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CONTRACTING ORGANIZATION: Minnesota, University of, Twin Cities

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14. ABSTRACT

Prostate cancer patients are often given androgen deprivation therapies (ADT) that block the function of the signaling protein, androgen receptor (AR). While effective initially, tumors typically transform into a more aggressive disease known as castration resistant prostate cancer (CRPC) while on ADT. One major cause of resistance in CRPC is thought to be the emergence of Androgen Receptor variants (AR-Vs) at the genomic level. AR-Vs are altered forms of AR that are not sensitive to these drugs. This proposal aims to develop a high-throughput platform that can identify at the protein level which AR-Vs are present in clinical samples including tumor tissues and Circulating Tumor Cells (CTCs). Recent evidence from this grant suggests that there are three novel AR-Vs expressed at the protein level which was not previously known in PCa cell lines and in LuCaP Patient derived xenografts. Our results indicates that the presence of AR-Vs might contribute to the ADT resistance and provides rational to further our investigation and identify if these variants are involved mechanistically in drug resistance and open avenues to develop new treatments approaches.

15. SUBJECT TERMS
Prostate Cancer (PCa), AR splice variants (AR-Vs), metastatic Castration Resistance Prostate Cancer (mCRPC), Single Cell, Proteomics, Targeted Mass Spectrometry

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INTRODUCTION

Prostate cancer (PCa) is the most commonly diagnosed cancer in men in the United States. Androgen Receptor (AR) dysregulation is one of the main drivers in PCa becoming the main target for treatment. If a patient has aggressive PCa, typical upfront therapy involves Androgen Deprivation hormonal Therapy (ADT)[2,3]. While effective initially, tumors typically progress to a more aggressive disease known as metastatic Castration Resistance Prostate Cancer (mCRPC)[3,4] and the administration of second generation ADT therapies including abiraterone, and enzalutamide[2] are the most common line of treatment. With the increased disease management using these drugs, more potent tumor phenotypes have begun to emerge and either lose AR signaling altogether or AR splice variants (AR-Vs)[2,4] genomics expression develops. We rationalized that this resistance is associated with the emergence expression of AR-Vs leading to drug resistance in mCRPC[4-7]. Our objectives are to 1.) Develop a targeted mass spectrometry-based platform to evaluate and measure if the AR splice are translated to protein expression, 2.) Determine the dynamic mRNA and protein expression of these variants and if they are being expressed in cell lines and clinical tumor and 3.) Evaluate if these variants are expressed in single and cluster circulating tumor cells using single cell proteomics technologies. The success of these work will significantly contribute to disease management and help inform clinicians the adequate treatment to prevent resistance and unnecessary drug administration.

KEYWORDS

Prostate Cancer (PCa); Androgen Deprivation hormonal Therapy (ADT); AR splice variants (AR-Vs) metastatic Castration Resistance Prostate Cancer (mCRPC); Enzalutamide; Abiraterone; Single Cell; Proteomics; Targeted Mass Spectrometry.

ACCOMPLISHMENTS

What were the major goals of the project?

The major goals for this DOD project were to:

Major Task #1:

1. Finalize the ARvT-MS platform using the CMSP core (University of Minnesota).
2. Finalize the ARvT-MS platform using the Dr. MacCoss Lab Collaboration (University of Washington, Seattle).

Major Task #2:

3. Measure temporal dynamic protein expression of AR-Vs during anti-hormonal therapy.

Major Task 4:

4. Evaluate the variable protein expression of AR-Vs in prostate cancer cells

What was accomplished under these goals?

Major Task #1:

1. **Refine and finalize the ARvT-MS platform.** To assess the dynamic protein expression of AR-Vs in prostate cancer samples, we employed a targeted mass spectrometry-based proteomics approach. We identified and designed sequences that are specific to each variant and commercially synthesized these peptides. We used these peptides to calibrate the mass spectrometer (MS) so that when clinical samples are evaluated, we can identify AR-Vs in high throughput without antibody enrichment steps. We finished running a library of 54 peptides that identifies all the AR-Vs. On our initial analysis, we identify that all the peptides' parameters cannot be analyzed in one mass spectrometer inclusion list method. Therefore, we prioritize the analysis by narrowing down the list of peptides based on the ability to detect these peptides in PDX tumor samples. Out of the 54 peptides identifying 17 AR-Vs plus AR-FL, we moved forward with a total of 9 peptides standards that includes two known AR protein isoforms (2 peptides per protein): AR-FL_1, AR-FL_2, AR-V7_2, AR-V7_3 and five novel isoforms: AR-V2, AR-V5, AR-V6, AR-V12 and AR-V23 (one peptide per protein since only a small

peptide sequence identifies the uniqueness of these variants). These standards were distributed equally to two different MS core facilities. Both Institutions, CMSP core (University of Minnesota, Twin Cities) and the Dr. MaCoss Lab Collaboration (University of Washington, Seattle) use two different instruments so we can validate our approach and that these peptides are reproducible and detectable in different locations with different technical handling individuals. We had achieved our goal and we have been able to observe several AR-Vs protein expressions in tumor samples of 5 novel ARVs that has not been observed previously.

2. **Subtask 1-4: Perform stability assay on the remaining peptides, perform Limit of Detection (LOD) and Limit of Quantification (LOQ) assays on the remaining peptides for relative peptide quantification, and measure peptide recovery by spiking in the synthetic peptides prior to sample processing into negative AR-Vs cells.** We rely on synthetic peptides standards to be able to run stability assay, limit of detection, limit of quantification and peptide recovery. Currently, MilliporeSigma the company that we use to generate the synthetic peptides sent us bad quality peptides. This is not normal, MilliporeSigma aqua peptides are typically of great quality and reproducible. We were expecting new batch of peptides in March of 2020. The new batch of peptides were ready but when MilliporeSigma were about to ship these peptides, the University of Minnesota was shut down due to a National emergency of COVID-19 pandemic. These peptides were held back in storage and were not delivered until August 2020. These peptides were again distributed to both institutions UMN and UW on August 25th. After both institutions received these peptides, they started to perform the analysis and previous mentioned assays. After a couple of months running and optimizing these peptides, they concluded that these peptides have high levels of polymer contamination which led to column and instrument clogging. Several of the peptides were of very low quality. I initiated and conducted a meeting with between both institutions and MilliporeSigma to present our challenges working with their synthesized peptides.

   To eliminate the peptide standards contamination, MilliporeSigma proposed to perform a peptide clean-up and re-send the same peptides. They did send the peptides cleaned and I distributed the peptides again to both institutions. Both Institutions came back indicating that these cleaned peptides are again of poor quality. I communicated back with MilliporeSigma, and we concluded that they will re-synthesize new peptides and deliver immediately so we can proceed with our studies. These is very unusual circumstance, and we are working forcefully to proceed with our studies timely.

   After extremely long time (1 ½ year) of optimization and waiting for new peptides, we decided to stop using MilliporeSigma peptide. The peptides standards were of extremely bad quality, and we wasted a lot of time working with these standards. We decided to use a different vendor, ThermoFisher Scientific. We purchase peptides from this company, and we are currently using this company and the peptides are of better quality. We performed calibration curves on each these peptides. We were able to test 7 out of the 9 peptides. We ordered two AR-V7 peptides and two AR-FL exon1 peptides. We selected one peptide per variant. AR-V23 is not significantly linear but this might be chromatography issues which we are working on it. **Figure 1A** shows the AR exon mapping structure and the other AR-variants that we are currently working on. The orange bar above the exon junctions indicates that this is the unique AR-V regions and where the peptide was designed from. **Figure B-H** shows the calibration curve of all the AR-Variants and each graphs contains the light (Top graph) and heavy (bottom) calibration curves and on the right side there three extracted ion chromatograms (XIC) indicated the relative abundance of the Light and Heavy peptides then the middle shows the transitions of the light and the Bottom shows the heavy peptide.

Major Task 2:

3. **Measure temporal dynamic protein expression of AR-Vs during anti-hormonal therapy.** We are currently working on transiently silencing AR FL and evaluating the dynamic expression of the ARVs. To measure the knock down efficiency, we developed a qRT-PCR based assay that measures absolute mRNA copy number per nanogram of RNA using gene-block standards. We have completed the

analysis on most targets including AR-exon1 (represents all variants), AR-Vs (AR-V7, AR-V2) in 22Rv1. AR-V5 and AR-V6 are challenging to measure at the qRT-PCR since these variants only differ by 1 to 3 amino acid(s). Therefore, these variants are only resolved at the mass spectrometry level since this technology can determine this mass different more efficiently. The remaining AR-FL, AR-V12, AR-V23 variants are on working progress currently since these variants are very similar so to determine if these probes are measuring the AR-v of interest, we use a degrader. **Figure 2. E and D.** Degraders are molecules that target the directed region of interest. The AR degrader targets the ligand binding domain. Using this degrader, we are able to evaluate if the larger protein molecules such as the AR-FL, AR-V23 and AR-V12 protein expression is reduced, and the relative abundance of the peptide's signal is reduced. We have some preliminary data for this, and we will continue working on this. **Figure 2 A-B.** We were able to silence four AR isoforms with more than 50% as indicated in **Figure 2C.** Currently we are working on running these samples where the given AR isoform will be measured. Then the knockdown efficiency is being measured using the targeted mass spectrometry platform. Preliminary data is very promising, and it shows that the AR targeted peptides are specific to its AR-isoform so were working on finishing these replicates.

Major Task 3:

4. **Evaluate the AR-Vs expression in single cells from PCa cell lines**. We have begun to assess the proteome expression from PCa cell lines in single cells. In order to start testing the single cell protocol, we first started to optimize the platform using single cell sorted from PCa cell lines. These cell lines are sorted and ready to proceed with MPOP-SCOPE assay. We will start working on it in the month of May-June of 2021.

We were able to analyze the proteome of single cells from 22Rv1. We were able to measure approximately 600 proteins per cell and we are working on increasing the number of proteins. We have preliminary data, and we are still working on running all the cell lines in replicates. Figure 3A shows the reproducibility of peptide abundance and 3B shows a Principal component analysis of the singles cells, control samples and carrier. Figure 3C and D shows the number of proteins and peptides identified from each cell.

Major Task 4:

Evaluate the variable protein expression of AR-Vs in CTCs. After establishing the single cell proteomics pipeline, we will proceed with the single cell proteomics from CTCs. We were able to evaluate the protein expression of single cells from prostate cancer cell line but not from single CTC. From a test sample of CTC from prostate cancer patients, we were able to observe the expression of AR-FL_exon1 and AR-V2 see **Figure 3E and 3F** shows the extracted ion chromatograms from a targeted MS analysis using a pool of CTCs of a prostate cancer patient. **Figure E-F** shows extracted ion chromatograms (XIC) indicating the relative abundance of the peak area between the endogenous and heavy peptides (top) then the Heavy peptides in the middle and the endogenous peptides coming from CTCs (bottom). This is preliminary data, and we are working on testing more samples such as patient derived xenografts tumors using the targeted-MS.

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

  Dr. Zoi Sychev had the opportunity to mentor and train two students. She was training them how to run the mass spectrometer and perform targeted mass spectrometer analysis.

- **How were the results disseminated to communities of interest?**
  - Dr. Zoi Sychev in the Drake Lab was able to attend a virtual conference at America Society Clinical Chemistry and presented preliminary data of her work from this proposal.
  - Dr. Zoi Sychev had the opportunity to present her work in conjunction with Dr. Scott Dehm Lab and Dr. Justin Drake lab.

- Dr. Zoi Sychev had the opportunity of presenting a poster at the American Association Cancer Research (AACR) prostate cancer conference in March 2023.
- Dr. Zoi Sychev had the opportunity to present her work at a local prostate cancer seminar known as TANGO.

- **What do you plan to do during the next reporting period to accomplish the goals?**
  - "Nothing to Report"

IMPACT

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

The overall impact of this work is to elucidate that we can measure the protein expression of AR-variants which has never been done before and will lead to potential identification of biomarkers for prostate cancer patient stratification approaches.

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?

This work will help patent stratification and provide support to clinicians to make inform decisions.

CHANGES/PROBLEMS:

Changes in approach and reasons for change

1. In **major task 1** of research specific tasks, we proposed to evaluate all the variants in a single mass spectrometry inclusion list. However, we determined that the mass spectrometer capacity is not more than nine peptides per run so, we narrowed down the list from 17 AR isoforms to 9 AR isoforms. We plan to follow the next 8 AR isoforms once we have accomplished our first round of tasks.

2. Due to the National emergency of COVID-19 pandemic, this has substantially delayed our work from all major task and milestones for at least 6-8 months. In addition, the issue with the peptide standard synthesis, has delayed our advancement even further which has significantly delayed us from accomplishing our proposed goals, however we are confident that we can move forward now that the country and schools are partially open, and we can go to the lab and perform the in-lab experimental work.

Actual or anticipated problems or delays and actions or plans to resolve them.

1. Since the Universities and lab has been partially open, we have been able to continue our work and communications with different core facilities to proceed with the mass spectrometry runs. Since the COVID pandemic, we had significant delays on being able to receive good quality peptides to use as standards for our method development. We were delayed approximately 1 ½ years but despite this and thankfully to the no cost extension we were able to make great progress.

Changes that had a significant impact on expenditures

None of the changes described above will result in 0% change in budget allocation

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to report.

PRODUCTS
Publications, conference papers, and presentations

Journal publications.
Nothing to Report.

Books or other non-periodical, one-time publications.
Nothing to Report.

Other publications, conference papers, and presentations.
Academy of Clinical Chemistry virtual conference 2020 and Society for Basic Urologic Research (SBUR) 2020 American Academy of Cancer research the AACR of prostate cancer in March 2023.

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

Other Products
Nothing to Report.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Dr. Justin Drake
Project Role:	Principal Investigator (PI)
Researcher Identifier (e.g. ORCID ID):	0000-0002-8329-7748
Nearest person month worked:	
Contribution to Project:	PI
Funding Support:	

Name:	Dr. Michael MacCoss
Project Role:	Principal Investigator (PI)
Researcher Identifier (e.g. ORCID ID):	0000-0003-1853-0256

Nearest person month worked:	
Contribution to Project:	PI
Funding Support:	

Name:	Dr. Zoi Sychev
Project Role:	Post-doctoral researcher
Researcher Identifier (e.g. ORCID ID):	0000-0002-9830-5245
Nearest person month worked:	12
Contribution to Project:	Post-doctoral researcher
Funding Support:	

Name:	Dr. Eric Huang
Project Role:	Senior Research Scientist
Researcher Identifier (e.g. ORCID ID):	0000-0002-8745-0025
Nearest person month worked:	4
Contribution to Project:	Research scientist
Funding Support:	

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.

SPECIAL REPORTING REQUIREMENT
Nothing to report

COLLABORATIVE AWARDS:
Nothing to Report.

APPENDICES

Figure 1-5

Figure 1. Calibration Curve of all the AR isoforms standards

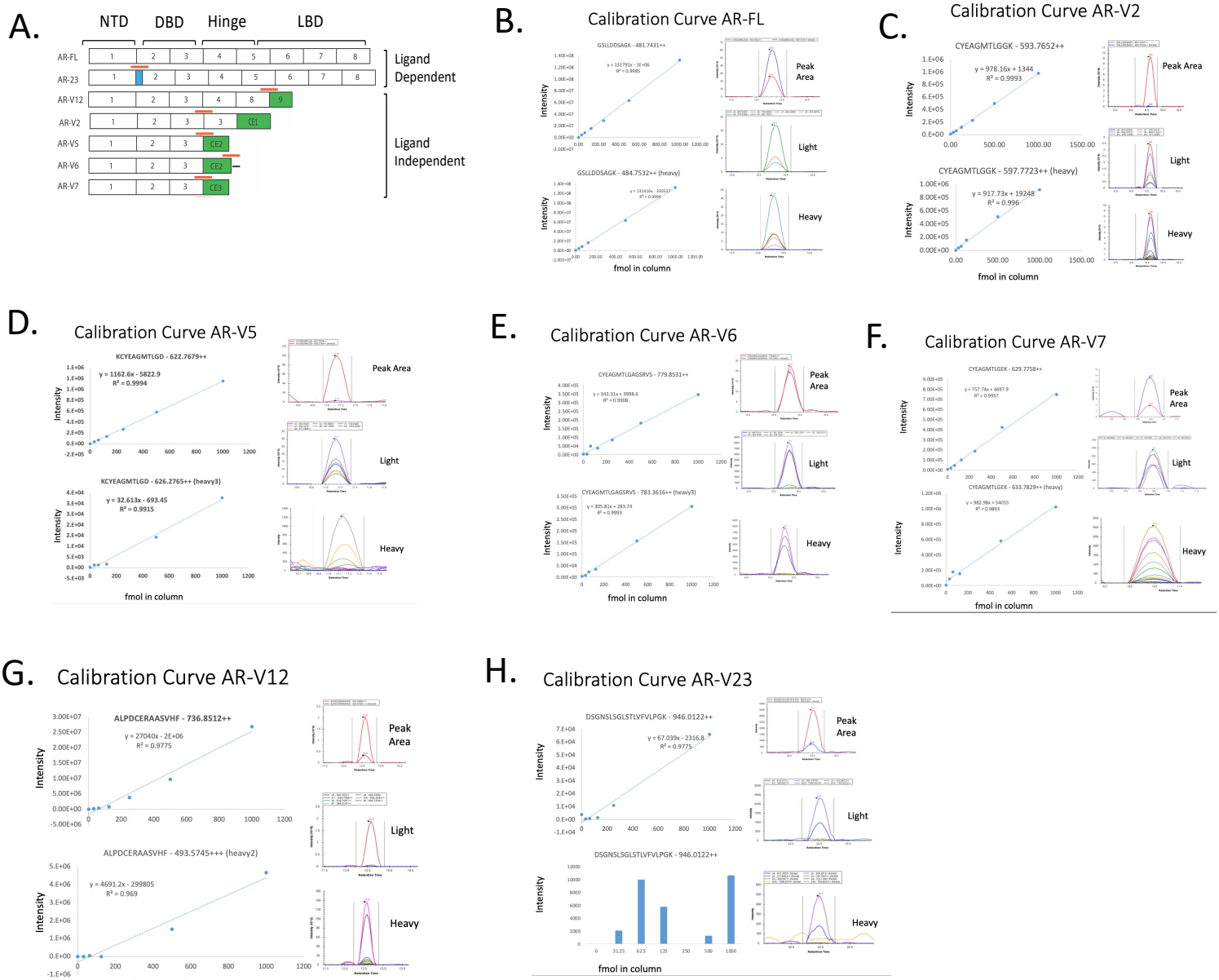
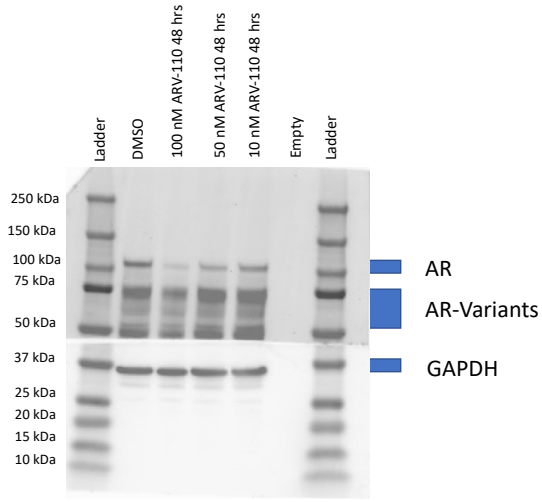


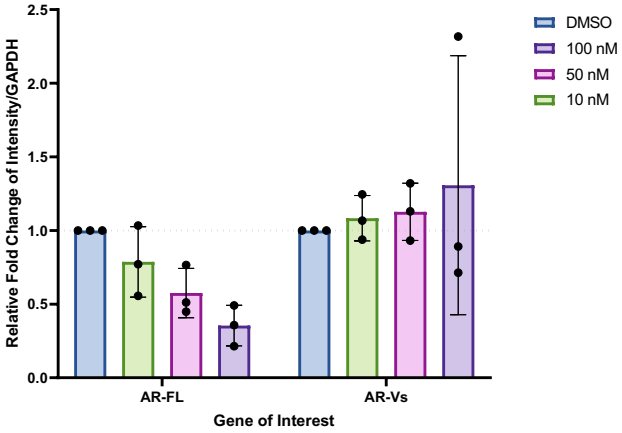
Figure 2. Transient silencing and degradation of AR-isoforms to evaluate peptide specificity.

Proteins expression inhibited using AR-Degrader in 22Rv1

A.



B.



Densitometry normalized via DMSO control, median of biological replicates is the bar, each point for biological replicates also graphed. n=3.

RNA silencing in 22RV1 using AR-Degrader in 22Rv1

C.

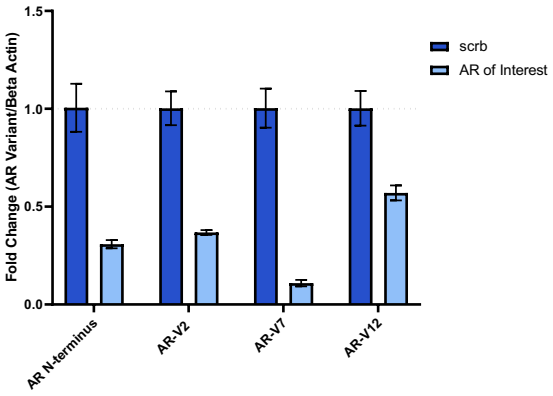


Figure 3. Analysis of the proteome of single cells by shotgun proteomics and targeted-MS