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In-depth proteomic analyses of exosomes isolated from expressed prostatic secretions in urine

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Abstract

Expressed prostatic secretions (EPS) are proximal fluids of the prostate that are increasingly being utilized as a clinical source for diagnostic and prognostic assays for prostate cancer (PCa). These fluids contain an abundant amount of microvesicles reflecting the secretory function of the prostate gland, and their protein composition remains poorly defined in relation to PCa. Using expressed prostatic secretions in urine (EPS-urine), exosome preparations were characterized by a shotgun proteomics procedure. In pooled EPS-urine exosome samples, ~900 proteins were detected. Many of these have not been previously observed in the soluble proteome of EPS generated by our labs or other related exosome proteomes. We performed systematic comparisons of our data against previously published, prostate-related proteomes and global annotation analyses to highlight functional processes within the proteome of EPS-urine derived exosomes. The acquired proteomic data has been deposited to the Tranche repository and will lay the foundation for more extensive investigations of PCa derived exosomes in the context of biomarker discovery and cancer biology.

Keywords

Exosome; Expressed Prostatic Secretion; Proteomics

Exosomes are small vesicles (30–100 nm) secreted from most cell types and are known to contain protein, RNAs and lipids [1]. In relation to cancer, there is evidence that exosomes play a role in carcinogenesis by affecting processes like angiogenesis, invasion/metastasis, anchorage-independent growth, immune system evasion and proliferation [2, 3]. Thus, exosomes could potentially serve as rich reservoirs of tumor-specific proteins capable of

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acting as biomarkers for disease detection and progression [4]. Specific to the prostate gland, prostasomes are exosome-related vesicles secreted by prostate acinar epithelial cells present in prostatic secretions, seminal fluid and ejaculate that function in normal reproductive physiology and sperm activities [5]. Structurally, prostasomes are heterogeneous in size (40–200 nm) and distinct from other exosomes in their membrane lipid composition, they primarily consist of high proportions of cholesterol, sphingomyelin and phosphatidylethanolamine lipids [6]. In addition to seminal fluid-derived sources [7], other studies have shown that prostate-derived vesicles can be isolated from plasma [8], urine [9], human tissues [10] and cell lines [11]. In the context of biomarker discovery, prostasomes derived from PCa patients likely contain a tumor-enriched repertoire of biomolecules (i.e. proteins, miRNAs, lipids, etc.) that could represent a novel and easily isolatable biomarker source. For this reason there is great interest in developing high-throughput strategies to isolate and characterize exosomes and related prostasomes from human bodily fluids [12].

Expressed prostatic secretions in urine, EPS-urine, are collected in voided urine after digital rectal examination (DRE) [13]. For EPS-urines, multiple genetic assays (PCA3, TMPRSS2) for PCa diagnostics are being developed [14], resulting in a recent FDA approval for the PCA3 commercial assay. EPS-urine contains both the exosome components found in urine, as well as prostasomes secreted from the prostate. For this study, we refer to both as EPS urine exosomes.

Our collaborative research group has recently published three proteomic studies characterizing EPS fluids associated with different stages of benign prostatic conditions and PCa [15–17]. Taking advantage of these previous studies and their associated proteomes, exosomes from EPS-urine samples were isolated by standard ultracentrifugation methods. Next generation ultra-performance liquid chromatography-mass spectrometry (UHPLC-MS) analysis was then performed across exosome pools isolated from low-grade PCa patients and non-cancer controls. One emphasis was on the global annotation of this large resource of EPS urine-derived exosomes, as well as the systematic comparison to previously published data on prostatic secretions [15–17] and urine-related exosomes [18].

All samples were collected from patients after informed consent following Institutional Review Board-approved protocols at Urology of Virginia, Sentara Medical School, and the Eastern Virginia Medical School along with the Research Ethics Board of the University Health Network, as previously described [13]. Pools (2 ml/sample) of EPS-urines were derived from 12 patients classified as having low grade, Gleason 6, organ-confined PCa and 12 non-cancer patients confirmed as biopsy negative for PCa. Each sample was selected for expression of PSA in the fluids at $>15 \mu\text{g/ml}$ (Supplemental Table 1). Exosomes were isolated by standard differential ultracentrifugation [19] with a dithiothreitol/sucrose wash step [12], processed using 2,2,2-trifluoroethanol (TFE) and prepared for MS analyses as previously described [20]. Each sample was analyzed in duplicate using an UHPLC system on-line to a Q-Exactive mass spectrometer equipped with a nano-electrospray ion source. Raw data were analyzed using the MaxQuant platform [21] to obtain peptide/protein identification (FDR $<1\%$). Functional annotation and comparisons to other published data was accomplished using the David bioinformatics resource [22] and the ProteinCenter software suite. See the Supplementary Methods for detailed information.

The analysis workflow is summarized in Figure 1A, and in total, close to 900 proteins were identified in the two EPS-urine exosome pools, representing the most comprehensive data for this clinically relevant fluid to date (Supplemental Table 2). These EPS-urine exosome protein lists were then compared to our previous analyses of prostatic secretions [15–17] to determine whether this fractionation step enriched for target proteins previously identified in the fluid studies, and whether novel vesicle-associated protein targets were identified. In

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addition, the generated EPS-urine exosome proteome was compared against a comprehensive healthy human urine exosome protein list [18]. This comparison could facilitate removal of exosomal proteins from the general urine background, hence enriching for prostatic proteins within our data. First, we performed global gene ontology (GO) analyses to obtain a general overview of enriched functional classes within the ~900 EPS-urine exosome proteins. In Figure 1B we show representative categories of the most significantly enriched GO terms. Within the molecular function (GO: MF) category, the top enriched terms were related to proteolytic enzyme function, highlighting the importance of exosomes in extracellular matrix modulation. In the cellular component (GO: CC) category, the top terms were linked to extracellular matrix, plasma membrane and endosomes, highlighting both the biogenesis and extracellular localization of exosomes. Interestingly, in the biological process (GO: BP) category, the top terms were involved in inflammation, defense response and complement activation. Next, we compared our EPS-urine exosome proteome against proteomic data from direct-EPS [15, 16] and EPS-urines [17] that our groups have recently published (Figure 1C). As expected, a large overlap was found between these independent proteomics data, with prostate enriched proteins such as prostate specific antigen (PSA), prostatic acid phosphatase (ACPP), prostate transglutaminase (TGM4) and prostate-specific membrane antigen (PSMA) found consistently among all studies. The majority of currently used exosome markers were also found in common among all analyzed proteomics data and included: membrane surface markers (CD9, CD63, CD81), programmed cell death 6-interacting protein (Alix), annexin-2 and 5 (ANXA2/5) and tumor susceptibility gene 101 (TSG101). This evidence suggests that, proteins found in urine exosome preparations are relatively abundant and thus also detected when the whole fluid is analyzed, or alternatively, some of these proteins could also exist as a soluble form. On the other hand, flotillin-1/2 was only detected in the enriched exosome samples, highlighting that exosome enrichment does indeed enrich for a different overall proteome.

Our second comparison was based on a recently published proteomic study of exosome proteins isolated from healthy human urine samples [18] (Figure 2A). The majority of proteins identified in the EPS-urine exosomes were in common with this extensive database of urine exosome proteins. Similar to our comparison above, all exosomal markers (CD9, CD63, CD81, Alix, TSG101, FLOT1, FLOT2, ANXA2, ANXA5) and prostate-enriched proteins (PSA, ACPP, TGM4, PSMA) were found in both datasets. Since both datasets were recorded on different mass spectrometry platforms, direct quantitative comparisons are not possible. We therefore focused on identifying proteins exclusively detected in the EPS-urine exosomes (127 proteins in Figure 2) that could represent proteins unique to prostatic secretions. Global GO enrichment analyses of these 127 proteins revealed that the most significant functional categories were related to inflammation: immune response (27 proteins), response to wounding (21 proteins), defense response (19 proteins), inflammatory response (14 proteins) and complement activation (7 proteins) (Figure 1B). Since our exosome isolation protocol was virtually identical to the study on healthy urine derived exosomes by Schey and colleagues [18] it is reasonable to conclude that the majority of inflammatory proteins detected in our study arise from the prostatic secretion component of EPS urine with a likely link to PCa [23]. Alternatively, it is possible that immune-related proteins such as complement factors and immunoglobulins are derived from immune cells or blood present in these complex clinical fluids. Certainly the presence of high abundant contaminating proteins, in exosome preparations from cancer-related biofluids such as EPS-urine, must be taken into account and further verified before generalizing their presence to a clinical association with the cancerous condition. This potential caveat will require more sophisticated isolation procedures in the future.

There is a long history of prostate-associated microvesicle research, stemming largely from the definition of prostatic structure and physiologic functions [5]. Defining how the

proteins and other molecules in prostasomes are altered or contribute to benign and PCa disease in older men has been an important research question [24]. More recently, there has been an increase in prostate-related microvesicle research and their protein content, and this corresponds to continued improvement in mass spectrometry-based applications. In this paper we thought to take the first step in defining the target proteins associated with exosomes isolated from proximal fluids of the prostate. The readily obtained EPS-urine samples that are used in current PCA3 diagnostic tests in PCa patients are an ideal fluid for future exosome-based investigations as they are already being collected for clinical applications. Exosomes derived from EPS-urines can be used to detect *PCA3* and *TMPRSS2:ERG* mRNA [9]. The goal of the current study was to define the exosome proteome from EPS fluids using the latest generation of LC-MS equipment. Virtually all currently used exosome markers and prostate-enriched proteins were readily detectable using this technology. Comparison to previously published proteomics data using direct-EPS and EPS-urines without enrichment for exosomes highlighted that significant number of additional proteins were identified. Similar results were obtained by comparison to a large resource of healthy male urine derived exosomes, highlighting the potential contribution of prostatic secretions and inflammatory components. Potential advantages of exosome isolation from EPS-urines, as compared to an analysis of the “whole” EPS-urine (which also contains exosomes) are based on the well-known heterogeneity of clinically obtained urine samples and the broad dynamic concentration ranges of proteins in these fluids. Using an exosome enrichment step could be a way to better define protein targets and standardize the comparison of samples obtained from many individuals [12]. An additional advantage would be the analyses of other cargo molecules within EPS-urine derived exosomes such as mRNA and miRNA. Defining the protein components that are unique to the prostate-specific prostatic vesicles in EPS-urine exosomes facilitates design of specific immunological or other affinity capture strategies.

In summary, we report the largest resource of exosomal proteins identified for EPS urines, to date. Using a combination of TFE-based protein extraction and heated column UHPLC-MS of pooled EPS-urines from controls and low-grade prostate cancer patients, almost 900 proteins were identified. Interestingly, these next-generation proteomics analyses strategies were sensitive enough to provide significant depth of detection from as little as 24ml of unprocessed EPS-urine. Using this proteomics technology on larger EPS-urine cohorts and other prostatic secretions [15–17], in combination with exosomal enrichment, represent powerful approaches for the identification of biomarkers for PCa. The current study will provide an important benchmark for such future investigations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

DRE	digital rectal exam
EPS	expressed prostatic secretions

FDR	false discovery rate
GO	gene ontology

References

- [1]. Vlassov AV, Magdaleno S, Setterquist R, Conrad R. Exosomes: current knowledge of their composition, biological functions, and diagnostic and therapeutic potentials. *Biochim Biophys Acta*. 2012; 1820:940–948. [PubMed: 22503788]
- [2]. Luga V, Zhang L, Vilorio-Petit AM, Ogunjimi AA, et al. Exosomes Mediate Stromal Mobilization of Autocrine Wnt-PCP Signaling in Breast Cancer Cell Migration. *Cell*. 2012; 151:1542–1556. [PubMed: 23260141]
- [3]. Peinado H, Aleckovic M, Lavotshkin S, Matei I, et al. Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. *Nat Med*. 2012; 18:883–891. [PubMed: 22635005]
- [4]. Duijvesz D, Luider T, Bangma CH, Jenster G. Exosomes as biomarker treasure chests for prostate cancer. *Eur Urol*. 2011; 59:823–831. [PubMed: 21196075]
- [5]. Ronquist G, Brody I. The prostasome: its secretion and function in man. *Biochim Biophys Acta*. 1985; 822:203–218. [PubMed: 2992593]
- [6]. Arienti G, Carlini E, Polci A, Cosmi EV, Palmerini CA. Fatty acid pattern of human prostasome lipid. *Arch Biochem Biophys*. 1998; 358:391–395. [PubMed: 9784255]
- [7]. Utleg AG, Yi EC, Xie T, Shannon P, et al. Proteomic analysis of human prostasomes. *Prostate*. 2003; 56:150–161. [PubMed: 12746840]
- [8]. Tavoosidana G, Ronquist G, Darmanis S, Yan J, et al. Multiple recognition assay reveals prostasomes as promising plasma biomarkers for prostate cancer. *Proc Natl Acad Sci U S A*. 2011; 108:8809–8814. [PubMed: 21555666]
- [9]. Nilsson J, Skog J, Nordstrand A, Baranov V, et al. Prostate cancer-derived urine exosomes: a novel approach to biomarkers for prostate cancer. *Br J Cancer*. 2009; 100:1603–1607. [PubMed: 19401683]
- [10]. Di Vizio D, Morello M, Dudley AC, Schow PW, et al. Large oncosomes in human prostate cancer tissues and in the circulation of mice with metastatic disease. *Am J Pathol*. 2012; 181:1573–1584. [PubMed: 23022210]
- [11]. Hosseini-Beheshti E, Pham S, Adomat H, Li N, Tomlinson Guns ES. Exosomes as biomarker enriched microvesicles: characterization of exosomal proteins derived from a panel of prostate cell lines with distinct AR phenotypes. *Mol Cell Proteomics*. 2012; 11:863–885. [PubMed: 22723089]
- [12]. Alvarez ML, Khosroheidari M, Kanchi Ravi R, DiStefano JK. Comparison of protein, microRNA, and mRNA yields using different methods of urinary exosome isolation for the discovery of kidney disease biomarkers. *Kidney Int*. 2012; 82:1024–1032. [PubMed: 22785172]
- [13]. Drake RR, White KY, Fuller TW, Igwe E, et al. Clinical collection and protein properties of expressed prostatic secretions as a source for biomarkers of prostatic disease. *J Proteomics*. 2009; 72:907–917. [PubMed: 19457353]
- [14]. Salagierski M, Schalken JA. Molecular diagnosis of prostate cancer: PCA3 and TMPRSS2:ERG gene fusion. *J Urol*. 2012; 187:795–801. [PubMed: 22245323]
- [15]. Drake RR, Elschenbroich S, Lopez-Perez O, Kim Y, et al. In-depth proteomic analyses of direct expressed prostatic secretions. *J Proteome Res*. 2010; 9:2109–2116. [PubMed: 20334419]
- [16]. Kim Y, Ignatchenko V, Yao CQ, Kalatskaya I, et al. Identification of differentially expressed proteins in direct expressed prostatic secretions of men with organ-confined versus extracapsular prostate cancer. *Mol Cell Proteomics*. 2012; 11:1870–1884. [PubMed: 22986220]
- [17]. Principe S, Kim Y, Fontana S, Ignatchenko V, et al. Identification of prostate-enriched proteins by in-depth proteomic analyses of expressed prostatic secretions in urine. *J Proteome Res*. 2012; 11:2386–2396. [PubMed: 22339264]

- [18]. Wang Z, Hill S, Luther JM, Hachey DL, Schey KL. Proteomic analysis of urine exosomes by multidimensional protein identification technology (MudPIT). *Proteomics*. 2012; 12:329–338. [PubMed: 22106071]
- [19]. Pisitkun T, Shen RF, Knepper MA. Identification and proteomic profiling of exosomes in human urine. *Proc Natl Acad Sci U S A*. 2004; 101:13368–13373. [PubMed: 15326289]
- [20]. Taylor P, Nielsen PA, Trelle MB, Horning OB, et al. Automated 2D peptide separation on a 1D nano-LC-MS system. *J Proteome Res*. 2009; 8:1610–1616. [PubMed: 19178303]
- [21]. Cox J, Mann M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat Biotechnol*. 2008; 26:1367–1372. [PubMed: 19029910]
- [22]. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc*. 2009; 4:44–57. [PubMed: 19131956]
- [23]. Sfanos KS, De Marzo AM. Prostate cancer and inflammation: the evidence. *Histopathology*. 2012; 60:199–215. [PubMed: 22212087]
- [24]. Ronquist KG, Ronquist G, Larsson A, Carlsson L. Proteomic analysis of prostate cancer metastasis-derived prostasomes. *Anticancer Res*. 2010; 30:285–290. [PubMed: 20332430]

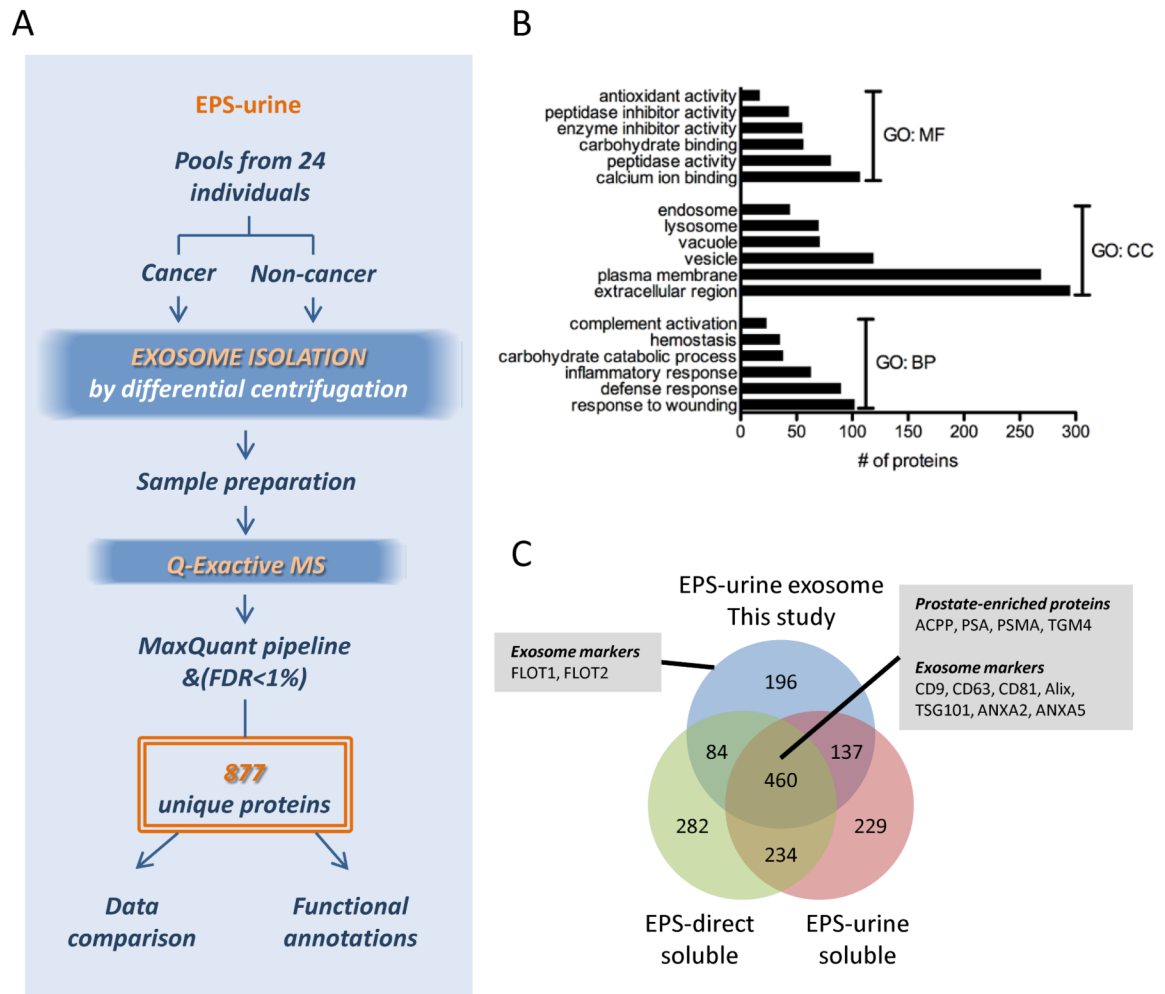


Figure 1. **A)** Workflow of the EPS-urine exosome proteome characterization. **B)** Selective, significantly enriched GO terms identified in the EPS-urine exosome proteome. **C)** Comparison of the current study to previously published prostatic secretion proteomics, published by our groups EPS-direct: [15, 16]; EPS-urine: [17].

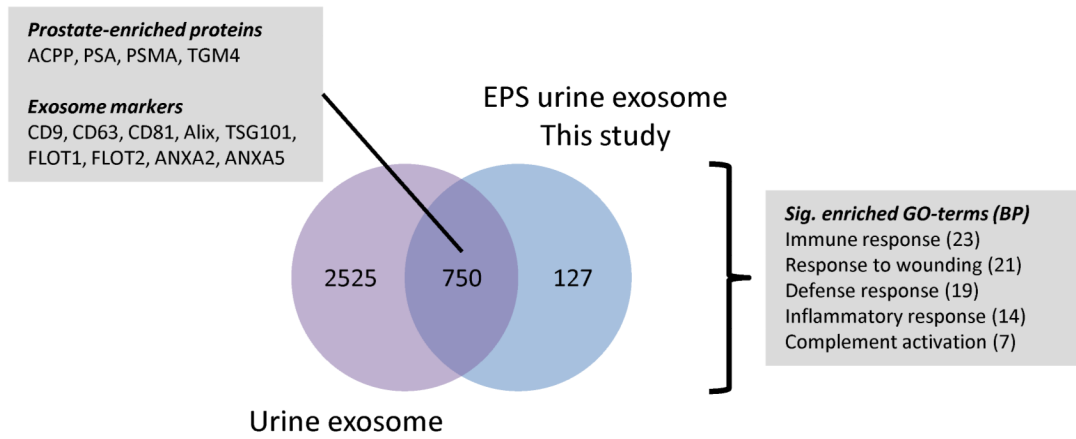


Figure 2.
Comparison of the current study to a previously published urine exosome proteome from healthy donors [18].