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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 (WBCs). 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. In order to measure GSTP1 and PRAC methylation from the same cells, we have made changes to this assay to allow for pre-amplification of target genes prior to qRT-PCR. We have tested this new version of the assay and ensured that the performance is similar to the original version. We have also tested a PRAC primer on cell line models and WBC DNA and found that WBCs may harbor some PRAC methylation, which will need to be addressed prior to use in patient samples. We have collected DNA from patients that will be used to assess GSTP1 methylation and may also be used to assess PRAC methylation if the primer can be optimized to exclude WBC methylation.

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

Major Task 2: Validate GSTP1 and PRAC methylation status from 40 patient samples with metastatic prostate cancer

What was accomplished under these goals?

Specific Aim 1, Major Task 1:

Subtask 2: We further optimized the digestion of DNA to be used for enrichment to allow us to detect multiple biomarkers from the same cells. This requires us to not use a methylation sensitive restriction enzyme, since finding an enzyme with a common methylated site within multiple promoters becomes prohibitively difficult. An example of how the presence of an unmethylated restriction site does not always correlate with hypermethylation within the region of interest is shown in Figure 1A for the gene HLA-A, where the HhaI restriction site is unmethylated in the otherwise heavily methylated exon 2. This results in lost signal in the HLA-A gene, even though the region assayed is heavily methylated (Figure 1B). GSTP1 signal from WBCs was increased slightly without the use of the methylation sensitive enzyme, but is still low enough to warrant use in this assay (Figure 1C). To ensure that the DNA is fragmented enough to reduce false positives from capture of methylation far away from the primer site, we added a second, non-methylation sensitive enzyme (HpyCH4V), which cuts outside of the primer regions.

Subtask 3: We have previously optimized the extraction of as little as 0.005ng of methylated DNA from cell lines (Figure 2 in 2018 Annual Report). This was performed using a SYBR based qRT-PCR method and standard oligo primers. Since then, we have also optimized this assay to work with TaqMan primers, which allows us to pre-amplify the enriched methylated DNA and look at more than a single biomarker in the same cells. TaqMan primers are also generally thought to be more specific because they contain an internal probe

that must also bind to the amplicon for signal to be produced. We performed the assay using the methylation sensitive enzyme and the TaqMan GSTP1 primer on serially diluted LNCaP DNA and used white blood cell (WBC) DNA as a negative control. We were able to detect GSTP1 from the enriched LNCaP DNA down to 0.005ng, similar to our results from the SYBR based method (Figure 2A). We also performed a similar experiment starting from whole cells, serially diluted from 1000 down to 1 cell, to ensure that our DNA extraction method for CTCs is compatible with the methylation enrichment assay. We were able to detect methylated GSTP1 from as little as 1 LNCaP cell (Figure 2B). The background detection from WBCs was similar to what we saw in previous experiments.

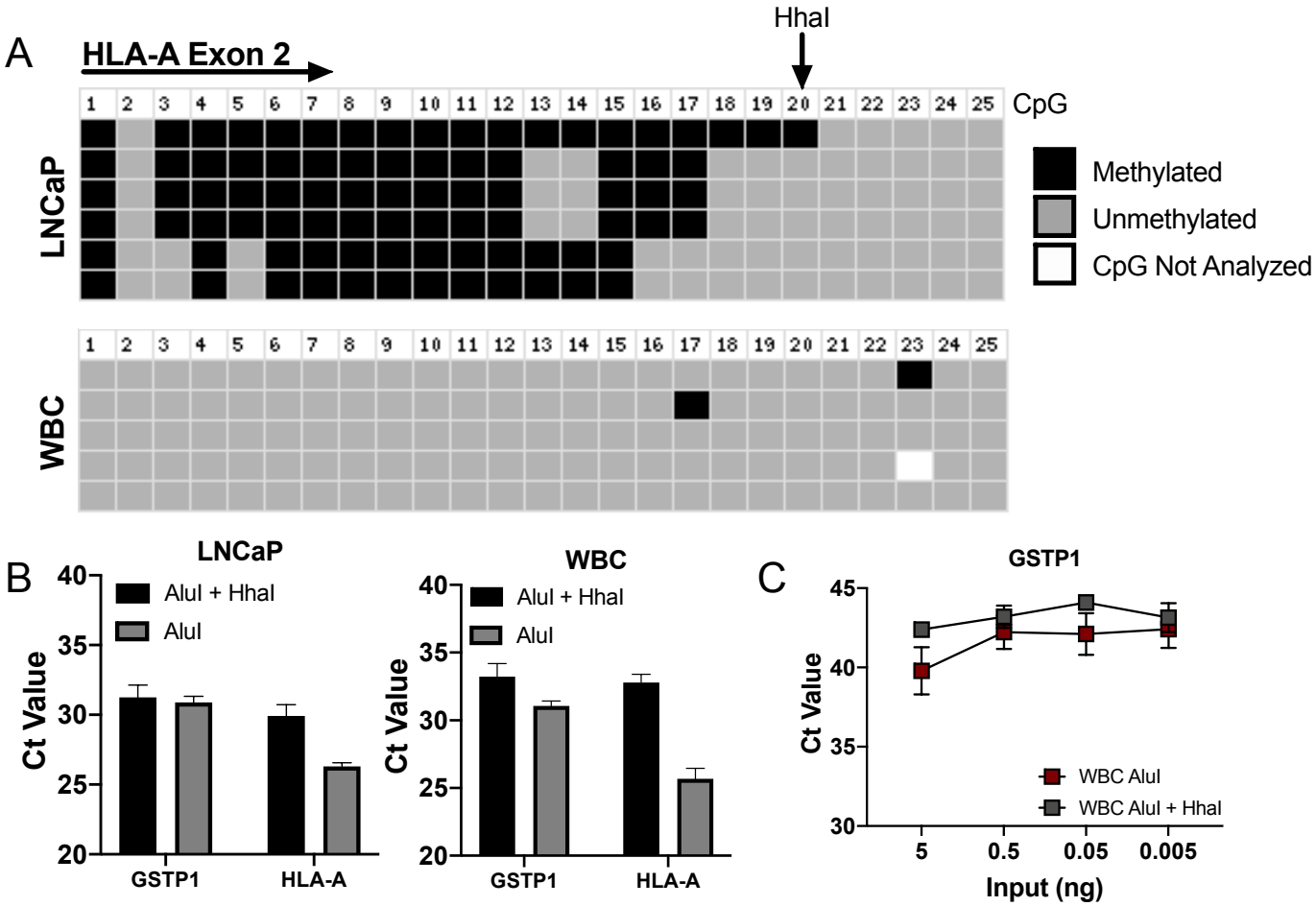

Figure 1: A) Bisulfite sequencing of a portion of HLA-A exon 2. Each square represents an individual CpG site. Black squares represent methylated CpG sites, gray squares represent unmethylated CpG sites, and white squares represent sites that were not analyzed due to base mismatch or poor base calling. The CpG contained within the HhaI restriction site is indicated. B) Raw Ct values obtained from qRT-PCR for the indicated genes after enrichment for methylated DNA are shown. DNA was digested with and without the metyhaltion sensitive enzyme, HhaI prior to enrichment. C) Serially diluted WBC DNA was digested with and without HhaI, enriched for methylated DNA and qRT-PCR was performed for GSTP1. Raw Ct values are shown for each dilution. A Ct value of 45 (equal to total cycles run) was given for any sample that did not amplify at the end of the qRT-PCR run.

In order to look at both GSTP1 and PRAC from the same cells, we decided to add a pre-amplification step to the process. Without this step, it would be easy to miss methylation from either gene when splitting a sample where only a few copies of either gene are present, as would be the case for samples with few CTCs. We performed this assay on serially diluted LNCaP, LACP4, and WBC DNA. LNCaP cells are methylated at GSTP1, but not PRAC, and LAPC4 cells are methylated at PRAC, but not at GSTP1. We were able to detect methylated GSTP1 in LNCaP cells from every dilution, however the background from WBC was slightly

higher at higher concentrations compared to the assay without pre-amplification (Figure 3A). We were able to detect PRAC in LAPC4 cells at every dilution, but we also detected PRAC in the WBCs at almost the same level (Figure 3B). To see whether this was real methylated PRAC we were detecting or if this was just background, we performed the same experiment using DNA from LNCaP cells, which do not have PRAC methylation. PRAC was detected at levels similar to GSTP1 in WBCs, indicating this is non-specific background detection (Figure 3B). The results of this experiment indicate that there may be methylation present in the WBC DNA at PRAC, which was unexpected and poses a problem for using PRAC as a biomarker in CTCs due to the presence of WBCs. Alternatively, this may be an issue with our PRAC primer. This will be explored further in future studies.

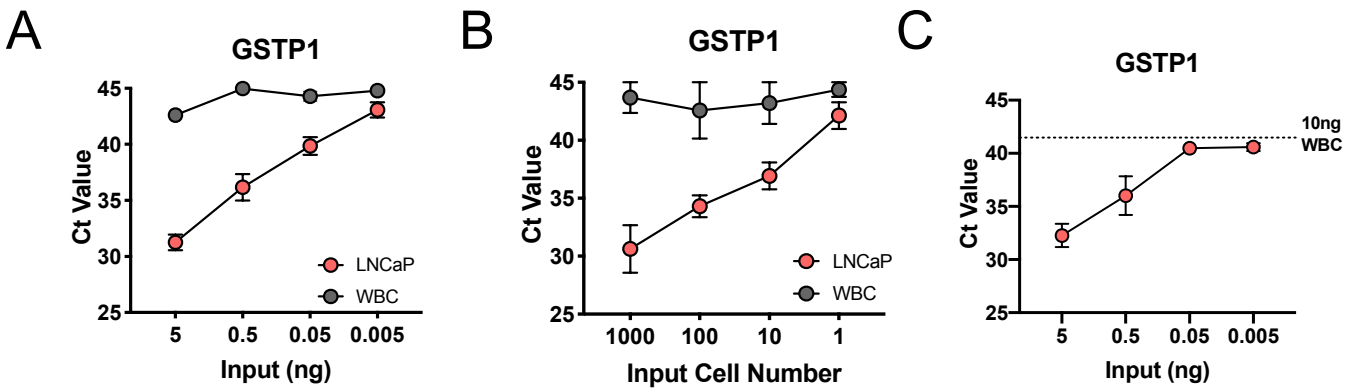

Figure 2: A) Serially diluted LNCaP and WBC DNA was digested with AluI and HhaI and enriched for methylated DNA. qRT-PCR for GSTP1 was perfomed on enriched DNA. Raw Ct values for each dilution are shown. B) LNCaP and WBC cells were serially diluted to indicated concentrations. Cells were lysed and DNA was captured by silica beads used a semi-automated protocol developed on the Gilson Pipetmax. DNA was digested with AluI and HhaI and enriched for methylated DNA. qRT-PCR for GSTP1 was perfomed on enriched DNA. Raw Ct values for each dilution are shown. C) LNCaP DNA was serially diluted and each dilution was added to 10ng of WBC DNA to mimic the CTC environment. DNA was digested with AluI and HhaI and enriched for methylated DNA. qRT-PCR for GSTP1 was perfomed on enriched DNA. Raw Ct values for each dilution are shown. A Ct value of 45 (equal to total cycles run) was given for any sample that did not amplify at the end of the qR T-PCR run.

Subtask 4: We spiked LNCaP DNA into 10ng of WBC DNA to mimic the CTC environment. We were able to detect GSTP1 from LNCaP cells even in a background of unmethylated WBC DNA (Figure 2C). This result is similar to the results from Figure 2B in the 2018 Annual Report, but we are were able to detect the spike-in DNA from 0.005ng in this experiment and were unable to previously. However, the detection in 0.005ng is at a similar level as 0.05ng, and not linear. We think it is possible the addition of the WBC DNA may act as carrier DNA for the methylated GSTP1 in the LNCaP cells, improving capture from this small amount. This will need to be further tested by seeing if the addition of actual carrier DNA improves capture.

Milestone 1: A manuscript describing the optimization of this assay and its use for detecting multiple biomarkers in CTCs is currently in preparation with plans to submit in early 2020.

Specific Aim 2, Major Task 1

Subtask 1, 2, 3: We previously performed this assay on a small patient cohort and detected GSTP1 methylation in 5/7 samples (Figure 4 in 2018 Annual Report). This was performed using the previous SYBR based method and will be repeated using our new qRT-PCR method.

Specific Aim 2, Major Task 2

Subtask 1, 2, and 3: We have collected blood, captured CTCs, and extracted DNA from patients with metastatic prostate cancer. These samples are ready to be used in this assay as soon as a decision is made regarding the high background seen in the PRAC optimization data. If WBC DNA does contain real PRAC methylation, we may need to use a single cell aspirator to isolate CTCs away from WBCs in order to assay for PRAC.

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 and at the 2019 Cell Symposia: Transcriptional Regulation in Evolution, Development, and Disease in Chicago, IL.

What do you plan to do during the next reporting period to accomplish the goals? Nothing to report.

IMPACT:

What was the impact on the development of the principal disciplines of the project? This work has led to new assays that will be published 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.

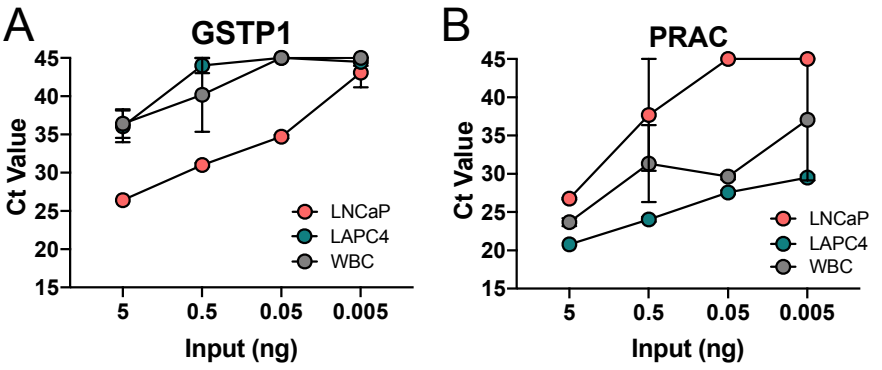

Figure 3: LNCaP, LAPC4, and WBC DNA was serially diluted and digested with AluI and HpyCH4V prior to enrichment for methylated DNA. A) qRT-PCR for GSTP1 was performed on enriched DNA. Raw Ct values are shown for each dilution. B) qRT-PCR for PRAC was performed. Raw Ct values are shown for each dilution. A Ct value of 45 (equal to total cycles run) was given for any sample that did not amplify at the end of the qR T-PCR run.

CHANGES/PROBLEMS:

Changes in approach and reasons for change. We changed the qRT-PCR chemistry for the assay from SYBR with standard DNA oligos as primers to the TaqMan system. The reason for this change is to be able to pre-amplify enriched methylated DNA so that we can detect more than one biomarker from the same cells (i.e. GSTP1 and PRAC from the same sample). Additionally, TaqMan primers contain a probe within the amplicon that improves specificity. We also optimized a version of the assay for multiple biomarkers that does not include a methylation sensitive enzyme and instead includes a second, non-methylation sensitive enzyme. The reason for this change is that it is very challenging to find a common methylated restriction site between multiple genes.

Actual or anticipated problems or delays and actions or plans to resolve them. The switch to TaqMan primers and new enzyme method resulted in needing to repeat assay optimization experiments, which has delayed the completion of Major Task 2, Subtask 4 and 5. Additionally, our discovery of apparent PRAC methylation in WBC DNA will likely result in the need to use a single cell aspirator to isolate CTCs so that WBC DNA does not confound the results. We will still use the DNA collected in Subtask 3 to assess GSTP1 methylation and other possible biomarkers as well.

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. Presented data on GSTP1 methylation that was collected during optimization of this assay at Cell Symposia: Transcriptional Regulation in Evolution, Development, and Disease.

Tamara S Rodems, Cole Gilsdorf, Duane Huang, Harshitha Gungurthi, Kris Carlson, Erika Heninger, David J. Beebe, Michael C. Haffner, Joshua M. Lang. *Epigenetic regulation of class I human leukocyte antigens (HLA I) in prostate cancer.* **Poster Presented** at Cell Symposia: Transcriptional Regulation in Evolution, Development, and Disease, October 2019.

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:

Portions of this poster presentation contained data collected for this project.

Tamara S Rodems, Cole Gilsdorf, Duane Huang, Harshitha Gungurthi, Kris Carlson, Erika Heninger, David J. Beebe, Michael C. Haffner, Joshua M. Lang. *Epigenetic regulation of class I human leukocyte antigens (HLA I) in prostate cancer.* Cell Symposia: Transcriptional Regulation in Evolution, Development, and Disease, Chicago, IL, October 2019.

Abstract: Expression of class I human leukocyte antigens (HLA-I) on the cell surface is required for T-cell recognition and the success of T-cell based cancer immunotherapies. HLA-I downregulation as a mechanism of immune evasion has been identified in more than 70% of patients with prostate cancer. However, the mechanism by which downregulation occurs remains unknown. Here we sought to determine the molecular

mechanisms involved in HLA-I transcriptional downregulation. We show that silencing of HLA-I gene expression and subsequent protein loss is regulated by epigenetic mechanisms including enrichment of repressive histone modifications near the transcription start site and DNA hypermethylation in the promoter and early exons. DNA methyltransferase (DNMT) and histone deacetylase (HDAC) inhibitors reverse these epigenetic signatures and induce HLA-I expression, suggesting epigenetic therapies may increase patient benefit from immunotherapies that rely on HLA-I expression. We also explore novel liquid biopsy biomarkers that may serve to monitor response to epigenetic therapies.

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).