Analysis of a Circulating Microvesicle-Based Assay in At-Risk Patients for the Detection of Prostate Cancer

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PURPOSE

To determine if a circulating microvesicle based immunoassay can be used to identify which patients have prostate carcinoma.

ABSTRACT

Introduction: While PSA-based testing has improved the ability to detect prostate cancer (PCa), it is limited by low sensitivity and specificity. Circulating microvesicles (cMV) are membrane-bound structures in the blood that carry material from their cell of origin. PCa patients have cMV with biosignatures that correlate with the presence of disease. The aim of this study was to evaluate a novel cMV-based assay for the detection of PCa.

Methods: The assay was developed by selecting antibodies to protein biomarkers based on their ability to differentiate between men with and without PCa. Sensitivity and specificity were evaluated using retrospective frozen plasma samples from men with biopsy-confirmed, non-metastatic PCa (n=331) and controls (n=197). Prostate-specific cMV were captured and analyzed. Samples were blindly classified as positive, negative, or borderline according to Median Fluorescence Intensity (MFI) values acquired by a flow cytometry-based method.

Results: The assay was successfully run on 528 of 649 (84%) of cases and controls. Pre-analytic sample collection conditions (e.g. centrifugation, temperature) resulted in 100 samples having anomalously high MFI, and there was no result for the remaining 21. The overall sensitivity, specificity, and Receiver Operating Characteristic Curve (ROC) area under the curve (AUC) in the validation cohort were 83%, 86%, and 0.92, respectively. In a subset of samples with known PSA values (n=487), the assay AUC for the cMV assay was 0.92, which was significantly higher ($P<10^{-10}$) than the 0.70 AUC for PSA in this cohort.

Conclusions: The cMV-based prostate cancer test is a promising new assay for the detection of PCa, with performance characteristics superior to serum PSA. While further validation in a larger prospective cohort is needed, this assay could significantly improve PCa detection, thus enabling physicians to make more informed decisions regarding invasive testing and therapies.

METHODS

Patient Population: Frozen plasma samples (N=649) were obtained from retrospective collections after IRB approval. 121 samples failed quality control and were not included in the analysis leaving a final cohort of 528. Biopsy-confirmed prostate cancer samples (N=331) were obtained from two commercial sources Asterand Detroit, MI and Innovative Research Novi, MI (N=23)]; and two academic sources [University of Washington, Seattle, WA (N=158); and Washington University, St. Louis, MO (N=150). Control samples (N=197) were obtained from four sources: (Asterand and Innovative Research)(N=106); The University of Alabama at Birmingham, AL (N=3); and The University of Arizona, Tucson, AZ (N=88). Samples from commercial sources and The University of Alabama at Birmingham originated from self-declared non-prostate cancer subjects. Samples from The University of Arizona were confirmed negative for prostate cancer by two negative biopsies.

Analysis: Blood was collected in K2-EDTA tubes and centrifuged at room temperature to isolate the plasma layer. Plasma samples were then immediately frozen and stored at or below -20ºC until tested. Samples were age range-matched and retrospective. A multiplex sandwich immunoassay was developed to detect microvesicles from prostate cancer cells. This assay is based on the antibody capture of microvesicles and subsequent detection of the captured microvesicles by phycoerythrin labeled anti-tetraspanin antibodies. Briefly, antibodies to specific protein biomarkers were selected for the assay that were either specific for cMV (tetraspanins CD9, CD63, and CD81), prostate cancer (PSMA and PCSA); and a cancer-associated biomarker (B7H3). The antibodies to PSMA, PCSA and B7-H3 were used to capture the microvesicles. Then the antibodies to the tetraspanins were used for the subsequent detection of the captured microvesicles . Signal thresholds that distinguishing between prostate cancer plasma and normal plasma were previously established in a training set. Samples scoring above the established thresholds for both a prostate marker (PSMA or PCSA) and the transformation marker (B7-H3) were classified as 'positive' while those scoring below thresholds were classified as 'negative'; and those that were indistinguishable from the threshold as 'borderline', thus resulting in a semi-qualitative determination of the correlation with prostate cancer.

Statistical Analysis: Performance statistics, measures of association, 95% confidence intervals and p-values were estimated in Matlab and R. AUC variance estimates were determined by sampling without replacement (jackknife). Samples classified as borderline or non-evaluable were not included in estimates of assay sensitivity, specificity and accuracy. Non-evaluable samples were also not included in ROC-AUC estimates.

RESULTS

Figure 1: *cMV Prostate Assay*

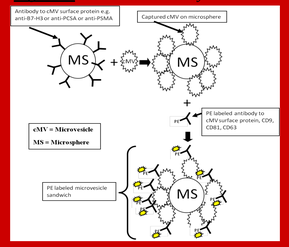


Figure 2: *SEM of Captured MV*

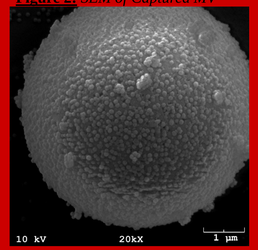


Figure 3: *Study Flow Chart*

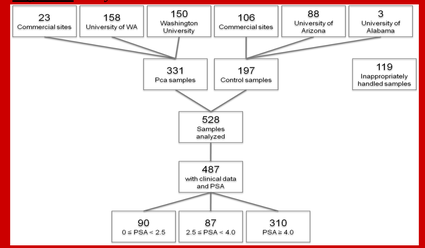


Table 1: *Demographics of Cases and Controls*

	Prostate Cancer (N=331) No. (%)	Control (N=197) No. (%)
Age (yr)		
<30	0(0)	3(1.5)
30-39	0(0)	4(2)
40-49	19(5.7)	23(11.7)
50-59	133(40.2)	40(20.3)
60-69	143(43.2)	99(50.3)
70+	36(10.9)	28(14.2)
Not reported	0(0)	0(0)
Ethnicity		
Caucasian	307(92.7)	97(49.2)
African American	19(5.7)	66(33.5)
Other	5(1.5)	13(6.6)
Not reported	0(0)	21(10.7)
PSA (ng/mL)		
0-2.5	15(4.5)	75(38.1)
2.5-4.0	59(17.8)	28(14.2)
4.0-10.0	197(59.5)	55(27.9)
10.0+	42(12.7)	16(8.1)
Not reported	18(5.4)	23(11.7)

Table 2: *Results*

Test Outcome	Assay data for entire cohort	for men with reported PSA	for men with 0 ≤ PSA < 2.5	for men with 2.5 ≤ PSA < 4	for men with PSA ≥4
TP	235	221	10	40	171
TN	155	134	60	18	56
FP	25	24	8	5	11
FN	48	47	2	10	35
Borderline*	43	41	4	10	27
Non-evaluable*	22	20	6	4	10
Total	528	487	90	87	310
Sensitivity (95% CI)	83 (78-87)	82 (77-87)	83 (52-98)	80 (66-90)	83 (77-88)
Specificity (95% CI)	86 (80-91)	85 (78-90)	88 (78-95)	78 (56-93)	84 (73-92)
Accuracy (95% CI)	84 (81-87)	83 (79-87)	88 (78-94)	79 (68-88)	83 (78-87)
Borderline rate (%)	8	8	4	11	9
Non-evaluable rate (%)	4	4	7	5	3
AUC (± S.E.)	0.923(± 0.013)	0.916 (± 0.015)	0.946 (± 0.025)	0.888 (± 0.044)	0.904 (± 0.025)
		P-value	0.31	0.54	0.68

Figure 4: *Performance of cMV assay*

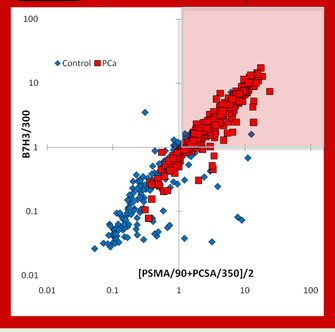


Figure 5: *ROC curves*

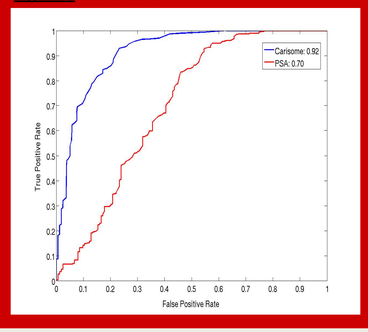
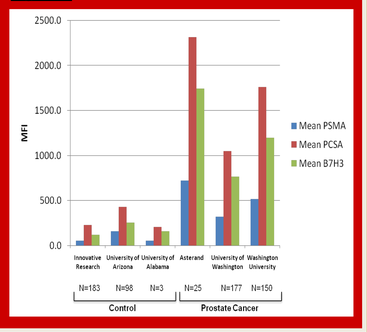


Figure 6: *Individual Site Data*



DISCUSSION

In part, the limitation of PSA for PCa screening is due to the fact that numerous nonmalignant processes, such as benign prostatic hyperplasia (BPH) and infection, can also result in PSA elevations. An assay which specifically detects biomarkers specific for cancer has the potential to dramatically improve our ability to detect malignancy. We have developed an assay that enables the isolation, capture and characterization of circulating microvesicles (cMV) specific for PCa. In this retrospective study, we demonstrated the cMV assay has high specificity and sensitivity for detecting PCa and outperforms PSA.

Circulating microvesicles (cMV) are heterogeneous membrane-bound structures approximately 30 to 1000 nm in diameter that are secreted into the blood and other body fluids by a variety of cell types in different tissues. They are involved in intercellular communication, inflammation, immune response modulation, and coagulation. Furthermore, they have been implicated in tumor progression, invasiveness and angiogenesis. Circulating microvesicles contain a variety of molecules that reflect their cellular origin (including membrane-bound proteins, mRNA and miRNA). cMV released by tumor cells may thus provide a source of potential tumor-associated biomarkers.

PSA derivatives such as PSA density and PSA velocity have been developed. While both play a role in prostate cancer detection, neither is cancer specific. Novel molecular forms of PSA such as free PSA and ProPSA are more cancer specific have improved sensitivity and specificity. Additional non PSA derived markers have been sought. The most promising markers are PCA3 and ETS fusion proteins. Both have a clear advantage in that they are PCa specific.

The shortcoming of this study is its retrospective nature. This not only introduces potential clinical confounder but importantly can influence which samples are available for analysis, since sample preparation is critical. A second issue is that cases and controls were from different institutions. However, in figure 6, one can see that readings are consistent across sites for cases and controls. A prospective trial is being designed to ensure that data can be replicated.

CONCLUSIONS

In this study, we report the performance of the first blood-based immunoassay that utilizes a unique biosignature based on circulating microvesicles (cMV) for detection of prostate cancer. The cMV assay substantially outperforms PSA in this retrospective cohort and shows considerable promise as an additional tool to aid clinical decision making.