**AWARD NUMBER:** W81XWH-19-1-0390

**TITLE:** A Nanotechnology Solution for Early Detection of Micrometastatic Prostate Cancer After Radical Prostatectomy

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**CONTRACTING ORGANIZATION:** Cedars-Sinai Medical Center

**REPORT DATE:** October 2022

**TYPE OF REPORT:** ANNUAL

**PREPARED FOR:** U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. REPORT DATE | 2. REPORT TYPE | 3. DATES COVERED |
|---|---|---|
| October 2022 | ANNUAL | 01SEP2021-31AUG2022 |

**4. TITLE AND SUBTITLE**

A Nanotechnology Solution for Early Detection of Micrometastatic Prostate Cancer After Radical Prostatectomy

**5a. CONTRACT NUMBER**
PC180192

**5b. GRANT NUMBER**
W81XWH-19-1-0390

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)**
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**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

Cedars-Sinai Medical Center
8700 Beverly Blvd
Los Angeles, CA 90048

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**

Approved for Public Release; Distribution Unlimited

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**

The overall objective of this research proposal is to conduct the initial development of a rapid circulating tumor cell-based blood test that can identify men with micrometastatic disease in order to facilitate patient selection for salvage radiotherapy. Aim 1 of this study was do perform a technical validation study and Aim 2 to embark upon testing of banked clinical specimens then to test the new assay in the setting of the NRG-GU-006 clinical trial (salvage radiotherapy +/- apalutamide). In the first year of work we have begun a series of key technical validation studies while completing the sample collection from the now fully accrued NRG-GU-006 trial. Due to COVID-19 there were delays in progress but due to rapid clinical accrual and regearing of our studies, this effort is still on time.

**15. SUBJECT TERMS**

Prostate cancer, circulating tumor cells, nanotechnology, mRNA, expression profiling, biochemical relapse

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
|---|---|---|---|---|---|
| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | USAMRDC |
| Unclassified | Unclassified | Unclassified | Unclassified | 21 | 19b. TELEPHONE NUMBER *(include area code)* |

**Standard Form 298 (Rev. 8-98)**
**Prescribed by ANSI Std. Z39.18**

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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

Biochemical relapse (BCR) after prostatectomy (i.e. a rising PSA after surgery) is a delicate and important clinical finding. Several men with BCR are potentially still curable with salvage radiotherapy, while others have been harboring occult micro-metastatic disease outside of a standard salvage field and will continue to progress. We have proposed to conduct advanced development of a rapid blood test that identifies the molecular footprint of circulating tumor cells (versus relying upon morphologic review alone) focusing on the expression of key genes (PSA, PSMA, SCHLAP1). Aim 1 of this study was do perform a technical validation study and Aim 2 to embark upon testing of banked clinical specimens then to test the new assay in the setting of the NRG-GU-006 clinical trial (salvage radiotherapy +/- apalutamide).

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

Prostate cancer, circulating tumor cells (CTCs), nanotechnology, mRNA, expression profiling, biochemical relapse (BCR)

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

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

*List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.*

- Major Task 1: Implement QA/QC protocols and carry out calibration studies for CTC capture efficiency and CTC recovery yield of the TR-NanoVelcro System.
  Timeline: Month 1-8 with 100% completion.
- Major Task 2: To carry out calibration studies to assess the performance of RNA quantification for ddPCR™.
  Timeline: Month 7-9 with 100% completion.
- Major Task 3: To carry out calibration studies to examine the complete CTC-RNA assay.
  Timeline: Month 10-12 with 100% completion.
- Major Task 4: Case-control study to verify the association between CTC-RNA markers and mPCa.
  Timeline: Month 13-24 with 100 % completion.
- Major Task 5: Parallel testing of the CTC-RNA assay.
  Timeline: Month 13-24 with 70% completion.
- Major Task 6: Testing of the CTC-RNA assay using patients' samples from NRG-GU-006 trial.
  Timeline: Month 13-35 with 60% completion.

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

*For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.*

1) Major activities
   - Exploration of other RNA markers in addition to the originally proposed *PSA, PSMA* and *SChLAP1*.
   - Modify and incorporate the well validated genomic classifiers, PAM50 and PCS, into the CTC-RNA assay
   - Calibration study of CTC-RNA assay in conjunction with the CTC-PAM50/PCS panels with parallel tests
   - Case-control study of CTC-RNA assay to verify the association between CTC-PAM50/PCS panels and the risk of developing metastatic PCa

- Case-control study of CTC-RNA assay to verify the association between CTC-PAM50/PCS panels and the detection of treatment resistance in metastatic PCa
- Sample collection from the NRG-GU006 trial.

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
   - **Exploration of other RNA markers in addition to the originally proposed *PSA, PSMA* and *SChLAP1***.
     - To explore other RNA markers that may have better distinguishing performance than the proposed *PSA, PSMA* and *SChLAP1*, we sought to expand the panel by exploring other existing transcriptomic signatures in PCa.
     - PAM50 is a transcriptome-based classifiers initially developed from breast cancer and is the basis for the commercially available Prosigna test.( Wallden B, Storhoff J, Nielsen T, et al. Development and verification of the PAM50-based Prosigna breast cancer gene signature assay. *BMC Med Genomics*. 2015;8:54.) This classifier and concept were then applied to PCa, in which the molecular subtyping by luminal and basal status is prognostic for clinical outcomes and may be associated with response to postoperative androgen deprivation therapy[1].
     - The Prostate Cancer Classification System (PCS)[2] is one of Dr. Sungyong You's achievements for improving prediction of prognosis and treatment sensitivity using datasets specific to gene expression in PCa/mCRPC[2]. PCS categorizes PCa into 3 subtypes, i.e., PCS1-3. Among them, PCS1 is associated with the worst prognosis, shortest time to metastasis, and highest risk of androgen receptor signaling inhibitor resistance. In comparison with PAM50, the PCS system exhibits greater separation in multiple clinical outcomes and provides better separation of prostate luminal and basal characteristics[3]. This result was published on *Prostate Cancer Prostatic Dis* this year.
   - **Modify and incorporate the well validated genomic classifiers, PAM50 and PCS, into the CTC-RNA assay**
     - To translate these tissue-based transcriptomic signatures to CTC-based signatures, a bioinformatics process was performed to filter out the background signals from WBCs. With an integrated data analysis framework using PCa and WBC datasets from the Prostate Cancer Transcriptome Atlas (PCTA, www.thepcta.org), PCa CTC RNA-seq (GSE67980)[4], Cancer Cell Line Encyclopedia (CCLE)[5], and Differentiation Map (DMAP)[6], 36 genes from PAM50 and 40 genes from PCS were shown to be highly expressed in PCa CTCs with low expression in WBCs and were selected for the CTC-RNA assay (**Figure 1**).

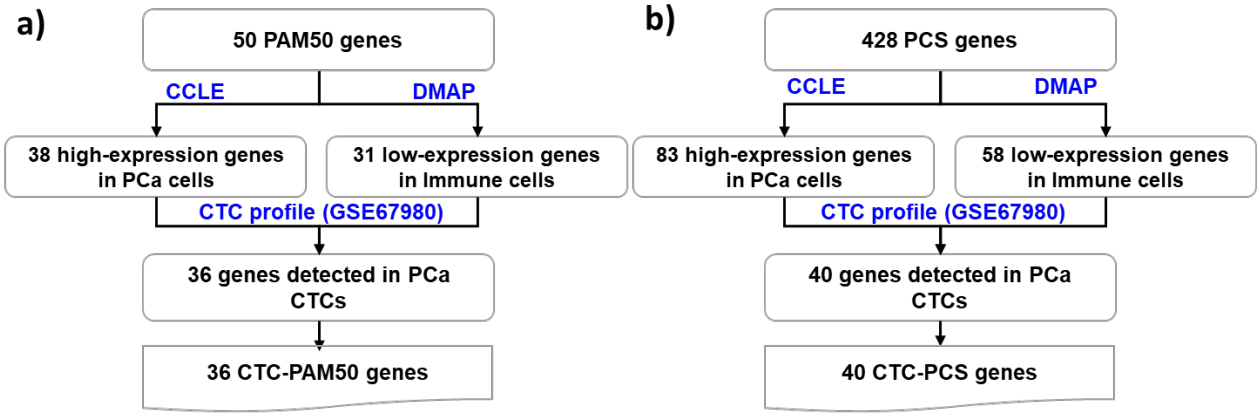


**Figure 1. Schematic flow of the development of CTC-PAM50 and CTC-PCS signature**. **a)** Using public gene expression databases including PCTA, CCLE, DMAP and GSE67980, a bioinformatics process was applied to enrich CTC-based signals and minimize background from leukocytes. This results in a CTC-PAM50 signatures containing 36 genes for being used with CTC-RNA assay. **b)** With similar methodology, the original 428 PCS subtype enriched genes were transformed into a CTC-PCS signature with 40 genes.

- The discriminatory performance of CTC- PAM50/PCS/ panel is compared with the original tissue-based PAM50/PCS classifier in the public database PCTA and GenomeDx GRID cohort.
  - For CTC-PAM50 panel, the AUC are 0.85, 0.92 and 0.87 for Luminal A, Luminal B and Basal, respectively in PCTA dataset. The AUC are 0.94, 0.96, 0.84 for Luminal A, Luminal B and Basal, respectively in GenomeDx GRID dataset. (**Figure 2a**).
  - For CTC-PCS panel, the area under curve (AUC) are 0.91, 0.86 and 0.80 for PCS1, PCS2 and PCS3, respectively in PCTA dataset. The AUC are 0.86, 0.90, 0.88 for PCS1, PCS2 and PCS3, respectively in GenomeDx GRID dataset. (**Figure 2b**).
  - These data suggested that the classification performance of the CTC-PAM50 and CTC-PCS panels were not mitigated by the previous gene filtering process.

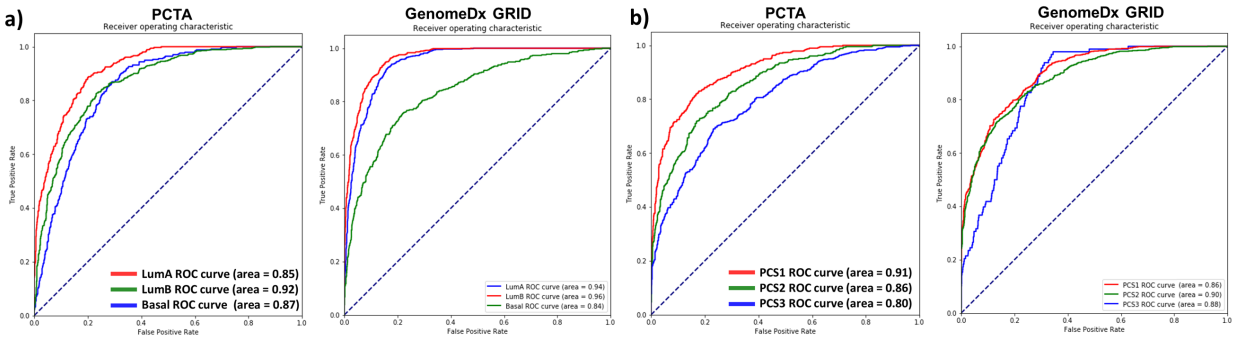


**Figure 2. Comparison of CTC-PAM50/PCS and their original PAM50/PCS classifier in the PCTA and GenomeDx GRID database. a)** ROC curve and the AUC of the CTC-PAM50 panel for distinguish the original tissue subtyping by the PAM50 panel. **b)** ROC curve and the AUC of the CTC-PCS panel for distinguish the original tissue subtyping by the PCS panel.

- The prognostic performance of these two CTC panels were validated in the GenomeDx GRID databases (**Figure 3a, 3b**). This confirmed the CTC-PAM50 and CTC-PCS panels retained their original prognostic performance. Patients with Luminal B and PCS1 subtype have the worst clinical outcomes, including prostate cancer specific mortality (PCSM), biochemical relapse (BCR) and metastasis free survival (MET).

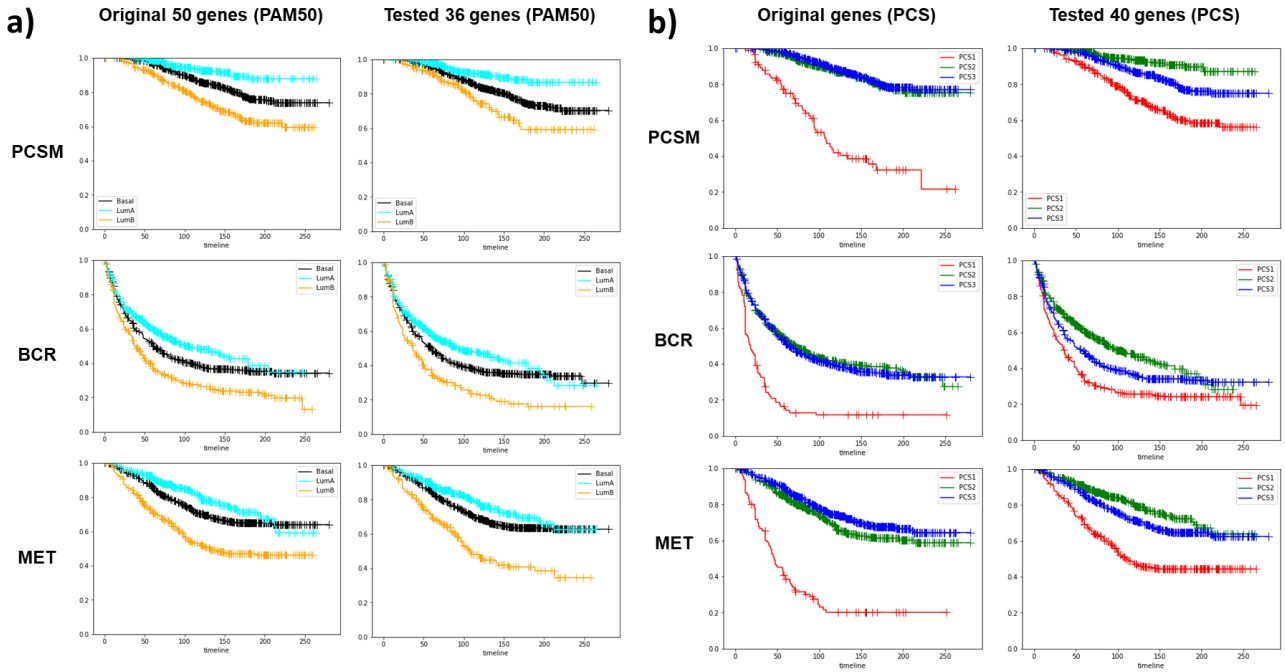


**Figure 3. Comparison of CTC-PAM50/PCS and their original PAM50/PCS classifier in the GenomeDx GRID database. a)** Prognostic performance of original PAM50 and CTC-PAM50 panel in the GenomeDx GRID database. **b)** Prognostic performance of original PCS and CTC-PCS panel in the GenomeDx GRID database. PCSM: prostate cancer specific mortality; BCR: biochemical relapse; MET: metastasis free survival.

- **Calibration study of CTC-RNA assay in conjunction with the CTC-PAM50/PCS panels**
  - Analytical validation of CTC-RNA assay was carried out parallelly at two different institutions, i.e., UCLA and CSMC, to ensure the reproducibility of the assay.
  - **Analytical validation studies of the CTC-RNA assay and CTC-PCS panel**.
    - To determine the sensitivity and dynamic range of the NanoVelcro CTC-RNA assay for quantification of the CTC-PCS1 signature, we first tested the assay with a PCa cell line, i.e., 22Rv1 using different cell numbers (n = 5, 10, 50, and 100 cells) that mimicked the CTC numbers present in 2-mL clinical blood samples.
    - We demonstrated that the NanoVelcro CTC-RNA assay can detect RNA transcripts of a housekeeping gene (i.e., HPRT) and the 16 genes in CTC-PCS1 panel with high sensitivity and linearity in the dynamic range of 5-100 cells (**Figure 2A, 2B**).
    - We then demonstrated that the CTC-PCS1 panel is capable of detecting PCa CTC-derived PCS1 signatures in the presence of WBC background by quantifying CTC-PCS1 RNA expression with NanoString nCounter platform using three sets of RNA samples extracted from 2 PCa cell lines (i.e., 22Rv1 and LNCaP), and healthy donor PBMCs with cell numbers mimicked the CTC and WBC numbers (i.e., 5-100 PCa cells and 50-1000 WBCs) in the CTC samples purified by the CTC-RNA from 2-mL of patient blood. We demonstrated that the RNA counts of the CTC-PCS1 panel genes in PCa cells were significantly higher than the RNA counts in WBCs in the given cell number range (**Figure 2C**). This further validated the bioinformatic process of developing the CTC-PCS1 panel.

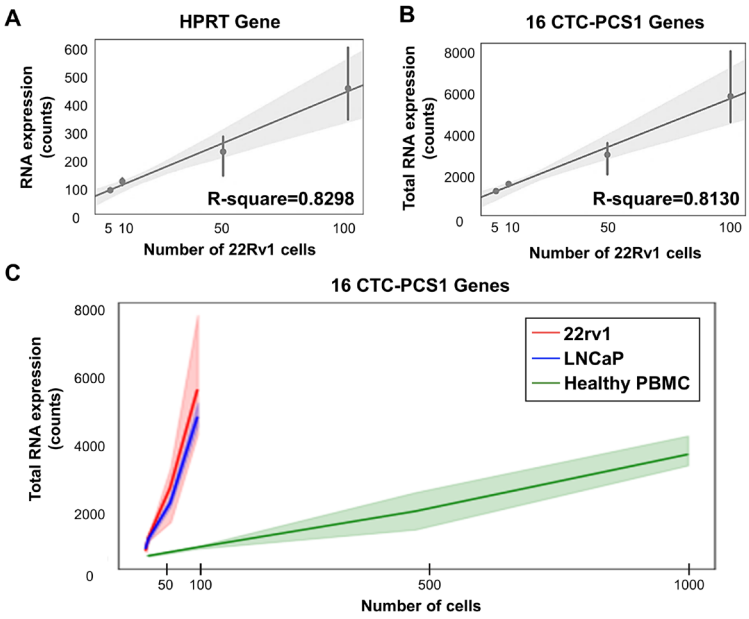


**Figure 4. Analytical validation studies of the NanoVelcro CTC-RNA assay and CTC-PCS1 panel. (A)** The HPRT RNA expression of PCa cell line 22Rv1 in different cell numbers measured by the NanoVelcro CTC-RNA assay (R-square= 0.8298). **(B)** NanoVelcro CTC-RNA assay quantification of the total CTC-PCS1 panel (16 genes) RNA expression of PCa cell line 22Rv1 in different cell numbers (R-square= 0.8130). **(C)** The total CTC-PCS1 panel (16 genes) RNA expression of 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.

  - **Analytical validation studies of the CTC-RNA assay and CTC-PAM50 panel**
    - To validate sensitivity and dynamic range of the CTC-PAM50 panel for quantification of CTC counts under the background of WBCs, a calibration study was performed. We analyzed the RNA samples with a mixing of different numbers of LNCaP cells and WBCs from healthy donors (HD).
    - LNCaP cells counts range from 0, 0.5, 1, 2, 5, 10, 20, 50, 100 cells in each sample, while healthy donor's WBC counts range from 0, 2.5k, 5k, 10k cells in each sample.

- **Figure 5** display the expression of the CTC-PAM50/PCS genes among different ratios of LNCaP cells and WBCs mixtures. The bottom two rows show the combination of LNCaP cells and WBCs that were analyzed. The top row shows the gene expression of the CTC-PAM50 and CTC-PCS genes. The middle panel shows the expressions of the housekeeping genes (dash lines), the top 10 genes associated with the number of LNCaP cells (Red lines) and the negative control genes (black lines).
- This data suggested genes in the CTC-PCS and CTC-PAM50 have a great dynamic range and sensitivity to detect 1-10 CTCs despite a huge background of WBCs, confirming the utility of these gene signatures in a liquid biopsy setting.

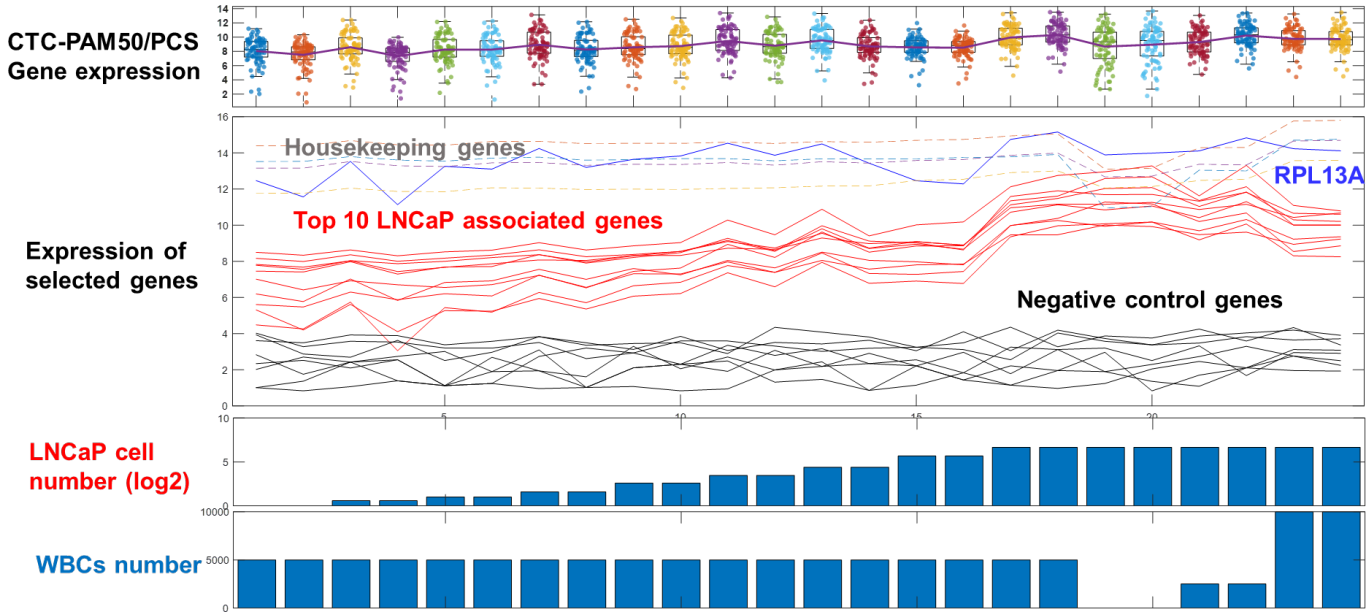


**Figure 5. Analytical validation studies of the CTC-RNA assay and CTC-PAM50 panel.** The bottom two rows of this figure show the combination of LNCaP cells and WBCs that were analyzed. LNCaP cells range from 0, 0.5, 1, 2, 5, 10, 20, 50, 100 cells, while healthy donor's WBC counts range from 0, 2.5k, 5k, 10k cells in each sample. The top row shows the gene expression of the CTC-PAM50 and CTC-PCS genes. The middle panel shows the expressions of the housekeeping genes (dash lines), the top 10 genes associated with the number of LNCaP cells (Red lines) and the negative control genes (black lines). The top 10 LNCaP associated genes correlated with the increase of LNCaP cells very well in the range of 1-100 LNCaP cells and were merely affected by the increase of WBC background from 0-10k WBCs.

- **Validation of the performance of CTC-PCS and PCS-PAM50 classification using PCa cell lines with different levels of androgen resistance**
  - PAM50 and PCS classifiers have been shown to be able to predict response to androgen deprivation therapy in PCa by analyzing the surgical or biopsy specimens of PCa. Among all subtypes, basal and PCS1 conferred the poorest response to androgen receptor signaling inhibitors (ARSI).
  - To validate the subtyping performance of CTC-PCS and CTC-PAM50 panel, well-characterized PCa cell lines with different biological properties (i.e., ARSI sensitive vs ARSI resistant) in a background of healthy donor's WBCs were tested with the CTC-RNA assay. The heatmap demonstrated the CTC-PCS and CTC-PAM50 expression profiles within the C42B parental cells, C42B resistant cells to Abiraterone (one type of ARSI) and C42B resistant cells to Enzalutamide (one type of ARSI) (**Figure 6a**).
  - CTC-PAM50
    - C4-2B ARSI-resistant cells had higher Luminal B and Basal probability, and lower Luminal A probability compared to the parental C4-2B cells. (**Figure 6b**)
  - CTC-PCS
    - C4-2B ARSI-resistant cells had significantly higher PCS1 and basal probability, and higher PCS1 Z scores compared to the parental C4-2B cells (p=0.02). (**Figure 6c, 6d**)

- This showed that the CTC-PAM50/PCS panel were able to differentiate the C4-2B ARSI-resistant cells from the C4-2B parental cells via the CTC-RNA assay.

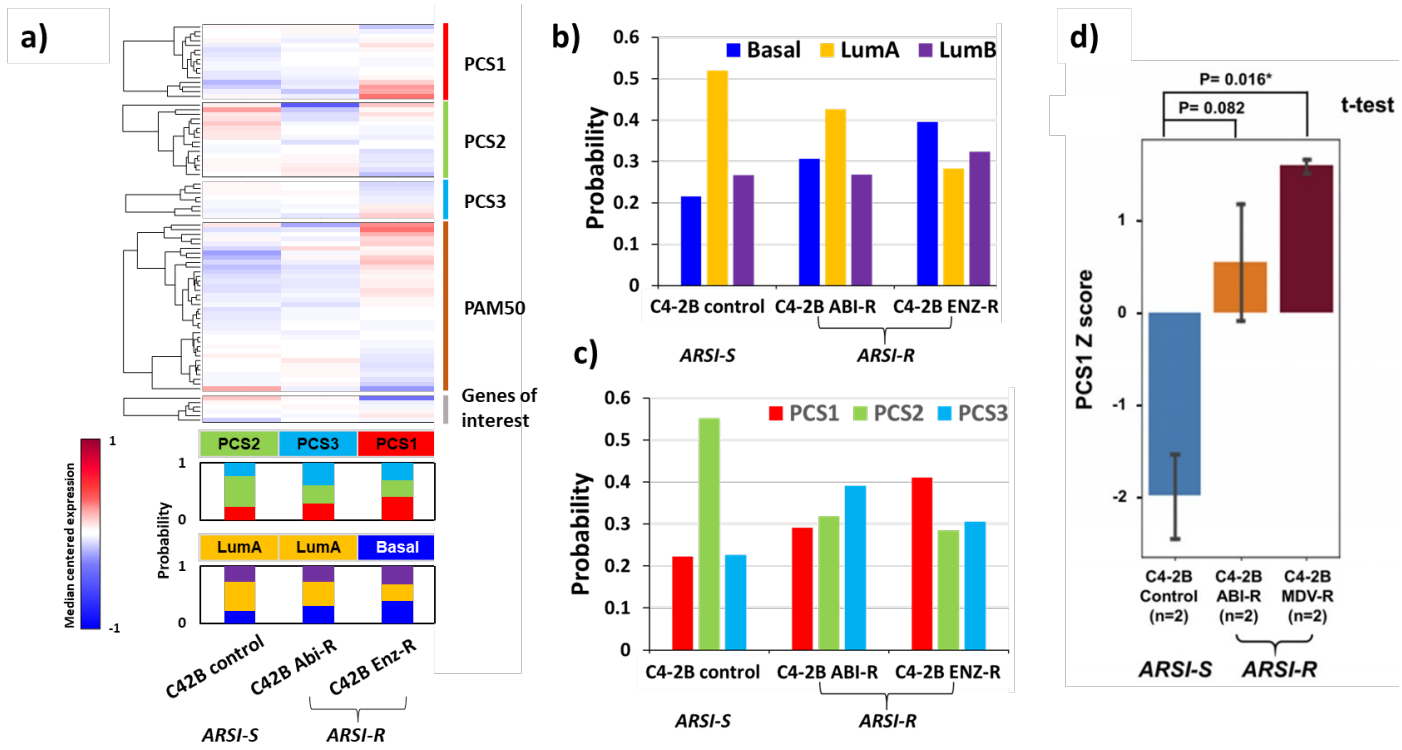


**Figure 6. The CTC-PCS/PAM50 subtype of cell lines is assigned using the nearest centroid method.** A) a heatmap displays the expression of CTC-PAM50/PCS genes in ARSI sensitive cell line (i.e., C42B control cell lines), and ARSI resistant cell lines (i.e., abiraterone resistant C42B and enzalutamide resistant C42B), …(a,b,c) Results of PCS/PAM50 subtypes in C4-2B cell lines. C4-2B treatment naïve (control, ARSI-S), C4-2B ABI-R (abiraterone-resistant, ARSI-R), and C4-2B ENZ-R (enzalutamide-resistant, ARSI-R)

- **Clinical study of the CTC-RNA assay for stratifying the risk of developing metastatic PCa**
  - We analyzed CTC samples from 18 PCa patients undergoing prostatectomy to investigate if the CTC-RNA signatures can distinguish high risk PCa patients from low risk PCa patients.
  - According to the National Comprehensive Cancer Network (NCCN), "high-risk" PCa was defined as men with stage T3a, Gleason ≥8, or PSA ≥20 disease, and "very high risk" was referred to as T3b or T4 disease. These "high risk" or "very high risk" PCa are aggressive cancers and often require multiple modalities of treatment. In addition, patients with high-risk disease have an increased risk of developing biochemical recurrence, metastases, and death from prostate cancer.
  - PAM50 and PCS classifiers have been used for predicting the risk of recurrence in PCa patients undergoing prostatectomy. We sought to investigate the performance of the CTC-RNA assay with the CTC-PAM50/PCS panels in risk stratification of these prostatectomy patients.
  - For this, banked PBMC samples of 18 localized PCa patients undergoing prostatectomy from Urologic Oncology Program Blood Biospecimens Bank (UOPBBB, CSMC IRB#Pro00042197) were tested with the CTC-RNA assay. Nanostring nCounter platform was used for the downstream RNA quantification of the 40 CTC-PCS genes and 36 CTC-PAM50 genes. Variance stabilizing normalization was applied. Batch effects were adjusted by ComBat normalization method.
  - The heatmap depicted the CTC-PAM50/PCS expressions from these 18 patients with different risk (**Figure 7**). For PCS1, a Z score for each patient was calculated using a weighted Z score method[7] to represent the likelihood estimate of being PCS1 subtype. However, there is no significant difference of PCS1 Z score in very-high-risk/high-risk vs intermediate risk group. More tests and further analysis will be carried out to re-train this model. In addition, we keep following up these patients to see if the CTC-PAM50/PCS can predict or detect the early metastasis, in comparison

with their clinical risk stratification. This longitudinal case investigation would help us identify markers for prediction/detection of metastatic PCa.

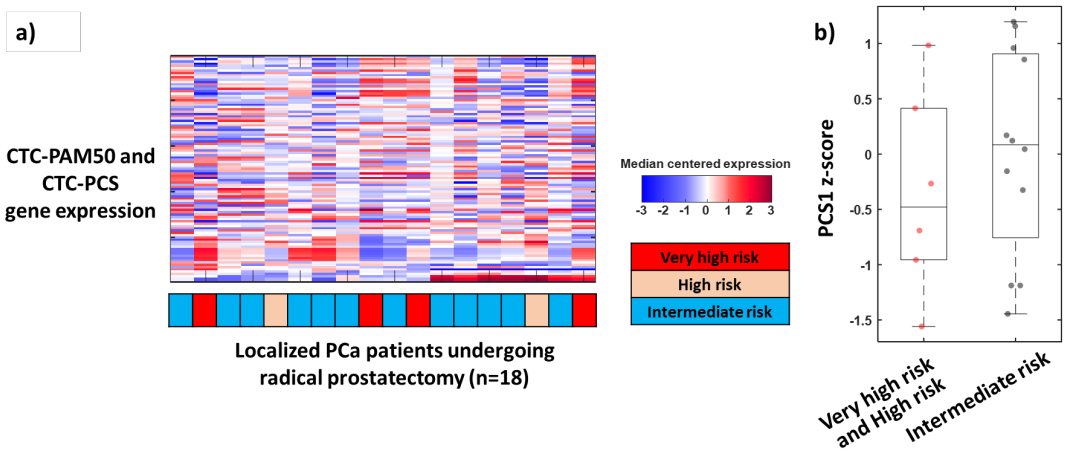


**Figure 7. CTC-PAM50/PCS expression profiles in 18 localized PCa patients with intermediate, high and very high risk. a)** Heatmap depicting the expressions of CTC-PAM50 and CTC-PCS panel in purified CTCs from 18 localized PCa patients with different risk. Data was normalized and batch corrected by combat and VSN method. b) Box plot showing PCS1 Z scores in patients with high to very high risk vs patients with intermediate risk.

- **Clinical study of CTC-RNA assay for detecting ARSI resistance in metastatic PCa**
  - In our previous study, it has been shown that PCS1 were highly expressed in CTCs from patients with enzalutamide (ARSI) resistant PCa.[2] To validate the performance of the CTC-RNA assay in identifying patients resistant to ARSI, we focus on PCS1, the most aggressive and ARSI-resistant PCS subtype, for analysis.
  - Thirty-one blood samples from a retrospective cohort of metastatic PCa patients receiving ARSI therapy (i.e., abiraterone acetate or enzalutamide) were analyzed with CTC-RNA assay. Variance Stabilizing Normalization (VSN) method and Log2 transformation were applied to entire data to reduce systematic variance.
  - **Figure 8a** shows the expression of genes in the CTC-PCS1 panel, MGH CTC panel (another gene set developed from a Massachusetts General Hospital (MGH) group, i.e., Miyamoto et al.[8]), as well as AR-V7. The 31 CTC samples were ordered by hierarchical clustering against CTC-PCS1 genes. The heatmap shows that samples from patients obtained during the emergence of drug resistance (i.e., ARSI-R, in red) have higher RNA expression in the CTC-PCS1 panel, while samples obtained from patients stable during therapy (i.e., ARSI-S, in blue) exhibited lower RNA expression in CTC-PCS1 panel.
  - The PCS1 Z score, which represents the likelihood estimate of being PCS1 subtype, was computed from the RNA expression of our CTC-PCS1 panel using a weighted Z score method.[7] The PCS1 Z scores represent the likelihood of the PCS1 phenotype in each sample (**Figure 8a**)
  - The PCS1 Z scores were higher in ARSI-R samples when compared with ARSI-S samples with statistical significance (Rank-sum test, P=0.003*, **Figure 8b**). Receiving Operating Characteristic (ROC) curve analysis of the PCS1 Z score was performed to test the sensitivity and specificity of the PCS1 Z score as a means of identifying sensitive and resistant patients. The area-under-curve (AUC) was 0.75, P=0.0192* (**Figure 8c**).
  - These findings demonstrate the CTC-PCS1 subtype can reflect the sensitivity to ARSI treatment.

- This shows the feasibility of the CTC-RNA assay as an noninvasive approach for detecting RNA expression relevant to clinical drug resistance, which can facilitate patient-specific treatment selection and early detection of drug resistance.

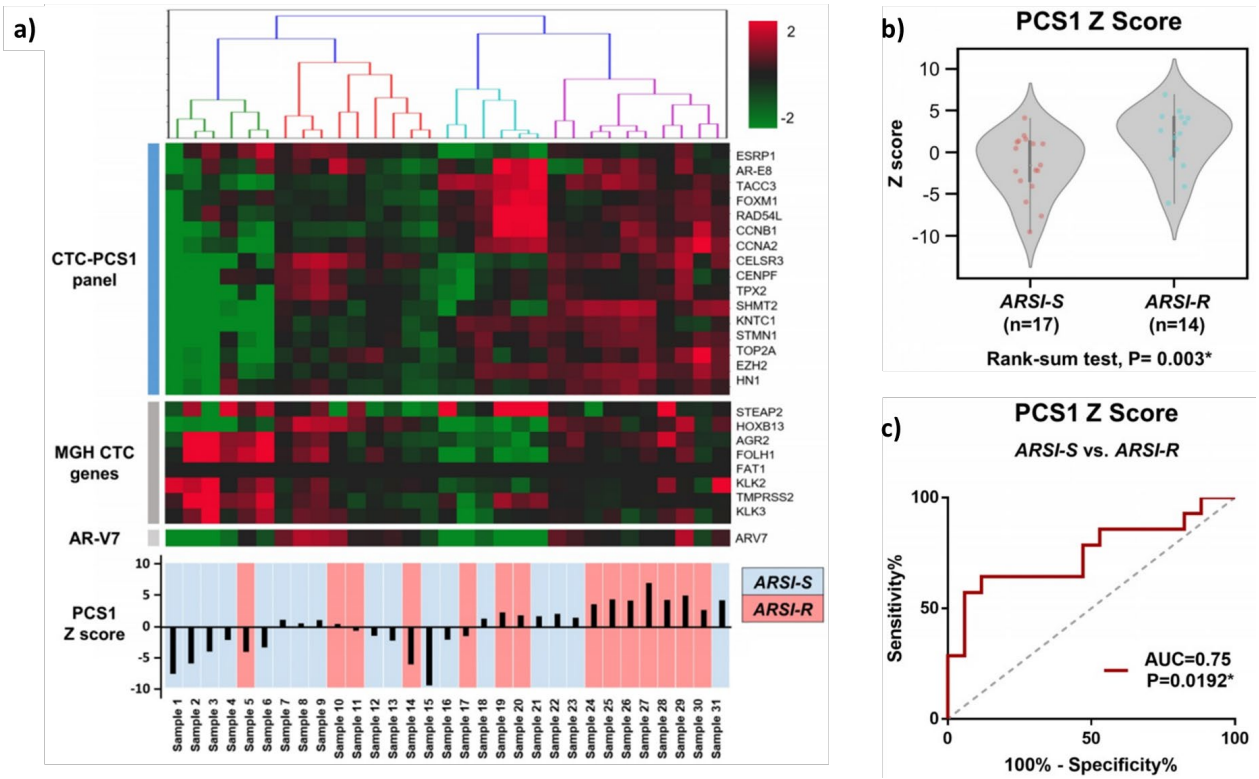


**Figure 8. CTC-RNA assay results in patient samples. a)** RNA expression of target genes in the PCS1 panel, MGH CTC panel, as well as AR-V7 are shown. Total of 31 mCRPC patient samples are labeled as abiraterone acetate and enzalutamide sensitive (ARSI-S, in blue) and abiraterone acetate and enzalutamide resistant (ARSI-R, in red). The PCS1 Z score generated by gene expression in PCS1 panel is also shown, which is highly correlated with patients' clinical drug sensitivity status. **b)** PCS1 Z 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). **c)** 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*.

- **Sample collection from the NRG-GU006 trial.**
  - As noted in the original application, through our participation in the NRG network, our laboratory group has been receiving specimens from NRG-GU-006: A Phase II, Double-Blinded, Placebo Controlled Randomized Trial of Salvage Radiotherapy With or Without Enhanced Anti-androgen Therapy With Apalutamide in Recurrent Prostate Cancer (BALANCE). This is an international study of salvage radiotherapy that is being conducted through NRG Oncology- an NCI supported cooperative group. This trial had a proposed sample size of 311 and completed accrual in March 2020. We have received 422 specimens from 238 unique patients on this study and await maturation of clinical outcomes.
  - All 422 blood specimens were processed to isolate the PBMC layer containing CTCs. All these CTCs were isolated by the CTC-RNA assay. RNA from the enriched CTCs were reverse transcribed into cDNA ready for genomic analysis.

**-What opportunities for training and professional development has the project provided?**
*If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."*

*Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency.*

*Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.*

Nothing to Report

**-How were the results disseminated to communities of interest?**
*If there is nothing significant to report during this reporting period, state "Nothing to Report."*

*Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.*

1. Teng PC, Jan YJ, Yoon J, Chen PJ, Chen JF, Yao N, Cheng S, Lozano A, Freeman M, You S, Tseng HR, Posadas EM. A circulating tumor cell specific RNA assay for assessment of androgen receptor signaling inhibitor sensitivity in metastatic castration-resistant prostate cancer. Journal of Clinical Oncology. 2019;37(15_suppl):5059-. doi: 10.1200/JCO.2019.37.15_suppl.5059. American Society of Clinical Oncology (ASCO) Annual Meeting 2019, Chicago, IL. (Poster presentation)
2. Teng PC, Jan YJ, Chen JF, Cook-Wiens G, Cheng S, Yao N, Lozano A, Chu GCY, Chen PJ, Ho H, Yang Y, Huang K, Li KC, Chung LWK, You S, Zhu Y, Freeman MR, Rogatko A, Yang JD, Tseng HR, Posadas EM. Very-Small-Nuclear Circulating Tumor Cells: Nuclear Size Reduction is Associated with Poor Clinical Outcomes in Metastatic Castration-Resistant Prostate Cancer. 2019 NCI Alliance of Nanotechnology in Cancer Principal Investigator Meeting, Rockville, MD. (Poster presentation)
3. Teng PC, Jan YJ, Yoon Junhee, Chen JF, Chen PJ, Kim M, Yao N, Cheng S, Lozano A, Freeman MR, You S, Tseng HR, Posadas EM. Preclinical Development of a Circulating Tumor Cell Based RNA-Classifier to Optimize the Treatment Selection in Patients with Metastatic Castration-Resistant Prostate Cancer. 2019 NCI Alliance of Nanotechnology in Cancer Principal Investigator Meeting, Rockville, MD. (Poster presentation)
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6. Teng PC, Kim M, Jan YJ, Chen JF, Yao N, Chu GC, Chen PJ, Wang JJ, Lee YT, Zhu Y, Chung LWK, Feng FY, Freeman MR, You S, Tseng HR, 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 (Poster presentation).
7. Wang JJ, Teng PC, Jan YJ, Chen JF, Cook-Wiens G, Yao N, Chu GC, Chen PJ, Yang Y, Yeo YH, Lee YT, Chung LWK, You S, Zhu Y, Freeman MR, Rogatko A, Yang JD, Tseng HR, 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 presentation).
8. Teng PC, Jan YJ, Kim M, Chen JF, Yoon J, Wang JJ, Chen PJ, Yao N, Lee YT, Lozano A, Gadilov R, Freeman M, You S, Tseng HR, 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 (Virtual meeting).

9. Wang JJ, Teng PC, Jan YJ, Chen JF, Cook-Wiens G, Yao N, Chu GCY, Chen PJ, Ho H, Yang Y, Lee YT, Huang J, Chung LWK, You S, Zhu Y, Freeman M, Rogatko A, Yang JD, Tseng HR, Posadas EM. Association of very small nuclear circulating tumor cell (vsnCTC) with clinical outcomes in metastatic castration-resistant prostate cancer. American Society of Clinical Oncology (ASCO) Annual Meeting 2020 (Virtual meeting).
10. Yoon J, Kim M, Posadas EM, Freedland SJ, Liu Y, Davicioni E, Den RB, Trock BJ, Karnes RJ, Klein EA, Freeman MR, You S. A comparative study of PCS and PAM50 prostate cancer classification schemes. Prostate Cancer Prostatic Dis. 2021 Sep;24(3):733-742. doi: 10.1038/s41391-021-00325-4. Epub 2021 Feb 2. PMID: 33531653; PMCID: PMC8326303.
11. Wang JJ, Cavassani KA, Teng PC, Chen JF, Jan YJ, Chu GC, Lee YT, Gao A, Di Vizio D, Chung LW, You **S**, Zhu Y, Freeman M, Rogatko A, Yang JD, Tseng H-R, Posadas EM. Nuclear size of circulating tumor cells in advanced prostate cancer to reveal a potential biomarker for clinical outcomes and androgen receptor indifference. Journal of Clinical Oncology 2021 39:6_suppl, 167-167. Genitourinary Cancers Symposium 2021, San Francisco, CA. (Poster presentation)

**-What do you plan to do during the next reporting period to accomplish the goals?**
*If this is the final report, state "Nothing to Report."*

*Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.*

we have strengthened our interactions with Dr. Sungyong You (co-I), an expert in prostate cancer computation biology, we have been revising our approach to molecular characterization of CTCs using the CTC-RNA assay. While the original proposal focused on digital RT-PCR of *PSA, PSMA*, and *SChLAP1* as a primary focus, we have expanded our exploration to other PCa transcriptomic classifiers including PAM50 and PCS in parallel to the directed RT-PCR to further optimize the performance of the assay. Although bioinformatics process has been done to improve the specificity of these panel to PCa CTCs when being used in a liquid biopsy setting, further investigation into the normalization methods to filter out the WBC background will be carried out to optimize the output signals. On the basis of the initial tests with CTC-PAM50 and CTC-PCS panels, further tests and analysis will be done to identify appropriate markers for better detection of metastatic PCa.
Then we will validate these genes using banked samples from our biobank and the patient samples collected from the NRG-GU006 trial.

***4.*** **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

**-What was the impact on the development of the principal discipline(s) of the project?**
*If there is nothing significant to report during this reporting period, state "Nothing to Report."*

*Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).*

**Post-RP BCR PCa patients.** PCa is the most common solid-organ malignancy and second leading cause of cancer death in American men[9]. Among the 190,000 of PCa cases diagnosed annually, about 92% of patients are diagnosed with localized cancers, which are commonly treated by radical prostatectomy (RP). After RP, approximately 35% of patients will experience biochemical recurrence (BCR)[10,11], clinically manifested as a rising serum prostate-specific antigen (PSA) concentration. For post-RP BCR patients without radiographic evidence of distant metastases, the mainstay of treatment is salvage radiotherapy (SRT) to the local prostate bed and the surrounding tissue, potentially salvaging the surgical failure and offering possibilities for cure[12].

**Clinical unmet need: optimizing BCR management by detecting distant micrometastases in post-RP BCR PCa patients**. Although SRT provides an opportunity for cure in post-RP BCR PCa patients, >50% of patients treated with SRT will experience disease progression[13-15]. Furthermore, patients receiving SRT often suffer from radiation toxicity, including long-term urinary incontinence and impotence[16]. The failure of SRT typically results from the presence of disease in the form of distant micrometastases outside the radiotherapy field. In this case, tumor cells will remain untreated by radiation[17]. Patients with distant micrometastases are best served by treating them as metastatic patients focusing on timely initiation of systemic therapy without the complications related to SRT. Current clinical imaging modalities (e.g., bone scan, CT, MRI, and/or PET) are helpful at times, but currently suffer from limited sensitivity and spatial resolution in detecting distant micrometastases[18,19]. As such, there is an urgent and unmet need to develop a diagnostic solution that will enable detection of distant micrometastases in post-RP BCR patients to personalize and optimize the use of SRT for better outcome.

**PCa CTC-RNA assay as a diagnostic solution to detect distant micrometastases**. It is known that as cancers progress and metastasize, increasing numbers of tumor cells are shed into the blood stream[20]. These cellular and clinical events are driven by alternations in changes in molecular pathways that govern growth and metastasis[21]. Our proposed PCa CTC-RNA assay will directly characterize these alterations through molecular profiling of the enriched CTCs. Applying this assay in post-RP BCR PCa patients, we are able to address the clinical unmet need to detect distant micrometastases, thereby improve treatment selection and clinical outcome.

**Others.** As the proposed assay is a combination of two existing technologies: the TR-NanoVelcro Assay and ddPCR™. The TR-NanoVelcro assay has been successfully deployed at test sites and ddPCR™ is now widely available. Thus, the merged assay can be deployed immediately following after our technical validation and QA/QC development. Expansion of FDA clearance of NanoVelcro platform to include the TR-NanoVelcro system which will allow for wider dissemination of this technology.

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

*If there is nothing significant to report during this reporting period, state "Nothing to Report."*

*Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.*

There are many platforms for enrichments or purifications of CTCs, which belong to the field of engineering. However, the subsequent studies of clinical applications are few. Our clinical validation of the CTC-RNA assay can provide positive feedback to the platform development. Indeed, our group has developed newer generations of NanoVelcro Chips which can purify CTCs with higher purity and throughput.[22,23]

Based on the success with PCa, we also utilized this platform in other diseases including melanoma[24], hepatocellular carcinoma[25], lung cancer[22], pancreatic cancer[26] and noninvasive prenatal diagnostics[27].

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

*If there is nothing significant to report during this reporting period, state "Nothing to Report."*

*Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:*

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report

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

*If there is nothing significant to report during this reporting period, state "Nothing to Report."*

*Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:*

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

The successful development of the proposed CTC-RNA assay is rapidly translatable, enabling a sensitive and biologically relevant CTC-based assay for optimizing the selection of salvage radiotherapy (SRT) candidates by identifying those who have micrometastases and will experience more harm than benefit from SRT. Such an approach will improve costs of care and, most important, quality of life for men dealing with BCR. Furthermore, Thermoresponsive (TR)-NanoVelcro Chips are expected to enable purification of CTCs from other solid tumors by targeting the corresponding surface markers, paving the way for the realization of a CTC-based RNA assay for cancer detection.

**5. CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

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

*Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.*

Expansion of the CTC-RNA assay: As a result of this project, we have strengthened our interactions with Dr. Sungyong You (Co-I), an expert in prostate cancer computation biology, we have been revising our approach to molecular characterization of CTCs using the CTC-RNA assay. While the original proposal focused on digital RT-PCR of *PSA, PSMA*, and *SCHLAP1* as a primary focus, we have gained the capacity to conduct other rapid genomic quantification approaches that we will explore in parallel to the directed RT-PCR to further optimize the performance of the assay. Using Nanostring nCounter analysis system for multiplexing gene quantification, two established PCa genomic classifiers, PCS and PAM50, were adopted into our CTC-RNA assay. These classifiers consist of genes enriched in multiple molecular pathways governing the tumorigenesis and progression of PCa. As such, they could provide more information regarding the biological properties of the tumor cells than the 3 gene markers we originally proposed. For this, a bioinformatic pipeline and analytical validation were applied to ensure these genomic classifiers suitable for being used for CTC gene expression analysis. With these two genomic classifiers incorporated into the CTC-RNA assay, we sought to explore the performance of the CTC-RNA assay in more clinical scenarios than we originally proposed, including risk stratification of localized PCa as well as detecting ARSI resistance in metastatic PCa patients. This exploration provided us more datasets to refine our data analysis methods to improve the signal-to-noise ratio of the CTC-RNA assay readouts.

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

*Describe problems or delays encountered during the reporting period and actions or plans to resolve them.*

COVID-19 related delays: During the up-ramping phase of our studies, both Cedars-Sinai and UCLA experienced a laboratory shut down which has negatively impacted the timelines of our proposed work. The restricted access to UCLA limited our implementation of major task 5, in which CTC-RNA assay should be tested parallelly at two institutions. Furthermore, the collection of patients' samples from NRG-GU-006 clinical trial for major task 6 has been slowed down due to the decreased manpower in the medical institutions. We are projecting that we will need an additional one year to complete these remaining project activities.

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

*Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.*

Nothing to Report

**-Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
*Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.*

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:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  *Report only the major publication(s) resulting from the work under this award.*

  **Journal publications.** *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  Nothing to Report

  **Books or other non-periodical, one-time publications.** *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  Nothing to Report

**Other publications, conference papers and presentations**. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

1. Teng PC, Jan YJ, Yoon J, Chen PJ, Chen JF, Yao N, Cheng S, Lozano A, Freeman M, You S, Tseng HR, Posadas EM. A circulating tumor cell specific RNA assay for assessment of androgen receptor signaling inhibitor sensitivity in metastatic castration-resistant prostate cancer. Journal of Clinical Oncology. 2019;37(15_suppl):5059-. doi: 10.1200/JCO.2019.37.15_suppl.5059. American Society of Clinical Oncology (ASCO) Annual Meeting 2019, Chicago, IL. (Poster presentation)
2. Teng PC, Jan YJ, Chen JF, Cook-Wiens G, Cheng S, Yao N, Lozano A, Chu GCY, Chen PJ, Ho H, Yang Y, Huang K, Li KC, Chung LWK, You S, Zhu Y, Freeman MR, Rogatko A, Yang JD, Tseng HR, Posadas EM. Very-Small-Nuclear Circulating Tumor Cells: Nuclear Size Reduction is Associated with Poor Clinical Outcomes in Metastatic Castration-Resistant Prostate Cancer. 2019 NCI Alliance of Nanotechnology in Cancer Principal Investigator Meeting, Rockville, MD. (Poster presentation)
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5. Teng PC, Jan YJ, Chen JF, Kim M, Yao N, Garraway I, Chu GCY, Chen PJ, Wang JJ, Lee YT, Zhu Y, Chung LWK, Feng FY, Freeman M, You S, Tseng HR, 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-. doi: 10.1200/JCO.2020.38.6_suppl.170. Genitourinary Cancers Symposium 2020, San Francisco, CA. (Poster presentation)
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- **Website(s) or other Internet site(s)**
  *List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.*

  Nothing to Report

- **Technologies or techniques**
  *Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.*

  Nothing to Report

- **Inventions, patent applications, and/or licenses**
  *Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.*

  UCLA Technology Development Group filed the first patent application entitled "Click Chemistry-Mediated Rare-Cell Sorting in Microfluidic Devices" (UCLA # 2018-441) to cover the IPs associated with the Click Chips and the related research and clinical applications.
  The second provisional patent application entitled "A Circulating Tumor Cell-RNA Assay for Assessment of Androgen Receptor Signaling Inhibitor Sensitivity in Metastatic Castration-Resistant Prostate Cancer" (UCLA # 2019-740) was to cover the IPs associated with the PCa CTC-based RNA profiling technology.

- **Other Products**
  *Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:*
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

  Nothing to Report

## 7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

**-What individuals have worked on the project?**
*Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".*

Name: Edwin Posadas (no change)
Project Role: Contact-PI
*No change*

Name: Jasmine Wang
Project Role: Postdoc
*No change*

Name: Kai-Han Tu
*No change*

Name: Arthur Salonga Jr.
*No change*

Name: Zijing Chen
*No change*

Name: Hsian-Rong Tseng
Project Role: PI
*No change*

Name: Tom Lee
Project Role: Collaborator
*No change*

**-Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
*If there is nothing significant to report during this reporting period, state "Nothing to Report."*

*If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.*

Nothing to Report

**-What other organizations were involved as partners?**
*If there is nothing significant to report during this reporting period, state "Nothing to Report."*

*Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved*

*with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.*

*Provide the following information for each partnership:*
*Organization Name:*
*Location of Organization: (if foreign location list country)*
*Partner's contribution to the project (identify one or more)*

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Organization Name: University of California, Los Angeles (UCLA)
Location of Organization: 500 Westwood Plz, California NanoSystems Institute (CNSI)
Partner's contribution to the project

- Financial support
- In-kind support
- Facilities
- Collaboration
- Personnel exchanges

## 8. SPECIAL REPORTING REQUIREMENTS

- **NONE**

## 9. APPENDICES:

*Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

Literature Cited:


1. Zhao SG, Chang SL, Erho N, et al. Associations of Luminal and Basal Subtyping of Prostate Cancer With Prognosis and Response to Androgen Deprivation Therapy. *JAMA Oncol.* 2017;3(12):1663-1672.
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