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PRINCIPAL INVESTIGATOR: Nanette H. Bishopric, MD

CONTRACTING ORGANIZATION: University of Miami
Miami, FL 33136

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Nanette H. Bishopric, M.D.

E-Mail: n.bishopric@miami.edu

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14. ABSTRACT
The purpose of this grant is to identify, isolate and characterize high affinity aptamers that distinguish between prostate cancers that are likely to remain organ-confined and those with potential to metastasize, The scope of this pilot is to generate DNA aptamers that selectively react with a prostate cancer cell line that remains confined to the prostate (LNCaP) vs. a subpopulation of this cell line that has acquired the ability to metastasize aggressively, employing Cell-Selex and Aptamer-Facilitated Biomarker Discovery (AptaBiD) technology. Major Findings and Progress: (1) Non-metastatic LNCaP-Pro-5 cells and metastatic LNCaP-LN3 cells have been provided by Curtis Pettaway, M.D. Anderson Cancer Center, Houston, Texas. (2) Parental LNCaP cells have been obtained from American Type Culture Collection. (3) Phenotypic validation (aggressive vs. indolent growth) has been carried out on all cell lines using the Boyden Chamber assay. (4) DNA 40 bp aptamer libraries have been generated. (5) 12 rounds of Cell-Selex have been initiated. (6) Plan for next-generation sequencing and bioinformatic analysis pathway has been established to yield desired phenotype-specific DNA aptamers. Next steps will include NexGen sequencing and testing of DNA aptamer families on human clinical prostate cancer specimens.

15. SUBJECT TERMS
Prostate cancer, Cell-selex, metastasis, prognosis, biomarker, biopsy, screening, prediction, tissue microarray

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Note: A version of this progress report was recently submitted to cover the period from March 2012-2013. No additional work was performed from March 2013-2014 because the award was on hold waiting for approval of an extension request.

1. Introduction:

Prostate cancer (PC) is the most common non-skin cancer diagnosed in American men, with about 1 out of every 7 men receiving this diagnosis during his lifetime. The American Cancer Society estimates that in 2014, about 233,000 new cases of prostate cancer will be diagnosed, and about 29,480 men will die of prostate cancer. For many men, prostate cancer is an incidental diagnosis that will never require treatment or affect length or quality of life, while unfortunately, for other men, prostate cancer is a catastrophic, aggressive disease that can metastasize to other tissues of the body well before it becomes symptomatic. Thus, early and accurate detection of PC is essential to the treatment and cure of this disease, and to avoiding both under- and over-treatment. There is a critical need to search for novel tumor-specific markers that may enable more accurate detection of PC, predict tumor aggressiveness and serve as molecular targets for imaging and effective therapies. Our research objective is to utilize novel technologies to identify, isolate and characterize high affinity nucleic acid oligomers (aptamers) that distinguish between prostate cancers that are likely to remain organ-confined and those with potential to metastasize. We are employing Cell-Selex [1-4] and Aptamer-Facilitated Biomarker Discovery (AptaBiD)[5] technologies to generate RNA aptamers that are selected to discriminate between PC cell lines that are either indolent or aggressive in their growth properties. In subsequent stages, these aptamers will be used A) to identify potential new biomarkers for indolent versus progressive prostate cancer, using affinity labeling, and B) for potential imaging applications including flow cytometry and fluorescence microscopy, in human prostate cancer microarrays.

2. Keywords: Prostate cancer, RNA aptamer, polymerase chain reaction, next-generation sequencing, biomarker, metastasis, risk stratification

A. Rationale:
It is critical to be able to identify the subset of prostate cancers that are likely to exhibit aggressive, metastasis-prone behavior. The highly metastasis-prone LNCaP-LN-3 prostate cancer cell was subcloned from a parental cell line (LNCaP) that almost never metastasizes[6, 7]. Thus these constitute a pair of cell lines that are genetically similar (derived from the same individual) but differ in metastatic potential. By identifying differences between these cell lines, we will be able to identify markers of aggressive behavior in prostate cancer.

B. Progress on Scope of Work

Task 1: Propagate non-metastatic (LNCaP-Pro-5) and metastatic (LNCaP-LN3) prostate cancer cell lines as well as control cell lines to initiate the DNA aptamer screening process. Status: Completed.
Task 1a. We have obtained LNCaP-Pro-5, LNCaP-LN3 and parental LNCaP cell lines from Dr. Pettaway of MD Anderson Cancer Center, Houston, TX[6] and American Type Culture Collection (http://www.atcc.org) respectively.
Task 1b. We have propagated and tested these cells for growth properties *in vitro* to confirm that they represent distinctive indolent (LNCaP and LNCaP-Pro-5) and aggressive (LNCaP-LN3) phenotypes. We have used several measures of aggressiveness: tumor doubling time, growth and invasiveness in 1- (scratch assay) and 2- dimensional (Boyden chamber) assays. These assays have indicated that there is little difference between the two indolent cell lines, but substantial difference between these two and the LNCaP-LN3 line, which is able to aggressively metastasize from the prostate *in vivo* (Pettaway ref). We also have propagated multiple controls: MCF-7, MCF-10A, MCF-12A, MDA-MB-231, HUVEC and HMEC cell lines, and primary rat and human fibroblasts, which will be utilized in the negative selection steps. By subtracting against these control cell types, we will eliminate aptamers that cross-react with non-prostate cancer cell types.

Task 2: Prepare dual-labeled, randomized aptamer pool for AptaBiD selection process. Status: Completed.

Task 2a. We previously generated and tested a pool of dual-labeled, 40-mer DNA aptamers (10^{33} randomized sequences; common 5' and 3' sequences for sequencing; biotinylated and labeled with Alexa Fluor 647). Following departure of the original PI of this project immediately after the start date, we have been re-assessing the strategic aspects of the project in light of the recommendations of local investigators with expertise in area. While we still retain this DNA aptamer library, we now have determined that RNA aptamers have practical and strategic advantages for our project [8-10].
Task 2b. We have obtained a second, RNA aptamer library from a collaborator (Dr. Paolo Serafini, University of Miami), thus its use will not add to the cost of the project. We therefore are currently in the process of performing Cell-Selex against a fluorinated RNA aptamer library with our indolent and aggressive LNCaP cells. Fluorination of the nucleic acid backbone stabilizes the RNA aptamers against RNAse degradation.

Task 3: Perform first round of "positive" RNA aptamer selection using the metastatic (LNCaP-LN3) prostate cancer cell line. Status: we expect this step to be completed in 1 week (April 2014).

Task 3a: Initial positive selection. As noted above, first-round selection is underway. Because the issue of clinical detection is most critical with respect to metastasis-prone tumors, we have opted to focus initially on a screen in which LNCaP-LN3 (aggressive) cells are used as the "positive" selection. In this approach, metastasis-prone LNCaP-LN3 cells are incubated with the complete RNA aptamer pool (30 minutes at 4°C) to initially select for RNA aptamers that interact with more aggressive prostate cancer cells. Unbound RNA aptamers are removed by media aspiration and washing in PBS.
Task 3b: Recovery of positively selected aptamers. Cells are heated (95°C for 5 minutes) to remove bound RNA aptamers.
Task 3c: Amplification of recovered aptamers. Released organ-confined or metastatic prostate cancer cell specific RNA aptamers are amplified via polymerase chain reaction (PCR) to expand this positively selected population.

C. Remaining tasks

1. **Task 4**: **Negative selection.** We will perform the first round of "negative" RNA aptamer selection using parental LNCaP cells, HPECs and non-prostate control cell lines with the enriched RNA aptamers pools (Timeframe = 1 month; April-May 2014).

Task 4a: Enriched metastatic prostate cell-specific RNA aptamer pools (generated in Task 3) will be incubated with cellular pools containing parental LNCaP cells, HPEC and non-prostate cells (30 minutes at 4°C).
Task 4b: Bound aptamers will be removed by centrifugation of the control cells to which they are bound. RNA aptamers that do not bind to these cells will be recovered in the cell supernatant and retained. The retained, "subtracted" RNA aptamer pool will be enriched for species that bind to metastatic LNCaP-LN-3 cells but not control cells.
Task 4c: The subtracted aptamer pool will be amplified by PCR.

2. **Task 5: Serial positive and negative selection cycles.** The positive and negative selection of RNA aptamers that bind either organ-confined or metastatic LNCaP cells will be repeated for 8 cycles. Sub-tasks outlined in Tasks 3 and 4 will be repeated 8x each. (Timeframe = 2 months; anticipated completion July 2012)

3. **Task 6: AptaBiD Biomarker discovery.** (see **E. Recommendations**, below). Our original scope of work calls for the use of aggressive PC-specific RNA aptamer pools to identify unique biomarkers to discriminate between organ-confined and metastasis-prone prostate cancer (Timeframe = 4 months; November 2014).

Task 6a: Binding of aptamers to biomarkers on PC cells. RNA aptamer pools, generated at the conclusion of task 5, will be labeled with biotin. Metastatic LNCaP-LN3 cells will be incubated with the biotin-labeled RNA aptamer pools (30 minutes at 4°C). Parental LNCaP and non-metastatic sister clone LNCaP-Pro-5 cells will be used as negative controls.
Task 6b: Capture of cell-bound aptamers. Following washing and centrifugation, aptamer-bound cells will be incubated with streptavidin-labeled magnetic beads (15 minutes at 4°C) that will bind with high affinity to the biotinylated RNA aptamers on the cells. Cell-RNA aptamer-magnetic bead complexes are then captured via a magnetic stand and

washed with PBS.
Task 6c: Recovery of specific aptamer-bound proteins. Captured cells are lysed (PBS+Mg with 0.1%v/v Triton X-100) for 10 minutes at 4°C. This releases biomarker protein-RNA aptamer-magnetic bead complexes from cell structures. The biomarker-containing complexes are captured on a magnetic stand and washed with cold PBS. Biomarker peptides are dissociated from the aptamer/bead complex using 8M urea (30 minutes at 0°C).
Task 6d: Purification of biomarker peptides. The biomarker peptide mixture is extracted and purified using a ready-to-go pipette tip filled with C18 spherical
silica reversed phase material (ZipTipC18 – Millipore).
Task 6e: Identification of biomarker proteins. Protein sequencing is performed by liquid chromatography and mass spectroscopy. When completed, this task will identify proteins specifically bound by the metastatic LNCaP-LN3 RNA aptamer pools but not by non-metastatic LNCaP parental and -Pro-5 subclones.

Task 7: Biomarker validation in clinical specimens. The potential biomarkers (both RNA aptamers and aptamer-identified proteins (AptaBIPs) for aggressive prostate cancer yielded in Task 6 will be examined across other prostate cancer cell lines as well as in banked tumor specimens. Direct reactivity of these aptamers and/or reactivity of antibodies against AptaBIPs in these additional PC types will be correlated with associated clinical data on prostate cancer progression and outcome. The ability of these new prostate cancer biomarkers to discriminate between progressive and indolent disease will be evaluated using fluorescent microscopy and immunohistochemistry with prostate cancer tissue microarrays (TMAs) (Timeframe = 4 months; end-February 2015).

Task 7a: Assembly of PC resources to be screened. Include 5 internal prostate TMAs (n=48 cores/slide including BPH (n=12), normal tissues (n=8), organ-confined prostate cancer (n=20) and several metastatic prostate tumors (n=8)) and 6 additional prostate TMAs (n=64 cores/slide) with non-prostate controls and prostate cancer cell lines will be screened for each novel biomarker. In addition, we have 163 sections of prostate samples including BPH, normal adjacent tissue, organ-confined tumors and a limited number of metastatic tumor sections. These TMAs and tissues sections will be analyzed for the presence of candidate biomarkers identified in Task 6.
Task 7b: Development of IHC strategy for biomarker identification. Where feasible, antibodies recognizing candidate biomarkers will be purchased and evaluated for marker detection using IHC. This will be completed at the Analytical Imaging Core Facility. Biomarker expression and distribution will be correlated to prostate cancer stage and progression as well as clinical outcome where known.
Task 7c: Reactivity of RNA aptamers with PC samples. Similar TMAs and sections will be exposed to Alexa Fluor 647-labeled metastatic RNA aptamer pools to evaluate specificity of these selected RNA aptamers as well as their potential use in imaging applications via fluorescent microscopy.

4. Impact

- We have obtained LNCaP-LN3 (aggressive, metastasis-prone), LNCaP-Pro-5 and parental LNCaP cell lines.
- We have propagated and tested these cells for growth properties *in vitro* to confirm that they represent distinctive indolent (LNCaP and LNCaP-Pro-5) and aggressive (LNCaP-LN3) phenotypes, using tumor doubling time, growth and invasiveness in 1- (scratch assay) and 2- dimensional (Boyden chamber) assays.
- We have propagated multiple control cell lines as noted in Task 1.
- We have refined, updated and validated the aptamer selection protocol in keeping with recent literature in this area.
- We have commenced Cell-Selex against a fluorinated RNA aptamer library with the indicated indolent and aggressive LNCaP cells described above.

Reportable Outcomes:

No publications or abstracts at the time of this report.

5. Changes/Problems

Next-Generation Sequencing in Task 6. Our original scope of work called for characterizing our metastasis-predictive aptamers by determining the unique proteins bound by these aptamers as outlined in Task 6. Based on recent technical improvements, accessibility, speed and cost-effectiveness of sequencing technology, we recommend next-generation sequencing to directly identify the aptamers themselves, to verify their nucleotide sequence relationships, and to allow us to focus on aptamer-specific PC detection in Task 7c of the proposal. We recommend replacement of Tasks 6a-6e as follows:

- **New Task 6a.** Perform next-generation sequencing on RNA aptamer pools after cycle 8 (replicate samples x 2), using Illumina HiSeq 2000 sequence detection system. Estimated timeline: 2 weeks.
- **New Task 6b.** Perform bioinformatic analysis on sequence information to identify core sequence motifs of selected RNA aptamers. Estimated timeline: 5-6 weeks.

Proceeding directly from Task 6 to Task 7c. In this approach the aptamers themselves serve as the reagents for identification of aggressive prostate cancer. This can be done instead of, or prioritized ahead of, the protein discovery steps previously proposed for Task 6. We anticipate considerable savings in time and cost as a result. Cost savings are realized at several levels:

- Fewer rounds of positive and negative selection would be required in Task 5 (we have already reduced cycles from 10 to 8 in our revised SOP based on this assumption.)
- We estimate that the 4 months of labor required to achieve protein isolation, purification and identification by mass spectroscopy could be replaced by 1-2 months of bioinformatic analysis of the sequence data recovered at the end of Task 5.
- Because the labeled aptamers themselves serve as reagents for imaging, the need for development or purchase of antibodies against biomarker proteins as described in Tasks 7a-b is obviated.
- Based on our selection protocol, the aptamers and aptamer pools will selectively bind to PC cells prone to aggressive growth and metastasis even in the absence of information about the specific protein biomarkers to which they are bound. Thus one is not limited to the universe of known proteins or protein complexes. This is also useful when antibodies are lacking for known proteins (see above).
- *A priori* validation of the RNA aptamers against other prostate cancer cell lines and tumor microarrays is likely to yield a limited number, possibly only 1-2 species, that are truly discriminatory for aggressive tumor growth. Eventually, identification of the protein targets of this small number of highly validated aptamers will be considerably less complex and more revealing of biologically relevant differences between indolent and progressive PC.

6. Products

On successful completion, the products of this project should include the following reagents which will have potential scientific utility and medical imaging and diagnostic uses, as shown by others [4, 9, 11, 12]:

- A pool of dual-labeled RNA aptamers able to discriminate between indolent and aggressive prostate cancer.
- One or more sequence-defined RNA aptamers able to discriminate between indolent and aggressive prostate cancer.
- A list of potential novel protein biomarkers for aggressive vs. indolent prostate cancer.
- A novel set of aptamer-based reagents for imaging applications via fluorescent microscopy.

The foundation for the identification and validation of critical biomarkers that may be involved in delineating indolent disease from aggressive or advanced prostate cancer is a critical challenge and focus area of the PCRP and would be addressed by the experiments outlined in this proposal.

7. Participants & Other Collaborating Organizations

at University of Miami:

- Nanette H. Bishopric MD
- Marc E. Lippman, MD
- Paolo Serafini, PhD
- Svetlana Speransky, BS

8. Special Reporting Requirements

Not applicable.

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