Supplementary Online Content

Scher HI, Lu D, Schreiber NA, et al. Association of AR-V7 on circulating tumor cells as a treatment-specific biomarker with outcomes and survival in castration-resistant prostate cancer. *JAMA Oncol*. Published online June 4, 2016. doi:10.1001/jamaoncol.2016.1828

This supplementary material has been provided by the authors to give readers additional information about their work.

Supplementary Material for the *Validation of AR-V7 on Circulating Tumor Cells as a Treatment Specific Biomarker at Decision Points in the Management of Castration-Resistant Prostate Cancer* for Online-Only Publication

Elements Included:

SUPPLEMENTAL METHODS

Stable AR-V7 expressing cell lines

A stable AR-V7 expressing cell line was generated by lentiviral infection of PC3 parental cell line cells. Transfected cell lines were selected via puromycin resistance, and AR-V7 expression was confirmed by quantitative real-time PCR and immunoblotting. For purposes of AR-V7 assay development, 22RV1 cell lines were used as a control expressing low AR-V7, while the stable transfected cell line was used as a high-expressing control.

Single cell quantitative real-time PCR (qPCR)

Single 22RV1 or DU145 cells were individually isolated and assayed for gene expression using a Single Cell-to-CT Kit (Ambion) as per manufacturer protocol. qPCR was performed with TaqMan probes for AR exons 1-2 (AR N Term) (Thermo Fisher Scientific) or a probe set specific to AR-V7 (custom design) with a QuantStudio 7 (Thermo Fisher Scientific). All samples were analyzed for 18S (Thermo Fisher Scientific) as an internal RNA quality control.

Immunoblotting

Whole cell lysates were collected from PC3, DU145, stable AR-V7 expressing and 22RV1 cell lines. In each lane, 20 ug of protein (assessed by Bradford assay) were separated by SDS-PAGE gel electrophoresis using a 4-12% Bis-Tris gel in MES buffer (Thermo Fisher). Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane with the iBlot2 system (Thermo Fisher). Membranes were blocked with tris-buffered saline Tween (1%) containing 5% nonfat dry milk and then incubated with primary antibody against AR-V7 or histone H3 as loading control. Bound antibodies were detected using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescent reagent.

Tissue microarray

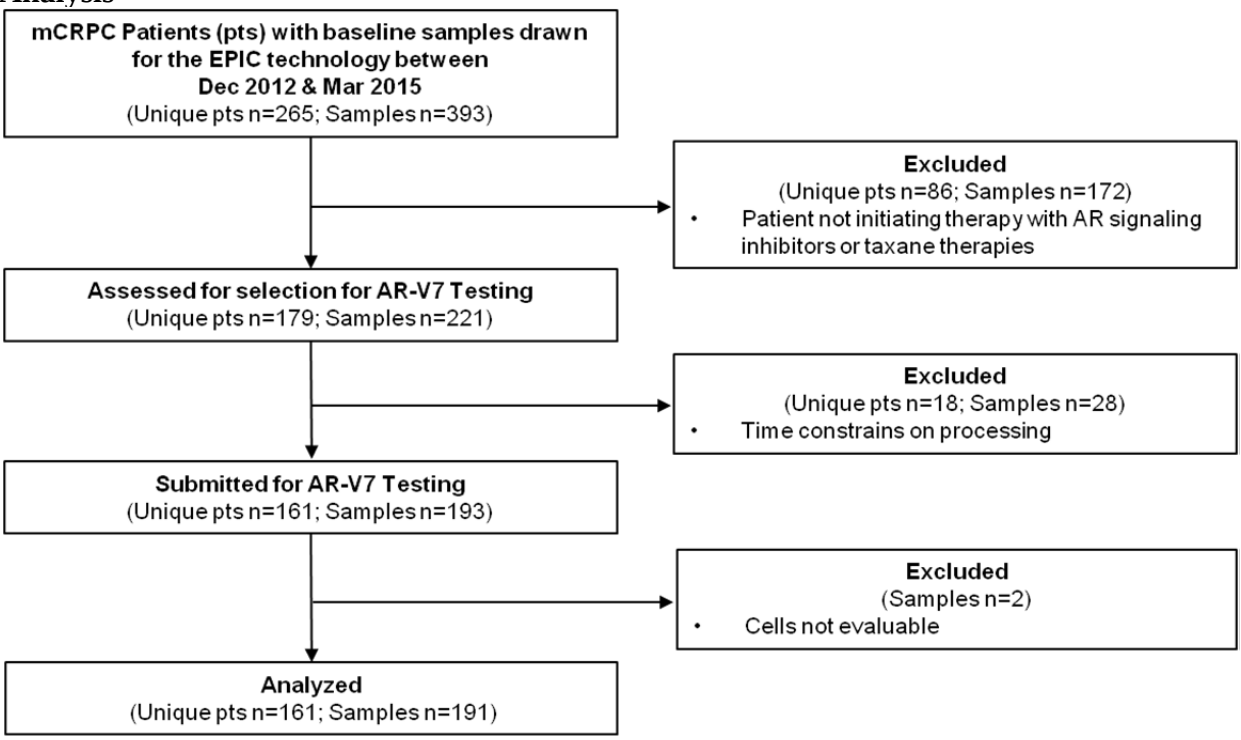
To assess antibody and assay specificity, immunohistochemical staining was conducted on tissue microarrays containing malignant, tumor-adjacent, and healthy tissues from prostate and other common sites of metastatic lesions. The same primary antibody used in the AR-V7 CTC assay herein described was tested, and a polymeric enzyme/chromogenic detection system was used. AR-V7 positive and negative tissues were used as respective controls. Stained slides were reviewed by a qualified, independent pathologist for the assessment of specific (nuclear-localized) staining and nonspecific background staining.

Supplemental Table 1. AR-V7 Status Prior to Initiation of ARS Inhibitors or Taxane Therapy in the 1st, 2nd, and 3rd or Greater Line of Therapy.

	AR Therapy (n=128)			Taxane Therapy (n=63)			Overall (n=191)		
Line of Therapy	AR-V7 Pos (n=16)	AR-V7 Neg (n=112)	Total (n=128)	AR-V7 Pos (n=18)	AR-V7 Neg (n=45)	Total (n=63)	AR-V7 Pos (n=34)	AR-V7 Neg (n=157)	Total (n=191)
1st line	2 (4%)	54 (96%)	56	0	11 (100%)	11	2 (3%)	65 (97%)	67
2nd line	6 (15%)	34 (85%)	40	3 (30%)	7 (70%)	10	9 (18%)	41 (82%)	50
3rd line or later	8 (25%)	24 (75%)	32	15 (36%)	27 (64%)	42	23 (31%)	51 (69%)	74
Test for association (Fisher's Exact)	P value = .0079			P value = .0539			P value < .0001		

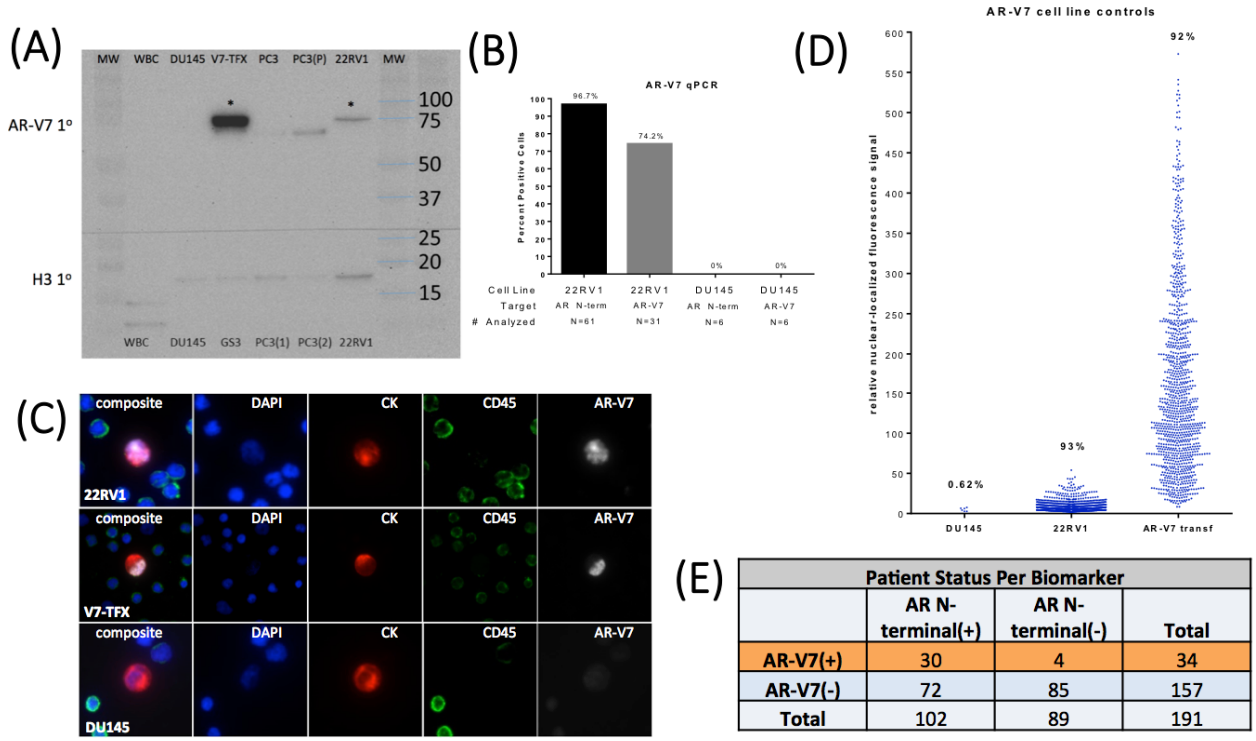
Note: 2 of 193 patient samples were not evaluable for AR-V7 status. All percentages are based on row totals.
Abbreviations: AR, androgen receptor.

Supplemental Figure 1: Patient and Sample Disposition Flow Chart for AR-V7 Analysis



Between December 2012 and March 2015, 265 mCRPC patients undergoing a change in systemic therapy were drawn for blood to be processed using the Epic Sciences CTC detection platform. Of these, 104 patients were excluded from this analysis because they were not starting abiraterone acetate, enzalutamide, ARN-509, docetaxel, cabazitaxel, or paclitaxel, or due to constraints on processing time and 2 samples had cells inevaluable for AR-V7 protein subcellular localization, leaving 191 evaluable samples from 161 unique patients for analysis.

Supplemental Figure 2: Cell-Level and Patient-Level Specificity of AR-V7 Antibody.



(E)

Patient Status Per Biomarker			
	AR N-terminal(+)	AR N-terminal(-)	Total
AR-V7(+)	30	4	34
AR-V7(-)	72	85	157
Total	102	89	191

(A) Western blot with anti-AR-V7 recognizes a 75 kDa band (denoted by *) corresponding to AR-V7 protein expressed in both 22RV1 and stable AR-V7 transfected cells (V7-TFX). AR-V7 protein is not detected in PC3 or DU145 negative control cells . The parental PC3 clones used to generate the stable AR-V7 expressing cells PC3(P) were also confirmed to be negative for endogenous AR-V7. Histone H3 serves as a loading control. (B) Single cell qPCR confirms the absence of AR N-terminal and AR-V7-specific mRNA in DU145 cells. AR N-terminal and AR-V7-specific transcript was found in 96.7% and 74.2%, respectively, of single 22RV1 cells assessed. (C) Representative images demonstrate nuclear-localized AR-V7 staining in 22RV1 and stable AR-V7 expressing cells (V7-TFX) but not in DU145 control cell lines. (D) Relative AR-V7 IF signals in individual control cell line cells are plotted. Only cells with nuclear-localized AR-V7 are shown. Nuclear-localized signal was observed in 0.62% (6/971) of DU145 (AR negative), 93% (1043/1121) of 22RV1 (AR-V7 positive), and 92% of AR-V7 transfected cell line cells (AR-V7 positive), respectively, as individually assessed by AR-V7 IF staining. (E) Sample-level specificity for AR-V7 positivity was assessed by additional sample testing utilizing an assay recognizing AR N-terminus on CTCs. (F) Tissue microarray IHC data as scored by an independent pathologist are summarized. Biopsy samples of malignant, tumor-adjacent, and healthy tissues representing common sites of malignant lesions are represented. Specific and background staining were qualitatively evaluated on a scale of 0 (no staining) to 4 (intense staining).

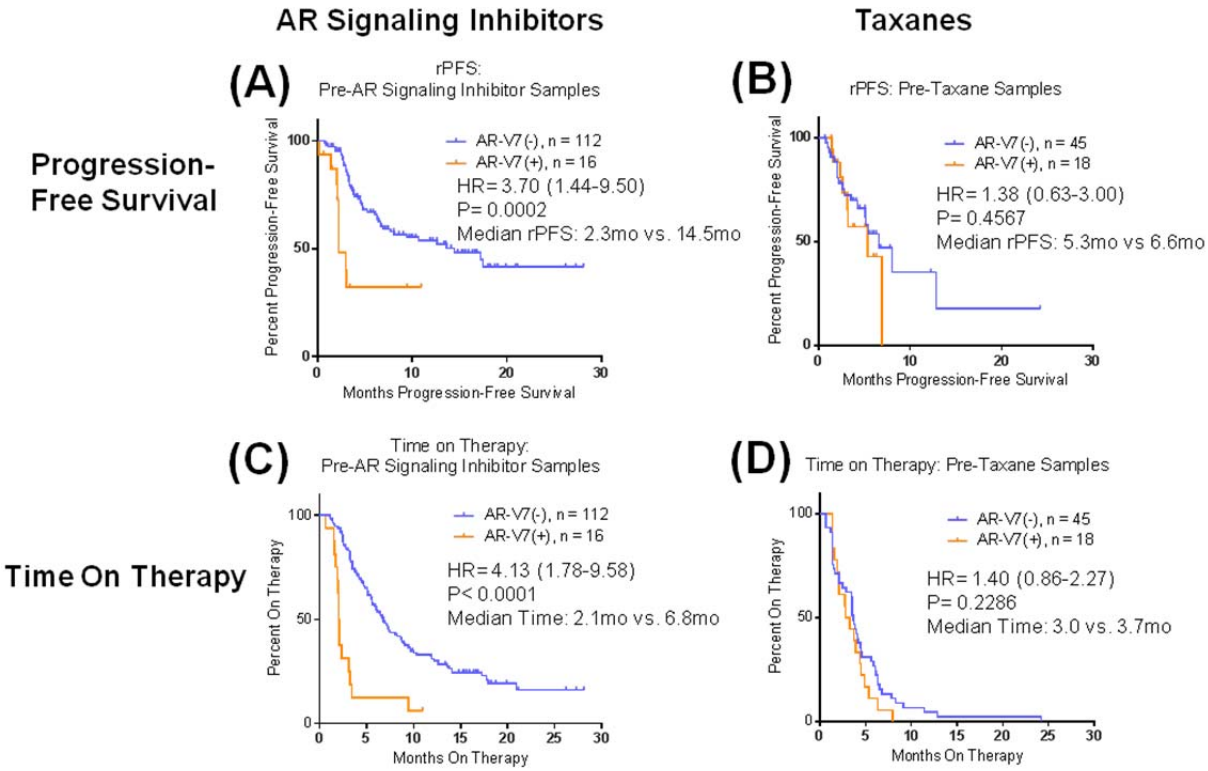
Supplemental Figure 2 (continued): Cell-Level and Patient-Level Specificity of AR-V7 Antibody.

(F)

Age	Sex	Organ	Pathology Diagnosis	Grade	Stage	TNM	Type	Score	Background
71	F	Bone	Osteosarcoma of right femur	1	IB	T2N0M0	Malignant	0	0
59	M	Brain	Pantomorphic glioblastoma of right occipital lobe	–	–	–	Malignant	0.5	0
30	M	Brain	Medulloblastoma of cerebellum	–	–	–	Malignant	0	0
F	58	Kidney	Papillary renal cell carcinoma	–	I	T1N0M0	Malignant	0	0
F	62	Kidney	Clear cell carcinoma	1	I	T1N0M0	Malignant	0	0
17	F	Liver	Hepatoblastoma	–	–	–	Malignant	0	0
56	M	Liver	Hepatocellular carcinoma	3	II	T2N0M0	Malignant	0	0
49	F	Lung	Adenocarcinoma	2	II	T2N1M0	Malignant	0	0
57	M	Lung	Squamous cell carcinoma	3	II	T2N1M0	Malignant	0	0
F	53	Lymph node	Diffuse B-cell lymphoma	–	–	–	Malignant	1R	0
M	21	Lymph node	Diffuse B-cell lymphoma of spleen	–	–	–	Malignant	0	0
M	40	Lymph node	Hodgkin's lymphoma of left clavicle	–	–	–	Malignant	1	0
77	M	Prostate	Adenocarcinoma (hyperplasia)	–	II	T2N0M0	Malignant	0	0
M	21	Spleen	Diffuse B-cell lymphoma	–	I	–	Malignant	0.5	0
70	M	Bone marrow	Cancer adjacent bone marrow tissue	N/A	N/A	N/A	NAT	0	0
35	M	Prostate	Cancer adjacent prostate tissue	N/A	N/A	N/A	NAT	0	0
21	F	Bone marrow	Bone marrow tissue	N/A	N/A	N/A	Normal	0	0
F	50	Cerebrum	Cerebrum tissue	N/A	N/A	N/A	Normal	0	0
M	50	Kidney	Kidney tissue	N/A	N/A	N/A	Normal	0	0
M	43	Liver	Liver tissue	N/A	N/A	N/A	Normal	0	0
M	30	Lung	Lung tissue	N/A	N/A	N/A	Normal	0	0
M	43	Lymph node	Lymph node tissue	N/A	N/A	N/A	Normal	0	0
31	M	Prostate	Prostate tissue	N/A	N/A	N/A	Normal	0	0
M	35	Spleen	Spleen tissue	N/A	N/A	N/A	Normal	1	0

N/A: Not Applicable
R: Positive cells are rare (<1%)
T1: Tumor invades submucosa
T2: Tumor invades muscularis propria
N0: No regional lymph node metastasis
N1: Metastasis in 1 to 3 regional lymph nodes
M0: No distant metastasis
NAT: Normal Adjacent Tumor
Grade 1 or well-differentiated: Cells appear normal and are not growing rapidly.
Grade 2 or moderately-differentiated: Cells appear slightly different than normal.
Grade 3 or poorly differentiated: Cells appear abnormal and tend to grow and spread more aggressively.

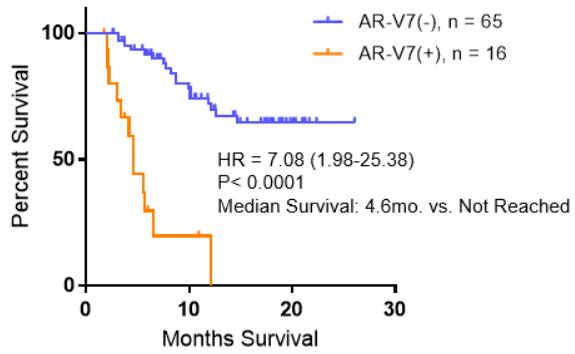
Supplemental Figure 3: Presence of AR-V7(+) CTCs Predicts Response to AR Signaling Inhibitors



Progression-free survival is shown, separated by AR-V7 status, for samples from patients receiving (A) ARS inhibitors or (B) taxanes. Time on therapy is shown, separated by AR-V7 status, for samples from patients receiving (C) ARS inhibitors or (D) taxanes.

Supplemental Figure 4: AR-V7 Identifies Worst Outcomes Among PSA Resistant Patients to AR Signaling Inhibitors

(A)

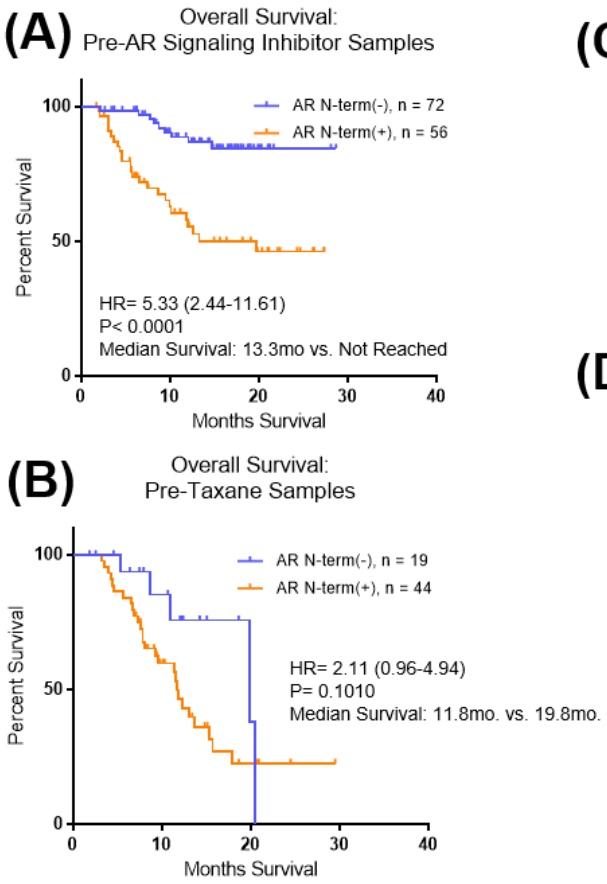


(B)

Multivariable Cox Proportional Hazard Analysis of Predictors of Overall Survival	
Effect	**P-value**
Line of Therapy (3rd or later vs 1st/2nd)	0.6889
Visceral Metastases Pre-Therapy	0.3958
LDH Pre-therapy (>250 vs ≤250 U/L)	0.0641
Patient Age (>65 vs ≤65 years)	0.6931
Hemoglobin (>12 vs ≤12 g/dL)	0.0236
AR-V7 Status	**<.0001**

(A) Overall survival of patients on ARS inhibitors is shown, stratified by pretherapy AR-V7 status. (B) Individual covariates were tested for additive predictive power to predict overall survival using the Cox proportional hazards model. The p-values are the result of compensating for the other factors listed.

Supplemental Figure 5: Presence of AR N-terminal(+) CTCs Predicts Response to AR Signaling Inhibitors



(C)

Multivariable Cox Proportional Hazard Analysis of Predictors of Overall Survival	
Effect	**P-value**
Line of Therapy (3rd or later vs 1st/2nd)	0.0107
Liver and/or Lung Metastases Pre-Therapy	0.0576
LDH Pre-therapy (>250 vs ≤250 U/L)	0.0717
Patient Age (>65 vs ≤65 years)	0.3120
Hemoglobin (>12 vs ≤12 g/dL)	0.0077
Therapy	0.4868
AR N-terminal Status	**0.0010**
AR N-terminal Interaction with therapy	**0.1113**

(D)

AR N-term : Therapy Interaction: Multivariable Cox PH Model		
	Comparison	**Hazard Ratio (95% CI)**
AR N-terminal Status & Therapy	AR N-term(+): Taxane vs AR	0.59 (0.32 to 1.08)
	AR N-term(-): Taxane vs AR	1.43 (0.52 to 3.89)

Overall survival of patients is shown, separated by AR N-terminus status, for patients going onto (A) ARS inhibitors or (B) taxanes. (C) Individual covariates were tested for additive predictive power to predict outcome using the Cox PH model. The p-values are the result of compensating for the other factors listed. (D) The interaction of therapy and AR N-terminus status was further investigated.