

AD _____

Award Number: W81XWH-05-1-0092

TITLE: Development of a Novel, Non-Invasive Diagnostic Test for Prostate Cancer

PRINCIPAL INVESTIGATOR: Robert Gardiner, M.D.

CONTRACTING ORGANIZATION: University of Queensland
Brisbane, QLD 4072 Australia

REPORT DATE: January 2007

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 01-01-2007		2. REPORT TYPE Annual		3. DATES COVERED 6 Dec 2005 – 5 Dec 2006	
4. TITLE AND SUBTITLE Development of a Novel, Non-Invasive Diagnostic Test for Prostate Cancer				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-05-1-0092	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Robert Gardiner, M.D. Email: f.gardiner@uq.edu.au				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Queensland Brisbane, QLD 4072 Australia				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 Original contains colored plates: ALL DTIC reproductions will be in black and white.					
14. ABSTRACT Hypothesis: The overall objective is to provide, for routine clinical use, a simple and reliable method for identifying patients with prostate cancer so that transrectal ultrasound guided biopsies can be restricted to men harboring PCa. Our broad strategy will involve the use of selected markers for sensitive detection of cancer cells in ejaculate. Objectives: (1) To recruit patients for a prospective clinical trial in a screening assay of patient ejaculate and urine samples from which PCa cells will be isolated by (2) immunocapture using antibodies to specific cell-surface markers and (3) detected by real-time PCR, with a combination of genes upregulated in PCa. These methods will be refined for application for routine laboratory use. Logistic regression analysis will be used with the above combination of PSMA, DD3 and Hepsin to determine each patient's probability of harboring PCa. This data will be correlated with clinical data obtained by conventional diagnostic methods. Relevance: It is our strong expectation that this research will lead to provision of a simple and reliable approach for diagnosing PCa, in the longer term reducing the need for the invasive, imprecise and unpleasant procedure of TRUS-guided prostatic biopsies. In addition we expect that the earlier detection will be associated with and even greater proportion of cancer localised to the prostate at time of diagnosis.					
15. SUBJECT TERMS Detection of Prostate Cancer in its earliest stages of development, Non-invasive, Clinical Trial, Molecular Biomarkers					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	7	19b. TELEPHONE NUMBER (include area code)

Table of Contents

Introduction.....4

Body.....4

REPORT ON SECOND YEAR'S PROGRESS

INTRODUCTION

In our report on December 2005, we indicated that there had been two changes to our research strategy since receiving the grant.

1. The first was the decision to collect prostatic fluid obtained in urine following Digital Rectal Examination (**DRE**) in addition to prostatic fluid obtained from ejaculate and urine immediately following ejaculation, for our assays. This decision was prompted by the published results of the multi-centre study by Fradet et al (**2004**) and the decision by Bostwick Laboratories provide a test commercially that assays for PCA3/DD3 RNA (<http://www.bostwicklaboratories.com/>) from prostatic cells in urine immediately following DRE (**Hessels et al, 2003; Tinzl et al, 2004**). These assays use NASBA (Nucleic-Acid Sequence Based Amplification (Compton, 1991) to amplify the PCA3/DD3 from sample RNA.

After submitting this report, we were contacted by the Human Research Protections Office (HRPO), Office of Research Protections U.S. Army Medical Research and Materiel Command because of their concern that we were including post-DRE urines in our study. Despite lengthy, though helpful and accommodating interactions with Debra de Paul for the Human Protection Office, we had to dispense with including this source of prostatic fluid for our analyses because we could not meet the requirements stipulated in terms of informed consent.

Clinical recruitment for this study has been halted for the best part of the past 12 months because of the above issue and other requirements of the Human Research Protections Office (HRPO), Office of Research Protections U.S. Army Medical Research and Materiel Command with which we inadvertently had not complied. We had initially commenced recruiting as planned in year one on the basis of an approval from Dr Mishra in his email of 19 November 2004. The, interactions to comply with the requirements of the DoD have been characterised by inevitable delays: we now have approval from 2 of our 3 institutional ethics committees for the stipulations agreed by DoD and ourselves.

2. The second was, in keeping with the use of NASBA for PCA3/DD3 above, to use this approach for PCA3/DD3 and PSMA, Hepsin and Gal-NAc-T3 in our prostatic fluid analyses. The NASBA approach provides the added advantage of greater sensitivity in comparison with that provided by antibodies, or RT-PCR as outlined in our application.

Evaluation of specimens obtained prior to stopping recruitment as directed: We continued our analyses with the samples collected in 2005 to enable us to optimise our biomarker detection protocol. Subsequently we have the NASBA analysis working for all prostate cancer biomarkers above.

While the samples collected in 2005 have permitted us to streamline our protocols, they are too few to allow significant statistical analysis of the data.

We have found that when just looking at PSA as a measure of cancer cell retrieval that the process of ejaculation seems to dislodge more prostate cells than the Digital Rectal Exam (See below):

	Cancer Positive	Cancer Negative
Ejaculate	10/12 (83 %)	5/5 (100%)
Urine	10/12 (83%)	5/5 (100%)
Post-DRE urine	7/11 (63%)	6/12 (50%)

We also noted that our detection rate with the cancer specific markers was disappointingly low, but later discovered that the RNA extraction protocol had not been followed correctly. This has been validated and corrected.

Aim 1. To continue to recruit patients for a prospective clinical trial.

(i) Recruitment: In the 3 years of the study, we plan to study specimens from 100 men whose TRUS biopsies reveal PCa and from 100 men whose ≥ 8 biopsies do not. At least 40 of the PCa patients selected will undergo RRP which will permit close correlation with histological parameters and markers of progression. At the time of submitting last year's report, we had recruited 40 men who provided prostatic fluid. Once we receive the approval from our 3 institutional ethics committees, these patients will be approached to provide (belated) informed consent consistent with the requirements of the Human Research Protections Office (HRPO), Office of Research Protections U.S. Army Medical Research and Materiel Command.

(ii) Sample collection: Because of the delay in the past 12 months, we have to recruit the planned 100 men whose transrectal ultrasound (**TRUS**) biopsies reveal a diagnosis of prostate cancer and the 100 men whose biopsies are benign in the last year of the 3-year programme. To achieve this aim, we have extended our network of collaborating urologists and will identify further patients via the database from a large, local, NHMRC-funded psychology project involving most of the urologists in south-east Queensland and on which I am a Chief Investigator. In the unlikely event that we will not accrue the required numbers by the end of the year, we will continue the study until this goal is achieved to enable the statistical calculations to be performed as planned in the application.

After initiating research into the use of seminal fluid for diagnosing prostate cancer in 1995, we have been working continuously towards the goal of evaluating a validated panel of RNA markers in patients: this constitutes the basis of this grant. Consequently, we are committed to realising the research plan with 100 patients in the tumour positive group and 100 in the tumour negative group.

(iii) Statistical considerations: With 200 male patients, 100 with PCa and 100 PCa-free, there is $>95\%$ power to detect a significant difference (at the 2-sided 5% level) in mean expression of DD3, PSMA, Hepsin, and Gal-NAc-T3 for cancer-affected and unaffected patients using a two group Satterthwaite t-test (due to the unequal variances).

Mean expression levels in genetic markers will be compared between PCa-affected and unaffected patients using t-tests and/or Wilcoxin non-parametric tests. Receiver-operator characteristic (ROC) curve analysis will be conducted to assess the usefulness of gene expression as a screening tool. This involves calculation of sensitivity and specificity for different thresholds of expression to determine the

optimal diagnostic cut-point. The area under the ROC curve is tested against a value of 50%: each marker will be assessed individually and then modelled as one of an array of covariates using logistic regression.

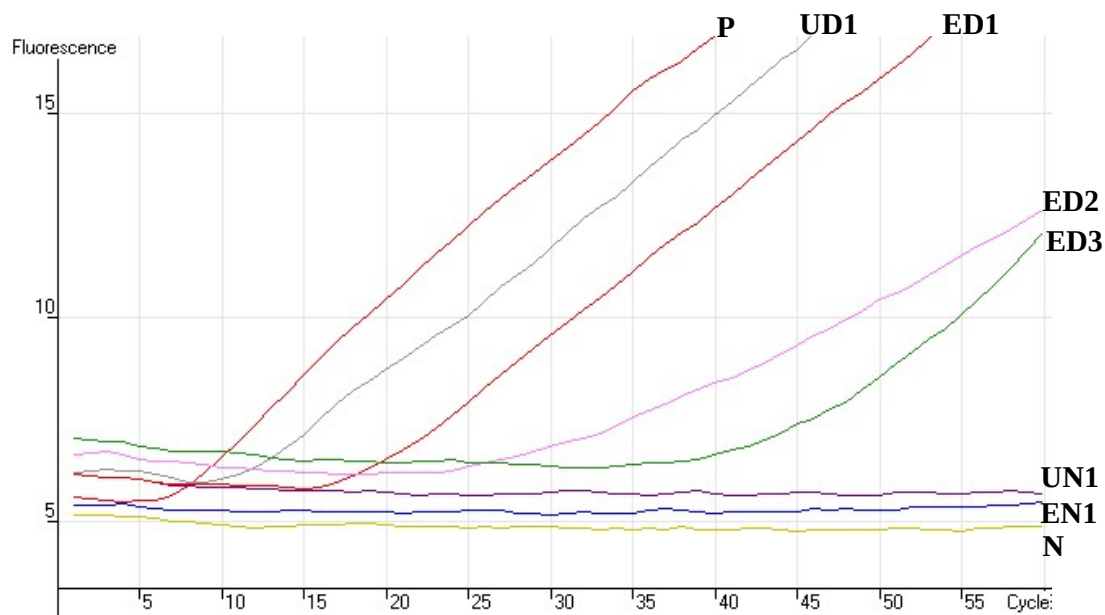


Figure Legend:

P; Positive Control RNA, **N**, Water only negative control: **ED1, ED2, ED3**, DD3 positive Ejaculate RNA; **UD1**, Post-ejaculate-Urine DD3 positive RNA: **EN1, UN1**, DD3 negative Ejaculate and Urine RNA (respectively).

Aim 2. To utilise immunocapture of PCa cells using antibodies to cell-surface markers

We have optimised our strategy of immunocapture of PCa cells by targeting PSMA alone as sufficient cells are retrieved for our real-time PCR.

(i) Cell enrichment and immunocapture from ejaculate. The initial stage of our enrichment and immunocapture protocol continues to involve isolating mononuclear cells from ejaculate. The ejaculate samples are collected in 20 mls of Hanks buffer (Invitrogen, Victoria, Australia) and 7 mls of the sample carefully layered over 2ml of 64% isotonic Percoll (Amersham Biosciences, NSW, Australia). The sample is centrifuged, for 30 minutes at 800 rpm at 4°C, after which the mononuclear cells are collected from the Percoll/Sample interface. Non-nucleated spermatocytes pellet below the Percoll and are discarded. The mononuclear cells are washed gently by centrifugation at 1000 rpm at 4°C and resuspension in 1ml of PBS. The first ~50 ml of voided urine immediately following DRE is collected and ferried at refrigeration temperatures to our laboratory for centrifugation and subsequent evaluation.

PCa cells are isolated from the mononuclear cell fraction by the addition of protein G coated Dynal Beads (Dynal Biotech Pty Ltd, Vic., Australia) and 5 µg of anti-PSMA antibodies. The samples are then incubated at 4°C for 1 hour on a rotating wheel. The Dynal Beads with capture PSMA expressing cells are collected to the side of the tube with a magnet and the supernatant removed. The beads are washed twice in 1ml of PBS and finally stored at -70 C in 20 µl of Cell Lysis buffer (0.15M NaCl; 10 mM Tris-HCl, pH 8; 5 mM DTT; 40 U RNasin (Promega Corporation, NSW, Australia)).

(ii) Cell enrichment and immunocapture from urethral washings: Urethral washings are also being collected by patients on this trial. The mononuclear cells in urine are being collected by centrifugation at 1000 rpm at 4°C and resuspended in 1ml of PBS.

PCa cells are isolated from the mono-nuclear cell fraction, as described above, by the addition of protein G coated Dynal Beads and 5 µg of anti-PSMA antibodies. The samples are then incubated at 4°C for 1 hour on a rotating wheel. The Dynal Beads with capture PSMA expressing cells are collected to the side of the tube with a magnet and the supernatant removed. The beads are washed twice in 1ml of PBS and finally stored at -70 C in 20 µl of Cell Lysis buffer (0.15M NaCl; 10 mM Tris-HCl, pH 8; 5 mM DTT; 40U RNasin) (Promega Corporation, NSW, Australia).

(iii) Polyclonal antibody production against other cell surface antigens. As indicated above, the NASBA technique is sensitive enough to detect a few positive cells in a cell mix and therefore, at this stage, we have not produced antibodies against additional markers. Rather, we have focussed on establishing the NASBA technique for for detection of a number of biomarkers including DD3, PSA Hepsin and PSMA as indicated by our recent publication (Landers et al 2005). As a consequence, we have not undertaken any work with animals. We have the assays working well and plan to multiplex the biomarkers using fluorescent molecular beacons specific for each marker in the New Year.