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14. ABSTRACT In the United States, prostate cancer has become the second most frequently diagnosed neoplasm and the second leading cause of cancer mortality in men. The main objective of this study is to develop a serum-based test for prostate cancer using DNA methylation of one or more candidate genes to improve the predictive value of prostate cancer screening, which is currently based on PSA > 4 ng/ml and/or abnormal DRE. Candidate genes include pi-class glutathione s-transferase (GSTP1), E-cadherin (ECAD), retinoic acid receptor β -2 (RAR β), and ras association domain family 1 protein isoform 1A (RASSF1A). 46 patients were enrolled in the study for the reporting period. DNA extracted from the serum of these patients was bisulfite treated and analyzed by MSPCR. For this, we optimized the MS-PCR conditions for analysis of methylation of candidate genes proposed in the study. We have summarized the histopathological diagnosis along with the methylation status of the candidate genes. However we are not able to report any outcome of the study since 46 samples are not sufficient for conducting statistical analysis.					
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Table of Contents

Cover.....	1
SF 298.....	2
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	8
Reportable Outcomes.....	8
Conclusions.....	8
References.....	8
Appendices.....	N/A

Introduction:

In the United States, prostate cancer has become the most frequently diagnosed neoplasm and the second leading cause of cancer mortality in men after lung cancer [1]. Approximately 80 percent of patients with prostate cancer are diagnosed while the disease is clinically confined to the prostate. The commonly employed tests for prostate cancer screening include digital rectal examination (DRE) and serum prostate specific antigen (PSA). *It is estimated that two out of three patients with PSA levels greater than 4.0 ng/ml will have a negative biopsy for prostate cancer [2-4]* . A biomarker that increases the specificity of current screening techniques can have a substantial economic impact by eliminating unnecessary prostate biopsies.

Several studies have shown the presence of tumor derived free DNA in the serum and/or plasma of cancer patients [5]. *Aberrant DNA methylation has been demonstrated in the serum/plasma of patients with non-small cell lung cancer, breast cancer, esophageal adenocarcinoma, and prostate cancer [6-9]. Further, aberrant serum/plasma DNA methylation appears to be a specific marker for cancer in small retrospective studies reported so far [6-9].*

None of the studies have prospectively examined the association between aberrantly methylated DNA in plasma/serum and cancer in a large number of patients. In this study, we propose to systematically study aberrant promoter methylation of certain genes in serum DNA as a biomarker in patients suspected to have prostate cancer.

The main objective of this study is to develop a serum-based test for prostate cancer using DNA methylation of one or more candidate genes to improve the predictive value of prostate cancer screening, which is currently based on PSA > 4 ng/ml and/or abnormal DRE. Candidate genes include pi-class glutathione s-transferase (GSTP1), E-cadherin (ECAD), retinoic acid receptor β -2 (RAR β), and ras association domain family 1 protein isoform 1A (RASSF1A). These four genes are commonly methylated and silenced in prostate cancer but not in benign conditions. A DNA-methylation test would be useful in conjunction with standard screening if (a) its false negative rate is low (i.e., sensitivity is high), so that relatively few prostate cancers are missed; and (b) it reduces substantially the number of false positives arising from standard screening (i.e., improves specificity), thereby reducing the number of patients having unnecessary prostate biopsies. Thus, we intend to develop a DNA-methylation profile of candidate genes that would be applied to patients who are positive on one or both of the standard prostate-cancer screening tests

Body:

Specific Aims

- 1. To determine the prevalence of candidate gene methylation in serum DNA of men who screen positive for prostate cancer using standard criteria (i.e., PSA > 4 ng/ml and/or abnormal DRE) at the University of Miami and affiliated hospitals:**
 - a. To compare the relative frequency of candidate gene methylation in serum DNA of men who are subsequently found to be biopsy-positive as opposed to biopsy-negative.

So far we have enrolled 46 patients. Histopathological diagnosis is available for the patients. Thirty ml of blood was collected from all patients (1 heparinised and 2 plain tubes). The samples were centrifuged at 3400 rpm for 15 min at room temperature. The serum was collected from the plain tubes and the buffy coat and plasma from the heparinised tubes. DNA was extracted from 2 ml of serum using QIAamp® UltraSens™ Virus kit as per the manufacturer's instructions. This kit was chosen as it can be used to extract DNA from large volume of serum. The DNA was eluted in 60µl of buffer AVE provided. Bisulfite treatment of the DNA was performed as per standard protocols. The bisulfite treated DNA was reconstituted in 100 µl of water. Of the 41 patients in whom biopsy was available 10 were positive for carcinoma.

Quantitative MS-PCR:

In the original proposal, we have mentioned that PCR amplification will be carried out using conventional method. When a real-time PCR machine became available in the laboratory (BioRad icycler™ RT-PCR system) we decided to use real time PCR for template quantification. Real-time PCR quantitates the initial amount of the template most specifically, sensitively and reproducibly, and is a preferable alternative to other forms of quantitative PCR, which detects the amount of final amplified product. Real-time PCR quantitation eliminates post-PCR processing of PCR products which reduces the chances of carryover contamination and removes post-PCR processing as a potential source of error. In comparison to conventional PCR, real-time PCR also offers a much wider dynamic range of up to 10^7 -fold (compared to 1000-fold in conventional PCR). It follows that the broader the dynamic range, the more accurate the quantitation.

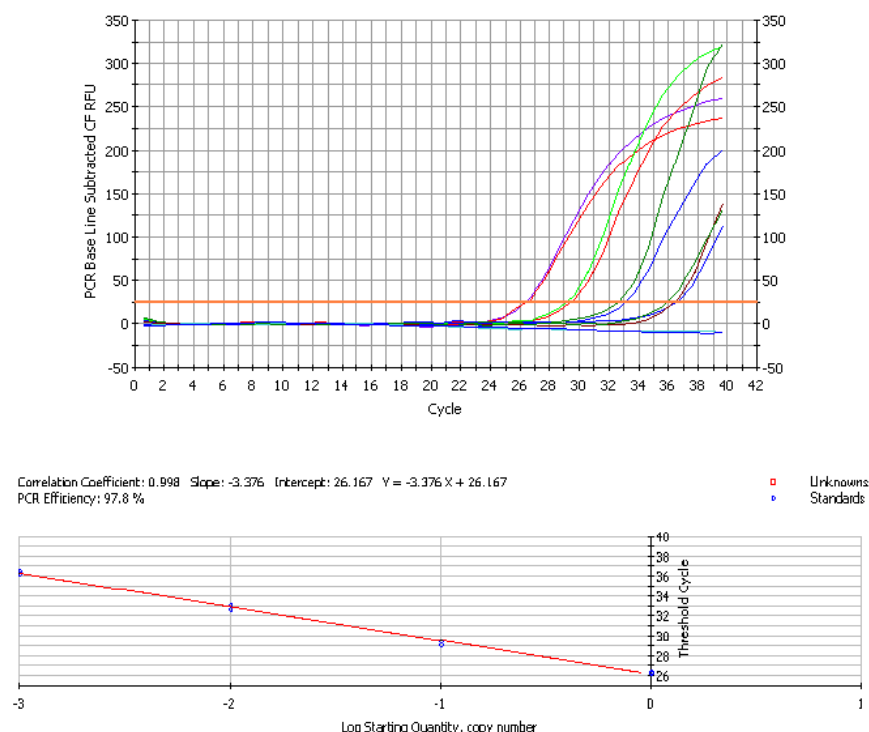


Figure 1. GSTPi MSPCR standard curve. Bisulphite treated DNA from LNCaP cells was diluted 10^0 , 10^{-1} , 10^{-2} , 10^{-3} and 5µl of each dilution was amplified with primers designed for bisulfite modified methylated GSTPi promoter sequence. Panel A. PCR quantification of the standards; Panel B. Standard curve.

Standard curves for RASSF1A, GSTPi and β -Actin were generated using bisulphite treated DNA from LNCaP prostate cancer cells. Input bisulfite treated DNA was diluted 10^0 , 10^1 , 10^2 , 10^3 , 10^4 and 5 μ l of each dilution was amplified with primers designed for RASSF1A, GSTPi and β -Actin. β -Actin is a housekeeping gene and used to calculate the normalized ratio. As an example we have shown the PCR quantification and standard curve for methylated GSTPi.

Patient samples were then analyzed for the three genes. MS-PCR products were analyzed by running on a 3% agarose gel and visualized under UV light. The results were inconsistent. Moreover, the values did not fall within the standard curve making it difficult to analyse the data. Optimisation of Quantitative MSPCR for patient samples turned out to be expensive because of the large number of subjects to be included in the study. Hence we switched to the conventional MSPCR method for methylation analysis.

Conventional MSPCR analysis:

We have optimized the MS-PCR conditions for analysis of methylation of candidate genes proposed in the study. We designed different primer sets for each gene and tested the best primer pair suited for carrying out the assay. We sequenced the PCR product to confirm that the primers amplify the specific sequence. Primer pairs that amplified the right sequence were chosen for MSPCR analysis of patient samples.

The panel below depicts a representative MSPCR carried out for the four candidate genes with 7 patient samples. Bisulfite treated DNA from LNCaP prostate cancer cells is used as positive control since all the four genes are methylated in LNCaP. To test the quality of bisulfite modified DNA, we carried out PCR with primers that amplify the bisulfite modified beta actin sequence (not shown).

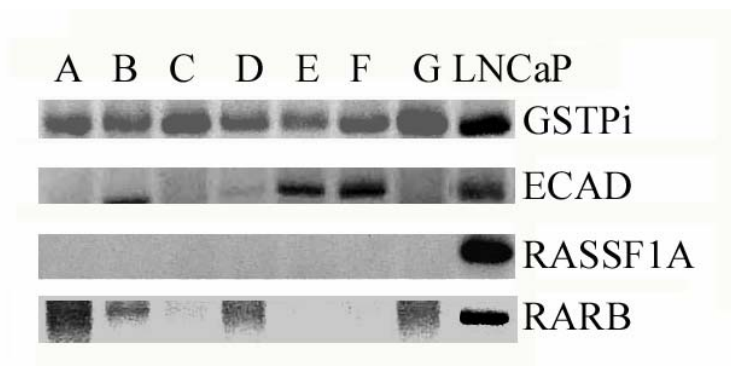


Figure 2. MSPCR analysis of patient samples. MSPCR was done using primers that amplify bisulfite modified methylated sequences. The PCR product was then run on a 3% agarose gel stained with Ethidium bromide and visualized by UV light.

Table 1: Methylation status of candidate genes in serum DNA of suspected prostate cancer patients.

SAMPLE NO	AGE	G.SCORE	G.SCORE (TOTAL)	BIOPSY	PSA	METHYLATION STATUS			
						GSTPI	RARB	ECAD	RASSF1A
1	63	3+4	7	+	19.81	+	+	+	-
2	67			A	11.69	+	-	+	-
3	61	3+4	7	P	5.13	-	-	+	-
4	77	3+4	7	+	18.01	-	-	+	-
5	56	3+3	6	+	6.44	+	-	-	-
6	60			P	1.57	+	+	-	-
7	64			H	3.24	-	-	-	+
8	60	3+4	7	+	75.66		-	-	-
9	65			B	11.41	-	-	-	-
10	72			B	12.45	-	-	-	-
11	64			B	13.02	+	-	-	-
12	75	4+5	9	+	9.51	+	+	-	-
13	61			B	4.41	+	-	-	-
14	75	3+3	6	+	9.91	+	-	-	-
15	63			**	24.85	+	+	+	-
16	63	3+3	6	+	10.65	+		-	-
17	68	3+3	6	+	14.18	+	+	-	-
18	75			N/A	4.27	-	-	-	-
19	62			P	6	+	+	-	+
20	60			P	11.55	+	-	-	-
21	60			P	10.62	+	-	+	-
22	67			P	23.76	+	-	+	-
23	58			P	7.19	+	-	+	-
24	62			N/A	2.08	+	-	+	-
25	64			P	8.89	-	-	-	-
26	68	5+3	8	+	5.63	+	-	-	-
27	66			B	0.44	+	-	+	+
28	64			P	5.65	-	-	-	+
29	65			B	7.3	-	-	+	-
30	67			B	5.04	-	-	+	-
31	62			P	4.71	+	-	+	-
32	61			P	N/A	-	-	+	-
33	57			B	8.2	-	-	-	-
34	63	3+3	6	+	13.34	+	-	-	-
35	69			P	1.21	+	+	+	-
36	84			B	N/A	-	-	-	-
37	56			B	4.43	+	-	-	-
38	61			B	5.53	+	-	+	-
39	61			N/A	5.43	+	-	+	-
40	74	3+3	6	+	5.78	+	+	+	-
41	72			B	12.79	+	-	+	-
42	66			B	8.84	-	-	-	-
43	63			B	9.74	-	-	-	-
44	69			P	4.54	+	-	-	-
46	64	3+3	6	+	12.95	+	-	-	-

N/A: Test not done
 P: Prostatitis
 H: Hyperplasia
 B: Benign
 A: Atypical
 (+): Prostate Cancer
 ** other cancer (bladder cancer)

} Benign

Key Research Accomplishments:

- We have enrolled 46 patients for this study so far.
- Serum DNA has been extracted from the blood. Bisulfite treatment of the DNA from 49 patients has been completed.
- We have optimized PCR conditions for GSTPi, ECAD, RASSF1A and RARB.
- We have performed MS-PCR for the four candidate genes on the serum DNA samples.

Reportable Outcomes:

It is too early to report any outcome of the study since 46 samples are not sufficient for conducting statistical analysis. We will need to analyze a sizable number of patients to arrive at any preliminary conclusion about the statistical significance of methylation in diagnosis of prostate cancer.

Conclusions:

This progress report covers period May 2004 – April 2005. However, since we received the IRB approval only at the end of December 2004 we could not enroll 200 patients as was proposed for this year. The above results indicate that we have made good progress towards meeting the goals set out in the statement of work. We will request a no-cost extension to complete the study.

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Appendices:

None