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Plasma *AR* and abiraterone-resistant prostate cancer

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Abstract

AR gene aberrations are rare in prostate cancer prior to 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 that covers all the *AR* coding bases and regions of the genome highly informative in prostate cancer. We here sequenced 274 plasma samples from 97 castration-resistant prostate cancer patients treated with abiraterone at two institutions. After controlling for the fraction of normal DNA in patients' circulation, we quantified *AR* copy number state and point mutations. *AR* aberrations by the two mechanisms were inversely correlated, supported further by the enrichment of non-synonymous versus synonymous mutations in *AR* copy number normal as

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Competing interests

The Institute of Cancer Research developed abiraterone and therefore has a commercial interest in this agent. G.A. is on the ICR list of rewards to inventors for abiraterone. J.S.d.B. has received consulting fees and travel support from Amgen, Astellas, AstraZeneca, Boehringer Ingelheim, Bristol-Myers Squibb, Dendreon, Enzon, Exelixis, Genentech, GlaxoSmithKline, Medivation, Merck, Novartis, Pfizer, Roche, Sanofi-Aventis, Supergen and Takeda, and grant support from AstraZeneca and Genentech. G.A. has received honoraria, consulting fees or travel support from Astellas, Medivation, Janssen, Millennium Pharmaceuticals, Ipsen, Ventana and Sanofi-Aventis, and grant support from Janssen, AstraZeneca and Arno. The other authors do not declare any competing interests.

opposed to *AR* gain samples. While *AR* copy number was unchanged from pre-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% at progression on abiraterone. Patients with *AR* gain or T878A or L702H pre-abiraterone (45%) were 4.9 times and 7.8 times less likely to have a decline in PSA by 50% or 90% respectively and had a significantly worse overall (HR 7.33, 95% CI 3.51-15.34) and progression-free (HR 3.73, 95% CI 2.17-6.41) survival. Evaluation of plasma *AR* using next-generation sequencing could identify cancers with primary resistance to abiraterone.

Introduction

Patients with metastatic prostate cancer commonly respond to androgen deprivation therapy (ADT). When patients relapse, a disease state known as castration-resistant prostate cancer (CRPC) develops, to 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 mutations of resistance 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 due to the logistics of obtaining repeated tumor biopsies and the observation of genomic heterogeneity in prostate cancer metastases (6). Our groups 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* associate 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 due to low circulating tumor DNA or normal DNA artifact. In order 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 reporting of the abiraterone Phase III regulatory trial (1), and October 2014. We performed sequencing on all patients who had available for analysis a minimum of 6ng DNA from plasma (three patients had <6ng DNA in 2mls plasma) collected 30 days before 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 novel AR targeting agents (namely, enzalutamide or orteronel). We obtained targeted next-generation sequencing data on 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 Cura dei Tumori (IRST), Meldola, Italy (characteristics described in Table 1). We included at least one sample eight weeks after treatment initiation from 70 patients (Fig. 1A). Across all samples, we achieved a median coverage of 1,434X (table S1).

Detection of AR gain in CRPC patients with circulating tumor DNA

Using the abundance of prostate cancer highly informative lesions, we calculated circulating tumor DNA fraction in 270 of 274 study samples (fig. S1). Based on false positive distributions built from *in silico* simulations and empirical data both for point mutations and common genomic monoallelic deletions (see Supplementary Materials), 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 S2). We observed a significantly lower circulating tumor DNA fraction in samples collected at response compared to after disease progression (fig. S2, $P = 0.02$). Using sequence data that covered all the AR coding bases (fig. S1), we proceeded to interrogate AR. Samples with evidence of AR gained copy number state (see Supplementary Materials and Methods) were classified as AR gain (table S3). In total, 81 of 217 samples had AR gain including pre-abiraterone samples from 32 of 80 patients. Using digital droplet PCR, we validated next-generation sequencing calls of AR gain and, by finding that ZXDB at Xp11.21 was copy number neutral in these cases, we confirmed that the area of gain did not involve the whole arm of chromosome X (fig. S3). 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, $P = 2.98 \times 10^{-4}$ and $P = 1.79 \times 10^{-5}$ respectively), total circulating cell-free DNA (fig. S4, $P = 1.58 \times 10^{-6}$) and plasma AR copy number (Fig. 1B, $P = 2.34 \times 10^{-2}$). Nonetheless, we also observed instances of high AR copy number state in the presence of low circulating tumor DNA fraction (Fig. 1B). We also confirmed that circulating tumor DNA fraction was associated with a significantly worse overall and progression-free survival (fig. S5A, $P = 0.008$ and $P = 0.011$ respectively) but patients with a fraction <0.075 did not represent a prognostically distinct group (fig. S5B).

Mutant *AR* alleles do not acquire copy number gain

We detected somatic *AR* non-synonymous point mutations described recently in sequencing studies of CRPC tissue (3) in 41 plasma samples (15%) from 16 patients (table S4). W742C and W742L *AR* mutations were observed in the same sample collected prior to initiation of abiraterone in a patient who had progressed on and discontinued bicalutamide 36 days previously. L702H was only observed in patients (five) receiving prednisolone. The L702H, H875Y and T878A mutations were validated using digital droplet PCR (fig. S6). Amongst samples with a circulating DNA fraction ≥ 0.075 , we observed a significant inverse correlation between detection of *AR* copy number gain and *AR* point mutations (Fig. 2A, $P = 0.004$) and no instances where the fraction of reads suggested gain of a mutant *AR* allele. As we had sequence data on all the bases in coding regions of the *AR*, we proceeded to identify a significantly higher rate of non-synonymous with respect to synonymous *AR* point mutations in the samples with no *AR* gain compared to those with gain (Fig. 2B, $P = 0.028$), supporting selection of non-synonymous mutations in the absence of gain. To identify *AR* point mutations that specifically associate with resistance to abiraterone, we selected lesions that were consistently detected and showed an increase or persistence in circulating abundance with disease progression. We included 59 patients with both baseline and progression samples (Fig 1A). *AR-L702H* (three patients) and *AR-T878A* (four patients) were the only two mutations that met these criteria (Fig. 2C). Both mutations are activated by non-androgenic ligands present at increased levels in patients treated with abiraterone (10, 17).

AR copy number is unchanged at progression on abiraterone

We then identified 44 patients with detectable tumor DNA in circulation sufficient for *AR* copy number assessments in both baseline and progression samples. We observed that 77% with gain at baseline and an equal fraction with no gain showed no change in *AR* status in their progression sample (Fig. 3). The equal conversion rate (23%) in both groups is overall in keeping with the heterogeneous genomic nature of CRPC metastases and their dynamic representation in circulation (10) and interestingly suggests that *AR* copy number status in individual metastases does not noticeably change with abiraterone.

Plasma *AR* gene aberrations strongly associate 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 *AR-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 decline in PSA $\geq 50\%$ and $\geq 90\%$ 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. S7). 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. 4B, C, $P = 1.3\text{e-}09$ and $P = 5.6\text{e-}07$ respectively). Similar significance was observed comparing overall and progression-free survival for *AR* normal to *AR* gain (fig. S8, $P = 5.7\text{e-}09$, $P = 3.1\text{e-}06$ respectively) and although unconfirmed due to limited patient numbers, a similar trend for *AR* mutants (table S5). Moreover, in support of these data, a swimmer's plot suggests that of the patients with both *AR* gain and a decline in

PSA, resistance potentially secondary to *AR* gain clones present prior to treatment emerged within a shorter time-frame than *AR* normal patients; of the two patients with *AR* gain and a decline in PSA 90% both developed radiological progression within 250 days (fig. S9). 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 associated with worse overall (HR 6.85, 95% CI 3.21-14.60) and progression-free survival (HR 3.58, 95% CI 1.92-6.69) in the final models (table S6).

Discussion

We sequenced all the *AR* coding regions in plasma from CRPC patients immediately prior to 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, 18). A smaller study has also suggested an association between *AR* gain and resistance to enzalutamide (19). In contrast, fluorescence *in situ* hybridization performed on prostate biopsies reported an association between *AR* amplification and sensitivity to first-generation anti-androgens (13). Future studies may shed light on the explanation for this discordance, including possibly genomic differences between prostate tumors and plasma DNA.

Although we observe a strong correlation between circulating tumor DNA fraction and indices of tumor load and outcome, our strategy may underestimate circulating tumor DNA fraction in some patients due to the emergence of more abundant lesions not included in our custom panel. Concurrent sequencing of more targets could therefore increase the proportion of patients with and the estimated levels of circulating tumor DNA. It is also possible that patients with normal plasma *AR* copy number harbor a sub-clone with *AR* gain which is 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 pre-treatment *AR* copy number was uncommon, also suggesting resistance to abiraterone commonly develops secondary to alternative mechanisms 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 putatively activated by non-androgenic ligands often observed several months before confirmed clinical progression (Fig. 2C). This suggests that analysis of plasma *AR* could complement other modalities for evaluating CRPC patients and allow early treatment change prior to overt radiological progression. The recent report of an association between *AR* splice variants and resistance to abiraterone (20) 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 *AR* splice variants and *AR* point mutations are also generally mutually exclusive (21), similar to our observation for *AR* copy number gain. We have not studied *AR* splicing variants in this study due to the absence of concurrent appropriately collected samples. *AR* gain has been

previously shown to associate with increased AR expression in prostate cancers (22) that *in vitro* can drive resistance to androgen suppression (23, 24). Future studies could shed light on the association between AR gene state and AR transcriptional profiles and their association with other aberrations that could drive abiraterone resistance.

In 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 $\geq 50\%$, in keeping with previous reports of cross-resistance (20). In addition to the prognostic importance, the association with a lower rate of PSA decline suggests 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.

Methods

Study design and patients

The primary aim was to determine the association between AR genomic aberrations in plasma from CRPC men starting abiraterone and a decline in serum PSA $\geq 50\%$ and $\geq 90\%$. 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 in two biomarker protocols, separately approved by the RM, London, UK, (REC 04/Q0801/6) and the IRST, Meldola, Italy (REC 2192/2013). The aims of the study were defined following sample collection. We aimed to analyse samples from the two institutions separately but group them together after confirming no substantial differences between the two data-sets. Patients needed to have histologically or biochemically confirmed prostate adenocarcinoma and were planned for initiation of 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 per milliliter or higher in the presence of castrate levels of serum testosterone (<50 ng per deciliter [1.73 nmol per liter]), consistent with Prostate Cancer Clinical Trials Working Group 2 (PCWG2) guidelines (25). Patients were required to receive abiraterone until disease progression as defined by at least two of a rise in PSA, worsening symptoms or radiological progression as defined previously (26), namely progression in soft-tissue lesions measured with the use of computed tomography (CT) imaging according to 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

abiraterone start, after 12 weeks treatment and 4-weekly thereafter. CT scans of the chest, abdomen and pelvis, and technetium-99m bone scans were performed up to 30 days prior to treatment initiation and every 2 to 4 months.

Circulating DNA was extracted from 1-2 ml of plasma using the QIAamp Circulating Nucleic Acid kit (Qiagen) and quantified using the Quant-iT high-sensitivity Picogreen double-stranded DNA Assay Kit (Invitrogen, CA, USA). Germline DNA was extracted from buccal swabs, saliva or white blood cells using the QIAamp DNA kit (Qiagen). Our custom Ampliseq library panel (fig. S1) was used in combination with the Ion Ampliseq library preparation kit v2.0 (Life Technologies) using a total DNA input of 10 ng. The samples were barcoded with IonXpress barcodes (LifeTechnologies) to enable sample pooling. Sequencing was performed on samples that passed quality control on the PGM Ion Torrent using a 316 or 318 Chip version 2.0 to account for 1000X expected coverage per target (N=6/8 of pooled samples) (table S1 and S7).

Computational Analysis

Data pre-processing included read counts for all genomic positions in the assay that includes informative SNPs (polymorphic positions at which the individual has heterozygous genotype) necessary for data analysis as previously described (10) using ASEQ (27). In-silico simulations based on study germline and healthy individuals samples were performed to assess sensitivity and specificity of point mutation detection and of tumor DNA fraction in circulation (tumor content) (table S2). Tumor content was estimated using a strategy similar to (10, 16) but relying on a modified approach for aberrant tumor reads percentage assessment that improves both computational and detection performances. Specifically, the new algorithm combines control samples 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% (fig. S10 and S11). To ensure unbiased *AR* gain calls, we first implemented a procedure modelling the stochastic noise of the copy number estimation with parameter distribution derived from control samples and utilized it to call *AR* copy number state in all the study samples. Extensive description of the procedures to estimate plasma DNA tumor fraction, copy number call and point mutation detection are delineated in Supplementary Materials and Methods.

Statistical Analysis

Pearson correlation statistics with significance level at 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 Exact Test using a significance level at 5% was used to measure the association between *AR* copy number gain and *AR* point mutation, non-synonymous to synonymous *AR* point mutations in *AR* gain compared to no *AR* gain, PSA decline for patients with either *AR* gain or *AR-L702H/AR-T878A* point mutation. Univariate overall survival and progression free survival analyses were performed using Kaplan-Meier estimator (log-rank test). Multivariate overall survival analysis was performed using a proportional hazard model with stepwise model selection by Akaike

information criterion using forward and backward directions. Continuous variables were categorized as per distributions quartiles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Detection of circulating tumor DNA in CRPC patients.

(A) Study profile showing the number of patients and samples with next-generation sequencing data and with a circulating tumor DNA fraction ≥ 0.075 . *26 and †1 patient(s) had a pre-abiraterone sample(s) only. RM, Royal Marsden. IRST, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori. (B) Correlation of circulating tumor DNA fraction with serum lactate dehydrogenase (LDH), serum alkaline phosphatase (ALP) and AR copy number.

Figure 2. AR gain usually occurs in a non-mutant AR allele.

(A) Distribution of AR point mutations in all samples and stratified by AR copy number (CN) status. (B) The prevalence of non-synonymous (Ka) and synonymous (Ks) substitutions rates in AR gain and AR CN neutral samples. Fisher's exact test was applied to test differences between the number of mutated versus wild-type samples across AR gain and AR CN neutral (A) and non-synonymous versus synonymous rates in AR gain versus AR CN neutral samples (B). (C) Presence of AR point mutations in serial plasma samples of study patients. For every patient, the temporal pattern of mutations detection is shown,

distinguishing baseline (green), on treatment (yellow), and progression (red) samples and fractions of circulating tumor DNA (TC) level. Mutations are marked with different color and symbol and corresponding allelic fractions corrected for tumor DNA fraction are reported. Temporal patterns observed for each specific patient/mutation combination are annotated as emergence (E), persistence (P) or loss of detection (L and marked with a red box). Stars are used to mark *AR* point mutations that are consistently detected with disease progression.

Figure 3. AR copy number is unchanged on abiraterone.

An illustration showing the changes in AR gene status for patients where both baseline and progression samples had detectable tumor DNA fraction. Samples that are AR copy number (CN) neutral (upper row) or AR CN gain (lower row) pre-abiraterone are split into AR CN neutral or AR CN gain at progression. The area of the circles is sized to represent the magnitude of the fractions. Orange represents AR CN neutral and blue, AR CN gain. The split of patients with a decline in PSA < 50% (light) or not (dark) is shown for every group.

Figure 4. AR gene status prior to start of abiraterone associates with treatment outcome.

(A) Water-fall plot showing the magnitude of PSA declines in patients with AR gain or an L702H or T878A point mutation or copy number (CN) neutral AR. The odds ratios for AR copy number neutral having a decline in PSA 50% or 90% were calculated using Fisher's exact test. Clinical site and prior treatment with the novel potent AR-targeting agents enzalutamide or orteronel are identified in the matrix. Overall survival (B) and progression-free survival (C) for AR CN neutral versus AR aberrant are shown with results of univariate analysis in the inset.

Table 1
Patient characteristics and treatment history.

Characteristics	Overall N = 97	IRST N = 26	RM N = 71
Age, years			
Median (range)	73 (41-92)	76 (63-92)	72 (41-87)
Gleason sum at diagnosis, n (%)			
7	26 (26.8)	3 (11.6)	23 (32.4)
8	57 (58.7)	18 (69.2)	39 (54.9)
Not available	14 (14.4)	5 (5.2)	9 (12.7)
Prior treatment for CRPC, n (%)			
Bicalutamide	97 (100)	26 (100)	71 (100)
Docetaxel	82 (84.5)	25 (96.1)	57 (80.3)
Enzalutamide	19 (19.6)	6 (23)	13 (18.3)
Cabazitaxel	6 (6.2)	2 (7.7)	4 (5.6)
Orteronel	4 (5.1)	0	4 (5.6)
Other	4 (4.1)	3 (11.5)	1 (1.4)
Pre-treatment PSA, µg/L			
Median (range)	127 (2-3211)	63.7 (2-1229)	141 (2-3211)
Pre-treatment LDH, U/L			
Median (range)	181 (97-968)	177.5 (97-968)	183 (98-950)
Pre-treatment ALP, U/L			
Median (range)	105 (39-1040)	99 (46-688)	124 (39-1040)
Sites of metastases, n (%), n with visceral metastases (%)			
5 bone metastases	39 (40.2), 13 (13.4)	12 (46.2), 4 (15.4)	27 (38), 9 (12.7)
6 bone metastases	50 (51.6), 7 (7.2)	13 (50), 5 (19.2)	37 (52.1), 3 (4.2)
Lymph node, no bone metastases	8 (8.2), 2 (2.0)	1 (3.8), 1 (3.8)	7 (9.9), 1 (1.4)
Time of follow-up, days			
Median (range)	608 (47-1439)	460 (47-608)	983 (78-1439)
Progression free survival, days			
Median (range)	196 (41-900)	224 (41-577)	171 (62-900)
Overall survival, days			
Median (range)	533 (48-1439)	NA (48-609)	415 (78-1439)

Abbreviations: IRST, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori; RM, Royal Marsden; PSA, prostate specific antigen; LDH, lactate dehydrogenase; pts, patients; ALP, alkaline phosphatase; CRPC, castration resistant prostate cancer.