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PRINCIPAL INVESTIGATOR: Jasmine Wang, M.D.

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Jasmine Wang, MD

E-Mail: Jasmine.Wang@cshs.org

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14. ABSTRACT

The proposed study illustrates the development of an entirely new class of non-invasive test in prostate cancer (PCa) that may address limitations of currently used methods (e.g. serum PSA testing, Gleason grading system) by bringing the power of RNA-based analysis to blood testing. Using the novel Prostate Cancer Classification System (PCS) developed by our collaborator, Dr. Michael Freeman, we can categorize PCas into subtypes that are related to survival, response to therapy and may even predict resistance to certain types of treatment. This RNA-based signature has been quantified in circulating tumor cells (CTCs) utilizing the nanostructure-embedded Thermoresponsive-NanoVelcro assay. By combining advances in nanotechnology and genomics, this **CTC-PCS assay** would allow for dynamic resolution of transcriptomic alterations over time providing new insights into dynamic cancer biology.

15. SUBJECT TERMS
Prostate cancer, circulating tumor cells, nanotechnology, mRNA, expression profiling

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1. Introduction

Our research collaborator, Dr. Michael Freeman (CSMC) has developed a new transcriptome-based subtyping: Prostate Cancer Classification System (PCS)[1]. PCS categorizes PCa into 3 subtypes which are related to survival, response to therapy and may even predict resistance to certain types of treatment. The limitation of these genomic assays, however, is the need for tissue biopsy. Given the risk and invasiveness of the procedure, a non-invasive assay with the reference to molecular features driving clinical behavior and outcomes would potentially address this unmet need in PCa. RNA-based molecular signatures can be detected in circulating tumor cells (CTCs), making this an opportune way to further use of these evolving genomic signatures. While promising, this process requires improvement and further development before it can be used in the clinic. Over the past decade, our team has pioneered the NanoVelcro CTC assay, in which capture agent-coated nanosubstrates are used to selectively enrich CTCs. Recently, we have introduced the ThermoResponsive (TR)-NanoVelcro CTC purification assay, which allows capture and release of CTCs with intact RNA. This allows for seamless coupling with NanoString nCounter® platform[2] to accurately quantify the expression of RNA transcripts. Using these tools, our goal is to develop CTC-based PCS panel that will measure the aggressiveness of PCa. This tool could be used not only for early detection, but also for continuous monitoring of disease in response to treatment as serial blood collection is safe and easy. This allows for timely detection of emerging drug resistance and progression that will be of particular benefit to those patients with advanced mCRPC and their treating physicians.

2. Key Words

Metastatic, castration-resistant prostate cancer (mCRPC), NanoVelcro Assay, Prostate Cancer Classification System (PCS)

3. Accomplishments

- **What were the major goals of the project?**

Training-Specific Tasks:

Major task: Training and educational development in prostate cancer research.

- Milestone(s) Achieved: Presentation of project data at a national meeting or preparation for publication.
- Timeline: Month 1-16 with 100% completion

Research-Specific Tasks

Specific Aim 1: Assess the feasibility of this CTC-PCS assay using artificial blood samples.

Major Task 1: Calibration studies of TR-NanoVelcro assay for CTC capture efficiency and release yield.

- Milestone(s) Achieved Achieve temperature-dependent recovery (capture then release) of purified CTCs in 20 min, with >90% capture efficiency and >80% recovery yield.
- Timeline: Month 1-4 with 100% completion

Major Task 2: Calibration studies to assess the performance of RNA quantification for NanoString nCounter® platform.

- Milestone(s) Achieved: Achieve a sensitivity of RNA detection down to the density of 1-5 cells/mL with an intra-class correlation coefficient (ICCC) greater than 0.9.

- Timeline: Month 1-4 with 100% completion

Major Task 3: Calibration studies to examine the complete CTC-PCS assay.

- Milestone(s) Achieved: Differential expression levels of PCS genes will be seen in different cell lines as an indicator of varying aggressiveness. This analysis can be used to validate the accuracy of the CTC-PCS classifier.
- Timeline: Month 5-8 with 100% completion

Specific Aim 2: Assess the performance of this CTC-PCS assay in annotated clinical blood samples.

Major Task 4: Assess the performance of this CTC-PCS assay in annotated clinical blood samples.

- Milestone(s) Achieved: Using the PCS classifier, we anticipate the clustering of ARSI sensitive and resistant patients. We predict those patients with aggressive subtypes of PCS signature will have more aggressive clinical behavior manifesting as shorter time to ARSI resistance and worse survival.
- Timeline: Month 9-16 with 40% completion

- **What was accomplished under these goals?**

1) Major activities
   - Training specific activities
     - Attended clinical and translation research workshops at UCLA and Cedars-Sinai Medical Center (CSMC)
       - 2020 spring UCLA- Quantitative computational biosciences workshop
       - 2020 fall CSMC- statistics in medical research
     - Attended national scientific meetings including 2020 Genitourinary Cancer Symposium, 2020 ASCO, 2020 AACR, and 2020 prostate cancer foundation (PCF) retreat.
     - Presented research at the weekly meeting with Drs. Posadas and Tseng, as well as monthly meetings with Drs. Freeman and Chung.
     - Presentation of project data at the national meetings and preparation for publication
   - Research-specific activities
     - Implement QA/QC protocols of TR-NanoVelcro CTC purification system.
     - Preparation of artificial samples (LNCaP, MCF7, SkBr3, Huh7, A549, 22Rv1, and PC3).
     - Calibration studies for CTC capture efficiency and recovery yield.
     - Calibration studies to assess the performance of RNA quantification with cDNA mixture of spiking different PCa cell lines into 5000 WBCs at densities of 400-500, 100-150, 40-50 and 1-5 cells per sample.
     - Calibration studies to examine the complete CTC-PCS assay by showing differential levels of PCS score in cell lines with different aggressiveness.
     - Assess the performance of this CTC-PCS assay in annotated clinical blood samples, including samples from mCRPC patients sensitive and resistant to androgen receptor signaling inhibitor (ARSI).
     - IRB protocol amendment to expand the patient cohort utilizing Cedars-Sinai network.

2) Specific objectives
    - Technical validation using artificial and patient samples.
    - Path to implementation and initial clinical test of the CTC-RNA assay.
3) Significant results
    - We reproduced the cell line study in PCa similar to the study described in our previous publication of the TR-NanoVelcro assay (which validated the performance using lung cancer cell line and patient samples).[3] We successfully captured C4-2B parental and treatment resistant lines with 90% of efficiency and recovered 80% of the captured cells.
    - We have developed a CTC-specific PCS1 panel through a rigorous bioinformatic process. Among the 3 PCS subtypes[1], PCS1 phenotype is likely to be independent of AR pathway and associated with the worst prognosis, visceral metastasis, and resistance to androgen receptor signaling inhibitor (ARSI). The performance of the TR-NanoVelcro chip as well as the CTC-PCS1 panel is well-validated in PCa cell lines (see Appendix A, Figure 1 & 2).
    - In cell line studies, the treatment resistant C4-2B cell lines had higher CTC-PCS1 Z scores compared to the parental lines (see Appendix A, Figure 3).
    - In an exploratory clinical analysis, ARSI-resistant patients had significantly higher CTC-PCS1 Z scores comparted to ARSI-sensitive patients (see Appendix A, Figure 4 & 5).
    - In addition to CTC-PCS1 panel, we also investigated the performance of CTC-PCS2 and CTC-PCS3 panels to better reflect the luminal-basal biology of prostate cancer. We validated the performance of these 3 CTC-PCS panels in the GenomeDx GRID database and compare the prognostic ability with its original tissue PCS. Kaplan-Meier analysis of prostate cancer specific mortality, biochemical progression free survival and metastasis free survival shows patients classified as PCS1 genotype has the worst clinical outcomes in both original and CTC-specific panels. (Figure 1)

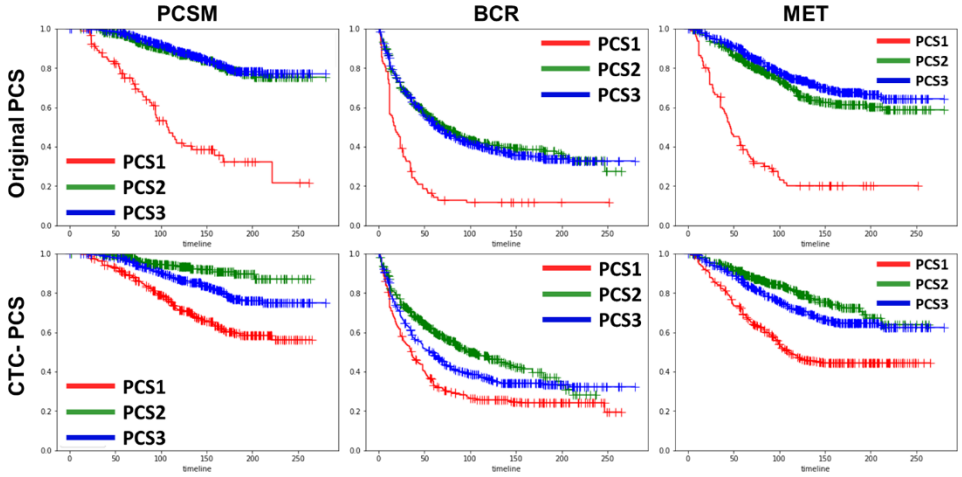

Figure 1. Performance of original PCS and CTC-PCS panel in the GenomeDx GRID database. PCSM: prostate cancer specific mortality; BCR: biochemical relapse; MET: metastasis free survival.

- Calibration studies of the CTC-PCS assay show the CTC-PCS panels can differentiate the aggressiveness and ARSI sensitivity of cell lines. The parental C4-2B cell line is assigned as PCS2, and the enzalutamide resistant cell line is assigned as PCS1.

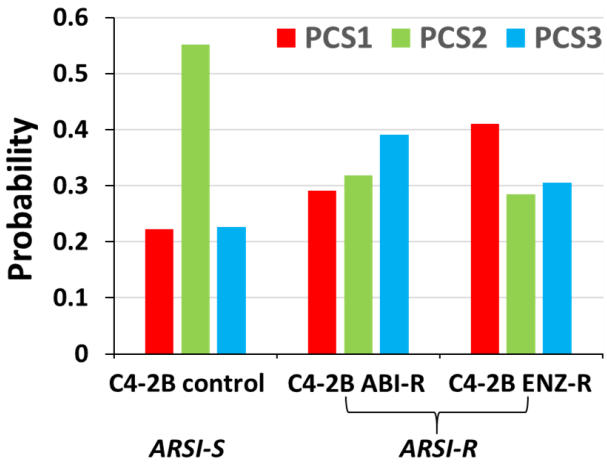

Figure 2. The PCS subtype of cell lines and patient samples is assigned using the nearest centroid method. C4-2B treatment naïve (control, ARSI-S), C4-2B ABI-R (abiraterone-resistant, ARSI-R), and C4-2B ENZ-R (enzalutamide-resistant, ARSI-R).

- We performed a pilot study to investigate the prognostic performance of PCS1, PCS2 and PCS3 in predicting overall survival of mCRPC patients using the CTC-PCS assay. Blood samples from 34 mCRPC patients prior to initiation of therapy with ARSIs (abiraterone, enzalutamide, or apalutamide) were analyzed with the CTC-PCS assay. Samples were classified as PCS1 (n=3), PCS2 (n=20), and PCS3 (n=11). The median overall survival for PCS1, 2, and 3 was 49, 149 and 157 weeks, respectively.

	PCS1 (n=3)	PCS 2/3 (c=31)	HR	p
bPFS on ARSI (wks)	13	53	6.9	0.0002
OS (wks)	49	149	4.1	0.014

Table 1. Biochemical progression free survival (PFS) on ARSI and overall survival (OS) for PCS1 vs. PCS2/3.

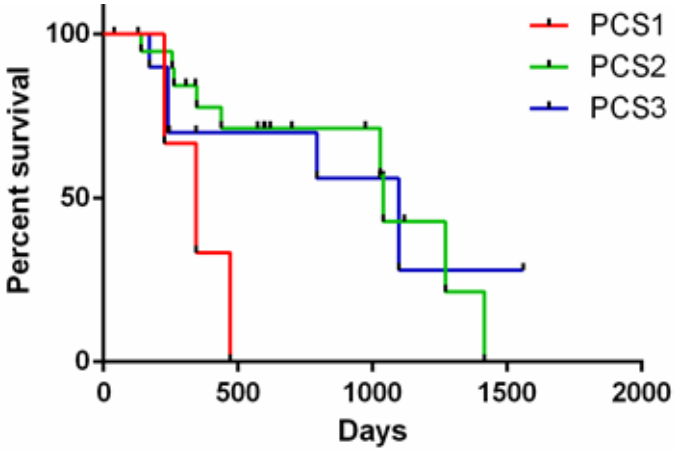

Figure 3. Kaplan-Meier analyses of OS according to CTC-PCS subtypes in 34 mCRPC patients prior to initiation of ARSI.

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

Training

- One-on-one work with mentor, Dr. Edwin Posadas, for clinical study design, execution, data collection, and interpretation
- One-on-one work with co-mentor, Dr. Hsian-Rong Tseng, for optimization of NanoVelcro CTC assay and development of subsequent approaches for CTC-based RNA measurement
- Monthly meeting with consultant, Dr. Leland Chung, for experimental design, data analysis and interpretation
- Quarterly meeting with consultant, Dr. Michael Freeman, for experimental design, data analysis and interpretation
- Attendance of Biostatistics courses at Cedars-Sinai Medical Center
- Attendance of Quantitative computational biosciences workshop at UCLA

Professional development

- Attendance of the Quantitative computational biosciences workshop in UCLA in spring of 2020.
- Attendance of national scientific meetings including 2020 Genitourinary Cancer Symposium, 2020 ASCO, 2020 AACR, and 2020 prostate cancer foundation (PCF) retreat.
- Submission of abstracts to 2020 Genitourinary Cancer Symposium, 2020 ASCO, and 2020 AACR.
- Poster presentation at 2020 Genitourinary Cancer Symposium and 2020 AACR.

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

Conference presentations:

- Teng P-C, Jan YJ, Chen J-F, Kim M, Yao N, Garraway I, Chu GCY, Chen P-J, **Wang JJ**, Lee Y-T, Zhu Y, **Chung LWK**, Feng FY, **Freeman M**, You S, Tseng H-R, **Posadas EM**. Prostate cancer CTC-RNA Assay: A new method for contemporary genomics and precision medicine via liquid biopsy. Journal of Clinical Oncology 2020 38:6_suppl, 170-170. GU Cancers Symposium 2020, San Francisco, CA.
- Teng P-C, Kim M, Jan YJ, Chen J-F, Yao N, Chu GCY, Chen P-J, **Wang JJ**, Lee Y-T, Zhu Y, **Chung LWK**, Feng FY, **Freeman M**, You S, Tseng H-R, **Posadas EM**. Gene expression of circulating tumor cells is predictive of treatment response in patients with advanced prostate cancer. American Association for Cancer Research (AACR) Annual Meeting 2020.
- Teng P-C, Jan YJ, Chen J-F, Kim M, Chen J-F, Yoon J, **Wang JJ**, Chen P-J, Yao N, Lee Y-T, Lozano A, Gadilov R, **Freeman M**, You S, Tseng H-R, **Posadas EM**. Development of a circulating tumor cell-based RNA classifier for patients with castration-resistant prostate cancer: CTC-PCS/PAM50. American Society of Clinical Oncology (ASCO) Annual Meeting 2020.

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

Generally, the majority of the experiments for the project has been done. Since our results revealed the potential of the CTC-PCS assay to be developed as a biomarker predicting ARSI

sensitivity, we have been in the progress of collecting more patients' samples for a larger scale of clinical validation.

In addition, since CTC-PCS assay has demonstrated its ability to identify the most aggressive subtype, PCS1, in mCRPC patients, we are also planning to expand the utility of this CTC-PCS assay to the cohort of localized and locally advanced prostate cancer with the goal of identifying aggressive prostate cancer at an early stage. The ultimate goal is to develope a noninvasive genomic classification to help physicians decide on the best treatment strategy for patients with early prostate cancer.

Finally, we plan to submit a bioinformatics-oriented article this year which will demonstrate our unique and rigorous bioinformatics pipeline to dissect molecular signals of PCa CTCs from background WBCs and translate a tissue-based genomic classifier into a liquid biopsy setting.

4. Impact

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

Success in this endeavor will produce tool for measuring dynamic, biological alterations that point to the emerging resistance to therapy. Optimally, this blood test will detect those changes responsible for the failure of particular cancer therapies in patients that define the biology of lethal PCa while providing new potential therapeutic targets for future exploration. This insight may also help refine the timing of changes in therapy to optimize outcomes and minimize toxicity.

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

Combination of engineering and informatics will create a means for translating other emerging gene/protein expression based signatures into blood tests that could be useful to developing blood-based companion diagnostics for PCa/CRPC.

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

Nothing to report.

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

The ultimate goal of this research is to pave the way for developing the use of CTC as a putative biomarker for aggressive prostate cancer, which will allow oncologists to implement therapy that will alter the natural history of advanced prostate cancer.

5. Changes/Problems

- **Changes in approach and reasons for change**

Nothing to report.

- **Actual or anticipated problems or delays and actions or plans to resolve them**

Nothing to report.

- **Changes that had a significant impact on expenditures**

Nothing to report.

- **Significant changes in use or care of human subjects**

Nothing to report.

- **Significant changes in use or care of vertebrate animals**

Nothing to report.

- **Significant changes in use of biohazards and/or select agents**

Nothing to report.

6. Products

- **Publications, conference papers, and presentations**

Journal publications

- Sun N, Lee YT, Zhang RY, Kao R, Teng PC, Yang Y, Yang P, **Wang JJ**, Smalley M, Chen PJ, Kim M, Chou SJ, Bao L, Wang J, Zhang X, Qi D, Palomique J, Nissen N, Han SB, Sadeghi S, Finn RS, Saab S, Busuttil RW, Markovic D, Elashoff D, Yu HH, Li H, Heaney AP, **Posadas E**, You S, Yang JD, Pei R, Agopian VG, **Tseng HR**, Zhu Y. Purification of HCC-specific extracellular vesicles on nanosubstrates for early HCC detection by digital scoring. Nat Commun. 2020 Sep 7;11(1):4489.
- Winograd P, Hou S, Court CM, Lee YT, Chen PJ, Zhu Y, Sadeghi S, Finn RS, Teng PC, **Wang JJ**, Zhang Z, Liu H, Busuttil RW, Tomlinson JS, **Tseng HR**, Agopian VG. Hepatocellular Carcinoma-Circulating Tumor Cells Expressing PD-L1 Are Prognostic and Potentially Associated With Response to Checkpoint Inhibitors. Hepatol Commun. 2020 Aug 4;4(10):1527-1540
- Jan YJ, Yoon J, Chen J-F, Teng P-C, Yao N, Cheng S, Lozano A, Chu GCY, Chung H, Lu Y-T, Chen P-J, **Wang JJ**, Lee Y-T, Kim M, Zhu Y, Knudsen BS, Feng FY, Garraway IP, Gao AC, **Chung LWK**, **Freeman MR**, You S, **Tseng H-R**, **Posadas EM**. A Circulating Tumor Cell-RNA Assay for Assessment of Androgen Receptor Signaling Inhibitor Sensitivity in Metastatic Castration-Resistant Prostate Cancer. Theranostics. 2019;9(10):2812-26.

Books or other non-periodical, one-time publications.

- Nothing to report.

Other publications, conference papers and presentations

- **Wang JJ**, Teng P-C, Jan YJ, Chen J-F, Cook-Wiens G, Yao N, Chu GCY, Chen P-J, Ho H, Yang Y, Lee Y-T, Huang J, **Chung LWK**, You S, Zhu Y, **Freeman M**, Rogatko A, Yang JD, **Tseng H-R**, **Posadas EM**. Association of very small nuclear circulating tumor cell (vsnCTC) with clinical outcomes in metastatic castration-resistant prostate cancer. Journal of Clinical Oncology 2020 38:6_suppl, 170-170. GU Cancers Symposium 2020, San Francisco, CA. (Poster Presenter)
- **Wang JJ**, Teng P-C, Jan YJ, Chen J-F, Cook-Wiens G, Yao N, Chu GCY, Chen P-J, Yang Y, Yeo YH, Lee Y-T, **Chung LWK**, You S, Zhu Y, **Freeman M**, Rogatko A, Yang JD, **Tseng H-R**, **Posadas EM**. Nuclear size of circulating tumor cells is associated with prognosis in metastatic, castration-resistant prostate cancer. American Association for Cancer Research (AACR) Annual Meeting 2020. (Poster Presenter)
- **Wang JJ**, Teng P-C, Jan YJ, Chen J-F, Cook-Wiens G, Yao N, Chu GCY, Chen P-J, Ho H, Lee Y-T, Huang J, Lee K-C, **Chung LWK**, You S, Zhu Y, **Freeman M**, Rogatko A, Yang JD, **Tseng H-R**, **Posadas EM**. Circulating tumor cells with small nuclear size: A novel biomarker for survival and clinical outcomes in advanced prostate cancer. Journal of Clinical Oncology 2020 38:15_suppl, e17512-e17512. American Society of Clinical Oncology (ASCO) Annual Meeting 2020.
- Teng P-C, Jan YJ, Chen J-F, Kim M, Yao N, Garraway I, Chu GCY, Chen P-J, **Wang JJ**, Lee Y-T, Zhu Y, **Chung LWK**, Feng FY, **Freeman M**, You S, **Tseng H-R**, **Posadas EM**. Prostate cancer CTC-RNA Assay: A new method for contemporary genomics and

precision medicine via liquid biopsy. Journal of Clinical Oncology 2020 38:6_suppl, 170-170. GU Cancers Symposium 2020, San Francisco, CA.

- Teng P-C, Kim M, Jan YJ, Chen J-F, Yao N, Chu GCY, Chen P-J, **Wang JJ**, Lee Y-T, Zhu Y, **Chung LWK**, Feng FY, **Freeman M**, You S, **Tseng H-R**, **Posadas EM**. Gene expression of circulating tumor cells is predictive of treatment response in patients with advanced prostate cancer. American Association for Cancer Research (AACR) Annual Meeting 2020.
- Teng P-C, Jan YJ, Chen J-F, Kim M, Chen J-F, Yoon J, **Wang JJ**, Chen P-J, Yao N, Lee Y-T, Lozano A, Gadilov R, **Freeman M**, You S, **Tseng H-R**, **Posadas EM**. Development of a circulating tumor cell-based RNA classifier for patients with castration-resistant prostate cancer: CTC-PCS/PAM50. American Society of Clinical Oncology (ASCO) Annual Meeting 2020.
- Lee Y-T, Sun N, Zhang RY, Kao R, Chen P-J, Teng P-C, **Wang JJ**, Yang Y, Kim M, **Posadas EM**, You S, Yang JD, Agopian VG, **Tseng H-R**, Zhu Y. Purification and mRNA profiling of extracellular vesicles for early detection of hepatocellular carcinoma. American Association for Cancer Research (AACR) Annual Meeting 2020.
- Lee Y-T, Sun N, Zhang RY, Teng P-C, **Wang JJ**, Kim M, You S, Yang JD, **Tseng H-R**, Zhu Y, Agopian VG. Purification and digital scoring of extracellular vesicles for detection of early-stage hepatocellular carcinoma. International Liver Cancer Association (ILCA) 2020 Virtual Conference. *One of the ten Basic/Translational top-scored posters.
-

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

Nothing to report.

- **Technologies or techniques**

In this research, we have developed the CTC-PCS Assay which can detect prostate cancer specific RNA signals in CTCs. This aggressive signature is correlated with treatment resistance (published in *Theranostics*. 2019;9(10):2812-26).

- **Inventions, patent applications, and/or licenses**

Nothing to report.

- **Other Products**

Nothing to report.

7. Participants & Other Collaborating Organizations

- **What individuals have worked on the project?**

Name: *Jasmine Wang, M.D.*
Project role: *PI*
Unchanged

Name: *Edwin M. Posadas, M.D.*
Project role: *Primary mentor*
Unchanged

Name: *Hsian-Rong Tseng, Ph.D.*
Project role: *Co-mentor*
Unchanged

Name: *Leland W.K. Chung, Ph.D.*
Project role: *Consultant*
Unchanged

Name: *Michael Freeman, Ph.D.*
Project role: *Consultant*
Unchanged

- **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?**

Organization Name: University of California, Los Angeles (UCLA)

Location of Organization: 500 Westwood Plz, California NanoSystems Institute (CNSI)

Partner's contribution to the project

- Facilities

8. Appendices

Appendix A: Abstract and figures regarding this project.

A Circulating Tumor Cell-RNA Assay for Assessment of Androgen Receptor Signaling Inhibitor Sensitivity in Metastatic Castration-Resistant Prostate Cancer

Background: Our objective was to develop a circulating tumor cell (CTC)-RNA assay for characterizing clinically relevant RNA signatures for the assessment of androgen receptor signaling inhibitor (ARSI) sensitivity in metastatic castration-resistant prostate cancer (mCRPC) patients.

Methods: We developed the NanoVelcro CTC-RNA assay by combining the Thermoresponsive (TR)-NanoVelcro CTC purification system with the NanoString nCounter platform for cellular purification and RNA analysis. Based on the well-validated, tissue-based Prostate Cancer Classification System (PCS), we focus on the most aggressive and ARSI-resistant PCS subtype, i.e., PCS1, for CTC analysis. We applied a rigorous bioinformatic process to develop the CTC-PCS1 panel that consists of prostate cancer (PCa) CTC-specific RNA signature with minimal expression in background white blood cells (WBCs). We validated the NanoVelcro CTC-RNA assay and the CTC-PCS1 panel with well-characterized PCa cell lines to demonstrate the sensitivity and dynamic range of the assay, as well as the specificity of the PCS1 Z score (the likelihood estimate of the PCS1 subtype) for identifying PCS1 subtype and ARSI resistance. We then selected 31 blood samples from 23 PCa patients receiving ARSIs to test in our assay. The PCS1 Z scores of each sample were computed and compared with ARSI treatment sensitivity.

Results: The validation studies using PCa cell line samples showed that the NanoVelcro CTC-RNA assay can detect the RNA transcripts in the CTC-PCS1 panel with high sensitivity and linearity in the dynamic range of 5-100 cells. We also showed that the genes in CTC-PCS1 panel are highly expressed in PCa cell lines and lowly expressed in background WBCs. Using the artificial CTC samples simulating the blood sample conditions, we further demonstrated that the CTC-PCS1 panel is highly specific in identifying PCS1-like samples, and the high PCS1 Z score is associated with ARSI resistance samples. In patient bloods, ARSI-resistant samples (ARSI-R, n=14) had significantly higher PCS1 Z scores as compared with ARSI-sensitive samples (ARSI-S, n=17) (Rank-sum test, P=0.003). In the analysis of 8 patients who were initially sensitive to ARSI (ARSI-S) and later developed resistance (ARSI-R), we found that the PCS1 Z score increased from the time of ARSI-S to the time of ARSI-R (Pairwise T-test, P=0.016).

Conclusions: Using our new methodology, we developed a first-in-class CTC-RNA assay and demonstrated the feasibility of transforming clinically relevant tissue-based RNA profiling such as PCS into CTC tests. This approach allows for detecting RNA expression relevant to clinical drug resistance in a non-invasive fashion, which can facilitate patient-specific treatment selection and early detection of drug resistance, a goal in precision oncology.

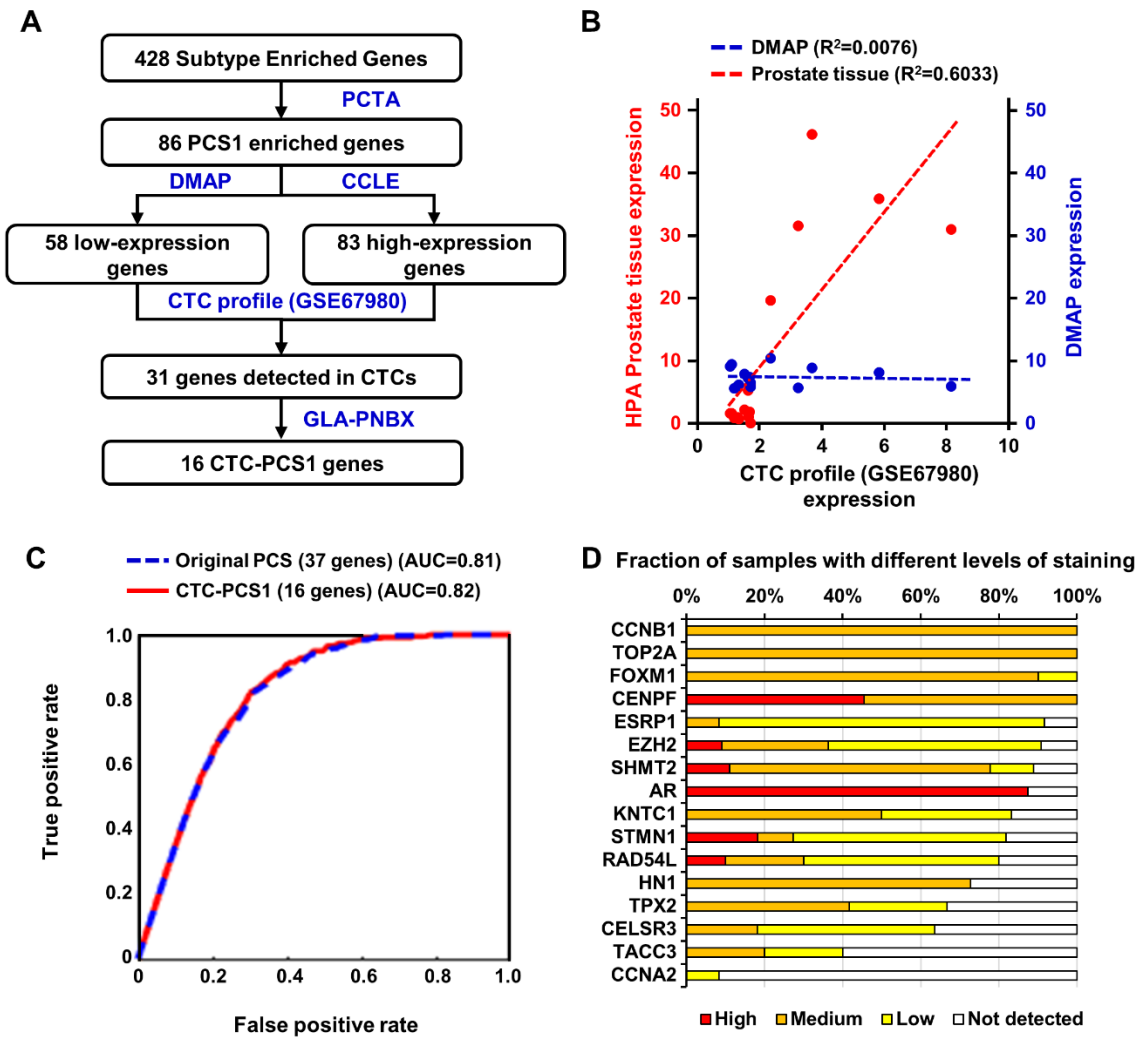

Figure 1. Selection and performance of the CTC-PCS1 gene panel. (A) Schematic flow of the selection of 16 CTC-PCS1 genes. **(B)** Scatter plot and regression lines shows expression of the 16 CTC-PCS1 genes in comparisons of CTCs (GSE67980) versus HPA prostate tissue, and CTCs (GSE67980) versus DMAP immune cells. Red dots and red dotted line indicate expressions in CTCs and prostate tissue, and blue dots and blue dotted line indicate expressions of CTCs and immune cells. **(C)** ROC curves of classifiers using the 16 CTC-PCS1 genes and original 37 PCS gene panel shows comparable level of performance of both classifiers. Red line indicates performance of 16 CTC-PCS1 genes and blue dotted line indicates performance of original 37 PCS gene panel for identification of PCS1 subtype. (D) Stacked bar graph depicts human PCa tissue staining of 16 CTC-PCS1 gene products in HPA database (https://www.proteinatlas.org/).

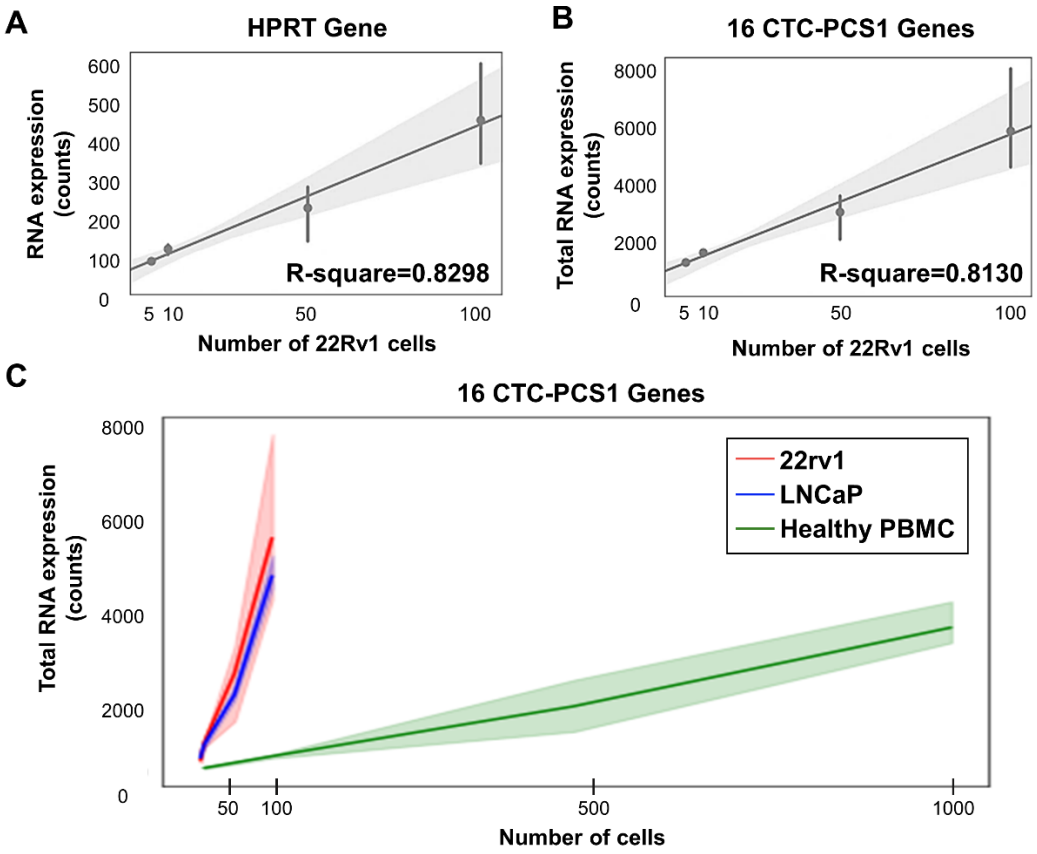

Figure 2. Analytical validation studies of the NanoVelcro CTC-RNA assay and CTC-PCS1 panel. (A) The HPRT RNA expression of PCa cell line 22Rv1 in different cell numbers measured by the NanoVelcro CTC-RNA assay. **(B)** NanoVelcro CTC-RNA assay quantification of the total CTC-PCS1 panel (16 genes) RNA expression of PCa cell line 22Rv1 in different cell numbers. **(C)** The total CTC-PCS1 panel (16 genes) RNA expression directly quantified by NanoString nCounter platform using PCa cell lines 22Rv1, LNCaP and healthy donor PBMCs in different cell numbers. Slopes of the curve- 22Rv1: 47 counts/cell, LNCaP: 44 counts/cell, healthy donor PBMC: 3 counts/cell.

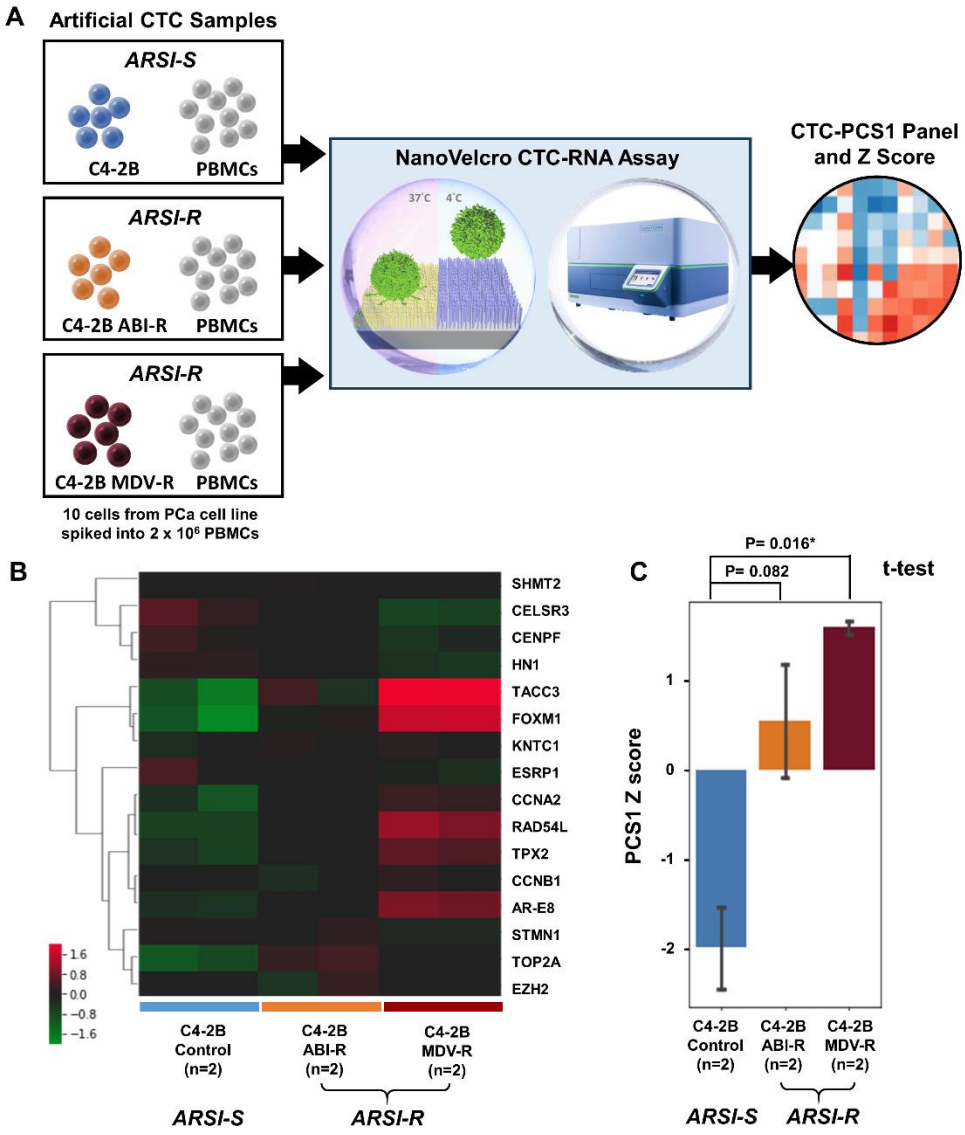

Figure 3. Cell line study of CTC-PCS1 panel for distinguishing ARSI sensitivity (A) Study workflow of the NanoVelcro CTC-RNA assay and CTC-PCS1 panel for profiling artificial blood samples of different ARSI sensitivities. Artificial CTC were prepared by spiking 10 cells of C4-2B treatment naïve (control, ARSI-S), C4-2B ABI-R (abiraterone-resistant, ARSI-R), and C4-2B MDV-R (enzalutamide-resistant, ARSI-R) into 2 x 106 healthy donor PBMCs respectively to simulate actual CTC blood samples. These artificial samples were then subjected to the NanoVelcro CTC-RNA assay, generating CTC-PCS1 RNA expression and Z scores. **(B)** Normalized and hierarchical clustered heatmap of PCS1 RNA expression in C4-2B treatment naïve (control, ARSI-S), C4-2B ABI-R (abiraterone-resistant, ARSI-R) and C4-2B MDV-R (enzalutamide-resistant, ARSI-R) artificial blood samples. (2 sets of 10 C4-2B control cells spiked in 2 x 106 healthy donor PBMCs, 2 sets 10 C4-2B ABI-R cells spiked in 2 x 106 healthy donor PBMCs and 2 sets 10 C4-2B MDV-R cells spiked in 2 x 106 healthy donor PBMCs). **(C)** PCS1 Z score comparison of C4-2B control artificial blood samples comparing to C4-2B ABI-R artificial blood samples and C4-2B MDV-R artificial blood samples. (T-test, P=0.082 and 0.016* respectively)

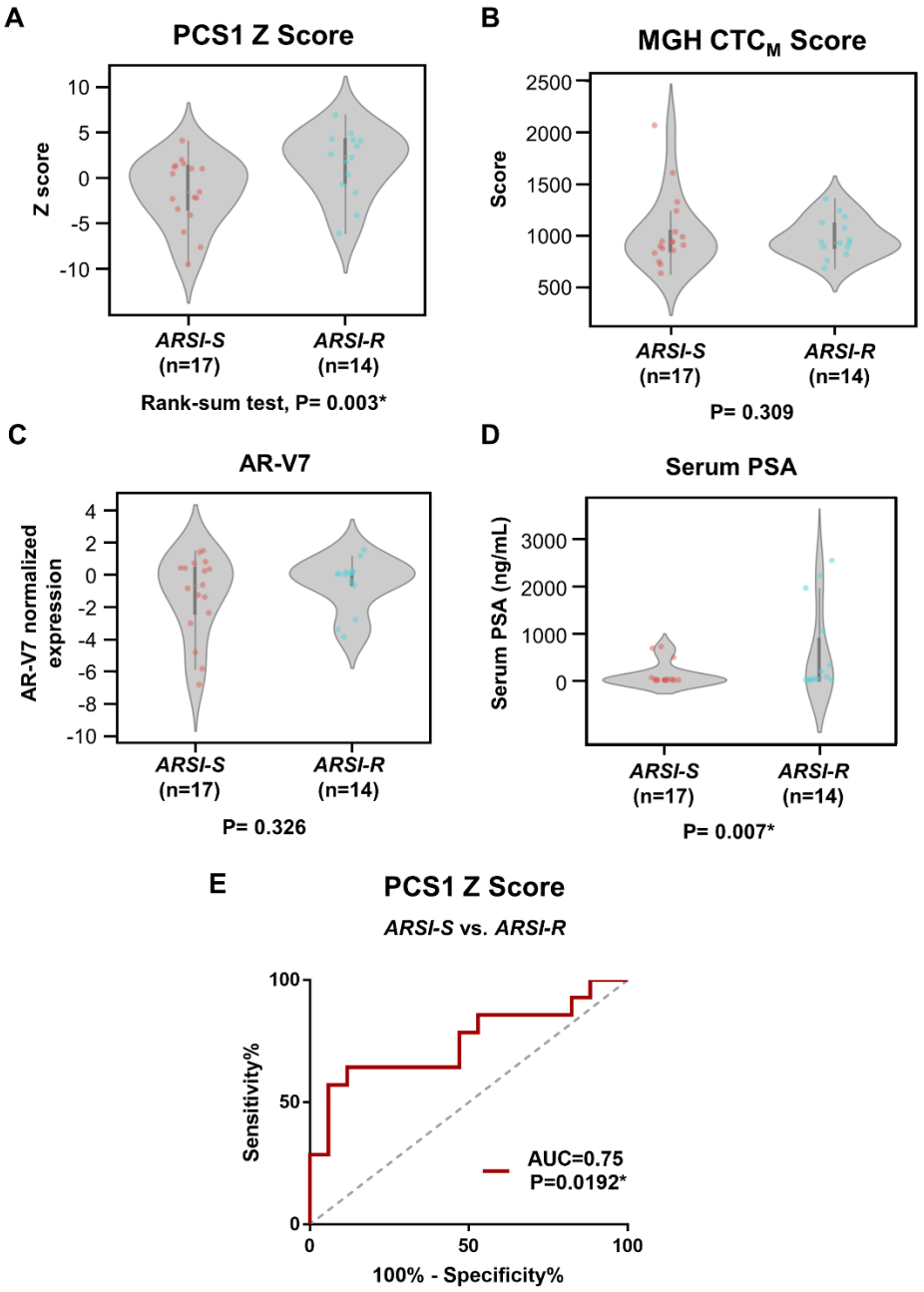

Figure 4. Analysis of gene scores between ARSI-S and ARSI-R samples. Comparison of **(A)** PCS1 Z score and **(B)** MGH CTCM score among 31 mCRPC samples, with 17 samples from ARSI sensitive state (ARSI-S), and 14 samples from ARSI resistant state (ARSI-R). PCS1 Z score is statistically significant higher in resistant patients (Rank-sum test, P=0.003*). No statistically significant trend was found in MGH CTCM score between the 2 groups (P=0.309). Similar tests performed using **(C)** AR-V7 expression and **(D)** serum PSA level are also shown. Serum PSA level exhibits statistically significant higher value in the resistant patients (P=0.007*). **(E)** Receiving Operating Characteristics (ROC) curve analysis of PCS1 Z score separating ARSI-S and ARSI-R patients. ROC curve exhibits Area Under Curve (AUC)= 0.75, P=0.0192*.

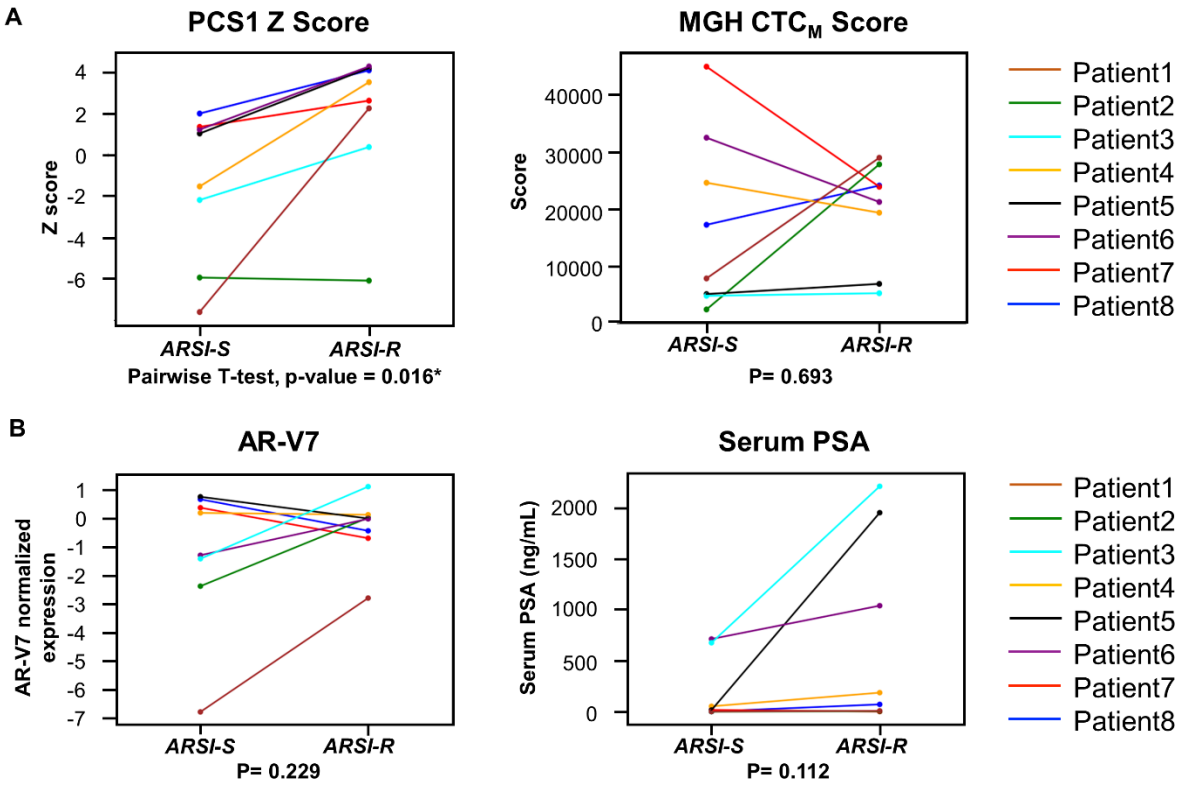

Figure 5. Analysis of gene signature score changes in continuous samples from individual patients. (A) Line plot depicts changes of PCS1 Z score for each patient from ARSI sensitive to resistant. Individual patients are displayed with different colors. Pairwise t-tests were conducted and PCS1 Z score showed a statistically significant increase from ARSI sensitive to resistant (P = 0.016*). No statistically significant trend of the MGH CTCM score was observed between the 2 timepoints (P = 0.693). **(B)** Same analysis was done with AR-V7 expression and serum PSA level. No statistically significant trend was observed in pairwise t-tests between the 2 timepoints (P = 0.229 and 0.112, respectively).

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