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TITLE: Androgen Receptor Splice Variants and Resistance to Taxane Chemotherapy

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<b>14. ABSTRACT</b>  During this reporting period, we have obtained approval for a no-cost extension of this award and change of research focus in the final year. This report will summarize the reseach activities and achievements up to date, organized based on the revised Statement of Work. In summary, we have 1) Identified two regions in the AR LBD that confer the microtubule-binding activity; 2) Further characterized the functional significance of the AR-microtubule interaction and provided compelling evidence to suggest that the microtubules play an important role in controlling the intracellular localization and activity of AR; 3) Provided a fundamental understanding of the constitutive nuclear localization and transcriptional activities of AR-V7 and ARv567es; and 4) Developed and validated a whole-blood assay for the detection of both AR-V7 and ARv567es.									
<b>15. SUBJECT TERMS</b> Castration-resistant prostate cancer; docetaxel; cabazitaxel; chemotherapy; androgen receptor splice variants; microtubule; ligand-binding domain; microtubule-associated sequence									
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## I. Introduction

Initially, we set out to test the central hypothesis that constitutively active androgen receptor splice variants (AR-Vs) are associated with resistance to taxane chemotherapy in castration-resistant prostate cancer (CRPC). However, this hypothesis has been challenged by several clinical studies after we started our project. Antonarakis et al first evaluated the associations between AR-V7 status and clinical responses in patients receiving taxane chemotherapy (1). The key response biomarkers, including PSA progression-free survival, clinical/radiographic progression-free survival, and overall survival, were comparable between patients with different AR-V7 expression status in circulating tumor cells (1). Furthermore, AR-V7-positive patients had superior responses to taxanes than to enzalutamide or abiraterone, suggesting taxane chemotherapy is a viable treatment option for these patients. Subsequently, a report by Scher et al confirmed that taxane is more efficacious than AR-directed therapies in AR-V7-positive patients, and that no significant differences in clinical responses to taxane are associated with AR-V7 status (2). Since our proposed clinical study in Aim 3 have a similar study design as these studies, we believe will be able to reach a different conclusion. In addition, we have encountered difficulty in patient accrual as few patients are treated with docetaxel or cabazitaxel alone at Tulane Cancer Center. Based on these considerations, we have requested to extend the study period for 12 months and to change the focus of the project. This request has been approved recently. The restructured proposal consists of the following specific aims:

Aim 1. To understand the fundamental difference in nuclear translocation mechanisms of AR-FL and AR-Vs.

Aim 2. To functionally characterize the microtubule association sequences.

Aim 3. To develop a blood-based assay for detecting the expression of AR-Vs.

In this report, we will provide a comprehension summary of the work completed to date, organized around the revised specific aims and major tasks. Please be noted that some of these have been reported in previous annual reports. We feel this is appropriate due to the reorganization of the State of Work.

## II. Keywords

Castration-resistant prostate cancer; docetaxel; cabazitaxel; chemotherapy; androgen receptor splice variants; microtubule; ligand-binding domain; microtubule-associated sequence

## III. Accomplishments

**What are the major goals/tasks of this project?**

**Major Task 1:** To identify the microtubule-associated sequence (MTAS) on AR.

**Major Task 2:** To develop a blood-based assay for detecting the expression of AR-Vs.

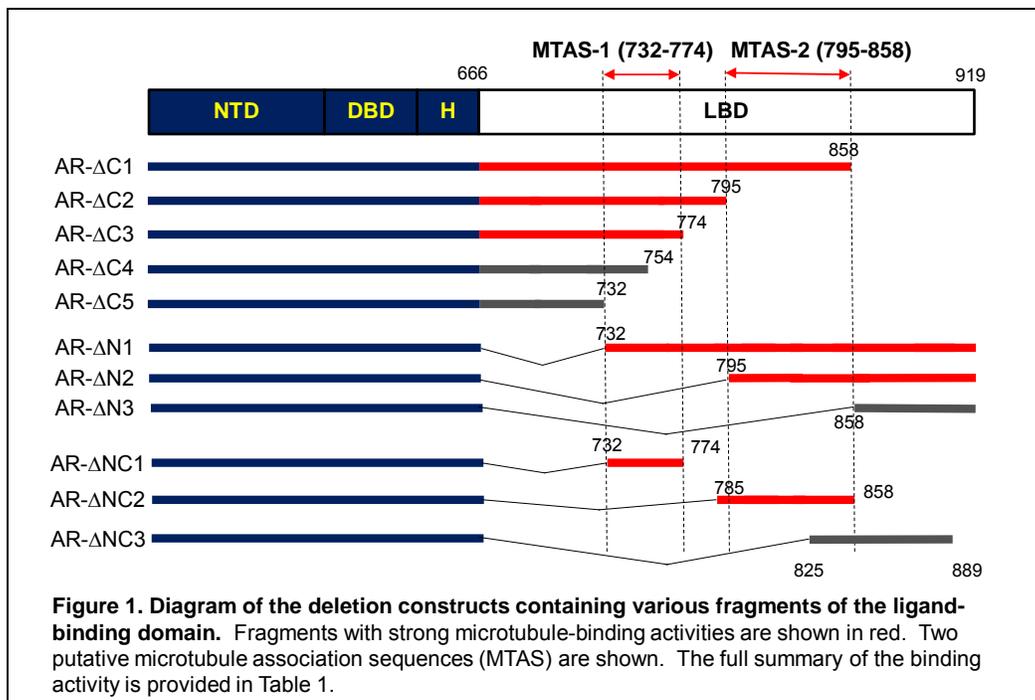
**Major Task 3:** To determine the transcriptional activity of MTAS-truncated AR constructs.

**Major Task 4:** To determine the influence of recurring AR LBD mutations on microtubule association.

## What was accomplished under these goals?

### **Major Task 1: To identify the microtubule-associated sequence (MTAS) on AR.**

1. Identification of the microtubule-associated sequences (MTAS) on AR. We have previously demonstrated that the nuclear import of full-length AR (AR-FL) depends on a dynamic microtubule, whereas that of the AR-Vs is microtubule-independent (3). We hypothesize that this fundamental difference is caused by the different binding capacities to the microtubule cytoskeleton by the two types of receptors. This hypothesis was confirmed by using an *in vivo* microtubule-binding assay (3). In addition, we generated a series of deletion constructs encompassing different domains of AR. By using the *in vivo* microtubule-binding assay, we have demonstrated that the microtubule-binding is mediated by the ligand-binding domain (LBD) of AR (3). Consistent with this finding, we found that the LBD-truncated AR-V7 and AR<sup>v567es</sup> both bind poorly to the microtubules (3).



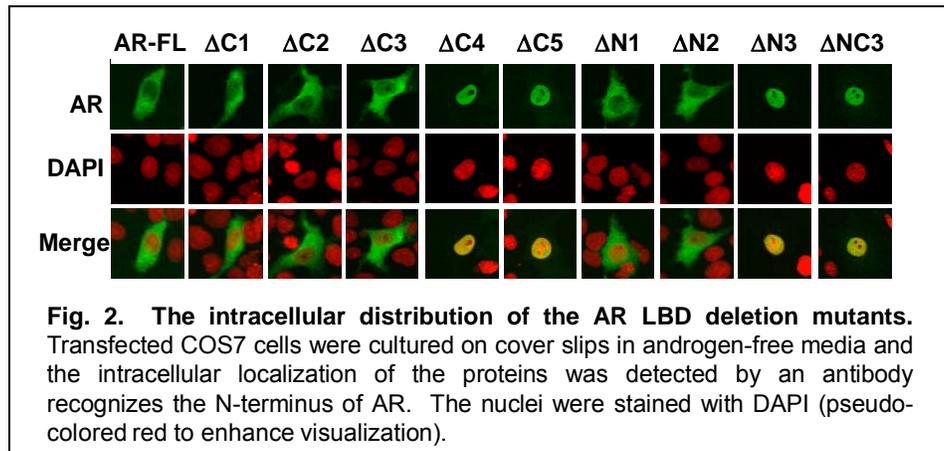
To further map the region(s) within the LBD that is responsible for microtubule association, we generated a series of deletion constructs within the LBD (Fig. 1). COS-7 cells were transiently transfected with these plasmids, cultured in an androgen-deprived condition, and lysed for the *in vivo* microtubule-binding assay. This deletion analysis revealed that two regions in the AR LBD could potentially mediate the association with the microtubules (Fig. 1). These regions were termed *microtubule association sequence 1* (MTAS1, a.a. 732-774) and *2* (MTAS2, a.a. 795-858). Constructs retained one or both MTAS displayed strong binding activities, whereas those with both MTAS deleted or disrupted lost the ability to bind to the microtubules (Fig. 1). Interestingly, all constructs that were capable of microtubule binding showed similar binding activities, regardless of which or how many copies of MTAS they contain (Table 1 and Fig. 1), suggesting there is functional redundancy between MTAS1 and MTAS2.

2. Correlation of the microtubule-binding activity and the intracellular localization of AR proteins in the absence of ligand stimulation. To determine the localization of the aforementioned AR deletional mutants, COS-7 cells were transfected with plasmids and cultured in an androgen-depleted condition. Following fixation, the intracellular localization of the AR proteins was analyzed by immunofluorescence using an antibody recognizing the N-terminus of AR. The complete results were compiled in Table 1 and representative images were shown in Fig. 2. In summary, we observed a strong correlation of intracellular localization and microtubule-binding activity of these proteins in the absence of androgen stimulation. All mutants with strong microtubule-binding activities were localized to the cytoplasm, whereas those with weak binding activities were found in the nucleus. These results provide evidence supporting the notion that association with the microtubules is a mechanism for retaining AR in the cytoplasm.

**Table 1. Summary of AR-LBD deletion analysis.**

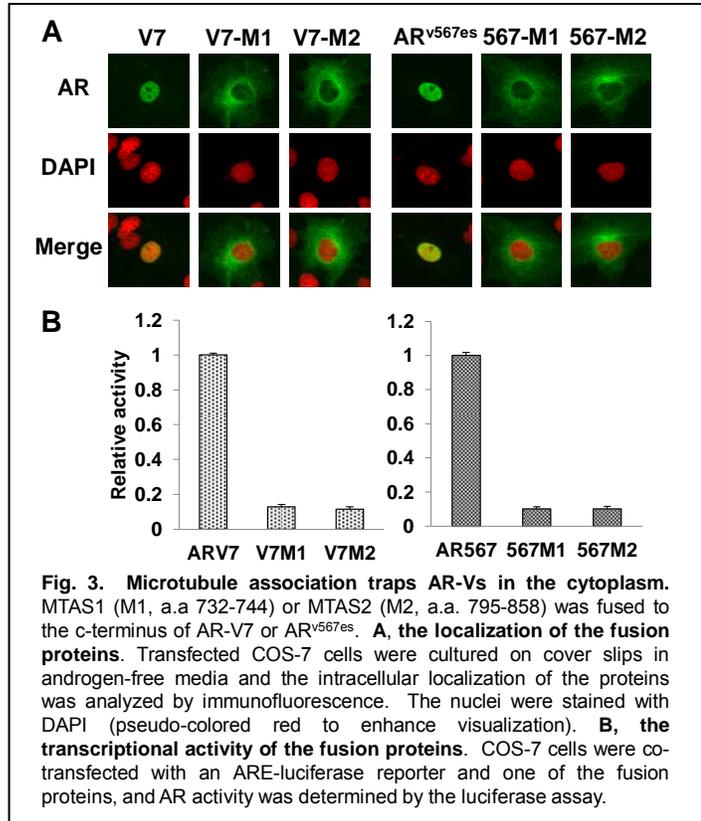
Name	LBD fragment	MT binding	Localization (w/o androgen)
AR-FL	666-919	++	Cytoplasmic
ΔC1	666-858	++	Cytoplasmic
ΔC2	666-795	++	Cytoplasmic
ΔC3	666-774	++	Cytoplasmic
ΔC4	666-754	-	Nuclear
ΔC5	666-732	-	Nuclear
ΔN1	732-919	++	Cytoplasmic
ΔN2	795-919	++	Cytoplasmic
ΔN3	858-919	-	Nuclear
ΔNC1	732-774	++	Cytoplasmic
ΔNC2	795-858	++	Cytoplasmic
ΔNC3	825-889	-	Nuclear

\*, scored based on relative MT-binding activity compared to that of AR-FL. ++, >75%; +, 50-75%; -, <50%.



3. Association with the microtubules traps nuclear-localized AR-Vs in the cytoplasm. The intracellular localization analysis above is consistent with what we have observed with AR-V7 and AR<sup>v567es</sup>, which possess weak microtubule-binding activities and are predominantly nuclear-localized (3). We hypothesize that AR-V7 and AR<sup>v567es</sup> gain constitutive nuclear localization by escaping microtubule-mediated cytoplasmic retention. To test this hypothesis we engineered chimeric proteins by appending MTAS1 (M1, a.a 732-744) or MTAS2 (M2, a.a. 795-858) to the

C-terminus of the variants. *In vivo* microtubule-binding assays confirmed that the ensuing fusion proteins gained microtubule-binding activities (data not shown). Next, the intracellular localization of the fusion proteins was examined in COS-7 cells by immunofluorescence as described above. As shown in Fig. 3A, AR-V7 was predominantly localized to the nucleus. However, after fusing M1 or M2, the chimeric protein were localized primarily to the cytoplasm. Similarly, the addition of M1 or M2 caused AR<sup>v567es</sup> to be trapped in the cytoplasm. In addition, AR-V7 and AR<sup>v567es</sup> lost its transcriptional activity when fused with M1 or M2 (Fig. 3B). Collectively, these results provide strong support for the role of MTAS in retaining AR in the cytoplasm.



#### 4. MTAS1 and MTAS2 are highly conserved among nuclear receptors.

MTAS1 is located within Exon 5, whereas MTAS is encoded by Exons 6 and 7. Sequence analysis revealed little homology between MTAS1 and MTAS2 (data not shown). However, both MTAS regions were highly conserved within the Type I nuclear receptor subfamily, particularly progesterone receptor (PR), mineralcorticoid receptor (MR), and glucocorticoid receptor (GR). In contrast, estrogen receptor alpha (ER $\alpha$ ) only contains a region with modest homology with MTAS1. This is possibly due to the short C-terminus of ER $\alpha$  as compared to other Type I receptors. Nonetheless, the sequence analysis suggests that both MTAS are present in other Type I nuclear receptors.

<b>MTAS1 (contained within Exon 5)</b>	
AR (732-774)	DDQMAVIQY <b>S</b> <u>WM</u> GLMV <b>F</b> AMG <b>W</b> RSFTNVNSR <b>M</b> LY <b>F</b> APDLVFNEY
PR (745-787)	DDQITLIQY <b>S</b> <u>WM</u> SLMV <b>F</b> GLG <b>W</b> RSYKHVSG <b>Q</b> M <b>L</b> Y <b>F</b> APDLILNEQ
MR (796-838)	EDQITLIQY <b>S</b> <u>WM</u> CLSS <b>F</b> ALS <b>W</b> RSYKHTNS <b>Q</b> FLY <b>F</b> APDLVFNEE
GR (590-632)	DDQMTLLQY <b>S</b> <u>WM</u> FLMA <b>F</b> ALG <b>W</b> RSYRQSSAN <b>L</b> LC <b>F</b> APDLIINEQ
ER $\alpha$ (373-403)	HDQVHLL <b>E</b> CAW <b>L</b> EILMIG <b>L</b> V <b>W</b> RS--MEHPG <b>K</b> LL <b>F</b> APNLLLDRN
<b>MTAS2 (contained by Exons 6 and 7)</b>	
AR (795-858)	FGWL <b>Q</b> IT <b>P</b> Q <b>E</b> FL <b>C</b> M <b>K</b> ALL <b>L</b> FS <b>I</b> IPVD <b>G</b> L <b>K</b> N <b>Q</b> K <b>F</b> DE <b>L</b> RM <b>N</b> Y <b>I</b> K <b>E</b> LD <b>R</b> II <b>A</b> CK <b>R</b> K <b>N</b> P <b>T</b> SC <b>S</b> R <b>R</b> F <b>Y</b>
GR (653-716)	LHRL <b>Q</b> VS <b>Y</b> E <b>E</b> Y <b>L</b> C <b>M</b> K <b>T</b> LL <b>L</b> LLSS <b>V</b> PK <b>D</b> GL <b>K</b> S <b>Q</b> E <b>L</b> F <b>D</b> E <b>I</b> R <b>M</b> T <b>Y</b> I <b>K</b> E <b>L</b> G <b>K</b> A <b>I</b> V <b>K</b> R <b>E</b> GN <b>S</b> S <b>Q</b> N <b>W</b> Q <b>R</b> F <b>Y</b>
PR (808-871)	FVK <b>L</b> Q <b>V</b> S <b>Q</b> E <b>F</b> L <b>C</b> M <b>K</b> V <b>L</b> LL <b>L</b> NT <b>I</b> P <b>L</b> E <b>G</b> L <b>R</b> S <b>Q</b> T <b>Q</b> F <b>E</b> E <b>M</b> R <b>S</b> S <b>Y</b> I <b>R</b> E <b>L</b> I <b>K</b> A <b>I</b> G <b>L</b> R <b>Q</b> K <b>V</b> V <b>S</b> S <b>S</b> Q <b>R</b> F <b>Y</b>
MR (859-922)	FVRL <b>Q</b> L <b>T</b> F <b>E</b> E <b>Y</b> T <b>I</b> M <b>K</b> V <b>L</b> LL <b>L</b> ST <b>I</b> P <b>K</b> D <b>G</b> L <b>K</b> S <b>Q</b> A <b>F</b> E <b>E</b> M <b>R</b> T <b>N</b> Y <b>I</b> K <b>E</b> L <b>R</b> K <b>M</b> V <b>T</b> K <b>C</b> P <b>N</b> S <b>G</b> Q <b>S</b> W <b>Q</b> R <b>F</b> Y

**Fig. 4. MTAS1 and MTAS2 are highly conserved among nuclear receptors.** Sequence analyses showed both MTAS1 and MTAS2 are highly conserved among members of the Type I nuclear receptor subfamily, including progesterone receptor (PR), glucocorticoid receptor (GR), mineralcorticoid receptor (MR). Identical amino acids among all members are highlighted in bold. The residue affected by the AR LBD hotspot mutation W742C is underlined.

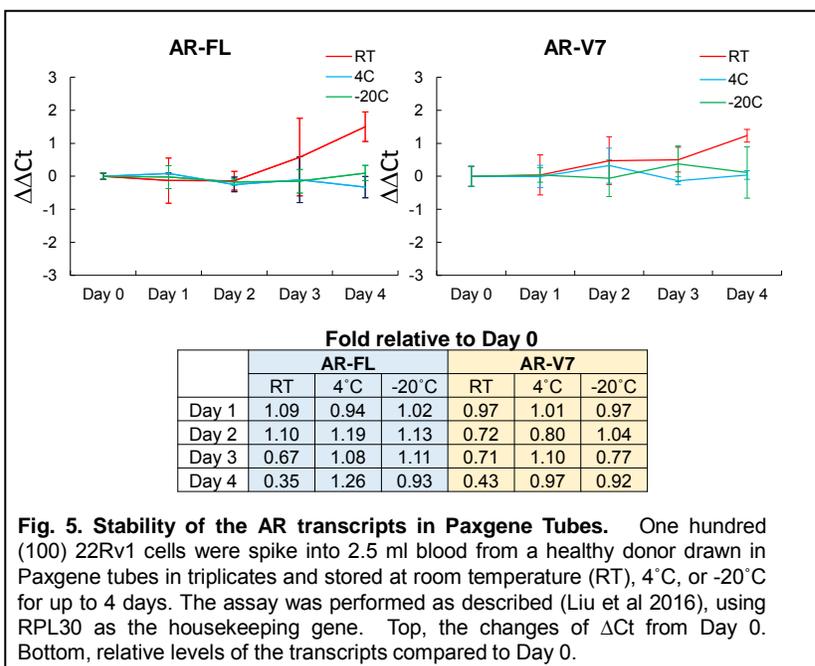
**Summary:** we have completed the proposed experiment listed under Major Task 1. In addition, we have extended the scope of this task with additional experiments to better understand the functional significance of AR association with the microtubules.

**Major Task 2: To develop a whole-blood-based assay for detecting the expression of AR-Vs.**

1. Development of a blood-based assay for AR-V7 and AR<sup>v567es</sup>. We have developed an assay for these variant using whole-blood collected in PAXgene blood RNA tubes. This method has been published (Liu et al, 2016, see below).

2. The stability of AR transcripts in PAXgene tubes. An important question for the blood-based assay is the stability of AR transcripts in the PAXgene tubes. To answer this question, we

spiked 100 22RV1 cells into PAXgene tubes containing 2.5 ml of blood from a healthy donor (AR-V negative). The tubes were stored at room temperature (RT), 4°C or -20°C for up to 4 days and the assay was performed as described. As shown in Fig. 5, AR-V7 transcript was fairly stable at room temperature for 24h, but suffered a 30% loss of signal after 48 h. In contrast, AR-FL was much more stable when stored at 4°C or -20°C, with more than 90% of signal remained after day 4. The stability of AR-FL transcript was similar to that of AR-V7.



**Fig. 5. Stability of the AR transcripts in Paxgene Tubes.** One hundred (100) 22Rv1 cells were spike into 2.5 ml blood from a healthy donor drawn in Paxgene tubes in triplicates and stored at room temperature (RT), 4°C, or -20°C for up to 4 days. The assay was performed as described (Liu et al 2016), using RPL30 as the housekeeping gene. Top, the changes of ΔCt from Day 0. Bottom, relative levels of the transcripts compared to Day 0.

These results showed that the AR transcripts remain stable in Paxgene tubes stored at 4°C or -20°C for at least 4 days.

**Summary:** By developing and validating the whole-blood-based assay for detecting the expression of AR-V7 and AR<sup>v567es</sup> in patients, we have completed Major Task #2. The addition work on the stability of AR-V7 in PAXgene tubes provides guidelines for sample collection from multiple centers, to be analyzed by a central lab.

***What opportunities for training and professional development has the project provided?***

Nothing to report.

***How were the results disseminated to communities of interest?***

Nothing to report.

*What do you plan to do during the next reporting period to accomplish the goals?*

**Major Task 3: To determine the transcriptional activity of MTAS-truncated AR constructs.**

These constructs will include  $\Delta C4$ ,  $\Delta C5$ ,  $\Delta N3$ ,  $\Delta NC3$  (Table 1), which have been shown to be defective in microtubule-binding and localized predominantly to the nucleus. COS-7 cells, which do not express any AR isoforms, will be transiently transfected with an androgen response element (ARE) luciferase construct (ARE-Luc) and pRL-TK, along with a plasmid encoding one of these deletion constructs. Cells will be cultured in the presence or absence of 1 nM R1881 for 24 h and dual-luciferase assay will be performed using the Dual-Luciferase Reporter Assay System (Promega). The transcriptional activity of AR, measured by the firefly luciferase activity, will be normalized by transfection efficiency (measured by Renilla luciferase activity). To corroborate the luciferase assay results, DU145 (AR-negative prostate cancer cell line) will be transfected with the MTAS-truncated AR plasmids, and the expression of endogenous AR targets (PSA and TMPRESS2) will be analyzed by qRT-PCR.

**Major Task 4: To determine the influence of recurring AR LBD mutations on microtubule association.**

Recent studies using cutting edge next generation sequencing analysis have confirmed AR LBD as a mutational hotspot. Particularly, four missense mutations (L702H, W742C, H875Y, and T878A) have been identified as recurring mutations in mCRPC specimens (4). Clinically, all these mutations have been implicated in resistance to AR-targeted therapies (5). Interestingly, the W742C mutation is located within MTAS1 and affects a residue conserved among all Type I nuclear receptors (Fig. 4). The other mutations are located in close proximity to MTAS1 (L702H) or MTAS2 (H875Y and T878A). To explore the possibility that these mutations may interfere with the microtubule-binding activity of AR, we will use site-directed mutagenesis to introduce these mutations to the AR deletion constructs. Specifically, the mutation will be introduced alone or in combination to the constructs containing a single MTAS as described by the following table:

<b>Template</b>	<b>Mutation(s) introduced</b>	<b>Designation</b>
AR- $\Delta C3$	L702H	AR- $\Delta C3$ L702H
AR- $\Delta C3$	W742C	AR- $\Delta C3$ W742C
AR- $\Delta C3$	L702H, W742C	AR- $\Delta C3$ L702H/W742C
AR- $\Delta N2$	H875Y	AR- $\Delta N2$ H875Y
AR- $\Delta N2$	T878A	AR- $\Delta N2$ T878A
AR- $\Delta N2$	H875Y, T878A	AR- $\Delta N2$ H875Y/T878A

The resulting plasmids will be analyzed for *microtubule-binding activity* by the *in vivo* microtubule-binding assay and *intracellular localization* by immunofluorescence microscopy. If these analyses show that at least some of these mutation(s) interfere with microtubule-binding and lead to nuclear localization, the protein(s) will be analyzed for *transcriptional activity* in the presence or absence of androgen.

**Citations (published journal articles):**

Xichun Liu, Elisa Ledet, Dongying Li, Ary Dotiwala, Allie Steinberger, Jianzhuo Li, Yanfeng Qi, Yan Dong, Jonathan Silberstein, Benjamin Lee, Oliver Sartor, and Haitao Zhang. A Whole Blood-

Based Detection Assay for AR-V7 and AR<sup>v567es</sup> in Patients with Advanced Prostate Cancer. Journal of Urology 2016;196:1758-1763.

#### **IV. Impact**

##### ***What was the impact on the development of the principal discipline(s) of the project?***

The impact of the project is several fold. First, we have developed and validate a whole-blood assay for the detection of AR-V7 and AR<sup>v567es</sup>, without the need for isolation of or enrichment for circulating tumor cells. This is important because constitutively active AR-Vs have been associated with resistance to hormonal therapies. Compared to other liquid biopsy assays for AR-V7, our assays are easy to implement due to its sensitivity, specificity, and cost effectiveness. Second, we have demonstrated that the AR transcripts remain stable in PAXgene tubes for at least 4 days at 4°C or -20°C, suggesting it is feasible to collect samples from multiple locations to be analyzed in a central lab, allowing more patients to have access to this assay. Finally, the work on the functional significance of AR-microtubule association provided strong evidence that the microtubule cytoskeleton plays an important role in regulating the intracellular localization and trafficking of AR. Constitutively active AR-Vs are able to escape such regulation due to the truncation of the ligand-binding domain.

##### ***What was the impact on other disciplines?***

Nothing to report.

##### ***What was the impact on technology transfer?***

Nothing to report.

##### ***What was the impact on society beyond science and technology?***

Nothing to report.

#### **V. Changes/Problems**

As mentioned in the Introduction, we have proposed to change the direction of the research to focus on the functional significance of AR-microtubule association. The previously proposed clinical study and animal experiment will not be carried out. These changes have been approved by the DOD-PCRP.

#### **VI. Products**

##### ***Publications, conference papers, and presentations***

- **Journal publications.** The following paper was published:

Xichun Liu, Elisa Ledet, Dongying Li, Ary Dotiwala, Allie Steinberger, Jianzhuo Li, Yanfeng Qi, Yan Dong, Jonathan Silberstein, Benjamin Lee, Oliver Sartor, and Haitao Zhang. A Whole Blood-Based Detection Assay for AR-V7 and AR<sup>v567es</sup> in Patients with

Advanced Prostate Cancer. Journal of Urology 2016;196:1758-1763. Status: Published; Acknowledgement of federal support: yes.

- **Books or other non-periodical, one-time publications.** Nothing to report.
- **Other publications, conference papers, and presentations.** Nothing to report

*Website(s) or other Internet site(s)*

Nothing to report.

*Technologies or techniques*

Nothing to report.

*Inventions, patent applications, and/or licenses*

Nothing to report.

*Others*

Nothing to report.

**VII. Participants & Other Collaborating Organizations**

*What individuals have worked on the project?*

<b>Name</b>	Haitao Zhang	Guanyi Zhang	Dongying LI	Jianzhuo Li
<b>Project role</b>	PI	Technician	Postdoctoral Fellow	Technician
<b>Researcher Identifier (ORCID ID)</b>	0000-0002-5969-1024	N.A.	N.A.	N.A.
<b>Nearest person month worked</b>	3	2	2	8
<b>Contribution to project</b>	Project design; data analysis; Study coordination; presentation; manuscript writing; report	Characterization of MTAS	Validate PAXgene assay in the cancer-free cohort	Functional characterization of MTAS
<b>Funding support</b>	DOD-PCRP, American Cancer Society	DOD-PCRP American Cancer Society	DOD-PCRP, American Cancer Society	DOD-PCRP, American Cancer Society

***Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?***

Nothing to report.

***What other organizations were involved as partners?***

Nothing to report.

**VIII. Special Reporting Requirements:** not applicable.

## **IX. Appendices**

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