Characterization of PSA associated with EVs from prostate cancer samples to develop a lateral flow diagnostic test.

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Background

Prostate Cancer is the most frequently diagnosed non-cutaneous urological cancer in men, accounting for 29% of diagnoses in the US[1]. Worldwide, PSA screening significantly reduces mortality[2], however PSA screening tests do not reliably discriminate between malignant (PCa) and benign (BPH) conditions, resulting in false positive results[3-4]. Recent work has demonstrated that serum PSA and EV-associated PSA are independent biomarkers, and EV-PSA can be used to classify samples as PCa, BPH, or healthy controls. Using the two-phase lateral flow EV-enrichment strategy, we are developing a point-of-care test for at-home screening using EV-associated PSA.

Objectives

1. **Demonstrate the basic function of a two-phase lateral flow prototype.**
2. **Demonstrate immunocapture of EVs from plasma samples on a two-phase lateral flow prototype.**
3. **Determine conditions for on-strip lysis of captured EVs.**
4. **Measure EV-associated PSA from healthy, PCa, and BPH samples.**

Methods

These experiments were performed following the MISEV2018 guidelines.

Antibodies and Antigens. Anti-tetraspanin (9PU, 63PU, 81PU) antibodies from Immunostep. When used for LF coating, used at 0.5 mg/mL. Two mouse anti-PSA monoclonal antibodies 8301 and 8311 (Medix) were used for capture and for conjugation to 40 nm gold. Goat anti-mouse IgG (Arista). Native human Prostate Specific Antigen ab78528 (Abcam).

Purified EV standards. EV standards from LNCaP, a metastatic prostate carcinoma that produces PSA, and PC3 cell lines, a bone metastasis of prostate cancer that does not produce PSA, and EV standards from Human Plasma were purchased from Novus Biologicals (now BioTechne). All EV standards were isolated by ultracentrifugation and microfiltration. EVs are quantified and validated for protein content and particle number by Nanoparticles Tracking Analysis (NTA). All standards underwent phenotyping assays for presence of CD9, CD63, and/or CD81 by flow cytometry.

Plasma samples. 10 normal healthy, 10 benign prostatic hyperplasia, and 10 prostate cancer plasma samples were acquired under IRB-approved collections. All samples are stored at -80°C until use.

Extracellular vesicle enrichment. Enrichment of EVs from human plasma was performed using a combination of silicon carbide resin matrix and size exclusion chromotography and concentration using the Norgen Biotek Plasma/Serum Exosome Purification Kit (Norgen). Each preparation underwent NTA analysis with a Nanosight (Malvern Panalytical).

Immunoassays. Total PSA ELISA was purchased from Alpco and run according to the manufacturer's instructions.

Nitrocellulose Strips. The EV Capture zone was made by striping three anti-EV capture lines with a mix of 0.5 mg/mL anti-CD9, anti-CD63, and anti-CD81.The Anti-PSA Test Line was made by striping 1.0 mg/mL of anti-PSA capture. The Control Line was made by striping 0.25 mg/mL of goat anti-mouse IgG capture.

Lateral Flow Assay. EV-PSA prototype lateral flow strips were used without housing for all experiments. Enriched EVs or plasma were diluted 1:2 in Sample Buffer (HEPES at pH 7.4+BSA) to a volume of 25 µL. Sample was pipetted directly onto the Sample Pad, allowed to flow for 1 min., then followed with 60 µL anti-PSA gold conjugate at 0.5 OD in Lysis Buffer (Tris buffer at pH 7.6 with NP40 and SDS).

Results

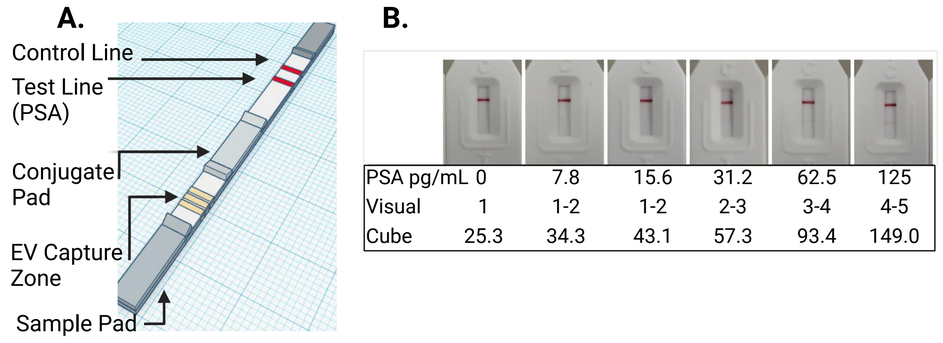

Figure 1. Two-Phase Lateral Flow for EV Capture and Lysis. A.Diagram of LFA with 3 striped lines of anti-tetraspanin antibodies in EV capture zone and Test (PSA) and Control (anti-species) lines upstream of conjugate pad. B. Images of assembled LFA with outer housing removed. Visual detection of less than 32 pg/mL of purified PSA in lysis buffer. A Cube reader (Chembio) was used to quantify test line signal intensity. Figure made in Biorender.

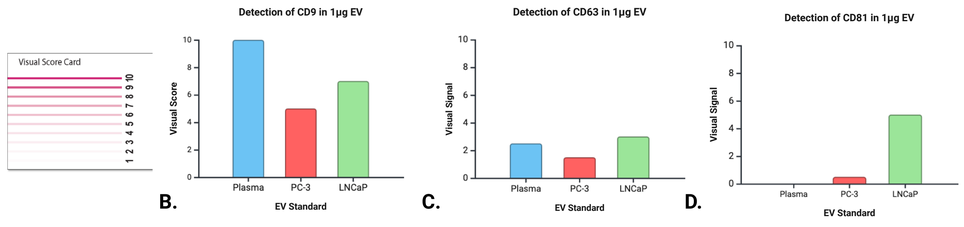

Figure 2. Differential Detection of EV Standards by Lateral Flow. A. Visual score hard used to determine semi-quantitative measurement of signal intensity. Three individual test lines were striped with antibodies to CD9, CD63, and CD81. A total of 1 µg in 20 µL were incubated with each strip, and detected with a mix of gold conjugated antibodies (CD9+CD63+CD81). Intensity of each line for each sample was recorded after 15 minutes. PSA-expressing LNCaP were differentially captured by CD81, but were similar by CD9 and CD63. Graphs made in Biorender.

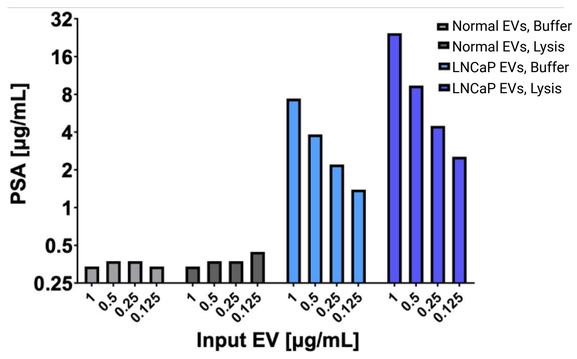

Figure 3. Quantification of PSA in LNCaP EV Standards by ELISA. Increasing amounts of Normal Plasma EVs or LNCaP EVs were tested for PSA levels by ELISA. Mean values of duplicate wells are graphed. Pre-incubation of EVs with a Lysis Buffer increased the detection of PSA in LNCaP EVs. Figure made in Biorender.

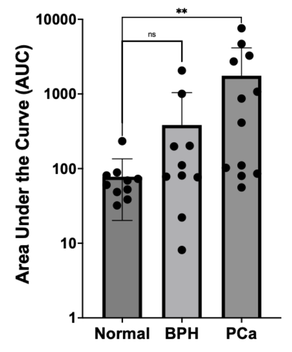

Figure 4. EV-PSA Lateral Flow on EVs. 20 µL EVs from plasma samples from Normal, BPH, and PCa samples were tested on EV-PSA strips. Images were quantified for test line intensity using ImageJ for AUC values. ** p<0.01

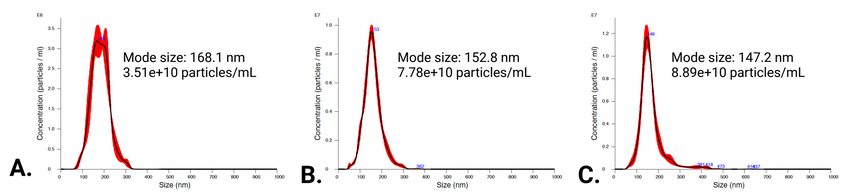

Figure 5. Nanosight Analysis of EVs Enriched from Plasma. A. One representative sample from Normal plasma. B. One representative sample from BPH plasma.C. One representative sample from PCa plasma. All enriched EV preparations undergo NTA analysis; one example from each group is shown here.

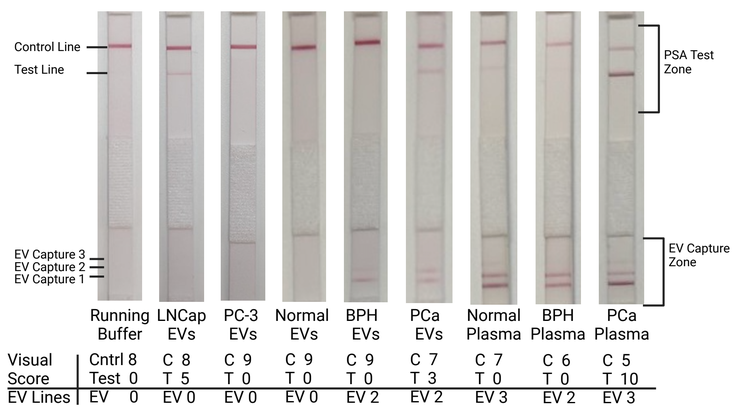

Figure 6. EV-PSA Testing of Enriched EVs and Plasma. Samples bind the EV capture lines prior to lysis with Lysis Buffer on the strip. Detection antibody is anti-PSA gold. The sample type is listed below the strip image, with the associated visual score of the Control and Test lines, indicating the detection of PSA released from EV after lysis. The visible line count is shown as EV Lines below each strip, indicating detection of PSA associated with the captured EVs. Figure made in Biorender.

Discussion

Several methods were used to demonstrate detection of PSA in EV standards and in human plasma. The two-phase lateral flow design was first tested with EV standards to demonstrate feasibility of the assay and to show the sensitivity of detection of PSA at the test line. We also demonstrated detection of EVs in the EV capture zone from about 1 µg of input material. Next, we demonstrated in purified LNCaP EV standards that addition of a lysis buffer increased the amount of detected PSA. Last, normal, BPH, or PCa plasma samples tested on the EV-PSA prototype using the anti-PSA gold conjugate showed that the PCa group had higher levels of detectable PSA. Interestingly, PSA was also detected on the EV capture lines at similar levels between BPH and PCa samples. This is consistent with the work by Sandúa *et. al.*[6], which showed EV-associated PSA measured by ELISA. Our results demonstrate that the PSA cargo released from the EV capture zone can distinguish between PCa and BPH, in addition to distinguishing from normal to PCa. This could result in lowering the false-positive rate of other PSA-based screening tests for prostate cancer. Future work will focus on characterizing the location of EV-associated PSA (cargo, internal but membrane associated, or external membrane associated) using super-resolution microscopy to identify internal and external markers of PSA-containing EVs in cell lines and in human plasma samples.

Conclusions

1. A two-phase (EV capture and cargo test) was designed for detection of EV-associated PSA.
   a. Fig. 1: LOD of EV-PSA assay 32 pg/mL.
2. EVs from Plasma, PC-3, and LNCaP cell lines were differentially detected by anti-tetraspanin lateral flow stripes.
   a. Fig. 2: EV capture zone binds EVs; LNCaP EVs had more detectable CD81 .
3. Lysis conditions determined for on-strip release of cargo.
   a. Fig. 3: Lysis buffers containing detergents increased the amount of detectable PSA from immunocaptured EVs.
4. EV-associated PSA from normal, BPH, and PCa plasma samples was measured by EV-PSA lateral flow prototype tests.
   a. Fig. 4: EVs enriched from PCa plasma samples had statistically higher EV-associated PSA levels.
   b. Fig. 5A-C: NTA characterization of enriched EVs had a median size of 180 nm.
   c. Fig. 6: EV-PSA testing of enriched EVs and plasma demonstrated detection of PSA in the Test Line after addition of lysis buffer.
      i. Higher concentration of PSA is seen in plasma
      ii. PSA is detected on EV capture lines as well.

Future Directions

- Optimize sample pad for red blood cell exclusion by testing LNCaP spiked whole blood.
- Test freshly collected samples, both plasma and whole blood, with paired PSA results.
- Further investigate PSA as cargo or EV membrane-associated.

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