Single Cell Analysis of AR N terminal, AR C terminal and the AR-V7 Splice Variant in the CTCs of metastatic Castration Resistant Prostate Cancer (mCRPC) Patients

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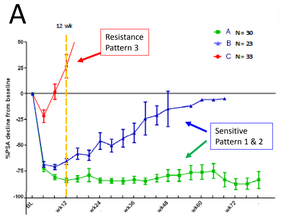
Background

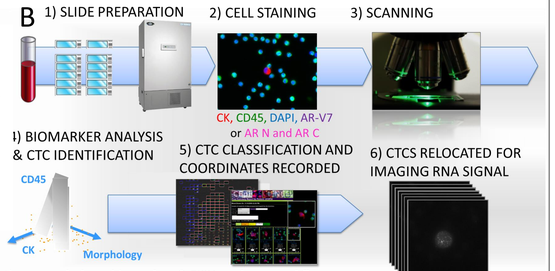
Androgen receptor signaling directed therapies (ARS Tx), including Abiraterone Acetate + Prednisone (A) and Enzalutamide (E), prolong survival in patients with mCRPC and are FDA approved. The presence of the splice variant AR-V7 mRNA in EpCAM selected CTCs has been prospectively linked to resistance to A & E[1] but not to taxane chemotherapy[2]. Alterations to the ligand binding domain (LBD) measured through the loss of the AR C terminus in bone[3] and CTCs[4] have also been associated with resistance to A & E but not taxanes. Together, measurements of AR-V7 and/or AR N & C termini (AR N & C) may provide clinical utility in therapy selection between A & E or taxanes.

Previous work showed high CTC phenotypic heterogeneity in patients resistant to A & E[5]. Understanding if AR-V7 is the predominant driver alteration of resistance in mCRPC patients is critical to the development of novel AR N terminal targeted therapies. A key limitation to assessing AR-V7 mRNA in CTCs is the diagnostic pre-analytic robustness of measurements in live cells (<2hr to processing, combined with 39 cycles of qPCR)[1]. To address both questions, we developed an AR-V7 rabbit MAb IF assay to assess fixed single CTCs utilizing samples from mCRPC patients annotated for sensitivity to these agents. The same samples were assessed with an AR N &C IF test to understand driver mechanisms in context of other AR negative CTC subtypes.

Methods

60 mCRPC patient blood samples were collected prior to starting Abiraterone (13); Enzalutamide (19), Docetaxel (22) and Cabazitaxel (6). Outcomes were recorded as Sensitive (S): Patterns **1** and **2**, or Resistant (R): Pattern **3** **(A)**[6]. Samples were processed utilizing the Epic Sciences platform **(B)**.



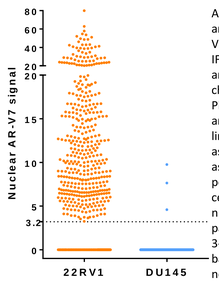


Schematic of Epic CTC Platform CTC enumeration, morphology, biomarker, & FISH analyses workflow:

1) Nucleated cells from blood sample placed onto slides and stored in a -80°C biorepository
2) Slides stained with cytokeratin (CK), CD45, DAPI, and either protein biomarker(s) of interest (AR-V7 or AR N and AR C combined) or RNA FISH target(s) of interest (RNA FISH performed concurrently with staining)
3) Slides scanned
4) CTC candidates detected by a multi-parametric digital pathology algorithm
5) Human reader confirmation of CTCs & quantitation of biomarker expression
6) CTCs relocated and RNA signals imaged along Z-axis of cells

AR-V7 IF and AR N & C Terminal Assay Development

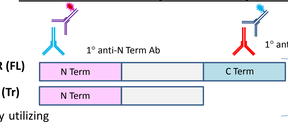
AR-V7-specific protein IF assay



A rabbit monoclonal antibody specific to the AR-V7 splice variant was used for IF staining of cell line controls and patient CTCs characterized by the Epic CTC Platform. Positive (22RV1) and negative (DU145) cell line controls were used to assess initial AR-V7 IF protein assay specificity. AR-V7 positive cells were defined as cells having a specific nuclear-localized staining pattern with signal intensity 3-fold higher than background staining from neighboring WBCs.

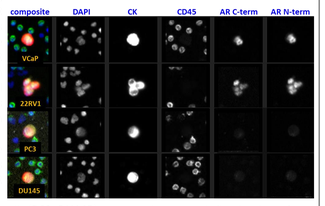
(Above) 52% (416/800) of individually characterized 22RV1 cells showed specific AR-V7 staining, while nuclear staining was seen in only 0.3% (3/963) of DU145 negative controls.

AR N & C terminal (C term loss) IF assay



A 5-plex IF assay utilizing antibodies specific to AR N and C termini was developed to detect the presence of truncated AR variants. N term positivity with decreased C term signal was indicative of C term loss/AR truncation.

(Right) Representative images of cell lines expressing full-length AR (VCaP), truncated AR (22RV1), and AR-negative cells (PC3, DU145) were used as controls for the detection of full-length and truncated AR variants .



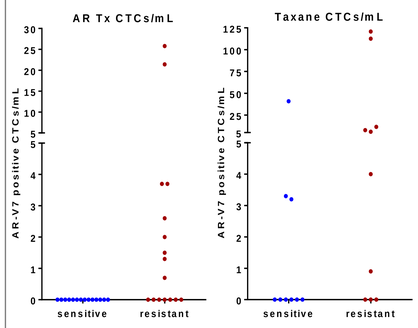
Patient Demographics

Characteristic	No. (%) or Median (range)
Number of Baseline Samples (unique patients)	60 (52)
Age, years	68 (48 – 91)
Blood Age, hours	27 (2 – 49)
Primary Treatment	
Prostatectomy	29 (49%)
Radiation	17 (28%)
None	14 (23%)
Hormone Therapies	
1 - 2 lines	23 (38%)
3 lines	13 (22%)
≥4 lines	24 (40%)
Chemo-naïve	33 (55%)
Chemo-exposed	27 (45%)
Metastatic Disease	
Bone	51 (85%)
Lymph Node	37 (62%)
Liver	5 (8%)
Lung	8 (13%)
Other Soft Tissue	4 (7%)
Laboratory Measures	
PSA, ng/mL	190.72 (0.51 – 2589.9)
Hgb, (g/dl)	11.15 (7.0 – 15.4)
ALK, (unit/L)	179 (42 – 2170)
LDH, (unit/L)	285 (151 – 1293)
ALB, (g/dl)	4.1 (3.4 – 4.9)
CTC, (cells/7.5mL)	80 (0 – >200)

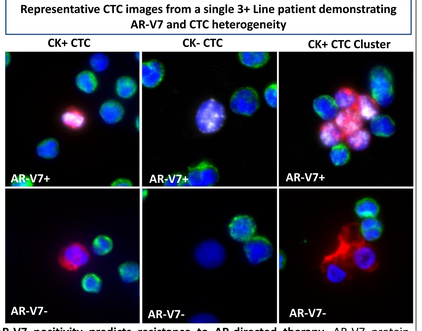
Patient Line of Therapy

	1st	2nd	3rd and 3+
A or E Baseline Blood Draw (n=32)	No Prior A or E Tx (1st Line) n= 12	Previous ARS Tx (A or E) (2nd Line) n= 9 Previous T (2nd Line) n= 1	Previous A & E (3rd Line) n= 2 Previous ARS Tx & T (3rd Line) n= 8
T Baseline Blood Draw (n=28)	No Prior A or E Tx (1st Line) n=3	Previous ARS Tx (A or E) (2nd Line) n= 3	Previous A & E (3rd Line) n= 8 Previous ARS Tx & T (3rd Line) n= 14

AR-V7 in CTCs Predicts Resistance to ARS Tx but not to Taxane Chemotherapy



Representative CTC images from a single 3+ Line patient demonstrating AR-V7 and CTC heterogeneity

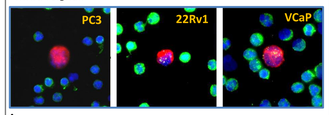


Patients with AR-V7+ CTCs			Clinical Specificity/Sensitivity		
	Sensitive	Resistant	Specificity	Sensitivity	OR
ARS Tx	0/14 (0%)	9/16 (56%)	1.00	0.56	Inf.
Taxane Tx	3/9 (33%)	7/10 (70%)	0.67	0.70	4.7

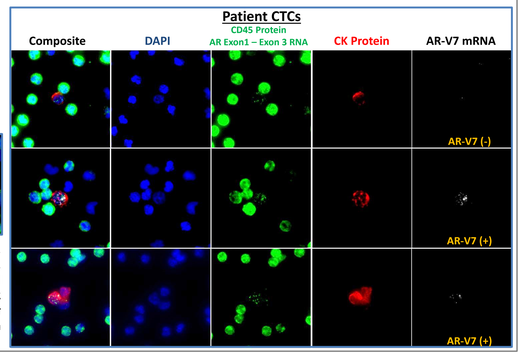
AR-V7 positivity predicts resistance to AR-directed therapy. AR-V7 protein expression was found in CTCs from 9/16 patients resistant to ARS Tx. While CTCs were identified in 13/14 ARS Tx sensitive patients (range: 0-418, median: 4), 0/14 harbored AR-V7+ CTCs. Of all patients in the ARS Tx cohort that lacked AR-V7+ cells, 67% were sensitive to ARS Tx. AR-V7 prevalence does not predict resistance to Taxane chemotherapy: AR-V7+ CTCs were found in 3/9 and 7/10 Taxane-sensitive and resistant patients, respectively.

Detection of AR-V7 mRNA in Patient CTCs

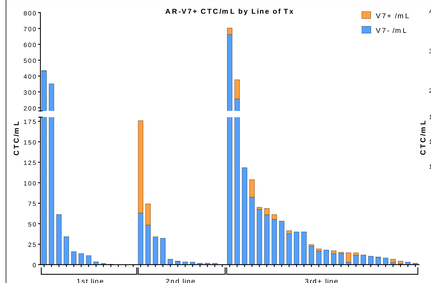
AR-V7 mRNA signal detected in patient CTCs. ➡ RNA FISH targeting AR-V7 mRNA and Exons 1-3 of full-length AR mRNA was performed on three patient samples. AR-V7 mRNA (white dots) and AR Exons 1-3 mRNA signal (green dots in same channel as CD45 protein signal) were found in a subset of patient CTCs. Not all patient CTCs with AR Exons 1-3 mRNA signal had AR-V7 mRNA signal.

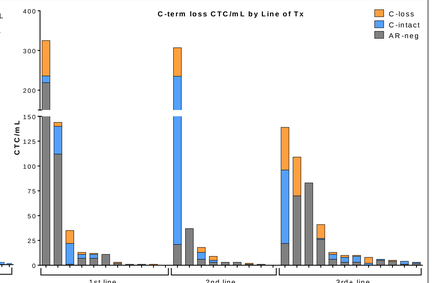


⬆**PC3, 22Rv1, and VCaP cell line cells used as controls.** Samples of healthy donor blood spiked with either PC3, 22Rv1, or VCaP cell line cells were processed in parallel with the patient samples. PC3 cells were negative for AR Exons 1-3 and AR-V7 mRNA, 22Rv1 cells were positive for both, and VCaP cells were positive for AR Exons 1-3 with a small subset of cells positive for AR-V7 mRNA.



Frequency and Heterogeneity of AR-V7 and C term Loss by Line of Therapy



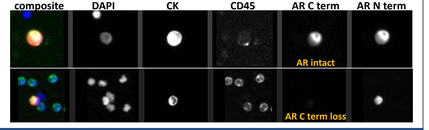


(%) AR-V7+ CTCs: range (median)				
Line of Tx	1	2	3	All lines
Total population	0-0.3 (0)	0-64 (0)	0-79 (8.6)	0-79 (0)
AR-V7+ pts only	0.3*	17-64 (35)	4.0-79 (12)	0.3-79 (15)

*only one AR-V7+ patient

(%) CTCs with C term loss: range (median)				
Line of Tx	1	2	3	All lines
Total population	0-100 (12)	0-50 (0)	0-75 (18)	0-100 (13)
C-term loss pts only	2.8-100 (27)	23-50 (36)	10-75 (25)	2.8-100 (28)

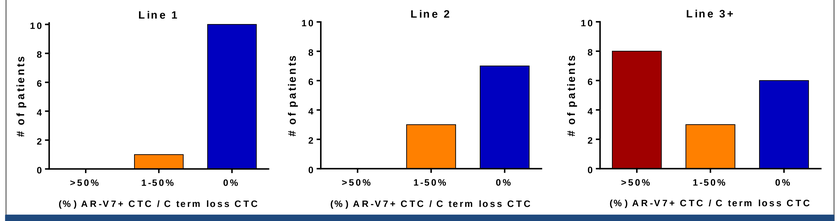
LBD splice variants, as detected by assays specific to AR-V7 and C term loss, detect AR LBD alterations that make up a subset of total CTCs. The proportion of LBD alterations (AR-V7 or other C term loss splice variants) vary across patients and may represent a predominant driving subpopulation in some, but not all patients. Additionally, heterogeneous expression of LBD alterations may have an association with therapy decision points.



AR-V7 Represents a Subset of Total AR LBD Alterations

Intra-patient analysis of AR-V7 compared to AR C terminal loss prevalence was performed in all patients with AR C terminal loss. AR-V7 and C term loss assays were run separately and compared between two equivalent slides. To assess if AR-V7 was the sole AR LBD alteration, a cut point of greater than 50% of AR-V7+/C terminal loss CTCs was utilized. Additionally, to determine if co-occurring AR LBD alterations are likely, a cut point of 1-50% AR-V7+/C terminal loss was analyzed. To determine if alternative AR LBD alterations should be considered, no AR-V7+ CTCs/AR C terminal loss CTCs was observed.

Evidence of other AR LBDs causing AR C terminal loss suggests patients may have **non AR-V7 alterations driving C term loss**. In many patients AR-V7+ CTCs make up a subset of AR C term loss cells suggesting intra-patient AR LBDs co-occurring within the same patient. 13/15 patients expressing AR-V7 positivity harbored C term loss supportive of strong assay concordance & specificity to AR LBDs:



Conclusions

- AR-V7 IF expression in CTCs of mCRPC patients assessed through the Epic Sciences platform is compatible with diagnostic workflows (median 27 hours from draw to processing) and specific to AR-V7 mRNA detected through AR-V7 RNA-FISH.
- AR-V7 prevalence increases with increased exposure to systemic therapy. The low median percentage (15%) of AR-V7+ CTCs in patients who have at least 1 AR-V7+ CTC shows disease heterogeneity and suggests that AR-V7 alone may not be the primary driver of disease progression.
- AR-V7 presence was highly specific to insensitivity of AR directed therapy, and AR-V7 negativity was associated with response (67%).
- The high number of patients with AR-V7:AR C term loss ratio of less than 50% suggests alternative or co-occurring AR LBD alterations: what ratio associates with a specific clinical phenotype or outcome is uncertain.
- Utilization of an AR C terminal loss assay may enable the identification of patients most likely to be sensitive to novel AR N terminal targeted therapies, particularly in the 1st and 2nd treatment decision point where the degree of heterogeneity is less than after additional lines of therapy.
- This is the first external clinical validation associating the presence of AR-V7 positive CTCs to A&E resistance but not to taxane, supporting the potential utilization of the AR-V7 biomarker to inform treatment selection in mCRPC patients.

References: 1) Antonarakis, Emmanuel S., et al. *New England Journal of Medicine* 371.11 (2014): 1028-1038. **2)** Antonarakis, Emmanuel S., et al. J Clin Oncol 33, 2015 (suppl 7; abstr 138). **3)** Efstathiou, E., et al. *Annals of Oncology* 2014 (25 suppl 4) **4)** Bambury, Richard M., et al. *Annuals of Oncology* 2014 (25 suppl 4) **5)** Scher, Howard I., et al ASCO GU Abstract #147 **6)** Scher, Howard I., et al. Cancer J. 2013 Jan-Feb;19(1):43-9

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