

AWARD NUMBER: W81XWH-16-1-0484

TITLE: Pharmacologic Dose Testosterone to Treat Castration-Resistant Prostate Cancer:
Mechanisms of Action and Drivers of Response

PRINCIPAL INVESTIGATOR: Michael Schweizer

CONTRACTING ORGANIZATION:

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REPORT DATE: October 2018

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE October 2018		2. REPORT TYPE Annual		3. DATES COVERED 30 Sep 2017-29 Sep 2018	
4. TITLE AND SUBTITLE Pharmacologic Dose Testosterone to Treat Castration-Resistant Prostate Cancer: Mechanisms of Action and Drivers of Response				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-16-1-0484	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Michael Schweizer E-Mail: schweize@uw.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Washington 4333 Brooklyn Ave Ne Seattle Wa 98195-0001				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Purpose: Single-arm studies have demonstrated preliminary signs of efficacy for intermittent pharmacologic dose testosterone (i.e. Bipolar Androgen Therapy; BAT) in treating advanced prostate cancer. In this project, we will conduct detailed molecular assessments on biospecimens (i.e. blood, metastatic tissue) from men receiving BAT to determine somatic and germline factors that predict for response/resistance. We will also evaluate additional PDT-based regimens (e.g. combinatorial treatments) in preclinical models. Scope: This annual technical progress report details progress made during the first year of funding for this project (30 Sep 2017 – 1 Oct 2018). Major Findings: During Year 2 we have continued to focus on biospecimen acquisition from men enrolled to a Phase II study testing BAT vs. enzalutamide. Results from correlative studies are not available at this time. Preclinical studies are ongoing.					
15. SUBJECT TERMS Castration-resistant prostate cancer, testosterone, bipolar androgen therapy, supraphysiologic testosterone, biomarker, mechanism of action					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT Unclassified	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified			19b. TELEPHONE NUMBER (include area code)

TABLE OF CONTENTS

	<u>Page</u>
1. Introduction	4
2. Keywords	4
3. Accomplishments	4
4. Impact	8
5. Changes/Problems	8
6. Products	8
7. Participants & Other Collaborating Organizations	9
8. Appendices	10

1. INTRODUCTION:

The androgen receptor (AR) is frequently upregulated as prostate cancer (PC) adapts to a low androgen environment – likely driving resistance to AR-signaling inhibition. Interestingly, some adapted PC cell lines display blunted growth when exposed to high androgen levels, potentially due to the induction of dsDNA breaks and errors in DNA relicensing. We hypothesized that the adaptive autoregulation of AR may serve as a therapeutic liability; sensitizing PC cells to supraphysiologic testosterone (SPT) induced cell death. To explore this concept further, we designed a mode of SPT therapy termed Bipolar Androgen Therapy (BAT), whereby men with castration-resistant prostate cancer (CRPC) are treated intermittently with very high Pharmacologic Dose Testosterone (PDT). In a proof of concept study, we showed that BAT resulted in PSA and radiographic responses in ~50% of men. However, this study did not incorporate biospecimen acquisition that would have allowed us to determine the molecular events driving these clinical responses. Recently, a large randomized trial testing BAT vs. enzalutamide in men with CRPC was launched. As part of our participation in this study, we will obtain blood and metastatic biopsies from men receiving BAT in order to identify biomarkers that predict for response/resistance to BAT, and understand the mechanisms of action underlying these responses.

2. KEYWORDS:

Castration-resistant prostate cancer, testosterone, bipolar androgen therapy, supraphysiologic testosterone, biomarker, mechanism of action

3. ACCOMPLISHMENTS:

Major goals of the project:

Major goals of the project as indicated in the Statement of Work. Milestones/target dates for important subtasks, with completion dates or percentage of completion. Note: months are from the start of the funding period (9/30/2016).

Training-Specific Tasks:

Major Task: Training and educational development in prostate cancer research	Months	Percent completed (date of completion)
Subtask 1: Audit select courses	24-48	0%
Subtask 2: Attend UW training seminars	1-18	100%
Subtask 3: Attend UW conferences/tumor boards	1-48	50%
Subtask 4: Attend National Conferences/Committees	1-48	50%
Major Task: Training and educational development in prostate cancer research	Months	Percent completed (date of completion)
Subtask 5: Present research at least once per year at Pacific Northwest Prostate Cancer SPORE research conferences	1-48	50%
Subtask 6: Provide direct care for patients with prostate cancer in my clinic. Supervise and educate medical residents and interns and medical oncology fellows	1-48	50%
Subtask 7: Prepare a grant submission to test a novel targeted therapy in the context of precision oncology trials	36-48	0%

Research-Specific Tasks:

Specific Aim 1: Identify somatic alterations in castration-resistant prostate cancers that associate with response and resistance to PDT and determine their causal roles in mediating treatment effects.

Major Task 1: Biospecimen Acquisition	Months	Percent completed (date of completion)
Subtask 1: Enroll ≥ 20 patients onto Phase II BAT vs. Enzalutamide Trial <i>Note:</i> 17 patients were enrolled and this study has now finished accrual. We continue to enroll to a separate Phase II study testing BAT plus olaparib.	1-24	90%
Subtask 2: Perform metastatic biopsies and collect blood samples from men receiving BAT	1-24	90%
Subtask 3: Isolate CTCs	1-24	90%
Subtask 4: Process plasma from ctDNA	1-24	90%
Major Task 2: Conduct Targeted Assays to Determine Somatic Features that Associate with Response to BAT		
Subtask 1: Conduct studies to assess for the presence of biallelic loss of DNA damage repair genes	24-40	0%
Subtask 2: Conduct studies to assess for alterations in AR at the genomic, transcript and protein levels	24-40	0%
Subtask 3: Conduct immunohistochemistry and immunofluorescence studies to assess for evidence of DNA damage and decreased cellular proliferation	24-40	0%
Subtask 4: Analyze results to determine if biallelic loss of DNA damage repair genes or alterations in AR associate with response to BAT	40-43	0%
Major Task 3: Conduct Molecular Profiling Studies to Determine Somatic Features that Associate with Response to BAT		
Subtask 1: Conduct transcriptome profiling (RNA-seq) studies on CTCs and metastatic tumors	24-40	0%
Subtask 2: Analyze transcriptome profiling results to assess for predictors of response to BAT	40-43	0%

Specific Aim 2: Evaluate the association of germ-line variations in genes contributing to AR activity and androgen metabolism with response and resistance to PDT.

Major Task: Evaluate the impact of germ-line variations in androgen transport genes on response and resistance to BAT

Subtask 1: Evaluate for the presence of germline <i>SLCO</i> polymorphisms using qRT-PCR	24-40	0%
Subtask 2: Perform liquid chromatography-mass spectrometry (LC/MS) assays on metastatic biopsy specimens to determine: intratumoral androgen (i.e. testosterone, DHT) and hormonal substrate (i.e. DHEA-S) levels	24-40	0%

Specific Aim 3: Conduct preclinical studies designed to augment the effectiveness of PDT including dosing schedules, testosterone concentrations, and drug combinations.		
Major Task: Conduct preclinical studies to evaluate different PDT schedules, testosterone concentrations and drug combinations		
Subtask 1: Establish LNCaP95 xenografts in castrated SCID-17B mice	12-18	100%
Subtask 2: Evaluate the effect of different PDT schedules, testosterone concentrations and drug combinations on LNCaP95 xenograft growth	18-30	50%
Subtask 2: Perform immunohistochemical studies to evaluate the effects of PDT on AR-FL, AR-SV and γ -H2AX.	18-30	0%

Year 2 Research Accomplishments:

The project entails conducting detailed molecular assessments on biospecimens obtained from men enrolled to a Phase II study testing bipolar androgen therapy (BAT) vs. enzalutamide. As outlined in the Statement of Work, Year 2 has focused on biospecimen acquisition. Accomplishments from this funding period are provided below.

- Year 2 Objectives: Complete biospecimen acquisition for downstream cell-free circulating tumor DNA (ctDNA), circulating tumor cell (CTC) and tumor tissue analyses.
- Major Activities: We have enrolled 17 out of a projected 20 subjects to the Phase II BAT vs. enzalutamide study. This study has now completed accrual and we do not anticipate enrolling any additional patients to this trial. I have recently initiated a separate Phase II study testing BAT in combination with the PARP inhibitor olaparib. Biospecimens are being obtained from this clinical trial and may be used to complete some of the described correlative work (e.g. measuring intratumoral androgens, etc).

Thirteen patients enrolled to the randomized BAT vs. enzalutamide study have received BAT to date, and we anticipate the others will cross over to the BAT arm in the near future. Three individuals enrolled to this randomized study have had metastatic biopsies performed (one patient underwent baseline and Day 8 biopsy). We have also received an additional 67 plasma samples for future ctDNA studies from our collaborators at Johns Hopkins. We have optimized UW-OncoPlex (a targeted next-generation DNA sequencing panel) for use on plasma samples (i.e. cell-free circulating tumor DNA; ctDNA) samples and are beginning to sequence these samples.

In vivo studies as described in Specific Aim 3 are underway. We expect that initial results from these studies will be available in the coming months.

- Results and Outcomes: We have surpassed our sample collection goal for plasma samples. Additional, blood and metastatic tissue collection is ongoing. Molecular assessments are planned to be completed in Years 3 and 4 of the project.
- Goals not Met: We are still collecting additional metastatic tissue and blood for CTC studies. We are still on track to complete molecular assessments in Years 3 and 4.

Year 2 Training and Professional Development:

This Training Award includes a multi-dimensional plan designed to endow me with the skills and practical knowledge that will allow me to recognize, develop and effectively exploit new approaches for treating prostate cancer. This training plan involves both didactic and 'hands-on' professional development experiences. Experiences from Year 2 are outlined below.

- Seminars and Conferences: As outlined in the Statement of Work, participation in a number of seminars is ongoing through the end of this Training Award. I continue to attend and present at the weekly Pacific Northwest Prostate Cancer SPORE conference series, as well as attend the weekly Oncology Center grand rounds. I regularly attend the Prostate Cancer Precision Tumor Board and GU Tumor Board, and actively participate in our Localized and Advanced GU Oncology Clinical Trials Conferences. In addition, I have attended seminars and workshops as outlined in the SOW. Due to scheduling conflicts, I was not able to attend the Translational Boot Camp in Year 2. I will plan to attend this in Year 3.
- National Conferences and Committees: This past year I have attended the annual ASCO meeting, the ASCO GU meeting and the Prostate Cancer Foundation annual retreat. I have also attended the 2018 Prostate Cancer Inter-SPORE Meeting and presented a poster there.
- Clinical Development: I see and manage men with localized and advanced prostate cancer on an outpatient basis 1.5 days per week. I also attend on the inpatient unit ~2-4 weeks per year. I receive clinical management advice from Dr. Nelson, Dr. Yu and other senior faculty. In my clinic I actively enroll patients onto clinical trials, including the Phase II BAT vs. enzalutamide study described in this project.
- Mentoring/Training: I interact regularly with medical residents, oncology fellows and medical students. I am also mentoring a first-year oncology fellow. We have published a few case reports/review articles and are in the process of completing a retrospective review project. We have also developed a prospective clinical trial that we anticipate will open in mid-2019. Finally, I have been working with two medicals students on a retrospective analysis and hope to have this submitted for publication in the next few months.
- Lectures: I have delivered several lectures over the past year. These include speaking locally at Grand Rounds, the SPORE Seminar Series and at the Seattle Cancer Care Alliance Comprehensive Hematology and Oncology Review Course. I have also given invited lectures at City of Hope, University of Utah and to the Society of Utah Medical Oncology.
- Peer Review Activities: Over the past year, I have reviewed a number of articles for peer-reviewed journals. I also served on a grant review committee for the DoD PCRP.

Results Dissemination:

Nothing to report.

Funding Year 3 Plans:

Over the next reporting period, I will work toward completing the molecular assessments described in Specific Aims 1 and 2. I will also continue to collect biospecimen as outlined in the Statement of Work, with a focus on metastatic tissue acquisition. Preclinical studies (Specific Aim 3) to assess combinatorial PDT-based regimens in LNCaP95 xenografts are ongoing and we

expect these will be completed in the coming months. We will then conduct immunohistochemical studies on LNCaP95 tumors as outlined in the SOW. We have also completed initial *in vitro* studies evaluating the mechanistic basis for BAT's clinical effects and we are in the process of preparing a manuscript for publication.

4. IMPACT:

Nothing to report.

5. CHANGES/PROBLEMS:

To date, we have had difficulty convincing patients to agree to on-treatment biopsies as part of the randomized BAT vs. enzalutamide trial. As an alternative approach, we plan to obtain metastatic biopsies as part of our BAT plus olaparib trial to use toward completing the work described in Specific Aims 1 and 2.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

Note: Federal funding was only acknowledged for papers that were at least partly related to the project serving as the foundation for this award. All papers published within the reporting period are included for completeness.

1. **Schweizer, M.T.**, Haugk, K., McKiernan, J.S., Gulati, R., Cheng, H.H., Maes, J.L., Dumpit, R.F., Nelson, P.S., Montgomery, B., McCune, J.S., Plymate, S.R. & Yu, E.Y. A phase I study of niclosamide in combination with enzalutamide in men with castration-resistant prostate cancer. *PloS one* 13, e0198389 (2018).
2. Hempelmann, J.A., Lockwood, C.M., Konnick, E.Q., **Schweizer, M.T.**, Antonarakis, E.S., Lotan, T.L., Montgomery, B., Nelson, P.S., Klemfuss, N., Salipante, S.J. & Pritchard, C.C. Microsatellite instability in prostate cancer by PCR or next-generation sequencing. *Journal for immunotherapy of cancer* 6, 29 (2018).
3. Teply, B.A., Wang, H., Lubner, B., Sullivan, R., Rifkind, I., Bruns, A., Spitz, A., DeCarli, M., Sinibaldi, V., Pratz, C.F., Lu, C., Silberstein, J.L., Luo, J., **Schweizer, M.T.**, Drake, C.G., Carducci, M.A., Paller, C.J., Antonarakis, E.S., Eisenberger, M.A. & Denmeade, S.R. Bipolar androgen therapy in men with metastatic castration-resistant prostate cancer after progression on enzalutamide: an open-label, phase 2, multicohort study. *The Lancet. Oncology* (2017).
4. Mohammad, O.S., Nyquist, M.D., **Schweizer, M.T.**, Balk, S.P., Corey, E., Plymate, S., Nelson, P.S. & Mostaghel, E.A. Supraphysiologic Testosterone Therapy in the Treatment of Prostate Cancer: Models, Mechanisms and Questions. *Cancers* 9(2017).
5. **Schweizer, M.T.**, Hancock, M.L., Getzenberg, R.H. & Yu, E.Y. Hormone levels following surgical and medical castration: defining optimal androgen suppression. *Asian journal of andrology* (2017).
6. Graham, L., True, L.D. & **Schweizer, M.T.** Metastatic Adenocarcinoma of the Epididymis: A Case Report and Brief Literature Review. *Clinical genitourinary cancer* (2017).

7. **Schweizer, M.T.** & Antonarakis, E.S. Prognostic and therapeutic implications of DNA repair gene mutations in advanced prostate cancer. Clinical advances in hematology & oncology : H&O 15, 785-795 (2017).
8. Bluemn, E.G., Coleman, I.M., Lucas, J.M., Coleman, R.T., Hernandez-Lopez, S., Tharakan, R., Bianchi-Frias, D., Dumpit, R.F., Kaipainen, A., Corella, A.N., Yang, Y.C., Nyquist, M.D., Mostaghel, E., Hsieh, A.C., Zhang, X., Corey, E., Brown, L.G., Nguyen, H.M., Pienta, K., Ittmann, M., **Schweizer, M.**, True, L.D., Wise, D., Rennie, P.S., Vessella, R.L., Morrissey, C. & Nelson, P.S. Androgen Receptor Pathway-Independent Prostate Cancer Is Sustained through FGF Signaling. Cancer cell 32, 474-489.e476 (2017).

Books or other non-periodical, one-time publications.

Nothing to report.

Other publications, conference papers and presentations.

Nothing to report.

- **Website(s) or other Internet site(s)**

Nothing to report.

- **Technologies or techniques**

Nothing to report.

- **Inventions, patent applications, and/or licenses**

Nothing to report.

- **Other Products**

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

<i>Name:</i>	<i>Michael Schweizer</i>
<i>Project Role:</i>	<i>PI</i>
<i>eRA Commons User Name:</i>	<i>mschwei9</i>
<i>Nearest person month worked:</i>	<i>6</i>
<i>Contribution to Project:</i>	<i>Coordinates all aspects of the research in this project, including: planning, data gathering and analysis.</i>
<i>Funding Support:</i>	<i>DoD PCRP PRTA (W81XWH-16-1-0484)</i>

Name: Peter Nelson
Project Role: Mentor
eRA Commons User Name: pnelson
Nearest person month worked: 1
Contribution to Project: Provides advice and resources to ensure this project is carried out as described.
Funding Support: PNW Prostate Cancer Spore (P50 CA097186-12)

Change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period:

Updated Support Document for the PI (Dr. Schweizer) is attached as an appendix.

Other organizations involved as partners:

Organization Name: Johns Hopkins School of Medicine

Location of Organization: Baltimore, MD

Partner's contribution to the project

- *Provided biospecimens from men receiving bipolar androgen therapy (BAT).*

8. APPENDICES:

OTHER SUPPORT
SCHWEIZER, MICHAEL T.

CURRENT

Title: A Neoadjuvant Clinical Trial to Assess the Effectiveness of Intense Combinatorial Targeting of AR-Signaling to Eradicate Prostate Carcinoma

Effort: 0.36 CM

Funding Agency: NCI/NIH (P30 CA015704)

Grants Officer: Heidi Tham; (206) 667-7245

Performance Period: 01/01/17 – 12/31/18

Funding Level: \$35,000

Goal: We will test a neoadjuvant AR-signaling ablative regimen that includes the AKR1C3 inhibitor indomethacin. Our primary objective will be to determine the pathologic complete response rate following 3-months of therapy in men with high-risk prostate cancer.

Specific Aims: Aim 1) Determine if high intensity short course combinatorial suppression of AR signaling with abiraterone, ARN-509, degarelix and indomethacin will eradicate prostate cancer cells within the prostate microenvironment and tumor cells that have disseminated to lymph nodes. Aim 2) Identify molecular features of prostate cancer cells and their microenvironments that associate with response and resistance to combinatorial AR pathway targeting.

Title: A Randomized Phase II Study Comparing Bipolar Androgen Therapy vs. Enzalutamide in Asymptomatic Men with Castration Resistant Metastatic Prostate Cancer: The TRANSFORMER Trial

Effort: 0.60 CM

Funding Agency: DoD (W81XWH-14-2-0189)

Contracting Officer: n/a (prime award to Johns Hopkins)

Performance Period: 09/01/15 – 03/29/19

Funding Level: \$137,705

Goal: To determine if treatment with supraphysiologic testosterone will improve radiographic progression free survival compared to enzalutamide in men with metastatic castrate-resistant prostate cancer post-treatment with abiraterone.

Title: Bipolar Androgen Therapy (BAT): Molecular Drivers of Response and Resistance

Effort: 0.60 CM

Funding Agency: Prostate Cancer Foundation

Grants Officer: Howard Soule, PhD; (310) 570-4596

Performance Period: 10/12/15 – 10/12/18

Funding Level: \$250,000

Goal: We will determine the drivers of response/resistance to supraphysiologic testosterone in men with castration-resistant prostate cancer. This will be accomplished by conducting molecular assessments on circulating tumor cells and metastatic biopsies obtained from men enrolled to a Phase II trial testing this therapy.

Specific Aims: Aim 1) Biospecimen acquisition from patients receiving BAT. Aim 2) Identify somatic features that associate with tumor response and resistance to BAT. Aim 3) Evaluate germ-line/host factors that may impact response and resistance to BAT

Overlap: PCF provides supplemental funding for PC150933. See "Overlap" section for details.

Title: Cancer Center Support Grant New Investigator Support

Effort: 0.60 CM

Funding Agency: NCI/NIH (P30 CA015704)

Grants Officer: Heidi Tham; (206) 667-7245

Performance Period: 12/15/15 – 12/14/18

Funding Level: \$75,000

Goal: Use next generation sequencing to determine the prevalence of genomic hypermutation within the general population of prostate cancer patients and determine if mismatch repair deficiency underlies this phenotype.

Specific Aims: Aim 1) Use mSINGS to estimate the frequency of hypermutated prostate cancer within the general population of prostate cancer patients. Aim 2) Use UW-OncoPlex to confirm hypermutation status and to evaluate for alterations in mismatch repair genes that may associate with this phenotype.

Title: A Phase III, Open-Label, Multicenter, Randomized Study of Atezolizumab (Anti-PD-L1 Antibody) Vs. Observation as Adjuvant Therapy in Patients with High-Risk Muscle-Invasive Urothelial Carcinoma After Surgical Resection

Effort: 0.60 CM

Funding Agency: F. Hoffmann-LaRoche / Genentech

Grants Officer: Jonathan Kursar; kursar.jonathan@gene.com

Performance Period: 10/14/16 – 06/30/19

Funding Level: \$142,000

Goal: To evaluate the efficacy of adjuvant atezolizumab treatment in patients with muscle- invasive urothelial carcinoma (UC), as measured by disease-free survival.

Specific Aims: Aim 1) To evaluate the efficacy of adjuvant atezolizumab treatment, as measured by overall survival. Aim 2) To evaluate the efficacy of adjuvant atezolizumab treatment, as measured by disease-specific survival.

Title: A Phase 2 Study of ARN-509 in Active Surveillance Patients

Effort: 0.60 CM

Funding Agency: Janssen Scientific Affairs, LLC

Grants Officer: Afrouz Bazmi; (724) 935-2140

Performance Period: 04/12/17 – 04/30/20

Funding Level: \$45,000

Goal: The main goal is to determine if a 90-day course of ARN-509 will lead to a negative repeat prostate biopsy in active surveillance patients.

Specific Aims: Determine the negative repeat biopsy rate by site directed and systematic prostate biopsy after 90-days of ARN-509.

Title: A Phase I Study of a DNA Vaccine Encoding Androgen Receptor Ligand-Binding Domain (AR LBD), With or Without Granulocyte Macrophage Colony-Stimulating Factor Adjuvant, in Patients with Metastatic Prostate Cancer

Effort: 0.36 CM

Funding Agency: Madison Vaccine Inc.

Grants Officer: Richard Lesniewski; rick@madisonvaccines.com

Performance Period: 01/30/17 – 01/29/21

Funding Level: \$99,000

Goal: The major goal of this study is to determine if a vaccine called pTVG-AR can enhance patients' immune response against prostate cancer.

Title: Pharmacologic Dose Testosterone to Treat Castration-Resistant Prostate Cancer: Mechanisms of Action and Drivers of Response

Effort: 4.8 CM

Funding Agency: Department of Defense

Grant Specialist: Mirlene Desir; (301) 619-7733; mirlene.desir.civ@mail.mil

Performance Period: 09/30/16 – 09/29/20

Funding Level: \$157,600 per year

Goal: This is a Physician Research Training Award. The goal of the program is to assist young investigators in their first years of research, and this project will focus on developing new therapeutic strategies that reduce the morbidity and mortality attributable to prostate cancer.

Specific Aims: Establish an intensive didactic training program, design and conduct early phase translational trials, and exploit findings from these trials to discover new pathways, targets, and strategies capable of improving prostate cancer outcomes.

Overlap: See "Overlap" section for details.

Title: A Phase 1 Safety and Tolerability Study of ZEN003694 in Combination with Enzalutamide in Patients with Metastatic Castration-Resistant Prostate Cancer

Effort: 0.60 CM

Funding Agency: Zenith Epigenetics, Ltd

Grant Specialist: Drew Davis, PCCTC, LLC.; (646) 477-2716

Performance Period: 07/27/18 – 04/30/23

Funding Level: \$344,738

Goal: The major goal of this study is to determine the appropriate dosage of ZEN003694 in combination with enzalutamide in patients with mCRPC.

Specific Aims: 1) To determine the safety, tolerability and maximum tolerated dose of ZEN003694 in combination with enzalutamide in patients with mCRPC who have progressed during prior treatment with enzalutamide or with abiraterone. 2) To confirm the safety and tolerability and MTD and recommended Phase 2 dose of ZEN003694.

COMPLETED

Title: Minimally Invasive Assessments of Tumor Molecular Composition for Precision Diagnostics and Monitoring Treatment Response

Effort: 0.36 CM

Funding Agency: NCI/NIH (P30 CA015704)

Grants Officer: Jennifer Jacyszyn; (206) 667-6250

Performance Period: 07/01/15 – 06/30/16

Funding Level: \$100,000

Goal: The goal of this project is to develop technology that will allow for the molecular assessment of circulating tumor cells. The assays developed will be utilized in a prospective biomarker trial to evaluate for determinates of response and resistance to prostate cancer therapies.

Specific Aims: Aim 1) Determine the molecular identity and genomic diversity between image-guided core tumor biopsies and parallel assessments of CTCs and ctDNA from the same patients. Aim 2) Determine if molecular assessments of AR-SVs and somatic alterations in DNA repair mechanisms (e.g. BRCA2 loss/mutation) from ctDNA or CTCs associate with clinical responses to: (i) agents targeting the AR pathway; or (ii) genotoxic therapeutics (e.g. carboplatin), respectively. Aim 3) Determine if quantitative measures of AR-SVs (for therapeutics targeting the AR pathway) or DNA repair gene defects (for genotoxic therapeutics) in CTCs or ctDNA can serve as dynamic measurements of responses and early indicators of treatment failures when measured over time during a course of treatment.

Title: Targeted Niche Therapy (TNT) to Cure Metastatic Prostate Cancer

Effort: 0.60 CM

Funding Agency: Prostate Cancer Foundation

Grants Officer: Howard Soule, PhD; (310) 570-4596

Performance Period: 08/01/14 – 08/17/17

Funding Level: \$19,000

Goal: The goal of this project is to determine the kinetics of prostate cancer cell mobilization from the bone marrow in response to CXCR4 inhibition as well as to explore the effect that docetaxel has on prostate cancer cells that have been mobilized. Molecular profiling studies have also been built into this project to further characterize prostate cancer cells both within the protective bone marrow niche and those that have been mobilized.

Specific Aims: Aim 1) Perform a Phase 0 trial to determine the kinetics of prostate cancer cell mobilization from the bone marrow in response to the CXCR4 inhibitor AMD3100. Aim 2) Define the phenotype of PCA cells that are mobilized from the HSC niche.

Title: A Phase I Study of Niclosamide in Men with AR-V Positive CRPC

Effort: 0.36 CM

Funding Agency: NCI/NIH (P30 CA015704)

Grants Officer: Jennifer Jacyszyn; (206) 667-6250

Performance Period: 01/01/15 – 12/30/17

Funding Level: \$49,239

Goal: The goal of this project is to determine the safety and tolerability of high-dose niclosamide when given in combination with enzalutamide. As a secondary objective, I will assess the effects of this therapy on androgen receptor splice variants (AR-Vs) pre- and post-treatment using a qRT-PCR assay.

Specific Aims: Aim 1) Conduct a Phase I study to assess the safety and pharmacokinetics of oral niclosamide in men with AR-V positive castration-resistant prostate cancer. Aim 2) Assess the impact of oral niclosamide on AR-V transcript levels.

Title: A Phase I Study of Niclosamide in Men with AR-V Positive CRPC: Molecular Correlates

Effort: 0.24 CM

Funding Agency: NCI/NIH (P50 CA097186)

Grants Officer: Samantha Farrell, farrellsa@mail.nih.gov

Performance Period: 04/01/15 – 08/31/17

Funding Level: \$29,300

Goal: The goal of this project is to determine the effects of high-dose niclosamide on the transcriptional program of androgen receptor splice variant (AR-V) positive prostate cancer cells.

Specific Aims: Aim 1) Determine the effect of oral niclosamide plus enzalutamide on the transcriptional program of circulating tumor cells (CTCs) through RNA-seq.

Title: Bipolar Androgen Therapy plus Olaparib in Patients with Castration-Resistant Prostate Cancer

Effort: 0.36 CM

Funding Agency: AstraZeneca

Contracting Officer: Gayle Ewing; Gayle.Ewing@astrazeneca.com

Performance Period: 09/01/18 – 08/31/21

Funding Level: \$430,010

Goal: The main goal of this study is to gauge the response rate in CRPC patients co-treated with Bipolar Androgen Therapy (BAT) and olaparib, with a focus on its effects on the subset of patients with DNA damage repair deficiencies.

PENDING

None

OVERLAP

The PRTA will support the PI's and the Research Coordinator's effort on this project, travel to relevant meetings, education fees, and publishing costs. The Program Announcement explicitly stated the PRTA award mechanism shall not be used for "research costs for studies on animals, human subjects, human biological substances, or clinical trials." Since this proposal has all four of those components, we plan to utilize the Prostate Cancer Foundation award to cover outpatient care costs, and the cost of purchasing, housing, and caring for the mice. Both sources are necessary to support this project as a whole, but there will be no budgetary overlap.

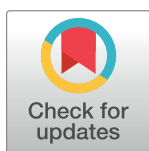
RESEARCH ARTICLE

A phase I study of niclosamide in combination with enzalutamide in men with castration-resistant prostate cancer

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OPEN ACCESS

Citation: Schweizer MT, Haugk K, McKiernan JS, Gulati R, Cheng HH, Maes JL, et al. (2018) A phase I study of niclosamide in combination with enzalutamide in men with castration-resistant prostate cancer. PLoS ONE 13(6): e0198389. <https://doi.org/10.1371/journal.pone.0198389>

Editor: Quan Sing Ng, National Cancer Centre Singapore, SINGAPORE

Received: March 14, 2018

Accepted: May 15, 2018

Published: June 1, 2018

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Data Availability Statement: All relevant data are within the paper.

Funding: We acknowledge research support from National Cancer Institute grants P30 CA015704, P30 CA033572 and R50 CA221836; Pacific Northwest Prostate Cancer SPORE CA097186, The Prostate Cancer Foundation (including Young Investigator Awards to MTS and HHC); and Department of Defense Award W81XWH-16-1-0484.

Abstract

Background

Niclosamide, an FDA-approved anti-helminthic drug, has activity in preclinical models of castration-resistant prostate cancer (CRPC). Potential mechanisms of action include degrading constitutively active androgen receptor splice variants (AR-Vs) or inhibiting other drug-resistance pathways (e.g., Wnt-signaling). Published pharmacokinetics data suggests that niclosamide has poor oral bioavailability, potentially limiting its use as a cancer drug. Therefore, we launched a Phase I study testing oral niclosamide in combination with enzalutamide, for longer and at higher doses than those used to treat helminthic infections.

Methods

We conducted a Phase I dose-escalation study testing oral niclosamide plus standard-dose enzalutamide in men with metastatic CRPC previously treated with abiraterone. Niclosamide was given three-times-daily (TID) at the following dose-levels: 500, 1000 or 1500mg. The primary objective was to assess safety. Secondary objectives, included measuring AR-V expression from circulating tumor cells (CTCs) using the AdnaTest assay, evaluating PSA changes and determining niclosamide's pharmacokinetic profile.

Results

20 patients screened and 5 enrolled after passing all screening procedures. 13(65%) patients had detectable CTCs, but only one was AR-V+. There were no dose-limiting toxicities (DLTs) in 3 patients on the 500mg TID cohort; however, both (N = 2) subjects on the 1000mg TID cohort experienced DLTs (prolonged grade 3 nausea, vomiting, diarrhea; and

Competing interests: The authors have declared that no competing interests exist.

colitis). The maximum plasma concentration ranged from 35.7–82 ng/mL and was not consistently above the minimum effective concentration in preclinical studies. There were no PSA declines in any enrolled subject. Because plasma concentrations at the maximum tolerated dose (500mg TID) were not consistently above the expected therapeutic threshold, the Data Safety Monitoring Board closed the study for futility.

Conclusions

Oral niclosamide could not be escalated above 500mg TID, and plasma concentrations were not consistently above the threshold shown to inhibit growth in CRPC models. Oral niclosamide is not a viable compound for repurposing as a CRPC treatment.

Clinical trial registry

Clinicaltrials.gov: [NCT02532114](https://clinicaltrials.gov/ct2/show/study/NCT02532114)

Introduction

Nearly 30,000 American men die as a result of their prostate cancer each year[1]. Since the 1940s, the treatment of advanced prostate cancer has focused almost exclusively on inhibiting the androgen receptor (AR)-signaling program[2]. Indeed, over the past decade it has been discovered that even in men with castration-resistant prostate cancer (CRPC)—a clinical state defined by disease progression in spite of medical or surgical castration (i.e., androgen deprivation therapy)—AR remains the primary driver[3, 4]. This realization has led to the further exploration of the AR-signaling axis as a therapeutic target in men with metastatic CRPC (mCRPC), and led to the development of effective new AR-directed agents like abiraterone and enzalutamide, which inhibit AR-signaling through disrupting the ligand-receptor interaction (abiraterone through ligand depletion and enzalutamide through receptor antagonism) [5–8]. These agents are unfortunately not curative, and resistance typically occurs in 1–2 years.

Several mechanisms of resistance to next-generation AR-directed therapies have been described, including: i) activation of canonical AR-signaling through AR amplification, AR overexpression and/or maintenance of intratumoral androgens; ii) AR-signaling activation via feedback pathways (e.g. AKT/mTOR/Pi3K, NF- κ B, Wnt/ β -catenin); and iii) activation of the AR program via mutations (e.g. AR ligand binding domain mutation) or AR substitutions (e.g. AR splice variants; Glucocorticoid Receptor-signaling)[9–22]. Of these mechanisms, the emergence of alternatively spliced AR variants (AR-Vs), which maintain constitutive activity in spite of lacking the AR ligand-binding domain, has received substantial attention. Inhibiting AR-V activity has been shown to be an effective strategy in preclinical models and the emergence of AR-V7, the most prevalent AR-V, has been associated with a lack of response to abiraterone and enzalutamide[9, 13, 23]. While the emergence of AR-Vs provides an elegant biologic rationale for why drugs that interfere with the AR-ligand interaction may not be effective, it remains unclear whether AR-V expression is a driver of disease progression or merely a reflection that a larger resistance program has been activated[13, 23–27].

Given that all the approved AR-signaling inhibitors work by preventing ligand-AR interaction, an agent that can effectively disrupt AR-V signaling, or inhibit other relevant resistance pathways, would be an invaluable therapeutic option for men with multi-drug resistant mCRPC. Niclosamide, an FDA-approved anti-helminthic drug, has been shown in several

preclinical models of CRPC to be a potent anti-neoplastic agent. It results in decreased cell proliferation across multiple cell lines, with a reported IC₅₀ of 330 ng/mL[28]. Most studies have tested concentrations below the IC₅₀, with decreased cellular viability occurring at concentrations from 81.8 to 327 ng/mL. Mechanistic studies have shown that niclosamide may exert its effect through degrading AR-Vs or through inhibiting other pathways, including AKT/mTOR/Pi3K, NF- κ B, and Wnt-signaling, which are implicated in prostate cancer resistance and progression[17, 28–32]. Interestingly, niclosamide does not appear to have an effect on full-length AR expression, providing a rationale for testing this drug in combination with next-generation AR-signaling inhibitors (i.e., abiraterone or enzalutamide).

Published data regarding the pharmacokinetics (PK) of niclosamide suggest that it has poor oral bioavailability, potentially limiting its use as a cancer drug. The standard oral dose of niclosamide used to treat anti-helminthic infections in adults is 2000 mg daily for 1–7 days, and in a cohort of healthy male and female volunteers administered a single 2000 mg oral dose of carbonyl-¹⁴C-labeled niclosamide, the maximum serum concentration of niclosamide (C_{max}) attained was estimated to be between 250 to 6000 ng/mL[33–35]. These data suggest that the standard anti-helminthic dose of niclosamide may not result in serum concentrations that are consistently in the therapeutic range shown to inhibit prostate cancer growth. On the basis of the aforementioned data, we launched a Phase I study to test oral niclosamide in combination with enzalutamide in men with mCRPC who have progressed on abiraterone. Our primary goal was to evaluate the safety, tolerability, and pharmacokinetics of niclosamide administered, in combination with enzalutamide, for longer (i.e., >7 days) and at higher doses (i.e., >2000 mg daily) than those used to treat helminthic infections.

Methods

Study design

This was an open label, Phase I dose-escalation study testing high-dose niclosamide in combination with the FDA-approved dose of enzalutamide (160 mg by mouth daily) [clinicaltrials.gov: NCT02532114]. This study was approved by the University of Washington/Fred Hutchinson Cancer Research Center Institutional Review Board and written informed consent was obtained from all enrolled subjects. Study participants were recruited through medical oncology clinics at the University of Washington and Seattle Cancer Care Alliance (both in Seattle, WA), and the study was open for recruitment from October 2015 to November 2017, with all patient follow up completing in December 2017. Niclosamide was given three-times-daily (TID) by mouth (PO) for four weeks at one of the following dosing cohorts: 500, 1000, or 1500 mg. All patients were required to have mCRPC (i.e., disease progression in spite of serum testosterone $\leq$ 50 ng/dL) and had received prior abiraterone. Patients were originally required to have detectable AR-V transcripts in circulating tumor cells (CTC) as determined using the AdnaTest assay[9]. However, due to very few AR-V+ patients in the initial screen, and recognition that niclosamide may exert antineoplastic effect through AR-V+ independent pathways, the protocol was modified to remove this eligibility criteria. Given that niclosamide has been shown to impair multiple mechanisms of resistance to enzalutamide, patients were permitted to start enzalutamide prior to the addition of niclosamide, and evidence of disease progression on enzalutamide was not exclusionary[17, 28–32]. Enrolled subjects were also required to have a creatinine clearance >30 mL/min, Eastern Cooperative Oncology Group (ECOG) performance status $\geq$ 2 and no signs of severe hepatic impairment (i.e., Child-Pugh Class C). The primary objective was to assess safety. Key secondary objectives were to assess the pharmacokinetic profile of niclosamide, evaluate prostate-specific antigen (PSA) changes following 4 weeks of niclosamide, and to assess pharmacodynamic effects on CTCs.

Pharmacokinetics

The pharmacokinetic samples were quantitated for plasma niclosamide concentrations using a modification of previously published methods[36]. Blood for pharmacokinetic analyses were drawn at the following time points after the first dose: 0.5 hr, 1 hr, 1.5 hr, 2 hr, 3 hr, 4 hr, 6 hr, 8 hr and on Day 15. In brief, patient plasma (25 μ L) was mixed with the internal standard solution (20 μ L containing 50 ng niclosamide $^{13}\text{C}_6$ in methanol) and methanol (200 μ L) in a 0.5 mL snap-cap micro-centrifuge tube. After vortexing, the samples were centrifuged at 20000 x G for 10 minutes at 4°C. The supernatant was transferred to a 96-well plate and 2 μ L were injected on the liquid chromatography mass spectrometry (LCMS) system. The LCMS system was an ultrahigh pressure liquid chromatography (Agilent (Santa Clara, CA) 1290 series) coupled to an Agilent G6410B triple-quadrupole mass spectrometer. The column was an Agilent Zorbax SB-C18 2.1 mm x 150 mm x 5 μ m maintained at 45°C. Mobile phase A was 10 mM ammonium formate (pH = 3) and mobile phase B was acetonitrile in the ratio of 35% A: 65% B at 0.4 mL/min.

The mass spectrometer was operated in the ESI-negative mode with the following transitions: 324.8 $\rightarrow$ 170.9 m/z (niclosamide) and 330.9 $\rightarrow$ 176.9 m/z (niclosamide $^{13}\text{C}_6$).

A ten-point calibration curve was created by spiking blank plasma with niclosamide to make standards in the range of 40.8 to 8160 ng/mL and processing the standards identically to samples. Calibration curves were based upon the height ratio of the niclosamide to the internal standard (niclosamide $^{13}\text{C}_6$) and were fitted using a polynomial fit with 1/x weighting. The correlation coefficient was used to evaluate the linearity of the calibration curves and was >0.99 in all experiments. The limit of quantitation was 40.8 ng/mL (CV = 11.3%; accuracy 104.9%) and the limit of detection was 16.3 ng/mL, with a signal to noise ratio of 53.75.

Splice variant determination

The presence of AR-V transcripts were determined from CTCs using qRT-PCR and primers designed to detect AR-V7 and AR-V567es (i.e., exon 5, 6 and 7 deleted AR-V) mRNA. The AdnaTest ProstateCancerSelect/ProstateCancerDetect assay (AdnaGen, Langenhagen, Germany) and methods similar to those described by Antonarakis and colleagues were used[9]. AR-V PCR product visualization was on 2% agarose gel. All variant PCR products were evaluated for size and concentration using the QIAxcel Advanced System (QIAGEN, Inc; Germantown, MD, USA).

Statistical considerations

We utilized the continual reassessment method (CRM) to evaluate dose-related toxicities and to determine the recommended Phase II dose[37]. We targeted a maximum of 30% of patients incurring dose-limiting toxicities (DLT) up to 30 days after the final (day-28) dose. Dose escalation and de-escalation were dictated by the CRM and based on posterior probabilities determined by: 1) the assumed dose-toxicity model, which was a 1-parameter power model with a Gamma(1,1) prior distribution; 2) assumed prior probabilities of DLTs of 5%, 10%, and 15% for dose levels 500, 1000 and 1500 mg PO TID, respectively; 3) a target DLT rate of $\leq 30\%$; and 4) accumulating toxicity data. Adverse events (AEs) were documented by incidence and their severity was graded according to the National Cancer Institute–Common Terminology Criteria for Adverse Events (CTCAE) version 4.0. The pharmacokinetic data underwent non-compartmental analysis using Phoenix WinNonlin version 8.0 (Certara USA, Inc; Princeton, NJ), and key pharmacokinetic parameters (e.g., C_{max} , C_{min} , C_{ss} and $t_{1/2}$) were extracted.

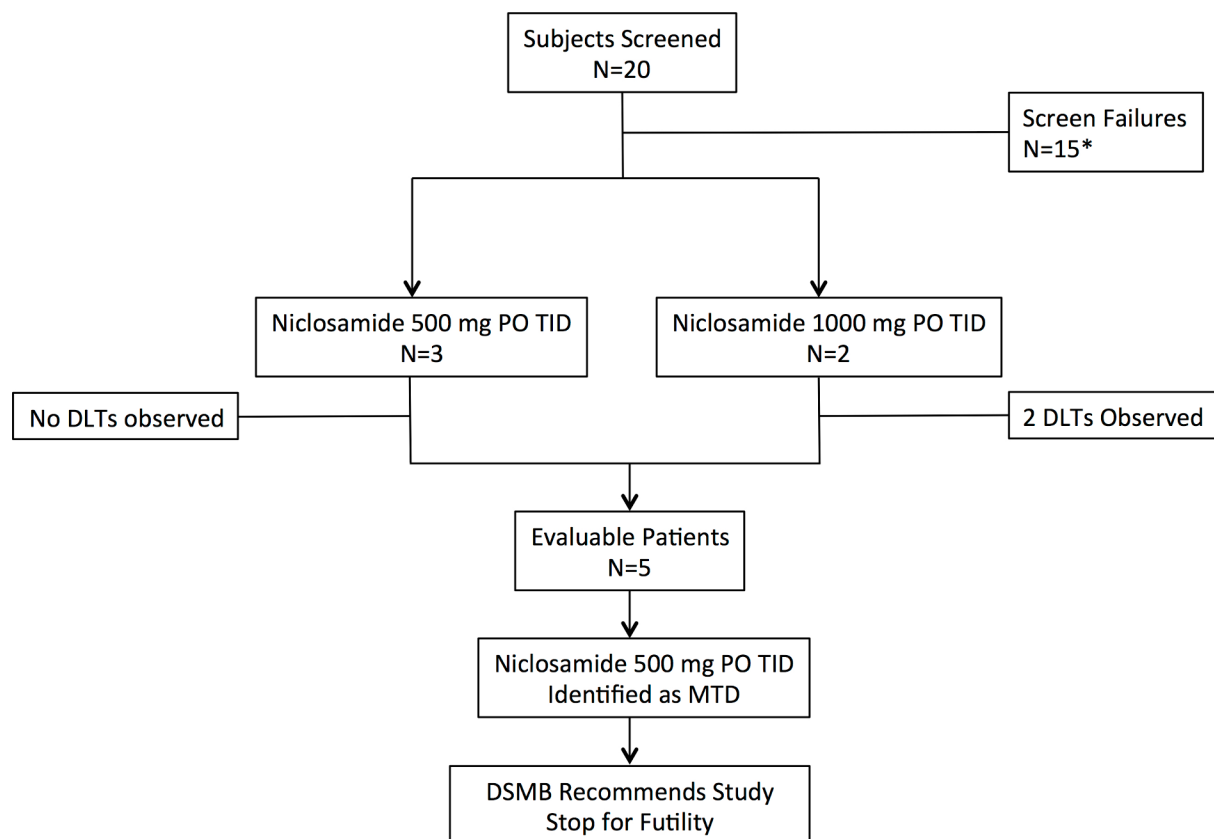


Fig 1. Study flow diagram. * All screen failures were due to undetectable androgen receptor splice variants, which were mandated to be present under protocol version 1. Protocol version 2 removed this criterion. MTD, maximum tolerated dose; TID, three times daily; PO, by mouth; DSMB, data safety monitoring committee.

<https://doi.org/10.1371/journal.pone.0198389.g001>

Results

Patients

From December 2015 to October 2017, 20 patients were screened and 5 passed all screening procedures and enrolled onto the study (Fig 1). The 15 screen failures occurred under protocol version 1 and were a consequence of undetectable AR-Vs. All patients previously progressed on enzalutamide, with 18/20 patients demonstrating a rising PSA on enzalutamide at the time of screening. Baseline patient demographics for those who enrolled in the trial are presented in Table 1. Two enrolled patients had previously documented AR-Vs as determined from prior

Table 1. Baseline characteristics of study population at time of enrollment. Patient characteristics at the time of screening. *PSA was previously rising on enzalutamide but began falling after palliative radiation to a painful bone metastasis, which was administered just prior to initiating niclosamide.

Pt#	Age	PSA (ng/mL)	Hemoglobin	Total Gleason Score	Sites of disease			Actively progressing on enzalutamide?
					Bone	Lymph nodes	Visceral	
1	84	70.77	10.6	9	Yes	Yes	No	Yes
17	75	63.66	13.6	9	Yes	No	No	Yes
18	71	100.99	11.7	9	Yes	Yes	Yes	Yes
19	68	1492.36	9.4	7	Yes	No	No	Yes
20	60	31.02	8.2	9	Yes	Yes	No	No*

<https://doi.org/10.1371/journal.pone.0198389.t001>

Table 2. AR splice variant detection using AdnaTest.

Pt#	Timepoint	Actin	PSA	PSMA	AR-FL	AR-V7	ARv567*	ARv56*
Screened and treated								
1	Screening	+			+		+	+
1	Day 29	+			+	+	+	+
17	Screening	+						
17	Day 29	+						
18	Screening	+	+	+	+			
18	Day 29	+			+			
19	Screening	+	+	+	+			
19	Day 29	+	+	+	+			
20	Screening	+						
20	Day 21**	+			+			
Only screened								
2	Screening	+			+			
3	Screening	+			+			
4	Screening	+	+		+			
5	Screening	+						
6	Screening	+						
7	Screening	+			+			
8	Screening	+						
9	Screening	+			+			
10	Screening	+						
11	Screening	+			+			
12	Screening	+			+			
13	Screening	+	+		+			
14	Screening	+						
15	Screening	+	+		+			
16	Screening	+			+			

Tumor-associated transcripts (PCR fragment) are detected by visualization on 2% agarose gel (+/-). Variant products with a concentration greater than or equal to 100 ng/mL are considered positive.

*ARv567 and ARv56 are indistinguishable on agarose gel. The presence of these variants was determined through sequencing the respective bands.

**Patient taken off study early secondary to a dose-limiting toxicity.

<https://doi.org/10.1371/journal.pone.0198389.t002>

transcript profiling studies; however, only one of these patients was AR-V positive (AR-V56) at the time of study enrollment. Of the 18 patients without prior evidence of an AR-V, none were found to be AR-V+. All patients were positive for actin (positive control) and 13/20 had detectable CTCs as indicated by the presence of at least one additional tumor-associated transcript (i.e., full-length AR, PSA, and/or prostate-specific membrane antigen) using the AdnaTest (Table 2). There was no evidence for declining PSA across any of the dose cohorts (Table 3).

Adverse events

Niclosamide was well tolerated in combination with enzalutamide in the first dose cohort (i.e., niclosamide 500 mg PO TID), and only one out of three patients at this dose level experienced any toxicities deemed at least possibly related to niclosamide (Grade 1 nausea, anorexia and weight loss). Both patients treated at the second dose level (i.e., niclosamide 1000 mg PO TID) experienced dose-limiting toxicities. Patient #19 had Grade 3 nausea, vomiting, and diarrhea lasting >72 hours. Symptoms began on Day 26 of treatment, and he discontinued the study

Table 3. Summary of on-study PSA changes and adverse events.

Pt#	Niclosamide cohort	PSA (ng/mL)		PSA Change (%)	Adverse Events Possibly/Probably Related to Niclosamide							
		Day 1	Day 29		Nausea (Grade)	Anorexia (Grade)	Vomiting (Grade)	Diarrhea (Grade)	Weight loss (Grade)	Lipase elevation (Grade)	Colitis (Grade)	Abdominal pain (Grade)
1	500 mg PO TID	70.77	78.54	9.9%	—	—	—	—	—	—	—	—
17	500 mg PO TID	63.66	114.77	44.5%	—	—	—	—	—	—	—	—
18	500 mg PO TID	100.99	188.8	46.5%	1	1	—	—	1	—	—	—
19	1000 mg PO TID	1492.36	3009.49	101.7%	3*	—	3	3	—	2	—	—
20	1000 mg PO TID	31.02	—	—	2	1	—	3	—	—	3**	3

*Constituted a dose-limiting toxicity given that these AEs lasted >72 hours.

**Dose limiting toxicity.

<https://doi.org/10.1371/journal.pone.0198389.t003>

drugs that day. He was ultimately hospitalized between Days 32 to 40 as a result of this AE. This patient also experienced a Grade 2 lipase elevation without other signs of pancreatitis, which was felt to be possibly related to niclosamide. At the time of discharge he had increased oral intake without nausea or vomiting. Patient #20 had Grade 3 colitis, abdominal pain and diarrhea, with the colitis constituting a DLT. Symptoms began on Day 8 of treatment and abdominal CT on Day 9 revealed evidence of colitis. He was subsequently admitted to the hospital between Days 9 to 12 where he received aggressive supportive care (i.e. IV hydration and antibiotics). This AE had returned to Grade 1 at the time of discharge. It should be noted that this patient had received an immune checkpoint inhibitor as part of a clinical trial immediately prior to enrolling onto this study, and a colonoscopy with biopsy was performed to evaluate for delayed onset immune-mediated colitis. Pathologic findings from this biopsy were not consistent with an immune-mediated adverse event, however, and it was felt that niclosamide was likely the causative factor. A summary of all AEs deemed at least possibly related to niclosamide are provided in Table 3.

Pharmacokinetic results

Considering the enthusiasm for developing niclosamide for AR-V+ mCRPC, we chose to include all pharmacokinetic data. Pharmacokinetic data were obtained after the first dose in all patients and after the morning dose on the 15th day of treatment in four patients (Table 4). It should be noted that the timing of the Day 15 blood draw in relation to niclosamide dosing was not recorded, and therefore this timepoint was excluded from the pharmacokinetic analyses. The maximum observed plasma concentration ranged from 35.7 to 182 ng/mL, which, for most patients, was below the minimum effective concentration in preclinical studies[28, 29, 38]. We also estimated the total area under the curve ($AUC_{0-\infty}$) after the first dose, which should be evaluated with caution in those participants whose $AUC_{0-\infty}$ was over 20% extrapolated. The apparent oral clearance of niclosamide, which is clearance divided by the oral bio-availability (i.e., CL/F), ranged from 9.09 to 37.8 L/hr per kg of ideal body weight. Because niclosamide plasma concentrations in the maximal tolerated dosing cohort (i.e., 500 mg TID) were below those expected to exert an anti-tumor effect, the study was closed for futility.

Discussion

Our main findings are: 1) that niclosamide doses could not be escalated above 500 mg PO TID because of toxicity; 2) niclosamide is not a viable oral compound for repurposing as a mCRPC

Table 4. Pharmacokinetic results summary.

Pt#	Dose	T _{max}	C _{max}	C _{max} /dose	T _{min}	C _{min} ^a	T _{1/2}	First dose AUC _{0-τ}	First dose AUC _{0-∞} ^a	% of AUC _{0-∞} extrapolated ^a	Apparent oral clearance ^b
(unit)	(mg)	(hr)	(ng/mL)	(ng/mL per mg dose)	(hr)	(ng/mL)	(hr)	(ng×hr /mL)	(ng×hr/mL)	%	(L/hr per kg of IBW ^c)
1 ^d	500	6	182	0.363	4	13.3	1.27	692	942	26.6	9.09
17	500	2	35.7	0.072	8	6.92	2.24	155	180	13.7	37.8
18	500	1	88.3	0.177	8	7.42	3.86	202	243	17.2	26.5
19	1000	1	182	0.182	8	45.5	5.61	676	1060	36.0	12.2
20	1000	6	149	0.149	8	90.0	2.75	629	986	36.2	14.9

^aC_{last}/elimination rate constant was used to estimate the AUC from the end of the dosing interval to time infinity (∞)

^bclearance divided by fraction absorbed

^cIBW = 50 kg + 2.3 kg per inch over 5 feet.

^dPatient had an aberrant pharmacokinetic profile with a second peak at 6h. Evaluate with caution.

<https://doi.org/10.1371/journal.pone.0198389.t004>

treatment because the dosing cohort with acceptable toxicity (i.e., 500 mg PO TID) does not consistently yield concentrations above those shown to inhibit tumor growth in mCRPC models; and 3) niclosamide pharmacokinetics had moderate variability with our C_{max} data agreeing with previous reports[17, 28–32]. Niclosamide has impressive preclinical activity across a range of malignancies, and is currently being investigated in several early phase clinical trials targeting patients with mCRPC as well as colorectal cancer [clinicaltrials.gov: NCT02687009, NCT03123978, NCT02519582, NCT02807805][17, 29–32]. Because niclosamide has been reported to have poor oral bioavailability, we sought to test higher doses of niclosamide than those used to treat helminth infections as a means to increase plasma niclosamide concentrations[35]. Ultimately, we were unable to reach a dose that resulted in plasma niclosamide concentrations predicted to exert an anti-tumor effect.

Prior preclinical studies testing niclosamide reported decreased DU145 proliferation at a half-maximal inhibitory concentration (IC₅₀) of 330 ng/mL, and niclosamide's anti-tumor effects are apparent across a range of prostate cancer models, with decreased cellular viability documented at concentrations from 81.8 to 327 ng/mL. Liu and colleagues have published a series of papers examining the effects of niclosamide in abiraterone and/or enzalutamide-resistant models, including several with AR-V7 expression[29, 38, 39]. They found that niclosamide concentrations ≥163.5 ng/mL consistently inhibited cell growth and that some AR-V7 + cell lines were inhibited by concentrations as low as 81.8 ng/mL. For instance, enzalutamide-resistant, AR-V7+ C4-2B cells were growth inhibited when co-cultured with 81.8 ng/mL of niclosamide; however, AR-V7+ CWR22Rv1 cells did not demonstrate significant growth inhibition when exposed to this concentration of niclosamide[29]. Similarly, enzalutamide-resistant LNCaP cells that had been engineered to maintain Stat3 activation were growth inhibited only when exposed to niclosamide concentrations ≥163.5 ng/mL[39]. It is worth noting that both of these studies demonstrated a growth inhibitory effect with lower concentrations of niclosamide when cells were treated concurrently with enzalutamide. On the basis of these data, we concluded that niclosamide plasma concentrations below 163.5 ng/mL were unlikely to exert a clinically meaningful effect. Overall, we observed one of three patients in the 500 mg PO TID cohort and one of two patients in the 1000 mg PO TID cohort achieve a C_{max} ≥163.5 ng/mL. There was no evidence of clinical activity in any patient enrolled and toxicity prevented dose escalation above the 500 mg PO TID cohort. Therefore, the Data Safety Monitoring Board for the trial recommended that the trial terminate prematurely for futility.

This study was originally designed to include AR-V positivity as a novel integral biomarker given that: 1) AR-V7 has been hypothesized to promote resistance to drugs that interfere with the AR-ligand/AR interaction (i.e., abiraterone and enzalutamide); 2) niclosamide has been shown to be effective in preclinical models of AR-V+ CRPC; and 3) prior studies documented AR-V positivity in >45% of mCRPC patients post-abiraterone and/or enzalutamide[9, 29, 38, 40, 41]. However, the proportion of AR-V+ patients in our study fell well short of the 45% prevalence mark, with only 5% of screened subjects testing positive using the AdnaTest[9, 40]. Because of low AR-V detection rates, we ultimately abandoned AR-V positivity as an inclusion criterion. It is notable that our experience with AR-V detection closely mirrors that of the ARMOR 3 Trial, a Phase III trial testing galeterone (an oral AR-signaling inhibitor that has also been shown to degrade AR-Vs), which documented 8% AR-V detection in men with treatment-naïve mCRPC[41, 42]. This study, as well as the ARMOR 3 Trial, highlights the importance of having assays that have been validated across institutions and have undergone rigorous pre-study confirmatory testing.

The small sample size of this trial represents its primary limitation, and while relatively few patients were treated per protocol, our data still represent the largest cohort of niclosamide-treated patients with contemporary safety and pharmacokinetic data. Prior conclusions regarding the pharmacokinetic profile of niclosamide all trace back to one internal Bayer document from 1971 in which an undisclosed number of healthy volunteers received a single dose of ^{14}C -labeled niclosamide[35]. Ultimately, the plasma niclosamide concentrations observed at the maximum tolerated dose (i.e., niclosamide 500 mg PO TID) were not consistently in the range shown to have activity in preclinical models of abiraterone and enzalutamide-resistant CRPC, and toxicity prevented us from escalating to a therapeutic dose. It is important to bear in mind, however, that because we did not evaluate enzalutamide pharmacokinetics, we are unable to exclude the possibility that there was a drug-drug interaction between enzalutamide and niclosamide. In addition, there was insufficient data to characterize niclosamide's steady state pharmacokinetics and it remains possible that niclosamide may accumulate over time or undergo more rapid clearance.

Overall, we conclude that the development of the current oral formulation of niclosamide as a cancer therapy should not be pursued. Attention should be turned to developing niclosamide analogs with improved oral bioavailability and enhanced antitumor effects.

Supporting information

S1 TREND Checklist. TREND statement checklist.
(PDF)

S1 Protocol. Clinical protocol.
(PDF)

Acknowledgments

We are grateful for the patients who participated in this study.

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Writing – original draft: Michael T. Schweizer.

Writing – review & editing: Kathleen Haugk, Jožefa S. McKiernan, Roman Gulati, Heather H. Cheng, Jessica L. Maes, Ruth F. Dumpit, Peter S. Nelson, Bruce Montgomery, Jeannine S. McCune, Stephen R. Plymate, Evan Y. Yu.

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RESEARCH ARTICLE

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Microsatellite instability in prostate cancer by PCR or next-generation sequencing

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Abstract

Background: Microsatellite instability (MSI) is now being used as a sole biomarker to guide immunotherapy treatment for men with advanced prostate cancer. Yet current molecular diagnostic tests for MSI have not been evaluated for use in prostate cancer.

Methods: We evaluated two next-generation sequencing (NGS) MSI-detection methods, MSIplus (18 markers) and MSI by Large Panel NGS (> 60 markers), and compared the performance of each NGS method to the most widely used 5-marker MSI-PCR detection system. All methods were evaluated by comparison to targeted whole gene sequencing of DNA mismatch-repair genes, and immunohistochemistry for mismatch repair genes, where available.

Results: In a set of 91 prostate tumors with known mismatch repair status (29-deficient and 62-intact mismatch-repair) MSIplus had a sensitivity of 96.6% (28/29) and a specificity of 100% (62/62), MSI by Large Panel NGS had a sensitivity of 93.1% (27/29) and a specificity of 98.4% (61/62), and MSI-PCR had a sensitivity of 72.4% (21/29) and a specificity of 100% (62/62).

Conclusions: We found that the widely used 5-marker MSI-PCR panel has inferior sensitivity when applied to prostate cancer and that NGS testing with an expanded panel of markers performs well. In addition, NGS methods offer advantages over MSI-PCR, including no requirement for matched non-tumor tissue and an automated analysis pipeline with quantitative interpretation of MSI-status.

Keywords: Prostate adenocarcinoma, Microsatellite instability, MSI, Promega, Capillary electrophoresis, Mismatch repair, NGS, Next-generation sequencing, mSINGS

Background

Microsatellite instability (MSI) is characterized by mutations in repetitive DNA sequence tracts, caused by a failure of the DNA mismatch repair system to correct these errors. Deficient DNA mismatch repair (dMMR) results from the bi-allelic mutational inactivation or epigenetic silencing of any of the genes in the MMR pathway (most commonly *MSH2*, *MSH6*, *MLH1*, and *PMS2*). Consequently, MSI status is used as a biomarker indicative of dMMR. In May of 2017, the U.S. Food and Drug Administration (FDA) granted accelerated approval of an immunotherapy-based anti-PD-1 cancer treatment

(pembrolizumab) for patients whose cancers have MSI or dMMR [1]. This is the first time the FDA has approved a drug based on the genetic characteristics of a tumor alone, regardless of the tumor's original location ("tumor agnostic").

MSI has been most closely studied in colorectal cancers, where it is present in up to 15–20% of cases [2]. However MSI has been found in many cancer types including endometrial (26–33%), ovarian (10%), cervical (8%), and gastric (8–22%) [3–9]. In prostate cancer, MSI and dMMR have been reported in a subset of tumors ranging from ~1% in primary to up to 12% of metastatic cancers [10–13].

Prostate cancer is the third leading cause of cancer death in American men and survival rates are low for prostate cancers that advance to metastatic castration-

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resistant disease [14]. Despite recent advances and a range of treatment options for metastatic castration-resistant prostate cancer (mCRPC), outcomes are varied and clinicians are not able to predict response to the available therapies [15]. Predictive biomarkers, which can tailor the treatment of mCRPC to individual patients, are urgently needed. MSI a promising marker for evaluation in prostate cancer, with robust responses to pembrolizumab reported in MSI advanced mCRPC [16]. However, current MSI-detection assays have not been evaluated for use in prostate cancer. The original National Cancer Institute (NCI) recommended 5-marker microsatellite panel, commonly known as the “Bethesda panel,” was developed in 2004 to screen patients for hereditary non-polyposis colorectal cancer (also known as Lynch syndrome) [17, 18]. The original NCI report makes clear that “the [Bethesda] reference panel is recommended for the characterization of MSI in colorectal cancer only” [17]. This panel is the basis for the most widely used MSI detection assay, the Promega MSI Analysis System [18]. While subsequent studies have shown the efficacy of using the Bethesda panel to test for MSI in other cancer types, specifically endometrial cancers [19], other studies have reported that the instability of microsatellite loci can vary greatly across different cancer types [3].

Yet regardless of tumor type, conventional MSI testing is still routinely performed using PCR and fragment analysis of the 5-marker Bethesda panel (BAT-25, BAT-26, MONO-27, NR-21 and NR-24) [17, 18] and/or through immunohistochemical (IHC) detection of MMR proteins. IHC also has diagnostic limitations [20]. For example, IHC cannot always detect loss of mutated proteins resulting from missense mutations and can have normal staining even for some protein-truncating mutations [21]. Consequently, there is an immediate need for a highly sensitive and specific diagnostic assay for MSI targeted to specific tumor types.

To address this need in prostate cancer, we evaluated two next-generation sequencing (NGS) MSI-detection methods, MSIplus [22] and MSI by Large Panel NGS [23], for efficacy in prostate cancer and compared the performance characteristics of each method to the MSI-PCR based on the 5-marker Bethesda panel [18] (Promega, Madison, WI, USA). Concurrently, we compared both NGS methods and MSI-PCR to targeted deep sequencing of MMR genes: because dMMR is caused by bi-allelic gene mutation rather than epigenetic silencing in prostate cancer [11], deep sequencing can provide a definitive “gold-standard” diagnosis.

Methods

Patients and specimens

A total of 91 prostate tumor samples were analyzed from 4 different sources: 1) Primary and metastatic prostate

cancer tissue from the University of Washington (UW) Prostate Cancer Donor Rapid Autopsy Program ($n = 31$) [11], 2) LuCaP patient-derived tumor xenografts (PDX) ($n = 23$) [11, 24], 3) UW-OncoPlex prostate cancer precision medicine program ($n = 28$) [25], and 4) Johns Hopkins University ($n = 9$) [26]. All tumors had $> 20\%$ neoplastic cellularity. Of the 91 total samples, 80 represent unique patients or xenografts. The 11 non-unique specimens represent primary and metastatic tissues from the same patient, LuCaP PDX subtypes (castration-resistant or not), or LuCaP PDX derived from patient samples. Genomic DNA was prepared from either formalin-fixed paraffin-embedded tissue (FFPE, $n = 71$) or fresh-frozen tissue ($n = 20$) with the Gentra Puregene DNA Isolation Kit (Qiagen, Catalog #158489). Clinical specimens were obtained in accordance with the declaration of Helsinki and the ethics guidelines of the human subjects division of the University of Washington and Johns Hopkins University.

Microsatellite instability by MSIplus

The MSIplus panel includes the 5 microsatellite markers that comprise the Promega MSI Analysis System plus an additional 13 discriminatory microsatellite markers which are frequently unstable in MSI positive tumors (Table 1). Detection of MSI status by MSIplus was performed by the CLIA-certified UW clinical genetics and solid tumors laboratory as described in Hempelmann et al. [22] following a standard operating procedure that was clinically validated to assess MSI status in colorectal samples (see Additional file 1 for detail). Based on established guidelines for small panels of selected discriminatory microsatellite markers, a fraction of ≥ 0.33 ($\geq 33\%$ unstable loci) was considered MSI-positive [17, 27–30].

Microsatellite instability by large panel NGS

MSI was assessed from BROCA or UW-OncoPlex sequence data [11, 31] using mSINGS as previously described by Salipante et al. [23]. This method evaluates microsatellite loci that are incidentally captured during targeted sequencing of gene panels (146 mononucleotide microsatellite loci captured by BROCA and 65 mononucleotide microsatellite loci captured by UW-OncoPlex) (Additional file 2: Table S1). Based on previous validation studies, a fraction of > 0.20 ($> 20\%$ unstable loci) was considered MSI-positive [23]. This threshold is lower than that used for MSIplus (> 0.33) because Large Panel NGS evaluates unselected microsatellites, whereas MSIplus evaluates loci selected for their ability to distinguish MSI-negative and MSI-positive samples, conferring high discriminatory power.

Microsatellite instability by MSI-PCR

Testing was performed by the UW clinical genetics and solid tumors laboratory using the 5-marker MSI Analysis

Table 1 MSIplus and MSI-PCR Microsatellite Loci

Target Loci	Panel(s)	Loci Coordinates (GRCh37/hg19)	Repeat Type	Gene
Bat25	MSI-PCR, MSIplus	chr4:55598212–55598236	(T)25	KIT, intronic
Bat-26	MSI-PCR, MSIplus	chr2:47641560–47641586	(A)27	MSH2, intronic
MONO-27	MSI-PCR, MSIplus	chr2:39564894–39564921	(T)28	MAP4K3, intronic
NR-21	MSI-PCR, MSIplus	chr14:23652347–23652367	(A)21	SLC7A8, exonic
NR-24	MSI-PCR, MSIplus	chr2:95849362–95849384	(T)23	ZNF2, exonic
MSI-01	MSIplus	chr1:201754411–201754427	(T)17	NAV1, intronic
MSI-03	MSIplus	chr2:62063094–62063110	(A)17	FAM161A, intronic
MSI-04	MSIplus	chr2:108479623–108479640	(T)18	RGPD4, intronic
MSI-06	MSIplus	chr5:172421761–172421775	(T)15	ATP6V0E1, intronic
MSI-07	MSIplus	chr6:142691951–142691967	(T)17	GPR126, intronic
MSI-08	MSIplus	chr7:1787520–1787536	(A)17	ELFN1, exonic
MSI-09	MSIplus	chr7:74608741–74608753	(T)13	GTF2IP1, intronic
MSI-11	MSIplus	chr11:106695515–106695526	(T)12	GUCY1A2, intronic
MSI-12	MSIplus	chr15:45897772–45897785	(T)14	BLOC1S6, intronic
MSI-13	MSIplus	chr16:18882660–18882674	(A)15	SMG1, intronic
MSI-14	MSIplus	chr17:19314918–19314935	(T)18	RNF112, intronic
HSPH1-T17	MSIplus	chr13:31722621–31722637	(A)17	HSPH1, intronic
EWSR1	MSIplus	chr22:29696469–29696484	(T)16	EWSR1, exonic

For coordinates of the loci captured by Large-Panel NGS see Additional file 2: Table S1

System v1.2 (Promega, Madison, WI, USA) following a clinically-validated standard operating procedure based on the manufacturers' protocol (see Additional file 1 for detail). All MSI-PCR electropherograms were analyzed by at least 2 independent reviewers for evidence of MSI. Specimens demonstrating instability within 2 or more of the 5 markers were considered 'MSI-positive' and all others were considered 'MSI-negative'. We did not classify any specimens as 'MSI-low' given the accumulation of evidence showing that it is not reliable when examining a small number of loci [3, 27, 32].

Limit of detection of MSI assays

Previous clinical validation of MSIplus, Large Panel NGS, and MSI-PCR completed by the UW clinical genetics and solid tumors laboratory established the limit of detection at 20% tumor-cellularity for all assays.

Targeted sequencing by large panel NGS

Targeted deep sequencing of DNA mismatch repair genes was performed by the UW clinical genetics and solid tumors laboratory using either the BROCA or UW-OncoPlex Large Panel NGS assays as previously described [11, 31].

Immunohistochemistry

A subset of 21 autopsy samples were tested for expression of MMR proteins by immunohistochemistry (IHC)

tissue microarray as described by Pritchard et al. [11] and Nghiem et al. [33].

Results

Establishing a validation sample set

We selected validation samples from 4 prostate cancer series comprising primary and metastatic disease. dMMR is caused by bi-allelic gene mutation rather than epigenetic silencing in prostate cancer [11]. Therefore, we established "gold standard" dMMR status based on targeted deep gene sequencing ($n = 91$ total; $n = 29$ with bi-allelic dMMR inactivating mutations, and $n = 62$ without) (Additional file 2: Table S2). For all further analyses, samples with bi-allelic dMMR mutations were considered true MSI-positives and samples without bi-allelic dMMR mutations were considered true MSI-negatives.

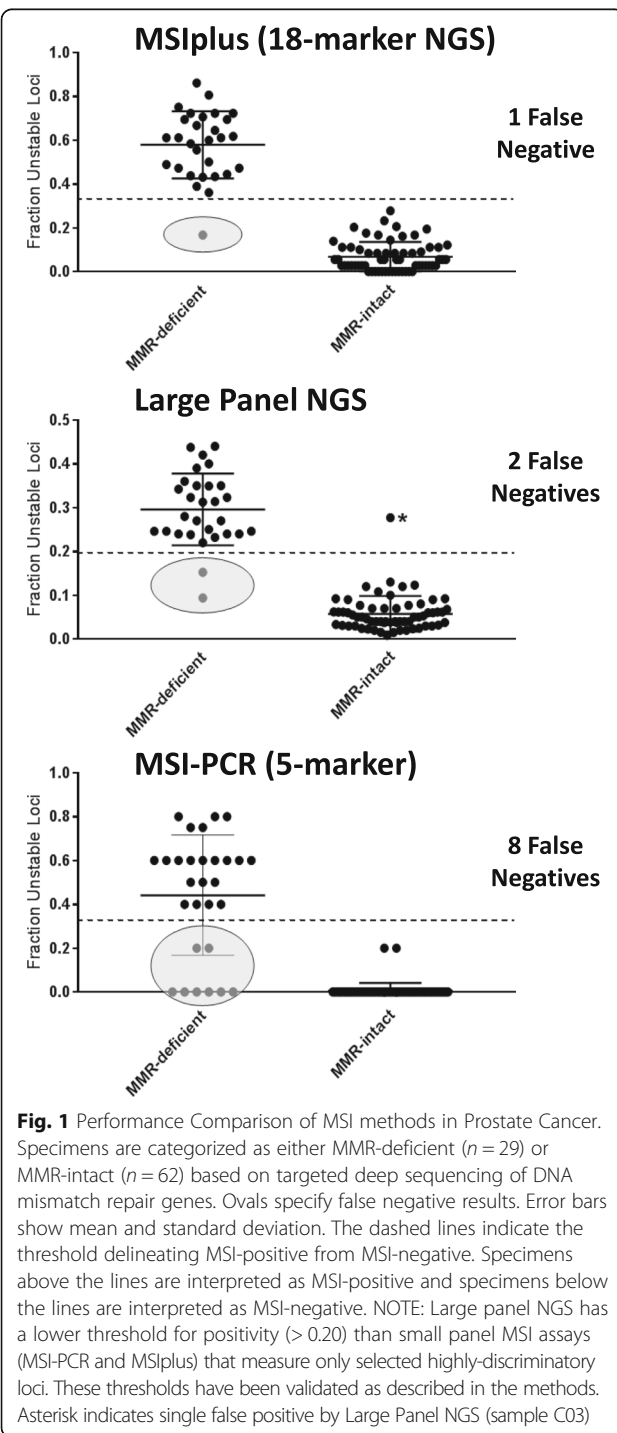
Microsatellite instability by MSIplus

MSIplus had a sensitivity of 96.6% (28 of 29 MSI-positive cases identified; 95% CI, 80.4%–99.8%), and a specificity of 100% (62 of 62 MSI-negative cases identified; 95% CI, 92.7%–100%) (Table 2) (Fig. 1a) (Additional file 2: Table S2).

All samples typed by MSIplus were run in duplicate, yielding a set of 182 separately prepared and sequenced within-run replicates with which to evaluate reproducibility (Additional file 2: Table S3). Thirty-five percent of the replicates had identical mSINGS scores. The average absolute difference between technical replicates was 0.06 mSINGS units. There were two instances of discordant

Table 2 Performance Characteristics of MSIplus, Large-Panel NGS, and MSI-PCR in Prostate Cancer

Assay	Sensitivity [95% CI]	Specificity [95% CI]
MSIplus	96.6% [80.4%–99.8%]	100% [92.7%–100%]
Large-Panel NGS	93.1% [75.8%–98.8%]	98.4% [90.2%–99.9%]
MSI-PCR	72.4% [52.5%–86.6%]	100% [92.7%–100%]



MSIplus interpretations between technical replicates (sample A10, mSINGS scores: 0.3529 and 0.1111; and sample L12, mSINGS scores: 0.3333 and 0.2222). After averaging the mSINGS scores of the replicates (see Additional file 1), both samples were correctly classified as MSI-negative.

Microsatellite instability by large panel NGS

Examining the same 91 prostate specimens, we found that Large Panel NGS had a sensitivity of 93.1% (27 of 29 MSI-positive cases identified; 95% CI, 75.8%–98.8%) and a specificity of 98.4% (61 of 62 MSI-negative cases identified; 95% CI, 90.2%–99.9%) (Table 2) (Fig. 1b) (Additional file 2: Table S2).

Microsatellite instability by MSI-PCR

For the same set of samples, MSI-PCR had a sensitivity of 72.4% (21 of 29 MSI-positive cases identified; 95% CI, 52.5%–86.6%) and a specificity of 100% (62 of 62 MSI-negative cases identified; 95% CI, 92.7%–100%) (Table 2) (Fig. 1c) (Additional file 2: Table S2). While a consensus was reached for all 91 specimens, initial interpretations produced discordant results for two MSI-negative samples (A10 and A14). Director-A interpreted both samples as MSI-negative and director-B interpreted both as MSI-positive (Additional file 2: Table S4). After three independent laboratory directors (A, B, & C) reviewed the discordant samples as a group, all directors interpreted the samples as MSI-negative. There were an additional 10 samples for which the directors' overall diagnosis was concordant (either negative or positive) but the total number of unstable loci was discordant (Additional file 2: Table S4).

Correlation with immunohistochemistry (IHC)

Although our primary objective was to compare the performance of expanded-marker NGS assays to MSI-PCR, we also evaluated the performance of IHC when this information was available (Additional file 2: Table S2). For the 21 samples with corresponding IHC data, gene sequencing confirmed that 7 were MMR-deficient and 14 MMR-intact. The results of MSI-testing by MSIplus, Large Panel NGS, and MSI-PCR were concordant for these 21 samples (7/7 MMR-deficient were MSI-positive; 14/14 MMR-intact were MSI-negative). The IHC results were consistent with the findings of the other approaches in all but 2 of the 21 cases. One MMR-intact, MSI-negative autopsy sample (A03) had isolated loss of MSH6 by IHC, and another MMR-intact, MSI-negative autopsy sample (A18) had isolated loss of PMS2 by IHC [11, 33].

Concordance between tissue sites

We evaluated concordance between tissue sites in one pair of autopsy samples (A24.1 & A24.2) from different metastatic sites, a second pair of autopsy samples (A28.1 & A28.2) from primary and metastatic tumor sites, and in 8 pairs of LuCaP PDX samples which included both the original xenograft lines and castration-resistant (CR) lines. In these samples MSI status was always concordant between metastatic sites, between primary and metastatic tumor, and between CR and non-CR PDX lines for all MSI detection methods (Additional file 2: Table S2).

Discussion

We found that the conventional MSI-PCR method developed and validated for colon cancer has inferior sensitivity when applied to prostate cancer, and that NGS testing with an expanded panel of markers performs more robustly. For our test set, the 5-marker Bethesda Panel (MSI-PCR) had a sensitivity of only 72.4% whereas the expanded 18-marker NGS assay MSIplus had a sensitivity of 96.6% and Large Panel NGS had a sensitivity of 93.1%. Specificity was high for all approaches: neither MSI-PCR nor MSIplus generated any false positive results, and Large Panel NGS generated only one false positive (Fig. 1). Predictive modeling estimates that by the year 2020, there will be over 40,000 men in the United States with mCRPC and that more than half of those patients will see their disease progress after exhausting standard treatment options [34]. A systematic review of available epidemiological data supports these estimates [14]. Even if MSI is present in only ~5% of these patients, using an MSI approach that is more sensitive could qualify many more men for life-extending immunotherapy.

In addition to improved sensitivity, NGS-based MSI testing with MSIplus or Large-panel NGS offers several advantages over conventional capillary electrophoresis MSI-PCR methods. 1) *they do not require matched non-tumor tissue*. MSI-PCR typically requires a matched-normal DNA sample for every specimen assayed because rare germline polymorphisms may be misinterpreted as positive microsatellite loci. Likewise, some NGS-based MSI-detection methods such as MANTIS [35] and MSI-sensor [36] require a matched-normal sample. Neither MSIplus nor Large Panel NGS require matched-normal DNA, which helps to simplify testing logistics and reduce cost. 2) *Interpretation is streamlined and semi-automated*. The interpretation of MSIplus and Large Panel NGS is completed by an automated analysis pipeline based on quantitative statistics (mSINGS) [23], whereas interpretation of MSI-PCR data is both manual and qualitative. Automation greatly reduces analysis time and is likely to reduce inter-observer and inter-laboratory variation. Conversely, the inter-laboratory variability of MSI-

PCR has been systemically demonstrated by the College of American Pathologists (CAP): results of the 2011 CAP microsatellite instability 5-marker capillary electrophoresis data interpretation survey which tested 88 independent laboratories revealed that only 78% of laboratories correctly identified an MSI-positive specimen [37].

Our study has several limitations that should be addressed in future work. It was not designed to assess the diagnostic accuracy of mismatch repair protein IHC in prostate cancer, which is a mainstay of testing for purpose of inferring MSI status in many pathology laboratories. In addition, we do not have information on responses to pembrolizumab or other clinical outcomes in our validation set. Our study design does not permit estimation of positive or negative predictive value because the samples are not part of a consecutive prospective series. Finally, we did not assess total mutational burden, which is another emerging biomarker which has been associated with mutations in MMR genes and sensitivity to immune checkpoint inhibitors [38–40].

Conclusions

In summary, our study demonstrates that expanded panel MSI NGS assays performed well in prostate cancer, and that the conventional 5-marker capillary electrophoresis MSI-PCR assay had inferior sensitivity. Both NGS assays we studied were superior to MSI-PCR, however MSIplus is probably most appropriate as first-line MSI screening in a low-prevalence population. MSIplus is an amplicon-based NGS assay, which takes less time, uses less sample material, and is much lower cost than Large Panel NGS. Given the ever-increasing demand for clinical MSI testing following the FDA's tumor agnostic approval of pembrolizumab for MSI-positive cancers, a low-cost, relatively fast, and highly sensitive/specific assay for MSI is urgently needed. MSIplus fulfills all these criteria and is now validated for both colorectal and prostate cancer. The MSIplus assay could be further improved by adding microsatellite loci that are especially informative in prostate cancer.

Additional files

Additional file 1: Supplementary Methods. (DOCX 20 kb)

Additional file 2: Table S1. Large-Panel NGS Microsatellite Loci. **Table S2.** Detail on Samples, Targeted Sequencing, Microsatellite Instability, and IHC (when available). **Table S3.** Detail on MSIplus Technical Replicates. **Table S4.** Discordant Interpretation of MSI-PCR (Promega 5-marker). **Table S5-A.** MSIplus Stage-1 PCR Primers. **Table S5-B.** MSIplus Stage-2 PCR Primers. **Table S5-C.** MSIplus Custom Index Sequencing Primer. (XLSX 143 kb)

Abbreviations

CAP: College of American Pathologists; CI: Confidence-interval; CLIA: Clinical laboratory improvement amendments; CR: Castration-resistant; dMMR: Deficient DNA mismatch repair; FDA: US Food and Drug Administration; FFPE: Formalin-fixed paraffin-embedded tissue; IHC: Immunohistochemistry; mCRPC: Metastatic

castration-resistant prostate cancer; MMR: DNA Mismatch repair; MSI: Microsatellite instability; NCI: National Cancer Institute; NGS: Next-generation sequencing; PDX: Patient-derived tumor xenograft; UW: University of Washington

Acknowledgements

We thank Rachel Slusher for help with MSI-PCR and all members of UW clinical genetics and solid tumors laboratory for their help in preparing the genomic DNA. We thank the patients and their families, Evan Yu, Elahe Mostaghel, Heather Cheng, Andrew Hsieh, Celestia Higano, Paul Lange, Daniel Lin, Funda Vakar-Lopez, Xiaotun Zhang, Martine Roudier, Lawrence True, Robert Vessella, Eva Corey, Colm Morrissey and the rapid autopsy teams for their contributions to the University of Washington Medical Center Prostate Cancer Donor Rapid Autopsy Program and the Development of the LuCaP PDX models.

Funding

Funding for this research was provided in part by the Prostate Cancer Foundation (CCP), a Department of Defense PCRP award PC131820 (CCP), DOD awards W81XWH-15-1-0430, W81XWH-15-1-0562 (PSN), Transformative Impact Award (TLL), and Prostate Cancer Biorepository Network (PCBN) awards (W81XWH-10-2-0056 and W81XWH-10-2-0046), support from the Institute for Prostate Cancer Research (IPCR), NIH support from the Pacific Northwest Prostate Cancer SPOR (P50CA97186) and PO1 CA163227, and support from the Richard M. LUCAS Foundation.

Availability of data and materials

The data generated or analyzed during this study are included in this published article [and its supplementary information files], or in previously published studies as referenced.

Authors' contributions

CCP conceived, designed, and supervised the study, and coordinated sample acquisition. JH performed the experiments and primary data analyses. CL, EQK, and SJS performed data analysis and assisted with the study design. CM, MS, ESA, TLL, BM, PSN, and NK assisted with tissue collection and selection. CM performed and analyzed the IHC studies. JH wrote the manuscript, with contributions from CCP. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Clinical specimens were obtained in accordance with the declaration of Helsinki and the ethics guidelines of the human subjects division of the University of Washington and Johns Hopkins University.

Consent for publication

Not applicable.

Competing interests

TLL has received research support from Ventana Medical Systems. CCP has no conflicts to disclose related to this work.

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Received: 9 February 2018 Accepted: 4 April 2018

Published online: 17 April 2018

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Bipolar androgen therapy in men with metastatic castration-resistant prostate cancer after progression on enzalutamide: an open-label, phase 2, multicohort study

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Summary

Background Prostate cancer that progresses after enzalutamide treatment is poorly responsive to further antiandrogen therapy, and paradoxically, rapid cycling between high and low serum testosterone concentrations (bipolar androgen therapy [BAT]) in this setting might induce tumour responses. We aimed to evaluate BAT in patients with metastatic castration-resistant prostate cancer that progressed after enzalutamide.

Methods We did this single-centre, open-label, phase 2, multicohort study in the USA. We included patients aged 18 years or older who had histologically confirmed and radiographically documented metastatic castration-resistant prostate cancer, with no more than two previous second-line hormonal therapies, and a castrate concentration of testosterone. Patients were asymptomatic, with Eastern Cooperative Oncology Group performance status of 0–2, and did not have high-risk lesions for tumour flare (eg, >5 sites of visceral disease or bone lesions with impending fracture). For the cohort reported here, we required patients to have had progression on enzalutamide with a continued prostate-specific antigen (PSA) rise after enzalutamide treatment discontinuation. Patients received BAT, which consisted of intramuscular testosterone cypionate 400 mg every 28 days until progression and continued luteinising hormone-releasing hormone agonist therapy. Upon progression after BAT, men were rechallenged with oral enzalutamide 160 mg daily. The co-primary endpoints were investigator-assessed 50% decline in PSA concentration from baseline (PSA₅₀) for BAT (for all patients who received at least one dose) and for enzalutamide rechallenge (based on intention-to-treat analysis). These data represent the final analysis for the post-enzalutamide cohort, while two additional cohorts (post-abiraterone and newly castration-resistant prostate cancer) are ongoing. The trial is registered with ClinicalTrials.gov, number NCT02090114.

Findings Between Aug 28, 2014, and May 18, 2016, we accrued 30 eligible patients and treated them with BAT. Nine (30%; 95% CI 15–49; $p < 0.0001$) of 30 patients achieved a PSA₅₀ to BAT. 29 patients completed BAT and 21 proceeded to enzalutamide rechallenge, of whom 15 (52%; 95% CI 33–71; $p < 0.0001$) achieved a PSA₅₀ response. During BAT, the only grade 3–4 adverse event occurring in more than one patient was hypertension (three [10%] patients). Other grade 3 or worse adverse events occurring during BAT in one [3%] patient each were pulmonary embolism, myocardial infarction, urinary obstruction, gallstone, and sepsis. During enzalutamide retreatment, no grade 3–4 toxicities occurred in more than one patient. No treatment-related deaths were reported during either BAT or enzalutamide retreatment.

Interpretation BAT is a safe therapy that resulted in responses in asymptomatic men with metastatic castration-resistant prostate cancer and also resensitisation to enzalutamide in most patients undergoing rechallenge. Further studies with BAT are needed to define the potential clinical role for BAT in the management of metastatic castration-resistant prostate cancer and the optimal strategy for sequencing between androgen and antiandrogen therapies in metastatic castration-resistant prostate cancer to maximise therapeutic benefit to patients.

Funding National Institutes of Health and National Cancer Institute.

Introduction

Metastatic castration-resistant prostate cancer remains dependent on androgen receptor signalling for growth.¹ Molecularly, this dependence is characterised by nearly universal upregulation of androgen receptor expression.^{2–4} Enzalutamide, an androgen receptor antagonist, was developed to inhibit androgen receptor signalling despite upregulated androgen receptor concentrations in patients with metastatic castration-resistant prostate

cancer.⁵ Enzalutamide is effective in inducing tumour responses and produces a survival benefit in these patients.^{6,7} Clinically, metastatic castration-resistant prostate cancer that has progressed after enzalutamide treatment is minimally responsive to further therapy that inhibits androgen receptor signalling. This resistance is often mediated by a reactivation of the androgen receptor-signalling programme, often through androgen receptor overexpression, gene amplification, mutations,

Lancet Oncol 2017

Published Online
December 13, 2017
[http://dx.doi.org/10.1016/S1470-2045\(17\)30906-3](http://dx.doi.org/10.1016/S1470-2045(17)30906-3)

See Online/Comment
[http://dx.doi.org/10.1016/S1470-2045\(17\)30907-5](http://dx.doi.org/10.1016/S1470-2045(17)30907-5)

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Research in context

Evidence before this study

We did a systematic PubMed review on Sept 30, 2017, which included all articles to date with no language restrictions, using the terms “supraphysiologic testosterone prostate cancer”, which identified 16 results. The concept of using high concentrations of hormones as treatment for hormone-driven cancers was first described by Charles Huggins in 1964. At the time of study conception and initiation, only two previous studies described the use of physiological doses of testosterone as therapy for castration-resistant prostate cancer. Preclinical and pilot trial results from our institution with supraphysiological testosterone supported the hypotheses tested in this study. However, no data have been published regarding the efficacy of cyclic parenteral supraphysiological testosterone for castration-resistant prostate cancer and no trials have been done prospectively investigating the efficacy of enzalutamide rechallenge after bipolar androgen therapy (BAT) or any other intervening therapy. Evidence has shown that adaptive upregulation of androgen receptor concentrations, via upregulation of expression, gene amplification, and splice variant expression, contributed to resistance to androgen receptor-signalling inhibitors in prostate cancer. Multiple clinical reports describe diminishing clinical response with sequential use of androgen receptor-signalling inhibitors, such as abiraterone and enzalutamide. This cross-resistance to androgen ablative therapies has emerged as a major clinical problem.

Added value of this study

To our knowledge, this study is the first prospective trial to investigate the activity and safety of administration of pharmacological doses of testosterone to rapidly cycle between the extremes of supraphysiological and near-castrate serum testosterone concentrations (ie, BAT) in patients with metastatic castration-resistant prostate cancer after progression on enzalutamide. Although paradoxical, the results of this trial suggest that BAT, as an androgen receptor-directed agonist therapy, can result in clinical responses, while transiently resensitising most patients to further enzalutamide therapy. Furthermore, BAT could be administered with a reasonable safety and tolerability profile. CTC-based analysis of AR-FL and AR-V7 mRNA expression showed that the profile of responders appears distinct from previous studies associating AR-FL and AR-V7 with poor response.

Implications of all the available evidence

The results of this trial suggest a role for BAT in sequence with androgen ablative therapies as part of hormonal treatment for prostate cancer aimed at disrupting androgen receptor signalling within prostate cancer cells. With a paucity of data regarding best sequencing of androgen receptor-directed therapies in patients, the efficacy of BAT and transient resensitisation to enzalutamide serve as a basis for development of sequential or combinatorial approaches to improve outcomes for patients with metastatic castration-resistant prostate cancer.

and expression of ligand-independent androgen receptor splice variants.^{8,9} Alternate approaches beyond androgen receptor-signalling inhibition are needed to produce clinical benefit in multidrug-resistant, metastatic castration-resistant prostate cancer.¹⁰

Although the adaptive overexpression of the androgen receptor by metastatic castration-resistant prostate cancer cells induces resistance to androgen receptor antagonists, this overexpression also represents a therapeutic vulnerability. In the setting of overexpressed androgen receptor, the administration of sufficient testosterone to achieve supraphysiological serum concentrations has paradoxically been shown to result in prostate cancer cell death and tumour regression in preclinical models.^{11,12} After an initial response to supraphysiological testosterone, castration-resistant prostate cancer cells can adaptively downregulate androgen receptors to allow for growth in an environment replete with testosterone.¹³ Theoretically, rapidly varying the androgen concentrations between the extremes of supraphysiological and near-castrate, a strategy termed bipolar androgen therapy (BAT),¹⁴ provides insufficient time for castration-resistant prostate cancer cells to adaptively regulate androgen receptor concentrations in response to androgen concentrations in the microenvironment. Therefore, castration-resistant prostate cancer cells with high

androgen receptor concentrations are vulnerable to cell death following exposure to high androgen concentrations, and the rapidly varying androgen concentrations might prevent readaptation and resistance.^{14,15} The possible mechanisms through which BAT might operate include disruption of the process of DNA relicensing required for cell division and introduction of double-strand DNA breaks that could induce genomic instability.^{16,17} On the basis of these preclinical mechanistic studies, we did a pilot study¹⁸ testing BAT in combination with etoposide for three cycles (28 days each) followed by BAT monotherapy in patients with metastatic castration-resistant prostate cancer who were responding. While the combination with chemotherapy showed toxicity, BAT monotherapy was well tolerated, and radiographic and biochemical responses were seen with and without etoposide. After treatment with BAT was completed, most patients went on to benefit from further androgen receptor-directed therapy, despite being heavily pretreated.

Given the encouraging outcomes of this pilot study with BAT, we sought to test BAT in patients with metastatic castration-resistant prostate cancer who previously progressed on androgen receptor-directed therapies, including enzalutamide. We hypothesised that BAT, when used after enzalutamide, would therapeutically

exploit adaptive androgen receptor upregulation resulting in tumour regression. We further hypothesised that patients progressing on BAT would have restored sensitivity to enzalutamide as the result of suppression of secondary resistance mechanisms (eg, androgen receptor splice variants or androgen receptor overexpression). To test these hypotheses, we did a phase 2 trial of BAT and subsequent enzalutamide rechallenge in a cohort of patients with asymptomatic metastatic castration-resistant prostate cancer after progression on enzalutamide.

Methods

Study design and participants

We did this multicohort, single-centre, open-label, phase 2 trial at Johns Hopkins University, Baltimore, MD, USA, to test BAT in cohorts of patients progressing on hormonal therapies, with the intention for each cohort to be analysed individually (protocol available in the appendix). For the post-enzalutamide cohort, we included patients aged 18 years or older if they had metastatic castration-resistant prostate cancer (disease progression despite a castrate concentration of testosterone [<50 ng/dL]). Evidence of metastatic disease by CT scan or nuclear medicine bone scan was required, but measurable disease by Response Evaluation Criteria in Solid Tumors (RECIST) was not a prerequisite for enrolment. Additionally, men were required to have radiographic or prostate-specific antigen (PSA) progression on enzalutamide, and a continuing PSA concentration rise during the 4 week washout from enzalutamide. We allowed up to two previous second-line hormonal therapies for metastatic castration-resistant prostate cancer (beyond first-generation antiandrogens and ketoconazole). We permitted previous docetaxel for castration-sensitive disease, radium-223, and sipuleucel-T. We also required patients to be asymptomatic or minimally symptomatic from their disease, and have an Eastern Cooperative Oncology Group performance status of 0–2. Adequate marrow and organ function was required for eligibility (ie, creatinine $<2.5 \times$ upper limit of normal [ULN]; bilirubin, aspartate aminotransferase, and alanine aminotransferase $<2.5 \times$ ULN; absolute neutrophil count $\geq 1500/\mu\text{L}$, platelets $\geq 100\,000$ per μL , and haemoglobin ≥ 9 g/L). No minimum estimated life expectancy was required.

Given the potential for flare after testosterone initiation, we excluded patients if they had cancer-related pain requiring the use of opioid analgesics, the presence of more than five sites of visceral disease, urinary obstruction requiring catheterisation, or other lesions deemed high risk (such as femoral or spinal metastases with impending pathological fracture or cord compression). Additional exclusion criteria were active uncontrolled infection (HIV, hepatitis B, or hepatitis C), haematocrit of more than 50%, thromboembolic event in the past 2 years if not on systemic anticoagulation,

myocardial infarction in the past year, uncontrolled heart failure, untreated severe obstructive sleep apnoea, or previous history of seizures while on enzalutamide. Previous treatment with docetaxel or cabazitaxel for metastatic castration-resistant prostate cancer was not allowed. This study was supervised by the Institutional Review Board at Johns Hopkins. All accrued patients provided written informed consent. Two additional cohorts (patients progressing after abiraterone treatment and patients with newly castration-resistant prostate cancer) are ongoing.

Procedures

After verification of eligibility and informed consent procedures, we treated patients with BAT, consisting of intramuscular testosterone cypionate 400 mg on day 1 of an every 28 day cycle while continuing luteinising hormone-releasing hormone agonist therapy. No stopping point was predefined and therapy could continue indefinitely. We did not modify testosterone doses. We assessed patients at each cycle for toxicity and with standard laboratory measures including complete blood count, comprehensive metabolic panel, and PSA. Staging CT and nuclear medicine bone scans were done every three cycles. The first response assessment did not occur until after three cycles. Patients who had a consistent rise of PSA concentration after three cycles stopped BAT if PSA was more than 25% higher than the baseline. We allowed patients who had an initial decrease in PSA with a subsequent rise of more than 25% from nadir to remain on BAT until a PSA increase over baseline. Patients with clinical or radiographic progression at any time stopped BAT. Patients with a treatment delay of longer than 2 weeks due to toxicity stopped treatment.

Patients discontinuing BAT proceeded to a 28 day washout period to allow testosterone concentrations to return to the castrate range. The PSA concentration after washout was considered baseline for purposes of evaluation of enzalutamide response. Treatment proceeded with oral enzalutamide 160 mg daily, with dose adjustments allowed per the US Food and Drug Administration's label. During treatment with enzalutamide, we clinically assessed patients at each cycle for toxicity with standard laboratory measures including PSA, and radiographic assessments occurred every three cycles. First response assessment occurred after three cycles, and patients with an increase in PSA concentration of more than 25% above baseline discontinued enzalutamide and completed the study. Patients with an initial decrease in PSA concentration with a subsequent rise of more than 25% from nadir were allowed to remain on enzalutamide until a PSA increase over baseline. Radiographic or clinical progression at any time required patients to stop enzalutamide. Patients were allowed to stay on the treatment indefinitely. PSA progression was defined by

See Online for appendix

Patients (n=30)	
Age (years)	74 (50–89)
Race or ethnicity	
White	27 (90%)
Asian	2 (7%)
African American	1 (3%)
ECOG PS	
0	22 (73%)
1	8 (27%)
Gleason grade	
6	3 (10%)
7	6 (20%)
8–10	20 (67%)
Not available	1 (3%)
PSA (ng/mL)	39.8 (2.4–245.3)
Testosterone <50 ng/dL	30 (100%)
Haemoglobin (g/dL)	12.8 (9.1–15.1)
Alkaline phosphatase	
Within normal limits	28 (93%)
>Upper limit of normal	2 (7%)
Albumin (g/dL)	4.3 (3.6–4.9)
Metastatic disease	
Soft tissue only	9 (30%)
Bone only	16 (53%)
Bone and soft tissue	5 (17%)
RECIST evaluable	12 (40%)
Previous therapy	
Enzalutamide	30 (100%)
Abiraterone	13 (43%)
Data are median (range) or n (%). ECOG PS=Eastern Cooperative Oncology Group performance status. RECIST=Response Evaluation Criteria in Solid Tumors.	
Table 1: Baseline characteristics for enrolled patients	

Prostate Cancer Working Group 2 (PCWG2) criteria.¹⁹ Clinical or radiographic progression was defined by RECIST 1.1²⁰ (soft tissue lesions) and PCWG2 (clinical and bone lesions). Objective responses for patients with measurable disease were defined using RECIST 1.1 criteria.

Clinical investigations were done at baseline and every three cycles, and included complete blood counts, serum hormone concentrations (total testosterone, free testosterone, dihydrotestosterone [DHT], oestradiol, dehydroepiandrosterone [DHEA], DHEA-sulphate [DHEA-S], and sex hormone-binding globulin [SHBG]), and metabolic parameters (fasting glucose, glycated haemoglobin A_{1c} [HbA_{1c}], fasting insulin, C-telopeptides, osteocalcin, HDL, LDL, triglycerides, and C-reactive protein). We collected all laboratory measures on the first day of each cycle, which corresponds to the testosterone nadir. We report safety and tolerability as incidence and severity of adverse events according to National Cancer Institute Common Terminology Criteria for Adverse Events 4.0, monitored each cycle.

Quality of life (QOL) questionnaires were completed at baseline and every three cycles and included RAND Short Form-36 (RANDSF-36), Functional Assessment of Chronic Illness Therapy-Fatigue Subscale (FACIT-Fatigue), Brief Pain Inventory (BPI), International Index of Erectile Function (IIEF), and Positive and Negative Affect Schedule Short Form (PANAS-SF). Study staff administered the QOL questionnaires during treatment visits. RANDSF-36 assessed multiple health domains, and each answer to the 36 questions was assigned a value from 0 to 100 (higher score indicates better), as previously described,²¹ and we present a global mean score. The FACIT-Fatigue subscale consisted of 13 questions assigned a value from 0 to 4 (higher score indicates better), and we present a sum score.²² The BPI queries pain severity and interference with activities on a scale from 0 to 10 (higher score indicates worse); we report mean scores for both.²³ The IIEF queries erectile and orgasmic function, sexual desire, and sexual satisfaction with 15 questions on a scale from 0 or 1 to 5 (higher score indicates better); we report sum scores.²⁴ The PANAS-SF consists of 20 questions, ten each for domains of positive (higher score indicates better) and negative (higher score indicates worse) affect, each on a scale of 1 to 5.²⁵ Both positive and negative affect questions were independently summed and reported. We did not follow up patients completing study treatments subsequently for QOL measures; thus QOL measures are reported only for patients actively on study treatment.

Blood samples for analysis of androgen receptor-full length (AR-FL) and androgen receptor splice variant 7 (AR-V7) mRNA in circulating tumour cells (CTCs) were drawn at baseline, after three cycles of BAT, and after three cycles of enzalutamide retreatment. AR-FL and AR-V7 transcript analyses were done and reported as previously described.⁸ Briefly, we isolated CTCs with antibody-conjugated magnetic beads, according to the Qiagen AdnaTest ProstateCancerSelect protocol (Germantown, MD, USA). We determined CTC-positivity according to Qiagen AdnaTest ProstateCancerDetect protocol, and determined AR-FL and AR-V7 copy numbers by quantitative RT-PCR using custom primers.⁸ Any amount of AR-FL was thus defined as CTC-positive, and any amount of AR-V7 was defined as CTC-positive and AR-V7-positive.

Outcomes

The co-primary endpoints for the study were investigator-assessed 50% declines in PSA concentration from baseline (PSA₅₀) for BAT treatment and for enzalutamide treatment. We defined the PSA₅₀ endpoints using the baseline value at initiation of BAT or enzalutamide, respectively, and therefore, patients with a 50% decline at any time while on treatment had met the endpoint.

Secondary endpoints were safety and tolerability, investigator-assessed PSA progression-free survival (from initiation of BAT or enzalutamide to the time of

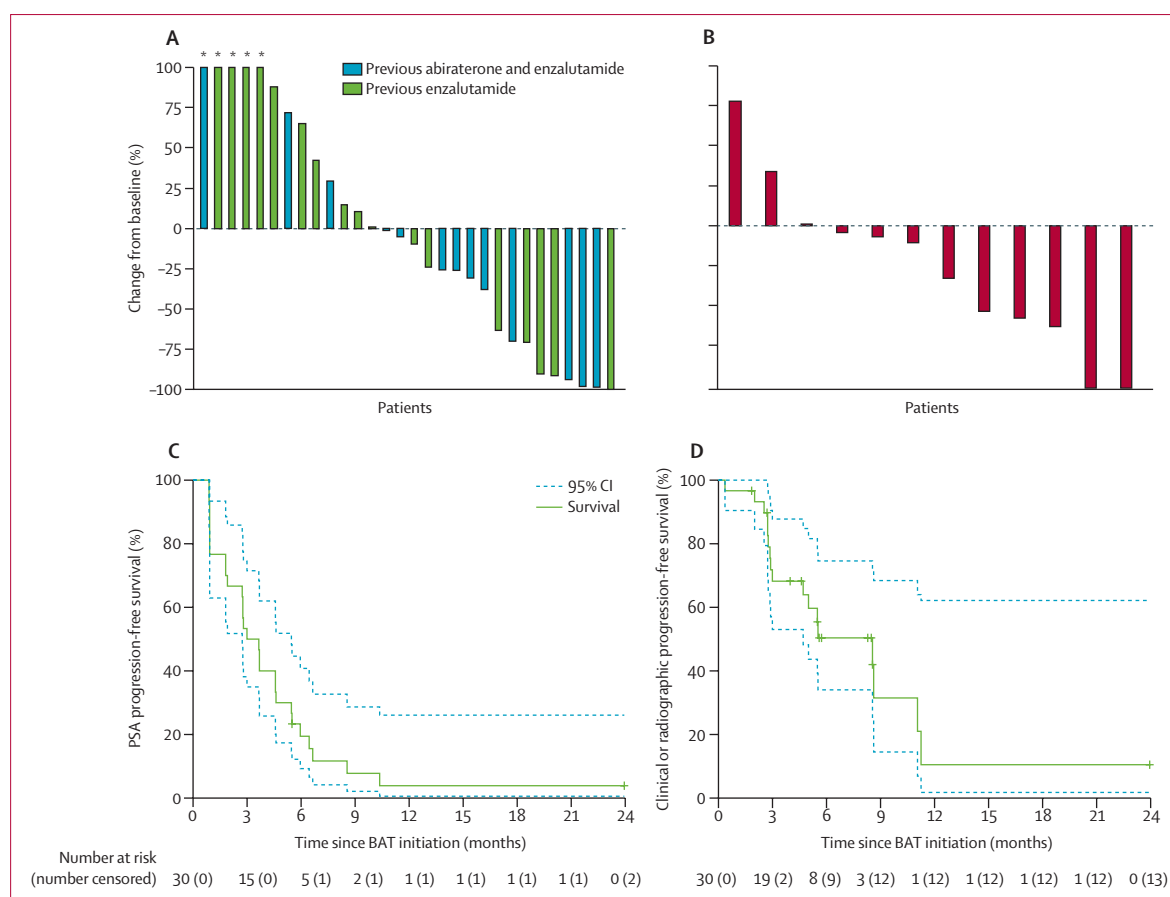


Figure 1: Responses to BAT

Responses characterised by best PSA response (A), best radiographic response in target lesions by RECIST 1.1 (B), PSA progression-free survival defined by PCWG2 (C), and clinical or radiographic progression-free survival defined by PCWG2 and RECIST 1.1 (D). Survival curves (solid lines) are bracketed by 95% CIs (dashed lines). 12 patients with RECIST-evaluable lesions were included in the best radiographic response analysis. BAT=bipolar androgen therapy. PSA=prostate-specific antigen. RECIST=Response Evaluation Criteria in Solid Tumors. PCWG2=Prostate Cancer Working Group 2. *Values truncated at 100%.

measured increase in PSA of at least 2 ng/dL and 25% from nadir values, confirmed with a second measurement at least 4 weeks later), investigator-assessed clinical or radiographic progression-free survival (from initiation of BAT or enzalutamide to the time of unequivocal clinical progression or radiographic progression), investigator-assessed objective response in measurable lesions, effect of BAT and enzalutamide on metabolic studies and QOL measures (at baseline, after three cycles of BAT, and after three cycles of enzalutamide), and time to initiation of cytotoxic chemotherapy (from study initiation [treatment with BAT] to either last known follow-up [resulting in censored for purposes of calculation] or receipt of chemotherapy). AR-FL and AR-V7 analyses were exploratory.

Statistical analysis

The null hypothesis for both co-primary endpoints was a PSA_{50} of 5%, with alternative hypotheses of PSA_{50} of 20% for BAT and 25% for enzalutamide. We required 30 patients for 83% power for BAT and 90% power for

enzalutamide, with an overall one-sided type I error of 0.1 that was split equally between the tests for the two endpoints (0.05 each). We included all patients receiving at least one dose of study therapy in the primary analysis for BAT, and we counted patients achieving a PSA_{50} at any timepoint during the study period as responders. For the enzalutamide primary endpoint, we did an intention-to-treat analysis regardless of whether patients proceeded to enzalutamide treatment, unless they remained on BAT. We calculated the CIs for objective responses as 95% exact binomial CIs.

One interim analysis for futility of enzalutamide rechallenge was done after PSA response data to enzalutamide rechallenge was available for the first nine patients. The study proceeded to completion based on the pre-established futility rule of at least one responder among the first nine patients.

We analysed all time-to-event endpoints with the Kaplan-Meier method. We evaluated changes in QOL scores and metabolic parameters before and after treatment by paired-sample *t* tests, with a two-sided

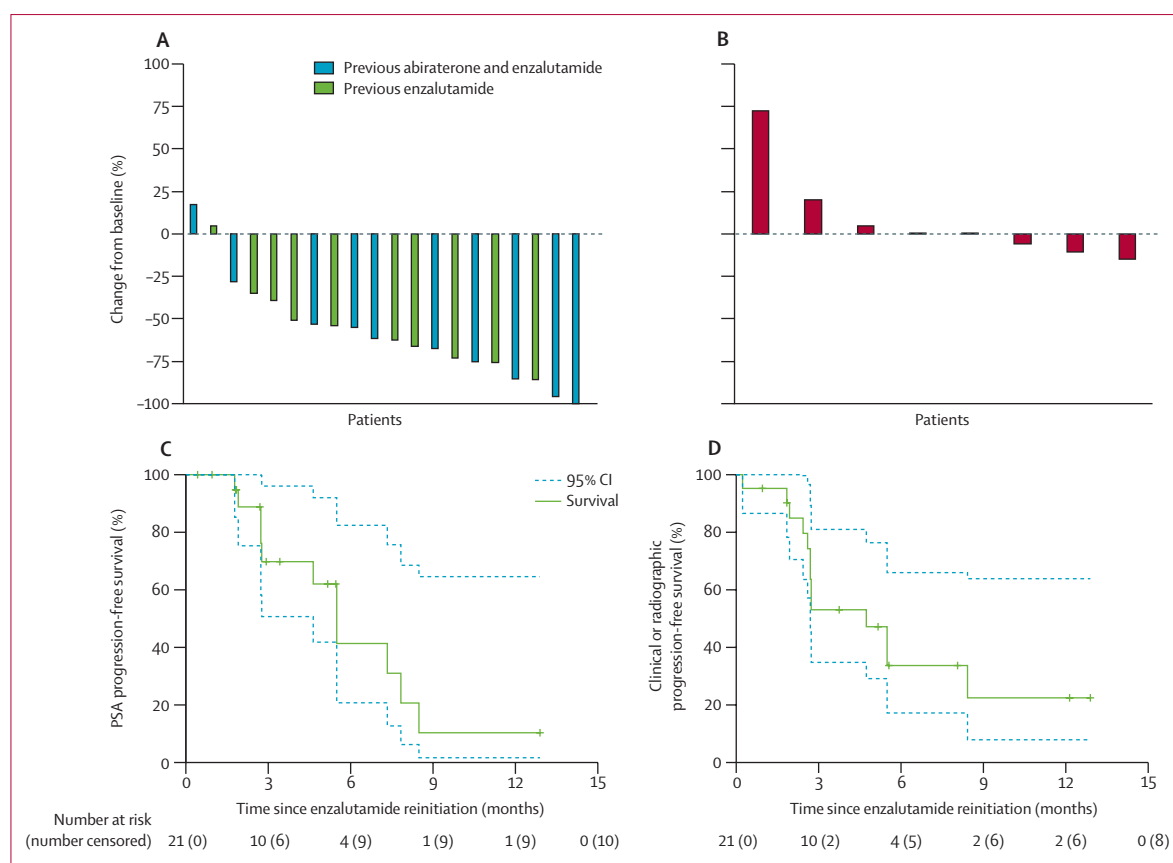


Figure 2: Responses to enzalutamide

Responses characterised by best PSA response among patients with at least one follow-up PSA on enzalutamide (A), best radiographic response in target lesions by RECIST 1.1 (B), PSA progression-free survival defined by PCWG2 (C), and clinical or radiographic progression-free survival defined by PCWG2 and RECIST 1.1 (D). Survival curves (solid lines) are bracketed by 95% CIs (dashed lines). 20 patients who proceeded to enzalutamide treatment and had at least one follow-up PSA on enzalutamide were included in the best PSA response analysis. Eight patients with RECIST-evaluable lesions were included in the best radiographic response analysis. BAT=bipolar androgen therapy. PSA=prostate-specific antigen. RECIST=Response Evaluation Criteria in Solid Tumors. PCWG2=Prostate Cancer Working Group 2.

p value of less than 0.05 threshold for significance, using complete case analysis to account for missingness of data. Summary statistics and plots were based upon all observed data for each timepoint. These data represent the final analysis for the post-enzalutamide cohort.

We did analyses with R statistical package (version 3.4.0). This trial is registered with ClinicalTrials.gov, number NCT02090114.

Role of funding source

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

We accrued 30 eligible patients from Aug 28, 2014, to May 18, 2016 (table 1). All patients had previous enzalutamide therapy and 13 had both previous abiraterone and enzalutamide therapy. All enrolled

patients were refractory to previous enzalutamide therapy, and 14 (47%) of 30 patients had previously achieved a PSA₅₀ response to enzalutamide with a median time on enzalutamide of 8 months (range 1–39).

Patients were treated with BAT for a median of six cycles (range 1–26). All patients received at least one cycle of BAT. Nine (30%; 95% CI 15–49) of 30 patients achieved a PSA₅₀ to BAT ($p < 0.0001$; figure 1A). 21 (70%) patients who completed BAT proceeded to enzalutamide treatment. Of the remaining nine patients, two had severe adverse events and were removed from the study (including one death assessed as not related to BAT), five were removed from the study by treating physician preference, one withdrew, and one remained on BAT at the time of analysis. The intention-to-treat population for evaluation of enzalutamide was thus the 29 patients that completed BAT, excluding the one long-term responder to BAT, because this patient could potentially be rechallenged with enzalutamide in the future. For enzalutamide rechallenge, 15 (52%; 95% CI 33–71) patients achieved a PSA₅₀ ($p < 0.0001$; figure 2A).

The median follow-up for patients on BAT was 4·9 months (IQR 2·8–7·6). Among 12 (40%) patients with RECIST-evaluable lesions, six (50%; 95% CI 21–79) had a partial or complete response (figure 1B). The remaining 18 (60%) patients were not RECIST-evaluable due to non-measurable bone-only metastatic disease (n=16) or lack of follow-up radiographic imaging (n=2). 28 (93%) of 30 patients had PSA progression events on BAT, leading to a median PSA progression-free survival of 3·3 months (95% CI 2·7–5·5; figure 1C). 17 (57%) of 30 patients had a clinical or radiographic progression event on BAT, and the median clinical or radiographic progression-free survival was 8·6 months (95% CI 4·7–not reached; figure 1D). After the 28-day washout period before restarting enzalutamide, 14 (70%) of 20 patients with measured testosterone values had castrate testosterone (<50 ng/dL) and the remainder had near castrate concentrations (median 30 ng/dL, range <20–133 ng/dL). 15 (68%) of 22 patients with PSA measured before and after the washout period had continued increases in PSA concentrations (appendix p 1).

The median follow-up after enzalutamide rechallenge was 2·7 months (IQR 2·4–5·5). 12 patients had RECIST-evaluable disease for BAT; however, four of these patients did not proceed to receive enzalutamide (one was a long-term responder to BAT and three terminated study participation after BAT); therefore, eight patients were evaluable for radiographic response (by RECIST) to enzalutamide. None (95% CI 0–37) of these patients had a radiographic response to enzalutamide (figure 2B). 11 (52%) of 21 patients rechallenged with enzalutamide had PSA progression events, and the median PSA progression-free survival was 5·5 months (95% CI 4·6–not reached; figure 2C). 13 (62%) of 21 patients rechallenged with enzalutamide had clinical or radiographic progression events and the median clinical or radiographic progression-free survival was 4·7 months (95% CI 2·7–not reached; figure 2D). Four patients remained on enzalutamide at the time of final analysis, all of whom had met the co-primary endpoint of PSA₅₀ response to enzalutamide.

14 (47%) of 30 patients were known to have proceeded to cytotoxic chemotherapy, and the median time to initiation of docetaxel chemotherapy for patients was 20·4 months (95% CI 11·3–not reached).

Adverse events reported during BAT treatment are in table 2. The most common grade 3–4 adverse event was hypertension (three [10%] patients). During the study, no dose-limiting toxicities occurred and no patients required dose adjustment on BAT. Three serious (grade 3–4) adverse events were potentially attributable to testosterone (pulmonary embolism, non-ST segment elevation myocardial infarction, and urinary obstruction; table 2). The pulmonary embolism was diagnosed as an incidental finding on CT scan after the sixth cycle of BAT. The non-ST segment elevation myocardial infarction required percutaneous coronary intervention and occurred during the second cycle of BAT. The episode of

	Grade 1–2	Grade 3	Grade 4	Grade 5
Musculoskeletal pain*	12 (40%)	0	0	0
Increased haemoglobin (>ULN)	11 (37%)	0	0	0
Hypertension	4 (13%)	3 (10%)	0	0
Anaemia	5 (17%)	0	0	0
Breast tenderness	5 (17%)	0	0	0
Rash	5 (17%)	0	0	0
Fatigue	5 (17%)	0	0	0
Nausea	5 (17%)	0	0	0
Gynaecomastia	4 (13%)	0	0	0
Hot flashes	4 (13%)	0	0	0
Pruritis	4 (13%)	0	0	0
Abdominal pain	3 (10%)	0	0	0
Oedema	3 (10%)	0	0	0
Fall	3 (10%)	0	0	0
Sinusitis	3 (10%)	0	0	0
Urinary obstruction	0	0	1 (3%)	0
Gallstone	0	1 (3%)	0	0
Sepsis	0	0	0	1 (3%)
Non-ST segment elevation myocardial infarction	0	1 (3%)	0	0
Pulmonary embolism	0	1 (3%)	0	0

Data are n (%) for 30 patients. Those adverse events occurring in 10% or more of the patients are listed for grades 1–2, and all events are listed for grades 3–5. BAT=bipolar androgen therapy. ULN=upper limit of normal. *Musculoskeletal pain not deemed to be tumour flare or clinical progression.

Table 2: Adverse events for BAT

urinary obstruction occurred during the first cycle of BAT, which is concerning for a flare phenomenon. Two patients had transient pain flares after initiation of BAT, characterised by onset of pain within hours of treatment that resolved within days (data not shown). One of these patients received further cycles of therapy without recurrence and achieved a PSA₅₀ response, and the other patient had recurrent flare each cycle that was managed with opioid analgesics, thus allowing him to stay on study. Two patients discontinued BAT because of adverse events, including one non-treatment-related patient death (severe sepsis with multiorgan dysfunction syndrome) and one myocardial infarction. No treatment-related deaths occurred.

Low-grade adverse events occurring during enzalutamide treatment were consistent with previous clinical experience (appendix p 2). No patient deaths happened during the enzalutamide treatment portion of the study. Five serious adverse events occurred on enzalutamide treatment in three patients (abdominal pain, pancreatitis, paresthesias, gastrointestinal bleed, and non-ST segment elevation myocardial infarction), and none of the serious adverse events were assessed as probably, likely, or definitely related to study treatment (appendix p 2).

29 patients completed at least one QOL instrument at baseline, 26 completed at least one after three cycles of BAT, and 15 completed at least one after three cycles

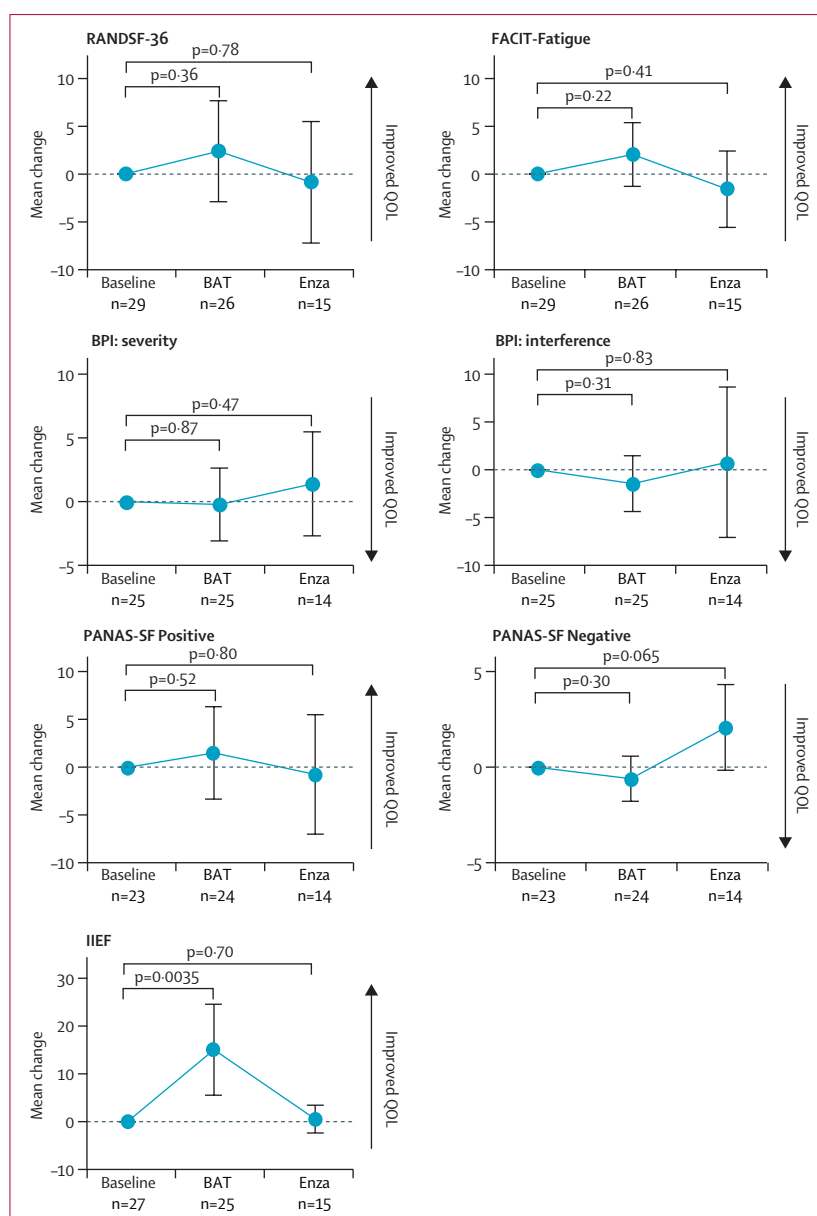


Figure 3: Quality of life metrics

Data are mean change from baseline after three cycles of BAT or three cycles of enzalutamide. Error bars are 95% CIs. RANDSF-36=Rand Short Form-36 item. FACIT-Fatigue=Functional Assessment of Chronic Illness Therapy-Fatigue Subscale. BPI=Brief Pain Inventory. PANAS-SF=Positive and Negative Affect Schedule Short Form. IIEF=International Index of Erectile Function. BAT=bipolar androgen therapy. Enza=enzalutamide. QOL=quality of life.

of enzalutamide treatment. Overall QOL (RANDSF-36), fatigue (FACIT-Fatigue), the degree of pain severity or interference (BPI), and positive and negative affect (PANAS-SF) after three cycles of BAT did not differ compared with baseline and after three cycles of enzalutamide (figure 3). Patients had significantly improved sum IIEF scores on BAT compared with baseline scores (figure 3).

After three cycles of BAT, nadir median total testosterone concentrations increased from less than

20 ng/dL (range <20–47.7) to 207.5 ng/dL (65–2339; $p<0.0001$), with corresponding increases in free testosterone (from 0.7 pg/mL [0–10] to 25.2 pg/mL [3.3–358.2]; $p<0.0001$), DHT (from <5 ng/dL [<5–5] to 12 ng/dL [7–81]; $p<0.0001$), and DHEA-S (from 32 µg/dL [6–207] to 58 µg/dL [3–268]; $p=0.068$; appendix pp 3–4). Concentrations of total ($p=0.59$) and free testosterone ($p=0.69$), DHT ($p>0.99$), and DHEA-S ($p=0.092$) after three cycles of enzalutamide were similar to baseline (appendix pp 3–4). DHEA was similar to baseline after BAT ($p=0.36$) and after enzalutamide ($p=0.11$; appendix pp 3–4). Oestradiol significantly increased after BAT ($p=0.0013$) and after enzalutamide ($p=0.042$) compared with baseline (appendix pp 3–4). SHBG was significantly decreased after BAT ($p=0.0006$) but significantly increased after enzalutamide ($p=0.0059$) compared with baseline (appendix pp 3–4). Haemoglobin was significantly increased from baseline after BAT ($p=0.0074$) whereas enzalutamide was similar to baseline ($p=0.84$; appendix pp 3–4). Glycaemic tolerance, as measured by fasting glucose and HbA_{1c}, significantly improved on BAT compared with baseline ($p=0.032$ and $p=0.0066$, respectively) whereas enzalutamide was similar to baseline ($p=0.53$ and $p=0.10$, respectively; appendix pp 3–4). HDL, LDL, and triglycerides were all significantly lower on BAT than baseline ($p=0.0014$, $p=0.043$, and $p=0.031$, respectively) whereas enzalutamide was similar to baseline ($p=0.83$, $p=0.080$, and $p=0.24$, respectively; appendix pp 3–4). Concentrations of C-telopeptides did not change from baseline on BAT ($p=0.72$) or enzalutamide ($p=0.25$) whereas osteocalcin was increased from baseline on BAT ($p<0.0001$), but not enzalutamide ($p=0.62$; appendix pp 3–4). Fasting insulin and C-reactive protein were not changed from baseline after either BAT ($p=0.85$ and $p=0.65$, respectively) or enzalutamide ($p=0.95$ and $p=0.58$, respectively; appendix pp 3–4).

All patients had samples available for CTC-based analysis of androgen receptor mRNA status (table 3). Responses to BAT were achieved in five (31%) of 16 patients who were baseline CTC-negative, three (27%) of 11 patients who were baseline CTC-positive and AR-V7-negative, and one (33%) of three patients who were baseline CTC-positive and AR-V7-positive (table 3). Responses to enzalutamide by intention-to-treat analysis were achieved in nine (60%) patients who were baseline CTC-negative, six (55%) who were baseline CTC-positive and AR-V7-negative, and no patients who were baseline CTC-positive and AR-V7-positive (table 3).

Discussion

Our study met its co-primary endpoints of showing PSA₅₀ responses to BAT after progression on enzalutamide and following enzalutamide rechallenge. The evaluation of PSA response in patients receiving BAT is complicated by the fact that PSA is an androgen-responsive gene. Importantly, in addition to PSA responses, radiographic

	Baseline CTC		Post-BAT cycle 3 CTC		Post-enzalutamide cycle 3 CTC		BAT response	Enzalutamide response
	AR-FL copy number	AR-V7 copy number	AR-FL copy number	AR-V7 copy number	AR-FL copy number	AR-V7 copy number		
Baseline CTC-negative								
1	Negative	Negative	Negative	Negative	Negative	Negative	Yes	No
4	Negative	Negative	Negative	Negative	Negative	Negative	Yes	Yes
27	Negative	Negative	Negative	Negative	NA	NA	Yes	NR
28	Negative	Negative	Negative	Negative	NA	NA	Yes	NR
40	Negative	Negative	Negative	Negative	1	0	Yes	Yes
7	Negative	Negative	Negative	Negative	Negative	Negative	No	Yes
9	Negative	Negative	Negative	Negative	Negative	Negative	No	Yes
24	Negative	Negative	Negative	Negative	Negative	Negative	No	Yes
34	Negative	Negative	NA	NA	NA	NA	No	NR
38	Negative	Negative	Negative	Negative	NA	NA	No	No
39	Negative	Negative	Negative	Negative	3	0	No	Yes
47	Negative	Negative	NA	NA	NA	NA	No	NR
51	Negative	Negative	Negative	Negative	NA	NA	No	Yes
53	Negative	Negative	7	0	Negative	Negative	No	Yes
54	Negative	Negative	NA	NA	NA	NA	No	Yes
57	Negative	Negative	1	0	NA	NA	No	NR
Baseline CTC-positive and AR-V7-negative								
18	1	0	Negative	Negative	2	0	Yes	Yes
42	7	0	Negative	Negative	NA	NA	Yes	NR
31	25	0	Negative	Negative	Negative	Negative	Yes	Yes
2	1	0	Negative	Negative	NA	NA	No	No
19	1	0	Negative	Negative	NA	NA	No	Yes
43	2	0	Negative	Negative	Negative	Negative	No	Yes
46	2	0	NA	NA	NA	NA	No	NR
48	3	0	1	0	8	0	No	Yes
49	4	0	NA	NA	NA	NA	No	NR
55	7	0	5	0	118	3	No	Yes
50	40	0	57	0	NA	NA	No	NR
Baseline CTC-positive and AR-V7-positive								
5	43	6	2	0	154	96	Yes	No
44	7	14	24	2	NA	NA	No	No
17	16	4	Negative	Negative	285	44	No	No

Individual patient data for each patient ID number are listed. Responders to BAT and enzalutamide are denoted. CTC=circulating tumour cells. AR-FL=androgen receptor full length. AR-V7=androgen receptor splice variant 7. BAT=bipolar androgen therapy. NR=not rechallenged. NA=not available.

Table 3: CTC-based measurements of mRNA copy numbers for AR-FL and AR-V7

Table 3: CTC-based measurements of mRNA copy numbers for AR-FL and AR-V7

responses to BAT occurred in 50% of patients with RECIST-evaluable disease and the clinical or radiographic progression-free survival was nearly 9 months. These data are promising considering published series^{26–29} have shown evidence of cross-resistance between abiraterone and enzalutamide, with objective responses of 0–11% and progression-free survival of 3–4 months in patients receiving abiraterone or enzalutamide after progressing on the alternate drug. Furthermore, our results support the hypothesis that an alternative strategy of therapeutically targeting the androgen receptor with BAT in the setting of progression on second-line androgen receptor-signalling inhibitors might be beneficial to patients.

Adaptive upregulation of androgen receptor signalling by prostate cancer cells in response to continuous exposure to the low testosterone microenvironment produced by androgen deprivation therapy is a key factor in the development and progression of resistance to androgen ablative therapies, such as castration and antiandrogens. High androgen receptor expression produces a therapeutic vulnerability that might sensitise castration-resistant prostate cancer cells to supra-physiological concentrations of testosterone. BAT is designed to take advantage of and disrupt castration-resistant prostate cancer cells' ability to adaptively upregulate or downregulate androgen receptor

expression in response to testosterone concentrations in the tumour microenvironment. A high PSA₅₀ response following enzalutamide rechallenge supports the ability of BAT to resensitise metastatic castration-resistant prostate cancer cells to androgen receptor-signalling inhibitors. This resensitisation to enzalutamide supports the hypothesis that BAT might modulate adaptive resistance mechanisms (eg, androgen receptor overexpression) that arise following androgen receptor-signalling pathway antagonism. However, the duration of response to enzalutamide was short in most patients, potentially reflecting a rapid readoption of a resistant phenotype. In future studies, potential strategies to address the transient resensitisation could include continually alternating between therapies to delay time to progression and maximise benefit to patients.

Testosterone was well tolerated in this study, and the side-effect profile was as expected for testosterone-replacement therapy. Other hormonal effects, including hot flashes, breast tenderness, and gynaecomastia, could have been the result of the fluctuating concentrations of testosterone and oestrogen through the BAT cycles. Despite the careful selection criteria intended to minimise risk for testosterone-induced pain flare, two patients had a post-BAT flare. Furthermore, low-grade musculoskeletal pain, not clearly related to prostate cancer disease burden, was observed during the trial. Serious (grade 3) cardiovascular events occurred during the trial, which included a myocardial infarction and pulmonary embolism on BAT. The metabolic parameters did not indicate a pattern of increased cardiovascular risk based upon the changes in inflammatory markers, lipid profile, or glucose metabolism. Although haemoglobin was increased above the upper limit of normal in 37% of patients on BAT, neither of the patients who had cardiovascular events while on BAT had increases in haemoglobin concentrations. Therefore, further close monitoring of cardiovascular risk during future trials of BAT is warranted. Nonetheless, this trial helps to show that study of BAT might be reasonably expanded to a broader population of patients with metastatic castration-resistant prostate cancer.

Some patients might have enrolled on this trial in the hope of mitigating the side-effects of androgen deprivation therapy. A fifth of patients had restored erectile function on BAT based upon paired IIEF evaluations—a finding consistent with the QOL changes observed with a modified version of BAT tested in the hormone-sensitive setting.³⁰ Other QOL metrics did not show any differences from baseline, and larger follow-up studies will further explore QOL changes during BAT versus androgen receptor-directed therapy in this patient population. Metabolic parameters including glycaemic control and lipid profile appeared to be improved on BAT compared with baseline.

No clear association between baseline CTC-derived androgen receptor status and response was observed.

The proposed mechanism for BAT requires high androgen receptor concentrations to be present for efficacy. Baseline androgen receptor mRNA copy numbers were likely to be indicative of overall disease burden and not necessarily representative of tumour-concentration androgen receptor expression, and the baseline status was not associated with response. However, the overall decreased AR-FL and AR-V7 concentrations in CTCs after three cycles of BAT is consistent with the hypothesis that BAT results in downregulation of androgen receptor expression or amplification. Patients who were CTC-negative after three cycles of BAT were most likely to be PSA responders. The subsequent increase in AR-FL and AR-V7 concentrations after enzalutamide rechallenge is also consistent with androgen receptor locus upregulation in response to androgen receptor antagonism. However, without concurrent tissue-based assessments, whether other mechanisms account for these changes, such as stabilisation of the tumour microenvironment leading to decreased tumour cell shedding, is not known. Importantly, presence of AR-V7 did not preclude response to BAT, although no patient who was CTC-positive and AR-V7-positive responded to enzalutamide retreatment. This study did not incorporate tissue-based assessments of androgen receptor expression nor genomic analysis. Future tissue-based assessment might yield a biomarker of response, by either identifying tissue-based androgen receptor overexpression or genetic alterations that potentially confer susceptibility to BAT, such as DNA repair defects.³¹

Limitations of the study included its single-institution, single-cohort analysis with strict entry criteria designed to mitigate against risks of adverse events. Randomisation is needed to define the QOL and metabolic differences between treatments, as sequential comparison is confounded by different times of follow-up on study, frequent removal of patients from study before completion of 3 months of enzalutamide, and symptoms related to disease progression.

In conclusion, for asymptomatic patients with progression after enzalutamide, BAT induces clinical responses and subsequent resensitisation to enzalutamide. Studies in progress, including a randomised trial comparing BAT to enzalutamide in patients progressing on abiraterone,³² will further define a potential clinical role for BAT in the management of metastatic castration-resistant prostate cancer as well as the clinical features of patients with the highest chance for benefit.

Contributors

HW, MTS, CGD, MAC, CJP, ESA, MAE, and SRD designed the study. BAT, RS, IR, AB, AS, MD, VS, CFP, JLS, MTS, CGD, MAC, CJP, ESA, MAE, and SRD collected data. BAT, HW, BL, CL, JL, and SRD analysed the data. BAT and SRD wrote the manuscript. All authors had access to the data, edited, and approved the final manuscript.

Declaration of interests

BAT reports grants from National Institutes of Health (NIH) and National Cancer Institute (NCI) and from Conquer Cancer Foundation, during the conduct of the study; and has a patent for Polymers for Functional Particles, System for targeted delivery of therapeutic agents with royalties paid to Pfizer, Selecta Biosciences, and Bind Therapeutics. CL reports a patent, C13162, with royalties paid to Tokai and Qiagen, and has a patent, C13084, pending. JL reports grants and personal fees from Astellas, Sanofi, and Gilead, personal fees from Janssen Oncology and Sun Pharma, and grants from Mirati and Orion, outside the submitted work, and has a patent, C10305, with royalties paid to A&G, Tokai, and Qiagen, a patent, C13126, with royalties paid to Tokai and Qiagen, and has a patent, C13084, pending. MAC reports personal fees from Pfizer, Astellas, Merck, and Abbvie, outside the submitted work. SRD reports grants from NIH and NCI, DOD Prostate Cancer Research Program, and One-in-Six Foundation, during the conduct of the study; and other from Medicenna, outside the submitted work. All other authors declare no competing interests.

Acknowledgments

This trial was funding by National Institutes of Health and National Cancer Institute grant number R01CA184012.

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Review

Supraphysiologic Testosterone Therapy in the Treatment of Prostate Cancer: Models, Mechanisms and Questions

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Received: 13 October 2017; Accepted: 29 November 2017; Published: 6 December 2017

Abstract: Since Huggins defined the androgen-sensitive nature of prostate cancer (PCa), suppression of systemic testosterone (T) has remained the most effective initial therapy for advanced disease although progression inevitably occurs. From the inception of clinical efforts to suppress androgen receptor (AR) signaling by reducing AR ligands, it was also recognized that administration of T in men with castration-resistant prostate cancer (CRPC) could result in substantial clinical responses. Data from preclinical models have reproducibly shown biphasic responses to T administration, with proliferation at low androgen concentrations and growth inhibition at supraphysiological T concentrations. Many questions regarding the biphasic response of PCa to androgen treatment remain, primarily regarding the mechanisms driving these responses and how best to exploit the biphasic phenomenon clinically. Here we review the preclinical and clinical data on high dose androgen growth repression and discuss cellular pathways and mechanisms likely to be involved in mediating this response. Although meaningful clinical responses have now been observed in men with PCa treated with high dose T, not all men respond, leading to questions regarding which tumor characteristics promote response or resistance, and highlighting the need for studies designed to determine the molecular mechanism(s) driving these responses and identify predictive biomarkers.

Keywords: high dose testosterone; supraphysiologic androgen; bipolar androgen therapy; biphasic; BAT; castration resistant prostate cancer; CRPC

1. Introduction

Since the landmark studies of Huggins and colleagues showed the androgen-sensitive nature of prostate cancer (PCa) [1], suppression of systemic testosterone (T) levels remains the most effective initial therapy for advanced disease. Although initially highly effective, standard androgen deprivation therapy (ADT) is characterized by the emergence of resistant tumors over a period of 18–20 months. Median survival after progression, termed castration resistant prostate cancer (CRPC), is between 1–2 years. An important aspect of CRPC is reactivation of androgen-receptor (AR) signaling, as demonstrated by analyses of metastatic tumors showing that essentially all known androgen regulated genes are expressed, including putative drivers of carcinogenesis (e.g., Transmembrane Protease, Serine 2-ETS-related gene (*TMPRSS2-ERG*) rearrangements).

From the inception of clinical efforts to suppress AR signaling by reducing AR ligands, it was also recognized that the administration of T to men with CRPC can result in substantial clinical

responses, though the reports were largely anecdotal and remissions were highly variable, potentially due to variability and inadequate increases in the levels of circulating T that were achieved [2–4]. In contrast, abundant data from preclinical models have reproducibly shown biphasic responses of hormone-sensitive cancers, whereby at physiological T concentrations proliferation is induced, but at higher, supraphysiological T (SPT) concentrations, proliferation is suppressed and in some instances apoptotic programs are engaged [5–8]. Though often considered to be an *in vitro* phenomenon, recent proof of principle trials using SPT therapy—two in men with CRPC and one in hormone sensitive PCa produced promising results, showing radiographic response rates of ~50% in men with CRPC, and favorable prostate specific antigen (PSA) responses in those with hormone naïve PCa [9–11].

Notably, biphasic responses to hormone concentrations are not unique to PCa and the AR: When exposed to estradiol (E2), estrogen receptor (ER)-responsive MCF-7 breast cancer cell line adapted to proliferate in the absence of estradiol undergo an apoptotic response [12–14]. E2 also has a biphasic effect on the growth of the rat pituitary line GH3 [6]. To date, there is a lack of unifying mechanisms to explain these effects, though cellular pathways, particularly involving cell cycle control, provide insights in some systems.

Many questions regarding the biphasic responses of prostate tumors to supraphysiologic androgen concentrations remain, primarily regarding the mechanisms driving this response and how best to exploit this phenomenon clinically. To date, efforts to enhance efficacy of SPT have focused on concomitant manipulation of the androgen receptor (AR), i.e., androgen cycling to induce AR upregulation and increased sensitivity, but preclinical studies suggest other approaches and drug combinations may be reasonable to pursue. Here we review the preclinical and clinical literature on androgen-mediated growth repression and discuss cellular pathways and mechanisms likely to be involved in mediating this response. Although meaningful clinical responses have now been observed in men with CRPC treated with SPT [9], not all men respond, leading to questions regarding which tumor characteristics promote response or resistance, highlighting the need for studies designed to determine the molecular mechanism(s) driving these responses and identify predictive biomarkers.

2. Physiologic Role of AR in Growth Repression

The AR plays a critical role in the normal development of the prostate gland, although initial morphogenic activity occurs via mesenchymal AR, not epithelial AR [15]. In the mature prostate, the small fraction of epithelial cells that are proliferating are localized to the basal compartment, and do not express AR protein, whereas luminal secretory cells which express AR are quiescent. Introducing the AR into benign prostate epithelial cells (PrEC), or activating AR function, results in cell growth arrest and subsequent differentiation toward a luminal phenotype. Detailed studies conducted by Isaacs et al. determined that the induction of AR activity in PrEC resulted in irreversible growth arrest in G0, with the maintenance of viability, metabolism, and the expression of proteins that are associated with terminally-differentiated prostate epithelium, such as PSA [16,17]. Studies using genetically-engineered mouse models (GEMs) showed that eliminating AR in prostate epithelium results in a hyper-proliferative cell state with loss of cell differentiation [18–20]. These and other studies provide compelling evidence that in benign cells with intact mechanisms for regulating cell proliferation, the AR functions to promote terminal differentiation and a quiescent, G0 state.

3. Oncogenic Role of AR in Prostate Cancer Progression

In contrast to its role in promoting differentiation in normal prostate epithelial cells, AR signaling in PCa acquires a critical oncogenic role and promotes growth and survival. The mechanism for this conversion is not fully understood but appears to involve in part a gain of function in AR induced regulation of myelocytomatosis oncogene cellular homolog (MYC) expression [16,17]. During progression to castration resistance, PCa acquire further changes directed at maintaining AR signaling in a low androgen environment. These changes include AR overexpression, AR mutations

that broaden ligand specificity and/or confer sensitivity to adrenal androgens, induction of constitutively active truncated AR splice variants, alterations in AR coactivators and/or corepressors that modulate AR stability and ligand sensitivity, and activation of the AR or downstream regulatory molecules by “cross talk” with other signaling pathways [21–43]. Restoration of full length AR expression and signaling in a xenograft model was both necessary and sufficient to drive progression from androgen-dependent to castration resistant growth, sensitizing cells to proliferate in 80% lower androgen concentrations [33]. Importantly, in these models ligand binding was required for castration resistant growth, and modest increases in AR expression were sufficient to support signaling in a low androgen environment.

The clinical relevance of continued AR signaling in promoting CRPC tumor growth is confirmed by the clinical responses to agents targeting residual androgen pathway activity including the striking clinical response observed with novel ligand synthesis inhibitors such as abiraterone (ABI), and potent AR inhibitors such as enzalutamide (ENZ) [44–50].

4. Historical Observations on Androgen Therapy of Prostate Cancer

Interestingly, despite his clear demonstration that androgens were a critical driver of PCa progression, Huggins himself proposed that both hormonal deprivation and hormonal excess (which he termed hormonal interference) might be used for therapeutic benefit [51]. A number of case reports and small series were published between 1950 and 1980. While demonstrating some evidence of clinical benefit, these studies also showed adverse effects of T administration, though the doses were generally low, ranging from 25 mg–100 mg daily [2,52]. In a series of three patients, two experienced temporary symptomatic benefit. In a series of 10 men with CRPC treated with 100 mg T propionate 3 times weekly, one individual, near death at the time of treatment initiation, experienced an objective response lasting approximately 1 year, although five patients had subjective and objective evidence of deterioration including pain and pathologic fracture [3]. Two case reports also detail responses to T therapy, one patient with progression despite orchiectomy and hypophysectomy who responded to T with a decrease in serum acid phosphatase and symptomatic improvement, and more recent patient with CRPC treated with T gel replacement with a sustained PSA response lasting for nearly a year [53,54].

Importantly, simply discontinuing ADT and allowing androgen recovery to a eugonadal state does not appear to enhance survival; studies of intermittent androgen suppression in metastatic disease, which also allows gradual T recovery to physiological levels, demonstrated a trend toward inferior survival compared to continuous ADT [55]. In this regard castration resistant VCaP cells treated with sequentially higher T doses had less significant apoptotic responses to androgen withdrawal than those seen in VCaP cells exposed to a single high T “boost” [56].

5. Preclinical Observations on Androgen-Mediated Growth Repression of Prostate Cancer

Although historical support for the clinical benefit of androgen therapy for PCa has been mixed, the paradoxical inhibitory response of PCa to supraphysiological androgens has been demonstrated in multiple in vitro and in vivo studies. These studies are summarized below, and a more detailed review of findings in each pre-clinical model is provided in Appendix A. The LNCaP cell line is widely used as a model for mechanistic studies of PCa molecular biology, including AR function. Several groups have reproducibly demonstrated a biphasic proliferative response to androgen, with minimal proliferation in the absence of any androgen, high rates of proliferation at concentrations of dihydrotestosterone (DHT) or the non-metabolizable androgen (R1881) of 0.1 nM, and cell cycle arrest with concentrations of DHT/R1881 exceeding 1.0 nM (equivalent to ~5–10 nM T) [7,8,57]. This effect has been observed in other PCa lines that natively express the AR as well as cells engineered to express the AR (summarized in Appendix A Table A1).

Within a tumor model, the response of cells to androgen-repressed growth is often more pronounced in castration resistant (CR) variants that have been derived after serial passage in

androgen-depleted media, whereby the growth repressing effects can occur at 10–100 fold *less* androgen. Progression to the CR state is often accompanied by an increase in AR levels and transcriptional activity, suggesting the elevated AR levels in the more refractory tumor cells sensitizes these models to repression at lower androgen doses. In the transition from the androgen sensitive (AS) LNCaP 104S variant to the androgen independent (AI) 104R line (after 8–11 months in charcoal stripped (CS) media), the repressive dose of R1881 is left-shifted two orders of magnitude, from 1 nM (10^{-9}) to 0.01 nM (10^{-11}), accompanied by increased AR expression and activity suggesting an increased sensitivity to androgen signaling [58]. An AI subline of MDA PCa 2ba shifts from being androgen-stimulated for growth to being androgen suppressed for growth with an increase in AR levels [59,60]. Similar to LNCaP, the growth of CWR22 cells is biphasic and the suppression is left shifted in the AI line CWR22R [61].

Exemplifying the known heterogeneity of advanced disease, PCa cell lines demonstrate a diversity of androgen-mediated growth responses. Importantly, an androgen-repressed growth phenotype is not exclusively associated with elevated AR levels, nor do low AR levels preclude androgen-repressed growth. Some PC cells with relatively low AR such as ARCaP still show a strong androgen-repressed phenotype [62]. In contradistinction to CWR22 cells, 22Rv1, another AI line derived from CWR22R, is AS for growth without a biphasic response [63]. The mechanism(s) underlying these responses and why the growth repressive effect of SPT is not uniformly observed in all AR + PC cells are not understood. In tumors where the repressive response is left shifted with increased AR it is not known whether additional mechanisms are operative. Nor is it known to what extent the mechanism of AR-mediated growth-suppression occurs by similar or different mechanisms in the different androgen-repressed cell lines.

Notably, despite compelling preclinical evidence that the androgen-repressive effect is often magnified in CR tumor variants, clinical observations do not necessarily support this. In the early studies of Fowler and Whitmore 45 of 52 men had unfavorable responses to exogenous T, and the proportion of men who had an unfavorable response was higher in those who had been on prolonged hormone suppression (94%) compared to castration-naïve men (25%), or men in early stages of hormone suppression (36%) [64]. It is possible that while repressive responses to SPT may be enhanced in CR tumors with upregulated AR, growth-promoting responses to physiologic T may be similarly enhanced in CR tumors.

6. Contemporary Clinical Studies of Testosterone Therapy for Prostate Cancer

Several contemporary trials of T treatment have been conducted (summarized in Table 1), two in which physiologic T levels were achieved and minimal responses were observed [65,66], and three in which T levels achieved were truly supraphysiologic and a clear subset of men showed clinical responses [9–11].

6.1. Studies of Continuous Testosterone Treatment

Morris et al. conducted a Phase 1 trial of 12 men with CRPC who were treated with T via 5 mg transdermal patch or 1% gel for 1 week, 1 month, or until disease progression. T treatment resulted in raising T levels to the normal physiological range although DHT levels were supraphysiologic in a subset of men. One patient achieved a PSA decline of >50% from baseline, but no objective responses were seen, with a median time to progression of 84 days (range 23–247 days) [65].

Szmulewitz et al. conducted a randomized trial evaluating transdermal T at 25, 50 or 75 mg/day in 15 men with CRPC but minimal metastatic disease [66]. Notably, serum T levels increased from castrate to ~300 ng/dL, essentially a eugonadal concentration. Increases in PSA, which may rise with T replacement, rather than other objective measures of disease progression were responsible for the majority of progression events. (20%) of patients demonstrated a decrease in PSA (largest was 43%), with a median time to progression of 9 weeks (range: 2–96 weeks). One patient experienced symptomatic progression. There was no significant improvement in quality of life (QoL).

Table 1. Contemporary Trials of High Dose Androgen Therapy.

Patient Population	No. of Patients	Treatment Regimen	Serum T Level	PSA Response	Objective Response	Median Time to Progression	Caner Related Adverse Effects	Ref.
CRPC (disease burden or symptoms not designated)	12	T via 5 mg transdermal patch or 1% gel for 1 week, 1 month, or until disease progression	physiologic (342–876 ng/dL)	1 patient had PSA decline >50% from baseline	none	84 days (23–247 days)		[65]
CRPC with minimal metastatic disease	15	transdermal T at 25, 5.0 or 75 mg/day	physiologic (94–824 ng/dL)	3/15 (20%) had PSA declines from baseline (largest decline 43%)	none	63 days (14–672 days)	one patient with symptomatic progression	[66]
Asymptomatic CRPC with low to moderate metastatic burden	16	T (400 mg IM day 1 of 28) and etoposide (100 mg oral daily; days 1 to 14 of 28)	T > 1500 ng/dL (~50 nM) at 2 days after T injection (range 920 to >3200 ng/dL), above 600 ng/dL at 2 weeks, and 150 ng/dL by 28 days	7/14 (50%) had PSA declines from baseline ($\geq 50\%$)	radiographic responses in 5/10 (50%), and 4 continued on treatment for ≥ 1 year	11 months (3 to not reached)	2 patients were not evaluable because they came off study after only one cycle of therapy due to toxicity	[9]
CRPC post progression on enzalutamide	30	alternating 3 month cycles of BAT (T 400 mg IM on days 1, 29 or 57), followed by 3 months of ADT alone	not reported	9/30 (30%) men achieved a $\geq 50\%$ decline in PSA from baseline	50% of patients achieving an objective radiographic response	8.6 months (4.7 to not reached)	3 patients progressed per RECIST criteria and 3 had unconfirmed progression on bone scan	[11]
Asymptomatic hormone naïve with low metastatic burden or biochemically recurrent disease, who achieved PSA < 4 ng/dL after 6 months of ADT	29	T 400 mg IM on days 1, 29, and 57	not reported	17/29 (59%) achieved primary endpoint of PSA < 4 ng/dL after 18 months	4 of 10 evaluable patients had complete and 4 had partial responses (80%)	not given	3 patients taken off study prior to completing 2 cycles due to concerns for early progression	[10]

CRPC: castration resistant prostate cancer; T: testosterone; PSA: prostate specific antigen; BAT: bipolar androgen therapy; IM: intra-muscular; ADT: androgen deprivation therapy.

6.2. Studies of Bipolar Androgen Therapy (BAT)

In contrast to these studies in which T was administered in continuous manner, the group of Denmeade and Isaacs has pioneered an approach termed Bipolar Androgen Therapy (BAT) [67]. Overexpression of AR is one of the most common molecular hallmarks of CRPC [68], and it was hypothesized that by rapidly cycling T levels between the supraphysiological (~1500 ng/dL) and near-castrate (~150 ng/dL) range, adaptive changes in AR expression would be blunted, thereby delaying the emergence of resistance. Data from these investigators has also suggested that AR becomes critically involved in the DNA replication licensing required for PCa cell proliferation. As discussed more fully below, increased ligand may over-stabilize AR on DNA, preventing its degradation and inhibits DNA relicensing, resulting in cell death in the subsequent cycle [69–71].

Schweizer et al. reported the first clinical experience with this approach: 16 men with asymptomatic metastatic CRPC were treated with 400 mg of T intramuscularly (IM) monthly [9]. Notably, 2 days after T administration, serum T levels exceeded 1500 ng/dL (~50 nM) and fell to <200 ng/dL at the end of each 28 day cycle. PSA declines ($\geq 50\%$) were observed in nearly one-third of patients, radiographic responses were observed in 50% of men, and four continued on treatment for ≥ 1 year. At progression, ADT or AR inhibitory therapy produced responses in 100% of men, suggesting that BAT may restore sensitivity to ADT [9]. Importantly, no patient developed worsening pain due to PCa, nor were there any other skeletal events or evidence of worsening urinary obstruction.

In a follow up study recently reported by Teply et al, men with CRPC who had progressed on ENZ went on to receive BAT ($n = 30$) [10]. This study documented similar activity in response to BAT, with 9 of 30 (30%) men achieving a $\geq 50\%$ decline in PSA from baseline and 50% of patients achieving an objective radiographic response. Twenty-nine patients progressed on BAT and went on to be re-challenged with ENZ. Fifteen of 29 (52%) had a PSA decline $\geq 50\%$, however, there were no objective radiographic responses and time to progression was generally short following ENZ re-challenge.

In another study in hormone naive patients, 29 men with low metastatic burden or biochemically recurrent disease who achieved PSA < 4 ng/dL after 6 months of ADT were treated with alternating 3 month cycles of BAT (given as IM injections of 400 mg T cypionate or enanthate on days 1, 29 or 57), followed by 3 months of ADT alone [10]. The primary endpoint was the percent of patients with a PSA < 4 ng/dL after two rounds of BAT-ADT (i.e., following the 18-month treatment period). Serum androgen levels were not reported. Three of 29 patients were taken off study prior to completing two cycles due to concerns for early progression. However, the 26 patients that completed the study as designed achieved a PSA below their pre-treatment baseline, with 17/29 (59%) achieving the primary endpoint of PSA < 4 ng/dL after 18 months, including three patients who had an undetectable PSA (<0.2 ng/mL) at the 18-month time point. Of 10 men with measurable disease, four complete and four partial responses were observed. Notably, five of seven patients who did progress to CRPC by the end of the study responded to subsequent treatment with anti-androgen, again suggesting that BAT may restore sensitivity to ADT. Treatment was associated with favorable improvement in QoL, although QoL diminished over the course of each cycle of BAT, presumably due to T levels falling below the normal range.

BAT is currently being tested in a large ($n = 180$) randomized trial (NCT02286921; TRANSFORMER) in asymptomatic mCRPC patients who have failed on abiraterone. In this study, BAT is being compared with ENZ with a primary end point of progression-free survival (PFS). While the more substantive clinical benefit observed in the studies of BAT vs. other contemporary and historical studies of androgen treatment may reflect the bipolar dosing strategy, it is likely that it also is related to the fact that these are the only studies in which supraphysiologic androgens have been achieved. Importantly, the relative dearth of patients experiencing clinical deterioration on T treatment compared to historical studies highlights the importance of patient selection, which, in modern studies, was limited to asymptomatic patients with limited metastatic disease.

7. Proposed Mechanisms of Androgen-Mediated Growth Repression

Numerous studies utilizing PCa models with endogenously expressed AR, as well as cell models with exogenously driven increases in AR, have shown that increased AR expression and/or ligand driven activation may result in growth inhibition, variably attributed to cell cycle arrest in G1/S or the subG0/G1 phase, and/or frank DNA fragmentation and apoptosis [18,36,70,72–77]. A number of mechanisms underlying the growth inhibitory effects of SPT have been proposed (summarized in Figure 1) including: I. Cell Cycle Arrest; II. Repression of MYC and S-phase kinase-associated protein 2 (SKP2); III. Apoptosis; IV. Disruption of AR-Mediated DNA Licensing; V. Transcriptional Repression of AR and AR Variants; VI. Transcriptional Reprogramming and Differentiation; and VII. Induction of Cellular Senescence or Quiescence; VIII. Induction of DNA Damage.

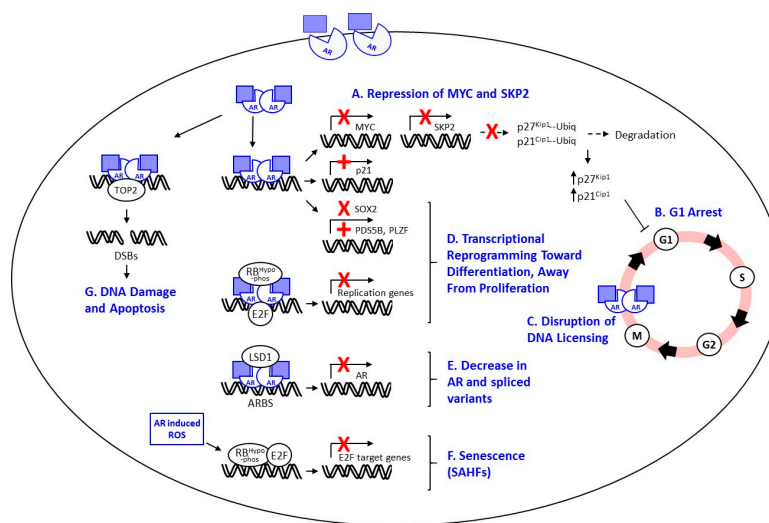


Figure 1. Potential mechanisms for repression of prostate cancer growth by high dose androgen. (A) AR activation in context of high dose androgen (denoted by light blue squares) may lead to transcriptional repression of myelocytomatosis oncogene cellular homolog (MYC) and its target gene S-phase kinase-associated protein 2 (SKP2), with loss of ubiquitin-mediated degradation of the G1 cyclin dependent kinase (CDK) inhibitors p21cip1 and p27kip1, leading to (B) G1 arrest. (AR can also directly induce expression of p21cip1 via an androgen response element (ARE) in its proximal promoter). (C) Ligand-dependent stabilization of AR during mitosis may inhibit AR degradation in M phase, preventing relicensing for DNA replication during G1 resulting in S phase arrest. (D) Androgen induced repression of genes that promote epithelial to mesenchymal transition (EMT) such as (sex determining region Y)-box 2 (SOX2), and expression of genes important in normal differentiation such as sister chromatid cohesion protein cohesion associated factor B (PDS5B (also known as androgen-induced proliferation inhibitor (APRIN)) and promyelocytic leukemia zinc finger protein (PLZF), may promote a more differentiated less aggressive cell state. Through recruitment of hypo-phosphorylated retinoblastoma protein (RB) to shared AR/RB/E2F binding sites, agonist-liganded AR represses genes involved in DNA replication, potentially leading to transcriptional reprogramming toward a less proliferative state. (E) Activated AR may act as a transcriptional repressor at certain AR binding sites (ARBS) via recruitment of lysine-specific histone demethylase 1 (LSD1) and demethylation of activating histone marks, resulting in decreased expression of full length AR and downstream generation of spliced variants. (F) AR-induced production of reactive oxygen species (ROS) leading to decreased RB phosphorylation and repression of E2F target genes may result in formation of senescence-associated heterochromatic foci (SAHF). (G) Androgen signaling leads to co-recruitment of AR and topoisomerase II beta (TOP2B) and TOP2B-mediated DNA double stranded breaks (DSBs) in regulatory regions of AR target genes, potentially leading to DNA damage and apoptosis, particularly in the setting of DNA damage repair (DDR) deficiency (such as mutations in ataxia-telangiectasia mutated gene (ATM) or breast cancer 2 (BRCA2)). X: inhibition of gene expression; +: induction of gene expression.

7.1. Cell Cycle Arrest

De Launoit et al. showed that at the maximal proliferative dose of DHT (0.1 nM), the fraction of androgen-sensitive LNCaP cells in G0–G1 phase significantly decreased (at 36 and 48 h), reflected by an increase in cells in the S and G2/M phases, whereas at growth inhibitory doses of DHT greater than 0.1 nM, an increase in the number of cells in G0–G1 phase was observed, with a significant decrease in cells in the S and G2/M phases [78,79]. G1 arrest was also shown in the androgen independent LNCaP 104-R1 and 104-R2 sub-lines treated with 10 nM R1881, with the maximum decline in the S phase fraction at 72 h after androgen treatment [57]. However, R1881 had no effect on cell cycle distribution in the related androgen-insensitive LNCaP R2-Ad subline [80]. Treatment with 1 nM R1881 yielded a cell cycle arrest in the androgen-repressed MOP, ME, and JAC LNCaP sublines, with an increase in the proportion of cells arrested in the G1 phase and a compensatory decrease in cells in the S and G2/M phases [76].

SPT in PC-3 cells with exogenous expression of AR has also shown a G0/G1 growth arrest. Litvinov et al. reported that overexpression of AR in PC-3 cells caused growth inhibition via G1 arrest, associated with increased expression of the cyclin dependent kinase (CDK) inhibitor p21 (WAF1/CIP1) (a known AR-regulated gene via an ARE in its proximal promoter [81]), as well as an increase in p27kip1 and suppression of p45/SKP2 (normally upregulated in G1 to target the CDK inhibitor p27kip1 for proteosomal degradation) [69,82]. These findings were confirmed by Kokontis et al., using PC3 cells re-expressing either the wild type AR (PC3-AR) or the mutant LNCaP AR (PC3 LNCaP-AR) [83].

7.2. Repression of SKP2 and MYC

Exposure of LNCaP and LNCaP-derived sublines to SPT has been shown to repress the expression of SKP2 [84]. SKP2 is a substrate-targeting subunit of the SCF E3 ubiquitin ligase complex which phosphorylates p21cip1 and p27kip1, targeting them for ubiquitination and degradation. p27kip1 is a cyclin-dependent kinase inhibitor which is encoded by *CDKN1B* gene [85]. The encoded protein was shown to inhibit the cyclin E/CDK2 complex in androgen-treated LNCaP cells [57,79]. LNCaP cells treated with SPT show elevated levels of p27kip1 protein, consistent with impairment in the degradation program following the reduction of SKP2 [57,79,80,86].

One proposed mechanism involves AR-mediated repression of MYC (transcription), with concomitant repression of MYC target genes, which include the ubiquitin ligase SKP2, and thereby upregulation of the G1 cyclin dependent kinase (CDK) inhibitor p27Kip1 which is regulated by SKP2-mediated degradation [16,80,83]. Although not shown in PCa, SKP2 is a direct transcriptional target of MYC in tumor cells including leukemia and neuroblastoma [87,88]. Conversely, SKP2 regulates MYC ubiquitination and stability and serves as a transcriptional coactivator for MYC [89].

In the androgen-sensitive LNCaP 104-S cells, R1881 increased levels of MYC and SKP2 expression and decreased levels of p27kip1 [80]. In contrast, R1881 decreased the levels of MYC and SKP2 and increased p27kip1 in the more strongly androgen-repressed LNCaP 104-R cell lines [80,83]. Moreover, overexpression of this protein along with MYC countered androgen-mediated growth suppression in castration resistant 104-R LNCaP cells [80,83]. In the androgen-insensitive R2Ad cell line, R1881 did not alter the levels of MYC, p27kip1, or SKP2 [90].

SKP2 is also a transcriptional target of E2F [91], and changes in its expression may reflect AR-mediated changes in RB induction of E2F. Jiang et al. demonstrated that SKP2 transcription is directly suppressed by an RB family member, the p107 pocket protein, following SPT treatment [82]. Repressing p107 partially blocked SKP2 repression following exposure to SPT, though whether SKP2 or p107 inhibition altered SPT growth arrest was not determined. Notably, SPT was shown to reduce p107 phosphorylation, though the AR-regulated phosphatase (potentially induced) or kinase (potentially repressed) responsible for this effect was not identified.

To date, these findings have not been validated across models that respond or resist SPT, and the mechanism(s) by which MYC or SKP2 are regulated by SPT have not been identified.

7.3. Apoptosis

While the role of androgen has been shown to be anti-apoptotic in androgen-dependent PCa cells [92], androgen may also induce apoptosis in castration resistant cell lines. Treatment of MOP cells, a castration resistant and androgen-repressed subline of LNCaP, with 100 nM R1881 resulted in an increase in the apoptotic index, with 37% of cells showing nuclear fragmentation and inter-nucleosomal DNA breaks after 6 days of treatment [75]. In the castration resistant 104-R1 LNCaP cell line, AR promoted B-cell lymphoma 2 (BCL2) associated x, apoptosis regulator (BAX)-mediated apoptosis, was involved in mitochondrial translocation of BAX, and addition of androgen potentiated AR-related BAX translocation and the induction of apoptosis [77]. BAX is a member of the BCL-2 family that when activated, initiates apoptosis [93]. Knocking down of AR by siRNA in LNCaP 104-R1 cells resulted in failure of BAX to induce apoptosis. Similarly, while UV induced apoptosis in 40% of the parental LNCaP cells, only 9% of the cells with AR knockdown underwent UV-induced apoptosis [77]. Moreover, addition of androgen potentiated BAX-mediated apoptosis, as was seen in 37% of cells treated with 1 nM R1881 compared to only 19% cell death induced by BAX alone. Androgen also induced apoptosis in PC-3 cells engineered to overexpress AR [73]. However, AR-dependent UV-induced apoptosis was also achieved in the same LNCaP cell line in an androgen independent manner, via AR transcriptional downregulation of p21cip1 expression [94]. p21cip1 is known to have anti-apoptotic and tumor promoting functions, and in the LNCaP 104R model AR was shown to prime cells for apoptosis via down-regulation of basal p21cip1 expression [94].

7.4. Disruption of AR-Mediated DNA Licensing

Normally, licensing factors are degraded in M phase or early G1 phase to allow for relicensing and re-initiation of DNA replication in the next cell cycle [95]. Litvinov et al. and Isaacs et al. have shown AR to interact with the pre-RC and DNA replication machinery in early G1 phase, suggesting it may act as a “licensing” factor for initiation of DNA replication in the subsequent S phase [69,96]. They suggest that excessive ligand-dependent stabilization of AR during mitosis, due to either increased AR or increased ligand, inhibits AR degradation in M phase. This results in a fraction of AR remaining bound to origin of replication sites, preventing relicensing during G1 and resulting in S phase arrest. As AR levels increase with ADT and decrease with normalization of androgen levels they have proposed that rapid androgen cycling between ADT and supraphysiologic androgen levels will prevent the adaptive down regulation of AR levels (that can occur in response to a slow rise in androgens) that would allow re-licensing and cause a poor inhibitory response to subsequent androgen treatment.

Using LNCaP, LAPC4 and CWR22Rv1 PCa cells, a cyclic proteasome-dependent degradation of AR during G1 was observed, along with co-immunoprecipitation (co-IP) of AR with replication complexes (RC), and co-IP of AR with origin of replication complex 2 (ORC2) in four of seven human CRPC metastases, suggesting AR may function as a licensing factor for DNA replication in cells that are androgen-sensitive for growth. In contrast, they do not observe binding of AR to RCs in cells in which liganded AR does not drive growth (the E006AA PCa line, and a prostate stromal line) [69,71]. The impact of high dose androgen on these parameters was not directly tested in LAPC4 or 22Rv1 cell lines. However, in parental LNCaP cells, a LNCaP derivative with castration resistant growth, in 22Rv1 cells overexpressing AR, and in PC-3 cells overexpressing AR, androgen-mediated growth repression was associated with a marked increase in the percent of mitotic cells with detectable AR expression (i.e., 85% vs. 0.5% in untreated), consistent with a role of AR degradation in permitting relicensing of DNA for subsequent replication. in these cells.

However, while cyclic degradation of AR in 22Rv1 cells, and association of AR with RC's in 22Rv1 and VCaP cells was similarly observed, a repressive effect of androgen on 22RV1 does not occur; androgen remains stimulatory at 10^{-6} M T in the 22Rv1 model [63]. These observations suggest that a potential impact of ligand on AR stabilization and interference with proper DNA re-licensing may not be the mechanism by which SPT mediates growth inhibition in all cases.

7.5. Transcriptional Repression of AR and AR Variants

To the extent that AR and AR splice variants are drivers of CRPC progression, mechanisms that repress their expression may lead to decreased cell growth. Studies by the Balk group have shown that AR gene expression is directly repressed by the AR through recruitment of lysine-specific histone demethylase 1 (LSD1) to an AR Binding Site (ARBS) region termed ARBS2 of which a segment of ~400 bp is highly conserved across species [97]. Agonist liganded AR was shown to decrease AR gene expression in castration resistant VCS2 cells (derived from VCaP cells) by functioning as a transcriptional repressor through recruitment of LSD1 and demethylation of histone 3 lysine 4 (H3K4)me1,2 [97]. AR also repressed aldo-keto reductase family 1 member C3 (AKR1C3) and hydroxysteroid 17-beta dehydrogenase 6 (HSD17B6) through a similar LSD1 dependent mechanism, indicating that the agonist liganded AR directly mediates a physiological intracellular negative feedback loop to regulate AR activity. Moreover, in all cases the androgen-stimulated down-regulation was decreased or abrogated by treatment with the LSD1 inhibitor pargyline. The role of LSD1 inhibition in the context of SPT has not been explicitly evaluated, although loss of LSD1 or LSD1 activity has the potential to abrogate SPT effects by inhibiting generation of AR repressive complexes on key targets, or may enhance SPT effects by increasing levels of AR itself.

Whether SPT results in dynamic changes of AR expression associated with growth inhibition in the clinical setting has not been assessed, but has been suggested in several pre-clinical models *in vivo*. Thelen et al. compared the growth of castration sensitive VCaP cells maintained in 10% fetal bovine serum (FBS) to a subline adapted to growth in 1 nM T after implantation in intact (non-castrate) nude mice [56]. Notably, the VCaP cells grown in FBS showed a significant growth disadvantage compared to the subline adapted to T in cell culture. Transcript levels of AR and ARV7 were rapidly downregulated by 1 nM T in the VCaP cells maintained in 10% FBS *in vitro*, and were also lower in the xenografts grown in intact mice than in the parental cells passaged in 10% FBS.

Nakata examined growth inhibition by androgen in JDCaP-hr, an AR and ARV7 positive cell line derived from the castration recurrent outgrowth of a JDCaP xenograft (generated from the skin metastasis of a Japanese CRPC patient) [98,99]. Expression of full length AR (AR-FL) and splice variant 7 AR (AR-V7) mRNA was upregulated by 10-fold in JDCaP-hr compared with that in JDCaP. T suppressed the growth of JDCaP-hr *in vitro* and *in vivo* in association with downregulation of AR and ARV7 expression, while silencing of AR-V7 but not AR-FL markedly suppressed cell growth.

A recent trial of BAT in patients who progressed on ENZ did not show a clear correlation between response and modulation of AR-FL or AR-V7 transcript levels in circulating tumor cells [11]. However, further studies, potentially with assessment of tumor AR expression, are needed to fully evaluate the association of AR modulation with response to SPT.

7.6. Transcriptional Reprogramming and Differentiation

As decreased AR signaling following ligand depletion leads to oncogenic changes in the AR-cistrome, transitioning back from low- to high-T conditions may reprogram the AR-cistrome toward a more differentiated state [97,100]. In addition to showing that AR may directly repress its own expression, the work of Cai et al. described above also showed that agonist liganded AR suppressed the expression of multiple genes mediating DNA synthesis and cell cycle progression, while it increased the expression of genes mediating synthesis of lipids, amino acids, and other metabolic processes. This led the authors to postulate a model whereby androgen levels in CRPC cells are adequate to stimulate AR activity on enhancer elements of genes mediating certain critical metabolic functions such as lipid synthesis (that are sensitive to lower levels of androgens), but are not adequate to effectively recruit AR and LSD1 to suppressor elements in multiple genes that negatively regulate AR signaling and cellular proliferation. In a subsequent publication this group demonstrated that AR causes transcriptional repression of multiple genes involved in DNA replication via interactions with RB1. In particular, SPT was able to induce AR binding to regulatory regions of genes involved in DNA replication and repress their transcription through recruitment of hypo-phosphorylated RB [101].

Alternatively, Ruan et al. have recently demonstrated that SKP2 can bind and stabilize expression of Twist protein, leading to acquisition of epithelial-mesenchymal transition (EMT) and cancer stem cell (CSC) characteristics [102]. Genetic or pharmacologic depletion of SKP2 reverted these effects and re-sensitized CRPC cells to chemotherapy. Whether AR-mediated repression of MYC, or AR-mediated changes in RB induction of E2F are the upstream factors responsible for the decreased SKP2 expression, these data provide another potential mechanism for the cellular reprogramming of CRPC cells treated with SPT toward a less aggressive tumor biology.

The repression or induction of specific genes involved in transcriptional programming and differentiation has also been linked to androgen induced proliferative arrest in PCa cells. SOX2 is an androgen repressed gene that is upregulated in CRPC and has been shown to stimulate epithelial-to-mesenchymal transition (EMT) as well as mediate lineage plasticity from (AR)-dependent luminal epithelial cells to AR-independent basal-like cells [103–105]. Endogenous expression of SOX2 in prostate epithelial cells, human embryonic stem cells, and PCa cells is repressed by AR signaling (via an enhancer element within the SOX promoter), and loss of SOX2 expression has been shown to inhibit growth of the castration-resistant CWR-R1 PCa cell line [103]. While not specifically evaluated, these data suggest a possible role for androgen induced repression of SOX2 in repressing tumor growth via promoting a more differentiated luminal epithelial cell state.

PDS5B (APRIN, AS3) was initially discovered as a gene induced in LNCaP PCa cell lines and rat prostate cells undergoing androgen induced proliferative arrest [5,106]. It was later shown that the basic features of PDS5B (lineage, domain architecture, unique high mobility group (HMG) domains and heterochromatin localization) were consistent with that of a chromatin regulator, suggesting PDS5B may serve as a regulator of chromatin architecture in hormonal differentiation [107]. Although not assessed in PCa models to date, PDS5B was shown to be a critical mediator of differentiation in embryonal carcinoma stem cells, with PDS5B silencing resulting in arrested differentiation at a transient, proliferative progenitor phase characterized by loss of contact signaling, hormone resistance, and continued proliferation [108].

Promyelocytic leukemia zinc finger protein (PLZF, ZBTB16) is another androgen induced transcription factor gene widely involved in regulation of proliferation, differentiation, and stem cell maintenance that could potentially play a role in androgen-mediated growth repression [109]. PLZF expression is rapidly induced by androgen in PCa cell lines, and expression of PLZF has been shown to inhibit proliferation in LNCaP and 22RV1 cells [110,111]. Notably, transcript expression of PDS5B ranges nearly an order of magnitude in CRPC tumors [112], while PLZF expression is reduced/lost in up to 86% of metastatic PCa specimens, with 5–7% of CRPC specimens harboring homozygous PLZF deletions [110,113], suggesting a possible role for androgen-induced expression of PDS5B or PLZF in promoting induction of a more differentiated cell state.

7.7. Induction of Cellular Senescence or Quiescence

Senescence is an irreversible cell cycle arrest associated with changes in cell morphology and gene expression that occurs during the normal embryogenesis [114]. Exogenous re-activation and induction of cellular senescence has been proposed as a potential target for cancer therapy [115].

Several mechanisms of induction of senescence by high dose androgens in PCa cells have been described. Roediger et al. demonstrated a dose-dependent induction of G1/G0 cell cycle arrest and senescence associated beta galactosidase activity (SA beta-Gal) in LNCaP, C4-2 and AR-expressing PC-3 PCa cell lines treated with R1881 at 1 nM or higher concentrations, and in malignant prostate tissues treated ex vivo with R1881 at 10 nM or higher concentrations [116]. Formation of senescence-associated heterochromatic foci (SAHFs) has been shown to coincide with stable repression of E2F target genes in a RB-dependent manner, and E2F1 regulates expression of its own gene by a positive feedback loop [117]. Accordingly, they showed E2F1 was localized to SAHF, and further that the p16-RB-E2F1 pathway was required for this effect, as knockdown of p16 by siRNA decreased formation of heterochromatic foci. SA beta-Gal activity was induced after only 3 h of androgen treatment suggesting a non-genomic

rapid signaling response, mediated in part via the Rous sarcoma oncogene (SRC)- phosphatidylinositol 3-kinase (PI3K)- serine/threonine kinase 1 (AKT) signaling pathway, as treatment with a SRC, PI3K or AKT inhibitor each abrogated the effect (whereas inhibitors of other factors downstream of SRC e.g., mitogen-activated protein kinase kinase (MEK), mitogen-activated protein kinase 14 (MAPK14, also known as p38), signal transducer and activator of transcription 3 (STAT3), and mechanistic target of rapamycin kinase (mTOR), did not). Mirochnik et al. demonstrated induction of senescence in LNCaP cells and PC-3 cells engineered to express AR via two mechanisms, first, via AR induced expression of p21cip leading, via an unidentified mechanism, to decreased expression of p63 (a p53-related protein that opposes cellular senescence) and second, via AR induced expression of reactive oxygen species (ROS) leading to decreased RB phosphorylation and repression of E2F target genes [118].

In contrast to senescence, quiescence is a reversible growth arrest in G0/G1 that generally requires persistence of an external growth condition for its maintenance. However, Bui et al. recently reported the androgen-mediated induction of a self-sustained quiescent state in LNCaP and VCaP cells that was dependent on culturing cells at low density, and associated with induction of oxidative stress, a sustained redox imbalance, and transforming growth factor-beta (TGF beta)/bone morphogenic protein (BMP) signaling [119]. Treatment with R1881 induced expression of stress, differentiation and mothers against decapentaplegic homolog (SMAD) signaling markers comprising a dormancy signature that the authors had previously identified in PCa cells rendered quiescent by culture at low density in hypertonic medium. Notably, transient treatment with R1881 at doses greater than 0.2 nM for 7 days caused a sustained decrease in cloning efficiency that persisted for at least 10 days after androgen withdrawal. However, growth arrest could be reversed by treatment with the anti-oxidants glutathione, or *N*-acetylcysteine, or by inhibition of TGF beta/BMP mediated SMAD phosphorylation. The authors propose that utilization of high dose androgen therapy as early as after radical prostatectomy, or possibly biochemical relapse, when cancer cells are still dispersed and solitary may have most efficacy as stable induction of the self-sustained quiescent state in their studies only occurred at low cell density.

7.8. Induction of DNA Damage

Although it is well established that ADT causes DNA damage, recent data have also shown that high androgen concentrations induce dsDNA breaks (DSB) in PCa cells that can lead to chromosomal rearrangements such as the TMPRSS2-ERG fusion [120], and may represent one mechanism whereby SPT inhibits proliferation. In particular, studies have shown that androgen signaling leads to co-recruitment of AR and topoisomerase II beta (TOP2B), and to TOP2B-mediated DNA DSBs at regulatory regions of AR target genes in PCa cells [120]. Moreover, treatment of PCa cells with etoposide, a TOP2-inhibitor that prevents resolution of TOP2B-induced DSBs, led to enhanced androgen-induced DSBs in the treated cells [120]. This provided rationale for inclusion of etoposide in the first study of BAT reported in men with CRPC [9]. Although the specific effect of this agent on the response to BAT was not fully dissected, a recent case report documented an extreme response following BAT in a patient with inactivating ataxia telangiectasia mutated (ATM) and breast cancer 2 (BRCA2) mutations, providing support for the concept that DNA damage can sensitize to SPT [121]. These data provide rationale for further studies to test the hypothesis that combining SPT and with DNA damaging agents such as PARP inhibitors (PARPi) will result in clinical responses in men with CRPC.

8. High Dose Estrogen Therapy for Breast Cancer—Clinical and Experimental Evidence

Similar to the dual growth-promoting and growth-repressing effects of androgens in PCa, estrogens occupy a similarly paradoxical role in the biology and treatment of breast cancer, although with a more substantial history of clinical use. The efficacy of synthetic estrogens for advanced breast cancer was first described by Haddow in 1944, followed by a number of clinical trials that made estrogens the standard of care in postmenopausal patients with advanced breast cancer

from the early 1960s onwards (reviewed in [122,123]). A critical observation in these early studies was the necessity of a ‘gap period’ following development of menopause, in that response rates to diethylstilbestrol (DES, 5–15 mg/day) or ethinyl estradiol (EE, 1.5–3 mg/day) were substantively higher (~30–40%) in women who were at least 5 years post-menopause compared to those who were not (~5–10%).

When the non-steroidal anti-estrogen tamoxifen was introduced in the 1970s for the treatment of advanced breast cancer, it was compared in clinical trials to DES or EE as the current standard of care. Notably, the response rate was generally comparable to that seen with estrogen treatment, but the consistently superior side effect profile of tamoxifen resulted in its uniform adoption for the first line treatment of advanced breast cancer. Estrogen therapy was essentially abandoned until studies in the 1990’s demonstrating the efficacy of this approach in patients who were resistant to anti-estrogens began to emerge. A number of studies evaluating DES or EE in heavily pre-treated post-menopausal women who were resistant to prior hormonal therapies including tamoxifen and/or aromatase inhibitors demonstrated objective response rates in approximately 30% of patients [122,123]. These observations renewed interest in the clinical and biological mechanisms underlying the activity of high dose estrogens in breast cancer.

Similar to the data for PCa, work by several investigators demonstrated that long term adaptation of breast cancer cells *in vitro* or *in vivo* to estradiol deprivation (and subsequently, to tamoxifen treatment) induces sensitivity to estradiol-mediated growth inhibition [124,125]. In contrast to the relative diversity of mechanisms proposed for androgen-mediated inhibition of PCa cell growth, the primary cause of estrogen-mediated growth inhibition in breast cancer cells appears to apoptosis, albeit via various effector mechanisms [126]. Notably, the conformation of the ER complex, which is dependent on the shape of the estrogenic ligand, can modulate the apoptotic effect, with class I planar estrogens (e.g., estradiol) triggering apoptosis after 24 h and class II angular estrogens (e.g., bisphenol triphenylethylene) delay the process until after 72 h [127].

A number of mechanisms for estrogen-induced apoptosis have been described. Work by the group of C. Jordan has delineated a SRC-dependent estradiol-mediated induction of endoplasmic reticulum stress and inflammatory responses that initiates an unfolded protein response, followed by apoptosis through the intrinsic (mitochondrial) pathway with subsequent recruitment of the extrinsic (death receptor) pathway to complete the process [122]. An estradiol mediated activation of protein kinase AMP-activated catalytic subunit alpha 1 (AMPK) in long term estrogen deprived (LTED) MCF-7 cells has also been described, with increased activity of forkhead box protein O3 (FOXO3) and upregulation of three FOXO3 target genes, Bcl-2-like protein 11 (BIM), fas cell surface death receptor (FAS) ligand (FASL), and Gadd45a (BIM and FASL mediate intrinsic and extrinsic apoptosis respectively and Gadd45a causes cell cycle arrest at the G2/M phase) [128]. Other signaling pathways identified in the apoptotic response to estradiol include estradiol mediated inhibition of PI3K/AKT signaling, nuclear factor kappa b subunit 1 (NF-κB) signaling and the c-Jun N-terminal kinase (JNK) pathway [126].

9. Potential Predictive Markers of Response to Androgen Therapy

9.1. Androgen Receptor

High AR levels appear to sensitize a number of PC preclinical models to the inhibitory effect of SPT, raising the possibility that tumor AR expression might be useful in predicting response to SPT. Consistent with this hypothesis, high baseline PSA levels were associated with response in the BAT CRPC study, suggesting that SPT responders may have more active AR-signaling [9]. However, as discussed above, not all high AR CRPC models are inhibited by SPT in preclinical studies. The ARCaP cell line expresses low levels of AR yet shows a strong androgen-dependent growth suppression [62], while, studies in AR-expressing PC-3 cells have shown that a growth-inhibitory effect was observed in clonal lines expressing low, moderate and high levels of AR [73], indicating that growth inhibition was not necessarily related to the level of AR overexpression. Furthermore, studies

in DU145, a castration resistant PCa cell line that lacks AR, have shown that ectopic overexpression of AR in this cell line failed to yield a growth response to androgen [129].

These data indicate that AR expression by itself is not necessarily correlated with androgen-driven growth suppression, and that a cell must possess the appropriate molecular mechanisms to engage AR as a growth suppressor or as an oncogene [69]. As discussed above, a clear association between response to BAT and modulation of AR-FL or AR-V7 transcript levels was not observed [11], suggesting AR-FL or AR-V7 levels are not a biomarker of SPT response, and the predictive value of AR signaling has yet to be fully evaluated.

9.2. DNA Damage Response Genes

As discussed above, preclinical data suggest the induction of dsDNA breaks may be a mechanism mediating the anti-tumor effects of BAT, and an extreme response to BAT in a patient with inactivating mutations in the DNA damage response (DDR) genes ATM and BRCA2 has been reported [121]. However, 50% of men on the BAT studies showed response and it is unlikely all of these had DDR deficiency. Thus the extent to which DDR deficiency predicts for response to SPT-based therapy remains unknown, but may identify a population of patients likely to show the strongest response as well as those most likely to benefit from the combination of SPT with PARP inhibition.

9.3. Steroid Metabolism and Transport Genes

Earlier trials using physiologic dose T showed limited clinical activity compared to the supraphysiologic levels achieved in the BAT studies, suggesting the anti-tumor efficacy of SPT may reflect the level of intratumoral androgens achieved on therapy, an effect that may be influenced by steroid transport and metabolizing enzymes such as SLCO1B3 and UGT2B17. Expression and/or genetic variation in SLCO1B3 can modulate cellular T uptake in PCa cells in vitro, and genetic variants of SLCO1B3 linked to more efficient T uptake were associated with a shorter time to progression in men with CRPC on ADT [130,131]. In context of SPT, more efficient tumoral T uptake might associate with an enhanced therapeutic response.

UGT2B17, responsible for the irreversible glucuronidation and ensuing elimination of T and DHT, is highly polymorphic and deletion variants of this enzyme (primarily expressed in the liver) are known to influence circulating steroid levels [132]. As such, patients with deletion variants might sustain higher serum androgen levels following exogenous T dosing. UGT2B17 is also expressed in primary and CRPC tumors, where its in situ tumor activity could also influence the maintenance of tumor androgen levels [133,134]. Thus, genetic variation in genes such as SLCO1B3 or UGT2B17 may serve as predictors of response to SPT.

10. Future Directions

Although a significant body of preclinical evidence, and emerging clinical data, support the concept of high dose androgen therapy for the treatment of CRPC, a number of important questions remain unanswered. What is the optimal dosing schedule of T therapy? Given the diversity of mechanisms observed in preclinical studies, what are the specific mechanisms mediating clinical anti-tumor efficacy in a particular individual? Can rational drug combinations be designed to improve efficacy or sensitize tumors that are not inhibited by high dose androgen alone? Can biomarkers be identified to predict response or resistance to SPT? What, if any, is the impact of stromal AR signaling on the response to high dose androgen therapy?

The relative success of the modern studies employing the BAT approach are encouraging, but the extent to which the clinical benefit observed in these studies reflects the bipolar dosing strategy or the documented achievement of truly supra-physiological androgen levels remains unclear. Preclinical studies have consistently found that SPT delivered on a continuous basis represses the growth of PCa cells, and no studies (preclinical or clinical) have directly compared continuously administered SPT with BAT. Whether rapid cycling of SPT (vs. continuous SPT) is necessary to achieve

the clinical benefit or delays resistance by preventing adaptive AR-downregulation remains to be determined. Ultimately, the predominant mechanism of SPT action may dictate the ideal dosing strategy, with continuous treatment likely providing improved efficacy if the main mechanism of SPT action relates to changing from a “low-T” oncogenic AR transcriptome to that of a more differentiating SPT transcriptome [97], and BAT demonstrating more activity if repeated cycles of DNA damage is a critical mechanism of action. However, continuous SPT can repress the expression of genes that repair DNA damage, and thus the continuous repression of these repair programs may also exceed the responses seen with BAT.

Understanding the mechanisms driving the anti-tumor efficacy of high dose androgen is particularly relevant for designing rational drug combinations. For example, the observation that androgens can induce DSB in conjunction with a recent report of an extreme response to BAT in a patient with inactivating ATM and BRCA2 mutations, provides support for the concept that DNA damage can sensitize to SPT [121]. These data provide a clear rationale for combining SPT with DNA damaging agents such as PARP inhibitors (PARPi) and such a trial is currently being designed. However, rational combinations based on other proposed mechanisms can also be conceived, including combinations with cell cycle inhibitors to promote the impact of high dose androgen in mediating cell cycle arrest, combinations with MYC inhibitors to promote the MYC-repressing effects of high dose androgens, or combinations with proteasome inhibitors to prevent AR degradation and promote the proposed stabilization of AR on DNA and inhibition of DNA licensing induced by high dose androgens.

A further consideration which merits discussion is the potential for aromatase-mediated conversion of exogenous T to E2. PCa cells can variably express one or both of ER-alpha (which can promote PCa proliferation) and ER-beta (which can inhibit PCa proliferation); thus the net effect of a possible increase in estrogen signaling may be adverse or beneficial depending on the relative level of each [66]. Whether this is a clinically relevant concern is unclear, as the extent to which T undergoes intra-tumoral conversion to E2 in men treated with SPT is unknown. However, studies testing the combination of T with an aromatase inhibitor (either upfront or at evidence of disease progression) would be informative.

The potential ability of T therapy to re-sensitize CRPC tumors to AR-axis inhibition is intriguing, and if borne out, may represent an important clinical approach for delaying disease progression. Illustrating the potential ability of androgen-repletion to ‘ug’ mechanisms of androgen sensitivity, culture of the androgen-repressed LNCaP 104-R1 cells in androgen rich media gave rise to a subline that was again androgen-sensitive for growth [80]. (Importantly, the related androgen-repressed LNCaP 104-R2 line passaged under similar androgen rich conditions gave rise to a subline that was androgen-insensitive for growth or repression, demonstrating that androgen induced effects on androgen sensitivity are not uniform.) In clinical studies a high response to AR-signaling inhibition was seen in men after BAT therapy [9,11] although whether this reflects a change to a more differentiated phenotype or specific changes in the AR/co-regulator signaling apparatus resulting in re-establishment of androgen-sensitivity remains to be determined.

Finally, preclinical studies of androgen-mediated PCa repression have largely been carried out in vitro or in subcutaneous xenograft models, systems which do not take into account the role of in situ stromal AR signaling on PCa behavior [135]. Notably, multiple studies have found that lower AR expression in PCa stroma is associated with disease progression and/or worse outcome, implying that stromal AR is protective [136]. Mechanistically, in the absence of stromal AR signaling, the fibroblast-derived extra-cellular matrix (ECM) was shown to have a decreased capacity to promote attachment of both myofibroblasts and cancer cells, and was less likely to impede cancer cell invasion [137]. In a separate study, AR-depleted cancer associated fibroblasts (CAFs) promoted increased stem cell marker expression in human PCa cells, apparently via increased levels of Interferon gamma (IFN- γ) and macrophage colony-stimulating factor (M-CSF) [138]. Thus, to the extent AR signaling in PCa stroma maintains an ECM microenvironment inhibitory to cancer cell invasion, or restrains induction of stem cell characteristics, enhanced AR-mediated stromal signaling may also

contribute to the anti-tumor activity of high dose androgen therapy observed in clinical studies. To date these hypotheses remain unexplored.

11. Conclusions

Meaningful clinical responses have now been observed in men with PCa treated with high dose T, but studies designed to determine the molecular mechanism(s) driving these responses and identify predictive biomarkers are needed in order to optimize this approach and identify rational treatment combinations.

The clinical utility of potential treatment combinations will clearly depend on the relative importance of the various proposed mechanism, and the extent to which the diversity of mechanisms observed in the laboratory is recapitulated in human tumor specimens. Clinical studies with built-in collection of biospecimens will be critical to assessing the molecular changes associated with response or resistance to SPT, and for identifying potential predictors of response. There may be subsets of men whose tumors will be variably responsive to SPT or particular combinations based on the status of steroid transport and metabolizing genes, DNA damage repair genes, AR expression, MYC dependence, and/or RB loss or alterations in other cell cycle regulators. Ultimately, high dose T therapy is likely to represent yet another avenue for applying the principles of precision medicine for optimizing the care of men with CRPC.

Acknowledgments: This work was supported by DOD grant W81XWH-15-1-0319 (to Elahe A. Mostaghel); NIH P01 CA163227 (Elahe A. Mostaghel, Peter S. Nelson, Stephen P. Balk, Eva Corey); Pacific Northwest Prostate Cancer SPORE P50CA097186 (Elahe A. Mostaghel, Peter S. Nelson, Michael T. Schweizer, Stephen Plymate, Eva Corey), R21 CA194798 (Eva Corey), DOD grant W81XWH-16-1-0206 (to Michael D. Nyquist), and DOD grant W81XWH-16-1-0484 and a PCF YIA (both to Michael T. Schweizer), including funds for covering the costs to publish in open access. The authors thank Accixx Biomedical Consulting (www.Accixx.com) for editorial assistance.

Author Contributions: Osama Mohammad, Elahe A. Mostaghel and Peter S. Nelson conceived the manuscript; Osama Mohammad, Michael D. Nyquist, Michael T. Schweizer, Stephen P. Balk, Eva Corey, Stephen Plymate, Peter S. Nelson and Elahe A. Mostaghel contributed to writing the manuscript, Elahe A. Mostaghel and Eva Corey performed final editing.

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Appendix A.1. Preclinical Observations on Androgen-Mediated Growth Repression of Prostate Cancer

Preclinical studies demonstrating the repressive effect of androgen on prostate cancer growth are summarized in the primary text of the manuscript. For the interested reader, and to more fully illustrate the similarities and heterogeneity of response observed, a more detailed review of findings in each pre-clinical model is provided in this Appendix A and summarized in Appendix A Table A1.

Table A1. Preclinical Responses to Androgen-Mediated Growth Repression.

Cell Line	Source	Derivation	In Vitro Growth Characteristics	In Vivo Growth Characteristics	Refs.
LNCaP	Lymph node metastasis in a 50-year-old Caucasian male with CRPC		Biphasic response in CSS (peak stimulation at 0.1 nM DHT, progressive growth suppression at 1 nM to 100 nM). Androgen repressed in 5% FBS		[78,139,140]
104-S	LNCaP	Parental Line	Similar to original report Biphasic response in CSS (peak stimulation at 0.1 nM R1881, growth suppression at higher doses)	In vivo growth stimulated by androgens	[7,58]
104-R1	LNCaP 104-S	Passage in CSS $\times$ 10 mo	Proliferated more rapidly than 104-S cells in CSS	In vivo growth inhibited by androgens	[7,57,58]
104-R2	LNCaP 104-S	Passage in CSS $\times$ 18 mo	Severely growth repressed by 0.1 nM or higher R1881 doses		
R1Ad	LNCaP 104-R1	Re-growth in castrate mice after T treatment in vivo	Lost androgen-repressed phenotype Androgen sensitive for growth		[141]
R2Ad	LNCaP 104-R2	Re-growth in castrate mice after T treatment in vivo	Lost androgen-repressed phenotype Androgen insensitive for growth-not affected by R1881 or bicalutamide		[80]
MOP	LNCaP	Passage (of LNCaP passage 25 cells) in CSS $\times$ 10–12 mo	Androgen insensitive for growth Dose-dependent growth suppression in response to R1881 at 0.1 to 10 nM	In vivo growth inhibited by androgens	[75]
JAC	LNCaP	Passage (of LNCaP passage 55 cells) in CSS $\times$ 10–12 mo			[76]
ME	MOP	Regrowth in castrate mice after T treatment in vivo	Still showed androgen repressed growth in vitro		[76]
LNCaP-abl	LNCaP	Long term passage in CSS	Biphasic response but with higher sensitivity than parental LNCaP (max proliferation at 0.001 nM R1881 vs. 0.01 nM)		[142]
CWR22	Primary PCa tumors initially injected subcutaneously into nude mice supplemented with T, then serially transplanted as cell suspension		Biphasic response to androgen, with optimal proliferation at 25 to 35 nM testosterone and growth repression at concentrations higher than 35 nM		[61,143,144]

Table A1. Cont.

Cell Line	Source	Derivation	In Vitro Growth Characteristics	In Vivo Growth Characteristics	Refs.
CWR22R	CWR22	Derived from a CWR22 tumor showing castration resistant re-growth in vivo	Not consistently stimulated by androgen Growth repressive effect left-shifted vs. parental CWR22 line, with repression induced at T levels of approximately 25 nM		[145]
22RV1	CWR22R		Androgen-sensitive for growth without a biphasic response		[63]
ARCaP (MDA PCa 1)	Isolated from the ascites fluid of an 83-year-old Caucasian man with metastatic CRPC		Highly androgen-repressed growth (starting as low as 100 pM DHT) despite relatively low AR expression	Grew 3 times faster in castrated hosts than in intact male hosts; growth in castrated hosts was suppressed by exogenous T	[62]
VCaP	From a vertebral metastatic lesion of patient with CRPC		40% repression at 10 nM R1881. Detachment and disintegration of cells passaged in low androgen conditions (10% FBS) when treated with 1 nM T in vitro	Poor growth in intact (noncastrate) SCID mice [56]	[56,67,146]
E006AA	From primary tumor of a 50-year-old African-American man with clinically localized PCa		Biphasic response, with proliferative response as low as 1 fM DHT and maximal proliferative at 0.1 pM DHT		[147]
MDA PCa 2b	From a bone metastasis of a patient with CRPC		Biphasic response, peak proliferation at 10 nM DHT with growth inhibitory effects at higher concentrations	Stopped growing or decreased in size after castration (response to high dose androgen not evaluated in vivo)	[59,60]
MDA PCa 2b-hr	MDA PCa 2b	culture of MDA PCa 2b in CSS for 35 weeks	Biphasic response to T concentrations ranging from 0.1 ng/ml to 1000 ng/ml, with maximal proliferation 1 ng/mL T		[60]
RC-77T	From primary tumor of a 63-year-old African American man with clinically localized PCa		Biphasic response, maximal growth at 0.1 nM R1881 and growth inhibition at higher doses		[148]
PC3-AR	From lumbar vertebral metastasis of a 62-year-old white man	PC3 with exogenous expression of AR	Androgen mediated growth repression at DHT 0.1 nM	In vivo growth inhibited by androgen levels presenst in intact male mice	[129,149–151]

CSS: charcoal stripped serum; DHT: dihydrotestosterone; FBS: fetal bovine serum; AR: androgen receptor; SCID: severe combined immunodeficiency; PCa: prostate cancer; MDA: MD Anderson.

Appendix A.1.1. LNCaPs

Derived from a supraclavicular lymph node metastasis in a 50-year-old Caucasian male [139], LNCaP is the most widely used cell line in prostate cancer research. A number of sublines have been derived under different culture conditions and with varying degrees of sensitivity to androgen induced growth and repression. Horoszewicz et al. originally reported the stimulatory effect of DHT on LNCaPs in androgen-depleted conditions 5% charcoal stripped serum (CSS) and the dose-dependent suppression in response to DHT in cells cultured in relatively androgen-rich conditions (5% FBS) [140]. De Launoit et al. also demonstrated a progressive decrease in the stimulatory effect of DHT (in 2% CSS) at doses higher than 0.1 nM, returning to basal levels with doses between 1 nM and 100 nM [78].

I. LNCaP 104-S, 104-R1, 104-R2, R1Ad, R2Ad

Similarly, androgen sensitive LNCaP 104-S cells showed maximal proliferation at 0.1 nM R1881 (with progressive growth suppression with higher doses), while 104-R1 and 104-R2 cells (derived after passages in androgen-depleted medium for 10 months and 18 months, respectively) proliferated more rapidly than 104-S cells in androgen-free conditions, and were severely inhibited by 0.1 nM or higher R1881 doses [57,58], illustrating the left-shift in androgen-repressed sensitivity frequently observed in the transition from androgen-sensitive to androgen-insensitive cell growth. Consistent with the in vitro data, proliferation of LNCaP 104-S tumors in vivo was stimulated by androgens but testosterone propionate (TP) pellets implanted in castrated nude mice bearing LNCaP 104-R2 resulted in tumor growth inhibition and a significantly reduced tumor size [7].

Interestingly, when re-passaged in androgen-rich conditions (castrated mice bearing T pellets), 104-R1 cells (now called R1Ad) lost the androgen-repressed phenotype and showed androgen-induced growth in vivo, illustrating the potential ability of androgen-repletion to ‘re-engage’ mechanisms of androgen sensitivity. In contrast, however, 104-R2 cells passaged in androgen-rich conditions gave rise to the androgen-insensitive R2Ad subline, the growth of which was not affected by R1881 or the anti-androgen bicalutamide [80], demonstrating that androgen-induced effects on androgen sensitivity are far from uniform.

II. MOP, JAC, ME

LNCaP variant MOP and JAC cells (derived, respectively, by continuous passaging of androgen-sensitive LNCaPs at passage 28 and 55 in 2.5–5% CSS for 10–12 months), were androgen-insensitive for growth, and showed dose-dependent growth suppression in response to R1881 at 0.1 to 10 nM [75,76]. Similarly, testosterone treatment delayed the take of palpable tumors following injection of LNCaP variant MOP cells in female nu/nu mice in vivo. Notably, tumors eventually escaped treatment, but ME cell lines, established from these non-testosterone repressed tumors still showed androgen repressed growth in vitro [76].

III. LNCaP-abl

Similar to androgen-sensitive LNCaP cells, LNCaP-abl, another subline derived from long-term passage in CSS, demonstrated a biphasic response to androgen, but with a higher sensitivity than the parental LNCaP [142], again illustrating the left-shift in androgen-repressed sensitivity observed in the transition from androgen-sensitive to androgen-insensitive cell growth. While the maximum proliferative rate of the parental LNCaP was achieved at 0.01 nM R1881, the R1881 dose that achieved maximal proliferation of the abl subline was left-shifted one order of magnitude to be at 0.001 nM. Moreover, at passages higher than 75, androgen treatment only induced an inhibitory growth effect on the LNCaP-abl subline [142].

IV. C4-2B

The C4-2B cell line was isolated from a mouse vertebral metastasis in 1994 as a subline of a LNCaP derivative established by Wu et al. [151]. Wu et al. co-injected LNCaP and osteosarcoma cell lines subcutaneously in intact mice, followed by castration, resulting in growth of an androgen-independent tumor. Cells cultured from these tumors, denoted C4 cells, were then subcutaneously injected in castrate mice, giving rise to tumors from which the C4-2 cells were cultured. Following subcutaneous or orthotopic injection of C4-2 cells into castrated mice, cells isolated from a vertebral bone metastasis were cultured and denoted C4-2B [152]. Although Thalmann et al. reported that the osseous metastases of the androgen-independent C4-2 cells (C4-2B) were enhanced in castrated hosts, suggesting androgen suppression of androgen-independent dissemination [152], Pfitzenmaier et al. later reported that the growth of C4-2 metastases was inhibited by androgen suppression i.e., castration [153].

Appendix A.1.2. CWR22 and CWR22R

CWR22 is a serially transplantable xenograft established along with other CWR lines from primary tumors excised through transurethral resection of the prostate or radical prostatectomies, and [143,144]. However, it has been since transplanted through injection of the cell suspension into testosterone supplemented nude mice [144]. After castration, the xenograft markedly regresses, but is often followed by tumor relapse 3 to 10 months after castration [61,145], from which the CWR22R line was derived.

Nagabhushan et al. reported the differential sensitivity of CWR22 and CWR22R cells to androgen stimulation in soft agar [61], again illustrating the left-shift in androgen-repressed sensitivity observed in the transition from androgen-sensitive to androgen-insensitive cell growth. CWR22 cells showed a biphasic response to androgen, with optimal proliferation at 25 to 35 nM testosterone and growth repression at concentrations higher than 35 nM [61]. Growth of CWR22 in FBS was parallel to CSS curves, with more overall cell proliferation in FBS. In both media, T concentrations higher than 35 nM inhibited proliferation [61]. In contrast, CWR22R cells were not consistently stimulated by androgen and the growth repressive effect was left-shifted compared to the parental CWR22 line, with repression induced at T levels of approximately 25 nM [61]. In contradistinction to CWR22 cells, 22Rv1, another AI line derived from CWR22R, is androgen sensitive for growth without a biphasic response [63].

Appendix A.1.3. ARCaP

Isolated from the ascites fluid of an 83-year-old Caucasian man with metastatic PCa, the highly metastatic ARCaP cell line, also known as MDA PCa 1, was first introduced and characterized by Zhau et al. in 1996 [62] and is notable for its highly androgen-repressed phenotype despite relatively low AR expression. ARCaP cells have been reported to demonstrate growth repression to DHT in vitro in a concentration-dependent manner (starting as low as 100 pM) [62]. Interestingly, overexpression of AR in these cells restored a biphasic response to androgen, with stimulation of proliferation in response to R1881 at 0.1 nM to 10 nM but suppression of proliferation at higher R1881 concentrations of 100 nM to 1 μ M R1881 [72]. ARCaP tumors, when maintained as subcutaneous xenografts, grew 3 times faster in castrated hosts than in intact male hosts, suggesting the sensitivity of these cells to suppression by physiological levels of androgen. Consistent with the in vitro assays, tumor growth in castrated hosts was suppressed by subcutaneous administration of either testosterone propionate [62].

Appendix A.1.4. VCaP

These cells were isolated in 2001 from a vertebral metastatic lesion of a patient with CRPC. Initial studies characterizing this line demonstrated it was androgen sensitive for growth in vitro and in vivo but did not report a biphasic response [146]. Subsequent studies by several groups have demonstrated the ability of high dose androgen to repress growth in this model. Denmeade showed 40% repression of growth after 5 days of exposure to 10 nM R1881 [67]. Thelen et al. demonstrated

detachment and disintegration of VCaP cells passaged in low androgen conditions (10% FBS) when treated with 1 nM T in vitro, and poor growth when injected subcutaneously in intact (noncastrate) severe combined immunodeficiency (SCID) mice [56].

Appendix A.1.5. E006AA

This cell line was established by Koochekpour et al. as spontaneously immortalized cells from a 50-year-old African-American patient who underwent radical retro-pubic prostatectomy for treatment of a clinically localized prostate cancer [147]. In vitro studies have shown a biphasic response of E006AA to androgen, with the cells being much more sensitive to DHT stimulation (in 1% CSS) than were LNCaP cells. The proliferative response of E006AA started at concentrations as low as 1 fM DHT, with a maximal proliferative effect at 0.1 pM, while higher DHT concentrations had an inhibitory effect on cell number compared with untreated cells [147].

Koochekpour et al. reported E006AA cells were non-tumorigenic in vivo when xenografted in athymic nude mice [147]. D'Antonio et al. subsequently showed that these cells tumorigenic in the NOD-SCID-IL2Rgamma (NSG)triple deficient mice, attributing the failure of growth in the original studies to the fact that nude mice possess high levels of activated natural killer T-cells, resulting in increased host-immunoreactivity towards a growing tumor [154]. Moreover, and despite having been isolated from a hormonally naïve primary prostate cancer, E006AA cells showed castration-resistant growth when xenografted in castrated vs. intact NSG mice [154]. However, the response of tumors to exogenous androgen in vivo, either in castrated or intact mice, has not been assessed.

Appendix A.1.6. MDA PCa 2b and MDA PCa 2b-hr

MDA PCa 2a and 2b were derived from a bone metastasis of a patient with castration resistant prostate cancer. Both lines express AR, grow in vitro and in vivo, and are androgen sensitive [59]. Both lines demonstrate peak proliferation in response to DHT at 10 nM with growth inhibitory effects at higher concentrations [59].

The MDA PCa 2b-hr cell line was derived from the androgen-dependent MDA PCa 2b cell line after prolonged culturing in androgen-depleted media [60]. Hara et al. reported the biphasic response of MDA PCa 2b-hr cells to testosterone concentrations ranging from 0.1 ng/mL to 1000 ng/mL, with the maximal proliferation rate achieved with 1 ng/mL of testosterone [60]. Navone et al. reported that MDA PCa 2b tumors formed in athymic mice stopped growing or decreased in size after castration [59]. Conversely, Hara et al. reported that in nude mice bearing MDA PCa 2b tumors, treatment with a 100 mg DHEA pellet (which achieved a 12.4 ng/mL serum testosterone levels, comparable with the physiological levels in uncastrated men) stimulated MDA PCa 2b growth. However, the effect of exogenous high-dose androgen on tumor growth in castrated conditions has not been assessed in this model.

Appendix A.1.7. RC-77N/E & RC-77T/E

These cell lines were first introduced by Theodore et al. in 2010 [155]. Tumor tissue (RC-77T) and non-malignant tissue (RC-77N) used for generating the cell lines that were obtained from a radical prostatectomy specimen of a 63-year-old African American patient with a clinical stage T3c adenocarcinoma with poor differentiation (Gleason 7) [155]. Androgen sensitivity assays were carried out in keratinocyte serum-free media (K-SFM) in the presence of 0, 0.1, and 1 nM R1881. RC-77T cells were shown to be more sensitive to androgen stimulation, reaching a peak growth with 0.1 nM R1881, than were RC-77N cells, which reached their peak growth with 1 nM. Higher doses (10–100 nM R1881) seemed to inhibit cell growth [155]. Effects of exogenous androgens on growth in vivo have not been reported.

Appendix A.1.8. PC-3

PC-3 cells were first derived by Kaighn et al. from a lumbar vertebral metastasis in a 62-year-old white man in 1979 [148]. Although the original PC-3 cell line lacks AR, multiple studies have used exogenous AR expression in PC-3 cells to investigate the role of AR in the androgen-mediated growth response of the cells. Early studies reported that ectopic expression of AR using a viral promoter in PC-3 cells led to androgen-mediated suppression of cell growth [73,156,157]. Yuan et al. first reported more than a 50% decrease in proliferation of PC-3 cells transfected with human full length AR when treated with 2 µg /mL (6.8 µM) DHT for 72 h, and more than 40% inhibition of cell proliferation with DHT doses as low as 0.1 nM after a 72 h incubation [156]. These findings were confirmed later by Litvinov et al. using a modified expression vector for the exogenous AR expression [129,149]. Interestingly however, Altuwaijri et al. reported an otherwise slight androgen-induced cell growth of PC-3 cells expressing AR driven by its natural human AR promoter, denoted PC-3(AR)9 cells, when treated with 1 nmol/L DHT [150]. To confirm the AR-mediated growth inhibition in animal models, Litvinov et al. xenografted Lenti-AR PC-3 cells and control cells into male nude intact mice. PC-3-Lenti-AR tumors in mice were profoundly growth inhibited in comparison with PC-3 control tumors [129].

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LETTER TO THE EDITOR

Prostate Cancer

Hormone levels following surgical and medical castration: defining optimal androgen suppression

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Asian Journal of Andrology (2017) 19, 1–2; doi: 10.4103/aja.aja_38_17; published online: 7 November 2017

Dear Editor,

In the 1940s, Charles Huggins discovered that surgical castration produced remarkable palliative benefits for men with advanced prostate cancer, an effect we now understand to be mediated through depriving the androgen receptor (AR) from its ligands (i.e., testicular-derived androgens). In the years since, medical forms of androgen deprivation therapy (ADT) have largely replaced orchiectomy as the predominate means of achieving castrate testosterone (T) levels, and currently, luteinizing hormone-releasing hormone (LHRH) agonists (e.g., leuprolide) are the most common form of ADT. Importantly, studies have shown that LHRH agonists are clinically efficacious and, similar to surgical castration, drive T below 50 ng dl⁻¹ for most patients.¹

While T <50 ng dl⁻¹ is the most frequently cited definition for what constitutes a castrate level T, it should be recognized that multiple studies have demonstrated better outcomes with lower T levels. For instance, Klotz *et al.*² reported that, in patients enrolled to the Phase III PR-7 study testing intermittent versus continuous ADT in nonmetastatic biochemically recurrent prostate cancer patients, a total T level <20 ng dl⁻¹ was associated with improved disease-specific survival and time-to-castration resistance compared those with a total T level >20 ng dl⁻¹. This observation has led to the European Association of Urology to recommend that a level of <20 ng dl⁻¹ should be used to define a castrate level of T.

It is important to note that the total serum T levels include both free and protein-bound (e.g., sex hormone-binding globulin, albumin) fractions, while older studies have shown that LHRH agonists suppress total T levels below castrate levels; it is widely understood that free, or unbound, T is the biologically and clinically relevant component.³ That being the case, the therapeutic goal of medical ADT should be to decrease free T levels to those achieved with orchiectomy. However, to date, free T has not been well studied in large cohorts of orchiectomized men. The purpose of this study was to examine total T and free T levels in men who have undergone orchiectomy or received medical ADT in the context of a prospective clinical trial. These determinations will help set expectations for future development of novel agents that effect androgen levels.

Baseline data were utilized from a double-blind, randomized, placebo-controlled trial (G300203) that was designed to determine the capacity of toremifene (a second-generation selective estrogen receptor modulator) to prevent bone fractures in men on ADT. This study included 1389 men from 150 sites in the US and Mexico. Patients were randomized in the ratio of 1:1 to receive toremifene 80 mg by mouth daily or matched placebo. Results from the primary analysis have already been published.⁴ Baseline characteristics, including whether men were on medical ADT or status postorchiectomy, were available.

The primary objective of this study is to describe baseline hormone levels in men receiving medical ADT or who underwent orchiectomy before initiating toremifene. Hormone levels were determined in a centralized clinical testing facility utilizing an Food and Drug Administration-approved radioimmunoassay (RIA) (Diagnostic Products Corporation, Los Angeles, CA, USA). Free testosterone levels were estimated using RIA and equilibrium dialysis as previously described.⁵ We also evaluated for differences in hormone levels between groups using the Wilcoxon rank-sum test.

This study was conducted in accordance with the Declaration of Helsinki and was approved by the local ethics committees of the participating institutions. Informed written consent was obtained from all patients before their enrollment in this study.

Between November 2003 and October 2005, 1284 men with prostate cancer receiving ADT were randomized between placebo and toremifene. Of this cohort, castrating therapy was administered as follows: 1191 received an LHRH agonist, 56 underwent bilateral orchiectomy, 27 underwent bilateral orchiectomy with androgen receptor blockade, and 10 underwent bilateral orchiectomy and also received an LHRH agonist. Details for why ten patients received LHRH agonist therapy and orchiectomy are not available as these data were gleaned from case report forms which are no longer available for review.

LH levels were significantly lower and estradiol levels were significantly higher in men receiving LHRH agonist therapy. There was no significant difference in total or free T levels between groups (Table 1). However, there was less variability in total and free T levels in orchiectomized patients compared to those receiving LHRH agonists. For instance, the total T ranged from 0.69 ng dl⁻¹ to 29.5 ng dl⁻¹ in men receiving LHRH agonist therapy alone compared to 0.69 ng dl⁻¹ to 13.01 ng dl⁻¹ in patients who underwent orchiectomy.

To our knowledge, this study is the largest to report free T levels in men who underwent orchiectomy. In this cohort, the mean serum-free

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Received: 02 May 2017; Accepted: 02 August 2017

Table 1: Baseline demographics and hormone levels

	LHRH agonist	Orchiectomy	Orchiectomy + androgen receptor blockade	Orchiectomy + LHRH agonist
Number of patients (n)	1191	56	27	10
The USA	1043	20	5	7
Mexico	148	36	22	3
Age (year, median with range in parentheses)	76 (48–93)	76 (61–90)	74 (62–87)	75 (51–82)
BMI, median (range)	27.84 (16.05–46.64)	27.70 (17.38–38.90)	26.48 (22.74–42.51)	24.49 (20.34–30.81)
Baseline PSA (ng ml ⁻¹ , median with range in parentheses)	0	0 (0–3.3)	0 (0–3.9)	0.1 (0–2.6)
Gleason score, n (%) ^a				
≤6	217 (42.2)	1 (3.8)	4 (44.4)	2 (66.7)
7	144 (28.0)	11 (42.3)	1 (11.1)	0
≥8	153 (29.8)	14 (53.8)	4 (44.4)	1 (33.3)
LH (mIU ml ⁻¹ , median with range in parentheses)	0.1 (0.1–52.6)	15.9 (3.3–36.9)	12.9 (2.8–34.9)	0.1 (0.1–5.8)
Estradiol (pmol l ⁻¹ , median with range in parentheses)	36.71 (0–330.39)	29.37 (18.36–187.22)	25.70 (18.36–62.41)	34.87 (18.36–69.75)
Free testosterone (pg ml ⁻¹ , median with range in parentheses)	1.01 (0.17–77.67)	1.14 (0.35–33.95)	1.25 (0.35–3.64)	0.35 (0.35–3.02)
Total testosterone (nmol l ⁻¹ , median with range in parentheses)	0.69 (0.69–29.50)	0.69 (0.69–13.01)	0.69 (0.69–1.32)	0.75 (0.69–1.21)

^a732 patients had missing Gleason score information. LHRH: luteinizing hormone-releasing hormone; BMI: body mass index; PSA: prostate-specific antigen; LH: luteinizing hormone. P values were determined using the Wilcoxon rank-sum test.

T was approximately 1.9 pg ml⁻¹. This value could be considered to be the optimal suppression of free T with orchiectomy and represents the goal of medical ADT. Reassuringly, there was no difference in free T levels between the orchiectomy cohort and those receiving an LHRH agonist.

An interesting observation from this study was that, while there was no difference in total or free T levels between treatment groups, LH levels were significantly lower and estradiol levels were significantly higher in patients receiving LHRH agonist therapy. Given that LHRH agonists inhibit testicular androgen biosynthesis by impairing LH release from the pituitary gland, it is not surprising that LH levels were lower in men receiving these drugs. Somewhat less clear is why estradiol levels are elevated in men receiving an LHRH agonist compared to other groups. LH receptors are present in the adrenal gland, and it is possible that LH may exert some influence on adrenal estradiol biosynthesis that has yet to be explained.^{6,7}

Given that mass spectrometry has been demonstrated to be more accurate means to quantitate low hormone levels, a limitation of this analysis was the use of RIA to measure circulating androgen levels.⁸ At the time this study was performed, RIA was considered to be the standard but has since been shown to underestimate free T levels by 20%–60%.^{9,10} Currently, equilibrium dialysis coupled with LC-MS/MS is the gold standard, but the results from this analysis provide us with increased understanding of the optimal level of free T in treating advanced prostate cancer.

This study provides greater clarity on the effects that LHRH agonists and orchiectomy have on circulating hormone levels. Importantly, as determined by RIA, there is no significant difference in total or free T levels irrespective of whether a man received surgical or medical ADT. These circulating hormone levels should serve as a benchmark for future studies investigating novel forms of medical ADT, with the goal being to meet or exceed this level of free and total T suppression.

AUTHOR CONTRIBUTIONS

This study was designed by MTS, EYY, and RHG. Statistical analysis was performed by MLH and all authors helped to analyze the data. The manuscript was prepared by MTS. All authors read and approved the final manuscript.

COMPETING INTERESTS

MLH and RHG are former employees of GTx, Inc, the sponsor of the clinical trial from which these data were derived. MTS and EYY have no competing interests to declare.

ACKNOWLEDGMENTS

We thank all the men who generously participated in this research. We acknowledge funding support from PNW Prostate SPORE CA097186 (MTS and EYY), DOD award W81XWH-16-1-0484 (MTS), and a Prostate Cancer Foundation Young Investigator Award (MTS).

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Metastatic Adenocarcinoma of the Epididymis: A Case Report and Brief Literature Review

Laura Graham,¹ Lawrence D. True,² Michael T. Schweizer^{3,4}

Clinical Practice Points

- Epididymal adenocarcinomas are rare and little is known about their biology or natural history.
- Some epididymal adenocarcinomas can behave indolently, and close surveillance may be reasonable in some instances.

Clinical Genitourinary Cancer, Vol. ■, No. ■, ■-■ © 2017 Elsevier Inc. All rights reserved.

Keywords: Epididymal adenocarcinoma, Epididymis cancer, Metastases, Next-generation sequencing, Paratesticular

Introduction

Neoplasms of the epididymis are rare, and benign tumors outnumber malignant ones by 3 to 1.¹ Of the benign neoplasms, adenomatoid tumors are most common. Less common neoplasms are leiomyomas, serous (non-papillary) cystadenomas, cavernous hemangiomas, and melanotic neuroectodermal tumors.² Primary malignant tumors of the epididymis include adenocarcinomas, mesotheliomas, and rhabdomyosarcomas. Herein, we report the case of a metastasizing adenocarcinoma of the epididymis — a clinical scenario that has been infrequently described in the literature.

Case Report

A 38-year-old male presented with painless right-sided scrotal swelling. He underwent spermatocelectomy, with pathology demonstrating a benign epididymal papillary cystadenoma. Five years later, the patient re-presented with recurrent swelling in the scrotum. A computed tomography (CT) scan demonstrated a 1.2-cm inguinal node, and he underwent a right epididymectomy for persistent pain. The mass consisted of an adenocarcinoma with papillary features and an associated papillary cystadenoma. A staging CT of the chest, abdomen, and pelvis demonstrated multiple sub-centimeter lung nodules and a 2.2 × 2.4 cm right inguinal mass. He subsequently underwent right radical

orchiectomy, scrotectomy, resection of the inguinal mass, and inguinal lymph node dissection. Histologically, the mass was a low-grade adenocarcinoma involving 3 of 8 lymph nodes (Figure 1A). Immunohistochemically, the carcinoma cells were variably positive for CK7 and CD10 and positive for mesothelin and CAIX, but negative for PSA and PROSAP, CK20, CDX2, WT1, SALL4, Glypican 3, CK5, calretinin, and S100. UW-OncoPlex, a next-generation deep sequencing panel, was used to look for mutations that could be potential therapeutic targets.³ A *TET3* frame-shift mutation was detected as was a low-level variant of a *MTHFR* splice site alteration.

Six months after his last surgery, the patient had another surveillance CT scan of the chest, abdomen, and pelvis that showed a right inguinal soft tissue density along the proximal femoral vessels. A positron emission tomography (PET)/CT scan revealed that the right inguinal mass was fludeoxyglucose-avid, as were several of the pulmonary nodules. Biopsy of the inguinal node confirmed metastatic adenocarcinoma. One year later, a repeat PET/CT scan demonstrated modest enlargement of the fludeoxyglucose-avid right inguinal node and stable pulmonary nodules (Figure 1B). The patient continues to have mild inguinal pain, but otherwise is doing well clinically in spite of no intervention in the preceding year.

Conclusion

Primary epididymal adenocarcinomas are rare. Only 25 cases have been reported in the English literature (Table 1).^{1,4-18} The actual number of cases may be smaller, given that some authors contest the diagnosis of epididymal adenocarcinoma.¹² Given the rarity of these tumors, their pathogenesis remains in question. Because our patient had a component of cystadenoma in his tumor, we suspect that the tumor arose as a malignant transformation of a benign papillary cystadenoma. It is not clear

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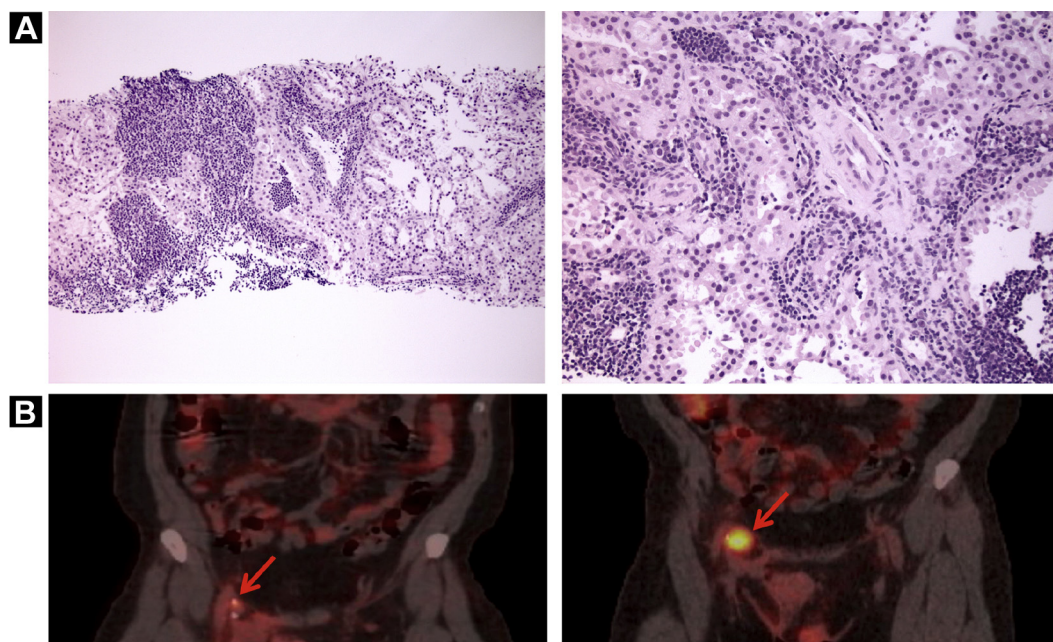
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Submitted: Sep 14, 2017; Accepted: Sep 21, 2017

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Epididymal Adenocarcinoma Case Report

Figure 1 A, Hematoxylin-eosin Stain, 40× (Left Image) and 200× (Right Image) Magnification. Tumor Cells Form Glands and Have a Pale Eosinophilic Cytoplasm and Small, Punctate, Round Nuclei. B, Representative Positron Emission Tomography/Computed Tomography Images at Baseline (Left) and 1 Year Later (Right). Arrow Indicates Hypermetabolic Inguinal Lymph Node, Which Increased From 11 × 9 mm (max Standardized Uptake Value, 3.3) to 28 × 19 mm (3/280) (max Standardized Uptake Value, 5.9) Over This Time Period.



whether the observed *TET3* or *MTHFR* mutations were transforming events, although this seems somewhat unlikely, given that these mutations have not been associated with malignant transformation in other tumor types. It seems likely that mutations in genes not included in the targeted sequencing panel used may have influenced tumor biology. It is worth noting that only one other published case of epididymal adenocarcinoma reported sequencing data.¹⁷ In this instance, a *TP53* mutation was revealed — a gene not altered in the present case.

These tumors have been reported in patients over a wide age range; from 24 to 82 years (Table 1).¹² Some tumors, which metastasized early, were fatal shortly after diagnosis, whereas in one case, the patient was alive 30 years after diagnosis despite the presence of para-aortic lymph node metastases at the time of diagnosis — paralleling the indolent course seen in our patient thus far.

As it stands, surgical resection of localized disease remains the mainstay for treatment of primary malignant epididymal tumors.

In patients with metastatic disease, the optimal management approach has not been defined. A handful of cases have reported > 1 year progression-free survival with systemic chemotherapy (eg, platinum- and taxane-based). Whether these periods of prolonged disease stability resulted from effective cytotoxic therapy or are a reflection of an indolent natural history is unknown.^{13,16}

Given the lack of data to guide clinical management, we opted for a conservative approach. Our patient has been monitored with serial CT scans and 2 PET/CT scans to evaluate for candidacy for metastasectomy. Although multidisciplinary consensus was that surgical resection of all metastatic disease was unlikely to be beneficial, we have not seen any evidence that his tumors are rapidly progressing, and he remains clinically stable. Because of a splice site alteration in the *MTHFR* gene, the tumor may respond to anti-folate chemotherapeutic agents. Consequently, we plan a trial of 5-fluorouracil-based chemotherapy if the tumor progresses more rapidly or he becomes symptomatic.

Table 1 Reported Cases of Primary Adenocarcinoma of the Epididymis in the English-language Literature

Case No.	Age, y	Clinical Presentation	Outcome	Treatment	Author	Year
1	78	Unilateral scrotal mass for 11 months	No recurrence at 8 months	Surgery	Hinman and Gibson	1924
2	73	Painless hydrocele and epididymal mass	Death at 18 months after diagnosis with widespread metastases	Surgery	Ferrier and Foord	1934
3	56	Painless epididymal mass for 15 years	No recurrence	Surgery	Thompson	1936
4	27	Painless epididymal mass for 7 years	No recurrence	Surgery	Thompson	1936
5	30	Painless epididymal mass for 10 years	No recurrence	Surgery and radiation	Thompson	1936
6	29	Painless epididymal mass	No recurrence	Surgery	Thompson	1936
7	54	Painless epididymal mass for 4 years	No recurrence	Surgery and radiation	Thompson	1936
8	24	Scrotal swelling for 3 months	Death at 36 months post-surgery with pulmonary metastases	Surgery and radiation	Chitkara et al	1960
9	35	Scrotal swelling for 1 month	Death at 9 months after surgery with widespread metastases	Surgery	Salm	1969
10	38	Testicular swelling for 3 years	Death shortly after diagnosis	Surgery and chemotherapy	Kher et al	1973
11	27	Testicular pain and swelling	Stable disease at 6 months	Surgery, radiation, and chemotherapy	Wang et al	1990
12	59	Epididymal mass for 4 months	No recurrence at 2 years	Surgery	Nistal et al	1992
13	27	Scrotal swelling for 1 year	No recurrence at 2 years	Surgery and radiation	Yu et al	1992
14	82	Scrotal swelling for 3 months	No recurrence at 8 months	Surgery	Kurhara et al	1993
15	77	Scrotal mass and hydrocele	Death 20 months after surgery with widespread metastases	Surgery	Jones et al	1997
16	81	Scrotal mass and hydrocele	Death 2.5 years after surgery with extensive abdominal disease	Surgery	Jones et al	1997
17	66	Scrotal mass and hydrocele	No follow-up reported	Surgery	Jones et al	1997
18	27	Scrotal mass	No evidence of disease at 30 years	Surgery	Jones et al	1997
19	62	Stable testicular mass for 5 years, followed by 1 month of testicular swelling and pain	No recurrence of disease at 18 months	Surgery	Ganem et al	1998
20	45	Scrotal mass for 2 years	Death at 30 months with widespread metastases	Surgery, radiation, and chemotherapy	Chauhan et al	2001
21	69	Testicular pain and epididymal enlargement	Death at 6 months	Surgery and chemotherapy	Arisan et al	2003
22	35	Epididymal mass	No evidence of disease at 3.5 years	Surgery and radiation	Soumarova et al	2012
23	61	Epididymal enlargement	No recurrence at 20 months	Surgery and chemotherapy	Stanik et al	2012
24	60	Painless testicular swelling for 6 months	Pulmonary metastases discovered 2 years after surgery, stable disease 2.5 years after surgery	Surgery and chemotherapy	Gupta et al	2015
25	43	Epididymal mass	No reported follow up	Surgery	Pindoria et al	2016

Disclosure

The authors have stated that they have no conflicts of interest.

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Epididymal Adenocarcinoma Case Report

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Androgen Receptor Pathway-Independent Prostate Cancer Is Sustained through FGF Signaling

Highlights

- The frequency of double-negative (AR-null; NE-null) prostate cancer is increasing
- FGF and MAPK pathways are active in AR-null prostate cancer
- Autocrine and paracrine FGF pathway activation can bypass AR dependence
- Targeting the FGF and MAPK pathways can repress AR-null prostate cancer

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In Brief

Bluemn et al. show that androgen receptor (AR) inhibition results in a phenotypic shift in castration-resistant prostate cancer, leading to tumors that are AR-null but not neuroendocrine (NE). Models for AR-null, non-NE tumors show elevated FGF and MAPK activity and are sensitive to blockade of these pathways.



Androgen Receptor Pathway-Independent Prostate Cancer Is Sustained through FGF Signaling

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<http://dx.doi.org/10.1016/j.ccell.2017.09.003>

SUMMARY

Androgen receptor (AR) signaling is a distinctive feature of prostate carcinoma (PC) and represents the major therapeutic target for treating metastatic prostate cancer (mPC). Though highly effective, AR antagonism can produce tumors that bypass a functional requirement for AR, often through neuroendocrine (NE) transdifferentiation. Through the molecular assessment of mPCs over two decades, we find a phenotypic shift has occurred in mPC with the emergence of an AR-null NE-null phenotype. These “double-negative” PCs are notable for elevated FGF and MAPK pathway activity, which can bypass AR dependence. Pharmacological inhibitors of MAPK or FGFR repressed the growth of double-negative PCs *in vitro* and *in vivo*. Our results indicate that FGF/MAPK blockade may be particularly efficacious against mPCs with an AR-null phenotype.

INTRODUCTION

Androgen deprivation therapy (ADT), achieved through surgical or pharmacological approaches, exploits the exquisite dependence of prostate carcinoma (PC) on androgen receptor (AR) signaling. Although initially highly effective as a treatment for metastatic PC, ADT is characterized by the predictable emergence of resistance, a disease state termed castration-resistant prostate cancer (CRPC). An important feature of CRPC is the reactivation of AR signaling, an event reflected by progressive rises in serum prostate-specific antigen (PSA), a gene product tran-

scriptionally regulated by the AR. A substantial body of evidence has documented that essentially the entire AR cistrome is re-expressed in most CRPCs, and several mechanisms capable of maintaining AR activity have been established (Carver et al., 2011; Montgomery et al., 2008; Nelson et al., 2002; Taylor et al., 2010).

The continued importance of AR signaling in most advanced PCs has prompted the development of therapeutics directed toward further suppressing AR ligands or the AR itself. Several drugs, including improved AR antagonists and inhibitors of androgen synthesis, extend survival (de Bono et al., 2011; Scher

Significance

Targeting AR signaling in metastatic PC commonly produces robust clinical responses. However, disease progression is nearly universal. Potent AR antagonists appear to be shifting the phenotypes of resistant PCs to tumors that are devoid of AR activity, but the drivers of these resistant carcinomas are not known. Here we report that autocrine and paracrine FGF signaling is capable of bypassing a requirement for AR, and find that FGF and MAPK pathways are active in metastatic AR-null PCs. Suppressing FGF and MAPK inhibits the growth of AR-null PC indicating that targeting the FGF axis may represent a therapeutic approach for those cancers resistant to AR-directed therapies and may circumvent treatment resistance if combined with initial AR pathway blockade.

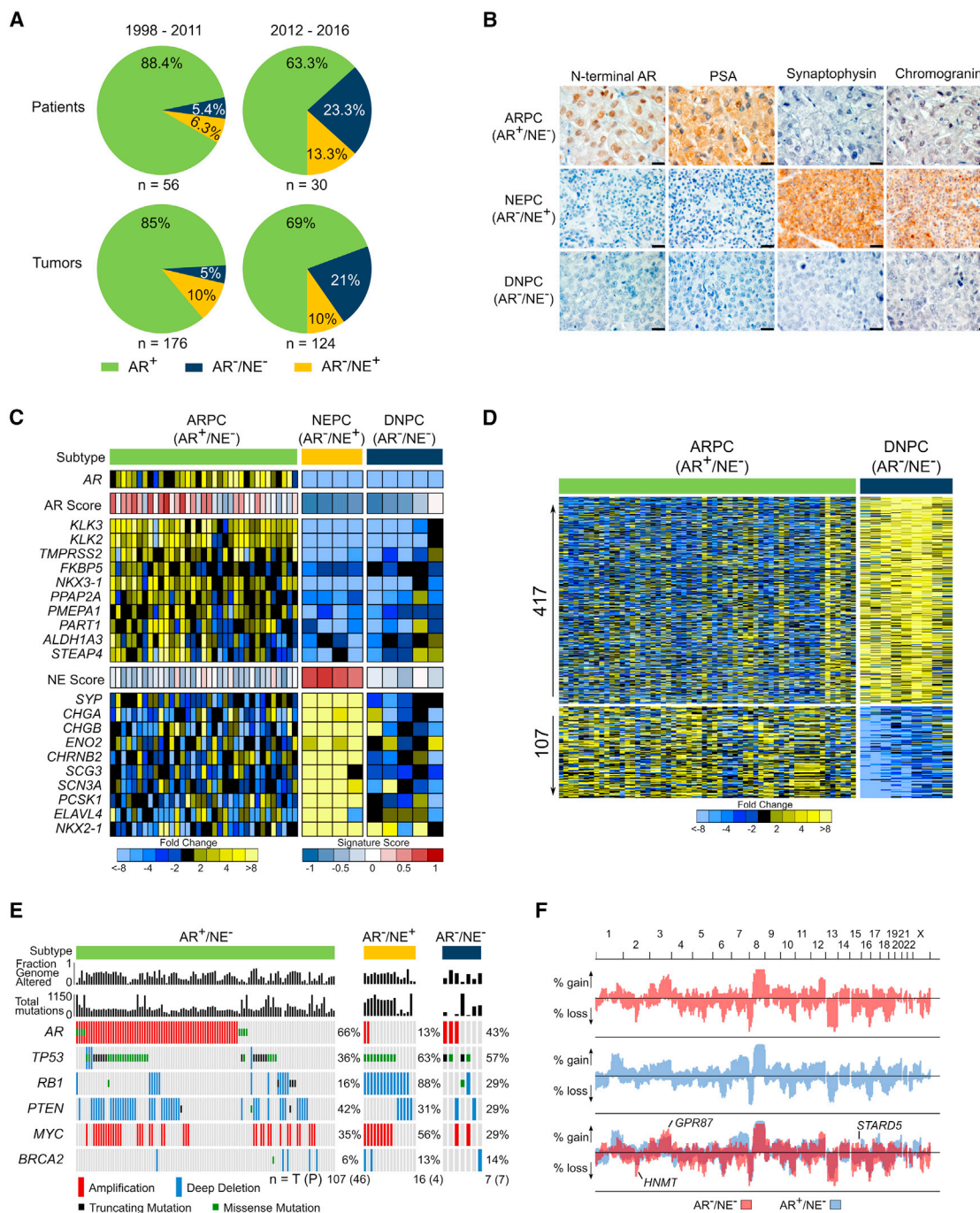


Figure 1. Molecular Features of AR-Null Neuroendocrine-Null Prostate Cancer

(A) The frequency of AR-active prostate cancers (ARPC), neuroendocrine prostate cancers (NEPC), and double-negative AR-null/neuroendocrine-null prostate cancers (DNPC) in men with metastatic CRPC evaluated in consecutive tissue acquisition necropsies from 1998 to 2016. Numbers of tumors and patients in each cohort is shown.

(B) Representative immunohistochemical stains for AR, PSA, synaptophysin and chromogranin used to classify metastases as ARPC, NEPC, or DNPC. Scale bars, 20 μ m.

(C) RNA sequencing-based measurements of transcripts comprising AR-regulated genes and neuroendocrine phenotype-associated genes in metastatic tumors from men with CRPC. Signature scores are shown above each gene set. Expression profile of one representative tumor per patient is shown, (AR⁺/NE⁻, n = 35; AR⁻/NE⁺, n = 4; AR⁻/NE⁻, n = 5.)

(D) Differentially expressed genes in ARPC compared with DNPC (5-fold difference; q value < 0.0001). Transcript abundance was determined by RNA sequencing and analyzed for differential expression using the Bioconductor edgeR software (ARPC, n = 58 tumors from 35 men; DNPC, n = 9 tumors from 5 men).

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et al., 2012), although to date complete remissions have been rare. While the intensive effort focused on completely repressing AR activity may completely eradicate a subset of PCs, this selective pressure has the potential to generate PCs reliant on survival mechanisms distinct from those regulated by AR or that substitute for vital AR functions.

Assessments of metastatic CRPCs have determined that patients may harbor tumor deposits that do not express AR following conventional ADT (Roudier et al., 2003; Shah et al., 2004). While a subset of AR-null tumors express markers of neuroendocrine (NE) differentiation, these neuroendocrine prostate cancers (NEPC) exist within a more complex spectrum of phenotypes ranging from anaplastic carcinomas, mixed prostatic adenocarcinomas with NE features, to pure small-cell carcinomas (Aparicio et al., 2011; Beltran et al., 2011; Tzelepi et al., 2012). Importantly, there are metastatic CRPCs that do not express the AR or markers of NE differentiation (Roudier et al., 2003; Wang and Epstein, 2008). Although conclusive data are lacking, evidence suggests that the widespread application of more effective AR pathway antagonists such as enzalutamide (ENZ) and abiraterone (ABI) is shifting the pattern of metastasis in patients with CRPC accompanied by alterations in their molecular landscapes (Beltran et al., 2014; Doctor et al., 2014). Anticipating that effective AR repression will more routinely result in CRPCs devoid of AR signaling, we sought to identify molecular pathways operating in CRPC that function to promote survival and growth in the absence of AR activity. The emergent signaling programs that confer resistance to AR-directed therapeutics may represent treatment targets for men with progressive CRPC.

RESULTS

Emergence of an AR-Null and Neuroendocrine-Null Prostate Cancer Phenotype in Patients Following AR-Directed Therapy

To evaluate the shifting phenotypic and molecular landscapes of metastatic CRPC (mCRPC), we characterized metastatic tumors acquired from a long-standing tissue acquisition necropsy program spanning two decades. We classified tumors from 84 consecutive patients as androgen receptor pathway active prostate cancer (ARPC) if they expressed AR and the AR-regulated gene PSA, or NEPC if they expressed the NE gene synaptophysin (SYP). In a small minority of patients both ARPC and NEPC tumors were evident. In the era prior to the approval of the AR pathway antagonists ENZ and ABI (1997–2011), most CRPCs were ARPCs (85%) with rare NEPCs (10%) and rarer AR[−]/NE[−] tumors (5%), hereafter classified as “double-negative” PCs (DNPC) (Figures 1A and 1B). In the contemporary era (2012–2016), we observed a shift in tumor phenotypes with a higher representation of DNPCs (Figure 1A). Gene expression programs

of the tumors classified by immunohistochemistry (IHC) supported these distinct subtypes using 10-gene signatures that were concordant with previously published gene sets indicative of NE and AR pathway activity (Figures 1C, S1A, and S1B) (Beltran et al., 2016; Hieronymus et al., 2006).

While molecular characteristics of CRPCs with active AR and NE programs are well described, those of DNPC are not established. We used RNA sequencing (RNA-seq) to quantitate gene expression differences between DNPCs and ARPCs and identified 417 and 107 mRNAs with substantially increased or decreased levels, respectively (5-fold; $q < 0.0001$) (Figure 1D). In comparison with NEPC, 162 and 594 genes were significantly increased or decreased, respectively in DNPCs (5-fold; $q < 0.0001$) (Figure S1C). Gene set enrichment analysis (GSEA) identified numerous biological processes that differed between ARPC and DNPC, which complicated efforts to identify a predominant driver event or signaling pathway (Figure S1D). To prioritize efforts defining causal mechanisms underlying DNPC, we evaluated tumors for genomic alterations and partitioned mCRPCs that we previously characterized for genome-wide copy-number and mutation status (Kumar et al., 2016) into categories of ARPC, NEPC, and DNPC based on their expression profiles (Figures 1E, 1F, S1E, and S1F). Common aberrations in CRPCs such as *TP53* mutation and *PTEN* loss did not differ significantly across groups with the exception of AR amplification, which was more frequent in ARPC (66%) compared with NEPC (13%) ($p = 5.6 \times 10^{-5}$) and *RB1* loss, a hallmark of NEPC, which differed between NEPC (88%) and ARPC (16%) ($p = 2.4 \times 10^{-8}$) (Figure 1E). Several genomic regions differed in copy number between ARPC and DNPC, but no genes in these regions varied in expression by more than 2-fold (Figure 1F). With the caveat of limited tumor numbers, these data indicate that recurrent genomic aberrations do not underlie the marked phenotypic differences between ARPC and DNPC.

AR Ablation Results in CRPC without Neuroendocrine Differentiation

To provide insights into causal mechanisms capable of promoting survival in an AR-null state, we developed a model system that recapitulated the transition from a tumor initially dependent on AR activity to one capable of AR-independent growth. We began with the LNCaP cell line, a widely studied androgen-sensitive *in vitro* model of PC. LNCaP derivatives capable of proliferating in the absence of AR ligands typically continue to exhibit AR signaling (Sobel and Sadar, 2005). Furthermore, targeting the AR in these cells with antibodies, ribozymes, or RNAi induces apoptosis or growth arrest, indicating that the AR maintains vital functions (Cheng et al., 2006; Zegar-Moro et al., 2002). To initiate the present studies, we used a LNCaP line stably transduced with a tetracycline (TET)-inducible anti-AR short hairpin RNA (shRNA) (Cheng et al., 2006), designated as LNCaP^{shAR}.

(E) The frequency of recurrent genomic aberrations in the CRPC subtypes of AR⁺/NE[−] (ARPC), AR[−]/NE⁺ (NEPC), and AR[−]/NE[−] (DNPC) determined by aCGH and exome sequencing. Status of individual tumors and percentage altered in each group is shown, with numbers of patients (P) and tumors (T) below the plot.

(F) Frequency of copy-number alterations (CNAs) determined by genome-wide array CGH. Copy-number gains and losses in ARPC (blue), DNPC (red), and shared (purple). Three genes (*HNMT*, *GPR87*, and *STARD5*) were significant by two-tailed Fisher's exact tests comparing the proportion of high copy gains or homozygous losses between the groups ($p < 0.05$) and also exhibited concordant differential mRNA expression by two-sample t test ($p < 0.05$). (DNPC, $n = 8$ tumors from 8 individuals; ARPC, $n = 118$ tumors from 52 individuals).

See also Figure S1.

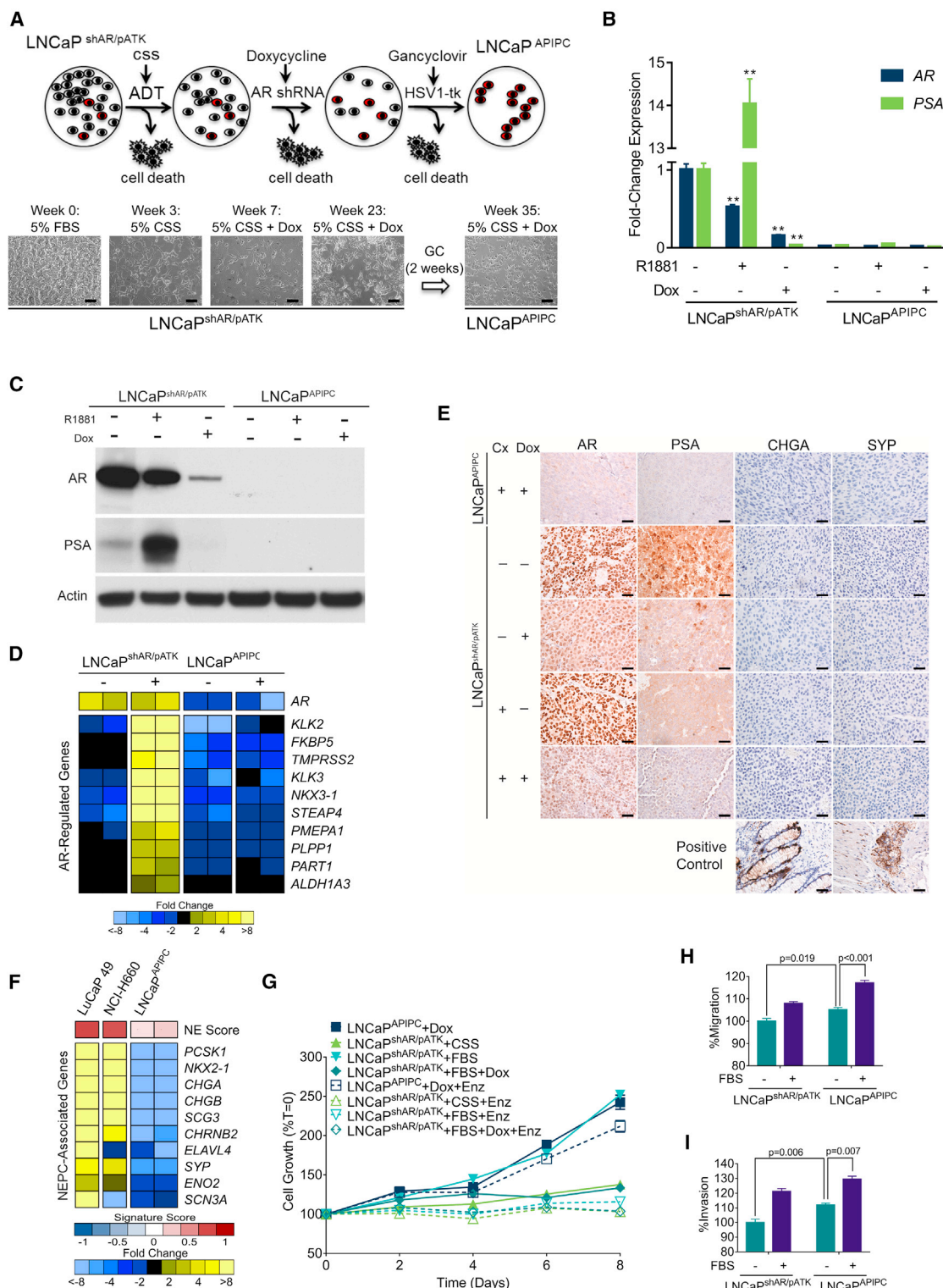


Figure 2. Characterization of a Model of AR Program-Independent Prostate Cancer

(A) LNCaP cells with a doxycycline (Dox)-inducible shRNA targeting the AR (shAR) and an androgen-driven thymidine kinase gene (pATK) were starved of androgens (ADT) and treated with Dox to induce the AR-directed shRNA, then treated with gancyclovir to eliminate cells with AR-driven thymidine kinase expression. Scale bars, 10 μ m.

(B) qRT-PCR analysis of AR and PSA expression in LNCaP^{shAR/pATK} and LNCaP^{AIPIC} with 1 nM R1881 or 1 μ g/mL Dox treatment. Significance was determined by Student's t test and data are presented as mean $\pm$ SEM ($n = 4$ replicates per data point); ** $p < 0.01$.

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Repressing AR in the setting of castration-resistant LNCaP^{shAR} growth results in tumor regression, but recurrent LNCaP^{shAR} tumors re-express AR, due to the selective loss or silencing of the AR-directed shRNA (Snoek et al., 2009). To enforce AR ablation, we introduced an androgen response element (ARE)-driven thymidine kinase suicide gene designated pATK. In the resulting LNCaP^{shAR/pATK} line, thymidine kinase is expressed in the setting of an active AR and induces cell death when treated with ganciclovir (Figures 2A and S2A–S2C).

We subjected LNCaP^{shAR/pATK} cells to increasingly severe AR pathway suppression (Figure 2A). After 2 weeks of androgen deprivation (ADT), medium was supplemented with 1 μ g/mL doxycycline (Dox) to induce the anti-AR shRNA, which produced >99% cell death. After 5 months, a residual population of viable cells remained. This colony was treated with a 2-week course of ganciclovir to eliminate cells expressing functional AR. Surviving cells were designated LNCaP-AR Program-Independent Prostate Cancer (LNCaP^{APIPC}). AR and PSA were nearly undetectable in LNCaP^{APIPC}; AR expression was 45-fold lower and PSA expression was 30-fold lower than LNCaP^{shAR/pATK} (Figures 2B and 2C). Transcripts comprising an AR activity signature were all substantially decreased in LNCaP^{APIPC} cells and showed no induction with androgen treatment (Figure 2D). We confirmed the absence of AR and PSA protein expression in LNCaP^{APIPC} grown *in vivo* as subcutaneous xenografts (Figure 2E).

Previous studies demonstrated that LNCaP cells grown in androgen-depleted medium or with AR antagonists display a transdifferentiated phenotype resembling NEPC (Mu et al., 2017; Zhang et al., 2003). NEPC is characterized by loss of AR expression and AR activity and increased expression of CHGA and SYP, and cells often exhibit small-cell morphology (Beltran et al., 2011). NE-associated genes were not upregulated in LNCaP^{APIPC} cells grown with or without androgen supplementation (Figure 2F). Furthermore, LNCaP^{shAR/pATK} and LNCaP^{APIPC} grown as murine xenografts do not express CHGA or SYP protein (Figure 2E).

To further evaluate the characteristics of LNCaP^{APIPC} cells, we determined the effects of AR pathway-targeted therapies. In contrast to parental LNCaP^{shAR/pATK}, LNCaP^{APIPC} grow robustly without androgen (Figure 2G). Furthermore, treatment of LNCaP^{shAR/pATK} with ENZ completely inhibited growth, while LNCaP^{APIPC} was highly resistant to ENZ treatment (Figure 2G). PC cells with low AR transcriptional activity that accompanies advanced Gleason grade exhibit invasive and metastatic phenotypes (Aihara et al., 1994; Erbersdobler et al., 2009). LNCaP^{APIPC}

cells displayed a slight but consistent increase in baseline migration (5%, $p = 0.019$) and invasion (12%, $p = 0.006$) when compared with LNCaP^{shAR/pATK}, and also responded to a transwell serum gradient with a higher number of migratory and invasive cells (Figures 2H and 2I).

FGFR and MAPK Signaling Pathways Are Activated in Androgen Receptor Pathway-Independent Prostate Cancer

The growth of LNCaP^{APIPC} cells in the absence of AR expression indicated that alternative survival pathways supplanted AR requirements and we next sought to identify them. We used RNA-seq to profile the gene expression program in LNCaP^{APIPC} and identified 548 differentially expressed transcripts relative to AR-intact LNCaP^{shAR/pATK} cells (≥ 10 -fold; $q < 0.001$) (Figure 3A). LNCaP^{APIPC} gained expression of basal cell genes such as *TP63* and *TRIM29*, and retained expression genes expressed in luminal cells such as *KRT8*, *KRT18*, and *HPN* (Figure 3B). We used array CGH to identify copy-number aberrations harboring genes that could bypass a requirement for AR signaling. Overall, the genomes of LNCaP^{APIPC} and parental LNCaP^{shAR/pATK} were nearly identical, with only seven regions differing in copy number between the two lines. Two genes, *MAT2B* and *KIAA1328*, exhibited concordant changes in copy number and expression, but transcript levels did not differ between ARPCs and DNPCs. Though located in the region of chromosome-3 copy gain, *WNT7A* transcripts were not measureable in LNCaP^{APIPC} cells (Figures S3A–S3C). Collectively, the few genomic aberrations identified do not explain the marked alterations in gene expression between LNCaP^{APIPC} and parental LNCaP^{shAR/pATK} cells.

To confirm lineage relationships, we compared the expression profiles of 15 PC cell lines with LNCaP^{APIPC} using unsupervised hierarchical clustering. LNCaP^{APIPC} grouped with other LNCaP derivatives, indicating that LNCaP^{APIPC} retains LNCaP characteristics even while lacking AR-regulated gene expression (Figure 3C). Notably, the removal of Dox from the culture medium of LNCaP^{APIPC} cells did not result in AR re-expression or a reversal of gene expression changes (Figure S4A). We also found no evidence of upregulation of the glucocorticoid receptor (*GR/NR3C1*), a nuclear hormone receptor previously shown to bypass AR requirements (Arora et al., 2013) (Figure 3D).

Phosphatidylinositol 3-kinase (PI3K)/AKT signaling can influence the progression of CRPC and effectively compensate for reduced AR activity in PC models via reciprocal feedback

(C) AR and PSA immunoblots of cell lysates from LNCaP^{shAR/pATK} and LNCaP^{APIPC} cultured in androgen-depleted conditions and treated with or without R1881 and with or without Dox.

(D) Quantitation of AR-regulated transcripts following treatment with the synthetic androgen R1881 (+) in parental LNCaP^{shAR/pATK} and LNCaP^{APIPC} cells. Measurements were made by RNA sequencing ($n = 2$ biological replicates per group).

(E) Immunohistochemical analysis of AR, PSA, CHGA, and SYP in parental LNCaP^{shAR/pATK} and LNCaP^{APIPC} xenografts. Cx, castration; Dox, doxycycline. Scale bars, 10 μ m.

(F) Expression of neuroendocrine-associated transcripts in the NEPC LuCaP49 PDX model, NEPC NCI-H660 cell line, and LNCaP^{APIPC} cells. Measurements were made by RNA sequencing (RNA-seq) ($n = 2$ biological replicates of LNCaP^{APIPC} cells, 1 each of LuCaP49 and NCI-H660).

(G) LNCaP^{APIPC} grown in androgen- and AR-depleted conditions were treated with vehicle (DMSO) or 5 μ M enzalutamide (ENZ). Growth was compared with parental LNCaP^{shAR/pATK} cells in charcoal stripped serum (CSS), fetal bovine serum (FBS), or FBS + 1 μ g/mL Dox $\pm$ ENZ. Solid lines, with DMSO vehicle; dotted lines, with ENZ. All values are normalized to day 0. Data are presented as mean $\pm$ SEM ($n = 5$ per data point).

(H and I) Transwell migration (H) and invasion assays (I) of LNCaP^{shAR/pATK} and LNCaP^{APIPC} at baseline (no FBS gradient) and in response to a serum (FBS) gradient. Significance was determined using Student's *t* test and data are presented as mean $\pm$ SEM ($n = 4$).

See also Figure S2.

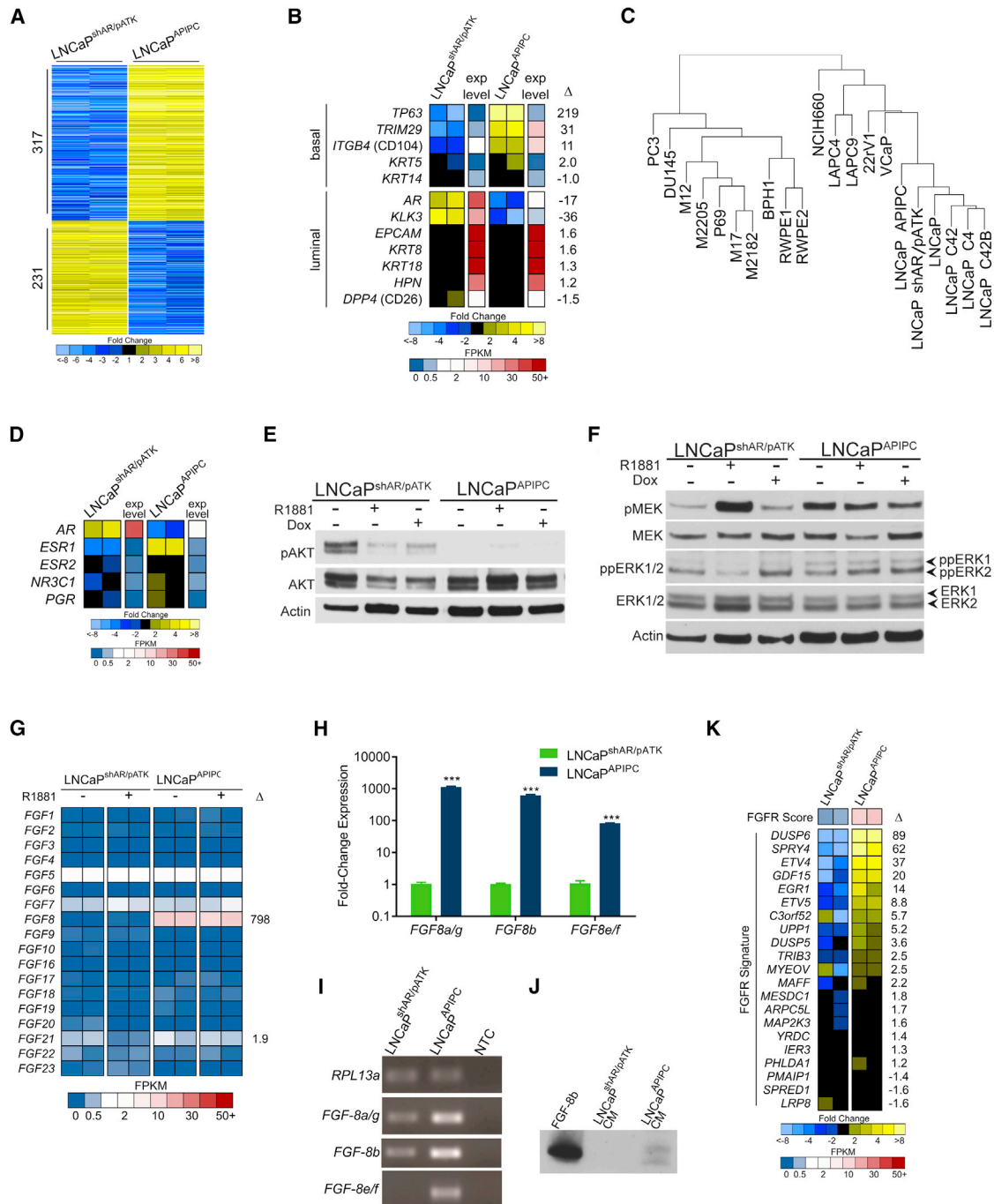


Figure 3. Assessments of AKT, MAPK, and FGF Pathway Activity in the LNCaP^{APIPC} Model of DNPC

(A) Genome-wide assessment of transcripts differentially expressed between LNCaP^{shAR/pATK} and LNCaP^{APIPC} cells as measured by RNA-seq. Shown are 548 genes with q values of <0.001 and fold changes of ≥ 10 (n = 2 biological replicates per group).

(B) Measurements of luminal and basal cell gene expression in LNCaP^{APIPC} cells. Relative ratios of RNA-seq transcript abundances are shown, along with mean FPKM (fragments per kilobase of transcript per million mapped reads) values (n = 2 biological replicates per group).

(C) Unsupervised cluster analysis of gene expression profiles across prostate cancer cell lines associates LNCaP^{APIPC} cells with LNCaP cells and sublines. One replicate of each cell line used to cluster RNA-seq profiles of the top 1,000 most variable genes.

(D) Expression of nuclear hormone receptors determined by RNA-seq of LNCaP^{shAR/pATK} and LNCaP^{APIPC} cells. Relative ratios of RNA-seq transcript abundances are shown, along with mean FPKM values. Two independent biological replicates were sequenced.

(E) PI3K pathway signaling was assessed by probing LNCaP^{APIPC} and LNCaP^{shAR/pATK} cell lysates with antibodies to AKT and phosphorylated AKT.

(F) MAPK pathway signaling was assessed by probing LNCaP^{APIPC} and LNCaP^{shAR/pATK} cell lysates with antibodies to MEK, phosphorylated MEK, ERK1/2, and dually phosphorylated ERK1/2.

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activation (Carver et al., 2011; Mulholland et al., 2011). Therefore, we hypothesized that PI3K pathway upregulation was supporting LNCaP^{APIPC} growth. Consistent with previous studies, pAKT levels increased in AR-intact LNCaP^{shAR/pATK} cells grown in androgen-depleted medium (Figure 3E). Surprisingly, pAKT was nearly undetectable in LNCaP^{APIPC}, suggesting that PI3K activity is not acting as a survival/growth pathway in these AR-null cells.

Increased mitogen-activated protein kinase (MAPK) signaling is also postulated to support CRPC proliferation (Aytes et al., 2013; Mulholland et al., 2012; Ueda et al., 2002). MAPK signal transduction is activated through a variety of stimuli, and is closely associated with receptor tyrosine kinase (RTK) activity. Phosphorylated MEK and dually phosphorylated ERK1/2 (ppERK1/2) were elevated in LNCaP^{APIPC} compared with LNCaP^{shAR/pATK} (Figure 3F). These data suggested that increased MAPK signaling may be sustaining AR-independent growth in LNCaP^{APIPC}. We evaluated RAS and RAF for alterations that could account for MAPK activation but found no evidence of altered expression or functional mutations (Figures S4B and S4C).

We next evaluated the LNCaP^{APIPC} transcriptome for mechanisms plausibly contributing to MAPK activity and found that *fibroblast growth factor 8* (FGF8) expression was substantially upregulated relative to AR-active LNCaP^{shAR/pATK} (>100-fold, $q < 0.001$) (Figure 3G). FGF8 is transcribed as eight distinct isoforms (FGF8a–h), and of these FGF8b has the most oncogenic effects (MacArthur et al., 1995). LNCaP^{APIPC} expressed all active FGF8 isoforms at substantially higher levels than LNCaP^{shAR/pATK} (FGF8a/g = 1,100-fold, $p < 0.001$; FGF8b = 600-fold, $p < 0.001$) (Figures 3H and 3I). FGF8 protein was detected in serum-free conditioned medium from LNCaP^{APIPC} but not from LNCaP^{shAR/pATK}, concordant with transcript expression results (Figures 3J and S4D).

To further assess FGF pathway activity, we measured a panel of transcripts shown to reflect the dynamic output of FGF receptor (FGFR) signaling (Delpuech et al., 2016). Several transcripts comprising this FGFR signature were increased more than 10-fold in LNCaP^{APIPC} cells including *DUSP6*, *ETV4*, and *EGR1*, and LNCaP^{APIPC} cells showed significant FGFR and MAPK pathway enrichment scores (Figure 3K). FGF pathway activation has been shown to occur in rare instances by FGFR genomic rearrangements in mCRPC (Wu et al., 2013), but we found no evidence of mutation, copy-number gain, or gene rearrangements involving FGF8 or FGFRs in LNCaP^{APIPC} (Figures S3B and S3C). Collectively, these data supported the hypothesis that an autocrine FGF signaling program is activated in LNCaP^{APIPC} in the absence of AR to maintain cell survival and growth via MAPK.

FGFR and MAPK Signaling Are Active in DNPC and Are Inversely Associated with AR Activity

We next sought to further evaluate FGF and MAPK signaling in DNPCs and confirm LNCaP^{APIPC} as a relevant model for this CRPC subtype. We determined that an LNCaP^{APIPC} gene signature is significantly enriched in DNPC metastases (false discovery rate [FDR] < 0.001) (Figure 4A), as are gene sets reflecting the activity of FGF signaling, MAPK activity, MEK/ERK, and EMT (Figure 4B). No single FGF ligand or receptor was universally increased across all DNPCs: individual tumors expressed high FGF1, FGF8, or FGF9, and different FGFRs. Each of these secreted FGF ligands has been shown to activate multiple FGFRs consistent with the finding that DNPCs exhibited consistently high MEK/ERK and FGF activity scores (Figures 4C and 4D). A small subset of ARPCs also expressed high MEK/ERK and FGFR pathway activity, and these tumors generally also had lower AR activity (Figure 4C). Across the full spectrum of CRPC metastases, AR activity was inversely associated with FGF8/9 expression, and FGFR activity (e.g., $r = -0.48$, $p < 0.001$ for FGF8) (Figure 4E). AR and FGF8/9 expression were inversely associated ($r = -0.13$) in an independent dataset of 150 metastatic CRPC tumors from the SU2C/PCF dataset (data not shown) (Robinson et al., 2015). Collectively, these results couple elevated FGF and MAPK signaling with a CRPC tumor phenotype, DNPC, which lacks AR activity and supports LNCaP^{APIPC} as a model that represents these attributes of DNPC.

To address the challenge of deriving a generalized understanding of DNPC from a single model, we sought to develop additional systems with which to evaluate drivers of DNPC and identify effective therapeutics. As with LNCaP^{APIPC}, our objective was to begin with an AR-positive PC and then repress AR activity. We were unable to successfully eliminate AR in the commonly used VCaP or 22Rv1 PC lines by shRNA or CRISPR-based approaches (data not shown). However, using the PacMet-UT1 PC line that expresses a functional AR (Troyer et al., 2008), albeit with attenuated activity, we were able to excise AR using CRISPR/Cas9 editing and generate multiple PacMet AR-null sublines (Figures 5A and 5B). AR loss was associated with 10-fold upregulation of FGF9 and enhancement of FGF and MAPK activity (Figures 5C and 5D). Notably, repressing AR activity in PacMet-UT1 cells did not result in an NEPC phenotype, and the expression of SOX2, a reprogramming factor associated with transdifferentiation to NEPC, was decreased (Figure 5C) (Mu et al., 2017).

We were also successful in generating a patient-derived xenograft (PDX) model of DNPC, designated LuCaP173.2, initiated from a tumor acquired from a rapid autopsy procedure. Metastatic tumors from this individual had phenotypic variability,

(G) Levels of transcripts encoding FGFs were assessed in LNCaP^{shAR/pATK} and LNCaP^{APIPC} cells by RNA-seq with or without R1881 androgen treatment. Two replicates of each line and treatment were measured, and fold difference between LNCaP^{shAR/pATK} and LNCaP^{APIPC} cells is shown for FGF8 and FGF21.

(H) Transcript levels of FGF8 mRNAs were measured by qRT-PCR in LNCaP^{shAR/pATK} and LNCaP^{APIPC}. Significance was determined by Student's t test and data are presented as mean $\pm$ SEM ($n = 3$ replicates per data point). *** $p < 0.00001$.

(I) qRT-PCR reaction products, visualized by agarose gel electrophoresis, confirms single-band amplification by each isoform-specific primer pair.

(J) Assessment of FGF8b protein in conditioned medium (CM) from LNCaP^{shAR/pATK} and LNCaP^{APIPC} by immunoblotting with an FGF8b-specific antibody.

(K) Expression of genes associated with FGFR pathway activity measured by RNA-seq of LNCaP^{shAR/pATK} and LNCaP^{APIPC} cells. Two independent biological replicates were sequenced.

See also Figures S3 and S4.

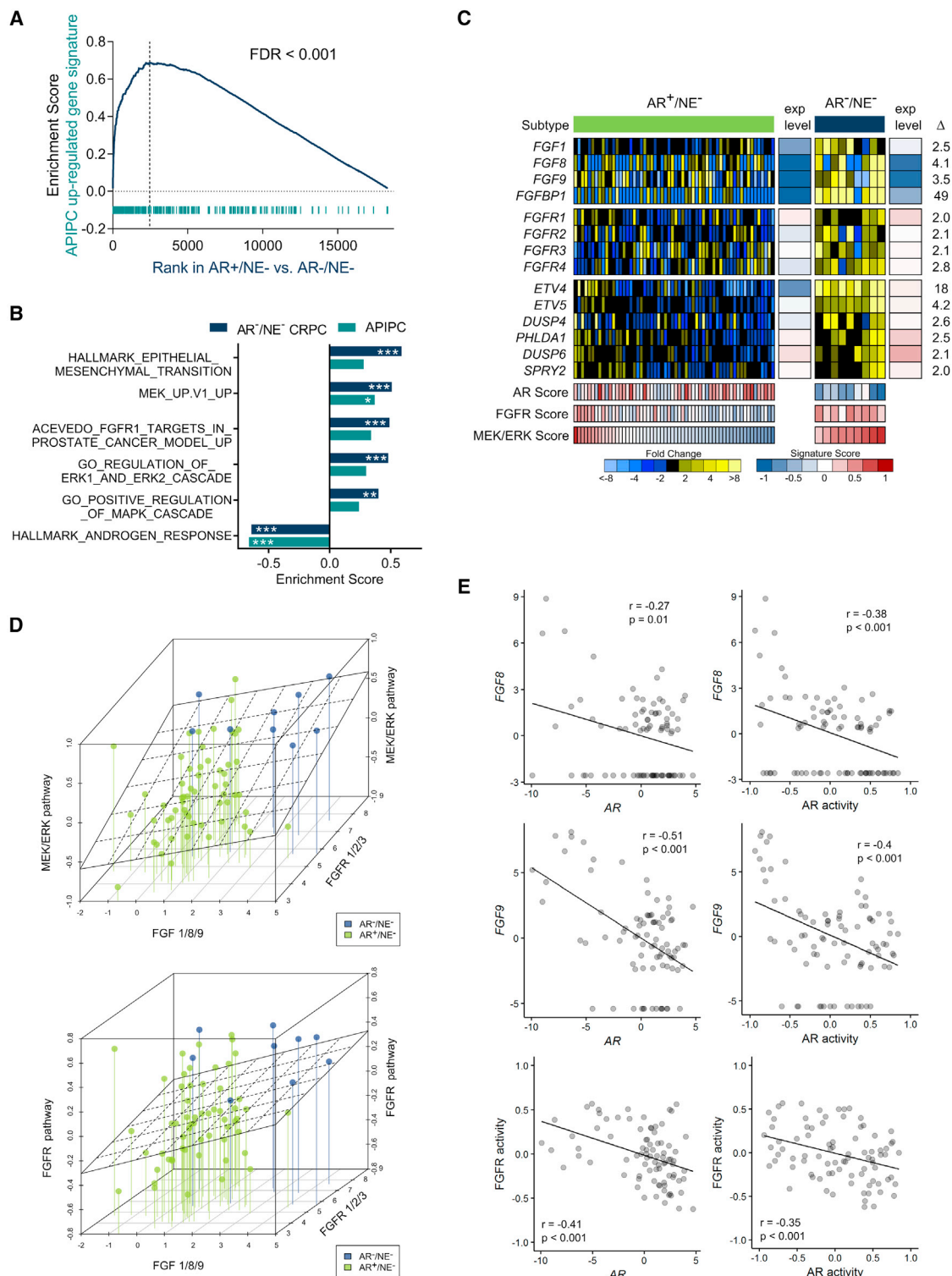


Figure 4. Assessments of FGF and MAPK Activity in Metastatic CRPC

(A) Analyses of transcripts differentially expressed between LNCaP^{shAR/pATK} and LNCaP^{APIPC} in DNPC and ARPC metastases (FDR < 0.001, pre-ranked GSEA). (B) GSEA demonstrates significant positive associations with FGF, MAPK, MEK/ERK, and EMT pathways and negative enrichment for AR response in DNPC metastases (***FDR < 0.0005, **FDR < 0.005, *FDR < 0.05, pre-ranked GSEA).

(C) Expression of FGF ligands, FGF receptors, and genes comprising an MEK/ERK activity signature. Relative ratios of RNA-seq transcript abundances are shown, along with mean FPKM values and signature scores (AR+/NE-, n = 58 tumors from 35 men; AR-/NE-, n = 9 tumors from 5 men).

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with one rib metastasis expressing AR and PSA and a second rib metastasis lacking AR or PSA staining (Figure 5E). We confirmed that the LuCaP173.2 PDX lacks AR and PSA expression and does not express classic NE markers such as chromogranin or synaptophysin, thus fulfilling criteria for DNPC (Figure 5F). However, other genes associated with an NE phenotype such as *EZH2* and *MYCN* are expressed in this PDX line, suggesting a continuum of tumor differentiation (Figure S5). In accord with findings in DNPC metastases, LuCaP173.2 expresses high *FGF9* and *FGFR1* levels with low AR and NEPC program scores and a high FGFR activity score (Figure 5G).

FGF Activates MAPK Signaling and Bypasses a Requirement for Androgens and the AR in Promoting Prostate Cancer Growth

We next sought to determine whether FGF signaling is necessary and sufficient for bypassing a requirement for AR activity. We hypothesized that the substantial upregulation of FGF8 in LNCaP^{APIPC} cells comprises an autocrine loop to sustain cell survival in the absence of AR. The introduction of FGF8-specific small interfering RNAs (siRNAs) reduced LNCaP^{APIPC} growth by 80% ($p < 0.001$) (Figure 6A). In contrast, siRNA knockdown of FGF9, which is not upregulated in LNCaP^{APIPC}, had no effect. Exogenous FGF8b increased the growth of parental LNCaP^{shAR/pATK} in androgen-depleted conditions ($p < 0.001$) and the addition of concentrated LNCaP^{APIPC} conditioned medium (CM) showed a small but statistically significant increase in proliferation (11%, $p = 0.01$), whereas LNCaP^{shAR/pATK} CM had no effect (Figure 6B). The addition of exogenous FGF8b increased ERK1/2 phosphorylation in both LNCaP^{shAR/pATK} and LNCaP^{APIPC}. FGF8-induced growth in androgen-depleted conditions and ERK1/2 phosphorylation were blocked by treatment with the FGFR inhibitor PD173074 (Mohammadi et al., 1998) (Figures 6C and 6D).

To demonstrate that FGF8 was sufficient to promote the growth of cells cultured under total AR pathway suppression, we treated parental LNCaP^{shAR/pATK} grown in androgen-depleted conditions with Dox to suppress AR expression, and added FGF8b. FGF8b maintained cell proliferation during AR pathway ablation (30% increase in cell number compared with untreated LNCaP^{shAR/pATK}, $p = 0.019$), albeit at a lower rate than AR-intact LNCaP^{shAR/pATK} (75% increase in cell number compared with untreated LNCaP^{shAR/pATK}, $p = 0.018$) (Figure 6E). In a parallel experiment, LNCaP^{shAR/pATK} cells were cultured in androgen-depleted medium and AR expression was suppressed by pre-treatment with Dox for 72 hr. Addition of exogenous FGF8b rescued the growth inhibition by ADT and AR suppression (58% increase in growth compared with untreated LNCaP^{shAR/pATK}, $p = 0.003$) (Figure S6A).

The FGFR antagonist PD173074 is a nanomolar inhibitor of FGFR1 but is also a submicromolar inhibitor of vascular endothelial growth factor receptor 2/kinase domain receptor (VEGFR2/

KDR) (Mohammadi et al., 1998). To confirm that FGFR antagonism is mediating the growth repression in DNPC, we treated LNCaP^{APIPC} with a second FGFR antagonist CH-5183284, which potently and selectively inhibits FGFR1–3 (IC_{50} of 8–22 nM) without significant biological effects toward VEGFR2/KDR or other kinases (Nakanishi et al., 2014). At concentrations of 0.1–1.0 μ M, CH-5183281 substantially inhibited the viability and increased apoptosis rates in LNCaP^{APIPC} with effects far exceeding those observed in wild-type LNCaP cells (Figures 6F and 6G). CH-5183281 also reduced the viability of AR-null PacMet-UT1 cells relative to the AR-intact parental line (Figure 6H). Confirming that MAPK activity is required for FGF8-mediated castration-resistant proliferation, the MEK1/2 inhibitor U0126 blocked the growth of LNCaP^{shAR/pATK} induced by FGF8 in androgen-depleted conditions ($p < 0.001$; Figure 6I) and repressed LNCaP^{APIPC} proliferation. Co-treatment of a second androgen-sensitive PC line, 22RV1, with U0126 and FGF8 led to a 46% decrease in cell number compared with cells treated with FGF8 alone ($p < 0.001$; Figure S6B).

We next sought to determine whether suppressing FGF signaling would inhibit the growth of DNPC *in vivo*. PD173074 significantly reduced LNCaP^{APIPC} xenograft growth rates: the study was terminated at 40 days due to large tumors in the control group at which time tumor volumes were 1,147 mm³ in the vehicle and 571 mm³ in PD173074 arms ($p < 0.001$) (Figure 6J). To confirm these findings, we treated LuCaP173.2 DNPC PDX tumors with CH-5183284. At the study endpoint of 24 days, tumor volumes were 814 mm³ and 170 mm³ in the vehicle and CH-5183284 arms, respectively ($p < 0.001$) (Figure 6K). The expression of FGFR pathway genes as well as composite FGFR and MEK/ERK pathway activity were significantly reduced in LuCaP173.2 tumors resected 3 days and 24 days on CH-5183284 treatment (Figure 6L).

FGF- and MAPK-Induced ID1 Contributes to AR-Null Prostate Cancer Growth

We next evaluated LNCaP^{APIPC} for downstream mediators of FGF/MAPK signaling that could promote the dedifferentiated phenotype of DNPC and support survival in the absence of AR activity. We identified a strong candidate for this role, *inhibitor of differentiation 1 (ID1)*, which was upregulated in LNCaP^{APIPC} compared with LNCaP^{shAR/pATK} (~10-fold by RNA-seq; $q < 0.001$; 5-fold by qRT-PCR) (Figure 7A). ID1 expression is induced by exogenous FGF and bone morphogenetic protein via MAPK pathway activation (Langenfeld and Langenfeld, 2004; Passiatore et al., 2011), prevents differentiation by binding cell lineage-specific transcription factors (Perk et al., 2005), and has been associated with poorly differentiated PC (Coppe et al., 2004; Sharma et al., 2012). Notably, other ID family members were also increased in LNCaP^{APIPC} and the LuCaP173.2 DNPC PDX (Figure 7B). ID1 levels are significantly higher in DNPC

(D) Plot of CRPC metastasis triangulated by the highest transcript level of FGF1, 8, or 9 (x axis), MEK/ERK pathway activity score or FGFR pathway activity score (y axis), and highest transcript level of FGFR1, 2, or 3 (z axis). Lines anchor MEK/ERK activity to lowest level to assist in visualizing activity on the y axis. A linear regression analysis of pathway score versus ligands and receptors is plotted as a plane (AR⁺/NE⁺, $n = 58$ tumors from 35 men; AR⁺/NE⁺, $n = 9$ tumors from 5 men).

(E) Correlation of *FGF8* and *FGF9* transcript levels and FGFR pathway activity and AR activity scores assessed in 85 CRPC metastases from 50 men by RNA-seq. Pearson's correlation coefficient and p value are indicated on each plot.

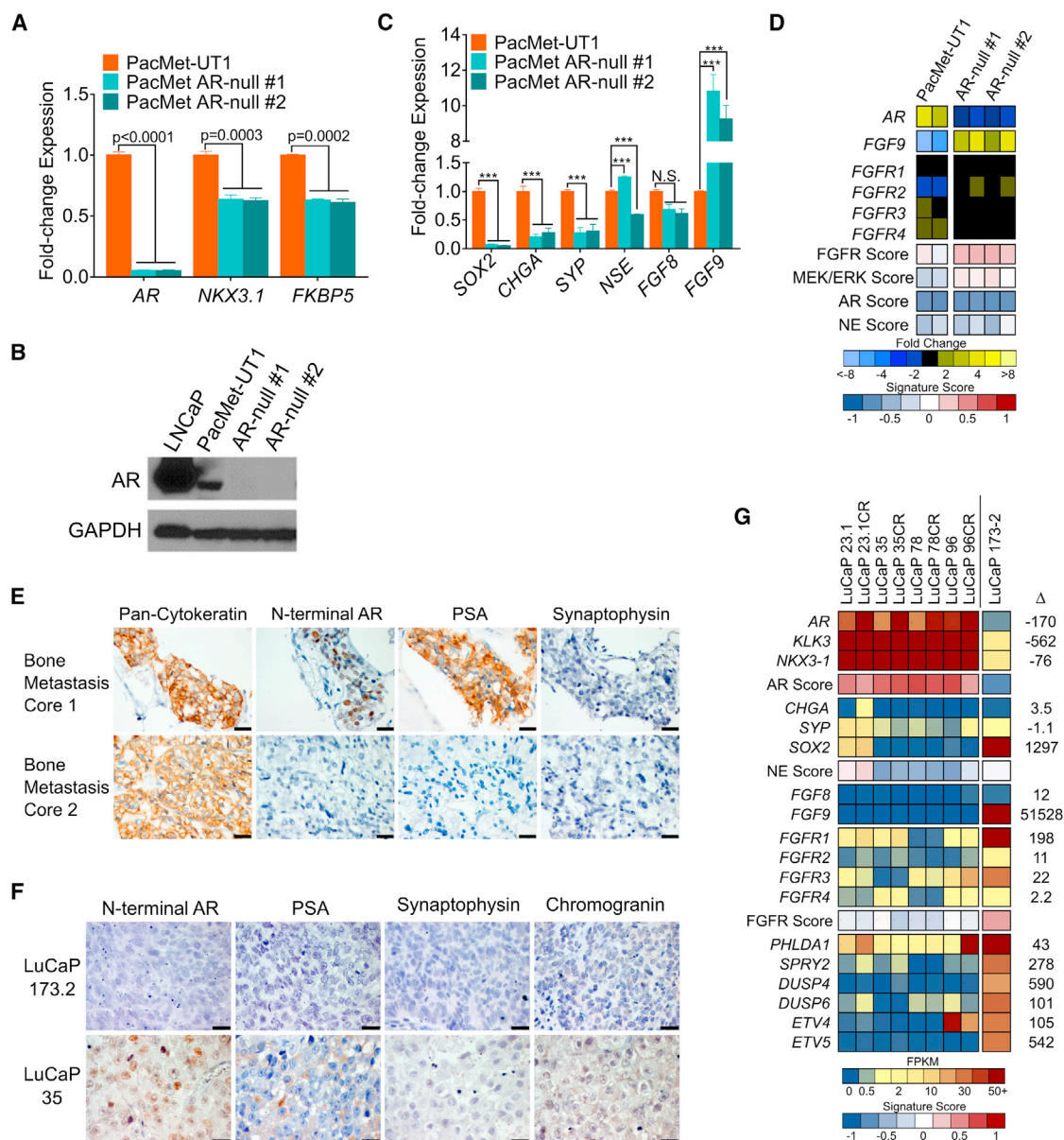


Figure 5. FGF Pathway and MAPK Activity in Cell Line and PDX Models of DNPC

(A) Quantitation of the indicated transcripts by qRT-PCR in parental PacMet-UT1 cells and two independent PacMet-UT1 clones propagated after CRISPR/Cas9-mediated AR deletion.

(B) Western immunoblot of AR protein in the indicated cell lines.

(C) Quantitation of the indicated transcripts by qRT-PCR in the indicated cell lines. *** $p < 0.0001$. N.S., not significant.

(D) Expression of genes reflecting the activity of AR, neuroendocrine (NE), FGFR, and MAPK signaling in parental PacMet-UT1 cells and AR-null sublines. Measurements were derived from RNA-seq ($n = 2$ biological replicates per group).

(E) Cytokeratin, AR, PSA, and synaptophysin IHC in two independent rib metastases obtained from a patient with mCRPC. Scale bars, 20 μ m.

(F) AR, PSA, synaptophysin, and chromogranin IHC in the LuCaP173.2 PDX model derived from rib metastasis core 2 (E) with comparisons with the AR-positive LuCaP35 PDX line. Scale bars, 20 μ m.

(G) Expression of genes comprising the AR program, neuroendocrine (NE) program, and FGFR program in AR-positive castration-sensitive and castration-resistant (CR) PDX models (LuCaP23.1, LuCaP35, LuCaP78, and LuCaP96) and the AR-null, NE-null LuCaP173.2 PDX line. Measurements were derived from RNA-seq ($n =$ one tumor from each LuCaP line.).

For (A) and (C), significance was determined by Student's t test and data are presented as mean $\pm$ SEM ($n = 3$ replicates per data point). See also Figure S5.

metastases relative to ARPCs ($p < 0.005$) (Figure 7C), and ID1 and AR expression are inversely associated in mCRPC (Pearson correlation = -0.39) (Figure 7D).

Stimulation of LNCaP^{shAR/pATK} cells with FGF8 resulted in a 4-fold ($p = 0.006$) increase in ID1 mRNA and protein (Figures 7E and 7F). MEK inhibition reduced FGF8-mediated

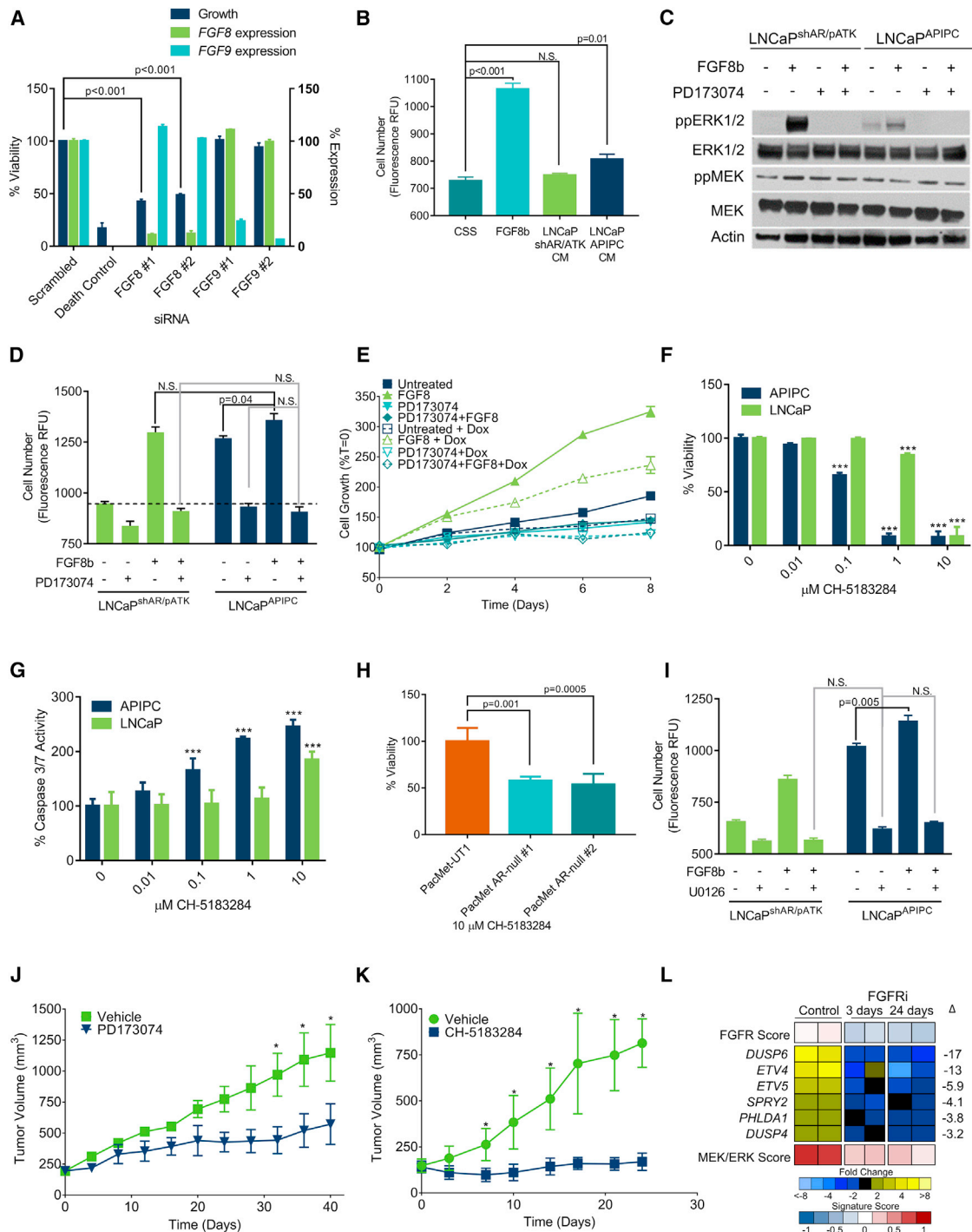


Figure 6. FGF Activates MAPK Signaling and Bypasses a Requirement for AR Activity in Promoting Prostate Cancer Growth

(A) Quantitation of cell viability and gene expression 96 hr after transfecting LNCaP^{APIPC} cells with siRNA pools specific for the indicated target. (B) LNCaP^{shAR/pATK} were cultured for 4 days in androgen-depleted medium and treated with 25 ng/mL FGF8b, CM from LNCaP^{shAR/pATK}, or LNCaP^{APIPC} cells. Cell number was determined using Cyquant. (C) LNCaP^{shAR/pATK} and LNCaP^{APIPC} were treated with 1 μ M PD173074 or vehicle and 25 ng/mL FGF8 or vehicle and cell lysates were evaluated for MAPK signaling via immunoblotting for ppERK1/2. (D) LNCaP^{shAR/pATK} and LNCaP^{APIPC} were cultured in androgen-depleted conditions and treated with $\pm$ 25 ng/mL FGF8b and $\pm$ 1 μ M PD173074. N.S., not significant. Dashed line indicates unstimulated LNCaP^{shAR/pATK} ($n = 3$ replicates per data point). (E) LNCaP^{shAR/pATK} cells were cultured in androgen-depleted medium $\pm$ 25 ng/mL FGF8, $\pm$ 1 μ M PD173074, and $\pm$ 1 μ g/mL Dox. Solid lines, no Dox; dotted lines, with Dox. Cell number was determined using Cyquant, and values were normalized to day 0. (F) LNCaP^{shAR/pATK} and LNCaP^{APIPC} were treated with 1 μ M PD173074 or vehicle and 25 ng/mL FGF8 or vehicle and cell lysates were evaluated for MAPK signaling via immunoblotting for ppERK1/2. (G) LNCaP^{shAR/pATK} and LNCaP^{APIPC} were cultured in androgen-depleted conditions and treated with $\pm$ 25 ng/mL FGF8b and $\pm$ 1 μ M PD173074. N.S., not significant. Dashed line indicates unstimulated LNCaP^{shAR/pATK} ($n = 3$ replicates per data point). (H) LNCaP^{shAR/pATK} cells were cultured in androgen-depleted medium $\pm$ 25 ng/mL FGF8, $\pm$ 1 μ M PD173074, and $\pm$ 1 μ g/mL Dox. Solid lines, no Dox; dotted lines, with Dox. Cell number was determined using Cyquant, and values were normalized to day 0. (I) LNCaP^{shAR/pATK} and LNCaP^{APIPC} were treated with 1 μ M PD173074 or vehicle and 25 ng/mL FGF8 or vehicle and cell lysates were evaluated for MAPK signaling via immunoblotting for ppERK1/2. (J) LNCaP^{shAR/pATK} and LNCaP^{APIPC} were cultured in androgen-depleted conditions and treated with $\pm$ 25 ng/mL FGF8b and $\pm$ 1 μ M PD173074. N.S., not significant. Dashed line indicates unstimulated LNCaP^{shAR/pATK} ($n = 3$ replicates per data point). (K) LNCaP^{shAR/pATK} cells were cultured in androgen-depleted medium $\pm$ 25 ng/mL FGF8, $\pm$ 1 μ M PD173074, and $\pm$ 1 μ g/mL Dox. Solid lines, no Dox; dotted lines, with Dox. Cell number was determined using Cyquant, and values were normalized to day 0. (L) LNCaP^{shAR/pATK} and LNCaP^{APIPC} were treated with 1 μ M PD173074 or vehicle and 25 ng/mL FGF8 or vehicle and cell lysates were evaluated for MAPK signaling via immunoblotting for ppERK1/2.

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ID1 induction by approximately 30% ($p = 0.005$) (Figure 7E). Although ID1 levels were already elevated, stimulation of LNCaP^{APIPC} with exogenous FGF8 resulted in a further 1.6-fold increase ($p < 0.001$), and treatment with U0126 alone decreased baseline ID1 expression by approximately 40% ($p = 0.006$) (Figure 7E). We also observed a significant upregulation of ID1 in response to FGF8 treatment in androgen-sensitive 22Rv1 cells (Figures S7A and S7B). The enhanced activity of specific RTKs is associated with ligand-independent activation of AR transcription in some models (Gregory et al., 2005; Yang et al., 2003); however, we did not observe a change in AR, PSA, or TMPRSS2 expression in response to FGF8b stimulation in androgen-depleted LNCaP^{shAR/pATK}, LNCaP^{APIPC}, or 22Rv1 cells (Figures 7G and S7C).

ID1 has been shown to influence PC differentiation and proliferation (Ling et al., 2011; Ouyang et al., 2002), and we hypothesized that ID1 could mediate a component of the growth-promoting effects of FGF/MAPK activity. In support of this hypothesis, levels of ID1–3 transcripts were diminished in the LuCaP173.2 DNPC PDX tumors treated with the FGFR inhibitor CH-5183284 (Figure 7H). ID1 knockdown did not significantly affect LNCaP^{shAR/pATK} growth compared with a scrambled control siRNA (siUNI). In contrast, two independent ID1-targeting siRNAs decreased LNCaP^{APIPC} growth by 32% ($p = 0.003$) and 43% ($p < 0.001$) (Figure 7I). When LNCaP^{shAR/pATK} were treated with FGF8, ID1 knockdown significantly attenuated FGF8-induced proliferation by ~35% ($p < 0.001$). The effect of ID1 knockdown was enhanced in LNCaP^{APIPC} with ID1 siRNAs suppressing FGF8-induced growth by 39%–50% ($p < 0.001$) (Figure 7I). These effects were replicated in 22RV1 cells grown in androgen-depleted conditions (Figure S7D).

DISCUSSION

Therapeutic approaches designed to impair AR activity remain first-line therapy for men with metastatic PC. While resistance to AR-directed therapeutics is usually accompanied by reactivation of AR signaling, newer drugs with potent AR pathway antagonism appear to be shifting the phenotypes of resistant PC to anaplastic and NE carcinomas that are devoid of AR activity (Figure 7J). The AR-null/NE-null tumors evaluated in the present study were acquired from men after initial responses to AR antagonists, indicating that these agents effectively eliminated tumor clones dependent on the AR, but failed to eradicate cell populations that no longer required AR signaling. Defining the

drivers of these resistant carcinomas is critical for the development of effective treatment strategies.

We determined that complete AR pathway independence was associated with elevated autocrine FGF signaling *in vitro* and elevated FGFR and MAPK pathway activity in mCRPC. FGF ligands and receptors have previously been shown to influence the development and progression of PC (Acevedo et al., 2007; Feng et al., 2015). Of relevance to the present study, a PDX model of PC devoid of AR signaling was shown to express high levels of FGF9, which promoted tumor growth, induced an osteoblastic tumor microenvironment, and responded to FGF-directed therapy (Li et al., 2008). MAPK signaling promotes poorly differentiated tumor growth in models of PC (Mulholland et al., 2012), and constitutive ERK1/2 activity is associated with castration resistance (Gioeli et al., 1999; Oka et al., 2005; Rodriguez-Berriguete et al., 2012). While there is evidence suggesting that MAPK can stimulate ligand-independent AR activity (Feldman and Feldman, 2001), FGF/MAPK signaling did not promote the re-expression of AR-regulated genes in our models and FGFR activity was inversely associated with the expression and activity of AR in CRPCs. At this time, the mechanism(s) influencing FGF expression in LNCaP^{APIPC} or other DNPCs is not known. As we found no recurrent genomic events involving FGFs/FGFRs, other processes capable of influencing FGF transcription, including epigenetic regulation, are likely operative. Notably, a small subset of CRPCs exhibiting FGFR/MAPK activity did not express high levels of FGF ligands, suggesting that in some circumstances paracrine FGF derived from microenvironment constituents may promote pathway activity and drive treatment resistance (Lawson et al., 2010).

While AR repression can allow for cellular reprogramming and transdifferentiation to NE carcinoma (Ku et al., 2017; Zou et al., 2017), our results indicate that the acquisition of NE characteristics may represent a continuum of differentiation from ARPC to DNPC to NEPC, although the acquisition of NE characteristics does not appear to be a certainty following AR ablation (Figure 7J). Importantly, alternative cell fates may associate with unique therapeutic vulnerabilities. Given that NE and anaplastic tumors are more common following sustained AR pathway suppression and appear to arise from adenocarcinomas *in vivo* based on shared genomic aberrations (Beltran et al., 2011, 2016), it is quite likely that the incidence of AR pathway-independent PCs will increase with the deployment of increasingly potent AR inhibition. Whether acute and more complete AR

(F and G) LNCaP and LNCaP^{APIPC} were treated with the indicated concentrations of CH-5183284, and cell viability (F) and apoptosis (G) were measured after 72 hr by ApoLive Glo ($n = 3$ replicates per data point). *** $p < 0.001$.

(H) PacMet-UT1 cells and AR-null derivatives were treated with 10 μ M CH-5183284, and cell viability was determined by CellTiter Glo after 72 hr.

(I) LNCaP^{shAR/pATK} and LNCaP^{APIPC} cultured in androgen-depleted conditions were treated with FGF8b or vehicle with or without 25 μ M U0126 or vehicle. Cell number was determined using Cyquant.

(J) LNCaP^{APIPC} cells were inoculated subcutaneously in castrate SCID mice receiving Dox-supplemented feed. When tumors reached 200 mm³ in size, treatment was initiated with the FGFR antagonist PD173074 or vehicle control. Tumor volumes were measured every 2 days ($n = 5$). * $p < 0.01$.

(K) LuCaP173.2 tumors were implanted subcutaneously in castrate SCID mice. When tumors reached 200 mm³ in size, treatment was initiated with the FGFR antagonist CH-5183284 or vehicle control. Tumor volumes were measured every 2 days ($n = 15$). * $p < 0.01$.

(L) Quantitation of FGFR and MEK/ERK pathway gene expression in LuCaP173.2 tumors treated with vehicle or CH-5183284 sampled 3 days or 24 days after the initiation of treatment. Transcripts were quantitated by RNA-seq of two independent tumors. Significance was determined by Student's *t* test and data are presented as mean $\pm$ SEM.

For (A), (B), and (D) to (I), $n = 3$ replicates per data point. See also Figure S6.

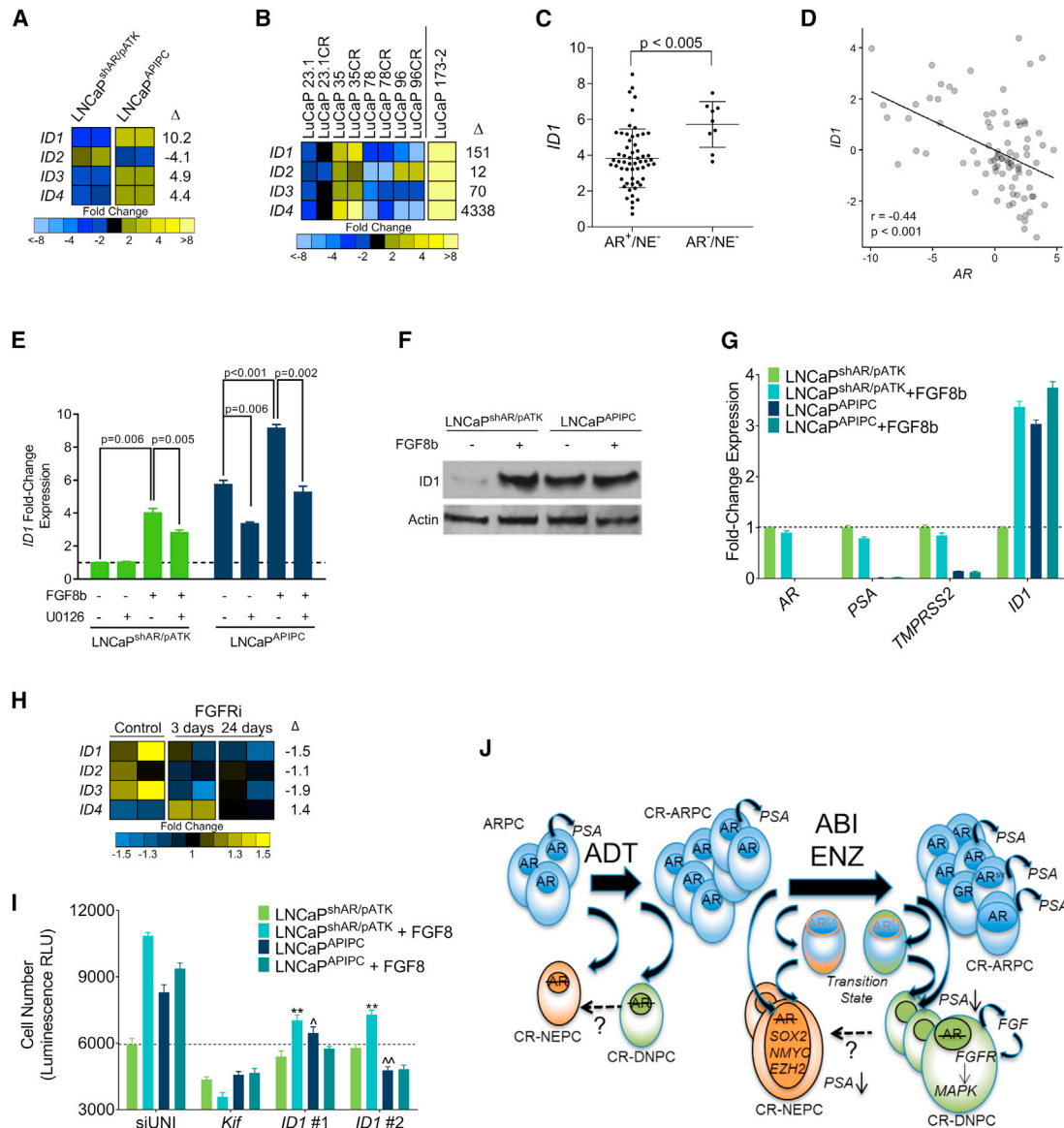


Figure 7. FGF8 Induces ID1 Expression and Castration-Resistant Growth via MAPK Pathway Activation

(A) Transcript levels of *ID1-4* in LNCaP^{shAR/pATK} and LNCaP^{APIPC} cells determined by RNA-seq in two independent cultures. Fold differences of gene expression levels between LNCaP^{shAR/pATK} and LNCaP^{APIPC} cells are shown.

(B) Expression of *ID1-4* in AR-positive castration-sensitive and castration-resistant (CR) PDX models (LuCaP23.1, LuCaP35, LuCaP78, and LuCaP96) and the AR-null, NE-null LuCaP173.2 PDX line. Measurements were derived from RNA-seq ($n =$ one tumor from each LuCaP line). Fold differences of gene expression between AR-positive and AR-negative groups are shown.

(C) Transcript levels of *ID1* in AR⁺/NE⁻ and AR⁻/NE⁻ CRPC metastases determined by RNA-seq transcript quantitation. Log² counts per million (CPM) mapped reads with mean $\pm$ SD are plotted. Groups were compared by unpaired, two-tailed t test (AR⁺/NE⁻, $n = 58$ tumors from 35 men; AR⁻/NE⁻, $n = 9$ tumors from 5 men).

(D) Association of *ID1* and *AR* transcripts in CRPC metastases. Each data point represents an individual metastasis ($n = 85$ tumors from 50 men). Transcript levels were quantitated by RNA-seq. Pearson's correlation coefficient $r = -0.39$; $p < 0.001$.

(E) *ID1* transcripts quantitated by qRT-PCR in LNCaP^{shAR/pATK} and LNCaP^{APIPC} treated with 25 ng/mL FGF8 or vehicle and the MEK inhibitor U0126 or vehicle. qRT-PCR values were normalized to RPL13a expression, and compared with unstimulated LNCaP^{shAR/pATK}.

(F) Immunoblot of cell lysates collected from LNCaP^{shAR/pATK} and LNCaP^{APIPC} treated with 25 ng/mL FGF8 or vehicle probed with anti-ID1 antibody.

(G) LNCaP^{shAR/pATK} and LNCaP^{APIPC} were cultured under androgen-depleted conditions and treated with vehicle (PBS) or 25 ng/mL FGF8. *ID1*, *AR*, *PSA*, and *TMPS2* transcripts were quantitated by qRT-PCR, normalized to RPL13a expression, and compared with unstimulated LNCaP^{shAR/pATK}.

(H) Quantitation of *ID1-4* in LuCaP173.2 tumors treated with vehicle or CH-5183284 sampled 3 days or 24 days after the initiation of treatment. Transcripts were quantitated by RNA-seq of two independent tumors.

(legend continued on next page)

repression will eliminate PCs or consistently generate AR-null variants remains to be determined. Early results from an ongoing clinical trial (NCT00831792) of the FGFR antagonist dovitinib in men with metastatic CRPC unselected for loss of AR activity reported a 26% response rate in bone and soft tissue lesions (Wan et al., 2014). Our results suggest that FGFR inhibition may have modest effects in AR-active CRPCs, but be particularly active in the subset of CRPCs with absent or limited AR function. A clinical trial of FGFR or MAPK antagonists may be fruitful in patients stratified by AR activity status. Furthermore, co-targeting of predictable AR bypass pathways capable of providing robust cell survival and proliferation signals may prolong responses to initial AR antagonism.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **LEAD CONTACT FOR REAGENT AND RESOURCE SHARING**
- **EXPERIMENTAL MODELS AND SUBJECT DETAILS**
 - Cell Lines
 - Tissue Acquisition
 - LNCaP^{APIPC} Xenograft Mouse Models
 - PDX Mouse Models
- **METHOD DETAILS**
 - Cell Culture
 - Migration and Invasion Assays
 - Cell Growth Assays
 - Conditioned Media
 - siRNA Transfection
 - RNA Collection and Quantitative Real-Time PCR
 - Protein Collection and Immunoblotting
 - Immunohistochemistry
 - AR CRISPR-Cas9 Editing
 - Transcript Profiling Methods
- **QUANTIFICATION AND STATISTICAL ANALYSIS**
- **DATA AND SOFTWARE AVAILABILITY**

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.jccell.2017.09.003>.

AUTHOR CONTRIBUTIONS

P.S.N., E.G.B., and C.M. conceived the project; E.G.B., S.H.-L., E.C., and H.M.N. performed mouse experiments; I.M.C., R.T.C., A.K., R.T., and D.W. performed sequencing and bioinformatics analyses; E.G.B., J.M.L., D.B.-F., R.F.D., A.N.C., Y.C.Y., M.D.N., and L.G.B. conducted molecular and cell biology experiments; X.Z., L.D.T., and M.I. provided pathology evaluation;

E.M., A.H., M.S., K.P., R.L.V., and P.S.R. provided biospecimens, model systems, and clinical expertise; P.S.N., I.M.C., and C.M. wrote the manuscript; all authors reviewed and edited the manuscript.

ACKNOWLEDGMENTS

We are grateful to the patients and their families who participated in this study. We thank members of the P.S.N. laboratory for helpful advice and criticism. We thank Steve Plymate for helpful advice and assistance with analyses. We thank the FHCRC Genomics shared resource for assistance with RNA-seq experiments. We would also like to thank Yoshito Nakanishi for his advice, Bryce Lakely and Belinda Nguyen for technical assistance, Celestia Higano, Bruce Montgomery, Evan Yu, Heather Cheng, Funda-Vakar Lopez, Martine Roudier, Kristine Von Maltzan, Maria Tretiakova, and the rapid autopsy teams in the Urology Department at the University of Washington for their contributions to the Prostate Cancer Donor Rapid Autopsy Program. This work was supported by NIH awards: Prostate SPORE grant P50CA097186, P01 CA163227, DOD awards PC140794 and PC160662, a DF/HCC Mazzone award, the Institute for Prostate Cancer Research, the Richard M. Lucas Foundation, and a Challenge award from Movember and the Prostate Cancer Foundation.

Received: June 18, 2017

Revised: August 1, 2017

Accepted: September 5, 2017

Published: October 9, 2017

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(I) LNCaP^{shAR/pATK} and LNCaP^{APIPC} were cultured in androgen-depleted medium and transfected with siRNA specific for target genes. Cells were treated with 25 ng/mL FGF8 or vehicle. siUNI, non-targeting control siRNA; Kif11, equimolar mixture of three siRNAs targeting Kif11 and a positive control for transfection efficiency; ID1 #1 and ID1 #2 are siRNAs targeting ID1. Relative cellular number was measured with the Cell Titer Glo luminescence assay.

(J) Schematic depicting the cellular differentiation states and underlying molecular drivers of cell survival and growth following AR pathway-directed therapy. ADT, androgen deprivation therapy; ABI, abiraterone; ENZ, enzalutamide; CR-ARPC, castration-resistant AR program active PC; CR-NEPC, castration-resistant NE program active PC; CR-DNPC, castration-resistant PC without AR or NE program activity.

For (E), (G), and (I), significance was determined by Student's *t* test and data are presented as mean ± SEM (*n* = 3–5 replicates per data point). See also Figure S7.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-AKT	Cell Signaling	Cat# 9272, RRID:AB_329827
Ant-Phospho-Akt (Ser473)	Cell Signaling	Cat# 4058, RRID:AB_331168
Anti-AR (N-20) antibody	Santa Cruz	Cat# sc-816, RRID:AB_1563391
Anti-Anti-MAP Kinase (ERK-1, ERK-2)	Sigma	Cat# M5670, RRID:AB_477216
Anti-Anti-MAP Kinase, Activated (Diphosphorylated ERK-1/2)	Sigma	Cat# M9692, RRID:AB_260729
Anti-FGF8 MAb	R&D Systems	Cat# MAB323, RRID:AB_2102956
Anti-Id1	Biocheck Inc.	Cat# 195-14; RRID: AB_115761
Anti-Anti-MAP Kinase (MEK 1/2)	Sigma	Cat# M5795, RRID:AB_260593
Anti-phospho-MEK1 (Ser298)	Millipore	Cat# 07-339, RRID:AB_310533
Anti-Prostate Specific Antigen	Dako	Cat# M0750, RRID:AB_2281105
Anti-Actin	Santa Cruz	Cat# sc-1616, RRID:AB_630836
Anti-Chromogranin A	Dako	Cat# M0869, RRID:AB_2081135
Anti-Synaptophysin	Dako	Cat# M0776, RRID:AB_2199013
Anti-Androgen Receptor	BioGenex	Cat# MU256-UC
Anti-Human Cytokeratin	Dako	Cat# M351501-2, RRID:AB_2631307
Anti-Synaptophysin	Santa Cruz	Cat# sc-17750, RRID:AB_628311
Biological Samples		
Patient-derived xenografts	University of Washington	LuCaP
Human localized and metastatic tumors	University of Washington Prostate Cancer Donor Autopsy Program	
Chemicals, Peptides, and Recombinant Proteins		
Power SYBR Green PCR Master Mix	ThermoFisher	Cat#4367659
DNeasy Blood & Tissue Kit	Qiagen	Cat#69506
Matrigel Matrix Growth Factor Reduced	BD Biosciences	Cat#354230
Teklad laboratory animal diets (doxycycline)	Envigo	Cat#td.04104
PD 173074	R & D Systems	Cat#3044/50
Ganciclovir	inVIVOgen	Cat#sud-gcv
MDV3100	Medivation Inc.	N/A
Recombinant Human/Mouse FGF-8b	R & D Systems	Cat#423-F8-025
Pierce Phosphatase Inhibitor Mini Tablets	ThermoFisher	Cat#88667
Pierce Protease Inhibitor Mini Tablets	ThermoFisher	Cat#88665
Goat anti-Rabbit IgG (H+L) Secondary Antibody	ThermoFisher	Cat#31460
Goat anti-Mouse IgG (H+L) Secondary Antibody	ThermoFisher	Cat#31430
SuperSignal West Pico Chemiluminescent Substrate	ThermoFisher	Cat#34087
Methyltrienolone (R1881)	Perkin Elmer	Cat#NLP005005mg
U0126	Cell Signaling Technology	Cat#9903
ESP3I	ThermoFisher	Cat#ER0451
RNeasy Plus Micro Kit	Qiagen	Cat#74034
TruSeq RNA Library Preparation Kit v2	Illumina	Cat#RS-122-2001
TruSeq Stranded mRNA Library Preparation Kit	Illumina	Cat# RS-122-2101
gentleMACS M Tubes	Miltenyi Biotec	Cat# 130-096-335
RNA STAT-60	Tel-Test	Cat# Cs-502

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical Commercial Assays		
CultreCoat Low BME Cell Invasion Assay, 96 well	R & D Systems	Cat#3481-096-K
CyQUANT Cell Proliferation Assay Kit	ThermoFisher	Cat#C7026
CellTiter-Glo Luminescent Cell Viability Assay	Promega	Cat#G7570
ApoLive Glo	Promega	Cat#G6411
Deposited Data		
Raw and analyzed Expression Microarray	Kumar et al., 2016	GEO: GSE77930
Raw and analyzed aCGH Microarray Data	Kumar et al., 2016	GEO: GSE77930
Expression microarray, aCGH Copy Number and Exome Sequencing Mutations MAF Data – FHCRC/ UW cohort	Kumar et al., 2016	http://www.cbiportal.org/study?id=prad_fhcrc
Analyzed RNAseq data – SU2C cohort	Robinson et al., 2015	http://www.cbiportal.org/study?id=prad_su2c_2015
RNAseq data	This study	GEO: GSE99381
Experimental Models: Cell Lines		
LNCaP	ATCC	ATCC CRL-1740
LNCaP ^{shAR}	Laboratory of P.S. Rennie	Cheng et al., 2006
LNCaP ^{shAR/pATK}	This Study	N/A
LNCaP ^{APIPC}	This Study	N/A
PacMetUT1	Laboratory of D.A. Troyer	Troyer et al., 2008
PacMet AR-null #1, #2	This Study	N/A
22RV1	ATCC	CRL-2505
NCI-H660	ATCC	CRL-5813
Experimental Models: Organisms/Strains		
NOD- <i>scid</i> IL2Rgamma ^{null}	The Jackson Laboratory	005557
CB-17 SCID	Charles River	236
Oligonucleotides		
esiRNA targeting human <i>KIF11</i> CAUUGACAGUGGCCGAUAA	Sigma	Cat#SASI_Hs01_00161689
esiRNA targeting human <i>KIF11</i> CUGUACUACAGGAAUUGAU	Sigma	Cat#SASI_Hs01_00161696
esiRNA targeting human <i>KIF11</i> CAACAAGGAUGAAGUCUAA	Sigma	Cat#SASI_Hs01_00161697
esiRNA targeting human <i>ID1</i> CCUCUCUGCACACCUACUA	Sigma	Cat#SASI_Hs01_00057899
esiRNA targeting human <i>ID1</i> GGGCGCUCCUCUCUGCACA	Sigma	Cat#SASI_Hs01_00246329
siRNA Targeting Human <i>FGF8</i> #1 CAAGAGCAACGGCAAAGGCAA	Qiagen	Cat#SI00145600
siRNA Targeting Human <i>FGF8</i> #2 GCGCUUCGAGUCCUCAACUA	Qiagen	Cat#SI02636725
siRNA Targeting Human <i>FGF9</i> #1 UUGGAUUAUACCUCGCCUAAUA	Qiagen	Cat#SI00031332
siRNA Targeting Human <i>FGF9</i> #2 CAGAGUCGGUUAGAGAGUAAA	Qiagen	Cat#SI04932011
sgRNA protospacer CTCCGGACCTTACGGGGACATG	This Paper	N/A
AR_Exon1_sgRNA+ caccgCTCCGGACCTTACGGGGACATG	This Paper	N/A
AR_Exon1_sgRNA- aaacCATGTCCCCGTAAGGTCCGGAGc	This Paper	N/A

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
AR-fwd CRISPR verification primer CGACTTCACCGCACCTGATG	This Paper	N/A
AR-rev CRISPR verification primer AGGGCAGCAGCAGAAATTAG	This Paper	N/A
qRT-PCR Primers	This Paper	Table S1
Recombinant DNA		
HSV1- <i>tk</i> gene in pORF vector	inVIVOgen	Cat#porf-hsv1tk
pATK	This paper	N/A
lentiCRISPRv2	Sanjana et al., 2014	Addgene Plasmid #52961
Software and Algorithms		
Prism v7	GraphPad	https://www.graphpad.com/scientific-software/prism/
TopHat v2.1.0	Kim et al., 2013	https://ccb.jhu.edu/software/tophat/index.shtml
Genomic Alignments v1.0.1	Lawrence et al., 2013	https://bioconductor.org/packages/release/bioc/html/GenomicAlignments.html
edgeR v3.16.5	Robinson et al., 2010	https://bioconductor.org/packages/release/bioc/html/edgeR.html
Ape v4.1	Paradis et al., 2004	https://cran.r-project.org/web/packages/ape/index.html
GSVA v1.24.0	Hänzelmann et al., 2013	https://bioconductor.org/packages/release/bioc/html/GSVA.html
Scatterplot3d v0.3-40	Ligges and Machler, 2003	https://cran.r-project.org/web/packages/scatterplot3d/index.html
GSEA v2.2.4	Subramanian et al., 2005	http://software.broadinstitute.org/gsea/index.jsp
MSigDB v6.0	Subramanian et al., 2005	http://software.broadinstitute.org/gsea/msigdb
Other		
RNAiMax lipofectamine	ThermoFisher	Cat#13778030
Lipofectamine 2000	ThermoFisher	Cat#11668019
FBS Charcoal Dextran Stripped	Gemini Bio-Products	Cat#100-119

LEAD CONTACT FOR REAGENT AND RESOURCE SHARING

For further information and requests for reagents may be directed to and will be fulfilled by the corresponding author Peter S. Nelson (pnelson@fredhutch.org).

EXPERIMENTAL MODELS AND SUBJECT DETAILS**Cell Lines**

All cells were maintained at 37°C in a 5% CO₂ incubator. LNCaP (ATCC), 22RV1 (ATCC), and PacMet-UT1 (gift of D.A. Troyer) prostate cancer cell lines were grown in RPMI1640 (Invitrogen) supplemented with 10% FBS (Invitrogen) and 1% PenStrep (Invitrogen). NCI-H660 (ATCC) cells were grown in RPMI 1640 supplemented with 0.005 mg/ml insulin, 0.01 mg/ml transferrin, 30 nM sodium selenite, 10 nM hydrocortisone, 10 nM beta-estradiol, 4 mM L-glutamine, 5% FBS and 1% PenStrep. LNCaP^{shAR} (gift of P.S. Rennie) were grown in RPMI1640 + 5%FBS + 1% PenStrep + 2.5 µg/mL Blasticidin (Invitrogen) + 1 µg/mL Puromycin (Invitrogen). LNCaP^{shAR/pATK} (this study) were maintained in RPMI1640 + 5% FBS + 1% PenStrep + 2.5 µg/mL blasticidin + 1 µg/mL puromycin + 25 µg/ml Zeocin (Invitrogen). LNCaP^{APIPC} (this study) were maintained in RPMI1640 + 5% CSS (Charcoal Dextran stripped FBS) (Gemini) + 1% PenStrep + 2.5 µg/mL blasticidin + 1 µg/mL puromycin + 25 µg/ml Zeocin + 1 µg/mL doxycycline (Clontech). Cell lines were authenticated by STR analysis by DDC Medical (Fairfield, OH).

Tissue Acquisition

Samples were obtained from male patients who died of metastatic CRPC and who signed written informed consent for a rapid autopsy performed within 8 hours of death, under the aegis of the Prostate Cancer Donor Program at the University of Washington. The Institutional Review Boards of the University of Washington and of the Fred Hutchinson Cancer Research Center approved this

study. LuCaP xenograft lines were established from specimens acquired at either radical prostatectomy or at autopsy, implanted, and maintained by serial passage in intact immune compromised male mice.

CRPC was assessed using immunohistochemical analysis to determine the distribution of adenocarcinoma (AR⁺), double-negative (AR⁻/NE⁺), and neuroendocrine (AR⁻/NE⁺) in these metastasis. Sites of metastases were ascribed a score between 0-200 for AR and SYP positivity. Any score <20 was categorized as negative to categorize each site.

LNCaP^{APIPC} Xenograft Mouse Models

NOD-scid IL2Rgamma^{null} mice were purchased from the FHCRC animal facility. LNCaP^{APIPC} cells were resuspended 1:1 in Matrigel (BD Biosciences) to a final concentration of 5×10^6 cells/ml and 100 μ l of cells were injected subcutaneously into the flank of castrated male mice. Xenografts were measured with digital calipers every 2 days and tumor volume was calculated using the formula $(\pi/6)(L \times W^2)$, where L is the length of the tumor and W its width. Animals implanted with LNCaP^{APIPC} xenografts were maintained on a diet supplemented with doxycycline (200 mg/Kg, Harlan). When tumors reached a total volume of 200 mm³ animals were enrolled into treatment arms consisting of PD173074 given at 50 mg/Kg/day by oral gavage five times per week or control vehicle 99% PBS with 1% DMSO used to dissolve PD173074. Each treatment group was composed of 8 animals. Animals were sacrificed when tumors reached a volume of 1500 mm³. All xenografts experiments were approved by the Fred Hutchinson Institutional Animal Care and Use Committee (File#1671).

PDX Mouse Models

The LuCaP 173.2 patient-derived xenograft (PDX) line is from a rib metastasis obtained at time of death from a patient with CRPC and implanted into 6 week old immunocompromised male mice. CB-17 SCID mice (Charles River) were implanted subcutaneously with LuCaP 173.2 tumor tissue. Animals underwent rolling enrollment once tumors reached 100 mm³ and were randomized into one of two groups (Control vs. Treatment). The FGFR inhibitor CH5183284 (Debio-1347) (Selleck chem) was dissolved in 100% DMSO and a 10-fold concentration of dosing solution was prepared. Then an equal volume of Cremophor EL was added to DMSO solution (5-fold concentration of dosing solution in 50 vol% DMSO/50 vol% Cr-EL). This solution was divided into daily usage amounts and stored at 4°C until each dosing day. For dosing, the stock solution was diluted with diluent (18.8 vol% PEG400 / 18.8% HPCD in distilled water) by 5-fold concentration on each day. The final concentration of vehicle was 10 vol% DMSO/10 vol% Cr-EL/15 vol% PEG 400/15% HPCD in distilled water as per [Nakanishi et al., 2014](#). LuCaP 173.2 tumor bearing animals received either vehicle (Control), while treated animals (Treatment) received 80 mg/kg CH5183284 4 days a week for 3.5 weeks via oral gavage. Note, 6 animals in the treated group received 100 mg/kg CH5183284 for 5 days/daily for one week before switching over to 80 mg/kg CH5183284 4 days a week due to loss of body weight. Tumor volumes (TV) were measured using digital calipers (calculated as $(\pi/6)(L \times W^2)$) and body weights (BW) were measured twice weekly. Animals were euthanized after 3.5 weeks, when tumors exceeded 1,000 mm³, or when animals became otherwise compromised. The tumors were then divided equally into paraffin blocks with the remainder flash frozen for subsequent sequencing analysis. All PDX experiments were approved by the University of Washington Institutional Animal Care and Use Committee (File #2110-03).

METHOD DETAILS

Cell Culture

LNCaP^{shAR} cells stably transfected with a tetracycline-inducible anti-AR shRNA, as previously described ([Cheng et al., 2006](#)), were obtained as a gift from Dr. Paul S. Rennie. These cells were further modified via Lipofectamine 2000 (Invitrogen) transfection of a plasmid encoding a triple-probasin-driven herpes thymidine kinase (HSV-TK) and a Zeocin resistance cassette. A clonal population of this cell line derived from Zeocin (Invitrogen) selection and serial dilution in a 96-well plate, which we refer to as LNCaP^{shAR/pATK}, was subjected to total AR pathways suppression (TAPS): for two weeks the cells were grown in RPMI1640+5%CSS; at week 3, media was supplemented with 1 mg/mL doxycycline. Media was changed every 3-4 days and LNCaP^{shAR/pATK} was maintained under TAPS for five months. A surviving colony of proliferating cells emerged. Following a 3-month expansion, this population of cells was treated with 50 μ M ganciclovir (GCV; InvivoGen) for two weeks to eliminate any cells still robustly expressing an AR transcriptional program. We referred to the surviving population as LNCaP^{APIPC}.

Migration and Invasion Assays

Migration and invasion assays were performed as per protocol in Cultrex 96-well cell invasion/migration transwell plates (R&D Systems). RPMI1640+/-10%FBS was added to the lower chamber and 100,000 cells suspended in serum-free RPMI1640 were added to the top chamber. For invasion assays, membranes were coated with 0.2x BME. Fluorescence was measured on a BioTek Synergy2 multiwell plate reader and normalized to LNCaP^{shAR/pATK} RPMI1640 serum-free control.

Cell Growth Assays

Cell growth was assayed by plating 5000 cells per well in a TC-treated 96-well black-sided, clear bottom plate (Corning). Media was changed every 48 hours and plates were collected at the reported timepoints and stored at -80°C. Plates for each experiment were assayed in batches using Cyquant (Invitrogen) to estimate cell viability as per manufacturer's protocol. Cells were treated with FGF8b

(25 ng/mL; eBioscience) or PD173074 (1 μ M; Tocris) or U0126 (25 μ M). Additionally, cells were treated with doxycycline (1 mg/mL) and enzalutamide (5 μ M) which was received as a gift from Medivation Inc.

Cells were plated as above and allowed to adhere for 24 hours then treated with various concentrations (as indicated in the figures) of CH-5183284 for 72 hours and assayed for apoptosis and viability using ApoLive Glo (Promega) following the manufacturer's instructions.

Conditioned Media

Serum-free phenol red-free OptiMem (Invitrogen) was added to 80% confluent LNCaP^{APIPC} and LNCaP^{shAR/pATK} cultured in a tissue culture-treated T75 flask (Corning). Twenty-four hours later, media was collected and centrifuged for 5 minutes at 5000 RPM to pellet cellular debris. The supernatant was added to an Amicon Ultracel 3K centrifugal filter (Millipore) and concentrated as per manufacturer's instructions.

siRNA Transfection

Cells were plated at 5000 cells/well in a 96-well plate in 100 μ l of phenol red-free OptiMem supplemented with either 3% FBS or 3% CSS +1% PenStrep. Twenty-four hours after cell plating, cells were transfected with siRNA (Sigma) using RNAiMax lipofectamine reagent (Life Technologies) in 20 μ l total volume. Cell viability was estimated 96 hours after transfection by adding Cell Titer-Glo (Promega) and measuring luminescence (RLUs) as per protocol on a BioTek Synergy2 multiwell plate reader. Luminescence measurements from wells transfected with an equimolar pool of 3xKif11 siRNAs was used to estimate transfection efficiency. Transfections performed in 6-well plates for RNA collection used scaled-up conditions from 96-well experiments, and cells were harvested 24 hours after transfection as described below. siRNA sequences can be found in the [Key Resource Table](#).

RNA Collection and Quantitative Real-Time PCR

Cell culture total RNA was isolated from 6-well plates using an RNEasy kit (Qiagen) as per protocol. qRT-PCR reactions were performed in triplicate using an Applied Biosystems 7900 sequence detector with SYBR Green PCR master mix (Invitrogen). Primers were designed using PrimerQuest (IDT, and all reactions were normalized to the expression of the housekeeping gene RPL13A. A water negative control did not produce significant amplification product. PCR primer sequences can be found in the [Table S1](#). Statistical analysis was performed using an unpaired two-sided Student's T-test to determine significance.

Protein Collection and Immunoblotting

Protein was collected from tissue culture by lysing adherent cells with a cell lysis buffer (1.5 M Urea, 1% SDS, 1% NP-40, 2% Tween20, 250 mM NaCl, PBS) supplemented with 1x phosphatase inhibitors (PhosStop, Roche Diagnostics) and a 1x protease inhibitor cocktail (Complete Mini, Roche Diagnostics). Protein was quantified per protocol using a bicinchoninic acid assay (Thermo Scientific). Normalized cell lysates were loaded onto a 12% NuPAGE Bis-Tris gel (Invitrogen) in MES buffer. Protein was transferred to nitrocellulose membranes using a semi-dry transfer apparatus and Tris/CAPS buffer. Immunoblots were probed with primary antibodies targeting AKT (Cell Signaling), phospho-AKT (Ser473; Cell Signaling), AR (Santa Cruz), Erk1/2 (Sigma), diphosphorylated-Erk1/2 (Sigma), FGF8b (R&D Systems), ID1 (Biocheck, Inc.), MEK1/2 (Sigma), phospho-MEK1 (Ser298; Upstate), or PSA (Dako). Horseradish-peroxidase conjugated secondary antibodies (Thermo Scientific) were used in conjugation with Supersignal West Pico Chemiluminescent substrate (Thermo Scientific) to visualize protein targets. Membranes were then stripped for 15 minutes in Stripping Buffer (Thermo Scientific) and re-probed with anti-Actin antibody (Santa Cruz Biotechnology) as a loading control.

Immunohistochemistry

PC metastases and xenograft tissues were fixed in buffered formalin (bone metastases were decalcified in 10% formic acid) and embedded in paraffin. Tissue microarrays (TMAs) were constructed using duplicate 1 mm diameter cores from these tissues. Five-micron thick sections of the TMAs were deparaffinized and rehydrated in sequential xylene and graded ethanol. Antigen retrieval was performed in 10 mM citrate buffer (pH 6.0) in a pressure cooker. Endogenous peroxidase and avidin/biotin were blocked respectively (Vector Laboratories Inc.). Sections were then incubated with 5% normal goat-horse-chicken serum, incubated with the following primary antibody dilutions: anti-Androgen Receptor (Biogenex) 1:60, anti-Prostate-specific Antigen (Dako) 1:1000, anti-Chromogranin A (Dako) 1:100, anti-Synaptophysin (Santa Cruz) 1:200 and anti-human cytokeratin (Dako) 1:100. They were subsequently incubated with biotinylated secondary antibody (Vector Laboratories Inc.), followed by ABC reagent (Vector Laboratories Inc.), and stable DAB (Invitrogen Corp.). All sections were lightly counterstained with hematoxylin and mounted with Cytoseal XYL (Richard Allan Scientific). Mouse or rabbit IgG were used as negative controls.

AR CRISPR-Cas9 Editing

To create the sgRNA targeting exon 1 of AR, an sgRNA protospacer of CTCCGGACCTTACGGGGACATG was cloned in to the ESP31 enzyme (Thermo Fisher) sites of the lentivirus expression vector lentiCRISPRv2 (Addgene Plasmid #52961) using annealed oligos and AR_Exon1_sgRNA+: caccgCTCCGGACCTTACGGGGACATG and AR_Exon1_sgRNA-: aaacCATGTCCCCGTAAGGTCCGGAGc. To confirm on-target cutting, cells were transfected with lentiCRISPRv2:AR-sgRNA or GFP control using lipofectamine 2000 (Thermo Fisher) according to manufacturer's recommendation. After five days of selection with 1.5 μ g/mL puromycin, genomic DNA was isolated using the DNeasy Blood & Tissue Kit (Qiagen) and amplified using Phusion High-Fidelity DNA Polymerase (New England

Biolabs) and primers: AR-fwd CGACTTCACCGCACCTGATG and AR-rev AGGGCACGCAGCAGAAATTAG. On target CRISPR cutting was confirmed using T7 endonuclease I (New England Biolabs) heteroduplex cleavage assay to measure insertion/deletions, introduced via NHEJ-mediated double strand break repair of CRISPR activity.

PacMetUT1 cells were seeded in 10 cm dishes and transfected with lentiCRISPRv2:AR-sgRNA using lipofectamine 2000 (Thermo Fisher) according to manufacturer's protocols and recommendations. Cells were supplemented with 25 ng/mL FGF8b (R&D Systems) or PBS + 0.1% human BSA solvent control during five days of selection with 1.5 μ g/mL puromycin. The surviving cells were replaced with fresh medium (RPMI 1640 10% FBS with or without FGF8b) and allowed to grow into colonies. Medium was changed once a week and FGF8b was replenished every three days. Approximately 5 weeks later, colonies were isolated from both FGF8b and PBS/BSA supplemented cells and allowed to expand for further analyses. Two colonies from cells treated with FGF8b were confirmed to be AR-negative by Western blot (GeneTex). These two colonies were referred to as the AR-null #1 and AR-null #2 sublines.

Transcript Profiling Methods

Cell line RNA was extracted using the Qiagen RNeasy Kit, (Qiagen Inc.), according to the manufacturer's protocol. On-column DNase digestion was performed. CRPC metastases RNA samples were prepared by reviewing a H&E of the frozen tissue block, followed by scoring the block with a razor so as to have as pure as possible sections of tumor. Cores were obtained from each of the bone metastases frozen tissue blocks that had been previously identified based upon review of H&E sections from corresponding paraffin embedded blocks; adjacent areas of tumor were cored out of the frozen tissue blocks using a 2 mm diameter tissue punch in a -20°C cryostat. Cores were homogenized in gentleMACS M Tubes (Miltenyi Biotec). Tissues were then isolated with RNA STAT-60 (Tel-Test). RNA concentration, purity, and integrity was assessed by NanoDrop (Thermo Fisher) and Agilent Bioanalyzer. Cell line RNA-seq libraries were constructed from 1 μ g total RNA using the Illumina TruSeq RNA Library Prep Kit v2 according to the manufacturer's protocol. CRPC Metastases RNA-seq libraries were constructed from 1 μ g total RNA using the Illumina TruSeq Stranded mRNA Library Prep Kit according to the manufacturer's protocol. Barcoded libraries were pooled and sequenced on the Illumina HiSeq 2500 generating 50 bp paired end reads. Sequencing reads were mapped to the hg19 human genome using TopHat v2.1.0. Gene level abundance was quantitated from the filtered human alignments in R using the Genomic Alignments v1.0.1 Bioconductor package.

QUANTIFICATION AND STATISTICAL ANALYSIS

Student's T-test was used to comparable significance between grouped quantitative data sets using GraphPad Prism 7.0 software. Differences were considered significant if $p \leq 0.05$. Differences in tumor volume (TV) between control and treated animals were calculated using unpaired t-tests with unequal variances, with significance set at $p \leq 0.05$.

Differential expression was assessed using transcript abundances as inputs to the edgeR v3.16.5 Bioconductor package in R. FDR and fold-change thresholds for significance are listed in the figure legends.

Unsupervised clustering of cell line expression profiles was performed in R on the 1000 most variable genes (calculated as the inter-quartile range of the $\log_2$ transcripts per million reads) using Euclidean distance and average-linkage. Clusters were visualized using the ape v4.1 Cran package.

The gene expression signature activity scores were calculated in R using the GSVA v1.24.0 Bioconductor package, using $\log_2$ transcripts per million reads as input. Pearson's correlation coefficient was used to study the relationships between variables shown in scatterplots using the cor.test function in R. The scatterplot3d v0.3-40 Cran package was used to plot three dimensional scatterplots.

Gene expression group comparisons were ranked by edgeR statistics and used to conduct Gene Set Enrichment Analysis using the GSEA v2.2.4 software to determine patterns of pathway activation in different phenotypic groups. We used the curated pathways and gene sets within MSigDBv6.0.

Genome-wide comparisons of copy number between DNPC and ARPC groups was performed using two-tailed fisher's exact tests using the fisher.test function in R. Proportions of tumors with somatic copy number alterations were compared, including high (greater than 1 copy) gain or homozygous loss.

DATA AND SOFTWARE AVAILABILITY

The RNA sequencing data has been deposited at the Gene Expression Omnibus (GEO) site: <https://www.ncbi.nlm.nih.gov/geo/> under accession number GSE99381.

Prognostic and Therapeutic Implications of DNA Repair Gene Mutations in Advanced Prostate Cancer

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Abstract: Recent work directed toward understanding the molecular features of advanced prostate cancers has revealed a relatively high incidence of both germline and somatic alterations in genes involved in DNA damage repair (DDR). Many of these alterations likely play a critical role in the pathogenesis of more aggressive prostate cancers—leading to genomic instability and an increased probability of the development of lethal disease. However, because the ability to repair DNA damage with a high degree of fidelity is critical to an individual cell's survival, tumor cells harboring alterations in DDR pathway genes are also more susceptible to drugs that induce DNA damage or impair alternative DNA repair pathways. In addition, because the genomic instability that results from these alterations can lead to an inherently higher number of mutations than occur in cells with intact DDR pathways, patients with genomic instability may be more likely to respond to immune checkpoint inhibitors, presumably owing to a correspondingly high neoantigen burden. In this review, we discuss the emerging molecular taxonomy that is providing a framework for precision oncology initiatives aimed at developing targeted approaches for treating prostate cancer.

Introduction

Failure to repair DNA damage and replication errors accurately can lead to the accumulation of mutations and an increased risk for cancer. It is therefore not surprising that mutations in DNA repair genes have been associated with several cancer predisposition syndromes.¹⁻⁵ Studies across a variety of malignancies have also shown that when DNA damage repair (DDR) deficiency occurs—often as a result of homozygous loss-of-function mutations in *BRCA1/2*, *ATM*, and other genes involved in homologous recombination (HR)—intrinsic genomic instability is present, which can render cells vulnerable to agents that induce DNA damage or inhibit alternative DNA repair pathways. Poly(ADP-ribose) polymerase (PARP) has been shown to be a key mediator in this respect, and strategies to inhibit PARP activity have been shown to be effective in a number of cancers with impaired HR.⁶⁻¹⁰ In addition, more

Keywords

Checkpoint inhibitor, DNA damage repair, homologous recombination, mismatch repair, PARP inhibitor, prostate cancer

recent data have shown that targeting PARP activity may be an effective strategy to augment the antitumor effects of other DNA-damaging agents (eg, alkylating agents and platinum chemotherapeutic agents) in cancers with intact DDR pathways.^{10,11} Tumors with homologous recombination deficiency (HRD) also appear to be exquisitely sensitive to DNA-damaging chemotherapeutic agents.^{12,13}

In addition, because alterations in mismatch repair (MMR) pathway genes can lead to the accumulation of vastly more mutations than occur in tumors with an intact MMR pathway (ie, hypermutation), it has been hypothesized that such tumors will have a higher neo-antigen burden, which renders them more susceptible to immune checkpoint inhibitors. A recent study testing this hypothesis has led to the first US Food and Drug Administration (FDA) tumor-agnostic approval for pembrolizumab (Keytruda, Merck) in patients with MMR gene mutations or microsatellite instability (MSI), a marker of genomic fragility.¹⁴⁻¹⁶

Alterations in the DDR pathway are present in upward of 20% of men with metastatic castration-resistant prostate cancer (mCRPC) and in up to 12% of men with metastatic prostate cancer harboring a germline alteration in one of these genes.^{5,17} Given how prevalent these mutations are, it is not surprising that a number of precision oncology approaches are being developed to treat patients who have advanced prostate cancer with impaired DDR. This review outlines the clinically relevant DDR pathways as they pertain to prostate cancer and discusses efforts to develop drugs targeting these pathways.

DNA Damage Repair: Overview

A multitude of events occur daily that lead to DNA damage that requires subsequent repair. The ability to repair DNA damage with a high degree of fidelity is both critical to an individual cell's survival and necessary to prevent malignant transformation. As such, germline alterations in DDR genes can increase replicative DNA stress, the accumulation of mutations, and the risk for cancer.^{18,19} Because of the critical role that DDR pathways play in maintaining cellular viability, a complex network of cellular pathways has evolved to deal with DNA damage by detecting and repairing it as it arises—herein referred to as DDR pathways.²⁰⁻²³

The DDR pathways are signal transduction pathways consisting of sensors, transducers, and effectors.^{24,25} The ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and RAD3-related (ATR) proteins are key kinases involved in sensing and regulating the response to DNA damage and are intimately involved in several DDR pathways.²⁶ If DNA damage is detected, cell cycle arrest occurs, providing an opportunity either for damaged

DNA to be repaired via a number of DDR pathways or for apoptosis to occur if catastrophic genomic instability has occurred.^{19,22,27} Some key proteins involved in regulating the cell cycle include the following: ATM (G1/S checkpoint), ATR (S-phase checkpoint), CHK1 (G2/M and S-phase checkpoints), CHK2 (G1/S checkpoint), DNA-PK (S-phase checkpoint), WEE1 (S-phase and G2/M checkpoints), and TP53 (G1/S checkpoint).²²

Following the detection of DNA damage, overlapping downstream DDR pathways are activated to resolve double-strand DNA (dsDNA) damage or single-strand DNA (ssDNA) damage.²⁷ The key pathways involved in ssDNA repair are MMR, base excision repair (BER), and nucleotide excision repair (NER). The main pathways involved in dsDNA damage repair are HR and nonhomologous end joining (NHEJ).²⁸⁻³⁸ A third pathway responsible for rescuing damaged dsDNA is called translesion DNA synthesis. Redundancies in these pathways ensure that even with loss-of-function mutations in one of these pathways, an individual cell may still be able to survive. Key proteins involved in these overlapping pathways are outlined in Table 1.^{1,25,26}

Given the complexity of the DDR pathways, an exhaustive review of the topic is beyond the scope of this article. Instead, we focus on the pathways that appear most clinically relevant to the prognosis and treatment of prostate cancer.

Targeting Homologous Recombination Deficiency

Mutations in the genes involved in HR are frequently observed in men with metastatic prostate cancer.^{5,17} Nearly 12% of unselected patients with metastatic prostate cancer have been found to have germline alterations in HR genes, and approximately 20% to 25% of patients with mCRPC harbor alterations in HR genes (somatic and/or germline), with *BRCA2*, *ATM*, and *BRCA1* the most commonly affected genes.^{5,17} Studies examining the effect of germline *BRCA1/2* mutations on prostate cancer risk have reported that *BRCA2* confers an 8.6-fold increased risk for prostate cancer and that *BRCA1* confers a 3.4-fold increased risk.³⁹⁻⁴³ *BRCA1/2* germline alterations have also been shown to be associated with a higher Gleason score, a higher T stage, nodal involvement, and metastases at diagnosis.⁴³ Rates of cause-specific overall survival and metastasis-free survival are also significantly lower for patients with localized prostate cancer and a germline alteration in *BRCA1* (hazard ratio, 2.6; $P=.01$) or *BRCA2* (hazard ratio, 2.7; $P=.009$).

The most genotoxic form of DNA damage is dsDNA damage because both strands of DNA are affected.^{22,44} The 2 key pathways involved in resolving dsDNA damage

Table 1. Key DNA Damage Repair Pathway Sensors, Transducers, and Effectors

	dsDNA Repair Pathways			ssDNA Repair Pathways		
	HR	NHEJ	alt-NHEJ	BER	MMR	NER
Causes of damage	Radiation, topoisomerase I inhibitors, nucleoside analogues	Radiation, topoisomerase II inhibitors	Radiation, topoisomerase II inhibitors	Radiation, alkylating agents, oxidation, deamination	Replication/recombination errors, alkylating agents	Ultraviolet light, polycyclic aromatic hydrocarbons, platinum chemotherapy
Sensors	MRN complex	Ku70-Ku80	PARP	APE1, 9-1-1 complex, PARP, RPA complex	MLH1, MSH2, MSH3, MSH6, PMS2	XPC, DDB2, CSA
Transducers	ATM, ATR, BRCA1, BRCA2, PALB2	ATM, DNAPK				RPA complex
Effectors	BLM, FANCI, PARI, POLQ, RAD51, RECQL5	LIG4, PAXX, XLF, XRCC4	LIG1, LIG3, POLQ, XRCC1	LIG1, LIG3A, POLB, XRCC	EXO1, LIG1, POLD	ERCC1, POLE, POLD1, LIG 1, LIG 3, XPG

alt-NHEJ, alternative NHEJ; BER, base excision repair; dsDNA, double-strand DNA; HR, homologous recombination; MMR, mismatch repair; NER, nucleotide excision repair; NHEJ, nonhomologous end joining; ssDNA, single-strand DNA.

are NHEJ and HR. It is important to note that although HR results in error-free repair of dsDNA damage and uses the undamaged sister chromatid as a template, NHEJ is an error-prone repair mechanism that can lead to a large number of chromatid breaks and aberrations, which can result in loss of cell viability.^{44,45} As mentioned earlier, HR is the major pathway for high-fidelity DNA repair following an insult that results in dsDNA damage. Cancers in which the tumor cells have biallelic loss-of-function mutations in genes involved in HR are sensitive to agents that induce DNA damage.

PARP Inhibitors in Prostate Cancer

PARPs (especially PARP1, PARP2, and PARP3) are key enzymes involved in BER and are required to repair ssDNA damage efficiently. Without PARP1 function, single-strand gaps in DNA persist, and degeneration to double-strand breaks can occur if a replication fork encounters these genomic defects.⁴⁵⁻⁴⁸ Under normal conditions, such dsDNA damage can be repaired via the HR pathway; however, in the case of HRD, replication forks collapse and chromatid breaks persist, leading a cell down a pathway toward apoptosis.⁴⁸⁻⁵⁰ In addition, PARP1 is involved in repairing dsDNA breaks through the alternative NHEJ pathway and can therefore further impair the ability to repair dsDNA breaks in HR-deficient tumors.⁵¹⁻⁵³ Preclinical studies have supported this model, demonstrating that *BRCA1/2*-deficient cell lines

are sensitive to pharmacologic PARP1 inhibition.^{45,48}

Proof of concept for this approach is derived from TOPARP (A Phase II Trial of Olaparib in Patients With Advanced Castration Resistant Prostate Cancer).⁶ This was a phase 2 study testing olaparib (Lynparza, AstraZeneca) at an oral dose of 400 mg twice daily in men with mCRPC. The primary endpoint was the response rate, which was defined as the presence of any of the following: an objective radiographic response per the Response Evaluation Criteria in Solid Tumors (RECIST) criteria v1.1, a reduction in the prostate-specific antigen (PSA) level of at least 50% from baseline (ie, a PSA₅₀ response), or a confirmed reduction in the number of circulating tumor cells (CTCs) from at least 5/7.5 mL of blood to fewer than 5/7.5 mL of blood. Of the 50 patients with mCRPC who were enrolled, all had received prior docetaxel, and 49 had received prior abiraterone acetate (Zytiga, Janssen Biotech) or enzalutamide (Xtandi, Astellas/Medivation). There were 16 patients (33%) who met the primary endpoint, achieving a response according to the composite definition. Most notably, responses to olaparib were enriched in the subset of patients with loss-of-function alterations (homozygous deletions, deleterious mutations, or both) in HR genes (eg, *BRCA1/2*, *ATM*, Fanconi anemia genes, *CHK2*); the observed response rate was 88% in this biomarker-positive cohort. Interestingly, genomic reversions of germline and/or somatic DNA repair mutations that restore the open reading frame (ORF) were

described as driving secondary resistance in this trial.⁵⁴ Several subsequent studies have since been launched to evaluate PARP inhibitors further in men with recurrent or advanced prostate cancer (Table 2).

DNA-Damaging Agents

The induction of DNA damage is one of the most common mechanisms by which chemotherapeutic agents exert their cytotoxic effects. Given the importance of HR in repairing dsDNA damage, it is intuitive that cells with impaired HR activity will be sensitive to any number of DNA-damaging agents. Indeed, preclinical models have shown that *BRCA1* and *BRCA2* are important mediators of platinum-induced DNA damage, and loss of function of these genes can enhance platinum sensitivity.^{45,55} Consistent with this finding is the observation that ovarian cancers with mutations in *BRCA1* or *BRCA2* are more susceptible to platinum chemotherapy.⁵⁶

Several older trials that did not include next-generation sequencing of tumor samples tested platinum-based chemotherapy regimens in men with advanced prostate cancer.⁵⁷⁻⁶¹ Because most of these studies tested combination regimens, it is difficult to estimate the contribution of the platinum agent to the observed response rate. Many studies have reported PSA₅₀ response rates of 15% to 30%—approximating the incidence of HRD in patients with CRPC.¹⁷ A phase 2 study reported by Ross and colleagues is particularly informative. In that trial, the authors reported that of 34 men with CRPC that had progressed during or within 45 days of completion of docetaxel-based chemotherapy, 18% had a decline in PSA of at least 50% following treatment with docetaxel (60 mg/m²) plus carboplatin (area under the curve [AUC], 4).⁵⁷ One can surmise that because this study enrolled only men with previously progression on docetaxel, the observed clinical effects were most likely the result of carboplatin activity.

Emerging data support HRD as a predictive biomarker for prostate cancer response to DNA-damaging agents. In a small case series, Cheng and colleagues reported on 3 heavily pretreated patients with mCRPC who had extreme responses to platinum-based chemotherapy; all of the men had deleterious alterations in HR genes.¹² Similarly, a recent retrospective analysis of patients with mCRPC who were receiving platinum-based chemotherapy revealed that PSA₅₀ response rates were higher in men with known pathogenic germline *BRCA2* alterations. In this study, by Pomerantz and colleagues, 6 of 8 carriers (75%) of a pathogenic *BRCA2* variant had a PSA₅₀ response following carboplatin plus docetaxel vs 23 of 133 men (17%) without a pathogenic *BRCA2* variant ($P < .001$).⁶² On the basis of these data, a precision oncology trial testing docetaxel plus carboplatin

in patients with mCRPC who have HRD was recently launched (NCT02598895).

Combination PARP Inhibitors and DNA-Damaging Agents

Because DDR inhibitors impair a cell's ability to resolve DNA damage, combining a PARP inhibitor with a conventional cytotoxic therapy could in theory potentiate the effects of the cytotoxic therapy. Consistent with this idea, PARP inhibitors have been shown across multiple preclinical tumor models to potentiate the antitumor effects of DNA-damaging cytotoxic agents (eg, alkylating agents, platinum chemotherapy) as well as of radiation.⁶³⁻⁶⁷ Importantly, many of these studies have shown that the observed antitumor effects are not restricted to cell lines with a biallelic loss of HR pathway genes.

On the basis of preclinical work demonstrating synergy between PARP inhibitors and temozolomide, a number of trials testing PARP inhibitors in combination with temozolomide have been launched.²² A pilot study testing low-dose veliparib with temozolomide in patients with mCRPC after docetaxel was previously reported by Husain and colleagues.⁶⁸ Of the 26 patients eligible for this study, 25 were evaluable for PSA₃₀ response (the primary endpoint). Overall, 2 of 25 patients (8%) had a confirmed PSA₃₀ response, and there were no objective radiographic responses in the 16 patients with RECIST-evaluable disease. The authors questioned whether the low dose of veliparib (40 mg twice daily) tested in this trial could have affected the overall efficacy of the combination. In addition, temozolomide is not particularly active in prostate cancer and may not have yielded sufficient DNA damage in this tumor type. Somatic tumor sequencing was unfortunately not performed in this study, and the underlying HRD status of the enrolled subjects is not known.

The more recent I-SPY 2 trial (Neoadjuvant and Personalized Adaptive Novel Agents to Treat Breast Cancer) tested veliparib in combination with carboplatin as a neoadjuvant therapy in patients with breast cancer.⁸ This study was a multicenter, randomized, phase 2 “platform” trial testing the addition of multiple experimental regimens to a control “backbone” regimen. Patients with high-risk primary breast cancer planning to undergo surgery were eligible. The control arm received 12 weekly cycles of paclitaxel followed by 4 cycles, every 2 to 3 weeks, of doxorubicin/cyclophosphamide. One of the experimental arms received a combination of 50 mg of veliparib by mouth twice daily and carboplatin (AUC, 6) concurrently with the weekly paclitaxel. The primary endpoint was the pathologic complete response (pCR) rate as assessed at the time of surgery. Among the patients with triple-negative breast cancer (ie, negative for human epidermal growth factor 2 [HER2], estrogen receptor [ER],

Table 2. Selected Ongoing Clinical Trials Testing PARP Inhibitors in Men With Prostate Cancer

Agents Being Tested	Trial Phase	Disease State	Key Eligibility Criteria	Sample Size	Primary Endpoint	Identifier
Olaparib +/- degarelix (Firmagon, Ferring Pharmaceuticals)	Phase 1	Localized	Intermediate- to high-risk disease Planning to undergo prostatectomy	20	Determination of PARP inhibition	NCT02324998
Olaparib +/- cediranib	Phase 2	mCRPC	Two or more prior lines of therapy for mCRPC	84	Radiographic PFS	NCT02893917
Rucaparib (Rubraca, Clovis Oncology)	Phase 2	mCRPC	HRD After taxane and 1-2 next-generation AR signaling inhibitors	160	Objective response rate PSA response rate	NCT02952534
Rucaparib vs abiraterone, enzalutamide, or docetaxel	Phase 3	mCRPC	HRD After next-generation AR signaling inhibitor	400	Radiographic PFS	NCT02975934
Niraparib (Zejula, Tesaro)	Phase 2	mCRPC	Progression on ≥ 1 taxane-based chemotherapy regimen and ≥ 1 AR signaling inhibitor	160	Objective response rate	NCT02854436
Niraparib + enzalutamide	Phase 1	mCRPC	—	—	MTD	NCT02500901
Olaparib	Phase 2	Biochemical recurrence	After prostatectomy Nonmetastatic disease	50	PSA response rate	NCT03047135
Olaparib + abiraterone	Phase 2	mCRPC	After docetaxel	159	Safety Radiographic PFS	NCT01972217
Abiraterone vs olaparib vs olaparib + abiraterone	Phase 2	mCRPC	HRD Before docetaxel	70	PFS	NCT03012321
Olaparib vs enzalutamide or abiraterone	Phase 3	mCRPC	HRD After abiraterone and/or enzalutamide	340	Radiographic PFS	NCT02987543
Olaparib + pembrolizumab*	Phase 1	mCRPC	After docetaxel	210	PSA response rate Safety	NCT02861573
Niraparib + radium-223	Phase 1	mCRPC	—	6	MTD	NCT03076203
Niraparib + apalutamide or abiraterone	Phase 1	mCRPC	After docetaxel	60	MTD Safety	NCT02924766

AR, androgen receptor; HRD, homologous recombination deficiency; mCRPC, metastatic castration-resistant prostate cancer; MTD, maximum tolerated dose; PARP, poly(ADP-ribose) polymerase; PFS, progression-free survival; PSA, prostate-specific antigen.

* This is a multiple-arm study testing pembrolizumab in combination with several prostate cancer therapies, including olaparib.

and progesterone receptor [PR]), the estimated pCR rates were 51% (95% Bayesian probability interval [PI], 36%-66%) in the veliparib/carboplatin arm and 26% (95% PI, 9%-43%) in the control group. It is notable that this study was not restricted to patients with DDR deficiency, although the percentage of patients in the veliparib/carboplatin arm with deleterious mutations in *BRCA1* or *BRCA2* (12/72, 17%) was higher than the percentage in the control arm (2/44, 5%). Given that platinum-based chemotherapy has shown promise in mCRPC, it would be reasonable to test platinum/PARP inhibitor combination strategies in men with advanced prostate cancer.

Although mounting evidence suggests synergistic efficacy when PARP inhibitors are combined with DNA-damaging agents, this likely comes at the expense of increased toxicity. For instance, in the aforementioned I-SPY 2 trial, grade 3 or higher neutropenia occurred in 71% of patients receiving paclitaxel in combination with veliparib and carboplatin compared with 2% in patients receiving only paclitaxel.⁸ Although some of the increased bone marrow toxicity observed in the experimental arm of I-SPY 2 was likely due to the addition of carboplatin, the stark difference in the rates of neutropenia cannot be completely explained solely by the addition of carboplatin, and it seems probable that veliparib compounded this risk. Similarly, increased toxicity was observed in a randomized phase 2 study, reported by Oza and colleagues, comparing olaparib, paclitaxel, and carboplatin followed by maintenance olaparib vs paclitaxel and carboplatin alone in women with recurrent platinum-sensitive ovarian cancer.⁶⁹ This study reported grade 3 or higher neutropenia in 43% of patients receiving PARP inhibitor combination therapy and in 35% of patients receiving chemotherapy only. Larger studies are needed to better define the clinical benefit, as well as overlapping toxicity, of PARP inhibitor/chemotherapy combinations.

Homologous Recombination Deficiency and Inhibition of Androgen Receptor Signaling

Hussain and colleagues recently reported on the activity of abiraterone, a cytochrome P₄₅₀ (CYP) 17 inhibitor able to decrease the production of androgens in extragonadal (eg, intratumoral and adrenal) sources with or without veliparib.⁷⁰ Their rationale for combining an inhibitor of androgen receptor (AR) signaling with a PARP inhibitor was based on preclinical data demonstrating that PARP is involved in the AR transcriptional machinery, and that inhibiting PARP can downregulate AR activity.⁷¹ Randomization to this study was stratified by expression of the ETS protein as determined by immunohistochemistry (IHC) on the basis of the hypothesis that the presence of AR-regulated *ETS* oncogene fusions would predict a response to PARP inhibition. The primary endpoint was

the PSA₅₀ response rate (ie, the proportion of patients with decreases in PSA of ≥50% from baseline). This trial accrued 148 subjects, with 72 randomly assigned to abiraterone alone and 76 to the combination arm. The study ultimately failed to meet its primary endpoint, with similar PSA₅₀ response rates in the 2 arms (63.9% with abiraterone vs 72.4% with the combination; *P*=.27), and ETS IHC status did not predict response to therapy. A secondary analysis involved next-generation sequencing of tumor samples (*N*=80) to evaluate for other genomic biomarkers that might predict response. This analysis revealed that 20 patients (25%) had alterations in HR genes (ie, *BRCA1*, *BRCA2*, *ATM*, *FANCA*, *PALB2*, *RAD51B*, and *RAD51C*), and interestingly, a post hoc analysis revealed that alterations in these genes predicted improved response rates irrespective of the treatment arm (PSA₅₀ response rates, 58% vs 39%; *P*=.013).

A contemporary phase 2 study reported by Chi and colleagues tested abiraterone vs the next-generation AR antagonist enzalutamide in patients with newly diagnosed mCRPC, with crossover following PSA progression.⁷² The coprimary endpoints were response and time to PSA progression following crossover. The study accrued 202 patients and randomized them equally between the groups. The PSA₅₀ response rates at 12 weeks were 53% for abiraterone and 73% for enzalutamide (*P*=.004). Circulating cell-free tumor DNA (ctDNA) was sequenced as part of this study, and in contrast to the results reported by Hussain and colleagues, the presence of deleterious *BRCA2* or *ATM* mutations (*n*=14) did not predict improved outcomes. Chi and colleagues instead found an association between HRD and shorter time to progression (hazard ratio, 5.34; *P*<.001).

We now have 2 studies with conflicting results regarding the use of HRD to predict response to AR-signaling inhibitors. To a certain extent, the study of Chi and colleagues confirms our biases derived from natural history studies that have revealed more aggressive biology in patients with DDR alterations.⁴³ Caution should be exercised, however, in relying too heavily on these results. Both analyses used exploratory secondary endpoints, with relatively small subsets of patients who had HRD in each trial. The assays used in these studies were also different; Hussain and colleagues relied on tissue sequencing, whereas Chi and colleagues used newer methods to sequence selected target genes from ctDNA samples. Finally, the definitions of a DNA repair lesion in the 2 studies may have been different, in terms of both the spectrum of genes included in the biomarker panel and the designation of pathogenicity (monoallelic vs biallelic).⁷³ Confirmatory studies to assess the efficacy of HRD as a predictive biomarker of response/resistance to AR-signaling inhibition are therefore needed.

Targeting Mismatch Repair Deficiency and Somatic Hypermethylation

The MMR pathway is responsible for correcting base-base mismatch and insertion-deletion loops, which occur during DNA replication and recombination. In tumors with MMR deficiency, long tracks of repetitive DNA sequences, known as microsatellites, are prone to strand slippage, which can result in persistent insertion-deletion loops and the rapid accumulation of mutations.¹⁸ As such, MMR-deficient tumors have been described as exhibiting a “mutator” phenotype, which is characterized by MSI (defined as differences in microsatellite tracks between normal germline DNA and somatic tumor DNA) and somatic hypermutation (≥ 10 mutations per megabase of coding DNA).⁷⁴

Lynch syndrome is a cancer predisposition syndrome characterized by germline loss of function of MMR genes and is a well-established risk factor for colorectal, endometrial, ovarian, and upper tract urothelial cancer in addition to other malignancies, including prostate cancer.^{18,75} This syndrome has most commonly been associated with alterations in genes involved in the MMR pathway, including *MLH1*, *MSH2*, *MSH6*, and *PMS2*, which occur in 32%, 39%, 15%, and 14% of cases of colorectal Lynch syndrome, respectively.⁷⁶ Clinically, this syndrome can be defined with the Amsterdam criteria, in which a germline alteration in an MMR pathway gene is assumed if a family meets the following criteria: (1) 3 or more family members with a Lynch syndrome–associated cancer; (2) 2 or more successive generations affected; and (3) 1 or more family members with cancer developing before the age of 50 years.^{77,78} The pathogenic role of MMR gene alterations in prostate cancer risk is not well defined, however. Pritchard and colleagues found deleterious germline MMR gene alterations in 4 of their cohort of 692 men (0.6%) with metastatic prostate cancer.⁵ Estimates of MMR mutations in metastatic prostate cancer (combined somatic and germline) are likely higher, however, with series reporting mutations in anywhere from 3% to 12% of cases. Rates of MMR deficiency may be higher in more aggressive histologic subtypes.^{17,79,80}

Defining the true incidence of MMR-deficient prostate cancer has been further challenged by the limitations of the assays commonly used to determine MSI status. Most MSI assays involve multiplex polymerase chain reaction (PCR) testing on a handful of genomic loci (the National Institutes of Health panel includes 5 microsatellite loci) and rely on comparisons of microsatellite loci amplified from tumors and matched normal controls.^{3,75,81} The loci tested in these assays and the threshold for declaring MSI have, for the most part, been validated and optimized to detect MSI only in colorectal cancer.

Because the performance of these PCR-based MSI assays for prostate cancer is unknown, clinicians should not rely too heavily on their results. Less-biased approaches for determining MSI status from next-generation sequencing data are available, and these tests may be more appropriate for noncolorectal histologies.⁸²

The determination of whether an MMR gene is altered in a prostate cancer is also challenged by the fact that hypermutated prostate cancers often occur as a consequence of complex structural genomic rearrangements in MMR genes.^{79,80,83} This contrasts with the inactivating mutations, loss of heterozygosity, and epigenetic silencing typical of colorectal cancers in patients with Lynch syndrome. Next-generation sequencing assays that sequence only the exons of target genes (which are the most common type of DNA-sequencing assays in clinical use) will therefore miss MMR gene alterations that arise as a result of rearrangements involving intronic regions. Assays that provide complete target gene coverage are more appropriate in this instance because they can accurately identify complex genomic rearrangements that may lead to MMR-deficient prostate cancer.⁷⁹ However, such assays are not in wide clinical use. A simpler screening approach could be to use standard IHC for MMR protein loss. For example, a recent paper used a validated IHC assay to screen 1176 primary prostate cancers for loss of MSH2, the most commonly inactivated MMR protein in prostate cancer. Although MSH2 deficiency was rare in the entire cohort (1%), MSH2 loss was enriched in patients with primary Gleason pattern 5 cancers (8%) and small cell prostate cancers (5%).⁸³ If these data can be replicated, screening for MSH2 inactivation in patients with primary Gleason 5 cancers and small cell prostate cancers might facilitate the identification of patients with MMR deficiency.

Because the loss of MMR gene function is often associated with a high mutational load, it has been hypothesized that individuals with this loss will have a higher tumor neoantigen burden, possibly predisposing them to respond to immune checkpoint inhibitors.^{14,84–86} Proof of concept that MMR-deficient tumors may respond well to checkpoint inhibition comes from a phase 2 study that tested the anti-programmed death 1 (anti-PD-1) agent pembrolizumab in patients who had metastatic carcinomas with and without MMR deficiency (ie, MSI-high and MSI-low carcinomas, respectively).¹⁴ In this study, 40% of the patients with MSI-high colorectal cancer had an immune-related objective response (irOR), compared with 0% of the patients with MSI-low colorectal cancer. Similarly, pembrolizumab was associated with a 50% response rate in patients with hypermutated noncolorectal gastrointestinal malignancies—supporting the hypothesis that mutational load may predict response to immune

checkpoint blockade in a range of malignancies. This study paved the way for the recent FDA approval of pembrolizumab in the treatment of patients with unresectable or metastatic MSI-high or MMR-deficient solid tumors that have progressed following prior treatment and who have no satisfactory alternative options. Of note, the approval of pembrolizumab for this indication is the FDA's first tissue-agnostic approval for a cancer therapy, which includes therapy for MMR-deficient advanced prostate cancer.¹⁵

Overall, immune checkpoint inhibitors have demonstrated only modest activity in unselected advanced prostate cancer, which may be a consequence of the relatively low mutational load observed in cohorts with unselected prostate cancer.⁸⁷ To date, the results of 2 phase 3 studies testing the anti-cytotoxic T-lymphocyte-associated antigen 4 (anti-CTLA-4) agent ipilimumab (Yervoy, Bristol-Meyers Squibb) in mCRPC have been negative.^{88,89} Similarly, rates of response to anti-PD-1 therapy in patients with unselected prostate cancer have been low, with no responses identified in the phase 1 study of nivolumab (Opdivo, Bristol-Meyers Squibb) and an objective response to single-agent pembrolizumab in only 13% of patients.^{90,91} It is worth noting, however, that a small trial testing combination enzalutamide plus pembrolizumab documented dramatic PSA declines in 3 of 10 patients.⁹² In that study, 2 responders had adequate tumor material for sequencing, and one of them was found to have underlying MSI—providing a partial explanation for the high response rate observed in that study. Cases of other patients with MSI-high prostate cancer responding to PD-1 pathway inhibitors have also been reported, and studies designed to determine the rate of response to immune checkpoint inhibitors in MSI-high mCRPC are planned (Durvalumab in Treating Patients With Metastatic Hormone-Resistant Prostate Cancer; NCT02966587).⁸⁰ In another recent study, 2 of 8 patients who had mCRPC and measurable disease achieved an objective response to a combination of ipilimumab and nivolumab; neither of the 2 responding patients had MSI or hypermutation.⁹³

Given that PARP inhibitors may be able to induce genomic instability, leading to neoepitope formation and enhanced sensitivity to checkpoint blockade, trials testing PARP inhibitors combined with PD-1 pathway inhibitors in advanced prostate cancer have also been launched. In an ongoing study testing the anti-programmed death ligand 1 (anti-PD-L1) agent durvalumab (Imfinzi, AstraZeneca) in combination with olaparib, 7 of 16 patients enrolled for longer than 2 months have had documented PSA₅₀ responses.⁹⁴ It should be noted that although most of the patients with a PSA₅₀ response had evidence of HRD, some patients with an intact HR pathway responded favorably to combination therapy. Therefore, the presence

of an HRD mutation or an MMR mutation may be neither necessary nor sufficient for a response to immune checkpoint inhibitors in prostate cancer.

Conclusion

During the past few years, our understanding of the recurrent molecular alterations defining advanced prostate cancer has increased dramatically. Somewhat unexpectedly, we have learned that a significant subset of men with this disease harbor alterations in DDR pathway genes, and precision oncology strategies designed to exploit these cellular vulnerabilities are being pursued actively, including in multiple large-scale efforts aimed at developing PARP inhibitors for patients who have prostate cancer with HRD. Several retrospective reports have also shown that platinum-based chemotherapy can be highly effective in patients with HRD, which is encouraging given that these drugs are readily available.^{12,62} In a similar vein, pembrolizumab has recently been approved for MSI-high or MMR-deficient advanced solid tumors, including prostate cancers, in patients who lack a reasonable alternative therapy. With this rapidly evolving treatment landscape, it is becoming increasingly important to define the genomic features of each patient's tumor so that all potentially beneficial therapies can be explored. However, as we strive toward a precision oncology framework for treating prostate cancer, critical issues surrounding the acquisition of tumor material for next-generation sequencing and the development of assays able that can accurately identify relevant somatic alterations are becoming apparent.

Currently, metastatic biopsy is the gold standard for obtaining tumor DNA for sequencing. Germline DNA assessments are insufficient because they do not capture all the relevant DDR pathway alterations used to guide therapeutic decision making. In addition, selective pressure during treatment can lead to clonal evolution, so that freshly obtained tumor DNA is preferred because it provides a snapshot of the current spectrum of mutations. Obtaining fresh tumor material is not a trivial matter, however. Prostate cancer is an osteotropic disease, and extracting DNA from osseous metastases for next-generation sequencing can be challenging.^{95,96} Metastatic biopsies are also painful, potentially morbid, and expensive. Fortunately, sequencing ctDNA is quickly becoming a viable alternative.⁹⁷ These so-called liquid tumor biopsies have the advantage of allowing genomic material to be sampled easily and repeatedly as needed.

Several commercial ctDNA sequencing assays are currently available; however, caution should be exercised before blood-based assays not optimized for use on prostate cancer samples are undertaken. For example, most com-

mercially available assays are not designed to identify accurately genomic copy number changes, which are some of the most frequent alterations found in mCRPC tumors.¹⁷ A number of groups are actively developing strategies to detect copy changes in ctDNA, and these approaches may provide a more accurate means for detecting the spectrum of mutational events that can lead to DDR pathway inactivation.⁹⁸⁻¹⁰⁰ Until these technologies are widely disseminated, however, metastatic biopsy should still be considered the standard for evaluating DDR pathway alterations.

Recurrent genomic rearrangements are another hallmark of prostate cancer, and many commercial sequencing assays—based on both ctDNA and tumor tissue—do not provide sufficient gene coverage to identify such changes accurately.¹⁰¹ This problem has specific relevance to MMR pathway genes because complex genomic rearrangements involving these genes have been described as a frequent cause of hypermutation in prostate cancers.⁷⁹ In addition, most PCR-based MSI assays rely on the testing of a limited number of microsatellite loci, which have been selected on the basis of data from colorectal cancer cohorts. Less-biased MSI assays that cover a larger number of microsatellite loci are currently available, however, and may be more appropriate for testing prostate cancers.¹⁰² With the recent approval of pembrolizumab for treating MSI-high and MMR-deficient tumors, it is increasingly important to choose tests that can accurately identify these alterations across a spectrum of tumor types.

Mutations affecting DDR pathway genes are both a liability—increasing the likelihood of cancer development—and potentially a therapeutic opportunity. Bridges first described the concept of synthetic lethality in the 1920s after observing that 2 mutations were necessary to induce lethality in a fruit fly, whereas either mutation in isolation had no effect on the insect's health.¹⁰³ Only recently have we applied these principles to treating prostate cancer, developing precision oncology strategies to select patients whose tumors have lost critical DDR pathway functionality. These tailored approaches for treating patients with advanced prostate cancer have tremendous potential and should provide hope that a wave of highly effective therapies are around the corner.

Disclosures

Dr Antonarakis has served as a paid consultant or advisor for Janssen, Astellas Pharma, Sanofi, Dendreon, Essa Pharma, Medivation, Merck, Clovis Oncology, and AstraZeneca; has received institutional research funding from Janssen, Johnson & Johnson, Sanofi, Dendreon, Genentech, Novartis, Tokai Pharmaceuticals, Merck, and AstraZeneca; and is a coinventor of a biomarker technology that has been licensed to Tokai Pharmaceuticals and Qiagen. Dr Schweizer has received institutional research funding from Janssen.

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