

Award Number: W81XWH-12-1-0323

TITLE: Advanced Lung Cancer Screening: An Individualized Molecular
Nanotechnology Approach

PRINCIPAL INVESTIGATOR: James Herman, Professor

CONTRACTING ORGANIZATION: Johns Hopkins University, The
Baltimore, MD 21287.

REPORT DATE: August 2013

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE August 2013		2. REPORT TYPE Annual		3. DATES COVERED 1 Aug 2012 – 31 July 2013	
4. TITLE AND SUBTITLE Advanced Lung Cancer Screening: An Individualized Molecular Nanotechnology Approach				5a. CONTRACT NUMBER W81XWH-12-1-0323	
				5b. GRANT NUMBER W81XWH-12-1-0323	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) James Herman E-Mail: hermaj@jhmi.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Johns Hopkins University, The Baltimore, Maryland 21218-2680				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel 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 This grant utilizes complimentary approaches to improve the early detection of lung cancer. Our goal is to examine whether detection of DNA methylation and enhanced CT evaluations will improve the specificity of lung cancer detection. In the first year of this proposal, we have developed an improved panel of genes hypermethylated in lung cancer, with extraordinarily high specificity and sensitivity. Initial panels demonstrate 100% specificity and 95-99% sensitivity in lung cancer in the United States. These novel genes have been used to develop sensitive methylation specific PCR assays suitable for biologic fluid testing (sputum and serum). We have optimized the processing of biologic samples to accomplish improved retention of DNA suitable for methylation detection, and have combined this improved method with newly developed PCR detection. This optimized processing has increased extraction efficiency by up to 5 fold, and analytical sensitivity improvements are over 25-fold. Evaluation of primary sputum and serum from patients with lung cancer and screen controls is ongoing. In combination with this molecular detection approach, we have examined alterations in air space for improving detection of lung cancer, with an evaluation of emphysema score in patients with lung cancer and non-cancer patients receiving CT evaluation.					
15. SUBJECT TERMS					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 8	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4-8
Key Research Accomplishments.....	8
Reportable Outcomes.....	8
Conclusion.....	8
References.....	8
Appendices.....	8

Introduction

This grant utilizes complimentary approaches to improve the early detection of lung cancer, with each aim having independent goals and thus separate utility. Our goal is to explore whether detection of DNA methylation changes and enhanced CT evaluations will add to the specificity of lung cancer detection. This is defined in our aims.

Specific Aim 1: To improve the clinical utility and effectiveness of a nested, gel based DNA methylation assay for sputum and plasma by increasing its sensitivity and specificity through nanotechnology. Hypothesis: Detection of DNA methylation from individuals with cancer can be used to determine lung cancer risk and can be enhanced through discovery of optimal hypermethylated genes and implementation of enhanced detection technologies.

Specific Aim 2: To use an in vitro molecular testing of sputum and serum with DNA methylation rather than simple demographics alone to select the highest risk smokers for an expensive screening modality such as CT scanning. Hypothesis: DNA methylation testing is more specific in selecting those at the highest risk for lung cancer than clinical demographics alone.

Specific Aim 3: To optimize low dose chest CT screening for lung cancer. Hypothesis: Valuable information on the chest CT scan, based on the severity, distribution, and pattern of low attenuation areas (“emphysema”), may be crucial to increasing our insights and effectiveness of determining lung cancer risk, the frequency of follow up scans, reducing false positives, and controlling costs compared to an annual chest CT screening for the sole use to detect lung cancer tumors after they occur.

Body

For specific aim 1, we have made significant progress on the two sub-aims of this proposal. They are specifically, to A) Develop optimal hypermethylated gene panels for detection of tumor DNA from lung cancer and B) Optimize nanotechnology based detection of DNA methylation for increased sensitivity and specificity.

The first efforts have been focused on the development of an optimal gene panel for detection of lung cancer, and the results of this effort are now in a manuscript under review, which will be summarized here. This approach has generated an extremely useful panel of genes with near universal methylation in a large cohort of lung cancers.

Non-Small Cell Lung Cancer (NSCLC) is the leading cause of cancer mortality in the world. Diagnostic biomarkers may augment both existing NSCLC screening methods as well as molecular diagnostic tests of surgical specimens to stratify and stage more accurately candidates for adjuvant chemotherapy. Hypermethylation of CpG islands is a common and important alteration in the transition from normal to transformed cells. Following previously validated methods for the discovery of cancer-specific hypermethylation changes from NSCLC cell lines, we identified >300 candidate genes. Using the Cancer Genome Atlas (TCGA) and employing extensive filtering to refine our candidate genes for the greatest ability to distinguish tumor from normal, we define a three-gene panel, CDO1, HOXA9, and TAC1, which we subsequently validate in two independent cohorts of primary NSCLC samples. This 3-gene panel is 100% specific, showing no methylation in 75 TCGA and 7 primary samples and is 83-99% sensitive for NSCLC depending on the cohort (Figure 1). The sensitivity for within the TCGA tumors is 99%, while in a validation cohort from the Johns Hopkins Hospital, the sensitivity is 95%. Slightly lower sensitivity is found in Asian

populations (a validation cohort from Japan has sensitivity of 83%), highlighting differences between lung cancer in the US and Asia. However, further utility of this panel will be explored in US populations. This degree of sensitivity and specificity would be of high value to diagnose the earliest stages of NSCLC. Furthermore, our experimental method revealed genes highly enriched for polycomb-associated, chromatin-bivalent genes in stem and progenitor cells that form clusters within NSCLC, genes which may also predict clinical outcomes in resected patients. The addition of this 3-gene panel to other previously validated methylation biomarkers holds great promise in both early diagnosis and prognosis of NSCLC.

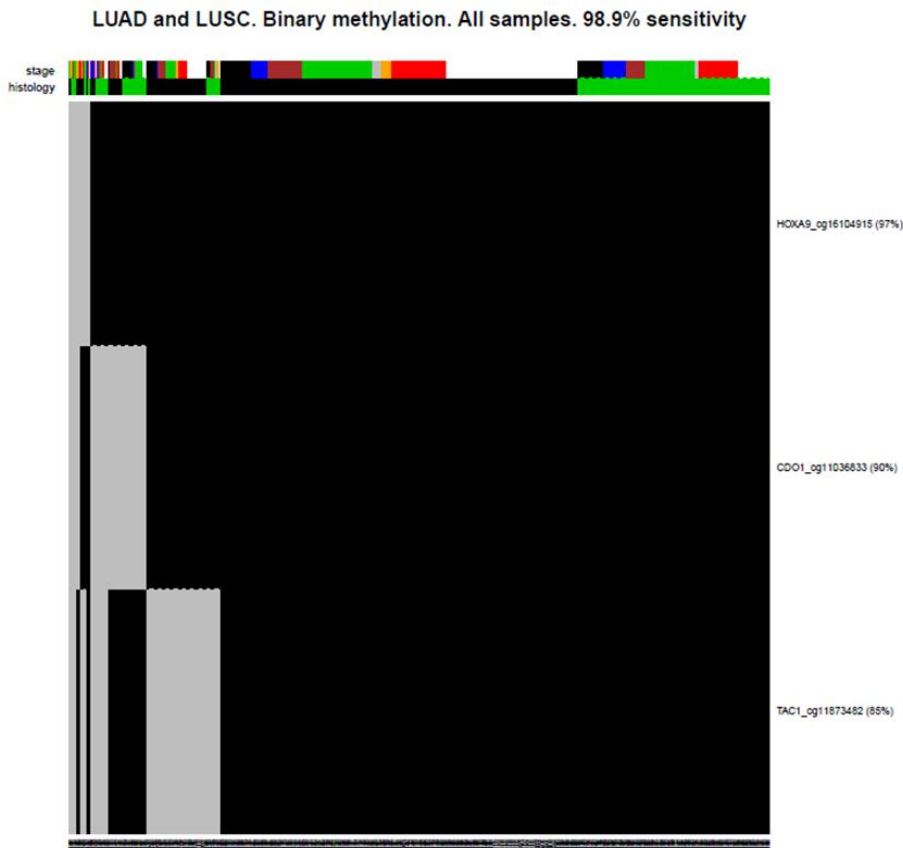


Figure 1. Methylation of *CDO1*, *HOXA9*, and *TAC1* is Highly Sensitive for NSCLC in the Cancer Genome Atlas. A single Infinium methylation probe with the best discriminative capacity between tumor and normal samples was selected for each of the 3 genes. A sample is considered methylated for a gene if its β -value was larger than three times the standard deviation of the mean of β -values of normal samples. Methylation of at least one gene-promoter among *CDO1*, *HOXA9*, and *TAC1* by Infinium array identifies 98.9% of NSCLC cases in 636 cases in The Cancer Genome Atlas.

This panel has been further expanded through the identification of additional genes with extremely high methylation frequencies in lung cancer. This panel now includes three additional genes, *HOXA7*, *SOX17* and *ZFP42*, which will complement the previous 3 gene panel to provide redundant tumor coverage to optimize detection. We developed primer and hybridization (TAQMAN detection) probes for all 6 genes previously identified using UCSC genome browser and primer 3. We have validated these new assays using normal lymphocytes and in vitro methylated bisulfite converted DNA, as well as no template controls and unmethylated DNA. We found high specificity to methylation in bisulfite converted DNA and no amplification in unmethylated and no template controls. We measured the amplification efficiency for all of these genes and found it to be 100 $\pm$ 20%, for 5 of the 6 genes, with assay optimization continuing for *ZFP42*. Validation in clinical samples: We have begun testing gene methylation in normal and cancer patients' sputum, as well as normal lung tissue and lung tumors. High levels of DNA methylation were found in tumor tissue, while no methylation has been found in normal lung tissues for all of these genes. Examination of sputum and serum is proceeding.

Subaim 2: The use of methylated tumor-specific circulating DNA has shown great promise as a potential cancer biomarker. Nonetheless, the relative scarcity of tumor-specific circulating DNA presents a challenge for traditional DNA extraction and processing techniques. Here we demonstrate a single tube extraction and processing technique dubbed “methylation on beads” that allows for DNA extraction and bisulfite conversion for up to 2 ml of plasma or serum. In comparison to traditional techniques such as phenol chloroform and alcohol extraction, methylation on beads yields a 1.5 to 5-fold improvement in extraction efficiency. The greatest enhancement in extraction efficiency is seen with small amounts of DNA, precisely matching the need for improved extraction in low DNA content samples such as plasma and serum. We also find analytical sensitivity improvements over 25-fold, thereby clearing the way for the detection of rare epigenetic events and the development of high sensitivity epigenetic diagnostic assays. This progress is demonstrated in the following figures (2-6)

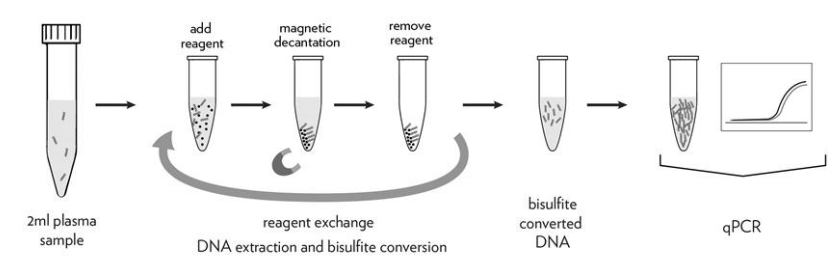


Figure 2 Overview of the Methylation-on-Beads (MOB) Process. Circulating DNA from up to 2 ml of plasma is extracted and purified via SSBs. The purified DNA is then subject to bisulfite conversion and analyzed via methylation specific PCR (MSP). The entire sample preparation

process can be performed in a single tube and consists of an iterative process of adding reagents, magnetic decantation, and removal of supernatant.

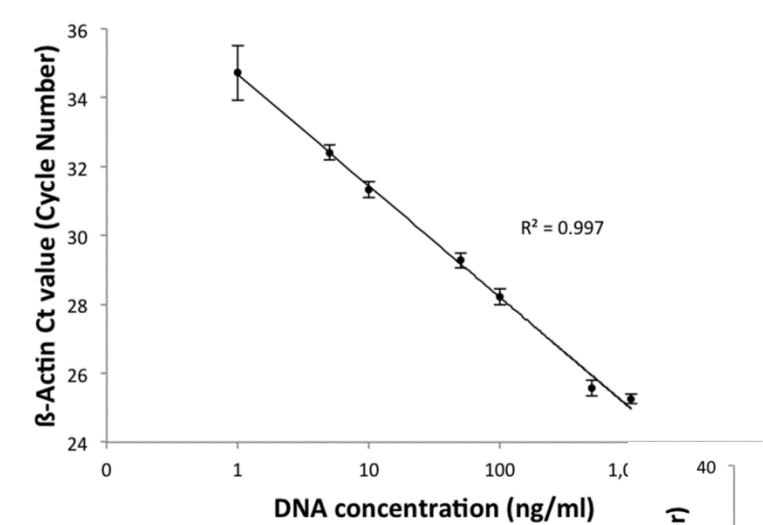
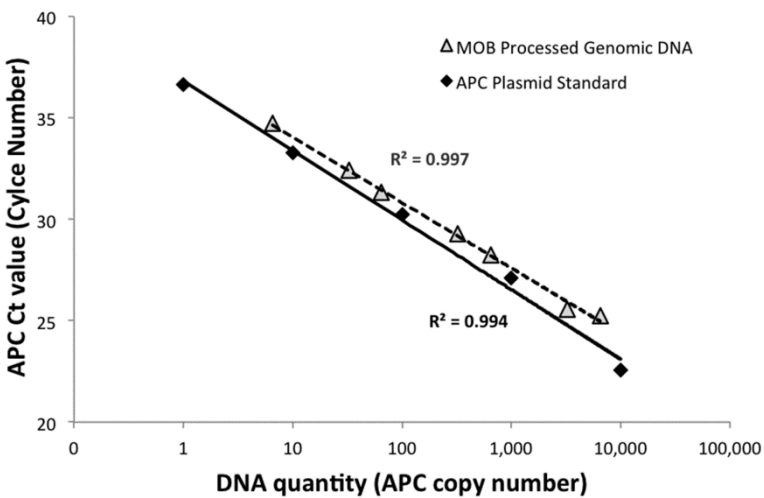


Figure 4 APC Gene Ct Values vs. Gene Copy Number for MOB-processed DNA. Ct values of MOB-processed genomic DNA were compared with known copy numbers of APC plasmid DNA. The Ct values show excellent rate of recovery and correlation with the plasmid DNA standard.

Figure 3 β-Actin Cycle Threshold (Ct) values of MOB processed DNA versus initial DNA concentration. The Ct value shows an inverse correlation with respect to starting DNA concentrations, thus demonstrating the linearity of the MOB process, from sample preparation to methylation specific PCR, of over 4 orders of magnitude.



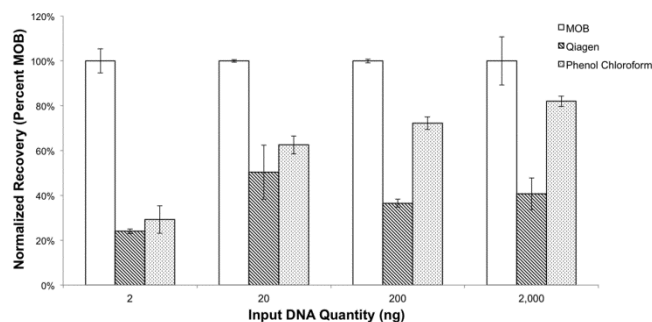


Figure 6 β -Actin Ct values for MOB processed vs. Phenol Chloroform extracted and traditionally processed plasma samples from 24 patients diagnosed with lung cancer. The MOB technique demonstrates consistently higher and less variable recovery, as demonstrated by the lower average Ct value (33.8 vs. 40.6 cycles) and Ct standard deviation (0.3 vs. 1.9 cycles), respectively.

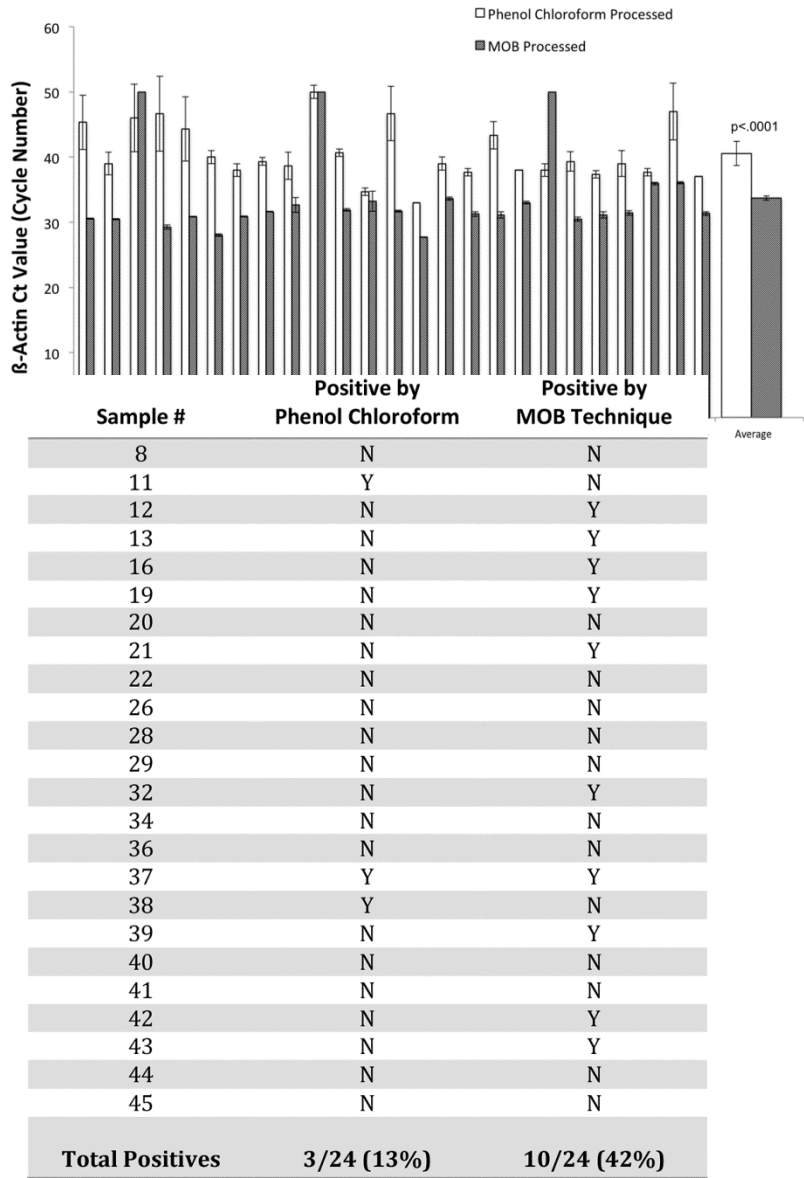
Table 1 Detection of methylation of the RASSF1A tumor suppressor gene in circulating DNA; MOB vs. Traditional Phenol Chloroform and Alcohol Extracted and processed plasma samples from 24 patients diagnosed with lung cancer. The MOB technique picked up 7 more methylation positive samples corresponding to over 3-fold higher clinical sensitivity than traditional phenol chloroform methods.

Current/ongoing work:

Subsequent to these studies, we have optimized MOB (Methylation on beads) for sputum samples. We have accounted for the high amounts of DNA in sputum, compared to serum, and mucin by using a high ratio of beads (to capture all available DNA) and increasing the concentration of proteinase K (to break down the mucin). With these improvements to both the processing of samples for DNA extraction and bisulfite conversion, as well as the development of a highly sensitive panel of methylation markers, we have made significant progress in completing aim 1 goals. The implementation of these assays now for proposed studies in aim 2 is ongoing.

For specific aim 3, to date we were able to identify 155 subjects in the SPORE data base that had CT scans performed prior to surgery which were adequate for analysis. We have completed measurement of the extent of emphysema in these subjects. Of the group, 130 of the subjects had cancer, and 25 did not. Also, 115 of the subject were current or former smokers with an average of 44 pk/yr history, and 38 of the cancer positive and 4

Figure 5 Normalized DNA recovery, as quantified by β -Actin qPCR, of MOB compared with traditional phenol chloroform and alcohol extraction and Qiagen Extraction Kit. The MOB technique exhibits superior recovery rates at all DNA concentrations tested.



of the cancer negative were classified as having COPD by spirometry. The emphysema score was based on the number of voxels with Hounsfield number less than -910. The percent emphysema of the lungs ranged from 0.1 to 56% among all the subjects. The mean emphysema score for the cancer positive subjects was 28%, and for the cancer negative subjects was 31%. The number of non-cancer patients was insufficient in for this comparison, so that we are now analyzing additional non-cancer controls. For this comparison, to increase the number of controls, we are currently analyzing 106 scans for another data base (SCCOR) all without cancer. These scans have been obtained and are currently being evaluated for emphysema score.

Key Research Accomplishment

- Development of Improved Panel of Genes with Cancer Specific Methylation, having 100% specificity and 95-99% sensitivity in lung cancer in the United States
- Optimized processing of biologic samples for methylation analysis, increasing extraction efficiency by up to 5 fold, and analytical sensitivity improvements over 25-fold.
- Initiating studies of emphysema score among cancer and control patients.

Reportable Outcomes

None

Conclusion

In summary, we have developed an improved panel of genes hypermethylated in lung cancer, with extraordinarily high specificity and sensitivity. These novel genes have been used to develop sensitivity methylation specific PCR assays suitable for biologic fluid testing (sputum and serum). We have further optimized the processing of biologic samples to accomplish improved retention of DNA suitable for methylation detection, and have now combined this improved method with the newly developed PCR detection. Evaluation of primary sputum and serum from patients with lung cancer and screen controls has now started. In combination with these molecular detection approaches, we have examined the alterations in air space for improving detection of lung cancer. Further work will increase the number of non-cancer CT scans analyzed to increase the power to detect differences between cancer and controls.

References

No papers have yet been submitted from the work done directly with support of this award.

Appendices

None