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Androgen Receptor Nuclear Localization correlates with AR-V7 mRNA expression in Circulating Tumor Cells (CTCs) from Metastatic Castration Resistance Prostate Cancer Patients

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Abstract

Androgen receptor (AR) signaling drives prostate cancer (PC) progression and remains active upon transition to castration resistant prostate cancer (CRPC). Active AR signaling is achieved through the nuclear accumulation of AR following ligand binding and through expression of ligand-independent, constitutively active AR splice variants, such as AR-V7, which is the most commonly expressed variant in metastatic CRPC patients. Most currently approved PC therapies aim to abrogate AR signaling and activity by inhibiting this ligand-mediated nuclear translocation. In a prospective multi-institutional clinical study, we recently showed that taxane based chemotherapy is also capable of impairing AR nuclear localization (ARNL) in circulating tumor cells (CTCs) from CRPC patients, whereas taxane induced decreases in ARNL were associated with response. Thus, quantitative assessment of ARNL in CTCs can be used to monitor therapeutic response in patients and help guide clinical decisions.

Here, we describe the development and implementation of quantitative high throughput (QHT) image analysis algorithms to aid in CTC identification and quantitative assessment of percent ARNL (%ARNL). We applied this algorithm to fifteen CRPC patients at the start of taxane chemotherapy, quantified %ARNL in CTCs, and correlated with expression of AR-V7 mRNA (from CTCs enriched via negative, CD45+ depletion of peripheral blood) and with biochemical (prostate specific antigen; PSA) response to taxane chemotherapy.

We found that CTCs from AR-V7 positive patients had higher baseline %ARNL compared to CTCs from AR-V7 negative patients, consistent with the constitutive nuclear localization of AR-V7. In addition, lower %ARNL in CTCs at baseline was associated with biochemical response to taxane chemotherapy. High inter- and intra-patient heterogeneity was also observed. As ARNL is required for active AR signaling, the QHT algorithms described herein can provide prognostic and/or predictive value in future clinical studies.

1. Introduction

Circulating tumor cells (CTCs) have emerged as a valid alternative source of tumor material in patients with solid tumors. Repeated tissue biopsies for genetic analysis are limited by the risk of complications and the possibility that one metastatic site is not representative of tumor heterogeneity. Many different technologies are available to isolate CTCs (liquid biopsy) and provide non-invasively obtained, representative tumor cells and real-time information regarding the molecular characteristics of the tumor [1]. This is particularly relevant in prostate cancer (PC) as archival primary prostate cancer tissue obtained at diagnosis is often not representative of more advanced stage metastases occurring years later.

The androgen receptor (AR) axis is critical to PC development and progression [2]. Upon ligand (dihydrotestosterone; DHT) binding, AR translocates to the nucleus where it activates a wide array of genes that promote tumor growth [3]. However, even after chemical or surgical castration, nearly all patients invariably progress to castration resistant prostate cancer (CRPC) characterized by increasing prostate specific antigen (PSA), a target gene of AR, while still maintaining low serum testosterone levels. In this setting, therapies targeting the AR pathway are routinely used such as AR signaling inhibitors, abiraterone and enzalutamide, designed to inhibit AR nuclear translocation and target-gene activation.

Taxane chemotherapies, which bind to β -tubulin and cause microtubule (MT) stabilization, are the only class of chemotherapy drugs to improve the survival of metastatic CRPC (mCRPC) patients [4, 5]. We and others have previously demonstrated that AR nuclear translocation is mediated by MTs and the motor protein dynein [6]. AR binds MTs via its hinge domain and depends on the presence of functional MTs for nuclear translocation. Taxane treatment stabilizes and disrupts MT function, inhibiting the nuclear translocation of AR and downstream signaling [6, 7]. We have clinically validated this mechanism in a prospective clinical trial of mCRPC patients by showing that taxane-mediated inhibition of nuclear AR correlated with clinical response to therapy [8]. Despite the clinical success of taxanes, patients still experience acquired or intrinsic resistance to chemotherapy, primarily due to continuous AR signaling via expression of AR splice variants.

AR splice variants lack the ligand-binding domain, are agnostic to the effects of AR-targeted therapies and, thus, have been implicated in clinical drug resistance [9, 10]. AR-V7 is the most common variant, constitutively active in the nucleus of CRPC tumors. Structurally, AR-V7 lacks both the ligand-binding domain and the MT-binding hinge domain, resulting in constitutive nuclear translocation in a MT independent manner [11]. The MT-independence and nuclear localization of AR-V7 has been correlated with taxane resistance *in vivo* [11].

With AR signaling inhibitors and taxanes alike, inhibition of nuclear AR can serve as a molecular biomarker of treatment efficacy. Here, we report a CTC based, high throughput quantitative image analysis algorithm that couples low-resolution CTC identification with high-resolution multiplex confocal imaging to quantify percent AR nuclear localization (%ARNL) in mCRPC patients. Correlation of %ARNL with AR-V7 mRNA expression assessed by digital droplet PCR (ddPCR) revealed that patients positive for AR-V7 had

higher %ARNL, consistent with this variant's constitutive nuclear localization. In addition, CTCs from patients who failed to respond to taxane chemotherapy had higher baseline %ARNL compared to the responders. Together these data suggest that %ARNL can be used as a surrogate for active AR signaling and that the assessment of %ARNL by QHT algorithms can provide prognostic and/or predictive value in future clinical studies.

2. Methods

2.1 Cell culture and reagents

Hormone sensitive human PC cell line, LNCaP, cells were maintained in RPMI 1640 (Corning) supplemented with 10% fetal bovine serum (FBS) (Corning) and 100 IU/mL penicillin plus 100 mg/mL streptomycin (Corning) at 37°C with 5% CO₂. Before any experiments, cells were cultured in RPMI 1640 supplemented with 10% charcoal stripped FBS (Gibco) for 72 hours.

The following drugs were used for drug treatment: docetaxel (DTX; Sigma Aldrich), dihydrotestosterone (DHT) analog methyltrienolone, R1881 (Sigma Aldrich).

The following antibodies were used for immunofluorescence staining: mouse monoclonal anti-CD45 directly conjugated with QDot 800 (ThermoFisher Scientific), rabbit monoclonal anti-ARN21, rat monoclonal anti-tyrosinated-tubulin (Millipore Sigma), and mouse monoclonal anti pan-cytokeratin (BioLegend) directly conjugated with CF594 (Biotium). All Alexa-conjugated secondary antibodies were from Invitrogen.

2.2 Drug treatment

For cells on coverslips, LNCaP prostate cancer cells were plated on Cell-Tak-(BD Biosciences) coated 8 mm glass coverslips (Electron Microscopy Sciences). Cells were treated overnight with DTX (100 nM) or DMSO, followed by exposure to R1881, (10 nM) or DMSO for 1 hour. Cells were subsequently processed for immunostaining.

For cells captured on a microfluidic device, LNCaP cells were pre-treated with DTX (100 nM) or DMSO, followed by exposure to R1881, (10 nM) or DMSO for 1 hour. Cells were then spiked into healthy donor blood and processed using a microfluidic device (geometrically enhanced differential immunocapture; GEDI chip) as previously described [12]. Cells were subsequently processed for immunostaining.

2.3 Blood collection and CTC enrichment

Following approval of the Weill Cornell Medicine Institutional Review Board (IRB), 20 ml of peripheral blood was collected in EDTA tubes (BD Vacutainer® Franklin Lakes, NJ) from 15 mCRPC patients before (baseline; cycle 1 day 1; C1D1) of taxane. Additionally, 10 ml of peripheral blood was obtained from four healthy donors.

Circulating tumor cells (CTCs) were enriched from the peripheral blood by CD45⁺ depletion (RosetteSep™ Human CD45 Depletion Cocktail; Stemcell Technologies) according to the manufacturer's suggestions. For each patient the enriched cells were equally divided into two halves, one for confocal microscopy and the other for mRNA extraction for ddPCR.

Briefly, the CTCs processed by microscopy were cytospun onto Cell-Tak-(BD Biosciences) coated 8 mm glass coverslips (Electron Microscopy Sciences), incubated for 30 minutes at 37°C, and subsequently processed for immunostaining. The CTCs processed for mRNA extraction were lysed in Buffer RLT (Qiagen) plus 1% 2-Mercaptoethanol and used as input for the ddPCR analysis.

2.4 Immunofluorescence

Cells on coverslips and microfluidic devices were fixed with pre-warmed (37°C) 2% formaldehyde in 1X PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM MgCl₂) for 15 minutes and blocked overnight in 10% Normal Goat Serum (Jackson ImmunoResearch) plus 6% Bovine Serum Albumin in PBS (Corning).

Cells were immunostained for leukocyte marker CD45 (QDot 800), androgen receptor (AR; 488), tubulin (647), epithelial marker cytokeratin (CK; 594) and DAPI. Coverslips were mounted on a glass slide by using Mowiol mounting media.

2.5 Low-resolution CTC identification

Multiplex confocal microscopy was performed using a Zeiss Axio Observer.Z1 microscope. Low resolution, multiplex scanning of coverslips was performed using a 10x/0.3 NA objective with a Hamamatsu ORCA-Flash 4.0 CMOS camera. Coverslips were scanned at a single, interpolated focal plane, resulting in approximately 49 tiles. Nucleated cells were segmented using a global threshold of the DAPI channel, resulting in individual binary masks, which were subjected to quantitative measurements including size (area, range 25-350 px), shape (form factor (circularity), range 0.75-1.00) and fluorescence intensity thresholds for maximum CD45 intensity (<7500 AU) and mean CK (>700 AU) and DAPI (>800 AU) intensities to identify putative CTCs with the established DAPI⁺/CK⁺/CD45⁻ phenotype. X-Y coordinates of putative CTCs, which fit the criteria were populated into a list and sorted by descending mean CK intensity for high resolution manual operator review.

2.6 Quantitative, high throughput image analysis of %ARNL

High resolution, multiplex confocal microscopy was performed using the same microscope with 63x/1.4 NA objective and a Yokogawa Electric Corporation Spinning Disk unit and Hamamatsu ORCA-R2 Digital CCD camera. Approximately 20-40 individual z-slices at 0.24 µm per z-plane were recorded for each cell. Imaging conditions (exposure time and laser intensity) were set to avoid saturation and were kept constant for all CTCs imaged across all patients.

Individual channel, multiplex confocal image stacks were exported as full resolution, grayscale, TIFF images and imported into MetaMorph (Molecular Devices Corp.). To generate a binary mask representing the total cell, individual channel maximum intensity projections (MIPs) were created from the individual channel image stacks and each was cropped using a manually created rectangular region of interest (ROI). Each MIP was segmented using an automatic thresholding algorithm. MIPs were used for thresholding rather than individual Z-planes due to the increased signal to noise ratio of MIPs, which improve the reproducibility of automatic thresholding algorithms. The resulting total cell

binary mask was defined by the union of the AR, CK, DAPI, and Tubulin regions detected by the automatic thresholding algorithm. To generate a binary mask representing the nucleus, the DAPI channel MIP was segmented using an automatic thresholding algorithm.

For quantitative analysis of AR, an average intensity project (AvgIP) was generated from the AR image stack. Background correction was performed by subtracting the average pixel intensity within a manually defined, extracellular region from the AR AvgIP. The AR AvgIP was segmented using the total cell and nuclear binary masks and pixel intensity was integrated within each. Based on a training set of images, a size filter omitting regions less than 50 pixels in area was applied and hole filling was enabled for the total cell and nuclear binary masks. Data was presented as %ARNL (integrated pixel intensity within nuclear binary mask/integrated pixel intensity within the total cell mask) for each individual CTC. For each patient and time point, mean %ARNL was calculated from up to 20 CTCs.

2.7 Digital droplet PCR (ddPCR) based analysis of AR variants in CTCs

Analysis of AR variant expression in patient CTCs was performed as previously described [13]. Briefly, total RNA was extracted from the enriched CTCs using the RNAeasy Plus Micro Kit (Qiagen) per the manufacturer's instructions. Primers and probes specific for AR-V7 were used to generate amplicons. Quantifications of transcripts were performed using a QX200 ddPCR system with automated droplet generation (Bio-Rad Laboratories). No threshold was applied to the ddPCR values.

3. Results

3.1 Low resolution, high throughput CTC identification algorithm

For the CTC identification algorithm, we used the established FDA-cleared CTC definition that identifies CTCs as nucleated (DAPI⁺), epithelial (CK⁺) and CD45⁻ (leukocyte) cells [14]. The overview of CTC enrichment and imaging is shown in Figure 1. CTCs enriched from patient peripheral blood following CD45⁺ depletion were subjected to multiplex immunofluorescence staining including DAPI, CK and CD45. Low resolution imaging identified putative CTCs on the basis of morphological features, and staining intensities for DAPI, CK, and CD45.

To develop the algorithm to identify putative CTCs at low resolution, we started with cultured LNCaP cells diluted in healthy donor blood. We developed thresholds for each fluorophore to maximize the rate of true-positive detection (sensitivity) and minimize the rate of false-positive detection (specificity). Our results revealed a 1.1% false-positive rate and a 2.9% false-negative rate (Supplemental Figure 1).

After benchmarking the CTC identifier in spike-in experiments, we moved to a training set of patient-derived CTCs. Due to the rarity of CTCs and considering that CK expression level in CTCs is usually lower than detected in cell lines [15], we examined the impact of lowering the mean CK intensity threshold on false-negative and false-positive rates.

To do so, three low-resolution fields of view containing a total of 60 patient derived cells were acquired from three different patients. High-resolution imaging of the 60 cells and

manual classification identified 31 cells as true CTCs (DAPI⁺/CK⁺/CD45⁻) and 29 cells as contaminating hematopoietic cells. The impact of varying mean CK intensity thresholds is shown in Supplemental Table 1. Higher CK thresholds (800-1000) led to very high specificity in CTC identification, at the expense, however, of sensitivity, as more than one-third of true-CTCs (11-18 CTCs) were excluded. Lowering the CK threshold improved sensitivity favoring the inclusion of nearly all CTCs. Favoring sensitivity due to the rarity of CTCs, we set the threshold to 700 AU, which missed only 1/31 (3.2%) CTCs, showing that the algorithm sensitivity was 96.8% (30/31). This threshold was applied to all subsequent patient samples.

This sensitive CTC-identification algorithm allowed us to routinely and reliably detect CTCs from CRPC patients. Given the role of active AR signaling in disease progression—which requires AR target-gene activation in the nucleus—we sought to develop an algorithm to quantitate the extent of AR nuclear localization (%ARNL) in detected CTCs and correlate with clinical outcomes.

3.2 %ARNL analysis in LNCaP cells

To benchmark our high resolution %ARNL algorithms, we used AR-positive, hormone sensitive LNCaP PC cells and exposed them to treatments that would modulate AR nuclear accumulation either by inducing AR nuclear translocation using the synthetic AR ligand R1881 or by sequestering AR in the cytoplasm using chemotherapy docetaxel (DTX) as we have previously shown [6]. R1881 binds AR and induces its nuclear translocation in a microtubule dependent manner. DTX impairs the function of the microtubule network by stabilizing MTs, thereby inhibiting full length AR (AR-FL) nuclear translocation, keeping the receptor inactive in the cytoplasm [6]. These chemical perturbations provided an appropriate dynamic range to test the performance of our high-resolution algorithm in detecting changes in %ARNL.

Cells were fixed and subjected to multiplex confocal microscopy (Figure 2(a)). High resolution, three dimensional images of individual cells were subjected to automatic thresholding algorithms to define total cell and nuclear compartments (Supplemental Figure 2). Percent ARNL was determined by the ratio of AR fluorescence intensity in the nucleus to total cell AR intensity.

LNCaP cells treated with R1881 (n = 19) exhibited a higher nuclear AR percentage, compared to untreated LNCaP cells (n = 21) (means: 91.2% vs. 88.2%; p-value = 0.0005) (Figure 2(b)). Cells treated with DTX followed by R1881 (n = 29) exhibited a lower nuclear AR percentage compared to cells treated with R1881 alone (medians: 72.0% vs. 92.4%; p-value < 0.0001). This result is in agreement with the expected on-target effect of taxanes, which is MT stabilization resulting in impaired AR nuclear translocation (Figure 2(a); arrows).

Highlighting the flexibility of the algorithm, similar results were observed using pre-treated LNCaP cells that were captured and imaged on a microfluidic device (Supplemental Figure 3). LNCaP cells treated with R1881 (n = 29) and captured on a microfluidic device exhibited a higher nuclear AR percentage compared to untreated LNCaP cells (n = 31) (medians:

81.6% vs. 75.3%; p-value = 0.0005. Cells treated with DTX followed by R1881 (n = 30) and captured on a microfluidic device exhibited a lower nuclear AR percentage compared to LNCaP cells treated with R1881 alone (n = 29) (medians: 74.1% vs. 81.6%; p-value = 0.0003).

3.3 High throughput CTC identification and high resolution %ARNL quantification in CTCs from CRPC patients

We next applied this algorithm to patient-derived CTCs, to quantify %ARNL and correlate with clinical outcomes. CTCs were enriched from the peripheral blood of 15 CRPC patients, before the start of taxane based chemotherapy. Putative CTCs, were subjected to high resolution imaging for operator review, to confirm true-CTC identity, based on the presence of 1) intact nucleus 2) positive CK staining 3) negative CD45 staining (Figure 3).

Patient clinical characteristics, including prior therapy and biochemical response (50% decline in 12-week (Cycle 5; C5) PSA according to Prostate Cancer Working Group 2 criteria) to taxanes is shown in Table 1.

Confirmed (high resolution) CTCs were detected in all patients (15/15; 100%). A total of 203 CTCs across the 15 patients were subjected to downstream %ARNL analysis (Figure 4(a)). The range of single CTC %ARNL was 21-97%, indicating high inter- and intra-patient heterogeneity (Figure 4(b)).

For some patients, low CTCs numbers were observed. To assess the impact of ARNL on clinical outcomes, we combined single CTC %ARNL measurements across patients based on clinical characteristics. First, we grouped CTCs from patients who received prior AR-targeted therapies (abiraterone or enzalutamide) and compared them to those from patients who were AR-targeted therapy naïve. No significant difference in %ARNL was observed in CTCs (n = 80) from six patients who were AR targeted therapy naïve compared to CTCs (n = 123) from nine patients who previously progressed on AR targeted therapy (means (standard deviation): 76.8% (11.9%) vs. 74.4% (15.0%); p-value = 0.4; Wilcoxon Rank Sum Test) (not shown).

Next, we looked at %ARNL in CTCs at baseline from patients treated with taxane chemotherapy in relation to biochemical response. CTCs (n = 103) from patients who did not achieve 50% PSA decrease at C5 (non-responders) had higher %ARNL compared to CTCs (n=100) from responders (means (standard deviation): 76.8% (15.0%) vs. 73.8% (12.6%); p-value = 0.0499; Wilcoxon Rank Sum Test) (Figure 5(a)).

These data suggest that baseline measurement of %ARNL in CTCs as a surrogate for active AR signaling in the nucleus, can be clinically meaningful. Currently, our results on %ARNL cannot differentiate between the presence of AR-FL and AR variants, whose expression will also contribute to the overall AR immunostaining. This is because the AR antibody routinely used for IF and IHC detection, is specific to the N-terminal domain of AR, which is common to both AR-FL and AR-variants. Thus, it is possible that the high %ARNL observed in patients who did not respond to taxane chemotherapy, could be partially due to the presence of constitutively nuclear AR variants, including the most prevalent, AR-V7.

3.4 AR-V7 expression and %ARNL in CTCs

To examine AR-V7 expression in the cohort of the 15 patients in this study, we measured AR-V7 mRNA expression in patient CTCs by ddPCR [13]. Twelve of the 15 patients had analyzable CTC mRNA. Of the 12 patients, nine were AR-V7 positive (9/12; 75%). We observed a significant correlation between higher %ARNL in CTCs ($n = 106$) from AR-V7 positive patients compared to CTCs ($n = 48$) from AR-V7 negative patients (means (standard deviation): 77.0% (13.6%) vs. 70.6% (13.5%); p -value = 0.007; Wilcoxon Rank Sum Test) (Figure 5(b)).

Although patient numbers were small, numerically lower biochemical response rates to taxane chemotherapy were observed in AR-V7 positive patients (3/9; 33%) compared to AR-V7 negative patients (2/3; 67%).

4. Discussion

Characterization of the molecular phenotypes of CTCs can provide a deeper understanding of a patient's current state of disease, which can be non-invasively and longitudinally assessed through the use of liquid biopsies. Personalization of prostate cancer therapy is dependent upon the development and validation of clinically relevant biomarkers but the lack of readily biopsied tumor tissue presents a major challenge and limits the use of biomarkers in understanding the mechanisms and markers of drug resistance. Here, we describe the development of a QHT image analysis technique that aims to aid in the detection and identification of CTCs from whole blood samples and in interrogation of AR subcellular localization in mCRPC patients as well as mRNA expression of the clinically prevalent AR splice variant, AR-V7. In PC, quantitative image analysis of %ARNL can elucidate clinically relevant information about mechanisms of drug resistance and sensitivity.

CTCs from patients who did not biochemically respond to taxane chemotherapy (did not achieve a 50% PSA decrease at cycle 5) had higher %ARNL compared to CTCs from patients who achieved biochemical response. Although statistically significant the utility of this test is limited by both low patient numbers and high CTC %ARNL variability. However, the increased nuclear AR may illustrate a possible mechanism of intrinsic resistance to taxane chemotherapy, involving aberrant AR signaling including AR amplifications, mutations, or expression of AR splice variants. Further analysis reveals that CTCs from patients who express AR-V7 mRNA also have higher %ARNL, supporting the constitutive nuclear localization of AR-V7. Although the patient numbers are small, a lower response rate to taxane chemotherapy was observed in AR-V7 positive patients compared to AR-V7 negative patients (33 vs. 67%). Taken together, this data supports the mechanism of AR-V7 having constitutive activity, allowing for its nuclear translocation in the absence of testosterone (ligand) in a MT-independent manner, resulting in AR-V7 mediated mechanisms of taxane resistance. Due to the limitations of the current study, especially the high intra- and inter-patient variability and small sample size, further work is necessary with larger patient populations to interrogate the effect AR variant expression and subcellular localization in conjunction with response to taxane chemotherapy.

Our CTC molecular interrogation is based on CD45+ depletion (RosetteSep™ Human CD45 Depletion Cocktail; Stemcell Technologies) of peripheral blood resulting in an antigen agnostic enrichment of CTCs (negative depletion). This approach differs significantly from the currently approved EpCAM-based methodologies for CTC isolation in prostate cancer. We have performed RNA-sequencing of CTCs enriched from mCRPC patients using the same negative-depletion methodology as well as from matching peripheral blood mononuclear cells (PBMCs; density gradient centrifugation alone). Gene expression analyses, revealed enrichment of pathways specific for prostate cancer in the CTC sample, such as AR signaling, epithelial-mesenchymal transition, and MYC signaling that were absent in the matching PBMC sample (unpublished). Taken together, these data support the notion that negative depletion of peripheral blood is a viable method to yield tumor tissue in mCRPC patients.

Investigations of taxane induced drug target engagement (DTE) and changes in %ARNL before and after treatment have been previously performed. The phase II, TAXYENERGY clinical trial utilized the algorithms for CTC identification and %ARNL quantification discussed within this manuscript to analyze taxane induced cytoplasmic sequestration of AR in CTCs one week after initiation of chemotherapy and explored the clinical utility of %ARNL as a biomarker for assessment of taxane efficacy. In that study, %ARNL was measured in CTCs from 26 mCRPC patients at both baseline (C1D1) and after one week (C1D8) of taxane treatment. The algorithm revealed that taxane-induced decrease in %ARNL (C1D8 vs. C1D1) was associated with a higher rate of 50% PSA decrease at cycle 4 (p-value = 0.009), showing that shifts in %ARNL may serve as a clinically relevant, early biomarker of response to taxane chemotherapy [8]. The clinical utility of change in %ARNL still requires further validation with prospective clinical trials designed to directly test its efficacy as a predictive biomarker, but preliminary data so far seems favorable.

The use of these described quantitative image analysis algorithms for %ARNL as part of the TAXYENERGY clinical trial demonstrates their flexibility and platform agnostic nature. In TAXYENERGY, patient CTCs were enriched using antibody coated microfluidic devices [12] whereas in our present patient cohort, coverslips following negative CD45 depletion were used. Furthermore, when using a prostate cancer cell line (LNCaP), these algorithms were able to detect both the expected increase in %ARNL induced by DHT and the abrogated effect when pre-treated with DTX for cells that were located on coverslips and microfluidic devices. The ability to use the algorithms for CTC identification and %ARNL analysis regardless of capture platform highlights their versatility and broad applicability.

Contrary to the data provided herein, the TAXYENERGY study did not show any significant correlations between baseline %ARNL and clinical response or AR-V7 status [8, 13]. The different methods utilized for CTC isolation (PSMA-based vs. CD45 depletion), and the different patient population in this manuscript, where almost all patients had prior AR signaling inhibitors and many prior taxane chemotherapy, both of which are shown to modulate ARNL, could partially explain these observed discrepancies. Specifically, the baseline %ARNL and AR-V7 analysis performed as part of the TAXYENERGY trial, required patients to have detectable CTCs not only at baseline (C1D1) but also one week after treatment (C1D8) [13]. Patients who lacked CTCs at C1D8, which may indicate

sensitivity to therapy, were omitted. The exclusion of these patients may affect the correlations of baseline %ARNL and AR-V7 status and further explain the differences observed between that study and the data provided herein.

However, previously unpublished analyses from the TAXYNERGY cohort examining %ARNL and AR variant status (AR-V7, ARv567es) at baseline, regardless of detectable CTCs at C1D8, showed that patients who did not expressed either variant (n = 7), measured using the same assays described herein, had lower %ARNL (mean = 56%) compared to patients who were positive for either or both variants (n=42; mean = 67%). This data is in line with the correlations provided in this manuscript, where AR-V7 positive patients had higher %ARNL at baseline compared to AR-V7 negative patients. Further studies in larger cohorts are necessary to examine the impact of both AR-V7 and ARv567es in patient CTCs.

The quantification of AR expression in CRPC patient CTCs using immunofluorescence has been employed by other studies [16-18]. Quantification of AR fluorescence intensity as a measure of expression, Reyes et al. showed increased expression in patients with prior exposure to abiraterone, suggesting that AR mediated mechanisms of resistance and maintained AR signaling are involved in resistance to abiraterone. However, when quantifying nuclear AR based on similarity index with the nuclear stain FxCycle, they did not see a significant difference between patients with and without prior abiraterone exposure [17].

Crespo et al. also did not detect a difference in nuclear AR expression in patients treated with abiraterone or enzalutamide compared to those who were treatment naïve [16, 17]. The lack of differences in nuclear AR in patients who progressed on AR targeted therapies compared to those that were treatment naïve in both reports is consistent with the data herein. It is likely that both patients with and without AR targeted therapy exposure exhibit increased nuclear AR, due to intact AR signaling, driving progression of PC. However, based on the current understanding of AR signaling, the factors contributing to the increased nuclear AR in each cohort is likely to be different, where the nuclear AR after exposure to AR targeted therapies can be attributed to AR splice variant expression [19].

In a smaller cohort of matched samples before and after treatment with AR signaling inhibitors, Crespo et al. did see an increase in nuclear AR expression at progression compared to pre-treatment. This longitudinal comparison of nuclear AR expression supports the notion that AR signaling is intact after progression on AR targeted therapies; emergent mechanisms of resistance involve increased AR signaling, which likely include AR aberrations, such as expression AR splice variants.

Also similar to the %ARNL quantitation presented herein, Sperger et al. reported higher %ARNL in pooled single CTCs from patients who progressed on AR signaling inhibitors compared to those who were responding [18]. However, unlike our data, no correlation was reported between AR-V7 mRNA expression and %ARNL, although both assays were employed in separate patient cohorts.

CTC analyses using immunofluorescence for AR-V7 specifically is currently commercially available (Epic Sciences). Studies using this platform have shown that AR-V7 nuclear

localization, and not simply expression, is associated with treatment response [20]. However, these studies were limited by the absence of simultaneous AR-FL staining within the same CTCs, which is the primary target of AR signaling inhibitors and secondary target of taxanes. Additionally, this AR-V7 liquid biopsy test does not provide quantitative measurements of the %ARNL, most likely due to limitations of low-resolution imaging, which may further narrow the scope of the analyses and overlook CTC heterogeneity and clinical correlations.

Although these data show a link between increased %ARNL, expression of AR-V7, and a lack of biochemical response to taxane chemotherapy, further work is necessary exploring AR splice variant protein expression and subcellular localization longitudinally during taxane chemotherapy. The development of specific antibodies for AR splice variants would allow for concatenated analysis of the localization of AR-FL and AR variants before and after chemotherapy while also looking at the organization of the MT network. Based on the current literature, it would be hypothesized that taxane induced MT stabilization would correlate with lower nuclear accumulation of AR-FL while AR-V7 nuclear localization would not be affected. The ability to resolve specifically AR-FL and AR-V7 in the same CTC would enhance our understanding of PC biology, tumor progression, and role in treatment resistance.

Overall, when looking at the CTCs from this cohort, a majority exhibited relatively high %ARNL, with a median of 78.3% ARNL for all 203 CTCs analyzed; the increased AR localized in the nucleus of these cells reveals AR signaling is intact within and remains a potential therapeutic target. Consistent with other reports [16-18], we observe inter- and intra-patient heterogeneity (Figure 4(b)). Dysregulated AR signaling in patients previously treated with AR targeted therapies may potentially explain the various levels of %ARNL observed within and between patients. Given that, studies in larger cohorts are necessary to identify the impact of %ARNL as a biomarker, but initial reports demonstrate the feasibility and promise that shifts in %ARNL can identify early responders to taxane chemotherapy [8].

5. Conclusion

We have developed a robust, quantitative, high-throughput CTC identification algorithm and subsequent high-resolution analysis algorithm, which are capable of identifying and analyzing biomarkers of interest from liquid biopsies. The expression of AR-V7 is associated with increased %ARNL and increased %ARNL is associated with lack of biochemical response to taxane chemotherapy. The aforementioned principles and algorithms can be applied to interrogate meaningful biomarkers of response and can potentially guide therapeutic decisions in the clinic for prostate cancer in regards to AR targeted and chemotherapies as well as to other solid malignancies where CTCs are present.

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Figure 1. Workflow of circulating tumor cell (CTC) enrichment, low resolution imaging, and high resolution CTC confirmation.

(a) Whole blood was collected from metastatic castration resistant prostate cancer (mCRPC) patients and subjected to CD45⁺ depletion, which utilizes an antibody cocktail that crosslinks CD45 expressing white blood cells (yellow) and glycophorin A expressing red blood cells (red) to form rosettes, leaving CTCs (green) unaltered. After density gradient centrifugation, CTCs remain in the buffy coat. The buffy coat is isolated, washed, and plated onto an 8 mm coverslip for immunofluorescence processing. Low resolution imaging is performed and putative CTCs (arrow) are identified on the basis of morphological features and positive cytokeratin (CK) staining and negative CD45 staining.

Figure 2. Measuring dihydrotestosterone (DHT) induced increase in androgen receptor nuclear localization (%ARNL) and docetaxel (DTX) induced cytoplasmic sequestration in LNCaP cells. (a) Androgen sensitive human prostate cancer (PC) cell line, LNCaP, were incubated overnight with DMSO (control), or docetaxel (DTX; 100 nM) followed by DMSO (control) or DHT analog (R1881; 10 nM) for 1 hour. High resolution images of LNCaP cells were acquired and subjected to quantitative image analysis for %ARNL. Scale bar = 20 μ m. Arrows indicated evidence of taxane drug target engagement (DTE) as microtubule bundling (b) DHT causes an increase in %ARNL while pre-treatment with DTX abrogates the DHT induced increase in %ARNL. *** = $p < 0.001$; **** = $p < 0.0001$.

Figure 3. High resolution circulating tumor cell (CTC) confirmation.

High resolution, three dimensional confocal microscopy is performed on putative CTCs to confirm identity. Leukocytes express CD45 but lack cytokeratin (CK). Confirmed CTCs express CK and lack CD45. Scale bar = 5 μ m.

Figure 4. Analysis of androgen receptor nuclear localization (%ARNL) in circulating tumor cells (CTCs) from patients

(a) Representative CTCs with %ARNL measurements. Scale bar = 5 μ m (b) Box plots showing range of and individual CTC %ARNL measurements (white circles) for each patient. Inter- and intra-patient heterogeneity is observed.

Figure 5. High AR nuclear localization (% ARNL) correlates with lack of response to taxane chemotherapy (a) and positive AR-V7 expression (b).

(a) CTCs (n = 103) from patients who did not achieve 50% PSA decrease at C5 (non-responders) had higher %ARNL compared to CTCs (n = 100) from responders. (b) CTCs (n = 106) from AR-V7 positive patients has higher %ARNL compared to CTCs (n = 48) from AR-V7 negative patients. * = p < 0.05; ** = p < 0.01.

Table 1.
Clinical and circulating tumor cell (CTC) characteristics of patients.

CTCs were analyzed from 15 patients before the initiation of taxane chemotherapy. Percent androgen receptor nuclear localization (%ARNL) was measured in up to 20 CTCs per patient and mean and range %ARNL was calculated. For 12 patients, CTCs were analyzed by ddPCR for AR-V7 expression. Patients positive for AR-V7 are shown in red, while those who were negative are shown in green. Patients with no available ddPCR data are shown in gray. Taxanes: C-Cabazitaxel, D-Docetaxel, P-Paclitaxel. AR targeted therapies: A-Abiraterone, E-Enzalutamide. Dashes indicate no prior therapies.

Current Taxane	Prior AR Targeted Therapies	Prior Taxanes	CTCs Analyzed	Mean %ARNL	Range %ARNL	PSA Response	AR-V7 Status
C	A, E	D, P	9	86%	44 - 95%	53%	
C	-	-	20	85%	50 - 96%	-30%	
P	A, E	C, D	20	84%	60 - 97%	94%	
D	A, E	C	7	80%	63 - 93%	41%	
D	A	-	3	79%	66 - 87%	-94%	
D	A, E	-	20	78%	45 - 93%	-52%	
D	-	-	7	78%	68 - 88%	-93%	
D	-	-	4	76%	64 - 85%	-21%	
C	-	D	20	76%	61 - 89%	-89%	
C	-	-	9	75%	33 - 89%	-59%	
D	-	-	20	71%	57 - 89%	-58%	
D	A	-	20	70%	48 - 84%	-5%	
C	A, E	D	19	70%	47 - 86%	-6%	
D	A, E	-	20	69%	35 - 85%	-65%	
D	A	-	4	47%	21 - 80%	-43%	