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TITLE: Blood Based Biomarkers for Lung Cancer Early Detection and Evaluation of CT Based Lesions

PRINCIPAL INVESTIGATOR: Stephen Lam, M.D.

CONTRACTING ORGANIZATION: British Columbia Cancer Agency
Vancouver, BC Canada V5Z1L

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Fort Detrick, Maryland 21702-5012

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15. SUBJECT TERMS
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INTRODUCTION:

The main objective of this multi-investigator, multi-site project is to evaluate, through blinded validation studies, candidate markers from genomic (mutation analysis, DNA methylation and microRNAs), proteomic (circulating proteins and autoantibodies) and metabolomic (altered glycans, metabolites and volatile organic compounds) studies that show promise for yielding blood based tests for lung cancer. Two specific blood biomarker application goals are addressed: (1) Discrimination of benign and malignant lung nodules between 5 mm to 30 mm in size identified by thoracic CT scans and (2) Detection of non-small cell lung cancer in high risk individuals up to 24 months prior to clinical diagnosis.

Led by Dr. Wan Lam and Dr. Stephen Lam, the specific tasks for the project at the BCCA are: (a) generate microRNA (miRNA), methylation and gene expression profiles of tumor and matched non-malignant tissues to identify differentially expressed miRNAs and differentially methylated genes for further testing and validation in archival and prospectively collected blood samples; (b) Collect blood samples, clinical data and final diagnosis from 100 individuals with lung nodules ≤3 cm being evaluated with PET/CT. Within the 100 subjects evaluated for lung cancer with PET/CT imaging prior to consideration for surgical resection, paired lung tumor and adjacent non-tumor lung tissue will be collected from a subset of 40 patients.

Collection of all tissue and blood samples, as well as genomic profiling of all 90 tumor and matched non-tumor tissues (methylation, gene expression and miRNA expression) has been completed. Integration of genomic dimensions to identify over-expressed genes that may be useful protein targets is currently underway, and we have already validated one of the blood biomarkers discovered by Dr. Hanash using blood samples from the Pan-Canadian Early Lung Cancer Detection Study. Candidate miRNA that accurately discriminate between tumor and non-malignant samples are currently being validated in the 100 blood samples to determine their concordance with tumor levels and effectiveness in discriminating benign from malignant lung nodules.

In Year 3, we will continue our integrative efforts and validation studies, while also determining if any of our biomarkers are capable of distinguishing between clinical features such as stage TIN0M0 vs T1N+/M+ and PET status. The clinical application of promising biomarkers that meet statistical criteria will also be assessed to determine if any of the identified biomarkers may be suitable for use in a blood test to screen for lung cancer.

BODY:

This section describes the research accomplishments associated with each BC Cancer Agency related task outlined in the approved Statement of Work.

Statement of Work for Year 2 (BC Cancer Agency component)

The tasks and timeline for the Vancouver site for Year Two are as follows:

1-Integration of miRNA, mutation status, methylation and gene expression as well as published data to deduce over-expressed genes to identify potential protein targets for investigation in blood by Dr. Hanash (Project #1).13-15 m

2- Select the best over-expressed miRNA for assessment using aliquots of specimens assigned for pre-validation studies for clinical applications 1 and 2. 13-24 m.

Work accomplished to date:

Overdue Work from Year One:

Due to a delay in IRB approval, most tasks from year one were not completed at the time of the first progress report. All tasks from year one have now been completed or are near completion, and the work accomplished on outstanding tasks from year one are summarized below.

Task 3 - *Collect an additional (40) paired lung cancer/blood specimens, PET imaging and other clinical data from patients undergoing resection (sub-set of patients in Task 4)*
Status: Completed
Work accomplished: At the end of year one only 21 tumor specimens had been collected due to the small size of some of the tumors and a lack of Tumor Tissue Biorepositories at other hospitals. Since then, the remaining 19 tumor and blood specimens have been collected from patients undergoing surgical resection, and biomarker validation is being performed using these samples.

Task 4 - *Collect additional (~100) blood samples and final diagnosis from patients undergoing PET/CT Imaging evaluation for solitary pulmonary nodules.*
Status: Completed.
Work accomplished: Blood samples and clinical data have been obtained from 70 subjects with lung nodules identified by CT screening. These nodules have been identified as benign by surgical resection or by stability of the lung nodule for a minimum of 2 years. The remaining blood samples were part of task 3 (40 paired lung cancer/blood) and have also been collected. In total, we have obtained clinical data and blood samples from 110 subjects. Additional blood samples from subjects with benign lung nodules were collected because they will serve as important controls for comparison with the cancer patients.

Task 5 - *Histological confirmation and micro-dissection of tumor samples and generate miRNA, methylation and gene expression profiles of tumor and normal tissues.*
Status: Completed
Work accomplished: Histological confirmation was performed on all samples, and specimens were manually micro-dissected for DNA and RNA extraction. Methylation, gene and miRNA expression profiling has been completed for all 60 samples(tumor and matched non-malignant tissue) in task 2 (30 locally invasive and 30 metastatic NSCLC), as have the miRNA expression arrays for the 30 screen detected lung cancer samples (Task 2).

Task 6 - *Assay over-expressed miRNA in blood from patients of the 100 tumor specimens and determine concordance with tumor tissue.*
Status: In progress. Expected to be completed in early 2013
Work accomplished: Due to the extended process of seeking in IRB approval, the time frame for Task 5 was shifted by over 5 months. With miRNA profiling of all 90 tumor samples completed, miRNAs overexpressed in tumor tissue relative to matched non-malignant tissue have been identified, validation in blood samples to determine the concordance of miRNA levels in blood is currently underway.

Task 7 - *Assay miRNA in blood from 50 patients with benign CT detected lung nodules and compare the levels with patients with lung cancer.*
Status: In progress. Expected to be completed in early 2013
Work accomplished: As PCR validation of overexpressed miRNA is completed (Task 6), validated miRNAs are being assessed in blood from patients with benign lung nodules detected by CT scans to determine if these miRNA are capable of distinguishing benign from malignant lung lesions. All samples for this validation set have been collected, RNA extracted and quality assessed and are thus ready for validation. Quantitative PCR assays for detecting microRNA levels in blood samples has been calibrated using miR-16.

Task 8 - *Select hypermethylated genes for further investigation in blood by Dr. Gazdar.*
Status: In progress but near completion
Work accomplished: Methylation profiling of tumors has identified a number of hypermethylated genes. A short list of candidate genes is being analyzed in conjunction with Dr. Gazdar at UTSW.

Year 2 Tasks:

Task 1- *Integration of miRNA, mutation status, methylation and gene expression as well as published data to deduce over-expressed genes to identify potential protein targets for investigation in blood by Dr. Hanash*
Status: In progress
Work accomplished: We performed a preliminary validation study on one of the blood biomarkers (Surfactant Protein B) identified by Dr. Hanash using an ELISA in 80 patients with cancer and 120 matched controls. Blood samples were made available by the Pan-Canadian Early Lung Cancer Detection Study at no cost to the project. A manuscript detailing the results of Dr. Hanash and our work is currently in progress. Integration of the multiple genomic dimensions has identified a number of candidate oncogenes and tumor suppressors with DNA level alterations and corresponding changes in gene expression. These include *EYA4*, *SIRPA, YEATS4,* and *ELF3* to name a few. As we continue to refine out list of candidates from integration, potential protein targets will be sent to Dr. Hanash for investigation in blood.

Task 2- *Select the best over-expressed miRNA for assessment using aliquots of specimens assigned for pre-validation studies for clinical applications 1 and 2.*
Status: Pending validation in blood samples from Year One Tasks 6 and 7
Work accomplished: Following the completion of technical validation outlined in year one, successfully validated miRNAs will be assessed in pre-validation specimens to determine their clinical application. Several miRNAs overexpressed in over 90% of tumor samples relative to matched-non malignant tissue, that are also detected in blood have been identified, suggesting they may be promising blood based biomarkers for the detection of lung cancer. Validation in this additional cohort will help identify the most robust and clinically relevant candidates.

Beginning of Year 3 Tasks:
As validation is completed and promising markers are identified, we will move into the Year three tasks of querying clinical information to determine if in addition to detecting lung cancer and discriminating benign from malignant lesions, any of our markers are predictive of stage (invasive vs. metastatic) and PET status.

KEY RESEARCH ACCOMPLISHMENTS:

Milestones accomplished in year 2:

- Completed all genomic profiling of tumors and matched non-malignant tissues; methylation, miRNA and gene expression profiling for all 30 locally invasive adenocarcinoma (TIN0M0) and 30 metastatic adenocarcinoma (TIN+MI) and miRNA expression profiling of the 30 screen detected Stage 1 lung cancers.

- Identified 15 microRNAs overexpressed in at least 90% of tumors for testing in blood samples as potential biomarkers

- Completed collection of the 40 paired lung cancer/ blood specimens in patients undergoing surgical resection, thus completing collection of the 100 blood samples (40 cancer, 60 benign solitary nodules) for validation. [The definition of specimen categories has been approved by USA MEDCOM USAMRMC. Please refer to e-mail correspondence with Ms Caryn L Duchesneau attached as **APPENDIX 1** (Caryn.Duchesneau@us.army.mil; subject: A-16471, Continuing Review Acceptance and Amendments Approval Memorandum (Proposal Log Number LC090634P2, Award Number W81XWH-10-1-0634) (UNCLASSIFIED); February-15-2013 7:16 AM)]

- Identified genes frequently hypermethylated in tumors vs. matched non-malignant tissue and are in the process of sending our results to Dr. Gazdar for validation

- Commenced validation of overexpressed miRNAs in blood to determine concordance with tumor results and identify those miRNA capable of discerning benign from malignant nodules

- Integration of multiple genomic levels (mutation, methylation and gene expression) has identified candidate oncogenes (*YEATS4, ELF3*) and tumor suppressors (*SIRPA, EYA4*) that are currently being validated in our lab.

REPORTABLE OUTCOMES:

2 manuscript have been published in peer-reviewed journals in year 2, and a number of other manuscripts are currently in preparation, and will be submitted early in the new year.

Published Manuscripts:
Enfield KS, Pikor LA, Martinez VD, Lam WL (2012). Mechanistic roles of noncoding RNAs in lung cancer biology and their clinical Implications. Genetics Research International 2012:737416 [PMID 22852089]

Yao Y, Fan Y, Wu J, Wan H, Wang J, Lam S, Lam WL, Girard L, Gazdar AF, Wu Z, Zhou Q (2012) Potential application of non-small cell lung cancer-associated autoantibodies to early cancer diagnosis. Biochemical Biophysics Research Communication 2012:423(3):613-9. [PMID 22713465]

***Manuscripts in Preparation*:**
Wilson I, Vucic EA, Enfield KSS, Zhang YA, Chari R, Thu KL, Lockwood WW, Radulovich N, Starczynowski D, Banath JP, Zhang M, Pusic A, Fuller M, Lonergan KM, Yee J, English JC, Buys TPH, Selamat SA, Laird-Offringa IA, Liu P, Anderson M, You M, Tsao MS, Brown CJ, Bennewith KL, MacAulay CE, Karson A, Gazdar AF, Lam S, Lam WL (2012) EYA4 is

inactivated biallelically at a high frequency in sporadic lung cancer and is associated with familial lung cancer risk. Submitted to *Oncogene*.

Pikor LA, Lockwood WW, Thu KL, Vucic EA, Lam S, Gazdar AF, Lam WL (2013). Integrative analysis identifies YEATS4 as a novel candidate oncogene in NSCLC that regulates the p53 pathway. In preparation

Vucic EA, Thu KL, Pikor LA, Ramnarine VR, Enfield KS, MacAulay CE, Lam S, Lam WL (2013). Conrtibution of smoking to miRNA deregulation in lung tumors, In preparation

Pikor LA, Thu KL, Vucic EA, Lam S, Lam WL (2013). miRNA sequencing identifies miRNAs that differentiate histology and distinguish tumor from non-malignant tissue in NSCLC (2013). In preparation

CONCLUSION:

Despite an approximate 6 month setback in year one due to delayed IRB approval, we have completed procurement and profiling of blood and tissue samples and are currently in the process of analyzing the data to identify candidate biomarkers, putting us back on track. Candidate miRNAs for early detection and discrimination of benign versus malignant lung nodules are being validated in blood samples, while data integration is well underway and has produced a list of candidate genes (overexpressed and or hypermethylated) for further analysis with partner groups. Although at a somewhat slower pace due to the extended process of obtaining ethics, all milestones are being met, and as validation continues we can proceed with the tasks outlined in year three. In year 3 we will complete the validation and integration tasks and interrogate the clinical data of our samples to determine the effectiveness of our markers at discriminating invasiveness and PET status and compare the test performance with other biomarkers.

PROPOSED CHANGES IN FUTURE WORK:

None proposed. The plan for Year 3 will be implemented as originally proposed and will focus on the following tasks:

1-For markers that show promise in distinguishing between cancer vs. non cancer CT lesions, pre-validate
whether the markers distinguish between TINOMO versus TIN+/MI lung cancer.

2-Test for discrimination between PET positive versus PET negative Stage IA lung cancers as well as PET positive benign lung nodules detected by screening spiral CT.

3- Test validation specimens for clinical applications I and 2 if the markers meet the statistical criteria
compared to other biomarkers in the pre-validation study.

REFERENCES:

None

APPENDIX 1

-----Original Message-----
From: Duchesneau, Caryn L Ms CIV USA MEDCOM USAMRMC [mailto:Caryn.Duchesneau@us.army.mil]
Sent: February-15-13 7:16 AM
To: Lam, Stephen
Cc: Drake, Carrie E Ms CTR US USA MEDCOM USAMRMC; Bennett, Jodi H Ms CIV USA MEDCOM USAMRMC; Katopol, Kristen R Ms CTR US USA MEDCOM USAMRMC; Lowery, Cheryl A Ms CIV USA MEDCOM USAMRAA; Wan Lam; Heather Saprunoff; Brosch, Laura R Dr CIV USA MEDCOM USAMRMC; Duchesneau, Caryn L Ms CIV USA MEDCOM USAMRMC; Shank, Patricia A CTR US USA MEDCOM USAMRMC; Drayton, Maria Ms CTR US USA MEDCOM USAMRMC; Rowe, Sheilah S CTR US USA MEDCOM CDMRP
Subject: A-16471, Continuing Review Acceptance and Amendments Approval Memorandum (Proposal Log Number LC090634P2, Award Number W81XWH-10-1-0634) (UNCLASSIFIED)

Classification: UNCLASSIFIED
Caveats: NONE

SUBJECT: Continuing Review Acceptance and Amendments Approval for the Protocol, "Blood Based Biomarkers for Lung Cancer Early Detection and Evaluation of PET/CT Lung Lesions," Submitted by Stephen C. Lam, MD, British Columbia Cancer Agency, Vancouver, British Columbia, in Support of the Proposal, "Blood-Based Biomarkers for Lung Cancer Early Detection and Evaluation of CT-Based Lesions," Research Ethics Board Study Number H10-01695, Proposal Log Number LC090634P2, Award Number W81XWH-10-1-0634, HRPO Log Number A-16471

1. The US Army Medical Research and Materiel Command (USAMRMC), Office of Research Protections (ORP), Human Research Protection Office (HRPO) approved the subject protocol on 4 February 2011.

2. The HRPO received a continuing review report for the subject protocol on 25 October 2012. Additional information regarding the continuing review was received on 9 November 2012. The University of British Columbia - British Columbia Cancer Agency Research Ethics Board (UBC BCCA REB) approved continuation of the protocol on 23 October 2012; this approval will expire on 23 October 2013.

3. The submitted continuing review report and supporting documentation have been reviewed by the HRPO and found to be in compliance with Federal, DOD, and US Army human subjects protection requirements. The report and supporting documents are accepted.

4. This study is currently approved for collection of data, blood, and tissue samples from 190 subjects (total includes both prospective and archival samples collected from previously approved BCCA REB studies H06-60001 and H08-01132). As of the date of the continuing review report submission, the total number of samples collected was 190.

5. Two amendments to this no greater than minimal risk protocol were received by the HRPO on 29 November 2012 and 18 December 2012. The amendments were approved

by the UBC BCCA REB on 23 November 2012 and 6 December 2012. The amendments allowed the following changes to clarify and adjust the accrual numbers to meet study objectives.

a. Corrected overall accrual goal from 1590 to 1540 and corrected local accrual from 280 to 230 (approved 23 November 2012).

b. Decrease in overall accrual from 1540 to 1500 and decrease in local accrual from 230 to 190 (approved 6 December 2012).

6. The changes proposed in the amendments have been reviewed by the HRPO and found to be acceptable. The protocol amendments are approved.

7. Please note the following reporting obligations.

a. Major modifications to the research protocol and any modifications that could potentially increase risk to subjects must be submitted to USAMRMC ORP HRPO for approval prior to implementation. All other amendments must be submitted with the continuing review report to the HRPO for acceptance.

b. All unanticipated problems involving risks to subjects or others, serious adverse events related to study participation, and deaths related to study participation must be reported promptly to the HRPO.

c. Any deviation to the subject protocol that affects the safety or rights of the subject and/or integrity of the study data must be reported promptly to the HRPO.

d. A copy of the continuing review report approved by the UBC BCCA REB must be submitted to the HRPO as soon as possible after receipt of approval. It appears the next continuing review by the UBC BCCA REB is due no later than 23 October 2013.

e. In addition, the current version of the protocol and consent form (if applicable) must be submitted along with the continuing review report and the UBC BCCA REB approval notice for continuation of the protocol.

f. The final study report submitted to the UBC BCCA REB, including a copy of any acknowledgement documentation and any supporting documents, must be submitted to the HRPO as soon as all documents become available.

8. Do not construe this correspondence as approval for any contract funding. Only the Contracting Officer/Grants Officer can authorize expenditure of funds. It is recommended that you contact the appropriate contract specialist or contracting officer regarding the expenditure of funds for your project.

9. The HRPO point of contact for this study is Patricia A. Shank, BSN, RN, CCRP, PMP, Human Subjects Protection Scientist, at 301-619-2282(email: patricia.a.shank.ctr@us.army.mil<mailto:patricia.a.shank.ctr@us.army.mil>.

CARYN L. DUCHESNEAU, CIP
Chief, Human Subjects Protection Review
Human Research Protection Office
Office of Research Protections
US Army Medical Research and Materiel Command

Note: The official copy of this memo is housed with the protocol file at the Office of Research Protections, Human Research Protection Office, 504 Scott Street, Fort Detrick, MD 21702. Signed copies will be provided upon request.

Classification: UNCLASSIFIED
Caveats: NONE