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Epigenetic Analysis of Circulating Tumor Cells

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14. ABSTRACT
Despite advances in the field, metastatic prostate cancer prognosis remains poor. Although genomic biomarkers are being assessed for clinical relevance, epigenetic modifications have been found to be much more common, some found in more than 90% of prostate cancer tumors. Two of these modifications are GSTP1 and PRAC DNA hypermethylation. Circulating tumor cells (CTCs) provide a minimally invasive way to monitor patient disease, however they are a rare cell population and current methods of epigenetic analysis are not sensitive enough to assay DNA methylation from such a population. We have developed an assay that can sensitively and specifically enrich for methylated DNA from rare cell populations and have optimized this assay using cell line models to enrich for methylated GSTP1 from as little as one cell. We have tested this assay in patient biopsies, either flow sorted by tumor compartment or unsorted populations, and found GSTP1 methylation in 100% of patient tissue tested, but not in white blood cells. We also chose 7 patients with castration resistance prostate cancer (CRPC) as a pilot study for GSTP1 methylation analysis in CTCs. 5 out of 7 (71%) CRPC CTC samples had GSTP1 methylation detectable above background.

15. SUBJECT TERMS
Methylation, circulating tumor cells, biomarker, castration resistant prostate cancer, epigenetics

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INTRODUCTION: Although prognosis for localized prostate cancer remains positive, the 5-year survival rate for metastatic prostate cancer is only 30%[1]. The development of new biomarkers that predict disease outcome and monitor treatment response is critically needed. Epigenetic alterations have been shown to be more prevalent than genomic alterations is prostate cancer, some found in more than 90% of prostate cancer cases, and may provide such a biomarker[2]. Two of the genes found to be methylated in prostate cancer but not in normal prostate tissue that may be useful as biomarkers are GSTP1 and PRAC[3, 4]. Circulating tumor cells (CTCs) in the blood of cancer patients collected by "liquid biopsy" would permit repeatable, minimally invasive sampling of the epigenetic signature of each individuals cancer. However, CTCs are a rare cell population and traditional methods of methylated DNA analysis are not suitable for rare cells. We are optimizing an automated platform for methylated DNA enrichment by a combination of methylation sensitive restriction enzyme digestion and methylated DNA precipitation with the methyl binding domain of methyl-CpG binding domain protein 2 (MBD2-MBD). The goal of this proposal is to optimize this technology for use in CTCs and use this technology to analyze methylation at GSTP1 and PRAC for use as biomarkers of castration resistant prostate cancer (CRPC).

KEYWORDS: Methylation, circulating tumor cells, biomarker, castration resistant prostate cancer, epigenetics

ACCOMPLISHMENTS:

What were the major goals of the project?

Major goals of this project as described in the Statement of Work:

Specific Aim 1: To refine the automated VERSA platform to extract methylated DNA from rare cell populations

Major Task 1: Develop the VERSA platform to extract methylated DNA

Specific Aim 2: To assess methylation at the GSTP1 and PRAC loci in prostate cancer CTCs from mCRPC vs. hormone naïve patients and potential of GSTP1 and PRAC as biomarkers

Major Task 1: Test previously extracted DNA from CTCs for GSTP1 and PRAC methylation status

What was accomplished under these goals?

Specific Aim 1, Major Task 1:

Subtask 1: LNCaP-abl cell line was acquired from the Yegnasubramanian lab in August 2016.

Subtask 2: Digestion of methylated DNA from cell lines was optimized to reduce length of digestion time to 15 minutes from the previously used time of 3 hours, following the enzyme manufacturers recommendation. Success of digestion was determined by RT-qPCR analysis of methylated and non-methylated DNA cut by the methylation sensitive enzyme in digested samples vs. un-digested samples. The 15-minute digestion was comparable to the 3-hour digestion as indicated by similar reduction in GSTP1 amplification in unmethylated DNA samples after digestion with the methylation sensitive enzyme (Figure 1).

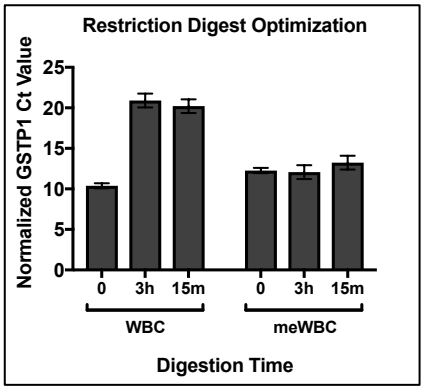

Figure 1. LINE1 normalized GSPT1 amplification in undigested samples vs. samples digested for 3 hours or 15 minutes. WBC: white blood cell DNA; meWBC: enzymatically methylated white blood cell DNA

Subtask 3: We have optimized extraction of methylated DNA and are able to specifically detect methylated GSTP1 from as little as 0.005ng of enzymatically methylated DNA or LNCaP DNA, equivalent to approximately 1 cell (Figure 2A).

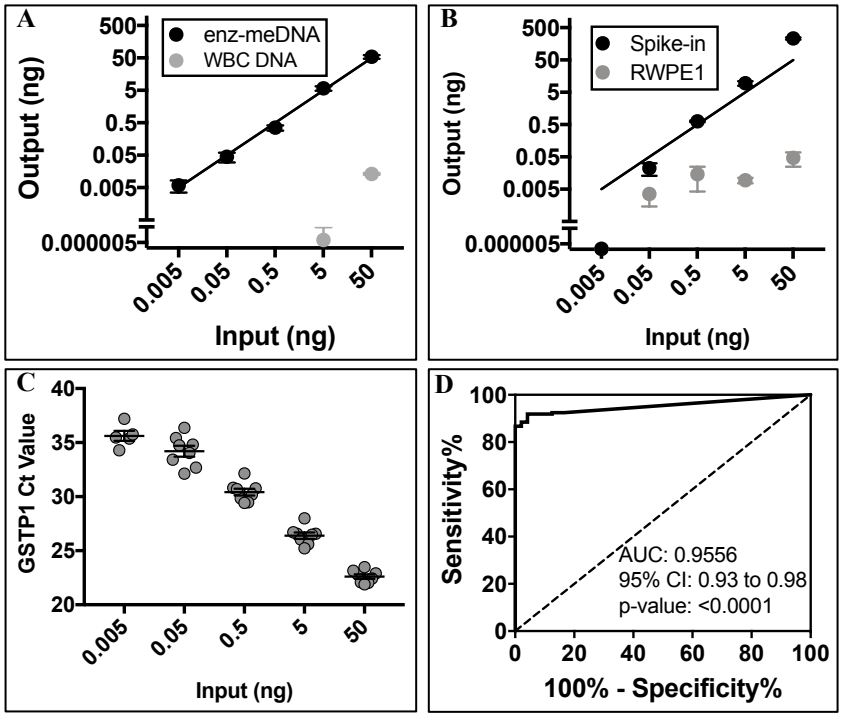

Figure 2. A) Assay performance for detection of GSTP1 in methylated DNA enriched from serial dilutions of input DNA. Output concentration is calculated from a standard curve for GSTP1. The black line represents perfect enrichment (A,B). Error bars represent SEM for n=7. Enz-meDNA: DNA methylated by SssI methyltransferase; WBC DNA: white blood cell DNA. **B)** Assay performance for detection of GSTP1 in methylated DNA enriched from varying amounts of LNCaP DNA (as indicated on the x-axis) spiked into 50ng of RWPE1 DNA. Spike-in: LNCaP DNA + RWPE1 DNA. **C)** Repeatability of assay across 8 different days. Each point represents the Ct value for GSTP1 amplification in methylated DNA enriched from the indicated input concentration of DNA methylated by SssI methyltransferase. **D)** ROC curve for this assays ability to distinguish between methylated and unmethylated DNA at GSTP1. Dotted line represents an assay that cannot distinguish methylated and unmethylated DNA. AUC: area under the curve.

Subtask 4: We have examined the sensitivity, specificity, and reproducibility of methylated DNA extraction by comparing enrichment of methylated DNA vs. enrichment of unmethylated DNA at the GSTP1 locus. We spiked varying amounts (50ng-0.05ng) of LNCaP DNA into 50ng of RWPE1 DNA to demonstrate that the assay is sensitive and specific enough to enrich for methylated DNA from a less methylated background (Figure 2B). LNCaP DNA was detected down to 0.05ng of input, equivalent to approximately 8 cells. Reproducibility and day to day precision of the assay is modeled in Figure 2C where each point represents a separate run of the assay across different 8 different days. On 3/8 of the days, no methylated DNA was detected from the 0.005ng input. This is likely due to difficulty in pipetting such dilute concentrations. An ROC curve based on methylated GSTP1 detection in enzymatically methylated and LNCaP DNA vs. WBC DNA is shown in Figure 2D. An assay that perfectly differentiates true positives (GSTP1 in enzymatically methylated and LNCaP DNA) from true negatives (GSTP1 in WBC DNA) would have an area under the curve (AUC) of 1.0. This assay has an AUC of 0.95 with a sensitivity and specificity of 91.91% and 95.83% respectively as dictated by the maximum value of Youden's index, where positive signal is a Ct value >36.65. To test this assay on patient samples, we first analyzed methylated GSTP1 in prostate cancer biopsies. GSTP1 methylation was detected in 8/8 biopsies tested (Figure 3A). To test assay specificity, cells from two tumor loci from one patient were flow sorted into epithelial and immune compartments using EpCAM and CD45 selection, respectively. GSTP1 methylation was detected in cells from the epithelial compartment, but not the immune compartment (Figure 3B).

Specific Aim 2, Major Task 1:

Subtask 1: We identified 7 CTC samples from patients with CRPC that have had DNA previously extracted using the VERSA (Table 1).

Pt Number	Capture Ab
274	Trop2
286	EpCAM
314	EpCAM
396	EpCAM
411	EpCAM
459	EpCAM
460	EpCAM

Table 1: Patient number and capture antibody used to enrich for circulating tumor cells from patient blood processed by Ficoll gradient.

Subtask 2: We successfully extracted methylated DNA from all 7 CTC samples, as evidenced by amplification of LINE1 in RT-qPCR. LINE1 values for each sample can be seen in Figure 4.

Subtask 3: GSTP1 methylation was analyzed by RT-qPCR in methylated DNA extracted from CTCs from 7 patients with CRPC and WBC DNA from 2 patients

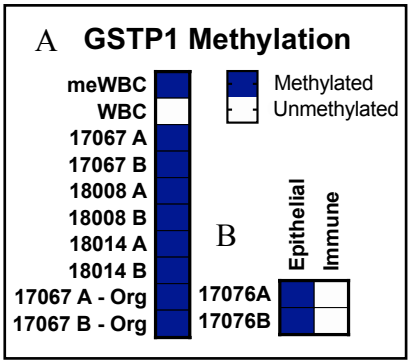

Figure 3: GSTP1 methylation in primary prostate biopsies. **A)** Two tumor loci were analyzed, marked A and B. Org – organoid culture from patient cells. **B)** Biopsy cells were flow sorted into epithelial and immune compartments.

with CRPC. GSTP1 methylation was detected in 5/7 CTC samples (Figure 4). A low level of GSTP1 methylation was detected in WBC DNA from one patient with prostate cancer when large amounts (10-50ng) of WBC DNA were added. This is likely due to a few CTCs getting stuck to the CD45 fractionation column during WBC purification rather than methylation in WBCs themselves. An ROC curve of the assay's ability to accurately distinguish cancer-derived DNA (biopsies or CTCs) and WBC DNA is shown in Figure 5. The maximum Youden's index for the ROC curve gives a cut off value of 44.33, sensitivity of 86.67%, and specificity of 80%.

What opportunities for training and professional development has the project provided? Nothing to report.

How were the results disseminated to communities of interest? Results from this project were presented to graduate students, post-docs, and PIs at the Cancer Biology student/post-doc seminar series.

What do you plan to do during the next reporting period to accomplish the goals? Each assay validation experiment shown here will be repeated for PRAC. CTC samples with DNA previously extracted will then be selected to screen for PRAC methylation. We will obtain samples from patients with hormone naïve prostate cancer to screen for GSTP1 and PRAC methylation. We will expand the patient cohort to include 40 patients with CRPC or hormone naïve prostate cancer to test the predictive value of GSTP1 and PRAC methylation in determining hormone resistance. We will also assess the feasibility of whole genome amplification of enriched methylated DNA in order to increase the genes we are able to screen for in patients.

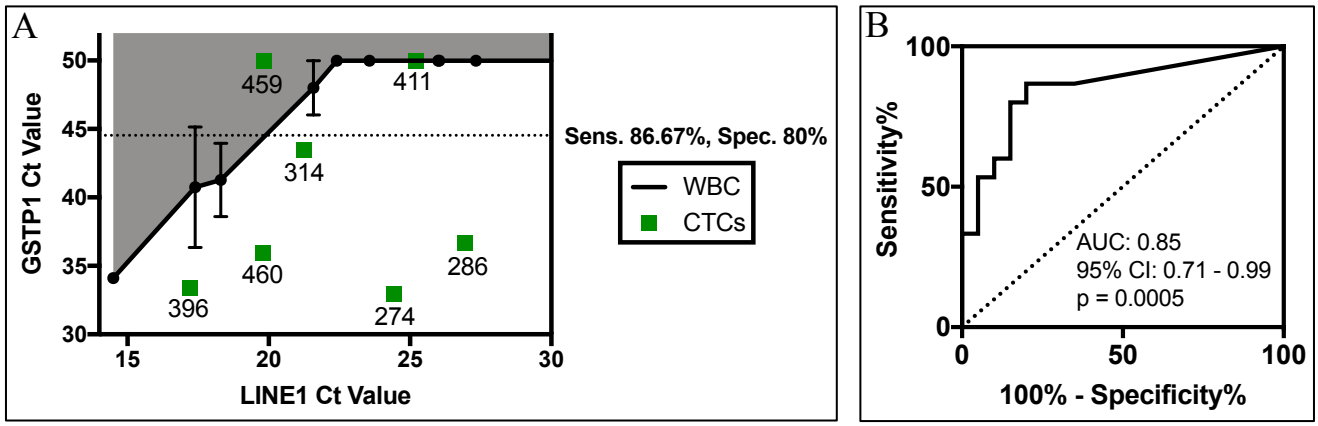

Figure 4: **A)** GSTP1 and LINE1 Ct values are plotted for each CTC sample and varying concentrations of WBC DNA. The shadowed area represents GSTP1 amplification below the threshold set by WBC across this range of LINE1 values. The cutoff Ct value for the indicated sensitivity and specificity determined by the highest Youden's index is shown by the dotted line. **B)** ROC curve for this assays ability to distinguish between prostate cancer and white blood cells at GSTP1. Dotted line represents an assay that cannot distinguish the difference. AUC: area under the curve.

IMPACT:

What was the impact on the development of the principal disciplines of the project? This work has led to new assays that will be presented at national conferences in the coming year.

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.

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, vertebrate animals, biohazards, and/or select agents. Nothing to report.

PRODUCTS:
Publications, conference papers, and presentations. Nothing to report.
Websites or other Internet sites. Nothing to report.
Technologies or techniques. Nothing to report.
Inventions, patent applications and/or licenses. Nothing to report.
Other products. Nothing to report.

PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS:
What individuals have worked on the project?

Name:	*Tamara Rodems*
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	4
Contribution to Project:	*Ms. Rodems has provided project oversight, has developed assays and technologies to enrich for methylated DNA from rare cell populations, assisted with securing regulatory approvals, and assisted with managing the grant budget and reporting.*
Funding Support:	

Has there been a change in the active other support of the PD/PI or senior/key personnel since the last reporting period? Nothing to report.
What other organizations were involved as partners? None

SPECIAL REPORTING REQUIREMENTS:
Collaborative awards: None.
Quad charts: None.

APPENDICES: None.

1. Howler, N.*, et al*. SEER Cancer Statistics Review. 1975 - 2013.
2. Yang, M. & Park, J.Y. DNA methylation in promoter region as biomarkers in prostate cancer. *Methods Mol Biol*. **863**, 67-109 (2012).
3. Lenka, G., Weng, W.H., Chuang, C.K., Ng, K.F. & Pang, S.T. Aberrant expression of the PRAC gene in prostate cancer. *Int J Oncol*. **43**, 1960-1966 (2013).
4. Yegnasubramanian, S.*, et al*. Hypermethylation of CpG islands in primary and metastatic human prostate cancer. *Cancer Res*. **64**, 1975-1986 (2004).