

AD _____

Award Number: DAMD17-98-1-8541

TITLE: The Assessment of Prostate cells in Semen Using Flow
Cytometry, for the Early Detection of Prostate Cancer

PRINCIPAL INVESTIGATOR: Sai Su, Ph.D.

CONTRACTING ORGANIZATION: Northwest Hospital
Seattle, Washington 98215

REPORT DATE: December 2002

TYPE OF REPORT: Final

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.

20030731 137

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

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 Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE December 2002	3. REPORT TYPE AND DATES COVERED Final (1 Sep 98 - 30 Nov 02)	
4. TITLE AND SUBTITLE The Assessment of Prostate cells in Semen Using Flow Cytometry, for the Early Detection of Prostate Cancer			5. FUNDING NUMBERS DAMD17-98-1-8541	
6. AUTHOR(S) Sai Su, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Northwest Hospital Seattle, Washington 98215 E-Mail: dwaters@gpmcf.org			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. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES Original contains color plates: All DTIC reproductions will be in black and white.				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				12b. DISTRIBUTION CODE
13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information) The goal of this research was to demonstrate the feasibility of using flow cytometry to detect prostate cancer cells in seminal fluid as an important step toward developing a new test for the non-invasive diagnosis of prostate cancer. Results obtained during the first 12 months of the project were promising. Tragically, the Principal Investigator, Dr. Gerald P. Murphy, died in the 16th month of the project period. After Dr. Murphy's death, a reassessment of the technique used to prepare seminal fluid samples for flow cytometry showed that these methods were not adequate for the repeatable detection of prostate cancer cells. As a result, a revised Statement of Work was approved which focused on developing a more robust method of epithelial cell detection in seminal fluid that would overcome sperm interference. Magnetic bead separation improved the recovery of prostate cancer cells from semen, but failed to consistently produce sufficient yield of epithelial cells from patient samples. Fixation of seminal fluid samples, a prerequisite for the practical application of this assay, significantly reduced prostate cell recovery using magnetic bead separation. In summary, our work failed to define conditions necessary to establish seminal fluid analysis using flow cytometry as a reliable assay for the detection of prostate cancer.				
14. SUBJECT TERMS: prostate, cancer detection, seminal fluid, flow cytometry			15. NUMBER OF PAGES 10	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	7
Reportable Outcomes.....	7
Conclusions.....	8
Appendices.....	8

INTRODUCTION

Prostate cancer continues to ravage the US male population, with almost 200,000 new cases diagnosed each year. The American Cancer Society suggests that men with elevated serum levels of the prostate cancer marker, prostate-specific antigen (PSA), undergo prostatic biopsy because they have an increased likelihood of harboring prostate cancer. However, elevations in serum PSA occur in men with benign prostatic hyperplasia, prostatitis, or prostate cancer and distinguishing between these malignant and non-malignant conditions currently requires prostatic biopsy and histopathologic evaluation of prostatic tissue. This year, over 1.5 million prostatic biopsies will be performed in the US, and *only two of five biopsies will be positive for prostate cancer*. Our *research purpose* was to minimize the need for prostatic biopsy by developing a technique that could identify the subpopulation of men with elevated serum PSA that have the greatest risk for prostate cancer. Our previous work showed that prostate epithelial cells (malignant and normal cells) could be detected in the seminal fluid of men with prostate cancer. The *objective* of our proposed research was to develop a test that distinguishes between prostate cancer cells and non-cancer cells in seminal fluid in order to identify men that are at highest risk for harboring prostate cancer. If successful, use of this test could significantly reduce the number of men without prostate cancer who are subjected to unnecessary invasive procedures. This research is central to our *long-term goal* which is to reduce prostate cancer mortality through developing new methods for improved early cancer detection and by identifying effective chemopreventive strategies.

BODY

In the original grant application, the Principal Investigator, Dr. Gerald P. Murphy, stated 3 research objectives: (1) define the parameters associated with the collection and preparation of semen samples and optimize procedures to maximize cellular quality; (2) using flow cytometry, perform semen cytology analysis on samples from volunteers with no evidence of prostate cancer, patients with BPH or prostatitis, and patients with biopsy proven prostate cancer; and (3) identify new markers and incorporate them into our current system.

In an Annual Report, submitted by Dr. Murphy in September 1999, the following key research accomplishments were reported:

- (1) Optimization of cellular quality by researching and selecting parameters for sample acquisition and processing
- (2) Development of a process to remove most sperm cells from seminal fluid, which reduces sperm interference during flow cytometry analysis
- (3) The PSMA:cytokeratin ratio tends to distinguish prostate cancer from patients with no evidence of cancer (**Figure 1**).

Dr. Murphy died during the 16th month of the project period, and a no cost extension was requested and approved in order to continue the research. Subsequent

studies using more rigorous controls called into question whether seminal fluid samples could be prepared reliably for the accurate detection of epithelial cells in the midst of sperm interference. Therefore, in November 2001, a revised Statement of Work was requested and approved by USAMRMC that re-focused our research efforts on optimizing seminal fluid preparation and assay conditions for the detection of prostate cancer cells in seminal fluid using flow cytometry.

The following provides a summary of the research activities based upon the revised Statement of Work:

Task 1. Optimize Conditions of Collection and Preparation of Seminal Fluid Samples for the Accurate Detection of Prostate Cancer Cells by Flow Cytometry

A. Assessment of Epithelial Cell Recovery from Seminal Fluid Samples Using “Swim Up” Method

Seminal fluid samples were slowly centrifuged and then warm RPMI media + 10% FBS were added to the pellet. The pellet was left undisturbed in a 37°C waterbath for 1 hour which allows the sperm to “swim up” from the pellet. Our results indicated that this technique improved the ability to detect epithelial cells in seminal fluid. However, not enough sperm are removed to allow accurate characterization of cells using flow cytometry. By using the patient’s own sample as a negative control (stained with negative isotype control goat anti-mouse antibody biotinylated to be stained with streptavidin-conjugated phycoerythrin), it was shown that many of the positive events counted by the flow cytometer were actually background events also counted under negative control conditions. A representative case is shown in **Figure 2**. In contrast to observations made in previous experiments, the “swim up” method suffered from an unacceptable degree of sperm interference. We concluded that the “swim up” method is not a reliable method of sample preparation for the accurate detection of prostate cells in seminal fluid using flow cytometry.

B. Minimizing Sperm Interference Through the Use of Magnetic Bead Separation of Sperm and Epithelial Cells

In these experiments, seminal fluid was mixed with warm media, centrifuged, and the remaining pellet was spiked with a known number of LNCaP prostate cancer cells. The sample was then divided into equal halves and half of the sample was subjected to MiniMACS magnetic separation procedure (Miltenyi Biotec, Auburn, CA). Flow cytometry showed a recovery rate of approximately 85%, i.e. 85% of live prostate cancer cells spiked in seminal fluid could be recovered. This was far superior to the recovery obtained using the “swim up” method sample preparation. However, because seminal fluid samples must be diluted during the magnetic separation procedure, few if any epithelial cells (cells positive for the epithelial marker CAM 5.2) could be identified in non-spiked patient samples.

C. Recovery of PSMA (+) Versus PSMA (-) Prostate Cells from Seminal Fluid

Experiments were conducted to determine the sensitivity of the assay in recovering prostate cells bearing the cell surface marker prostate specific membrane antigen (PSMA). Previous experiments performed in our laboratory had successfully used the Anti-PSMA antibody 7E11. Because the manufacturer no longer made the 7E11 antibody available to Murphy Foundation investigators, we tested 2 polyclonal antibodies to PSMA provided by another manufacturer. In spiking experiments, in which known PSMA (+) LNCaP and PSMA (-) PC-3 prostate cancer cells were added to seminal fluid, one of these antibodies showed acceptable specificity to detect PSMA (+) cells.

D. Assessment of Anti-PSA Antibody for Detection of Prostate Cells in Seminal Fluid

Experiments were conducted to determine if anti-PSA antibody could be used to identify prostate epithelial cells in seminal fluid. Our results indicate that PSA staining could not discriminate between CAM 5.2 (+) epithelial cells and non-epithelial cells in seminal fluid. A representative experiment is shown in **Figure 3**. We concluded that the high concentration of extracellular PSA in seminal fluid samples obscures the accurate detection of prostate cells in this assay.

E. Recovery of Fixed Prostate Epithelial Cells from Seminal Fluid Specimens

In separate experiments, we assessed the ability of the seminal fluid assay to detect prostate cancer cells in seminal fluid samples that were subjected to chemical fixation, rather than fresh samples. The justification for these experiments was that evaluation of batched samples or samples prepared at other centers that would be transported to a central laboratory requires that the assay can utilize fixed (i.e. non-fresh) samples. Experiments were performed using ethanol or paraformaldehyde. Results with ethanol fixed cells were poor, because ethanol fixed cells became too rigid for optimal binding to beads resulting in low cell recovery. LNCaP prostate cancer cells fixed with 1% paraformaldehyde and spiked into seminal fluid had the highest recovery among the fixation protocols evaluated.

Task 2. Determine Whether Seminal Fluid Assay Using Most Promising Markers Can Distinguish Between Men With and Without Prostate Cancer

Task 2 was not initiated because our experiments failed to establish assay conditions that could reliably detect prostate cancer cells in seminal fluid samples.

Task 3. Complete Data Analysis From This Phase I Study

No manuscripts have been published regarding this work. Based upon the negative results of this study, the use of flow cytometry for the early detection of prostate cancer is not currently being pursued by investigators at the Gerald P. Murphy Cancer Foundation. We are focusing on other aspects of prostate cancer control, including the further development of selenium as a practical means of prostate cancer prevention. This

research is currently being supported by a Phase II Idea Development Award from the USAMRMC.

KEY RESEARCH ACCOMPLISHMENTS

- PSMA:cytokeratin ratio in seminal fluid tends to distinguish patients with prostate cancer from patients without prostate cancer but results are significantly influenced by sperm interference, rendering this assay unreliable.
- PSA cannot be used as a marker for the detection of prostate cancer cells in seminal fluid samples because of high extracellular concentrations of PSA in seminal fluid.
- Magnetic bead separation techniques may be useful in diminishing sperm interference but frequently renders patient samples with an inadequate number of epithelial cells for diagnostic assessment.

REPORTABLE OUTCOMES

Manuscripts

None

Patents

None

Career Development / Research Opportunities

Throughout his career, the Principal Investigator, Dr. Gerald P. Murphy, made important contributions to the field of prostate cancer research. In particular, Dr. Murphy's work focused on the use of markers such as PSA and PSMA in the early detection of prostate cancer. After Dr. Murphy's untimely death, the Foundation has continued to pursue novel approaches to reduce prostate cancer-related morbidity and mortality. (Support from the USAMRMC has made this possible.) Under the direction of its new Executive Director, Dr. David J. Waters, the Foundation is actively engaged in prostate cancer chemoprevention research. The Foundation's cancer prevention research is funded by a Phase II Idea Development Award from USAMRMC as joint research between the Murphy Foundation and Purdue University.

CONCLUSIONS

In this Idea Development Award, the USAMRMC funded a high risk-high gain project to determine the feasibility of developing a new prostate cancer detection method using flow cytometric evaluation of seminal fluid. The initial results of this project provided optimism that the analysis of seminal fluid might hold great promise as a prostate cancer detection test. However, subsequent experiments using more stringent controls suggested that the accurate and repeatable discrimination of cell types within seminal fluid samples was significantly impeded by sperm interference. Efforts to establish seminal fluid preparation techniques and assay conditions that would yield reliable results were unsuccessful. We conclude that, in our hands, flow cytometry does not provide a reliable method to accurately detect prostate cancer cells in seminal fluid. A major difficulty encountered in this project was the untimely death of the Principal Investigator, Dr. Gerald P. Murphy, which significantly hindered the completion of the originally proposed work. No additional studies to further develop this assay have been proposed by our research group. Instead, we intend to actively pursue other research approaches to achieve our long-range goal of reducing prostate cancer mortality.

APPENDIX

Figure 1. Comparison of flow cytometry dot plots of seminal fluid from a prostate cancer patient and a patient without any evidence of disease

Figure 2. Flow cytometry dot plot showing sperm interference

Figure 3. Flow cytometry dot plots showing failure of PSA staining to identify prostate epithelial cells within seminal fluid

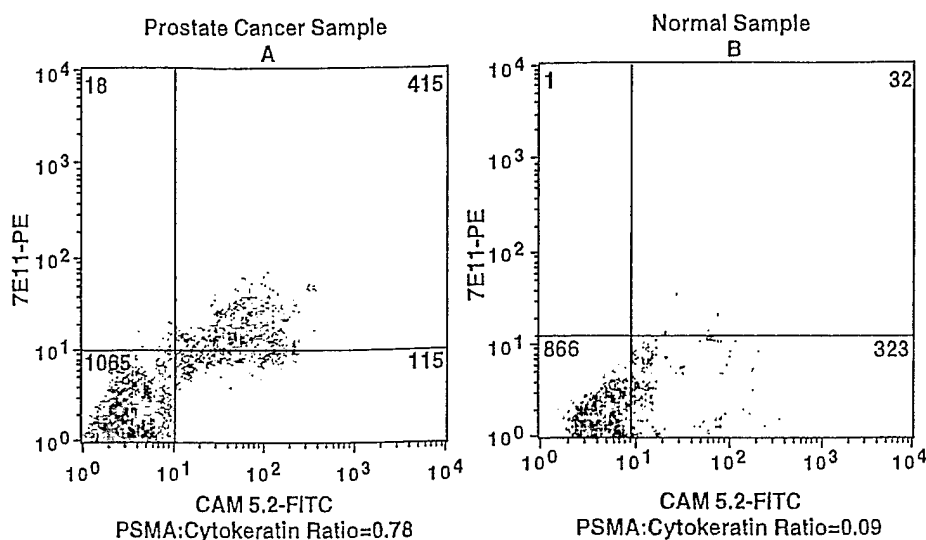


Figure 1. Representative flow cytometry dot plots of seminal fluid from (A) a prostate cancer patient, and (B) a patient without any evidence of disease. These graphs were generated from the DNA-cycling populations as determined by DNA pulse width and area graphs. Quadrants are based on the staining of the control cell lines LNCaP (CAM 5.2⁺, 7E11⁺, PSA⁺) and PNCf 007 (CAM 5.2⁻, 7E11⁻, PSA⁻), run in conjunction with the patient samples. The prostate cancer sample demonstrates the 7E11-positive staining of the cytokeratin population and the relative lack of staining in the normal patient.

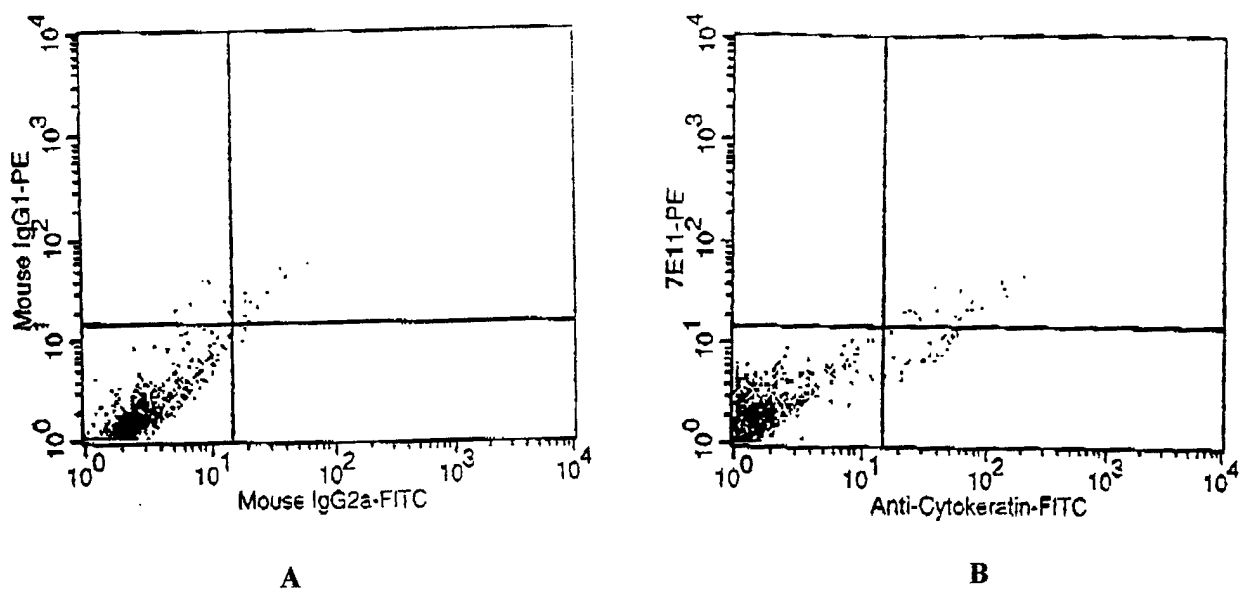


Figure 2. (A) Flow cytometry dot plot of a patient sample negatively stained with isotype controls used to set the parameters for the flow cytometer for the positively stained sample. (B) Corresponding positively stained patient sample using the settings determined by the negative control. There is an increase in the events positive for cytokeratin (epithelial marker), but not enough to represent a significant positive population for characterization. The interference from sperm prevents the cytometer from accurately selecting positive events from this sample.

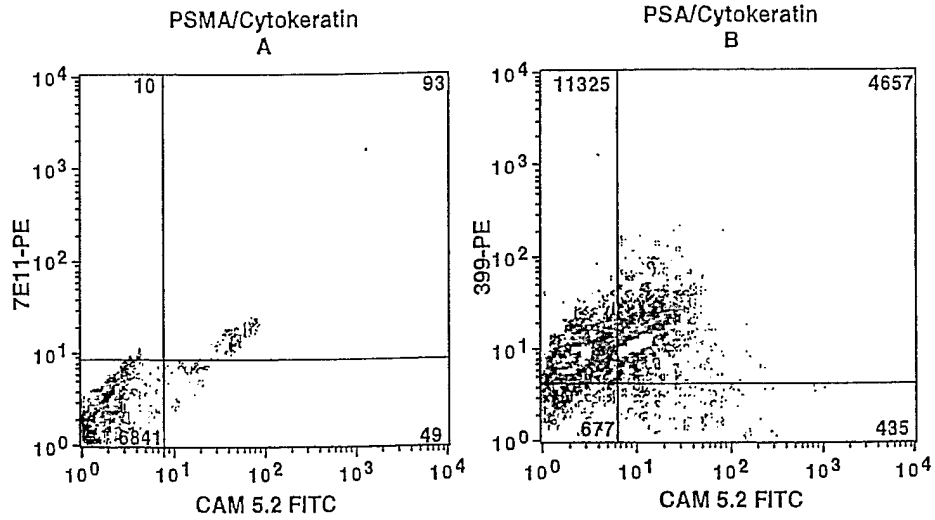


Figure 3. Flow cytometry dot plots representing a prostate cancer patient showing the comparative staining between PSMA and PSA. These graphs were generated from the DNA-cycling populations as determined by DNA pulse width and area graphs. (A) PSMA/cytokeratin staining shows that only cells positive for CAM 5.2 will stain positive for PSMA. (B) PSA/cytokeratin staining shows that PSA staining is indiscriminate for CAM-positive or negative cells.