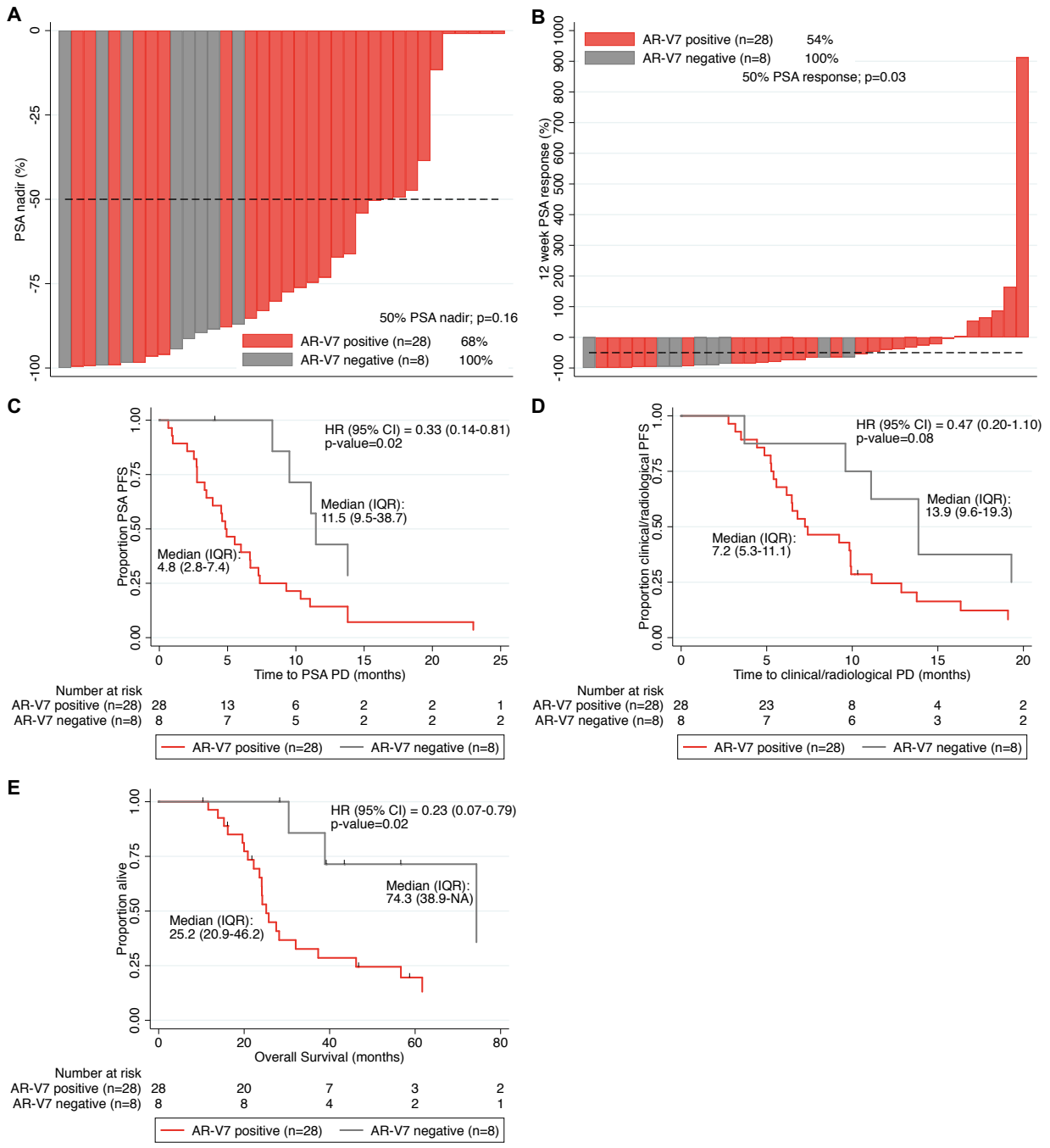

Figure 4: AR-V7 status and response to AR targeting therapies (abiraterone acetate or enzalutamide) prior to chemotherapy in castration resistant prostate cancer.

Thirty-six patients received AR targeting therapies (abiraterone acetate or enzalutamide) prior to chemotherapy for CRPC. **(A)** Percentage PSA nadir on AR targeting therapies for AR-V7 negative (H-score ≤ 10; grey) and AR-V7 positive (H-score > 10; red) CRPC patients is shown. 50% PSA nadir rate is shown. p-value was

calculated using Fisher's exact test. **(B)** Percentage 12-week 50% PSA response rate on AR targeting therapies for AR-V7 negative (grey) and AR-V7 positive (red) CRPC patients is shown. Twelve-week 50% PSA response rate is shown. p-value was calculated using Fisher's exact test. **(C-E)** Kaplan-Meier curves show time to PSA progression (C), time to clinical/radiological progression (D) and overall survival (E) from starting AR targeting therapy. Hazard ratios (HR) with 95% confidence intervals (CI) are shown. p-value was calculated using univariate Cox proportional hazards model.

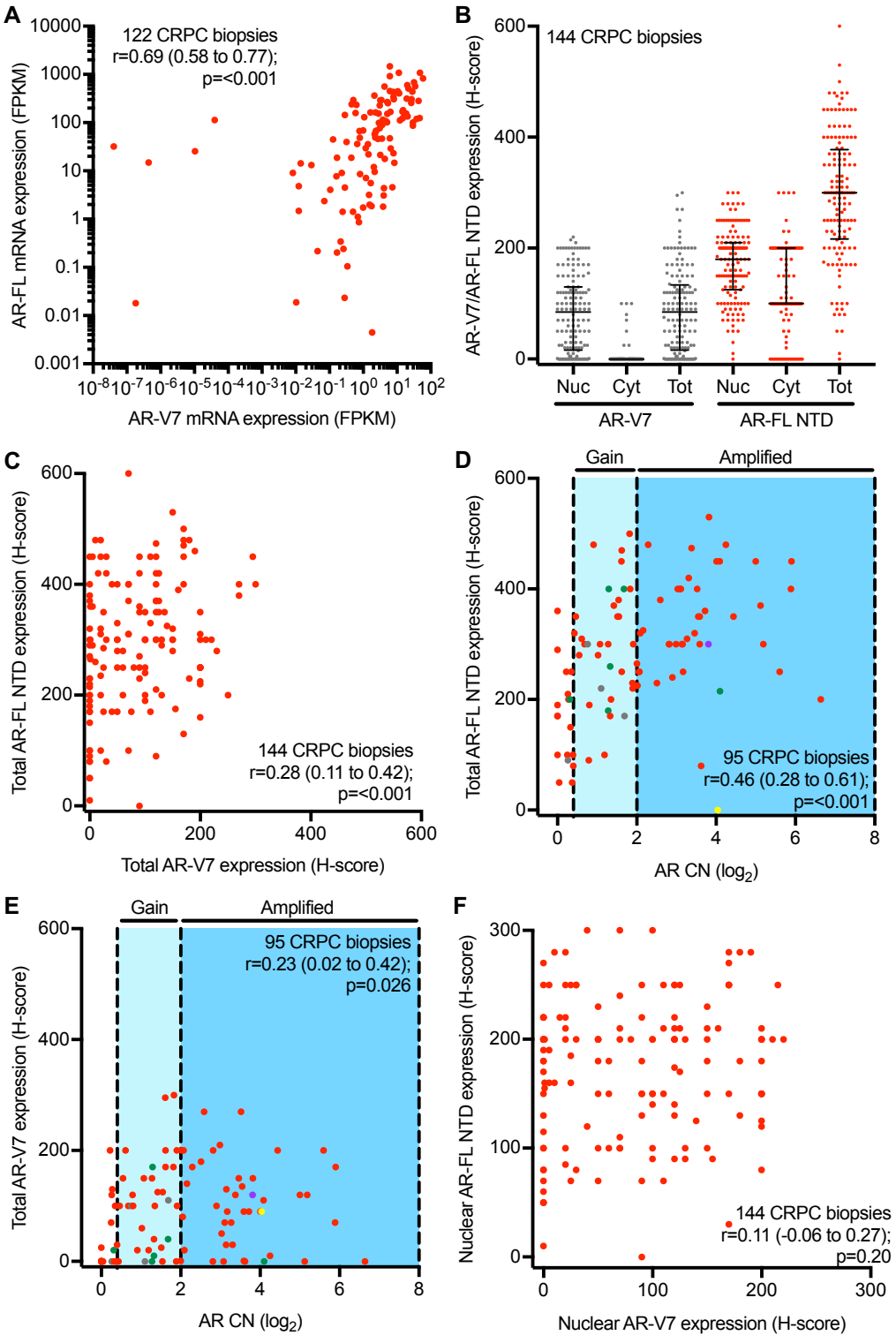

Figure 5: AR-FL and AR-V7 mRNA and protein quantification with AR copy number analysis in castration resistant prostate cancer.

(A) AR-FL and AR-V7 mRNA expression in fragments per kilobase of transcript per million mapped reads (FPKM) for 122 CRPC transcriptomes from the PCF/SU2C cohort is shown. Spearman's rank correlation is shown. **(B)** Expression (H-Score; HS) for nuclear, cytoplasmic and total (nuclear + cytoplasmic) AR-V7 (grey) and AR-FL N-terminal domain (NTD; red) is shown. Median HS and interquartile range is shown. **(C)** Expression (HS) of total AR-FL NTD protein and total AR-V7 protein in 144 CRPC biopsies from the ICR/RMH CRPC cohort is shown. Spearman's rank correlation is shown. **(D)** Expression (HS) of total AR-FL NTD protein and AR copy number (log_2) in 95 CRPC biopsies from the ICR/RMH CRPC cohort is shown. Cases with AR mutations are shown (L702H grey, T878A green, H875Y purple, K313E yellow). Spearman's rank correlation is shown. **(E)** Expression (HS) of total AR-V7 protein and AR copy number (log_2) in 95 CRPC biopsies from the ICR/RMH CRPC cohort is shown. Cases with AR mutations are shown. Spearman's rank correlation is shown. **(F)** Expression (HS) of nuclear AR-FL NTD protein and nuclear AR-V7 protein in 144 CRPC biopsies from the ICR/RMH CRPC cohort is shown. Spearman's rank correlation is shown.

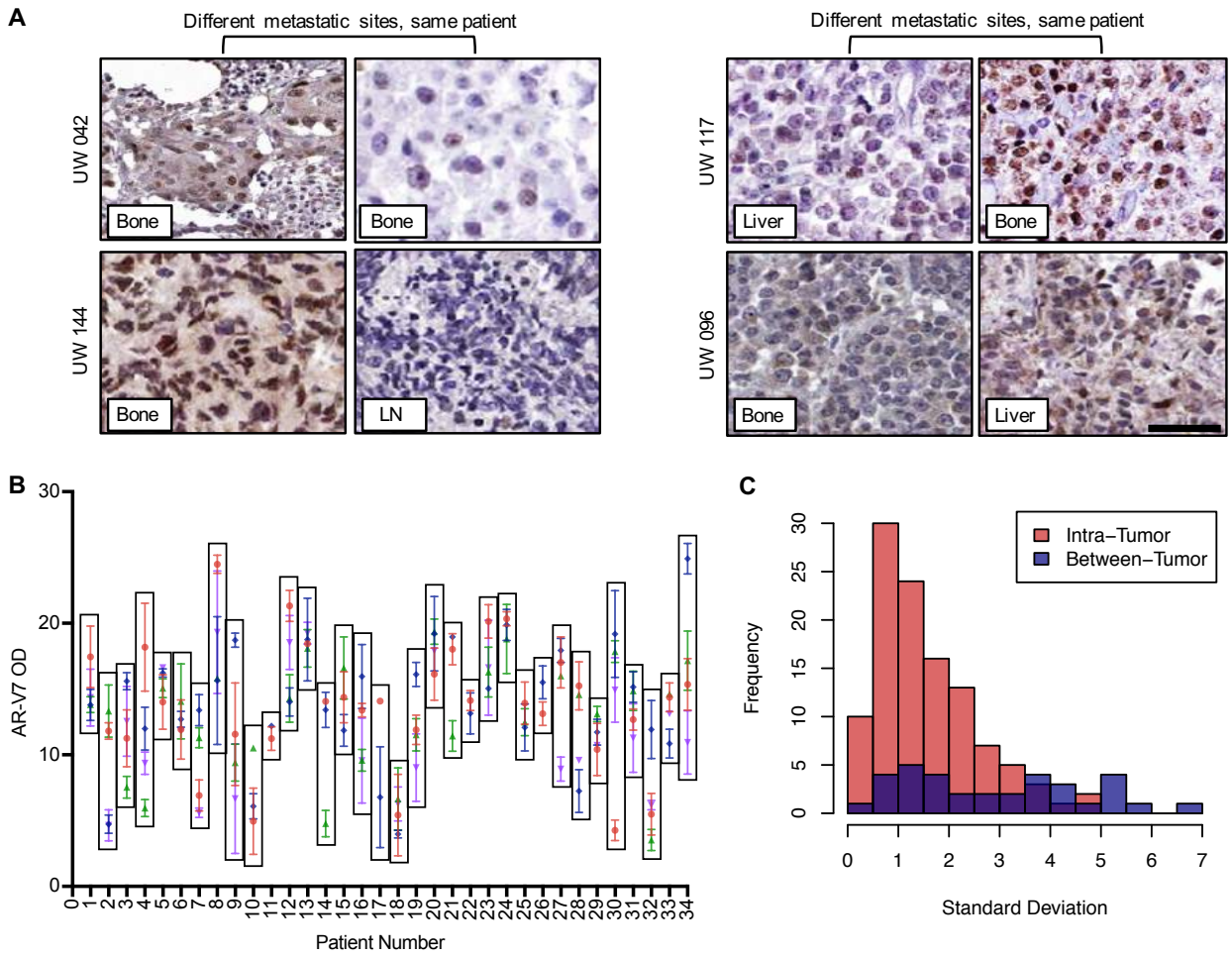

Figure 6: AR-V7 protein expression variability within metastasis and between metastases from individual patients with castration resistant prostate cancer.

(A) Representative micrographs of AR-V7 detection by immunohistochemistry in four UW patients with multiple castration resistant prostate cancer (CRPC) biopsies (magnification 200x; scale bar 50 μm). **(B)** Expression (optical density; OD) of nuclear AR-V7 expression in 133 metastases from 34 CRPC patients from UW CRPC cohort. Mean OD and standard deviation (SD) for three measurements from each metastasis is shown. Each box encloses all metastases from a patient. Different colors for each patient represent an individual metastasis. **(C)** Frequency distribution of SD within a metastasis (Intra-Tumor; comparison of triplicates in a metastasis; red) and between metastases (Between-Tumor; comparison of multiple metastasis within a patient; blue) is shown. Median SD for Intra-Tumor measurements is 1.2 and Between-Tumor measurements 2.9.

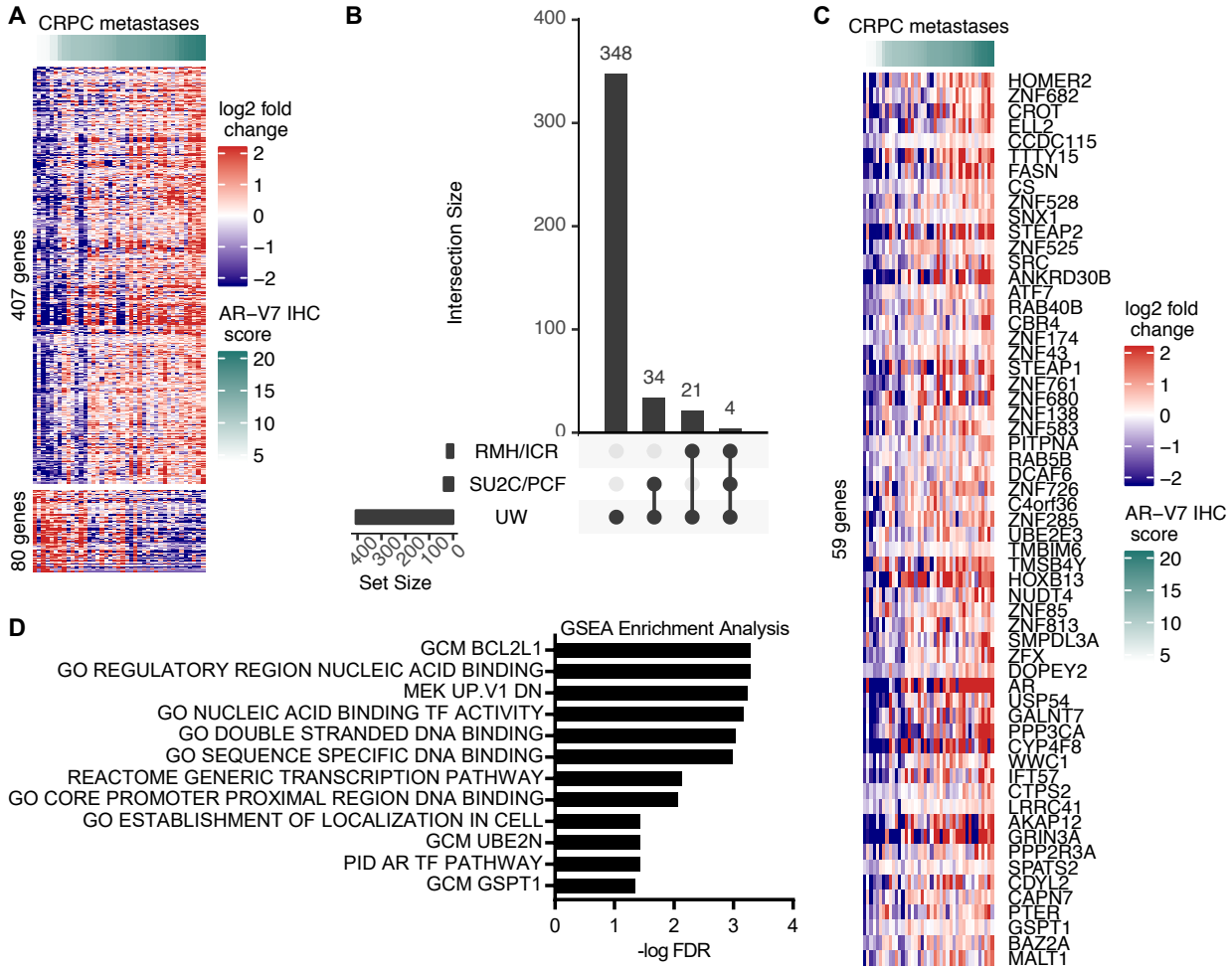

Figure 7: AR-V7 protein expression is associated with a unique gene signature in metastatic castration resistant prostate cancer.

(A) Expression (optical density; OD) of nuclear AR-V7 protein correlated (q<0.05) with gene mRNA expression of 487 (407 upregulated and 80 downregulated) genes in 41 metastases from 24 patients from UW CRPC cohort. Heatmap shows metastasis ranked in order of nuclear AR-V7 expression (OD) and mean-centered $\log_2$ fold change in gene mRNA expression. **(B)** Fifty-nine of the 407 upregulated genes were validated in either 21 CRPC metastasis from ICR/RMH CRPC cohort or 122 CRPC transcriptomes from SU2C/PCF cohort. Figure shows overlap of significantly correlated genes between the three cohorts. **(C)** Heatmap shows metastasis ranked in order of nuclear AR-V7 expression (OD) and mean-centered $\log_2$ fold change in gene mRNA expression of the 59-gene signature in the UW CRPC cohort (n = 41). **(D)** Pathway over-representation analysis using MSigDBv6.2 (H – Hallmark, CP – Canonical Pathways, C4 – Computational Gene Sets, C5 – GO and C6 – Oncogenic Pathway) in the 59-gene set. Pathways with FDR <0.05 are shown.

10. Table

	AR-V7 negative (H-score ≤ 10) N=8	AR-V7 positive (H-score > 10) N=28	p value
Age, years			
Mean (SD)	63.0 (4.8)	69.0 (7.5)	0.04[1]
Performance status, N (%)			
0	4 (50)	14 (50)	
1	4 (50)	14 (50)	1.00[2]
2	0 (0)	0 (0)	
>2	0 (0)	0 (0)	
Bloods			
Hb (g/L), Mean (SD)	133.0 (8.7)	127.6 (12.2)	0.25[1]
ALT (U/L), Mean (SD)	21.4 (6.1)	22.0 (8.5)	0.84[1]
ALP (U/L), Median (IQR)	90.0 (80.3-170.5)	90.0 (63.5-176.8)	0.83[3]
Albumin (g/L), Mean (SD)	36.5 (2.9)	38.4 (3.3)	0.16[1]
LDH (U/L), Median (IQR)	163.0 (144.0-169.0)^	168.0 (156.0-184.0)^^	0.29[3]
PSA (ng/mL), Median (IQR)	154.0 (8.9-238.3)	87.5 (35.5-272.5)	0.62[3]
Metastatic, N (%)			
Node only	3 (38)	4 (14)	
Visceral (with/without bone)	1 (13)	1(4)	0.12[2]
Bone	4 (50)	23 (82)	
AR therapy N (%)			
Abiraterone	5 (63)	19 (68)	1.00[2]
Enzalutamide	3 (38)	9 (32)	
Prior CRPC treatments, N (%)			
Abiraterone	0 (0)	0 (0)	
Enzalutamide	0 (0)	0 (0)	NA
Docetaxel	0 (0)	0 (0)	
Cabazitaxel	0 (0)	0 (0)	

Table 1: Baseline characteristics at time of staring AR targeting therapy (pre-chemotherapy).

N – number, SD – standard deviation, IQR – interquartile range, Hb – hemoglobin, ALT – alanine aminotransferase, ALP – alkaline phosphatase, LDH – lactate dehydrogenase, PSA – prostate specific antigen, NA – not applicable

^1 patient missing LDH value
^^1 patient missing LDH value

1 t-test
2 Fisher's exact test
3 Rank-sum test

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Research Paper

Ipilimumab plus nivolumab and DNA-repair defects in AR-V7-expressing metastatic prostate cancer

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ABSTRACT

AR-V7-expressing metastatic prostate cancer is an aggressive phenotype with poor progression-free survival (PFS) and overall survival (OS). Preliminary evidence suggests that AR-V7-positive tumors may be enriched for DNA-repair defects, perhaps rendering them more sensitive to immune-checkpoint blockade. We enrolled 15 metastatic prostate cancer patients with AR-V7-expressing circulating tumor cells into a prospective phase-2 trial. Patients received nivolumab 3 mg/kg plus ipilimumab 1 mg/kg every 3 weeks for four doses, then maintenance nivolumab 3 mg/kg every 2 weeks. Targeted next-generation sequencing was performed to determine DNA-repair deficiency (DRD) status. Outcomes included PSA response rates, objective response rates (ORR), PSA progression-free survival (PSA-PFS), clinical/radiographic PFS and OS. Median age of participants was 65, median PSA was 115 ng/mL, 67% had visceral metastases, and 60% had ≥4 prior systemic therapies. Six of 15 men (40%) had DRD mutations (three in *BRCA2*, two in *ATM*, one in *ERCC4*; none had microsatellite instability). Overall, the PSA response rate was 2/15 (13%), ORR was 2/8 (25%) in those with measurable disease, median PSA-PFS was 3.0 (95%CI 2.1–NR) months, PFS was 3.7 (95%CI 2.8–7.5) months, and OS was 8.2 (95%CI 5.5–10.4) months. Outcomes appeared generally better in DRD+ vs. DRD− tumors with respect to PSA responses (33% vs. 0%; *P*=0.14, nonsignificant), ORR (40% vs. 0%; *P*=0.46, nonsignificant), PSA-PFS (HR 0.19; *P*<0.01, significant), PFS (HR 0.31; *P*=0.01, significant), and OS (HR 0.41; *P*=0.11, nonsignificant). There were no new safety concerns. Ipilimumab plus nivolumab demonstrated encouraging efficacy in AR-V7-positive prostate cancers with DRD mutations, but not in the overall study population.

INTRODUCTION

Androgen-receptor splice variant 7 (AR-V7) is a constitutively-active isoform of the androgen receptor that is associated with a particularly aggressive form of advanced prostate cancer [1]. Because AR-V7 lacks the androgen-receptor ligand-binding domain, AR-V7-positive prostate cancers are generally resistant to novel hormonal therapies including abiraterone and enzalutamide [2, 3]. In addition, prostate cancers expressing AR-V7 often show poor responses to taxane chemotherapies including docetaxel and cabazitaxel [4, 5]. To this end, patients with AR-V7-positive prostate cancer generally have a median progression-free survival (PFS) of only 3-4 months and a median overall survival (OS) of 7-9 months. Therefore, developing effective therapies for AR-V7-expressing advanced prostate cancer represents an urgent unmet need.

Immune-checkpoint blockade may be one potential strategy to treat such patients. In many cancer types, inhibition of cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) and/or the programmed death 1 (PD-1) receptor has resulted in meaningful antitumor responses [6]. In some settings, combined blockade of both PD-1 (mediating T-cell exhaustion in peripheral tissues) and CTLA-4 (involved in earlier phases of T-cell activation) has proven more efficacious than inhibition of either pathway alone [7, 8]. Furthermore, tumors harboring DNA mismatch-repair defects or those with hypermutation may be particularly sensitive to immune-checkpoint inhibition [9, 10]. While prostate cancer is generally regarded as a low–mutation-burden tumor [11] and immune-checkpoint blockade has resulted in only modest benefits as a monotherapy [12, 13], recent data have suggested that AR-V7-expressing prostate cancers may be associated with a greater number of DNA-repair gene mutations and a higher mutation load [14].

We hypothesized that metastatic castration-resistant prostate cancer patients with AR-V7-positive circulating tumor cells (CTCs) would be susceptible to treatment with combined immune-checkpoint blockade, and that this approach would be safe and tolerable. We also sought to determine (in an exploratory fashion) whether treatment efficacy was associated with presence of DNA-repair gene mutations. To test these hypotheses, we conducted a phase-2 clinical trial testing ipilimumab plus nivolumab in patients with AR-V7-positive advanced prostate cancer.

RESULTS

Patient characteristics

From March 2016 through December 2016, a total of 36 patients underwent clinical-grade AR-V7 testing for eligibility purposes, 26 (72%) had detectable CTCs, and 16 men (44%) were AR-V7-positive. One patient failed screening, leaving 15 patients that comprised our study cohort. Supplementary Table 1 summarizes the baseline characteristics of the study participants. Median age was 65 years, 47% had ECOG performance-status of 1, median PSA was 115 ng/mL, 67% had visceral (liver or lung) metastases, and 60% had received ≥4 prior regimens for metastatic castration-resistant prostate cancer (mCRPC). All patients received at least one dose of the study drugs. At the time of data cutoff (October 2017), median follow-up was 8.6 (range, 1.9–17.9) months, and two patients remained alive.

Overall clinical outcomes

All patients were evaluable for efficacy (summarized in Table 1, Supplementary Figure 1). Overall, 2 of 15 men (13.3%, 95%CI 3.7–37.9%) achieved a PSA response. Among the 8 patients with measurable soft-tissue disease, the objective response rate (ORR) was 25.0% (95%CI 7.2–59.1%). Median PSA-PFS was 3.0 (95%CI 2.1–NR) months, and median PFS was 3.7 (95%CI 2.8–7.5) months. Three of 15 patients (20.0%, 95%CI 7.1–45.2%) achieved a "durable PFS". Median OS was 8.2 (95%CI 5.5–10.4) months.

DNA-repair defects and outcomes

Six of 15 patients (40%) harbored potentially deleterious somatic and/or germline mutations in a least one DNA-repair gene (Table 2, Supplementary Table 7C), and were considered DNA-repair deficient (DRD+). Patient 3 had a germline *BRCA2* mutation, patient 4 had somatic mutations in both *BRCA2* and *MSH6*, patient 6 had a somatic *ATM* mutation, patient 8 had a germline *BRCA2* and a somatic *FANCM* mutation, patient 9 had a somatic *ATM* mutation, and patient 14 had a somatic *ERCC4* mutation. Baseline characteristics and clinical outcomes of the DRD+ and DRD– patients are summarized in Supplementary Tables 1 and 2. Two patients (3 and 8) had germline mutations in *BRCA2*, and two patients (4 and 8) had biallelic *BRCA2* alterations resulting from LOH of the wild-type allele. No patient demonstrated microsatellite instability. Mean tumor mutational load was estimated at 3.2 (range, 0.8–7.8) mutations/Mb in DRD+ patients and 1.6 (range, 0.8–3.1) mutations/Mb in DRD– patients.

To further examine whether DNA-repair defects are enriched in AR-V7-positive patients, we interrogated the StandUp2Cancer (SU2C) database comprising whole-exome and transcriptome sequencing from 150 mCRPC biopsies [11], of which 143 had adequate RNA yields. Of these, 17.5% of cases (25/143) had AR-V7/AR-FL ratios on RNA sequencing of >10%, and were designated as AR-V7-high; while the remaining 82.5% (118/143) were designated as AR-V7-low. This threshold was set so that the prevalence of an AR-V7-positive tissue-based test would be broadly similar to that of a positive CTC-based AR-V7 test. To this end,

Table 1: Overall outcomes for all patients, and according to DNA-repair deficiency (DRD) status

	Overall (N=15)	DRD Negative (N=9)	DRD Positive (N=6)	HR (95%CI)	*P* value
PSA_{50}, N (%) (95% CI)	2/15 (13.3%) (3.7–37.9)	0/9 (0%) (0–29.9)	2/6 (33.3%) (9.7–70.0)	–	0.14
ORR, N (%) (95% CI)	2/8 (25.0%) (7.2–59.1)	0/3 (0%) (0–56.2)	2/5 (40.0%) (11.8–76.9)	–	0.46
Durable PFS (95% CI)	3/15 (20.0%) (7.1–45.2)	0/9 (0%) (0–29.9)	3/6 (50.0%) (18.8–81.2)	–	0.044
PSA-PFS (mo), (95% CI)	2.96 (2.07–NR)	2.07 (1.74–NR)	5.82 (4.24–NR)	0.19 (0.06–0.62)	0.0003
PFS (mo), (95% CI)	3.68 (2.76–7.52)	2.83(1.87–NR)	6.51 (3.88–NR)	0.31 (0.10–0.92)	0.014
OS (mo), (95% CI)	8.18 (5.52–10.41)	7.23 (3.45–NR)	9.04 (8.18–NR)	0.41 (0.14–1.21)	0.11

NR: upper 95% confidence limit of survival probability not reached.

pathogenic DRD mutations were found in 36.0% (9/25) of AR-V7-high cases but only in 18.6% (22/118) of AR-V7-low cases (*P*=0.056), suggesting a possible (but non-significant) association between AR-V7 and DNA-repair defects. In the AR-V7-high SU2C cohort, the altered DNA-repair genes were *BRCA2* (x4), *ATM* (x2), *CDK12* (x2) and *MSH2* (x1).

We then compared clinical outcomes in DRD+ and DRD– patients from our trial (Table 1). Response measures appeared generally better in DRD+ versus DRD– cases (Figure 1) with respect to PSA responses (33% vs. 0%; *P*=0.14, nonsignificant), ORR (40% vs. 0%; *P*=0.46, nonsignificant) and "durable PFS" (50% vs. 0%; *P*=0.04, significant). Interestingly, both patients who achieved PSA responses (4 and 8) had biallelic *BRCA2* alterations. Similarly, time-to-event outcomes also appeared better in DRD+ versus DRD– patients (Figure 2) with respect to PSA-PFS (HR 0.19, 95%CI 0.06–0.62; *P*<0.001, significant), PFS (HR 0.31, 95%CI 0.10–0.92; *P*=0.01, significant), and OS (HR 0.41, 95%CI 0.14–1.21; *P*=0.11, nonsignificant).

Other biomarkers and outcomes

To examine the prognostic impact of CTC phenotypic heterogeneity, we compared outcomes in patients with a high (≥1.5) versus low (<1.5) Shannon index (Supplementary Table 3). Five (33%) and 10 men (67%) were classified as Shannon-high and Shannon-low, respectively. There were numerically more Shannon-high cases among DRD+ compared to DRD– patients (50% [3/6] vs. 22% [2/9] respectively, *P*=0.26, nonsignificant). Outcomes appeared generally better in Shannon-high vs. Shannon-low patients with respect to PSA responses (20% vs. 10%; *P*=1.0, nonsignificant), ORR (100% vs. 0%; *P*=0.04, significant), "durable PFS" (40% vs. 10%; *P*=0.24, nonsignificant), PSA-PFS (HR 0.67, 95%CI 0.23–1.99; *P*=0.44, nonsignificant), PFS (HR 0.43, 95%CI 0.15–1.22; *P*=0.11, nonsignificant), and OS (HR 0.34, 95%CI 0.11–0.99; *P*=0.07, nonsignificant) (Figure 3). Interestingly, both men with RECIST-defined objective responses (6 and 14) had high Shannon indices. CTC pleomorphism (high vs. low) was also assessed in relation to clinical outcomes. No statistical trends were observed (Supplementary Table 4, Supplementary Figure 2), although both patients with PSA responses (4 and 8) were classified as pleomorphism-high.

Eight patients underwent new metastatic biopsies and were evaluable for PD-L1 status. Five (62%) and 3 men (38%) were PD-L1–positive and -negative, respectively. Representative immunostains are shown in Supplementary Figure 3. There were numerically more PD-L1–positive cases among DRD+ compared to DRD– tumors (80% [4/5] vs. 33% [1/3] respectively, *P*=0.19). No statistical trends between PD-L1 status and clinical outcomes were observed (Supplementary Table 5, Supplementary Figure 4), although both patients with objective responses (6 and 14) had PD-L1–expressing tumors.

Safety and adverse events

The most common toxicities that developed during or after treatment were fatigue, AST elevation, diarrhea and anorexia (Supplementary Table 6). Seventeen grade 3-4 adverse events occurred in 7 of 15 patients (46%). There were two cases of grade 3-4 fatigue, two cases of grade 3-4 diarrhea/colitis, and two cases of grade 3-4 elevated lipase. Immune-related adverse events were of

Table 2: Summary of DNA-repair deficiency (DRD) status among the 15 patients treated with ipilimumab plus nivolumab

Patient no.	DRD status	DNA-repair gene	Pathogenic DNA-repair mutations	Germline vs. somatic	Loss of heterozygosity (LOH)	MSI markers shifted	Mutational load (muts/Mb)	Source of tumor DNA
1	–	-	-	-	-	N/A	1.1	Plasma
2	–	-	-	-	-	N/A	2.4	Prostate
3	+	*BRCA2*	E1646Qfs*23	Germline	No	0/5	1.6	Liver mass
4	+	*BRCA2* *MSH6*	P3189H E192X	Somatic Somatic	Yes No	0/5	7.8	Lymph node
5	–	-	-	-	-	N/A	3.1	Plasma
6	+	*ATM*	D2708N	Somatic	No	0/5	1.6	Lymph node
7	–	-	-	-	-	0/5	1.4	Epidural mass
8	+	*BRCA2* *FANCM*	D3095E R579H	Germline Somatic	Yes No	0/5	0.8	Prostate
9	+	*ATM*	E2039X	Somatic	No	0/5	1.1	Plasma
10	–	-	-	-	-	N/A	1.1	Plasma
11	–	-	-	-	-	0/5	1.3	Prostate
12	–	-	-	-	-	0/5	0.8	Prostate
13	–	-	-	-	-	0/5	1.3	Lymph node
14	+	*ERCC4*	D762V	Somatic	No	0/5	5.6	Lymph node
15	–	-	-	-	-	0/5	1.8	Liver mass

particular interest. There were five events (affecting 33% of patients) that were possibly or probably related to autoimmune phenomena and that required treatment with corticosteroids: two episodes of colitis, two episodes of pneumonitis, and one episode of hepatitis; hypophysitis was not observed. There were no treatment-related deaths.

DISCUSSION

Prostate cancer expressing AR-V7 represents a lethal phenotype with inadequate treatment options. Here, we report data from the first trial specifically targeting AR-V7-positive disease and the first trial of ipilimumab plus nivolumab in prostate cancer. Although sufficient clinical activity was not observed in the overall study population (and the primary endpoint was not met), encouraging clinical activity using combined immune-checkpoint blockade was seen in the subset of patients harboring germline and/or somatic mutations in DNA-repair genes (and not restricted to mismatch-repair genes). Moreover, there appeared to be a positive correlation between AR-V7 detection and the presence of sequence alterations in DNA-repair genes, further supporting an immunotherapy approach in these patients.

It is now appreciated that approximately 20-25% of metastatic prostate cancers harbor somatic mutations involving DNA-repair genes, primarily homologous-recombination repair genes (e.g. *BRCA2*, *ATM*) and, to a lesser extent, mismatch-repair genes (e.g. *MSH2*, *MSH6*) [11, 21]. The current study, coupled with our secondary analysis of the StandUp2Cancer dataset, suggests that DNA-repair defects (DRD) may be further enriched in AR-V7-positive prostate cancers with a prevalence approaching 40%. These DRD+ patients may benefit from alternative treatment strategies including poly–ADP-ribose polymerase (PARP) inhibitors [22] or other genetically-targeted approaches [23, 24]. The potential association between AR-V7 detection and DRD mutations has also been suggested by a previous study,[14] but still requires further confirmation.

The correlation between DNA mismatch-repair deficiency (resulting in microsatellite instability) and

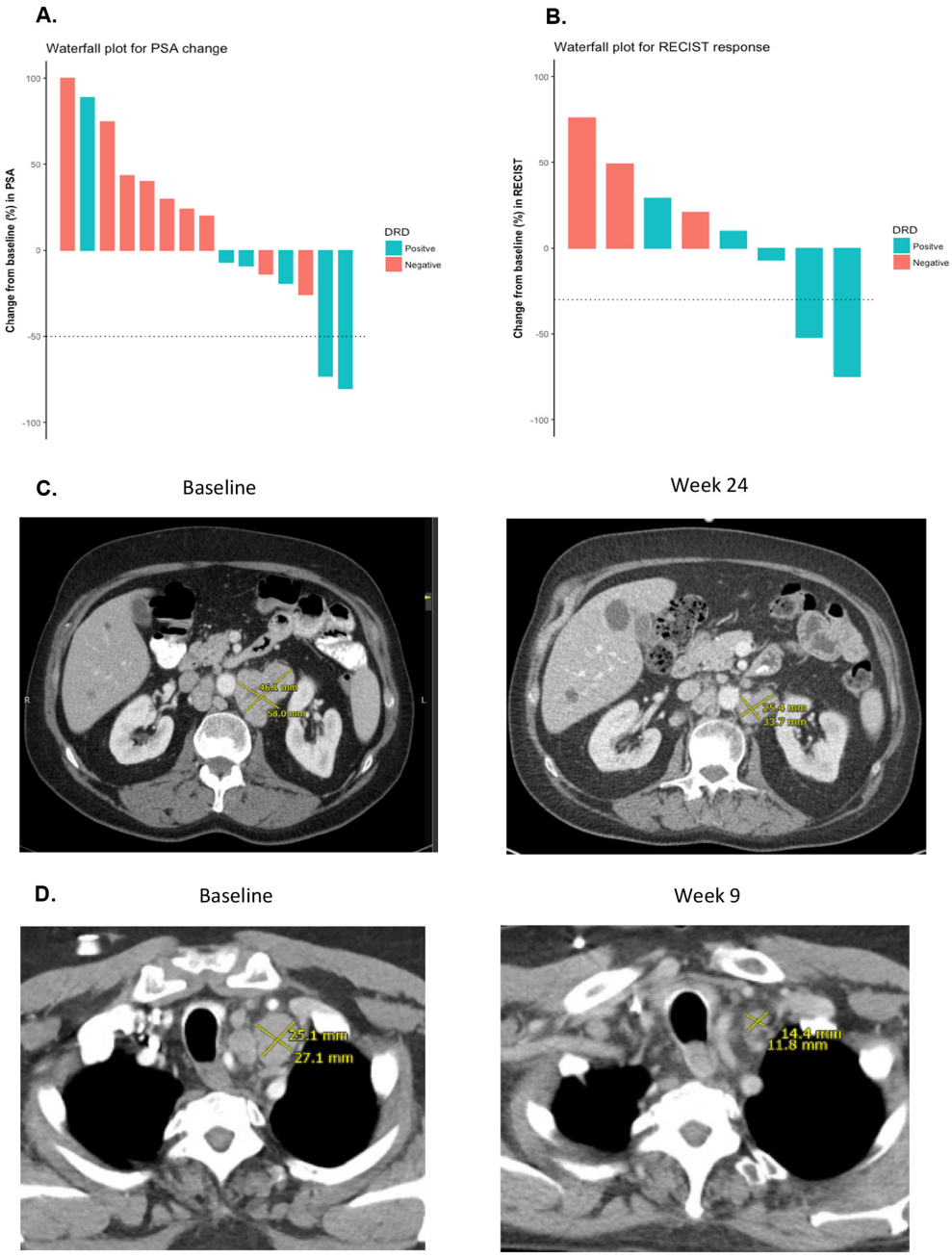

Figure 1: PSA responses and radiographic responses according to DRD status. (A) Waterfall plot showing PSA responses according to DRD status. The two patients with PSA_{50} responses (#4 and #8) both had biallelic *BRCA2* gene mutations. Patient #4 had a mixed soft-tissue response (some measurable lesions decreased while others increased) and achieved a durable PFS. Patient #8 did not have any measurable disease, but also achieved a durable PFS, and experienced complete resolution of malignant bone pain (pain score 7/10 decreased to 0/10 after 12 weeks of therapy); he is still alive after 17.5+ months of follow-up. **(B)** Waterfall plot showing objective RECIST responses according to DRD status. The two patients with soft-tissue responses (#6 and #14) had mutations in *ATM* and *ERCC4*, respectively. Patient #6 achieved a durable PFS, and is still alive after 17.9+ months of follow-up. **(C)** CT scan of radiographic response for patient #6 (with somatic *ATM* mutation) at baseline and after 24 weeks of treatment. The sum diameter of his target lesions decreased by 52% at the time of his best response. **(D)**. CT scan of radiographic response for patient #14 (with somatic *ERCC4* mutation) at baseline and after 9 weeks of treatment. The sum diameter of his target lesions decreased by 75% at the time of his best response.

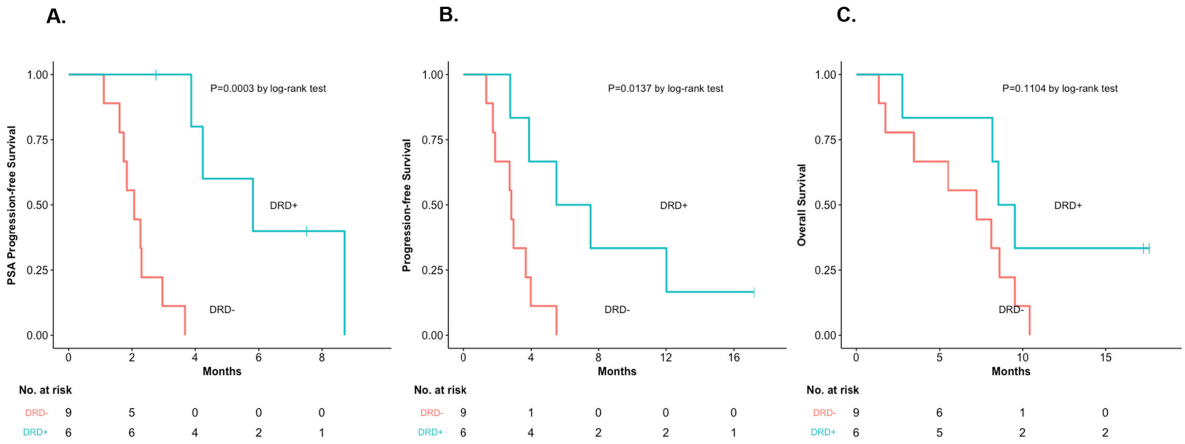

Figure 2: Time-to-event outcomes, according to DRD status. **(A)** PSA-PFS, according to DRD status [HR 0.19, 95%CI 0.06–0.62, *P*=0.0003]. **(B)** PFS, according to DRD status [HR 0.31, 95%CI 0.10–0.92, *P*=0.014]. **(C)** OS, according to DRD status [HR 0.41, 95%CI 0.14–1.21, *P*=0.11].

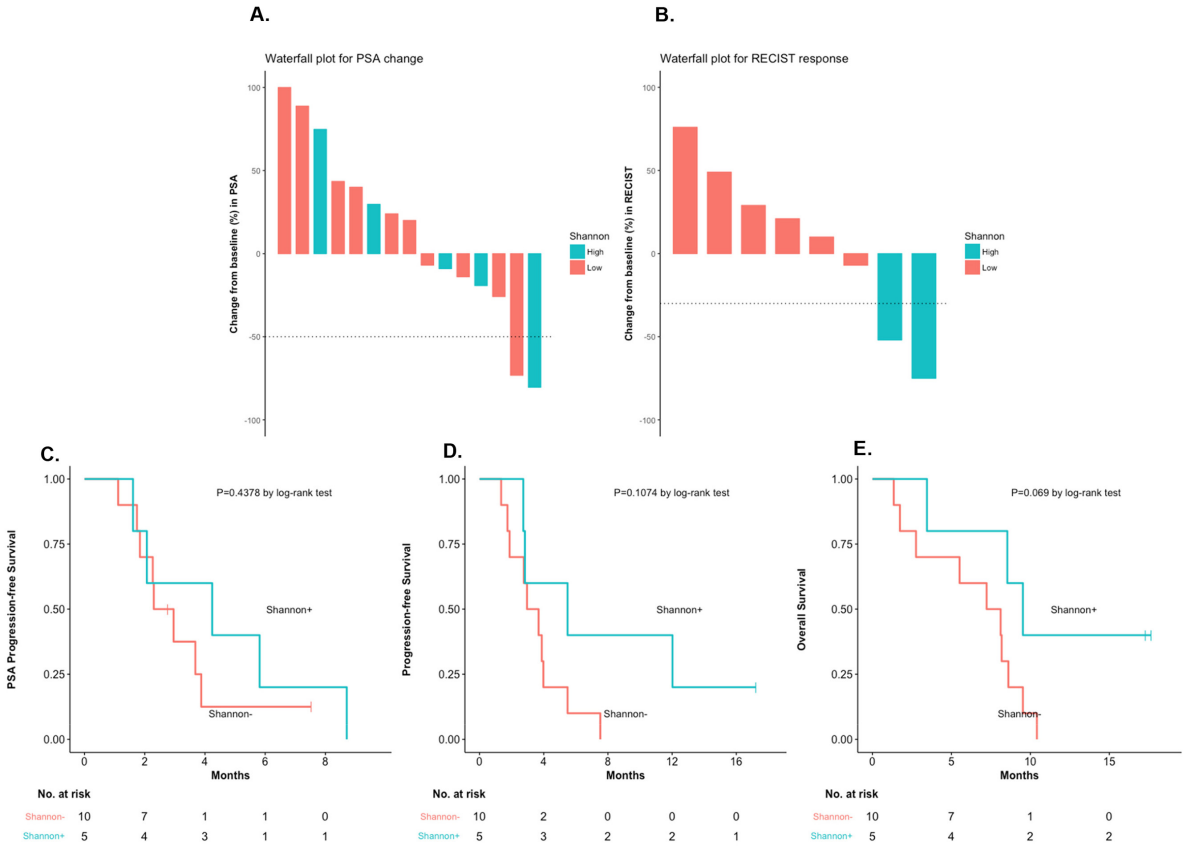

Figure 3: Clinical outcomes, according to Shannon index (low vs. high). **(A)** PSA responses, according to Shannon Index. **(B)** RECIST responses, according to Shannon index. **(C)** PSA-PFS, according to Shannon index [HR 0.67, 95%CI 0.23–1.99, *P*=0.44]. **(D)** PFS, according to Shannon index [HR 0.43, 95%CI 0.15–1.22, *P*=0.11]. **(E)** OS, according to Shannon index [HR 0.34, 95%CI 0.11–0.99, *P*=0.07].

responsiveness to PD-1 inhibitor therapy is now well established, although MMR mutations are only observed in 2-3% of advanced prostate cancers [9]. Our data suggest that sensitivity to immune-checkpoint inhibitors may perhaps be expanded to other types of DNA-repair alterations, particularly homologous-recombination deficiency (HRD) mutations. Among the six DRD+ patients in this study, five had HRD lesions (three in *BRCA2*, two in *ATM*) and one had a nucleotide-excision repair (*ERCC4*) lesion. Interestingly, mean tumor mutational burden was approximately 2-fold higher in DRD+ versus DRD– cancers, although none of these patients demonstrated microsatellite instability. These findings are consistent with two prior studies (including one in prostate cancer) that reported a modestly higher mutational load in *BRCA2*-mutant vs. wild-type tumors [25, 26]. Two other studies in *BRCA1*-deficient breast cancers and *BRCA1/2*-deficient ovarian cancers, respectively, demonstrated that these tumors may have higher predicted neoantigen loads, more tumor-infiltrating lymphocytes and increased expression of PD-1 and CTLA-4 as compared to their homologous-repair–proficient counterparts [27, 28]. Furthermore, a recent study combining durvalumab (a PD-L1 inhibitor) with olaparib (a PARP inhibitor) in mCRPC patients reported high response rates in men with HRD mutations [29]. Finally, a recent clinical study in advanced urothelial carcinoma suggested that outcomes to PD-1 or PD-L1 inhibitors were superior in patients with vs. without HRD mutations [30]. Taken together, these data imply that HRD alterations, not just MMR alterations, may sensitize patients to immune-checkpoint blockade. In addition, the current study is the first to suggest that defects in nucleotide-excision repair (e.g. *ERCC4*) may also be associated with immunotherapy sensitivity.

We also observed a trend between high phenotypic CTC heterogeneity (Shannon index) and favorable responses to combination immunotherapy. In addition, DRD+ patients demonstrated a trend towards higher CTC heterogeneity compared to DRD– patients. Previous studies showed that mCRPC patients with Shannon-high CTCs respond poorly to novel hormonal therapies and better to taxane chemotherapies [20]. Interestingly, the two patients with the highest Shannon indices (6 and 14) both had objective tumor responses, both harbored DRD alterations, and both expressed PD-L1. This suggests a theoretical model whereby DRD mutations result in greater genomic heterogeneity, manifesting as greater phenotypic CTC heterogeneity, and increasing the likelihood of a favorable response to immune-checkpoint inhibition. This hypothesis remains to be proven.

In conclusion, our data suggest that the combination of nivolumab plus ipilimumab demonstrates acceptable safety and encouraging efficacy in men with AR-V7-expressing advanced prostate cancer who also harbor DNA-repair alterations, but not in the overall study population. Moreover, the prevalence of these DNA-repair defects appears to be higher in AR-V7-positive patients. Both of these findings require large-scale prospective validation.

MATERIALS AND METHODS

Patient eligibility

Eligible patients had histologically confirmed, progressive, metastatic castration-resistant prostate cancer (mCRPC) with detectable AR-V7 transcripts using the Johns Hopkins CTC-based clinical-grade AR-V7 assay (see below) [15, 16]. Additional eligibility criteria included an ECOG performance-status of 0-1, at least 18 years of age, serum testosterone <50 ng/dL with ongoing androgen-deprivation therapy, adequate organ (liver, kidney, bone marrow) function, and availability of new or archival tumor tissue for biomarker analysis. Key exclusion criteria included a second active malignancy within 5 years, prior immune-checkpoint inhibitor therapy, active brain or meningeal metastases, history of autoimmune disease, or requirement for systemic corticosteroids. Complete eligibility criteria are available in the Supplementary Materials.

Study design

This was a single-institution one-arm open-label phase 2 study conducted at Johns Hopkins. Patients received treatment by intravenous infusion consisting of 3 mg per kilogram of nivolumab plus 1 mg per kilogram of ipilimumab every 3 weeks for 4 doses, followed by a maintenance regimen of 3 mg per kilogram of nivolumab every 2 weeks thereafter. Treatment continued until radiographic progression, unequivocal clinical progression, development of unacceptable toxicity, or withdrawal of consent. Suspected immune-related toxicities were managed using available guidelines. Patients were not permitted to receive nivolumab maintenance therapy unless they tolerated all four doses of combination immunotherapy.

The primary endpoint was the PSA response rate, defined as a ≥50% decline in PSA from baseline maintained for ≥4 weeks. Secondary endpoints included freedom-from-PSA-progression (PSA-progression-free-survival; PSA-PFS), freedom-from-clinical/radiographic-progression (progression-free-survival; PFS), objective response rate (ORR) according to RECIST1.1 criteria [17] in patients with measurable disease, PFS lasting >24 weeks (termed "durable PFS"), and overall survival (OS). PSA-progression was defined as a ≥25% increase in PSA from baseline or nadir, requiring confirmation ≥4 weeks later (PCWG2 criteria [18]). Clinical/radiologic-progression was defined as unequivocal symptomatic progression (worsening disease-related symptoms or new

cancer-related complications), or radiographic progression (CT scan showing ≥20% enlargement in sum diameter of soft-tissue target lesions [RECIST1.1]; bone scan showing ≥2 new osseous lesions not related to bone flare) or death, whichever occurred first. Safety and adverse effects were also assessed.

Study assessments were prospectively defined. PSA measurements were obtained at baseline and every 4 weeks on study. Radiographic evaluations (CT of chest/abdomen/pelvis and technetium-99 bone scans) were performed at baseline and every 12 weeks. Physical examination, toxicity assessments, and laboratory studies (complete blood count, comprehensive metabolic panel, thyroid function) were performed every 4 weeks. Safety was assessed by collecting and grading adverse events according to CTCAE v4.0 criteria.

This was an investigator-initiated trial (NCT02601014) designed by the principal investigators (E.S.A. and C.G.D.) and funded by Bristol Myers-Squibb who also provided both study drugs free of cost. The study was approved by the Johns Hopkins University IRB, and was overseen by an independent scientific review committee and an independent data and safety monitoring committee. All patients provided written informed consent before participation.

DNA sequencing

All 15 patients underwent prospective tumor DNA sequencing. Details of targeted next-generation sequencing methods performed on pre-treatment tumor, matched normal and circulating-tumor (ct)DNA samples, and bioinformatic analyses, are provided in the Supplementary Materials. We performed targeted sequencing on 8 matched tumor-normal and 3 tumor-only cases (Supplementary Table 7A). In 4 patients, where tumor tissue was not available, we performed next-generation sequencing of cell-free ctDNA (Supplementary Table 7B). In addition to examining sequence alterations and microsatellite instability, we generated estimates of mutation burden for each tumor. We subsequently focused on sequence alterations in DNA-repair genes, identified somatic and germline variants and assessed allele-specific copy-number and loss-of-heterozygosity events for these loci. Putative pathogenic variants were determined by an ensemble of bioinformatic platforms, as described in the Supplementary Materials. To correlate genomic findings with clinical outcomes, patients were classified as "positive" or "negative" for potentially pathogenic mutations in DNA-repair genes. Patients were considered to be DNA repair-deficient (DRD-positive [DRD+]) if they had at least one pathogenic mutation in a gene involved in DNA-damage repair [22]; otherwise they were classified as DRD-negative (DRD–).

AR-V7 and CTC analyses

A modified AdnaTest assay (Qiagen, Hannover, Germany) conducted in our CLIA-certified laboratory was used to interrogate CTCs for AR-V7 mRNA detection [15], and a positive test was required for eligibility. Briefly, this employs EpCAM-based CTC capture followed by multiplexed reverse-transcription polymerase-chain-reaction (qRT-PCR) using custom primers to detect full-length androgen receptor (AR-FL) mRNA and AR-V7 mRNA, as previously described [2, 15]. In addition, all patients underwent collection of CTCs at baseline using the Epic Sciences platform (San Diego, CA) [19], and these cells were analyzed for phenotypic heterogeneity (Shannon index)[20] and degree of pleomorphism, as described in the Supplementary Materials. Clinical outcomes were compared among patients with high versus low CTC heterogeneity and high versus low pleomorphism.

PD-L1 analysis

In patients undergoing a new metastatic tumor biopsy, expression of PD-L1 protein was assessed using immunohistochemistry (rabbit monoclonal antibody, Ventana, Tucson, AZ), as described in the Supplementary Materials. A positive test was defined as any percentage of PD-L1 staining on tumor cells.

Statistical analyses

The primary endpoint was PSA response, and a response rate above 5% was considered clinically meaningful in this AR-V7-positive population. Accordingly, a sample size of 15 patients with ≥3 PSA responses would produce a 90% confidence interval of 6–44%, which would be above the 5% threshold. A positive study would therefore be defined as ≥3 of 15 patients achieving a PSA response.

Analyses of response endpoints (*e.g.* PSA response, ORR) were expressed as proportions with 2-sided Wilson binomial 95% confidence intervals. Time-to-event endpoints (*e.g.* PFS, OS) were analyzed using the Kaplan-Meier method and 95% confidence intervals were generated using the generalized Brookmeyer-Crowley method after log-transformation. Clinical outcomes were compared among patients who were DRD+ and DRD– (primary biomarker analysis), as well as according to other biomarker categories (CTC heterogeneity, CTC pleomorphism, tumor PD-L1 expression). To examine associations between clinical outcomes and biomarker status, response endpoints were compared using Fisher's exact test, and time-to-event endpoints were compared using the log-rank test with Cox proportional-hazards models to derive hazard

ratios. All tests were two-sided, and P values ≤0.05 were considered significant; we did not correct for multiple hypotheses. Statistical analyses were performed using *R* (version 3.4.3).

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CONFLICTS OF INTEREST

E.S.A. is a paid consultant/advisor to Janssen, Astellas, Sanofi, Dendreon, Medivation, ESSA, AstraZeneca, Clovis and Merck; has received research funding to his institution from Janssen, Johnson & Johnson, Sanofi, Dendreon, Genentech, Novartis, Tokai, Bristol Myers-Squibb, AstraZeneca, Clovis and Merck; and is the co-inventor of a biomarker technology that has been licensed to Qiagen. S.A.T. has served as a consultant and received honoraria from Roche/ Ventana Medical Systems, Almac Diagnostics, Janssen, AbbVie and Astellas/Medivation; he is also a co-founder of, consultant for and Laboratory Director of Strata Oncology. Y.W., A.J., R.P.G. and R.D. are employees of Epic Sciences. V.E.V. is a founder of Personal Genome Diagnostics (PGDx), is a member of its Scientific Advisory Board and Board of Directors, and owns PGDx stock, which is subject to certain restrictions under university policy; he is also on the Scientific Advisory Board for Ignyta; the terms of these arrangements are managed by the Johns Hopkins University in accordance with its conflict of interest policies. The remaining authors disclose no relevant conflicts of interest.

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