Plasma AR and abiraterone-resistant prostate cancer

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Androgen receptor (AR) gene aberrations are rare in prostate cancer before primary hormone treatment but emerge with castration resistance. To determine AR gene status using a minimally invasive assay that could have broad clinical utility, we developed a targeted next-generation sequencing approach amenable to plasma DNA, covering all AR coding bases and genomic regions that are highly informative in prostate cancer. We sequenced 274 plasma samples from 97 castration-resistant prostate cancer patients treated with abiraterone at two institutions. We controlled for normal DNA in patients’ circulation and detected a sufficiently high tumor DNA fraction to quantify AR copy number normal as opposed to AR gain samples. Whereas AR copy number was unchanged from before treatment to progression and no mutant AR alleles showed signal for acquired gain, we observed emergence of T878A or L702H AR amino acid changes in 13% of tumors at progression on abiraterone. Patients with AR gain or T878A or L702H before abiraterone (45%) were 4.9 and 7.8 times less likely to have a ≥50 or ≥90% decline in prostate-specific antigen (PSA), respectively, and had a significantly worse overall hazard ratio (HR), 7.33; 95% confidence interval (CI), 3.51 to 15.34; P = 1.3 × 10−9 and progression-free (HR, 3.73; 95% CI, 2.17 to 6.41; P = 5.6 × 10−7) survival. Evaluation of plasma AR by next-generation sequencing could identify cancers with primary resistance to abiraterone.

INTRODUCTION

Patients with metastatic prostate cancer commonly respond to androgen deprivation therapy. When patients relapse, a disease state known as castration-resistant prostate cancer (CRPC) develops, against which agents such as abiraterone or enzalutamide, targeting the androgen receptor (AR) axis, are highly effective (1, 2). Abiraterone- and enzalutamide-resistant CRPC is now common and a major challenge in the management of prostate cancer. Ongoing whole-exome and transcriptome sequencing studies of metastatic tumor biopsies obtained in this setting are starting to give insights into the complexity and distribution of genomic aberrations (3, 4). Metastatic biopsies have provided important information on the emergence of resistance mutations in other diseases, such as EGFR mutations in lung cancer (5). However, monitoring for mutations of resistance with serial samples is challenging in CRPC as well as other tumor types because of the logistics of obtaining repeated tumor biopsies and the observation of genomic heterogeneity in prostate cancer metastases (6). Our group and others have advocated using “liquid biopsies” (7–11). By using next-generation sequencing on circulating tumor DNA obtained from plasma through a minimally invasive blood test, we have demonstrated the capacity to interrogate for disease evolution and identify genomic aberrations that emerge with drug resistance (10). This approach allows surveillance of genetic material potentially representative of multiple metastases (12).

Progression to CRPC is associated with AR copy number gain or somatic point mutations (13–15). We hypothesized that genomic aberrations involving the AR were associated with resistance to abiraterone, potentially through allowing reactivation of AR signaling. We therefore aimed to use circulating tumor DNA extracted from repeated plasma samples to interrogate the circulating AR genomic landscape in CRPC resistant to potent AR-targeting drugs while controlling for circulating tumor DNA fraction through a combination of assay design and computational means. Key to this strategy is the concurrent sequencing and analysis of common prostate cancer somatic variants and deletions, similar to the approach we designed to assess tumor tissue purity to account for cell admixture (16). This reduces bias secondary to missing circulating AR genomic lesions caused by low circulating tumor DNA or normal DNA artifact. To evaluate associations with clinical outcome that would support the clinical utility of this strategy, we used samples prospectively collected independently at two institutions in correlative biomarker protocols that stipulated the molecular analysis of plasma DNA to identify associations with treatment resistance in prostate cancer.

RESULTS

Selection of CRPC patients for evaluation of AR genomic aberrations

We selected patients starting standard-of-care abiraterone between January 2011, after the reporting of the abiraterone phase 3 regulatory trial (1), and October 2014. We performed sequencing on all patients who had available for analysis a minimum of 6 ng of DNA from plasma (three patients had <6 ng of DNA in 2 ml of plasma) collected...

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within 30 days of starting treatment. To have a population representative of patients commonly seen in clinical practice, we allowed patients who had received prior treatment with docetaxel or new AR-targeting agents (namely, enzalutamide or orteronel). We obtained targeted next-generation sequencing data from 274 samples from 97 patients: 71 patients treated at the Royal Marsden (RM), London, UK, and 26 at the Istituto Scientifico Romagnolo per lo Studio e la Curaglia dei Tumori (IRST), Meldola, Italy (characteristics described in Table 1). We included at least one sample obtained 8 weeks after treatment initiation from 70 patients (Fig. 1A). Across all samples, we achieved a median coverage of 1434X (table S1).

Detection of AR gain in CRPC patients with circulating tumor DNA
We used the abundance of highly informative lesions in prostate cancer to calculate circulating tumor DNA fraction in 270 of 274 study samples (table S2). On the basis of false-positive distributions built from in silico simulations and empirical data for point mutations and common genomic monoallelic deletions, we identified a threshold of 0.075 as the lower circulating tumor DNA fraction amenable to accurate estimation of absolute AR copy number. Of 274 evaluable samples, 217 had a confirmed circulating tumor DNA fraction greater than 0.075, including pre-abiraterone samples from 80 of 97 patients (82%) (table S3). We observed a significantly lower circulating tumor DNA fraction in samples collected at the time of initial treatment response compared to that after disease progression (fig. S1, *P* = 0.02).

Using sequence data that covered all the AR coding bases (table S2), we proceeded to interrogate AR. Samples with evidence of AR gained copy number state were classified as AR gain (table S4). In total, 81 of 217 samples had AR gain, including pre-abiraterone samples from 32 of 80 patients. We used digital droplet polymerase chain reaction (PCR) to validate next-generation sequencing calls of AR gain, and by finding that ZXDB at Xp11.21 was copy number neutral in these cases, we

Table 1. Patient characteristics and treatment history. ND, not determined.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Overall (n = 97)</th>
<th>IRST (n = 26)</th>
<th>RM (n = 71)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>73 (41–92)</td>
<td>76 (63–92)</td>
<td>72 (41–87)</td>
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<tr>
<td>Gleason sum at diagnosis, n (%)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>≤7</td>
<td>26 (26.8)</td>
<td>3 (11.6)</td>
<td>23 (32.4)</td>
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<tr>
<td>≥8</td>
<td>57 (58.7)</td>
<td>18 (69.2)</td>
<td>39 (54.9)</td>
</tr>
<tr>
<td>Not available</td>
<td>14 (14.4)</td>
<td>5 (5.2)</td>
<td>9 (12.7)</td>
</tr>
<tr>
<td>Prior treatment for CRPC, n (%)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Bicalutamide</td>
<td>97 (100)</td>
<td>26 (100)</td>
<td>71 (100)</td>
</tr>
<tr>
<td>Docetaxel</td>
<td>82 (84.5)</td>
<td>25 (96.1)</td>
<td>57 (80.3)</td>
</tr>
<tr>
<td>Enzalutamide</td>
<td>19 (19.6)</td>
<td>6 (23)</td>
<td>13 (18.3)</td>
</tr>
<tr>
<td>Cabazitaxel</td>
<td>6 (6.2)</td>
<td>2 (7.7)</td>
<td>4 (5.6)</td>
</tr>
<tr>
<td>Orteronel</td>
<td>4 (5.1)</td>
<td>0</td>
<td>4 (5.6)</td>
</tr>
<tr>
<td>Other</td>
<td>4 (4.1)</td>
<td>3 (11.5)</td>
<td>1 (1.4)</td>
</tr>
<tr>
<td>Pretreatment PSA, µg/liter</td>
<td>127 (2–3211)</td>
<td>63.7 (2–1229)</td>
<td>141 (2–3211)</td>
</tr>
<tr>
<td>Pretreatment LDH, U/liter</td>
<td>181 (97–968)</td>
<td>177.5 (97–968)</td>
<td>183 (98–950)</td>
</tr>
<tr>
<td>Pretreatment ALP, U/liter</td>
<td>105 (39–1040)</td>
<td>99 (46–688)</td>
<td>124 (39–1040)</td>
</tr>
<tr>
<td>Sites of metastases, n (%), n with visceral metastases (%)</td>
<td></td>
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<tr>
<td>≤5 bone metastases</td>
<td>39 (40.2), 13 (13.4)</td>
<td>12 (46.2), 4 (15.4)</td>
<td>27 (38), 9 (12.7)</td>
</tr>
<tr>
<td>≥6 bone metastases</td>
<td>50 (51.6), 7 (7.2)</td>
<td>13 (50), 5 (19.2)</td>
<td>37 (52.1), 3 (4.2)</td>
</tr>
<tr>
<td>Lymph node, no bone metastases</td>
<td>8 (8.2), 2 (2.0)</td>
<td>1 (3.8), 1 (3.8)</td>
<td>7 (9.9), 1 (1.4)</td>
</tr>
<tr>
<td>Time of follow-up, days</td>
<td>608 (47–1439)</td>
<td>460 (47–608)</td>
<td>983 (78–1439)</td>
</tr>
<tr>
<td>Progression-free survival, days</td>
<td>196 (41–900)</td>
<td>224 (41–577)</td>
<td>171 (62–900)</td>
</tr>
<tr>
<td>Overall survival, days</td>
<td>533 (48–1439)</td>
<td>ND (48–609)</td>
<td>415 (78–1439)</td>
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</table>
confirmed that the area of gain did not involve the whole arm of chromosome X (fig. S2). We observed a significant dosage effect between circulating tumor DNA fraction and serum indices of tumor burden [lactate dehydrogenase (LDH) and alkaline phosphatase (ALP)] (Fig. 1B). We also confirmed that circulating tumor DNA fraction was in keeping with the heterogeneous genomic nature of CRPC metastases (3). The equal conversion rate (23%) in both groups is overall in agreement with the heterogeneous genomic nature of CRPC metastases and their dynamic representation in circulation (10) and suggests that AR copy number status in individual metastases does not noticeably change with abiraterone.

**Plasma AR gene aberrations strongly associated with clinical outcome on abiraterone**

We then proceeded to evaluate the rate of prostate-specific antigen (PSA) decline for patients with either AR gain or an AR (L702H or T878A) point mutation in the pre-abiraterone plasma sample [AR aberrant, 36 of 80 patients (45%)]. Plasma AR aberrant patients were 4.9 and 7.8 times less likely to have a ≥50 or ≥90% decline in PSA, respectively (Fig. 4A; P = 0.002 and P = 0.004, respectively). No trend for PSA decline was observed when considering circulating tumor DNA fraction (fig. S6). Patients with normal plasma AR also had a significantly longer overall and progression-free survival when compared to patients with AR gain or mutation (Fig. 4, B and C; P = 1.3 × 10^{-3} and P = 5.6 × 10^{-5}, respectively). Similar significance was observed comparing overall and progression-free survival between AR normal and AR gain (fig. S7; P = 5.7 × 10^{-9} and P = 3.1 × 10^{-5}, respectively), and, although unconfirmed because of limited patient numbers, a similar trend was also observed for AR mutants (table S6). Moreover, in support of these data, a swimmers’ plot suggests that among patients with both AR gain and a decline in PSA, resistance that may be secondary to AR gain clones present before treatment emerged within a shorter time frame than for AR normal patients; both patients with AR gain and a ≥90% decline showed no change in AR status in their progression samples (Fig. 3). The equal conversion rate (23%) in both groups is overall in agreement with the heterogeneous genomic nature of CRPC metastases and their dynamic representation in circulation (10) and suggests that AR copy number status in individual metastases does not noticeably change with abiraterone.
in PSA developed radiological progression within 35 weeks (fig. S8). In multivariate regression analysis considering AR gene status, serum LDH and ALP, total circulating DNA (categorized into quartiles), and prior use of enzalutamide or orteronel (binary), aberrant AR remained the only significant variable that was associated with worse overall (HR, 6.85; 95% CI, 3.21 to 14.60) and progression-free survival (HR, 3.58; 95% CI, 1.92 to 6.69) in the final models (table S7).

**DISCUSSION**

We sequenced all the AR coding regions in plasma from CRPC patients immediately before starting abiraterone, on treatment, and after progression, concurrently evaluating both copy number and somatic point mutations. We provide clinical qualification of preliminary data reported by us supporting an association between AR copy number gain and resistance to abiraterone (10, 20). A smaller study has also suggested an association between AR gain and resistance to enzalutamide (21). In contrast, fluorescence in situ hybridization performed on prostate biopsies reported an association between AR gain and resistance to abiraterone (10, 20). A smaller study has also suggested an association between AR copy number gain and resistance to enzalutamide (21). In contrast, fluorescence in situ hybridization performed on prostate biopsies reported an association between AR gain and resistance to abiraterone (10, 20). In contrast, fluorescence in situ hybridization performed on prostate biopsies reported an association between AR gain and resistance to abiraterone (10, 20). In contrast, fluorescence in situ hybridization performed on prostate biopsies reported an association between AR gain and resistance to abiraterone (10, 20). In contrast, fluorescence in situ hybridization performed on prostate biopsies reported an association between AR gain and resistance to abiraterone (10, 20). In contrast, fluorescence in situ hybridization performed on prostate biopsies reported an association between AR gain and resistance to abiraterone (10, 20). In contrast, fluorescence in situ hybridization performed on prostate biopsies reported an association between AR gain and resistance to abiraterone (10, 20). In contrast, fluorescence in situ hybridization performed on prostate biopsies reported an association between AR gain and resistance to abiraterone (10, 20).
The magnitude of the fractions. Orange represents AR copy number neutral, and blue represents AR copy number gain. The split of patients with a PSA decline ≥50% (light) or not (dark) is shown for every group.

present in too low a frequency relative to overall circulating tumor DNA to be detected. This could explain a proportion of the cases with normal plasma AR who did not respond to abiraterone. Nonetheless, after controlling for circulating tumor DNA fraction, emergence of AR gain at progression in patients with normal pretreatment AR copy number was uncommon, also suggesting that resistance to abiraterone commonly develops secondary to mechanisms that do not involve AR gain in these patients.

Resistance in up to 30% of patients with no detectable AR gain at progression was associated with an AR somatic point mutation, which is often observed several months before confirmed clinical progression and putatively activated by nonandrogenic ligands. This suggests that analysis of plasma AR could complement other modalities for evaluating CRPC patients and allow early treatment change before overt radiological progression. The recent report of an association between AR splice variants and resistance to abiraterone (22) introduces the possibility of a link between a gain in AR copy number, increased AR transcripts, and the presence of AR splice variants. Also, a recent study reports that AR splice variants and AR point mutations are generally mutually exclusive (23), similar to our observation for AR copy number gain. We have not studied AR splicing variants in this study because we did not have concurrent appropriately collected samples. AR gain has been previously shown to be associated with increased AR expression in prostate cancers (24), which can drive resistance to androgen suppression in vitro (25, 26). Future studies could shed light on the association between AR gene status and AR transcriptional profiles and their association with other aberrations that could drive abiraterone resistance.

Among patients who had previously received enzalutamide or orteronel, 10 of 20 had AR gain and only 3 (all plasma AR normal) had a PSA decline ≥50%, in keeping with previous reports of cross-resistance (22). In addition to the prognostic importance, the association with a lower rate of PSA decline suggests that AR gene state may be predictive for abiraterone resistance. This could suggest that patients with aberrant plasma AR should be selected for treatments such as chemotherapy or radiopharmaceuticals. A similar association may also be observed with other potent AR-targeting strategies such as enzalutamide or the experimental agents ODM-201, ARN-509, and galeterone. These data now warrant prospective validation in randomized clinical trials. Overall, the association of genomic aberrations with clinically meaningful end points in our study suggests that circulating tumor DNA is representative of tumor clones that are driving disease progression in CRPC.

MATERIALS AND METHODS

Study design

The primary aim was to determine the association between AR genomic aberrations in plasma from CRPC patients starting abiraterone and a ≥50 or ≥90% decline in serum PSA. Secondary aims included the association with radiological progression-free survival, overall survival, and changes in AR genomic status after disease progression. Samples were collected prospectively under two biomarker protocols, separately approved by the RM (REC 04/Q0801/6) and IRST (REC 2192/2013). The aims of the study were defined after sample collection. We aimed to analyze samples from the two institutions separately, but we grouped them together after confirming no substantial differences between the two data sets. Patients needed to have histologically or biochemically confirmed prostate adenocarcinoma and were planning to initiate a new treatment for progressive CRPC, as defined by a minimum of three or more rising serum PSA values obtained two or more weeks apart, with the last value being 2.0 ng/ml or higher in the presence of castrate levels of serum testosterone [<50 ng/dl (1.73 nM)], consistent with the Prostate Cancer Clinical Trials Working Group 2 (PCWG2) guidelines (27). Patients were required to receive abiraterone until disease progression as defined by at least two of the following: a rise in PSA, worsening symptoms, or radiological progression as defined previously (28), namely, progression in soft tissue lesions measured by computed tomography (CT) imaging according to the modified Response Evaluation Criteria in Solid Tumors or progression on bone scanning according to criteria adapted from the PCWG2. All patients provided written informed consent.

Procedures

Peripheral blood samples for plasma DNA extraction were obtained within 30 days of treatment initiation, and patients were given the option for further blood draws every 8 weeks on treatment and after progression. Serum PSA, LDH, and ALP were checked before the start of abiraterone treatment, after 12 weeks of treatment, and every 4 weeks thereafter. CT scans of the chest, abdomen, and pelvis, and technetium-99m
Bone scans were performed up to 30 days before treatment initiation and every 2 to 4 months. Circulating DNA was extracted from 1 to 2 ml of plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen) and quantified using the high-sensitivity Quant-iT PicoGreen double-stranded DNA Assay Kit (Invitrogen). Germline DNA was extracted from buccal swabs, saliva, or white blood cells using the QIAamp DNA kit (Qiagen). We used our custom AmpliSeq library panel (table S2) in combination with the Ion AmpliSeq Library Preparation Kit version 2.0 (Life Technologies), with a total DNA input of 10 ng. The samples were barcoded with IonXpress barcodes (Life Technologies) to enable sample pooling. Sequencing was performed on samples that passed quality control on

![Fig. 4. Association of AR gene status before abiraterone with treatment outcome. (A) Waterfall plot showing the magnitude of PSA decline in patients with AR gain, an L702H or T878A AR point mutation, or AR copy number neutral. The odds ratios for AR copy number neutral having a ≥50 or ≥90% decline in PSA were calculated using Fisher’s exact test. Clinical site and prior treatment with the potent AR-targeting agents enzalutamide or orteronel are identified in the matrix. (B and C) Overall survival (B) and progression-free survival (C) for AR copy number neutral versus AR aberrant (AR gain or point mutation, L702H or T878A) cancers are shown with results of univariate analysis in the inset.](image-url)
survival analysis was performed using a proportional hazard model using the Kaplan-Meier estimator (log-rank test). Multivariate overall survival and progression-free survival analyses were performed in all the study samples. An extensive description of the procedures to estimate plasma DNA tumor fraction, copy number call, and point mutation detection is provided in Supplementary Materials and Methods.

Statistical analysis
Pearson correlation statistics with significance level of 5% was used to measure the association between serum indices of tumor burden, plasma copy number gain and copy number state.

REFERENCES AND NOTES

**REPORT**

The personal Genome Machine (PGM) Ion Torrent using a 316 or 318 Chip version 2.0 to account for 1000X expected coverage per target (n = 6 of 8 pooled samples) (tables S1 and S8).

**Computational analysis**
Data preprocessing included read counts for all genomic positions in the assay, which included informative single-nucleotide polymorphisms (SNPs) (polymorphic positions at which the individual has a heterozygous genotype) necessary for data analysis as previously described (10), using ASEQ (29). In silico simulations with germline samples from study patients and healthy individuals were performed to assess sensitivity and specificity detecting point mutations and tumor DNA fraction in circulation (tumor content) (table S3). Tumor content was estimated using a strategy similar to (10, 16) but relying on a modified approach for assessing the percentage of aberrant tumor reads to improve both computational and detection performances. Specifically, the algorithm combined control sample data features, in silico simulation, and Mann-Whitney statistics to provide a fast and precise tumor content estimation. For 50 or more informative SNPs and coverage greater than 1000X, mean sensitivity values of 97 and 99.9% were achieved for 8 and 10% of aberrant tumor reads, respectively, with a mean specificity of 99% (figs. S9 and S10). To ensure unbiased AR gain calls, we first implemented a procedure modeling the stochastic noise of the copy number estimation with parameter distribution derived from control samples and used it to call AR copy number state in all the study samples. An extensive description of the procedures to estimate plasma DNA tumor fraction, copy number call, and point mutation detection is provided in Supplementary Materials and Methods.

**Statistical analysis**
Pearson correlation statistics with significance level of 5% was used to measure the association between circulating tumor DNA fraction, serum indices of tumor burden, plasma AR copy number, and total circulating-cell-free DNA. Fisher’s exact test with a significance level of 5% was used to measure the association between AR copy number gain and AR point mutation, nonsynonymous to synonymous AR point mutations in the copy number estimation compared to no AR gain, and PSA decline for patients with either AR gain or AR point mutation (T878A or L702H). Univariate overall survival and progression-free survival analyses were performed using the Kaplan-Meier estimator (log-rank test). Multivariate overall survival analysis was performed using a proportional hazard model with stepwise model selection by Aikake information criterion using forward and backward directions. Continuous variables were categorized as per distribution quantiles.

**SUPPLEMENTARY MATERIALS**

www.sciencetranslationalmedicine.org/cgi/content/full/7/312/312re10/DC1

Materials and Methods
Fig. S1. Circulating tumor DNA fraction at response compared to after-disease progression.
Fig. S2. Validation of next-generation sequencing calling of AR gain.
Fig. S3. Total cell-free DNA concentration versus tumor DNA fraction.
Fig. S4. Overall and progression-free survival analysis of AR normal versus AR gain.
Fig. S5. Overall survival and progression-free survival analysis of AR normal versus AR gain.
Fig. S6. Swimmer plot of patients with a PSA decline.
Fig. S7. Survival analysis of aberrant tumor read percentage estimation.


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Detecting resistance before it starts

Androgen receptor targeting is the cornerstone of prostate cancer treatment. Even when the tumors become “castration-resistant” or no longer sensitive to androgen deprivation, androgen signaling can still be effectively targeted by newer drugs such as abiraterone and enzalutamide, which also inhibit the androgen signaling axis. Romanel et al. analyzed tumor DNA samples from the blood of 97 patients with castration-resistant prostate cancer at different times during the course of treatment with abiraterone. Although some new mutations emerged during therapy, the authors found that androgen receptor amplifications were present from the beginning and correlated with abiraterone resistance, suggesting that detection of these amplifications should be useful for identifying abiraterone-resistant cancers before starting treatment.