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14. ABSTRACT

Our proposal exploits a new technology, MagSweeper, for isolation of Circulating Tumor Cells (CTCs) from patients with prostate cancer. MagSweeper is an automated immunomagnetic separation technology that gently extracts CTCs from whole blood, allowing the possibility of molecular characterization of the isolated cells. In this grant, we proposed 3 specific aims and made significant progress in all of them. For the first aim we now have optimized a protocol that captures a high EpCAM expressing cell line LNCaP and a low EpCAM cell line PC3 with high purity and efficiency. For the second aim, we applied the optimized protocol on isolating circulating tumor cells from 40 prostate cancer patients and found CTCs in 15 of those patients. To demonstrate purity and specificity of the isolated CTCs, we used RT PCR to demonstrate the presence of Prostate Specific Antigen (PSA) and Androgen Receptor (AR) transcripts in a subset of these cells. Finally, we have begun applying nextgen sequencing technologies to develop a whole transcriptome sequencing protocol for pooled and single CTCs. In the second year of funding, we plan to increase our sample size of patients with metastatic disease and attempt to isolate CTCs from patients with localized prostate cancer.

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Introduction:

Our proposal exploits a new technology, MagSweeper, for isolation of Circulating Tumor Cells from patients with prostate cancer. MagSweeper is an automated immunomagnetic separation technology that gently extracts CTCs from whole blood, allowing the possibility of characterization of the cells molecularly. The MagSweeper isolates completely purified CTCs while preserving the integrity and viability of these fragile cells and eliminating contamination from nonspecific adsorption or entrapment of other blood cells. We proposed 3 specific aims in our grant proposal and we have made significant progress in each of them. Below are the details of the progress.

Specific Aim 1: To optimize MagSweeper technology for purification of prostate CTCs:

Our main objective for last year was to optimize a robust isolation protocol for isolation of live Circulating Tumor Cells (CTC) in both cell lines and patient blood samples with maximum capture efficiency and purity. We have made significant progress in creating a standard operating procedure that could be utilized for isolating CTCs using MagSweeper. MagSweeper utilizes EpCAM protein expression on cell surface for the isolation of circulating tumor cells from patients with prostate cancer. However, any other cell surface marker can be used in the future if it is expressed on the cell surface and there are specific, high-affinity antibodies for the protein. In our first set of experiments, we used prostate specific cell lines that express high and low levels of EpCAM on their cell surface to demonstrate purity, isolation efficiency and specificity of the MagSweeper. We used LNCaP and PC3 cell lines which are respectively high and low EpCAM expressing cell lines to initiate the isolation protocol. The sensitivity of the capture and purity was examined by spiking 100 cells in 7.5ml of human blood sample followed by counting of numbers of spiked cell line and WBC (confirmed with CD45 staining). In LNCaP, the capture efficiency was found to be 63% ± 25% with a purity of 99%. Similarly, in PC3 spiking studies, the capture efficiency was found to be 23% ± 20% with 99% purity. This step followed by a manual cell picking resulted in isolation of 100% pure preparation of the isolated cells. To minimize loss of genetic information following collection a cryopreservation protocol was developed and tested. Cell recovery and purity was assessed by spiking 10 LNCaP cells spiked in 3.75 ml of normal blood followed by processing in CPT tubes (Becton Dickenson). Plasma containing leukocytes and spiked cells was then cryopreserved for at least 48 hours prior to thawing, MagSweeping and single cell isolation. Mean live LNCaP cell recovery following MagSweeper and isolation was 52% ± 27%. The final purity of LNCaP cells among contaminating white blood cells was 99% (Figure 1).

Specific Aim 2: To test whether MagSweeper can successfully isolate CTCs from patients with localized and metastatic prostate cancer:

We have tested this same SOP on isolating circulating tumor cells from metastatic prostate cancer patients. So far we have recruited 40 metastatic prostate cancer patients and found live CTCs in 15 of the patients. 12 samples were sent to Quest Diagnostics for comparison and

independent analysis of CTC counting using CellSearch technology. CellSearch is an FDA approved technology to quantitate numbers of CTCs from patients with prostate and breast cancer (although it does not allow isolation of cells or genetic analysis as we have proposed). Our CTC counts correlated with (or exceeded) CTC numbers measured by the CellSearch assay (Figure 2). Live CTCs isolated from patient blood samples were examined for PSA and AR expression to discern the origin of the CTCs. We tested 6 CTCs and found all expressed PSA while 5/6 were positive for AR expression demonstrating that these CTCs are prostate derived.

Specific Aim 3: Perform gene expression biomarker assays on individual CTCs isolated from patients:

In our initial application we proposed doing a multiplex qPCR analysis of CTC using prostate specific marker genes. This includes finding ways to use Fluidigm technology which can accommodate up to 384 genes for comparison of gene expression profiles. Since our proposal started, there has been tremendous progress in DNA sequencing both in terms of technology and cost. We have begun using technologies for nextgen sequencing developed at Illumina Inc. (Hayward, CA) to develop a single cell sequencing protocol for CTCs (Figure 3). So far, using their protocol we have done whole transcriptome amplification and mRNA seq on 6 single CTCs from patient blood, a single T24 bladder and LNCaP prostate cancer cells, a pool of 8 prostate CTCs, and one leukocyte isolated from the blood of a metastatic prostate cancer patient. Our initial analysis of the data obtained from sequencing studies suggests that we can reproducibly amplify 66% of mRNA pool from a single cell. Clustering analysis does differentiate CTCs from LNCaP and T24 bladder cell lines (Figure 4). At present we have between 2-24 live CTCs from 10 patients that are compatible with nextgen sequencing protocols. Our aim this year is to create sequencing libraries from the isolated CTCs and collect sequencing profiles from these single cells. Additionally we plan to recruit more patients, collect CTCs and perform additional single cell sequencing profiles.

In our application we also hypothesized that there would be heterogeneity in gene expression between CTCs from a single patient and that this heterogeneity might correlate with disease state. Our initial analysis of the sequencing data does demonstrate both intra and inter patient heterogeneity (Figure 4). For instance, when clustered with LNCaP and T 24 cell lines we found that 5/7 CTC cells cluster together, while 2/7 CTCs show very different expression profiles. The heterogeneity was also apparent in the expression levels for individual genes. For example, 7/7 CTC cells expressed KLK3, AR and EpCAM, 6/7 expressed CD24 and AMACR, 5/7 expressed MYC and 0/7 expressed CD45 (a white blood cell marker). On the other hand, the WBC did not express any of these genes except CD45. At this point we have not correlated the heterogeneity to grade and stage nor have we got the sample size to perform detailed analysis on the gene expression profiles. There is certainly a need for expansion of CTC pool and data to understand whether heterogeneity in itself can provide clinical or biological insights into prostate cancer.

KEY RESEARCH ACCOMPLISHMENTS:

Specific Aim 1: To optimize MagSweeper technology for purification of prostate CTCs:

- Developed a protocol to isolate high EpCAM expressing LNCaP and low EpCAM expressing PC3 cell lines with maximum efficiency and purity.

Specific Aim 2: To test whether MagSweeper can successfully isolate CTCs from patients with localized and metastatic prostate cancer:

- We were able to isolate live circulating tumor cells from prostate cancer patients.
- Counts obtained from MagSweeper correlated with or exceeded counts obtained from Cell Search system.
- We were able to establish the purity and specificity of CTC using prostate specific marker assay.

Specific Aim 3: Perform gene expression biomarker assays on individual CTCs isolated from patients:

- We were able to test single cell whole trancriptome amplification and sequencing protocol on 6 CTCs.
- Using this protocol we could reproducibly amplify 66% of mRNA pool from a single cell.
- Clustering analysis done using gene expression profiles could distinguish a CTC from prostate cancer cell line LNCaP and T24 bladder cancer cell line.
- There was intra and inter patient heterogeneity observed in the gene expression profile of single CTC

REPORTABLE OUTCOMES:

A poster titled "**Transcriptome sequencing of circulating tumor cells reveals their heterogeneity"** was presented at the Annual American Association of Cancer Research (AACR) meeting held in Orlando, FL in April 2011.

Overall:

We have made significant progress on all 3 specific aims. We have an optimized protocol for prostate specific CTC isolation which has been tested in number of metastatic prostate cancer patients. In addition, we are improving protocols for whole transcriptome sequencing analysis of single CTCs which could provide significantly richer datasets and insights into CTC biology and clinical utility. In the second year of funding, we plan to increase our sample size of patients with metastatic disease and attempt to isolate CTCs from patients with localized prostate cancer.

Supporting Data

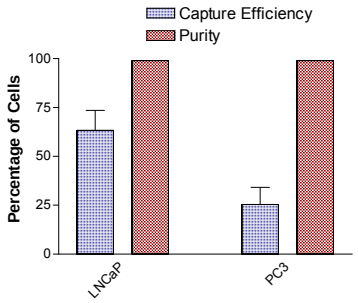

Figure 1: **Isolation of spiked LNCaP and PC3 from normal human blood.** 100 cells were spiked into 3.75ml normal human blood with 1micron EpCAM labeled beads. Bar 1 represent retrieval of percentage of LNCaP and PC3 cells respectively from blood using MagSweeper. Bar 2 represent purity of cell population obtained after MagSweeper cycle. Purity was estimated by counting the number of CD45 positive WBCs found compared to number of WBCs found in 3.75ml of blood.

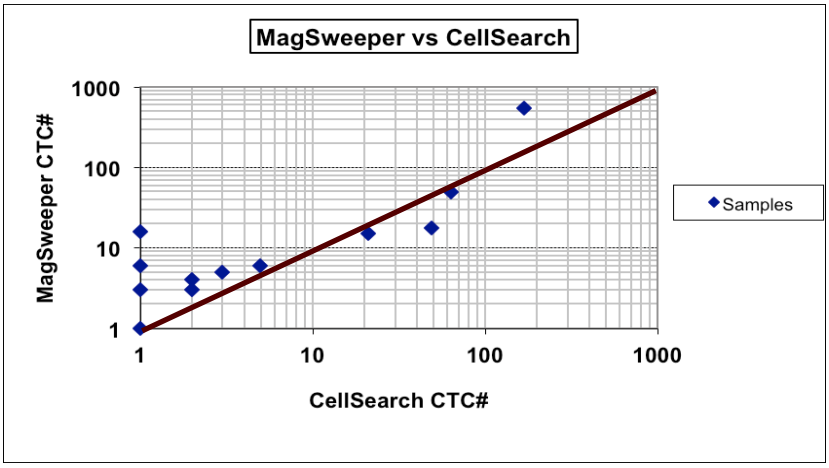

Figure 2: **Comparison of CTC isolation platforms; MagSweeper versus Veridex CellSearch System.** To directly compare CTC isolation capabilities between MagSweeper and the FDA–cleared CellSearch system, 7.5 ml replicate blood samples were drawn from 12 prostate cancer patients. Samples for CellSearch were collected in CellSave tubes and processed by Quest Diagnostics, while MagSweeper isolation was done on live samples. Number of CTC isolated using MagSweeper correlated or exceeded compared to CellSearch assay (samples with 0 CTCs were plotted as 1).

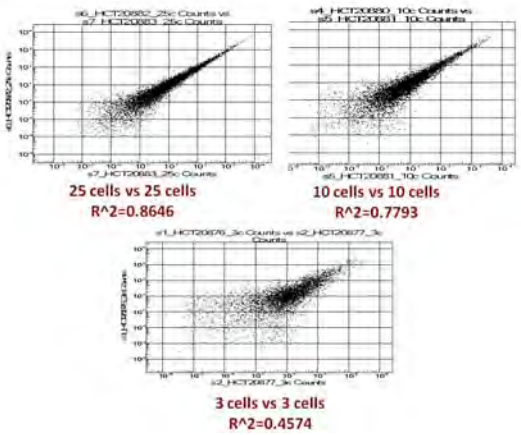

Figure 3: **Performance evaluation of small input numbers of whole cells into a proprietary whole transcriptome amplification and mRNA seq protocol.** Replicate pools of LNCaP cells were directly lysed, amplified, libraries prepared and sequenced. Transcripts detected in matching pools of cells were then plotted against each other to understand stochastic effects using small inputs of cells in this protocol.

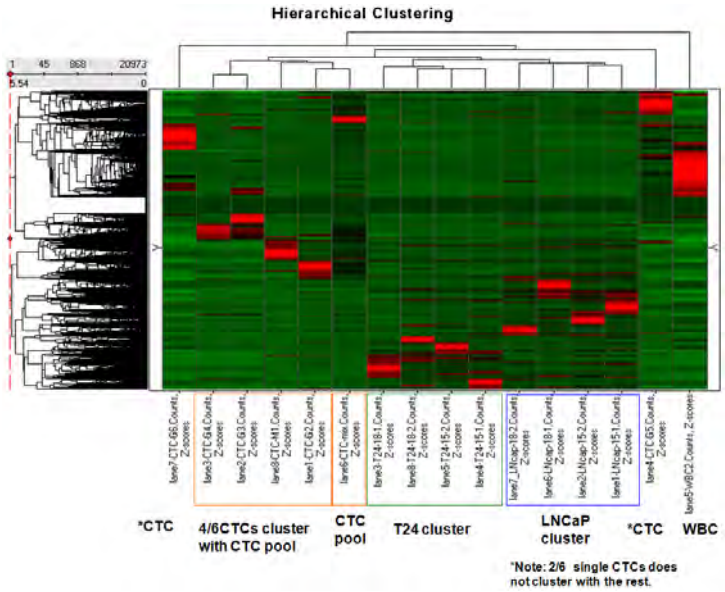

Figure 4: **Single Cell Gene Expression Profiling of CTCs from Prostate Cancer Patients.** mRNA seq data for 6 single prostate CTCs, 8 cell prostate CTC pool, single T24 bladder and LNCaP prostate cancer cells and a leukocyte were clustered to find relationships between samples. 4/6 prostate CTCs cluster. Hierarchical Clustering analysis does differentiate CTCs from LNCaP and T24 bladder cell lines. There were both inter and intra patient heterogeneity observed. 5/7 CTC cells cluster together, while 2/7 CTCs show very different expression profiles.