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

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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 (33% vs. 17%; vs. 21%, P = 0.02, χ²). 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 > 30% (P = 0.013, χ²) 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.

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
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) ≥ 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 WGA kit (Cat No WGA-10x or WGA-50x) 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 WGA-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′-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 CytSNP 8x60 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 CISTICAL 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 log₂ (signal intensity of tumor vs. reference) for gain and loss, respectively. A log₂ 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 5′ 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 TCC AGT CAG GGT GTC TCT ATC AAC CCT GTT TTG CTC CTT ATG ATG TAG GGT TCC CAT CAA TGC TTC ACT. The resulting 279 base-pair ampiclons 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...
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 χ² test. In patients switched onto enzalutamide after cfDNA collection, univariate analysis was performed to identify variables associated with PSA response (χ² for categorical variables or logistic regression for continuous variables) and clinical and/or radiographic progression-free survival (PSF; Cox proportional-hazards modeling). Multivariate analysis of PSF 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 LHHR 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

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Median (IQR)</th>
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<tr>
<td>Age, n (%)</td>
<td>72 (49–92)</td>
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<tr>
<td>Gleason score, n (%)</td>
<td>14 (23)</td>
</tr>
<tr>
<td>6–7</td>
<td>38 (61)</td>
</tr>
<tr>
<td>8–10</td>
<td>10 (16)</td>
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<tr>
<td>Disease sites, n (%)</td>
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<tr>
<td>Bone</td>
<td>60 (97)</td>
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<tr>
<td>Lymph nodes</td>
<td>33 (53)</td>
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<tr>
<td>Visceral</td>
<td>8 (13)</td>
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<tr>
<td>ECOG, n (%)</td>
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</tr>
<tr>
<td>0–1</td>
<td>41 (66)</td>
</tr>
<tr>
<td>≥2</td>
<td>21 (34)</td>
</tr>
<tr>
<td>Laboratory</td>
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</tr>
<tr>
<td>Hemoglobin (median, g/L; IQR)</td>
<td>122 (108–128)</td>
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<tr>
<td>≤100, n (%)</td>
<td>12 (19)</td>
</tr>
<tr>
<td>LDH (median, U/L; IQR)</td>
<td>209 (174–316)</td>
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<td>Elevated (≥250), n (%)</td>
<td>26 (42)</td>
</tr>
<tr>
<td>ALP (median, U/L; IQR)</td>
<td>144 (93–320)</td>
</tr>
<tr>
<td>Elevated (≥360), n (%)</td>
<td>30 (48)</td>
</tr>
<tr>
<td>Albumin (median, g/L; IQR)</td>
<td>39 (32–41)</td>
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<td>Low (&lt;35), n (%)</td>
<td>14 (23)</td>
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<td>Normal, n (%)</td>
<td>33 (53)</td>
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<tr>
<td>Unknown, n (%)</td>
<td>15 (24)</td>
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<tr>
<td>Current treatment</td>
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<tr>
<td>Abiraterone acetate</td>
<td>29 (47)</td>
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<td>19 (31)</td>
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<tr>
<td>Bicalutamide</td>
<td>6 (10)</td>
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<td>Prednisone</td>
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<tr>
<td>Docetaxel</td>
<td>3 (5)</td>
</tr>
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<td>Outcomes on current treatment</td>
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<tr>
<td>Best PSA response</td>
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<tr>
<td>PSA decline ≥ 50%</td>
<td>26 (42)</td>
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<td>PSA decline &lt; 50%</td>
<td>20 (32)</td>
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<td>All treatments received</td>
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<tr>
<td>Enzalutamide</td>
<td>19 (31)</td>
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<tr>
<td>Docetaxel</td>
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<td>10 (16)</td>
</tr>
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<td>Other</td>
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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-naive 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%, $P = 0.02$; $\chi^2$; 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 (WCDDT). 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 ($P = 3.764e-05$; 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
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; $P = 0.38$; $\chi^2$). However, all 7 patients with H874Y or T877A
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-
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 ≥ 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 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 ≥ 30% (21% vs. 60%; P = 0.013) on enzalutamide with a strong trend toward significance for PSA decline ≥ 50% (21% vs. 50%; P = 0.06). No other variables were significantly associated with PSA decline ≥ 30% or ≥ 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 interval: 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...
Abiraterone platform-specifically overcame dilution effects, but can be compromised by low-level cfDNA 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 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 progesterone-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 Jansen. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

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