Androgen Receptor Gene Aberrations in Circulating Cell-Free DNA: Biomarkers of Therapeutic Resistance in Castration-Resistant Prostate Cancer

Arun A. Azad[1,2], Stanislav V. Volik[1], Alexander W. Wyatt[1], Anne Haegert[1], Stephane Le Bihan[1], Robert H. Bell[1], Shawn A. Anderson[1], Brian McConeghy[1], Robert Shukin[1], Jenny Bazov[1], Jack Youngren[3], Pamela Paris[4], George Thomas[5], Eric J. Small[3], Yuzhuo Wang[1,6], Martin E. Gleave[1,6], Colin C. Collins[1,6], and Kim N. Chi[1,2,6]

Abstract

Purpose: Although novel agents targeting the androgen–androgen receptor (AR) axis have altered the treatment paradigm of metastatic castration-resistant prostate cancer (mCRPC), development of therapeutic resistance is inevitable. In this study, we examined whether AR gene aberrations detectable in circulating cell-free DNA (cfDNA) are associated with resistance to abiraterone acetate and enzalutamide in mCRPC patients.

Experimental Design: Plasma was collected from 62 mCRPC patients ceasing abiraterone acetate ($n = 29$), enzalutamide ($n = 19$), or other agents ($n = 14$) due to disease progression. DNA was extracted and subjected to array comparative genomic hybridization (aCGH) for chromosome copy number analysis, and Roche 454 targeted next-generation sequencing of exon 8 in the AR.

Results: On aCGH, AR amplification was significantly more common in patients progressing on enzalutamide than on abiraterone or other agents (53% vs. 17% vs. 21%, $P = 0.02$, χ^2). Missense AR exon 8 mutations were detected in 11 of 62 patients (18%), including the first reported case of an F876L mutation in an enzalutamide-resistant patient and H874Y and T877A mutations in 7 abiraterone-resistant patients. In patients switched onto enzalutamide after cfDNA collection ($n = 39$), an AR gene aberration (copy number increase and/or an exon 8 mutation) in pretreatment cfDNA was associated with adverse outcomes, including lower rates of PSA decline $\geq 30\%$ ($P = 0.013$, χ^2) and shorter time to radiographic/clinical progression ($P = 0.010$, Cox proportional hazards regression).

Conclusions: AR gene aberrations in cfDNA are associated with resistance to enzalutamide and abiraterone in mCRPC. Our data illustrate that genomic analysis of cfDNA is a minimally invasive method for interrogating mechanisms of therapeutic resistance in mCRPC. *Clin Cancer Res; 21(10); 2315–24. ©2015 AACR.*

[1]Vancouver Prostate Centre, Vancouver, British Columbia, Canada. [2]Department of Medical Oncology, British Columbia Cancer Agency, Vancouver, British Columbia, Canada. [3]Helen Diller Family Comprehensive Cancer Center, University of California at San Francisco, San Francisco, California. [4]Department of Urology, UCSF, UCSF Diller Comprehensive Cancer Center, San Francisco, California. [5]Department of Pathology and Laboratory Medicine, OHSU Knight Cancer Institute, Portland, Oregon. [6]University of British Columbia, Vancouver, British Columbia, Canada.

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

A.A. Azad, S.V. Volik, and A.W. Wyatt contributed equally to this article.

C.C. Collins and K.N. Chi share senior authorship.

Corresponding Authors: Kim N. Chi, Department of Medical Oncology, BC Cancer Agency, 600 West 10th Avenue, Vancouver, BC V5Z 4E6, Canada. Phone: 604-877-6000; Fax: 604-877-0585; E-mail: kchi@bccancer.bc.ca; and Colin C. Collins, Vancouver Prostate Centre, 899 12th Avenue West, Vancouver, BC V5Z 1M9, Canada. Phone: 604-779-9287; Fax: 604-875-5654; E-mail: ccollins@prostatecentre.com

doi: 10.1158/1078-0432.CCR-14-2666

©2015 American Association for Cancer Research.

Introduction

Persistent activation of the androgen–androgen receptor (AR) axis underlies resistance to androgen deprivation therapy and progression to metastatic castration-resistant prostate cancer (mCRPC; ref. 1). This knowledge has underpinned the development of novel agents targeting the androgen–AR axis, including abiraterone acetate and enzalutamide. Both agents have proven clinical efficacy in mCRPC (2–5); however, disease progression on treatment is inevitable and poses a major clinical challenge. Although incompletely understood, resistance to abiraterone and enzalutamide has been attributed to a variety of mechanisms, including reactivation of the androgen–AR axis via AR gene aberrations such as AR amplification and AR exon 8 mutations (6–10). A key barrier to better understanding mechanisms of treatment resistance in mCRPC is the difficulty of obtaining biopsies from bone and deep intra-abdominal lymph nodes, the commonest sites of metastatic disease. Therefore, developing less invasive means of sampling tumor material is essential for investigating therapeutic resistance in mCRPC.

In recent years, circulating cell-free DNA (cfDNA) has emerged as a promising and minimally invasive biomarker for profiling the

Translational Relevance

Analysis of circulating cell-free DNA (cfDNA) is a promising and minimally invasive approach for characterizing the tumor genome in metastatic castration-resistant prostate cancer (mCRPC). We performed chromosome copy number analysis and androgen receptor (AR) exon 8 sequencing on cfDNA extracted from plasma of 62 mCRPC patients progressing on systemic therapy, including abiraterone acetate and enzalutamide. Aberrations in the AR (copy number increase and/or mutations) were observed in over half of our cohort, with AR amplification being linked to enzalutamide resistance. In patients who commenced enzalutamide following cfDNA collection, detection of an AR gene aberration in pretreatment cfDNA was significantly associated with lower PSA response rates and shorter time to progression. Our data collectively identify AR aberrations in cfDNA as biomarkers linked to treatment resistance and illustrate the potential for cfDNA to serve as a liquid biopsy in mCRPC patients.

tumor genome (11). An estimated 3% of tumor DNA is released into the blood daily, most likely originating from apoptosis and necrosis of cancer cells (12). Elevated levels of cfDNA have been described in patients with malignancies, including prostate cancer, being four to six times that of healthy controls (12). Importantly, studies using targeted and whole-genome approaches in metastatic prostate cancer patients have identified somatic genomic alterations in cfDNA, including amplification (13) and mutations (7, 8). However, the utility of cfDNA for interrogating resistance mechanisms in mCRPC remains uncertain.

We hypothesized that AR gene aberrations, including amplification and mutations in the ligand-binding domain (LBD), are detectable in cfDNA and would be associated with resistance to novel therapies targeting the androgen–AR axis. Using plasma samples collected from mCRPC patients progressing on systemic therapy, including abiraterone and enzalutamide, the aim of this study was to elucidate possible mechanisms of treatment resistance through chromosome copy number analysis and AR exon 8 sequencing of cfDNA.

Materials and Methods

Patient cohort

Sixty-two patients with mCRPC were recruited at the British Columbia Cancer Agency (BCCA) - Vancouver Centre, Vancouver, Canada between August 2013 and March 2014. End-of-treatment samples were collected from patients with evidence of biochemical or radiologic progression (as defined by Prostate Cancer Working Group 2 criteria; ref. 14) or clinical progression [defined as worsening disease-related symptoms necessitating a change in anticancer therapy and/or deterioration in Eastern Cooperative Group Performance Status (ECOG PS) $\geq$ 2 levels; ref. 15] on systemic therapy for mCRPC. Thirty-nine of 62 (63%) patients were switched onto enzalutamide after collection of cfDNA. Clinicopathological characteristics, including prior and subsequent therapies, were recorded for each patient. The University of British Columbia (Vancouver, British Columbia, Canada) Research Ethics Board approved this study and all patients provided informed written consent.

Collection and processing of peripheral blood samples

Four EDTA tubes (6 mL each) of whole blood were collected per patient. Within 1 to 2 hours of collection, 150 µL of 10% buffered formalin was added to each tube. Plasma was removed after centrifugation at 1,600 rpm at 4°C (2 × 10 minutes) and stored in 1-mL aliquots at −80°C.

DNA extraction and quantification

cfDNA extraction, amplification, sequencing, and analyses were performed at the Vancouver Prostate Centre by investigators who did not have access to clinical data and were blinded to patient outcome on therapy. DNA was isolated from plasma with the Qiagen Circulating Nucleic Acids Kit (Cat No 55114), using 6 mL of plasma per patient as per manufacturer's instructions. DNA quantification was performed on a Qubit 2.0 Fluorometer (Life Technologies) using Qubit dsDNA HS Assay Kit according to the manufacturer's instructions.

Array comparative genomic hybridization of cfDNA

Plasma DNA was amplified with the Sigma WGA2 kit (Cat No WGA2-10rxn or WGA2-50rxn) as per manufacturer's instructions. Up to 50 ng of sample DNA was amplified with an equal amount of normal reference DNA amplified concurrently. Following quantification by the NanoDrop Spectrophotometer, 2.5 µg of WGA DNA was used as input for labeling for CGH (to control for WGA2-related artifacts). Samples and reference were labeled using the NimbleGen method, which uses Cyanine dye labeled random nonamers (Cy5 for samples and Cy3 for reference) and Klenow 3′-5′exo-to enzymatically label and amplify the DNA. Labeled samples were purified by isopropanol precipitation, and quantified by the NanoDrop spectrophotometer. Equal amounts (5 µg) of Cy5-sample and Cy3-reference were applied to Agilent CGH microarrays (Agilent SurePrint G3 Human Catalog CGH 8 × 60 K, Design ID 021924), hybridized overnight at 65°C, washed, scanned with the Agilent DNA Microarray Scanner, and quantified with Agilent Feature Extraction software. Comparative genomic hybridization (CGH) data were visualized and analyzed using Biodiscovery Nexus 7 software, which includes the GISTIC algorithm. To account for dilution of tumor DNA with "normal" circulating DNA, we relaxed the default thresholds for copy number calls to 0.1 and −0.1 log$_2$ (signal intensity of tumor vs. reference) for gain and loss, respectively. A log$_2$ ratio of >1.2 was termed high-level gain (i.e., amplification). Raw array comparative genomic hybridization (aCGH) data are available at Gene Expression Omnibus accession number GSE61134 (16).

Androgen receptor exon 8 sequencing

PCR was used to amplify a fragment of the AR (encompassing exon 8) using Q5 Hot Start High-Fidelity DNA Polymerase (NEB M0493L) and the following primers, which contain the relevant MID and fusion sequences necessary for the Roche 454 GS FLX+ system, software version 2.9: Forward (5′ to 3′) CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG CGT GTC TCT ATC AAC CCT GTT TTT CTC CCT CTT ATT; Reverse (5′ to 3′) CCT ATC CCC TGT GTG CCT TGG CAG TCG AAA TAG GGT TTC CAA TGC TTC ACT. The resulting 279 base-pair amplicons were run on an agarose gel (1.5%) and imaged. Normalization and pooling of each amplicon were accomplished by quantifying the fluorescence intensities of each amplicon (from the gel) and adding the relative amount (in µL) needed to reach highest intensity. The separate pools were

then subjected to AMPure (Agencourt A63881) clean-up to ensure no primers or nucleotides were carried into downstream workflows. Each pool was processed through the Lib-L Roche 454 emulsion PCR protocol and subsequently sequenced using the XLR70 sequencing kit as outlined by the manufacturer. For data analysis, raw reads were mapped to the human genome (hg19) using BWA and visualized using the Integrative Genomics Viewer (IGV). Mutation frequency was quantified with SAMTOOLS. For positive controls, we used three prostate cancer cell lines [LNCaP (harbors T877A), 22rv1 (harbors H874Y), and LAPC4 (wild-type AR)]). DNA was extracted directly from these cell lines and processed through the same pipeline as patient samples (amplification, sequencing, mapping, and mutation calling). Matched white blood cell (WBC) DNA served as negative controls. On three random spots on the plate, we used blanks (water) to control for contamination. Roche 454 technology is susceptible to specific artifacts (typically adjacent to homopolymer runs) that tend to be present in all sequence libraries. To filter these artifacts, we excluded variant calls that were detected in more than 75% of sequenced samples (this arbitrary filter could be varied significantly with the same end result), since no true somatic mutation is likely to exist at such high recurrence. For mutation calling, we set a threshold of 12 nonreference reads, which was three times the maximum nonreference frequency (i.e., noise) observed in WBC DNA samples. For samples with multiple mutations, we performed an estimation of haplotype frequency through careful manual curation of aligned reads in IGV. Reads that had >7 mismatches with reference sequence were considered uninformative and were excluded. The remaining high confidence reads were counted and used for frequency estimation. To validate the 454 results, we resequenced AR exon 8 amplicons on the Illumina MiSeq platform. An additional PCR was carried out to adapt these amplicons to the Illumina MiSeq platform using the following primers: Forward (5′-3′) TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCA TCT CAT CCC TGC GTG TCT CCG AC and Reverse (5′-3′) GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GCC TAT CCC CTG TGT GCC TTG GCA GTC (the overlap with the 454 primers is shown in bold). After adding index sequences to allow multiplexing of libraries and sequencing, the resulting amplicons were purified by AMPure (twice) and quantified via KAPA Library Quantification Kit (cat# KK4835). The libraries were then normalized to 2 nmol/L. Sequencing, using a 500 cycle, 1M read kit (cat# MS-103-1003) was carried out as per manufacturer's instructions.

Circulating tumor cell enumeration

Circulating tumor cell (CTC) enumeration was performed as previously described (17). Seven and one-half milliliters of whole blood was collected in evacuated blood tubes (CellSave; Janssen), stored at room temperature for no more than 96 hours, and then processed as per manufacturer's instructions on the CellSearch System (Janssen). CTCs were defined as nucleated cells expressing cytokeratin but not CD45.

Array comparative genomic hybridization of tumor biopsies

Approximately 50 cells were obtained from each biopsy sample through laser microdissection. Five microliters of cell extraction buffer was pipetted onto the tissue to dislodge cells and then transferred to a PCR tube. The cells were amplified using the Picoplex WGA kit (New England Biosciences) and the amplified

product was purified using the QIAquick PCR purification kit (Qiagen). The DNA concentration was quantified using Nanodrop and the quality of DNA was verified by gel electrophoresis. Of note, 500 ng of amplified tumor sample was run on SurePrint G3 human CGH 8 × 60 K arrays (Agilent Technologies) along with commercially available reference DNA (Promega) according to manufacturer's protocol. Feature Extraction and Cytogenomics software (Agilent Technologies) was used to analyze and detect copy number aberrations. To compare the copy number profiles of tumor biopsies and cfDNA, we calculated correlation coefficients for every possible pair. Because normality could not be assumed, we then applied a Wilcoxon rank-sum test to determine whether matched pairs differed from nonmatched.

FISH of tumor biopsies

FISH was performed with AR (Xq12) and X chromosome centromere probes. Fifty interphase cells were scored. Cutoff for amplification: ≥2.0.

Human cancer cell lines

The LNCaP cell line was kindly provided by Dr. Leland W.K. Chung (1992, MDACC, Houston TX) and tested and authenticated by whole-genome and whole-transcriptome sequencing in July 2009. 22Rv1 cells were originally obtained from ATCC (2008 and 1989, ATCC authentication by isoenzymes analysis). During the course of our study, each cell line was also authenticated using aCGH.

Statistical analysis

Frequency of copy number changes and AR exon 8 mutations in end-of-treatment samples were compared between patients progressing on abiraterone, enzalutamide, or other agents using χ^2 test. In patients switched onto enzalutamide after cfDNA collection, univariate analysis was performed to identify variables associated with PSA response (χ^2 for categorical variables or logistic regression for continuous variables) and clinical and/or radiographic progression-free survival (PFS; Cox proportional-hazards modeling). Multivariate analysis of PFS was performed using factors that were significant ($P < 0.05$) on univariate analysis.

Results

Patient cohort

Plasma was collected from 62 mCRPC patients with evidence of disease progression on abiraterone ($n = 29$), enzalutamide ($n = 19$), or other systemic agents ($n = 14$). A summary of patient characteristics at collection of cfDNA is presented in Table 1. Median time from diagnosis of CRPC to cfDNA collection was 28.3 months [interquartile range (IQR) range: 16.3–40.9]. Patients had been treated with a median of three lines of systemic therapy (range: 1–8) beyond LHRH antagonists/agonists. Mean and median DNA concentration was 6.38 ng/μL and 1.47 ng/μL, respectively (IQR range: 0.9–4.0).

AR gene amplification in cfDNA associated with treatment resistance

We used aCGH to profile the genome copy number of all 62 cfDNA samples (Supplementary Table S1). Profiles were strikingly concordant with published prostate cancer datasets (Fig. 1A), strongly implying sampling of tumor DNA (18). Over half of the

Table 1. Baseline characteristics at collection of cfDNA

Characteristic (*n* = 62)	
Age	
Median (IQR)	72 (49–92)
Gleason score, *n* (%)	
6–7	14 (23)
8–10	38 (61)
Unknown	10 (16)
Disease sites, *n* (%)	
Bone	60 (97)
Lymph nodes	33 (53)
Visceral	8 (13)
ECOG, *n* (%)	
0–1	41 (66)
≥2	21 (34)
Laboratory	
Hemoglobin (median, g/L; IQR)	122 (108–128)
≤100, *n* (%)	12 (19)
LDH (median, U/L; IQR)	209 (174–316)
Elevated (>250), *n* (%)	26 (42)
ALP (median, U/L; IQR)	144 (93–320)
Elevated (>160), *n* (%)	30 (48)
Albumin (median, g/L; IQR)	39 (32–41)
Low (<35), *n* (%)	14 (23)
Normal, *n* (%)	33 (53)
Unknown, *n* (%)	15 (24)
Current treatment	
Abiraterone acetate	29 (47)
Enzalutamide	19 (31)
Bicalutamide	6 (10)
Prednisone	3 (5)
Docetaxel	3 (5)
Other	2 (3)
Outcomes on current treatment	
Best PSA response	
PSA decline ≥ 50%	26 (42)
PSA decline < 50%	20 (32)
No PSA decline	16 (26)
Median treatment duration (mo)	5.4
All treatments received	
Abiraterone acetate	46 (74)
Enzalutamide	19 (31)
Docetaxel	33 (53)
Orteronel	4 (6)
Bicalutamide	58 (94)
Flutamide	10 (16)
Nilutamide	10 (16)
Other	27 (44)

cohort (34/62) exhibited at least one prostate cancer-associated copy number variation including 8p loss (19/62, 31%), 8q gain (27/62, 44%), AR gain/amplification (28/62, 45%; Fig. 1B), and *RB1* loss (18/62, 29%), all of which occurred at a frequency expected in a CRPC cohort (18–20). Other copy number variations included copy number increase of *CCND1*, *CCNE1*, *MYC*, and *MET* (Fig. 1C), which were present in 16% (10/62), 10% (6/62), 39% (24/62), and 16% (10/62) of patients, respectively.

Although AR copy number gain is rarely present in hormone-naïve primary disease, elevated AR gene copy levels in CRPC are a common aberration linked to disease progression (21). Reassuringly therefore, focal AR copy number increase in cfDNA was highly significant by GISTIC analysis across the patient cohort (Q-bound significance = 3.36E-6; G-score = 11.941). However, the frequency of AR amplification was significantly higher in patients progressing on enzalutamide than patients progressing on abiraterone or other agents (53% vs. 17% vs. 21%, $P = 0.02$; χ^2;

Supplementary Table S2). This was in contrast with the frequency of other prostate cancer-associated copy number changes (e.g., 8q gain), which showed no significant difference between treatment groups. Furthermore, median cfDNA concentration exhibited only minimal variation between patients progressing on abiraterone (1.64 ng/µL; IQR 1–3.46) and enzalutamide (1.77 ng/µL; IQR 0.98–4.85). Although 16 of 19 patients progressing on enzalutamide had previously received abiraterone, these data point to AR amplification as a key mechanism of resistance to enzalutamide.

To determine whether cfDNA data (generated inherently from "dead" cells) are representative of actual metastases, we analyzed matched metastatic tumor biopsies collected from 7 patients enrolled on the SU2C/PCF/AACR West Coast Prostate Cancer Dream Team study (WCDT). All 5 patients with AR amplification in cfDNA had AR amplification detected by FISH on tumor tissue. The remaining 2 patients had single copy gain of AR in cfDNA and as expected, neither had AR amplification present on FISH. For 5 of these 7 patients, we performed additional copy number profiling of tumor biopsies using aCGH. Notably, matched pairs (cfDNA and biopsy from the same patient) were significantly more correlated than nonmatched pairs ($P = 3.764e\text{-}05$; Wilcoxon rank-sum test) and AR status was completely concordant across all 5 samples (Supplementary Fig. S1). Two patients, VC-008 and VC-013, had *CCND1* and *MYC* amplification, respectively, in their cfDNA and both amplifications were detectable in their matched tumor biopsy (Supplementary Fig. S1). A third patient (VC-051) had *CCNE1* amplification in cfDNA that was not detectable in his biopsy (despite similar copy number profiles overall), possibly reflecting tumor heterogeneity and highlighting recognized sampling limitation of biopsies.

Deep sequencing reveals AR mutations in cfDNA

Mutations of the LBD of the AR can modulate ligand and cofactor affinity, and result in gain-of-function through increased sensitivity to other steroid ligands (e.g., progesterone, adrenal androgens) or the conversion of direct inhibitors (e.g., bicalutamide) into agonists (22). Although primary prostate tumors seldom demonstrate AR mutations (7), they are found in 10% to 20% of patients with late-stage CRPC (19, 20), most commonly in the LBD (22). Several of these mutations are localized to exon 8 in the AR, including H874Y, T877A, and F876L, which have been linked to enzalutamide resistance *in vitro* and was detected in cfDNA from 3 of 27 prostate cancer patients progressing on the novel AR antagonist ARN-509 using PCR-based BEAMing technology (8, 9). We hypothesized that mutations in exon 8 of the AR may contribute to resistance in a subset of our patients progressing on enzalutamide or abiraterone and would be detectable in their cfDNA at progression. Given the low quantity of cfDNA and the high "dilution" with normal circulating DNA, we adopted a targeted approach using Roche 454 technology to provide deep sequencing coverage (>10,000X) across the coding region of AR exon 8 (52 codons). Unlike aCGH, DNA was not amplified for the purposes of sequencing. Samples from 14 of 62 patients were not amenable to sequencing due to low DNA quantity or concentration.

To ensure integrity of our sequencing data, several different controls were used, including water blanks (which produced < 5 reads), seven matched WBC DNA samples (which had a maximum of four nonreference reads at any base), and three prostate
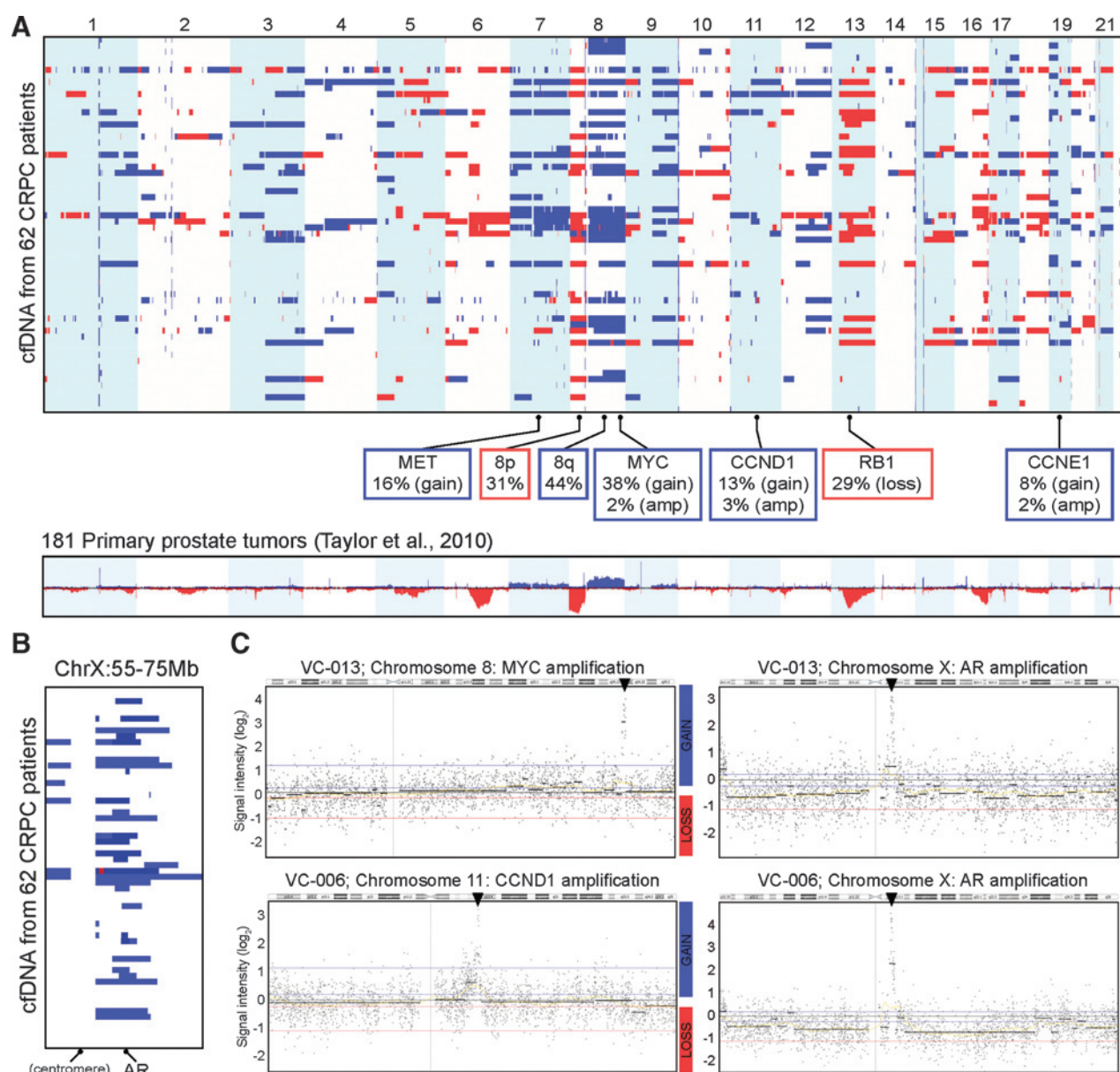
Figure 1.
Copy number analysis of cfDNA. A, a pile-up of the aCGH results from all patients showing gains (blue) and losses (red). Important genes are annotated together with their aberration frequency across the cohort. Note the broad similarity to the aggregate of a published dataset (18) of copy number calls from fresh-frozen DNA of prostate tumors (below). B, aCGH results of a 20 Mbp region on Chromosome X showing focal gain/amplifications (blue bars) at the AR loci in almost half the cohort. C, examples of focal amplifications of oncogenes robustly observed in two different patients.

cancer cell lines: 22RV1, LNCaP, and LAPC4. Importantly, expected mutations were detected at 100% frequency in 22RV1 (H874Y) and LNCaP (T877A) cells, whereas no mutations were found in AR wild-type LAPC4 cells. A threshold of 12 nonreference reads was set for calling mutations as this was three times the maximum nonreference frequency (i.e., noise) observed in WBC DNA samples.

In total, six different base changes in the coding region of exon 8 were robustly detected in 11 of 62 (18%) patients (Table 2). Further underlining the validity of results, all six changes result in an amino acid substitution: H874Y ($n = 5$), T877A ($n = 2$),

D879E, L881I, E893K, and M895V (all $n = 1$; Fig. 2A). Mutation frequency in cfDNA ranged from 0.09% to 17.75%, likely reflecting the significant dilution effect of nontumor DNA. Of note, AR exon 8 mutations were mutually exclusive with AR copy number increase in 8 of 11 (73%) patients, whereas three of the mutations detected (D879E, L881I and E893K) have not been previously identified in patients with prostate cancer. In contrast with AR copy number gain described above, the incidence of mutations was not significantly different between patients progressing on abiraterone (7/29), enzalutamide (3/19), or other agents (1/14; $P = 0.38$; χ^2). However, all 7 patients with H874Y or T877A

Table 2. Summary of mutations detected in AR exon 8 in cfDNA

Sample	Base change	Amino acid change	Mutant read count	Wild-type read count	Total read count	Percent mutant	ARDB	Validated (MiSeq)
VC-005	ChrX:66943600 G>A	E893K	170	11,969	12,148	1.40	—	Yes
VC-012	ChrX:66943606 A>G	M895V	1271	6,167	7,447	17.07	PCa	Yes
VC-015	ChrX:66943552 A>G	T877A	231	9,720	9,955	2.32	AIS/PCa	n/a
VC-017	ChrX:66943552 A>G	T877A	101	12,854	12,960	0.78	AIS/PCa	No
VC-022	ChrX66943560 C>A	D879E	12	11,165	11,180	0.11	—	No
VC-023	ChrX:66943543 C>T	H874Y	12	12,917	12,929	0.09	PCa	n/a
VC-040	ChrX:66943543 C>T	H874Y	488	7,570	8,058	6.06	PCa	n/a
VC-041	ChrX:66943543 C>T	H874Y	1,570	7,277	8,847	17.75	PCa	n/a
VC-053	ChrX:66943543 C>T	H874Y	140	9,105	9,245	1.51	PCa	n/a
VC-063	ChrX:66943543 C>T	H874Y	279	14,879	15,158	1.84	PCa	Yes
VC-064	ChrX:66943564 C>A	L881I	17	15,699	15,716	0.11	PAIS	n/a
VC-012 [2nd sample]	ChrX:66943543 C>T	H874Y	49	10,364	10,413	0.47	PCa	Yes
VC-012 [2nd sample]	ChrX:66943549 T>C	F876L	146	10,242	10,388	1.41	PCa[1]	Yes
VC-012 [2nd sample]	ChrX:66943552 A>G	T877A	511	9,762	10,280	4.97	AIS/PCa	Yes
VC-012 [2nd sample]	ChrX:66943585 A>G	S888G	158	9,143	9,313	1.70	—	Yes
VC-012 [2nd sample]	ChrX:66943606 A>G	M895V	38	9,206	9,254	0.41	PCa	Yes
VC-041 [2nd sample]	ChrX:66943543 C>T	H874Y	4743	15,683	20,426	23.22	PCa	n/a

NOTE: ARDB column: The AR mutation database was downloaded from http://androgendb.mcgill.ca/ARDB.xls.
Abbreviations: PCa, a mutation was reported to be present in prostate cancer; AIS, a mutation was reported to be present in Androgen Insensitivity Syndrome; PAIS, a mutation was reported to be present in Partial Androgen Insensitivity Syndrome; AIS/Pca, a mutation was reported to be present both in PCa and in AIS. Validated (MiSeq) Column: Yes, mutation validated; No, mutation not validated; n/a, validation not performed.

mutations were progressing on abiraterone at cfDNA collection or had been treated with prior abiraterone.

To validate our data, samples with sufficient remaining DNA were subjected to AR exon 8 resequencing using the Illumina MiSeq platform. All tested mutations were redetected, with the exception of two that had very low frequency in the discovery run (Table 2). To further validate mutation findings, we obtained a second cfDNA sample from 2 patients (VC-012 and VC-041), this time at progression on enzalutamide (the first cfDNA sample was collected at progression on abiraterone before commencing enzalutamide). Reassuringly, the initial mutations were redetected for both patients using Roche 454 sequencing (Table 2). For VC-012, four additional mutations arose in the second sample, including F876L (the first instance of this mutation being detected in an enzalutamide-resistant patient) and S888G (not previously reported in patients with prostate cancer). Interestingly, although five total mutations were found in the second sample from VC-012, no sequencing read contained more than two mutations simultaneously (Fig. 2B). Manual curation of high quality reads suggested that there were six different AR gene variants, likely reflecting diverse intra-patient AR heterogeneity. Of note, VC-012 experienced rapid disease progression (2.3 months) on enzalutamide.

### Integrated copy number and AR mutation profile of cfDNA

A summary of copy number changes and AR exon 8 mutations detectable in cfDNA for each patient is presented in Fig. 3. Overall, 40 of 62 (65%) patients had a prostate cancer-associated copy number change and/or an AR exon 8 mutation present in cfDNA, strongly implying the presence of tumor-
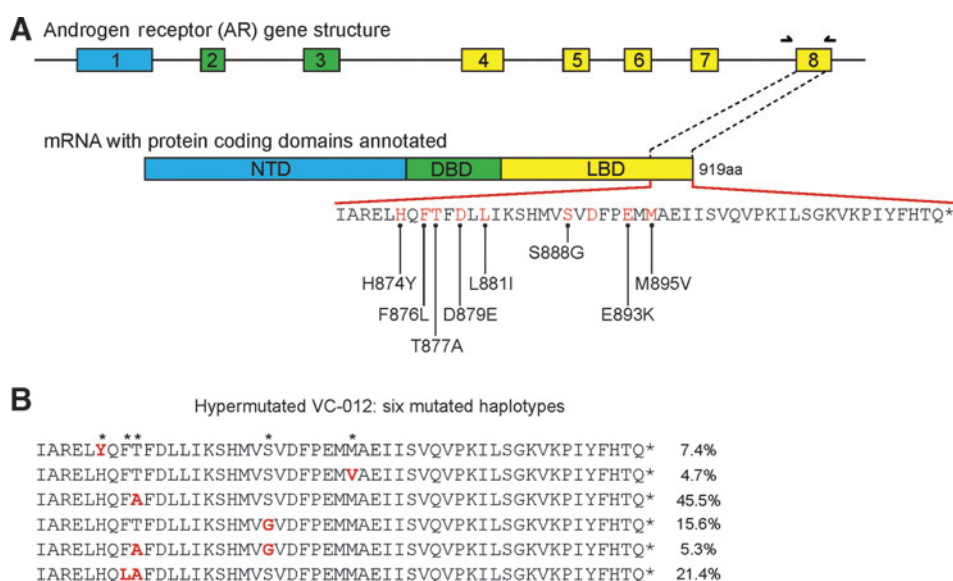
Figure 2.
A, mutations in the LBD of the AR gene detected in cfDNA. Representation of the AR domain structure, indicating the amino acid sequence that was sequenced for this analysis (NTD, N-terminal domain; DBD, DNA-binding domain). The mutations identified in this study are annotated on the amino acid sequence. B, the different AR LBD haplotypes predicted in VC-012 from examining individual sequence reads from his second cfDNA sample. The relative frequency of each haplotype is indicated.

Figure 3.
Integrated copy number and AR exon 8 mutation data in cfDNA for 62 patients on study. Left column, prior treatments denoted by black squares, treatment at time of cfDNA collection denoted by "P". Middle column, aCGH findings. Copy number loss and increase denoted by red and blue squares respectively. Up-pointing arrow denotes amplification. Right column, 454 sequencing data. Green squares denote mutations detected. Shaded grey rows denote patients with insufficient cfDNA for analysis.
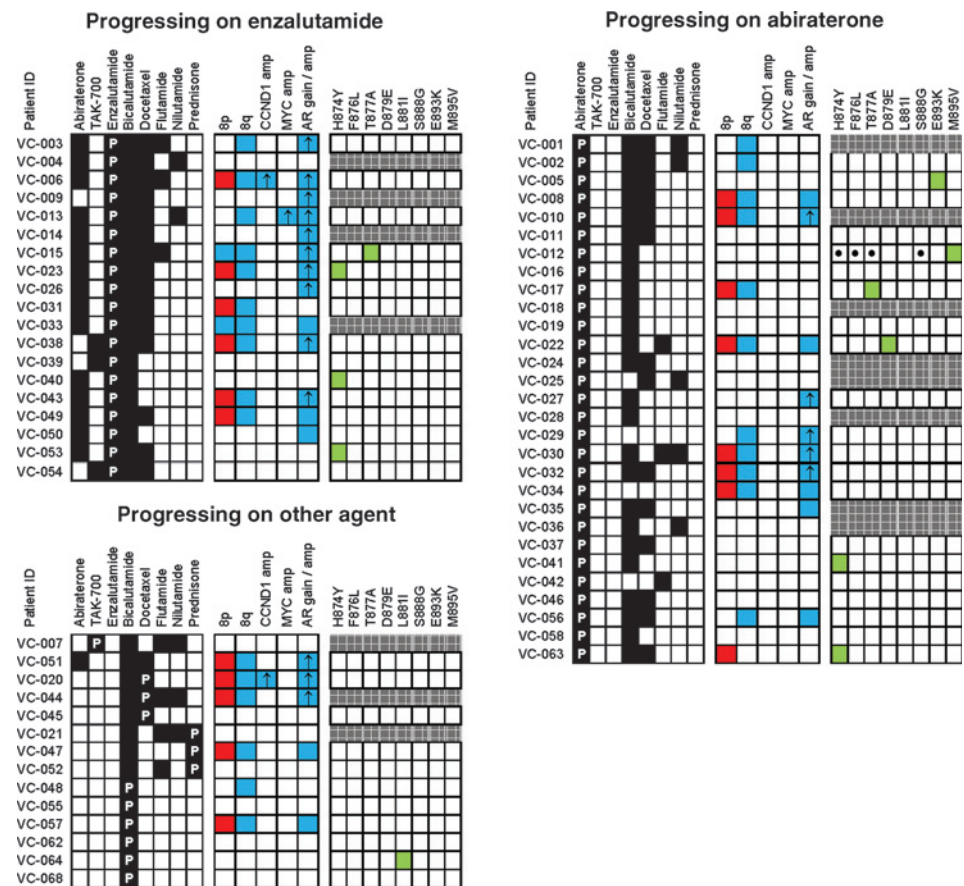
derived cfDNA. AR gene aberrations (copy number increase and/or mutation) were observed in 36 of 62 (58%) patients. Clinicopathological factors significantly associated with the presence of tumor-derived cfDNA included treatment with any of docetaxel, abiraterone, or enzalutamide, serum ALP > 160 IU/L and CTC count $\geq$ 5 cells/7.5 mL blood (Supplementary Table S3). In patients without detectable ctDNA, the median CTC count was 1 (IQR 0–3).

AR aberrations as predictive biomarkers in pretreatment cfDNA

Of the 62 patients on study, 39 were switched from abiraterone or other systemic therapy onto enzalutamide after cfDNA collection. Of these patients, 19 of 39 (49%) possessed an AR gene aberration in cfDNA, including 14 with copy number increase and 5 with the following mutations: H874Y ($n = 2$), E893K, M895V, and T877A (all $n = 1$). To identify predictive biomarkers in this cohort, genomic changes of the AR in pretreatment cfDNA and other baseline clinicopathological variables were correlated with outcomes on enzalutamide. As shown in Supplementary Table S4, an AR gene aberration in pretreatment cfDNA was significantly associated with lower rates of PSA decline $\geq$ 30% (21% vs. 60%; $P = 0.013$) on enzalutamide with a strong trend toward significance for PSA decline $\geq$50% (21% vs. 50%; $P = 0.06$). No other variables were significantly associated with PSA decline $\geq$ 30% or $\geq$50%. The PSA waterfall plot stratified by AR gene status is shown in Fig. 4A.

In patients with and without an AR gene aberration, median clinical/radiographic PFS was 2.3 months (95% confidence inter-

vals; CI, 1.6–3.1) versus 7.0 months (95% CI, 2.7–11.3), respectively ($P < 0.001$, log-rank) on Kaplan–Meier analysis (Fig. 4B). Significantly, although H874Y-mutated prostate cancer cells are sensitive to enzalutamide *in vitro* (8), both patients with an H874Y mutation in pretreatment cfDNA progressed rapidly on enzalutamide (2.4 and 2.9 months, respectively). On univariate Cox proportional hazards regression analysis (Supplementary Table S5), the following factors were significantly associated with PFS on enzalutamide: presence of an AR gene aberration, AR copy number increase, prior docetaxel, visceral metastases, ECOG PS, serum ALP, and CTC count. Incorporating these variables into a multivariate model (with the exception of AR copy number increase), only an AR gene aberration in cfDNA remained a significant predictor of PFS (Supplementary Table S5).

Discussion

In this study, we identified widespread genomic aberrations in cfDNA from a large cohort of mCRPC patients progressing on systemic therapy. Over half of our cohort had an AR gene aberration, with enzalutamide resistance linked to AR amplification. Likewise, in a cohort of patients switched onto enzalutamide after cfDNA collection, the presence of pretreatment AR gene aberrations was predictive for adverse clinical outcomes.

The interrogation of cfDNA shows great promise for advancing precision oncology, particularly in prostate cancer where tissue biopsies are logistically challenging. However, the low overall yield and variable dilution of tumor DNA with "normal" DNA
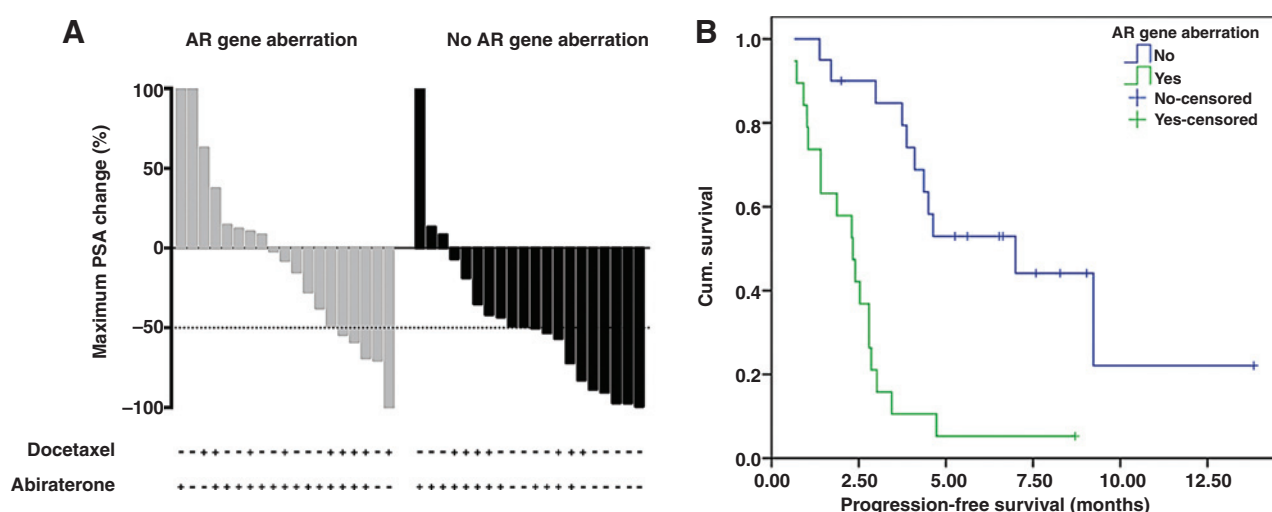
Figure 4.
A, waterfall plot of maximum % PSA change from baseline stratified by AR gene status. Prior treatment with abiraterone or docetaxel as indicated. B, Kaplan–Meier analysis of radiographic/clinical PFS in patients (*n* = 39) switched onto enzalutamide after collection of cfDNA. PFS is shown for patients with and without an AR gene aberration (copy number increase and/or an exon 8 mutation) in pretreatment cfDNA.

poses a significant challenge for clinical integration. In our study, robust detection of copy number changes was only possible in patients where tumor DNA was not heavily diluted. Although this represented the majority of our patients, in those samples with no apparent copy number changes, it is not possible to distinguish between the absence of copy number changes in a patient's tumor, or insufficient tumor DNA yield (although the burden of copy number changes in typical CRPC suggests the latter explanation more likely). As demonstrated recently (7), this problem may be overcome in patients by obtaining several temporal samples (maximizing the chance that at least one will contain tumor DNA), although this is not always feasible in late-stage disease. Nevertheless, even in the solitary samples obtained here, we observed remarkable concordance to matched tissue biopsies where available, suggesting that cfDNA copy number analysis will accurately inform on *in situ* disease in most patients. Mutation profiling provides a different challenge, since deep sequencing can overcome dilution effects, but can be compromised by low-level platform-specific noise. Use of two sequencing platforms with different types of artifacts, as used in this study, can mitigate this concern, and provide a level of validation. We drew additional comfort from temporal sampling in two patients confirming mutation presence, and the complete lack of synonymous mutations across the cohort, implying selection.

A key finding of our study is that AR amplifications appeared to be associated with resistance to enzalutamide. Although enzalutamide progressing patients were more heavily treated than abiraterone patients, we observed no significant difference in the detection rates of other copy number changes, suggesting that the enrichment of AR amplifications in enzalutamide-resistant patients was not driven merely by tumor DNA yield. Similarly, the WCDT study reported that 4 of 6 (67%) enzalutamide-resistant but only 1 of 11 (9%) abiraterone-resistant patients had AR amplification on FISH of metastatic tumor biopsies (6). Anecdotal evidence also linked AR exon 8 mutations in cfDNA with enzalutamide resistance, as evidenced by the emergence of four new mutations (including F876L) in a posttreatment sample

collected from a patient with rapid disease progression on enzalutamide. This finding may reflect the presence of a mutation in mismatch repair genes such as *MSH2* and *MSH6*, which were recently linked to a hypermutated phenotype in CRPC patients (23).

Although AR amplification did not appear to correlate with resistance to abiraterone, it is notable that all patients with an H874Y or T877A AR mutation were progressing on abiraterone or had previously received abiraterone. In tumor biopsies from mCRPC patients, the emergence of progresterone-responsive T877A mutations was recently reported to be associated with abiraterone resistance (10). Together with our data, this implicates T877A mutations as a possible factor in the development of resistance to abiraterone. To the best of our knowledge there are currently no data linking H874Y mutations with abiraterone resistance; however, it is well recognized that this mutation promotes AR promiscuity and activation by antiandrogens, adrenal androgens, and nonandrogenic steroids (24–26). In keeping with this, two abiraterone-resistant patients with H874Y mutations subsequently had rapid disease progression on enzalutamide. This finding contradicts previous *in vitro* data suggesting that enzalutamide maintains antitumor activity in H874Y-mutant prostate cancer cells (8).

The identification of predictive biomarkers in mCRPC is critical to improving the clinical utility of currently available systemic agents. Importantly, our data indicate that AR gene aberrations (amplification and/or exon 8 mutation) may be a predictive biomarker for adverse outcomes in patients commencing enzalutamide. We found that an AR gene aberration in pretreatment cfDNA was the only independent predictor of PFS and the only factor associated with PSA decline ≥30% on enzalutamide. Similarly, AR amplification in cfDNA has been associated with lower response rates to abiraterone (7). Expression of AR splice variants in CTCs was also recently associated with primary resistance to abiraterone or enzalutamide (27) although AR amplification was not evaluated in this study, which may be significant since full-length AR is typically coexpressed with AR-V7 (28) and may be

required for tumor growth mediated by AR splice variants (29). Nevertheless, it is likely that divergent aberrations of the AR underpin cross-resistance between different novel agents targeting the androgen–AR axis (30–37), as exemplified by our observation that AR exon 8 mutations were mutually exclusive with AR copy number gain in 73% of cases. With this in mind, real-time serial analysis of circulating biomarkers, including cfDNA, could aid optimal therapy sequencing for individual patients.

A priority for future cfDNA studies will be to confirm the relevance of AR aberrations using pre- and posttreatment samples obtained from a homogenous group of patients. This would overcome key limitations of our study, which included collection of cfDNA at a single timepoint and recruitment of a heterogeneous cohort with differing baseline characteristics and variable clinical follow-up. Furthermore, although AR exon 8 sequencing proved very informative here, it will be important to sequence the entire AR, especially in light of recent data suggesting that a L702H mutation can convert glucocorticoids to AR agonists (7). The spectrum of AR mutations, including the four novel mutations reported here, will need to be accurately functionalized to determine their precise relevance to therapeutic resistance and disease progression. Finally, other genomic aberrations identified in our study, such as *CCND1* amplification (potentially sensitizing to CDK4/6 blockade; ref. 38), may serve as predictive factors guiding treatment selection and warrant further exploration.

Disclosure of Potential Conflicts of Interest

K.N. Chi reports receiving commercial research grants and honoraria from Astellas and Janssen. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: A.A. Azad, S.V. Volik, R. Shukin, M.E. Gleave, C.C. Collins, K.N. Chi

Development of methodology: A.A. Azad, S.V. Volik, A.W. Wyatt, B. McConeghy, J. Bazov, M.E. Gleave, C.C. Collins, K.N. Chi
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.A. Azad, S.V. Volik, A. Haegert, S. Le Bihan, J. Youngren, P.L. Paris, G.V. Thomas, M.E. Gleave, C.C. Collins, K.N. Chi
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.A. Azad, S.V. Volik, A.W. Wyatt, R.H. Bell, S. Anderson, J. Bazov, P.L. Paris, G.V. Thomas, Y. Wang, M.E. Gleave, C.C. Collins, K.N. Chi
Writing, review, and/or revision of the manuscript: A.A. Azad, S.V. Volik, A.W. Wyatt, J. Youngren, P.L. Paris, G.V. Thomas, E.J. Small, M.E. Gleave, C.C. Collins, K.N. Chi
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A.A. Azad, A. Haegert, S. Le Bihan, S. Anderson, R. Shukin, J. Bazov, C.C. Collins
Study supervision: S.V. Volik, C.C. Collins, K.N. Chi

References

1. Sridhar SS, Freedland SJ, Gleave ME, Higano C, Mulders P, Parker C, et al. Castration-resistant prostate cancer: from new pathophysiology to new treatment. Eur Urol 2014;65:289–99.
2. Beer TM, Armstrong AJ, Rathkopf DE, Loriot Y, Sternberg CN, Higano CS, et al. Enzalutamide in metastatic prostate cancer before chemotherapy. N Engl J Med 2014;371:424–33.
3. de Bono JS, Logothetis CJ, Molina A, Fizazi K, North S, Chu L, et al. Abiraterone and increased survival in metastatic prostate cancer. N Engl J Med 2011;364:1995–2005.
4. Scher HI, Fizazi K, Saad F, Taplin ME, Sternberg CN, Miller K, et al. Increased survival with enzalutamide in prostate cancer after chemotherapy. N Engl J Med 2012;367:1187–97.
5. Ryan CJ, Smith MR, de Bono JS, Molina A, Logothetis CJ, de Souza P, et al. Abiraterone in metastatic prostate cancer without previous chemotherapy. N Engl J Med 2013;368:138–48.
6. Small EJ, Youngren J, Thomas G, Olson S, Toschi A, Foye A, et al. Androgen receptor (AR) amplification in patients with metastatic castration-resistant prostate cancer (mCRPC) refractory to therapy with abiraterone acetate or enzalutamide: Preliminary results from the SU2C/PCF/AACR West Coast Prostate Cancer Dream Team (WCDT). J Clin Oncol 32:5s, 2014 (suppl; abstr 5020).
7. Carreira S, Romanel A, Goodall J, Grist E, Ferraldeschi R, Miranda S, et al. Tumor clone dynamics in lethal prostate cancer. Sci Transl Med 2014;6: 254ra125.
8. Joseph JD, Lu N, Qian J, Sensintaffar J, Shao G, Brigham D, et al. A clinically relevant androgen receptor mutation confers resistance to second-generation antiandrogens enzalutamide and ARN-509. Cancer Discov 2013;3: 1020–9.
9. Korpal M, Korn JM, Gao X, Rakiec DP, Ruddy DA, Doshi S, et al. An F876L mutation in androgen receptor confers genetic and phenotypic resistance to MDV3100 (enzalutamide). Cancer Discov 2013;3:1030–43.
10. Chen E, Sowalsky AG, Gao S, Cai C, Voznesensky O, Schaefer R, et al. Abiraterone treatment in castration-resistant prostate cancer selects for progesterone responsive mutant androgen receptors. Clin Cancer Res 2014 Oct 15. [Epub ahead of print].
11. Schwarzenbach H, Alix-Panabieres C, Muller I, Letang N, Vendrell JP, Rebillard X, et al. Cell-free tumor DNA in blood plasma as a marker for circulating tumor cells in prostate cancer. Clin Cancer Res 2009;15: 1032–8.
12. Schwarzenbach H, Hoon DS, Pantel K. Cell-free nucleic acids as biomarkers in cancer patients. Nat Rev Cancer 2011;11:426–37.
13. Heitzer E, Ulz P, Belic J, Gutschi S, Quehenberger F, Fischereder K, et al. Tumor-associated copy number changes in the circulation of patients with prostate cancer identified through whole-genome sequencing. Genome Med 2013;5:30.
14. Scher HI, Halabi S, Tannock I, Morris M, Sternberg CN, Carducci MA, et al. Design and end points of clinical trials for patients with progressive prostate cancer and castrate levels of testosterone: recommendations of the Prostate Cancer Clinical Trials Working Group. J Clin Oncol 2008; 26:1148–59.
15. Azad AA, Eigl BJ, Leibowitz-Amit R, Lester R, Kollmannsberger C, Murray N, et al. Outcomes with abiraterone acetate in metastatic castration-resistant prostate cancer patients who have poor performance status. Eur Urol 2014. Jan 31. [Epub ahead of print
16. Available from: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token= ehgnwukmjpultoj&acc=GSE61134.

17. Shiota M, Bishop JL, Nip KM, Zardan A, Takeuchi A, Cordonnier T, et al. Hsp27 regulates epithelial mesenchymal transition, metastasis, and circulating tumor cells in prostate cancer. Cancer Res 2013;73:3109–19.

18. Taylor BS, Schultz N, Hieronymus H, Gopalan A, Xiao Y, Carver BS, et al. Integrative genomic profiling of human prostate cancer. Cancer Cell 2010;18:11–22.

19. Beltran H, Yelensky R, Frampton GM, Park K, Downing SR, MacDonald TY, et al. Targeted next-generation sequencing of advanced prostate cancer identifies potential therapeutic targets and disease heterogeneity. Eur Urol 2013;63:920–6.

20. Grasso CS, Wu YM, Robinson DR, Cao X, Dhanasekaran SM, Khan AP, et al. The mutational landscape of lethal castration-resistant prostate cancer. Nature 2012;487:239–43.

21. Waltering KK, Urbanucci A, Visakorpi T. Androgen receptor (AR) aberrations in castration-resistant prostate cancer. Mol Cell Endocrinol 2012;360: 38–43.

22. Egan A, Dong Y, Zhang H, Qi Y, Balk SP, Sartor O. Castration-resistant prostate cancer: adaptive responses in the androgen axis. Cancer Treat Rev 2014;40:426–33.

23. Pritchard CC, Morrissey C, Kumar A, Zhang X, Smith C, Coleman I, et al. Complex MSH2 and MSH6 mutations in hypermutated microsatellite unstable advanced prostate cancer. Nat Commun 2014;5:4988.

24. Duff J, McEwan IJ. Mutation of histidine 874 in the androgen receptor ligand-binding domain leads to promiscuous ligand activation and altered p160 coactivator interactions. Mol Endocrinol 2005;19:2943–54.

25. Shi XB, Ma AH, Xia L, Kung HJ, de Vere White RW. Functional analysis of 44 mutant androgen receptors from human prostate cancer. Cancer Res 2002; 62:1496–502.

26. Fenton MA, Shuster TD, Fertig AM, Taplin ME, Kolvenbag G, Bubley GJ, et al. Functional characterization of mutant androgen receptors from androgen-independent prostate cancer. Clin Cancer Res 1997;3:1383–8.

27. Antonarakis ES, Lu C, Wang H, Luber B, Nakazawa M, Roeser JC, et al. AR-V7 and resistance to enzalutamide and abiraterone in prostate cancer. N Engl J Med 2014;371:1028–38.

28. Hu R, Lu C, Mostaghel EA, Yegnasubramanian S, Gurel M, Tannahill C, et al. Distinct transcriptional programs mediated by the ligand-dependent full-length androgen receptor and its splice variants in castration-resistant prostate cancer. Cancer Res 2012;72:3457–62.

29. Watson PA, Chen YF, Balbas MD, Wongvipat J, Socci ND, Viale A, et al. Constitutively active androgen receptor splice variants expressed in castration-resistant prostate cancer require full-length androgen receptor. Proc Natl Acad Sci U S A 2010;107:16759–65.

30. Thomsen FB, Roder MA, Rathenborg P, Brasso K, Borre M, Iversen P. Enzalutamide treatment in patients with metastatic castration-resistant prostate cancer progressing after chemotherapy and abiraterone acetate. Scand J Urol 2014;48:268–75.

31. Azad AA, Eigl BJ, Murray RN, Kollmannsberger C, Chi KN. Efficacy of enzalutamide following abiraterone acetate in chemotherapy-naive metastatic castration-resistant prostate cancer patients. Eur Urol 2015;67:23–9.

32. Noonan KL, North S, Bitting RL, Armstrong AJ, Ellard SL, Chi KN. Clinical activity of abiraterone acetate in patients with metastatic castration-resistant prostate cancer progressing after enzalutamide. Ann Oncol 2013;24: 1802–7.

33. Loriot Y, Bianchini D, Ileana E, Sandhu S, Patrikidou A, Pezaro C, et al. Antitumour activity of abiraterone acetate against metastatic castration-resistant prostate cancer progressing after docetaxel and enzalutamide (MDV3100). Ann Oncol 2013;24:1807–12.

34. Schrader AJ, Boegemann M, Ohlmann CH, Schnoeller TJ, Krabbe LM, Hajili T, et al. Enzalutamide in castration-resistant prostate cancer patients progressing after docetaxel and abiraterone. Eur Urol 2014;65:30–6.

35. Bianchini D, Lorente D, Rodriguez-Vida A, Omlin A, Pezaro C, Ferraldeschi R, et al. Antitumour activity of enzalutamide (MDV3100) in patients with metastatic castration-resistant prostate cancer (CRPC) pre-treated with docetaxel and abiraterone. Eur J Cancer 2014;50:78–84.

36. Badrising S, van der Noort V, van Oort IM, van den Berg HP, Los M, Hamberg P, et al. Clinical activity and tolerability of enzalutamide (MDV3100) in patients with metastatic, castration-resistant prostate cancer who progress after docetaxel and abiraterone treatment. Cancer 2014;120: 968–75.

37. Cheng HH, Nadal R, Gulati R, Azad A, Twardowski P, Vaishampayan UN, et al. The effect of prior abiraterone (Abi) use on the activity of enzalutamide (Enza) in men with mCRPC. J Clin Oncol 32, 2014 (suppl 4; abstr 18).

38. Dean JL, Thangavel C, McClendon AK, Reed CA, Knudsen ES. Therapeutic CDK4/6 inhibition in breast cancer: key mechanisms of response and failure. Oncogene 2010;29:4018–32.