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TITLE: Analysis of the Genetic Landscape in the Evolution of Oligometastatic Prostate Cancer Through Peripheral Bioanalytes

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CONTRACTING ORGANIZATION: The University of Texas MD Anderson Cancer Center

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Approximately 1 in 9 men will develop prostate cancer during their lifetime. Although cure rates for early-stage (localized) disease can be high, the proportion of men presenting with metastatic disease has substantially increased. The standard of care for metastatic prostate cancer continues to be treatment with life prolonging, yet non-curative, systemic therapy. However, emerging data suggest an intermediate state of "oligometastatic" disease that represents a transition from localized to widely disseminated "polymetastatic" disease. We are currently conducting a trial (EXTEND, NCT03599765) in men with castration- sensitive (n=87) and -resistant (n=87, with lead-in phase [n=8] total n=182) metastatic prostate cancer to explore the hypothesis that oligometastatic disease represents a state of limited metastatic potential amenable to local consolidative therapy with curative intent. Current definitions of oligometastatic disease rely on conventional imaging, which can provide information about the number and location of lesions but not tumor biology. In this proposal, we aim to fill this gap in knowledge by leveraging banked tissue and longitudinally collected plasma samples from EXTEND by using recent advances in genomic and epigenomic techniques to develop sensitive assays to query changes in the prostate cancer genome, gene expression patterns, and DNA methylation profile to develop a multi-faceted understanding of the disease biology over time.

15. SUBJECT TERMS

NONE LISTED

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2. **KEYWORDS:** Oligometastatic disease, prostate cancer, metastasis directed therapy (MDT), liquid biopsy, predictive biomarker, genetic sequencing, tumor educated platelets (TEP), circulating tumor DNA (ctDNA), genomic methylation, and cell free DNA (cfDNA).

3. **ACCOMPLISHMENTS:**

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

	Months
Specific Aim 1: Establish multi-transcriptome sequencing of oligometastatic prostate cancer tumor samples**.** Establish multi-transcriptome sequencing of oligometastatic prostate cancer tumor samples	1-24
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   - **What was accomplished under these goals?**

- Specific Aim 1, Subtask 1: Collection, cutting, and marking of biopsy and prostatectomy tissue samples. During grant year 1 we have completed collection of 42 metastasis and 22 prostatectomy tissues.

- Specific Aim 1, Subtask 2: DNA and RNA extraction for sequencing and methylation analysis. We have extracted DNA from 60 samples. DNA yield is variable but enough for various forms of genomic analysis including DNA methylation and whole exome sequencing, although we often cannot run both assays on the same sample.

- Specific Aim 1, Subtask 3: Gene expression analysis. As noted during year 1, we have examined the contribution of tumor derived RNA on tumor educated platelet TEP signatures (346 healthy samples and 89 patients with glioblastoma [GBM]) utilizing data available from Best et al. *Cancer Cell* 2015. We applied deconvolution algorithms to cluster gene expression into platelet clusters, comprising gene expression signatures known to be involved in platelet function, and non-platelet clusters containing signatures derived from non-platelet cell types.

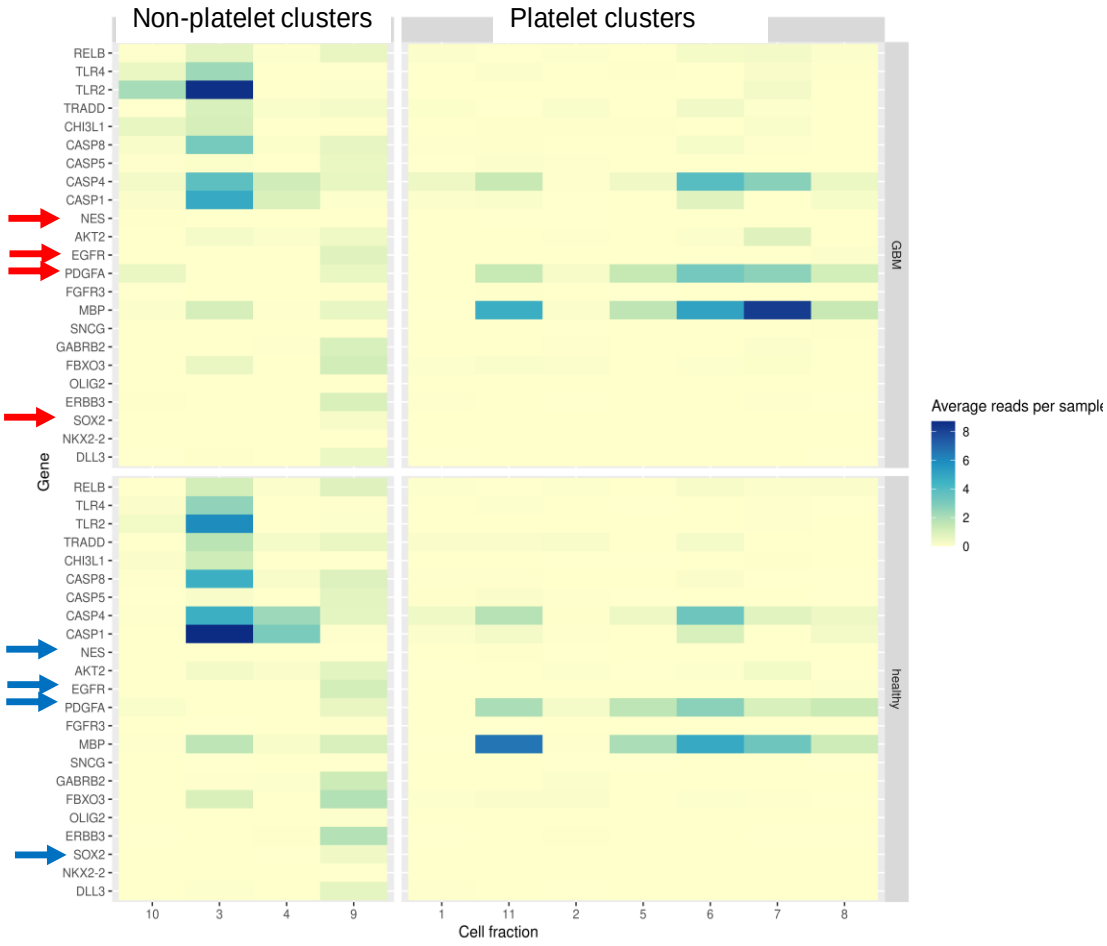

Figure 1: RNA expression deconvolution from TEPs derived from 346 healthy volunteers (bottom half of rows) and 89 patients with a diagnosis of GBM (top half of rows). X-axis displays RNA expression clustering into 11 groups labelled "cell fractions". Cell fractions derived from platelet sources are demarcated on the right side while those derived from non-platelet sources are on the left. Y-axis displays the genes of interest with canonical GBM altered genes highlighted with red or blue arrows. Note that highlighted genes are not differentially expressed between TEPs from GBM patients and healthy volunteers.

In both clusters, GBM specific RNAs such as EGFR, PDGFA, Nestin, or SOX2 were not differentially detected in TEPs between GBM patients and healthy volunteers (**Fig. 1**). However, this algorithm did identify signatures that could differentiate TEPs from GBM patients and from healthy volunteers that consistent of genes that were not necessarily tumor derived (data not shown). Therefore, we infer that gene expression analysis from the prostate primary and metastases will not reflect TEP RNA profile. We will continue to profile TEPs to identify signatures that can provide meaningful biological insights and can be pursued as biomarkers. In grant year 1 we proposed to transition the allocated funding from tissue gene expression analysis to funding additional methylation analyses. Our early experience investigating cfDNA methylation has led to incorporate additional control samples to verify that the signal that we have identified is real. These include input controls and methylation analysis of the adjacent prostate non-cancer tissues.

- <u>Specific Aim 1, Subtask 4: Whole exosome sequencing of extracted DNA samples</u>: We have conducted whole exome sequencing on 30 with prostate cancer. Sequencing reveals alterations as shown in the Oncoplot (**Fig. 2**). As typical for prostate cancer, common mutations include TP53 (37%), CDK12 (17%), and APC (17%). As these patients are predominately castration sensitive, we identified an expectedly low rate of AR mutations (3%).

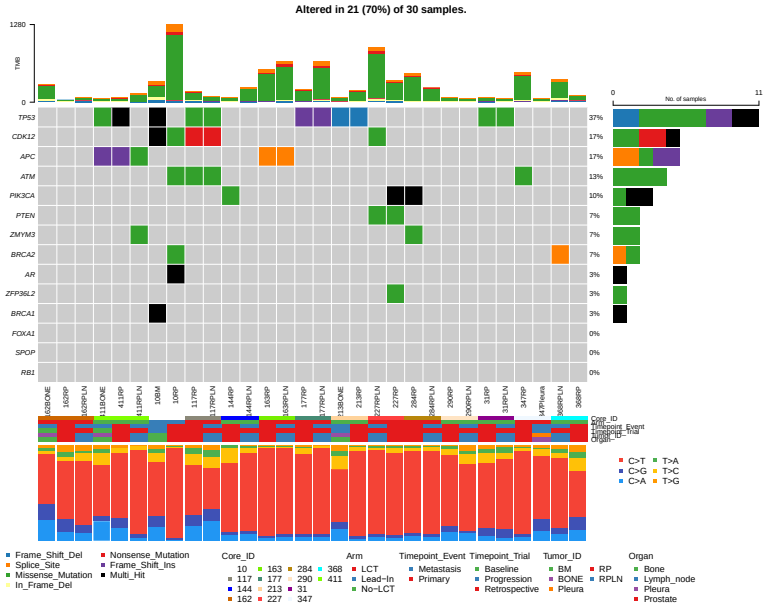

Figure 2: Oncoplot of whole exome sequencing results from 30 oligometastatic prostate patients, focusing on frequent seen alterations in prostate cancer.

- Specific Aim 1, Subtask 5: Tumor methylation profiling: We have completed tumor methylation analysis from 10 prostatectomy samples and 10 metastasis samples in addition. As a control we utilized adjacent normal prostate tissue also derived from prostatectomy sample and 10 plasma samples from healthy age and gender matched controls. For all the raw cf-MeDIP-seq (cell-free methylated DNA immuno-precipitation sequencing) files quality and quantity assessment was performed using FastQC (version 0.11.8) and MultiQC (version 1.8). Raw reads were then trimmed for contaminating adaptors using Trim Galore (version 0.6.5) with default settings in paired-end mode. The trimmed reads were then aligned to human reference genome *hg38* by implementing Bowtie2 in paired-end mode (version 2.4.1) with all other settings as default. Conversion of sequence alignment map (SAM) format files to binary alignment map (BAM) format, sorting, indexing, and removal of PCR duplicates was achieved using the SAMtools suite (version 1.15). In concordance with previous studies, 5mC-enriched regions were identified from BAM files using R package QSEA (Quantitative Sequencing Enrichment Analysis). 5mC peaks with human RefSeq genes annotation were visualized using the UCSC Genome Browser. Feature enrichment analysis demonstrates a preference for gain of methylation o CPG islands for both prostatectomy (**Fig. 3A**) and metastasis samples (**Fig 3B**).

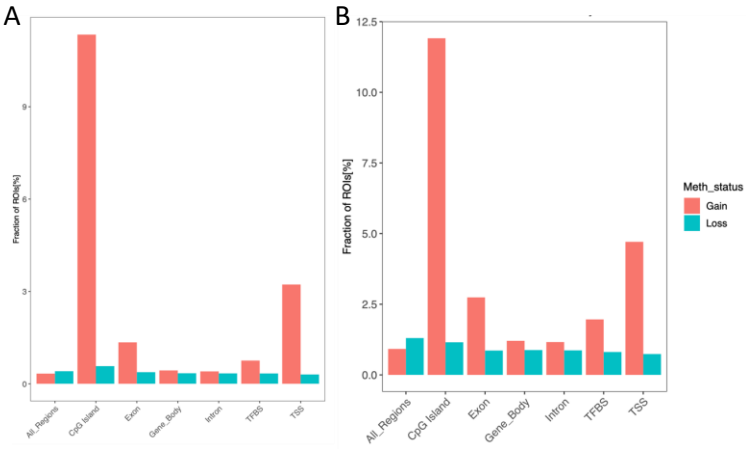

Figure 3: Feature Enrichment analysis from (**A**) prostatectomy and (**B**) metastasis samples obtained from oligometastatic patients enrolled on the EXTEND trial.

Full-length Sequencing				
PTEN	1731	SPOP	1125	**Hotspots Only**
AR	2763	CDKN2B	417	HRAS
TBX3	2232	CDKN2A	504	KRAS
MYC	1365	ATM	9171	APC
CDKN1B	618	BAP1	2190	PIK3CA
NKX3-1	705	MSH2	2817	CTNNB1
TMPRSS2	1590	CDK12	4473	CCND1
TP53	1233	PMS2	2589	ZFHX3
RYBP	684	CHEK2	1761	KMT2C
CCND1	888	MSH6	4083	BRAF
FOXA1	1419	BRCA1	5655	NCOR1
BRCA2	10257	PALB2	3561	KDM6A
RB1	2787	ATRX	7479	MED12

Table 1: Left 4 columns list genes that will be subject to full-length sequencing and their corresponding size in base pair. The right-most column lists genes in which sequencing will be limited to hotspots only.

- Specific Aim 2, Subtask 1: Creation of a candidate gene/exome list for custom ctDNA panel: As noted in year 1, we utilized all publicly available prostate cancer sequencing data from cBioportal and an applied an algorithm to iteratively select candidate genes, we have created the following list of candidate genes/exosomes (**Table 1**) which will be utilized in our custom prostate ctDNA panel (PCAN assay). **Table 1** lists genes that will be sequenced for the custom PCAN assay including those that will be sequenced in their entirety and those in which sequencing will be limited to hot spots only.

- Specific Aim 2, Subtask 2: Creation of a ctDNA panel and validation: To assess the performance of the PCAN assay and conduct analytical validation of controls and patient samples, we constructed an automated a custom bioinformatics pipeline (**Fig. 4**). The pipeline consists of demultiplexing of Fastq reads (bcl2Fastq v.2.20.0) aligned (bwa-mem v.0.7.17) to the hg19 human genome

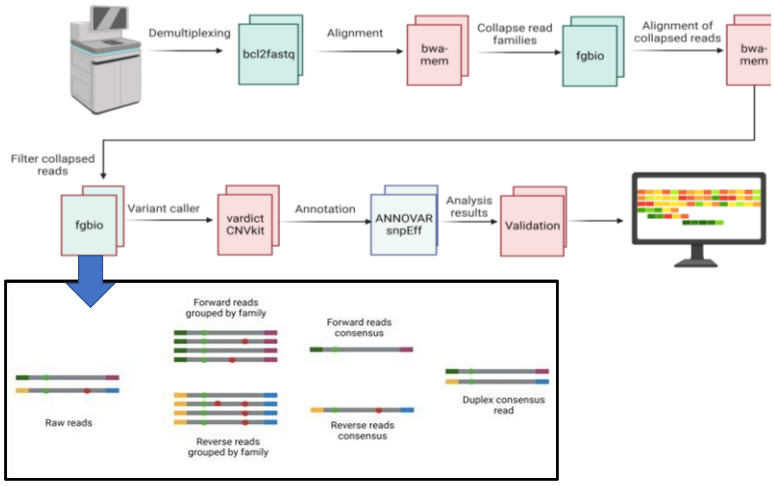

Figure 4. Bioinformatics analysis workflow.

and subsequently collapsed, realigned, and filtered (fgbio v. 1.3.0). The reads are then grouped into "families", from which there is a consensus for the mutations detected in comparison to the reference genome and to ensure that the same sequence is identified among forward and reverse reads independently. Afterwards, duplex consensus reads are used for the variant calling for SNV (vardict v. 1.8.2), and CNV detection (CNVkit v.0.9.6), and subsequent annotation (Annovar v.2019.10.24, snpEff, v. 5.1d).

Specific Aim 2, Subtask 3: ctDNA panel utilization in plasma samples: An initial test batch of 6 samples were sequenced using the PCAN assay. After applying background error correction, 142 SNVs (Single Nucleotide Variants), deletions, insertions and complex mutations were found across the sample cohort. **Figure 5** details the mutations detected within plasma samples. All 6 samples had been sequenced prior with the Guardant360 ctDNA panel. We identified a high degree of concordance between mutation detection in the PCAN and Guardant panels, providing further validation of the PCAN panel function. In the next year we plan to sequence multiple batches of plasma samples from oligometastatic prostate cancer patients enrolled on the EXTEND trial.

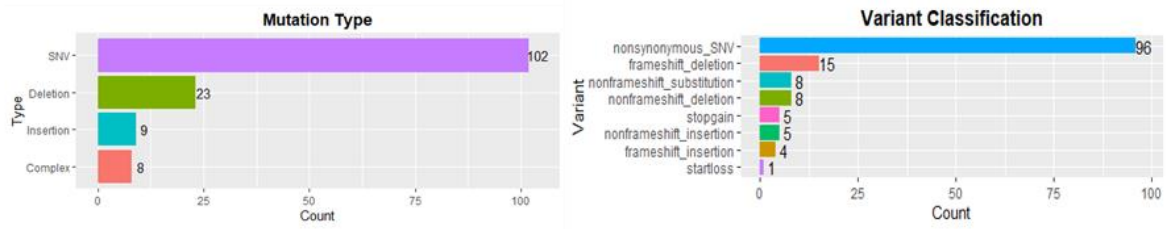

Fig 5. Count for mutation type and variant classification across the cohort of six prostate cancer patients. Among the mutations, SNVs are the most common with 102 counts while insertions and complex mutations are below 10. On the other hand, the most frequent variant classification accounts for nonsynonymous SNVs and start loss function was only for one variant

- Specific Aim 2, Subtask 4: Statistical modeling to develop "metastatic score": As noted in the SOW, work on this subtask will begin grant year 3 after we have run assays from additional samples.

- Specific Aim 3, Subtask 1: RNA extraction from tumor educated platelets: We have extracted RNA from tumor educated platelets (TEPs) from 148, including 136 done this grant year. All were collected from sequential time points drawn from oligometastatic prostate cancer patients enrolled on the EXTEND trial.

- Specific Aim 3, Subtask 2: RNA sequencing from tumor educated platelets: We have sequenced RNA extracted from TEPs from 148 samples, including 136 done this year. We are currently in the middle of the analyses of the current batch of 136 samples which has been analytically intense. We plan to compare this analysis to an online repository of platelets from 350 control patients without a diagnosis of cancer. Currently we have only completed analysis of the initial 12 samples. Multiple comparisons from the initial 12 samples, all of which were drawn from different patients, are described here in **Figure 6**. Principle component (PC) analysis comparing RNA expression from TEPs from prostate cancer patients to TEPs from 55 healthy donors available online (Best et al. *Cancer Cell* 2015) revealed segregation of the two patient populations (**Fig. 6A**). We next compared differential gene expression between TEPs from prostate cancer patients to those drawn from glioblastoma multiforme (GBM) patients (done as part of a separate research study by our group). Volcano plots visualizing this comparison revealed numerous differentially expressed genes and significant segregation of the patient populations (**Fig. 6B**). In the upcoming year we plan to conduct and refine analysis from the most recent batch of 136 samples.

- Specific Aim 3, Subtask 3: cfDNA purification from plasma samples: We have extracted cfDNA from plasma collected from 48 baseline prostate cancer patients and 10 healthy age matched donors.

- Specific Aim 3, Subtask 4: cfDNA methylation analysis: We are in currently conducting cfDNA methylation analysis of 148 plasma samples. Comparisons of differentially methylated regions (DMRs) between plasma samples, 10 prostatectomies, and10 metastases samples identified 789 genes for cfDNA methylation analyses (**Fig. 7A**). We will focus our cfDNA methylation analysis on the top 300 DMRs from this list. Evaluation of cfDNA methylation utilizing the top 300 DMRs yielded a promising area under the curve (AUC) of 0.91 when differentiating buffy coat from plasma (**Fig. 7B**).

- Specific Aim 3, Subtask 5: Statistical modeling of cfDNA and TEP expression results. Integration of results into "metastatic score": As noted in the SOW, work on this subtask will begin grant year 3.

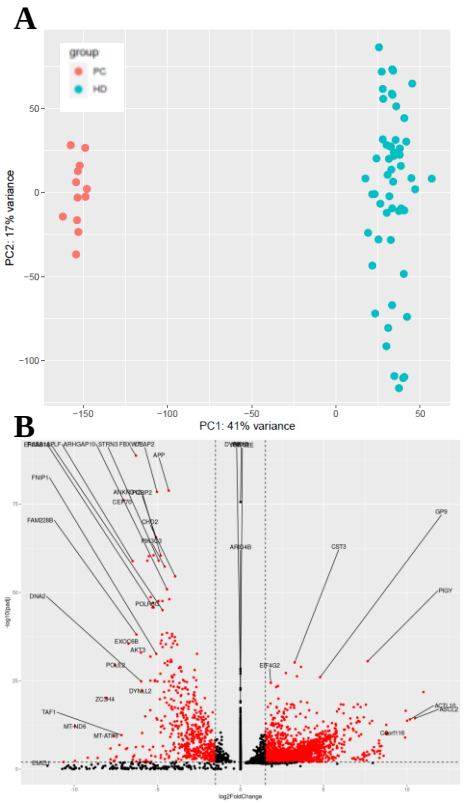

Figure 6: TEP analysis initial run. (A) Evaluation of RNA expression using principal component (PC) analysis to separate TEPs collected from prostate cancer (PC) patients to those from health donor (HD) controls. (B) Volcano plot comparing gene expression differences between prostate cancer (left side) versus GBM (right side)

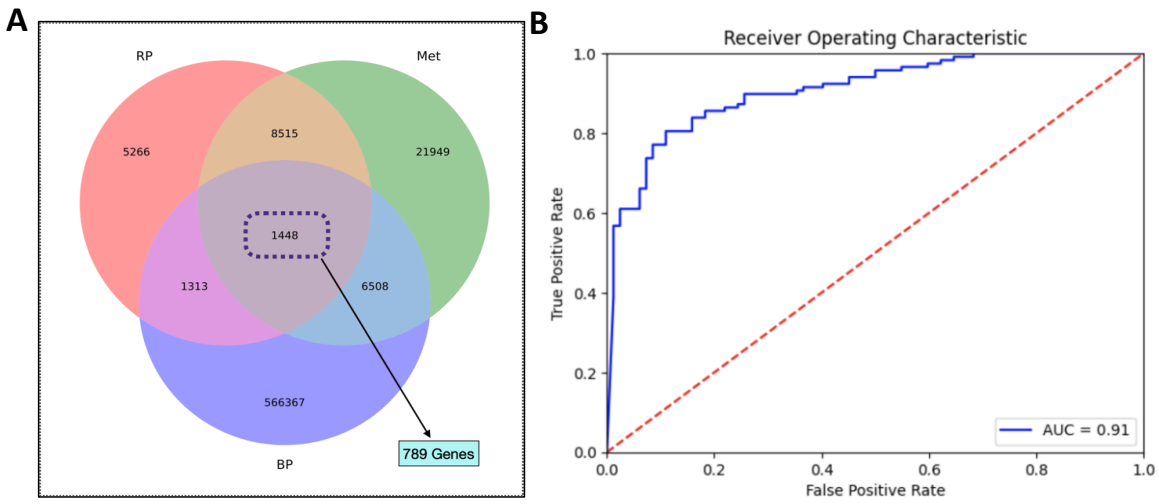

Figure 7: (A) Shared differentially methylated regions (DMRs) between radical prostatectomy (RP), metastases (Met) and baseline plasma (BP) yielding a shared 789 genes overlap between these 3 sample types. (**B**) Receiver Operating Characteristics utilizing 300 top DMRs to differentiate between plasma and buffy coat from oligometastatic prostate cancer patients enrolled on EXTEND.

- **What opportunities for training and professional development has the project provided?**
  - We have continuous met with our mentors Dr. Felix Feng and Dr. Michael Ittman during this project and will continue to communicate with them on regarding ongoing issues. All collaborators and staff involved on work from this grant study collaborators meet on a monthly basis to review grant activities and provide feedback on their own projects as well as others.

- **How were the results disseminated to communities of interest?**
  - Nothing to Report

- **What do you plan to do during the next reporting period to accomplish the goals?**
  - **Specific Aim 1**: Continue whole exome sequencing bioinformatics analysis from DNA samples extracted from prostate and metastases tissues. Integrate data from whole exome and DNA methylation analyses to inform analyses of Specific Aims 1 and 2.

  - **Specific Aim 2:** Run ctDNA samples from patients on EXTEND exploring baseline and post treatment changes from plasma samples utilizing the newly constructed custom prostate cancer panel (**Table 1**).

  - **Specific Aim 3**: Conduct bioinformatics analysis on the most recent batch of 136 TEP samples. Continue to refine bioinformatic analysis for the cfDNA methylation assay. As soon as the bioinformatic analysis is complete for cfDNA methylation we will run cfDNA methylation on additional plasma samples.

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

- Nothing to Report

- **What was the impact on other disciplines?**
  - Nothing to Report

- **What was the impact on technology transfer?**
  - Nothing to Report

- **What was the impact on society beyond science and technology?**
  - Nothing to Report

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**
     - **Specific Aim 1, Subtask** 2: We have rectified the delay in grant year #1 and have been able to conduct DNA extraction for whole exome and tumor methylation profile analysis from the appropriate number of tissues. We will continue to identify additional tissues if additional samples are required.
     - **Specific Aim 3, Subtask 1:** We have rectified the delay in grant year 1 and have run TEP samples in one large batch during this grant year.
     - **Specific Aim 3, Subtask 3:** Nothing to report

   - **Changes that had a significant impact on expenditures**
     - As noted in Grant Year 1, we proposed transitioning allocated funds for RNA sequencing of tumor tissue to cfDNA methylation analysis.

   - **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
     - 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:**
   - Nothing to Report

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**
   - **What individuals have worked on the project?**

Name:	Chad Tang

Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1
Contribution to Project:	Oversight of all aspects of the grant and integration of the molecular assay results and the clinical component.
Funding Support:	N/A

Name:	Krishna Bhat
Project Role:	Collaborator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1
Contribution to Project:	Supervision of sample preparation, DNA/RNA extraction, and conduction of molecular assays.
Funding Support:	N/A

Name:	Alexander Sanchez Espitia
Project Role:	Laboratory technician
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	3
Contribution to Project:	Completion of whole exosome sequencing, developing the ctDNA assay, and applying the ctDNA assay to research samples.
Funding Support:	N/A

Name:	Rakesh Trivedi
Project Role:	Post Doc
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	2
Contribution to Project:	Sample preparation, DNA/RNA isolation, and RNA transcription analysis.
Funding Support:	N/A

Name:	Yi Zhong
Project Role:	Post Doc
Researcher Identifier (e.g. ORCID ID):	N/A

Nearest person month worked:	2
Contribution to Project:	Completion of bioinformatic analyses pertaining to molecular tests conducted in this grant.
Funding Support:	N/A

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
  No

- **What other organizations were involved as partners?**
  - *Nothing to Report.*

8. **SPECIAL REPORTING REQUIREMENTS**

   - **COLLABORATIVE AWARDS:** Not Applicable

   - **QUAD CHARTS:** Not Applicable

9. **APPENDICES:** ***N/A***