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Proteomic Analysis of Urine Exosomes by Multidimensional Protein Identification Technology (MudPIT)

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Abstract

Exosomes are membrane vesicles that are secreted by cells upon fusion of multivesicular bodies with the plasma membrane. Exosomal proteomics has emerged as a powerful approach to understand the molecular composition of exosomes and has potential to accelerate biomarker discovery. Different proteomic analysis methods have been previously employed to establish several exosome protein databases. In this study, TFE solution phase digestion was compared with in-gel digestion and found to yield similar results. Proteomic analysis of urinary exosomes was performed by multidimensional protein identification technology (MudPIT) after TFE digestion. 3280 proteins were identified from nine human urine samples with 31% overlap among nine samples. Gene ontology (GO) analysis, coupled with detection of all of the members of ESCRT machinery complex, supports the multivesicular origin of these particles. These results significantly expand the existing database of urinary exosome proteins. Our results also indicate that more than 1000 proteins can be detected from exosomes prepared from as little as 25 mL of urine. This study provides the largest set of proteins present in human urinary exosome proteomes, provides a valuable reference for future studies, and provides methods that can be applied to exosomal proteomic analysis from other tissue sources.

Keywords

microvesicles; exosomes; ESCRT pathway

Introduction

Exosomes are small (40–100 nm) intralumenal vesicles (ILVs) released into the extracellular environment when multivesicular bodies (MVBs) fuse with the plasma membrane [1, 2]. Exosomes are enriched in membrane and cytosolic proteins and contain an array of lipids, mRNAs, and microRNAs [3]. Most cell types can release exosomes into cell culture media *in vitro*, and recently it has been shown that exosomes can be found in a variety of body fluids such as blood, urine, saliva, gastric fluids and breast milk [4–8]. Exosomes from different cell origins share common groups of proteins that are involved in exosome biogenesis, and thus reflect many common biological functions of these particles such as mediating intercellular communication [9–11] or membrane material transfer [9–10, 12] and

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genetic information transfer [3, 12]. In addition, cell-type specific proteins are present that may reflect the origin and biological functions of the parent cells [9, 13]. Exosomes can be easily isolated from a variety of body fluids making them amenable to proteomic and transcriptomic analyses, which supports the hypothesis that their analysis may provide valuable information about disease diagnosis and monitoring. [14–16].

Urine is an ideal source for the discovery of new biomarkers because it can be collected noninvasively in large amounts. Urinary exosomes are derived from epithelia throughout the urogenital tract [5]; therefore, analysis of urine exosomes may provide potential biomarkers for kidney diseases involving every segment of the nephron, including podocytes [5, 17–18]. Urine exosome biomarker studies may also have potential diagnostic value for non-kidney diseases [19–20]. Pisitkun et al. first reported the presence of exosomes in urine, isolated them by ultracentrifugation, and characterized them by electron microscopy [5]. In later studies, they report optimized collection, storage, and preservation conditions for urine exosome isolation [21] and additional approaches have been introduced [18, 22–23]. Urine exosome proteomics has been studied by LC-MS/MS techniques and more than 1000 proteins have been identified in normal human urine exosomes [5, 17–18, 24]. In addition, several proteins that are present in exosomes have been identified in other studies as biomarkers of renal and urogenital diseases [14, 16, 25–26]. For example, the level fetuin-A was reported to be elevated 50-fold within hours following nephrotoxin exposure [14]. Urinary ATF3 was detected in AKI patients but not in normal subjects or patients with chronic kidney disease (CKD) and urinary WT-1 was detected in animal models before significant glomerular sclerosis [25]. 5T4 [16], prostate cancer gene 3 (PCA-3) and TMPRSS2: ERG [26] were all detected in prostate cancer-derived urine exosomes.

Multiple methods of analysis of urine exosome proteins have been reported including: SDS-PAGE (GeLC/MS), 1D LC/MS/MS, and 2D LC/MS/MS. In GeLC/MS analysis, urine exosome proteins were separated by SDS-PAGE and 40 gel bands were digested by in-gel digestion in the previous urine exosome analysis [24]. Considering the large numbers of samples needed for identification of disease-related biomarkers, alternative, more streamlined methods are needed. Nevertheless, compendia of exosomal proteins have been assembled as a resource for proteomics and biomarker investigations [5, 24, 27]. In this report, urine exosomal proteins from normal human urine were isolated and analyzed by Multidimensional Protein Identification Technology (MudPIT) following the method reported previously [6] with minor changes. Several methodological comparisons were made to achieve an optimized method that provides the largest number of confidently identified proteins. Digestion conditions were compared where trifluoroethanol (TFE) solution phase digestion was compared to the in-gel digestion approach that has been used by most exosome studies. In addition, a new generation of high efficieincy ion trap instrumentation (LTQ Velos) was compared to its predecessor, the LTQ. Lastly, the number of protein identifications was determined as a function of the starting urine volume to provide a guide for sample collection in clinical studies. The combination of TFE digestion and MudPIT techniques reported here have resulted in identification of a larger number of proteins beginning with a smaller and more easily handled volume of urine. In addition, our results significantly expand the known protein components of human urinary exosomes.

Material and Methods

Collection and isolation of urinary exosomes

Urine was collected from nine healthy adults (mean age 26.4±3.9 years, 6 Caucasian/3 African American, 5 male/4 female) in the Vanderbilt Clinical Research Center after written informed consent was obtained. All study procedures were approved by the Vanderbilt Institutional Review Board. We controlled for diurnal variability by collecting samples at

the same time of day in all subjects. The subjects were on a standardized sodium diet to control for inter-individual differences in self-selected dietary sodium intake and renin-angiotensin system activation. All subjects were relatively young, healthy volunteers without diabetes or elevated blood pressure and were screened to exclude cases of hematuria and proteinuria. About 200 mL of urine was collected from each person and urine samples from different persons were not mixed. Protease inhibitors were added immediately after urine collection as described previously [5], and specimens were stored at −80°C. Exosome isolation was accomplished using the protocol reported by Gonzales et al with minor changes [24]. Briefly, the urine samples (around 200 mL unless otherwise indicated) were first centrifuged at 17,000 g for 15 min at 4°C. The supernatant was then centrifuged at 200,000 g for one hour at 25°C. The pellets in each of 12 Beckman centrifuge tubes were suspended in 50 μL of 200 mg/mL of DTT in 10 mM triethanolamine and 250 mM sucrose, pH 7.4. The resuspended exosome pellets from six tubes were combined together in an eppendorf tube and heated at 95°C for 2 min. Then the suspension in each eppendorf tube was transferred to a centrifuge tube and 8 mL of 10 mM triethanolamine and 250 mM sucrose, pH 7.4 was added. The tubes were centrifuged at 17,000 g for 15 min. at 25 °C to remove any pellets left from the previous spins or newly formed during DTT reduction process. The supernatant was then centrifuged at 200,000 g for one hour at 25 °C. Pellets in the bottom of the tube were visible, but were clear and without any particle-like residues. The pellets were pooled and suspended in 200 μL of water and saved at −80 °C until further analysis.

Electron Microscopy

An exosome pellet from the last step of ultracentrifugation was re-suspended in 200 μL of phosphate buffered saline (PBS) and an aliquot (25 μL) was placed on a copper Formvar grid. After 1 minute, excess fluid was wicked off and 25 μL of phosphotungstic acid (1%), pH 5.1, was added. After 1 minute excess fluid was wicked off and the sample allowed to air dry. Electron micrographs were acquired on a Philips CM-12 microscope operated at 80.0 kV.

Sample preparation for proteomics analysis

Protein concentrations were measured by Bradford Assay and 50 μg of protein was used for each analysis unless less than 50 μg of protein was obtained from one preparation. If less than 50 μg of protein was obtained, the entire sample was used. Exosome suspensions were concentrated to around 20 μL by SpeedVac. The samples were further processed for TFE solution digestion or in-gel digestion. For in-gel digestion, the exosomes were solubilized in lithium dodecylsulfate (LDS) sample buffer (Invitrogen, Carlsbad, CA). The samples were loaded onto 4–12% NuPAGE Novex Bis-Tris gel and MOPs running buffer was used for separation (Invitrogen, Carlsbad, CA). The samples were allowed to run into the gel for 1.5–2 cm. The gel was fixed and stained with Colloidal Blue Staining kit (Invitrogen, Carlsbad, CA) then washed and the stained area was excised and diced to 1 mm cubes. Proteins were reduced with 45 mM DTT for 20 minutes at 55 °C, followed by alkylation with 100 mM iodoacetamide for 30 min at room temperature in the dark. The volume of iodoacetamide added was the same as the volume of DTT added. After reduction and alkylation, gel pieces were destained with three consecutive washes with a 50:50 mixture of 50 mM ammonium bicarbonate and acetonitrile for 10 min, dehydrated with 100% acetonitrile for 10 min, and dried in a SpeedVac. The gel pieces were rehydrated in a 50 μL of solution containing 10 ng/μL trypsin (Promega, Madison, WI) in 25 mM ammonium bicarbonate (pH=8.0) for 15 min. 100 μL of 25 mM ammonium bicarbonate buffer was added to each sample and the samples were incubated at 37 °C for 18 hours. Peptides were extracted using 60%ACN/ 0.1%TFA twice. The extracted samples were pooled and dried in a SpeedVac and reconstituted in 0.1% formic acid for subsequent analysis. For TFE solution phase digestion,

the exosomes were resuspended in 20 μL of 50%TFE in 100 mM Tris HCl, pH 8.0. Proteins were reduced by adding 0.8 μL of 0.5 M TCEP and incubated for 45 min. at room temperature and alkylated by adding 1.6 μL of 1 M iodoacetamide and incubated at room temperature for one hour in the dark. The sample then was diluted 10-fold with 100 mM Tris buffer (pH=8.0), followed by addition of 1μg of trypsin, and digested at 37 °C for 18 hours.

LC-ESI/MS/MS

Tryptic peptides from either in-gel digestion or TFE in-solution digestion were loaded onto a custom packed biphasic C18/SCX trap column (4 cm × 150 μm, Jupiter C18, 5 μm, 300 Å media followed by 6 cm × 150 μm, Luna SCX, 5 μm, 100 Å media). The trap column was coupled to a nanoflow analytical column (20 cm × 100 μm, Jupiter C18, 3 μm, 300 Å media). MudPIT analysis was done with a 11-step salt pulse gradient (25 mM, 50 mM, 75 mM, 100 mM, 150 mM, 200 mM, 250 mM, 500 mM, 750 mM and 1 M ammonium acetate). Peptides were eluted from the analytical column after each salt pulse with a 105 minute reverse phase solvent gradient (2% – 45% ACN containing 0.1% formic acid) for the first ten salt pulses and a 105 minute reverse phase solvent gradient (2%–95% ACN containing 0.1% formic acid) for the last salt pulse. The eluate was directly electrosprayed into an LTQ or LTQ Velos mass spectrometer. Both instruments were operated in a 6-step data dependent mode with one precursor scan event to identify the top five most abundant ions in each MS scan, which were then selected for fragmentation. Dynamic exclusion (repeat count 1, exclusion list size 150, and exclusion duration 60s) was enabled to allow identification of less abundant ions.

Data Analysis

Tandem mass spectra were analyzed using a suite of custom-developed bioinformatics tools. For database searching, all MS/MS spectra were converted to DTA files by Scansifter, a tool under development at Vanderbilt University Medical Center and searched on a 2,500 node Linux cluster supercomputer using a custom version of the Sequest algorithm [28]. The protein database was a concatenated forward and reversed (decoy) Uniprot human database (Version 155, Dec 31, 2009). All searches were configured to use a variable modification of carbamidomethylation of cysteine and oxidation of methionine residues. The combination of Xcorr and DeltaCN was used for score optimization and results were filtered to a 5 percent peptide false discovery rate (FDR) with the requirement for a minimum of two peptides per reported protein using IDPicker [27]. For single protein identification by IDpicker, the parameters used include: 1) minimum peptide length of 5; 2) maximum false positive rate (FDR) of 5%; 3) minimum unique peptides per protein of 2 and modifications to cysteines or methionines were not considered distinct from the ummodified peptides; 4) minimum additional peptides to establish a unique protein group of 2. Only one protein is reported per protein group and proteins that shared the same set of peptides (indiscernible from each other based on available data) belong to a single protein group. The protein-level FDR was also calculated and controlled below 5% by controlling the minimum spectra per protein.

Results

Proteomics analysis of urinary exosomes

Exosomes were prepared from a total of nine normal human urine samples. Most samples were isolated from approximately 200 mL of urine and the total protein was obtained from Bradford assay. Both BCA assay and λ280 absorption methods were tested to measure protein concentration. Results from the λ280 absorption method supported the Bradford assay results; however, the BCA results were more than ten times higher than Bradford assay results which could be due to interference by remaining DTT from the reduction process. Therefore, Bradford assay results were used to determine protein loading amounts.

Exosomal total protein concentration in the urine varied between samples from 0.11 μg/mL to 0.635 μg/mL (see Table 1). Isolated exosomes were also characterized by electron microscopy. Figure 1 shows a representative electron micrograph of isolated urine exosomes having diameters of approximately 100 nm. Based on proteomic analysis, significant amounts of uromodulin (Tamm-Horsfall protein) and serum albumin were present in some samples. Table 1 lists the exosome protein concentration in urine of the seven samples analyzed by LTQ and also lists the normalized spectral count (normalized to total spectral counts) for uromodulin and serum albumin. The high protein concentration correlates well with high abundance of uromodulin and serum albumin in most cases. Therefore, the significant association with these two proteins to exosomes in some urine samples at least partially contributes to the large variations of exosome protein concentration in urine.

Two digestion conditions, in-gel digestion and solution phase digestion (TFE digestion) were compared to determine the most efficient method of exosome protein digestion. For this comparison, 50 μg of exosome proteins from one urinary exosome preparation was split and either digested in solution or run into a short stack gel followed by in-gel digestion. The experiment was repeated using a separate exosome sample. After digestion, multidimensional protein identification technology (MudPIT) was used to identify the proteins in the urinary exosomes. A total of 11 raw data files were collected from each MudPIT analysis and a total of 22 raw files of one exosome sample (both in-gel and in-solution) were searched together. Beside the filters listed in the data analysis methods, a minimum of three spectra per protein were required to obtain less than 5% FDR. The actual in protein-level FDR was below 1% for both samples. The results are shown in Figure 2, which indicates that in-gel digestion and solution phase digestion are similar. The number of proteins identified in sample 1 was 1462 for in-gel digestion and 1491 for solution phase digestion, respectively. The number of proteins identified in sample 2 was 2028 for in-gel digestion and 2179 for solution phase digestion, respectively. The overlap of identified proteins between in-gel digested sample and solution phase digested sample was 90% for sample 1 and 94% for sample 2. Considering the easier procedure of solution phase digestion, all of the remaining comparisons were carried out by solution phase digestion.

A total of 9 samples were digested by solution phase digestion and analyzed by MudPIT. Seven samples were run on an LTQ mass spectrometer and two samples were run on an LTQ Velos mass spectrometer. The search results (a total of 9 MudPIT) were combined and minimum 5 spectra were required per protein to control the FDR below 5%. After removing decoy protein identifications, a total of 3280 proteins were identified with the actual protein-level FDR of 4.4%. On average, 2220 proteins can be identified from a single MudPIT analysis on an LTQ mass spectrometer whereas an average of 2599 proteins can be identified from one MudPIT analysis on an LTQ Velos mass spectrometer. An average of 10,237 peptides was identified from the LTQ analysis and an average of 12,388 peptides was identified from the LTQ Velos analysis. This result indicates that analysis on an LTQ Velos mass spectrometer yielded 17% more protein identifications, likely due to a 2-fold increase in scan speed and 5- to 10-fold enhanced sensitivity. In addition, due to the faster scan speed, analysis on an LTQ Velos increases the spectral counts dramatically. The average spectral count for one MudPIT analysis on LTQ Velos is 81,491 which is more than three times higher than an average spectral count of 24,284 for one MudPIT analysis on an LTQ instrument. Since the average protein identifications increased by only 17%, the high spectral count obtained from the Velos mass spectrometer was mainly due to repeated fragmentation of the same peptides, however, the high spectral count will be beneficial for quantitative analysis based on spectral counting.

The present study extensively expands the known urinary exosome proteome. The combined list of proteins identified in nine samples can be found in the supplemental materials.

Among the 3280 proteins identified from nine exosome samples from normal human urine (Supplemental Table 1), 2480 proteins (75%) were identified in at least five exosome samples and 1014 proteins (31%) were identified in every exosome sample analyzed. The protein-level FDR was reduced to 1% by increasing the minimum number of spectra to nine resulting in a total of 2620 protein identifications with 39% proteins (1025) common among all samples; however, all of the analysis in this paper was based on the protein list generated at 4.4% FDR. The variability of protein identifications among patient samples is likely due to patient-to-patient variability as well as the stochastic nature of the data dependent method used to acquire the data.

Despite the observed variability in protein identifications among patient samples, a series of common groups of proteins in exosomes were detected. Most of these proteins are relatively abundant in the exosome samples since they have high spectral counts and they are normally detected in every exosome sample analyzed. Thirty four proteins that have been reported in the ESCRT (Endosomal Sorting Complex Required for Transport) pathway [29] were detected. These proteins are summarized in Table 2. In this study 28 of the reported ESCRT pathway proteins were consistently detected, i.e. in each of the nine exosome samples. This result further demonstrates that the microparticles we isolated are from multivesicular bodies. A large number of proteins were detected which are involved in membrane trafficking, protein transport and exosome docking such as 12 annexin proteins, 32 RAB proteins, 5 RAP proteins, GDI1, GDI2 and RAPGEF3. A number of proteins that are involved in signal transduction are also highly abundant such as EGF, heterotrimeric G proteins (GNB1, GNB2, GNAI1, GNAI2, GNAI3, GNAS) and 14-3-3 proteins (zeta, epsilon, sigma, alpha, theta, eta and gamma). Beside these common proteins detected in exosomes, proteins indicating renal origin were also detected such as aquaporin-1, aquaporin-2, aquaporin-7, solute carrier family 12 member 1 (Kidney-specific Na-K-Cl symporter), solute carrier family 12 member 3 (Thiazide-sensitive sodium-chloride cotransporter), neutrophil gelatinase-associated lipocalin and sodium-hydrogen exchanger. Some of these proteins are potential urine biomarkers currently under investigation elsewhere [30], including neutrophil gelatinase-associated lipocalin, sodium-hydrogen exchanger, transferrin, gamma-synuclein, calreticulin, interleukin-18 and cystatin C.

Comparing this protein list with previously published urine exosome protein list (http://dir.nhlbi.nih.gov/papers/lkem/exosome/), 980 out of 1160 listed proteins (84.5%) were detected in our study. Among the 180 proteins not detected in our study, 39 proteins were no longer present in the Uniprot database used in this study. Comparing our results to the protein list in the ExoCarta database (http://exocarta.ludwig.edu.au/, Release date: 12 October 2010), 1231 unique proteins in ExoCarta human database were detected.

In total, 1788 new proteins that are not present in either above two databases were detected from nine exosome samples in the present study (Supplementary Table 2). The complete protein list can be found in the supplemental materials. Among the previously unreported urine exosomal proteins, 253 proteins were present in each of these 9 samples and another 221 proteins were present in eight of these 9 samples. 1276 proteins were present in more than half of the nine samples. Among the 143 most commonly found exosomal proteins as reported in the Exocarta database [10], 95% were detected in our analysis and 91% of proteins in this list were detected in each of these nine exosome samples.

Gene Ontology Analysis

The identified proteins were converted to their EntrezGene IDs resulting in 3183 unique EntrezGene IDs. These proteins were functionally categorized by WebGestalt [31]. The results indicate that proteins identified in the exosomes include a large proportion of membrane proteins (44%). The major processes that these proteins are involved in include

metabolic processes (51%), biological regulation (41%), localization (27%) and cell communication (24%). The protein list was also analyzed by the DAVID Bioinformatic Resources [32] and 163 proteins were characterized as plasma proteins and among them, 22 proteins were plasma specific. Functional category enrichment in exosome samples was evaluated by a hypergeometric test against the entire human genome using WebGestalt. The *p*-value was adjusted for multiple comparisons and a p-value of < 0.05 is considered an enriched GO category. The top 10 significantly enriched GO categories under biological process, molecular function, and cellular component are indicated in Figure 3. In the biological process category, GO terms related to vesicle-mediated transport (252 genes), protein transport (300 genes), catabolic process (495 genes) and protein metabolic process (799 genes) etc. are enriched in the exosome samples. In the molecular function category, GO terms related to purine ribonucleotide binding (559 genes) and pyrophosphatase activity (264 genes) etc. were overrepresented. Enriched pyrophosphatase activity includes numerous members of ATP-binding cassette transporters, G proteins and ATPases. In the cellular component category, enriched terms include cell fraction (344 genes), cytosol (532 genes), endosome (147 genes), membrane-bounded vesicle (222 genes) and pigment granule (66 genes) etc. Results of functional enrichment analysis further support the exosome origin of identified proteins since a number of processes related to protein transport, membrane-bounded vesicle and vesicle-mediated transport are enriched.

Urine volume study

Protein concentration in normal urine is very low compared with other body fluids and normal protein excretion is less than 150 mg/day or 100 mg/L [33]. The protein content of urine exosomes is estimated to be about 3% of total urine protein based on a previous report [21]. Isolation of exosomes by ultracentrifugation is currently the standard method to isolate uninary exosomes, but this step is time consuming because large volumes of urine are needed to produce sufficient amounts of protein for proteomics analysis. It is important to know the volume needed to obtain significant information from the urinary exosome analysis both for the analysis requirements and the urine volumes available (e.g. from catheterized patients). To test the relationship between the volume used and the number of proteins identified, two urine collections were pooled together and then sub-divided to 25 mL, 50 mL, 100 mL and 200 mL volumes, as described above. The spectral counts and number of proteins identified are listed in Table 3. As indicated by the results, the number of proteins identified did not significantly decrease with decreasing the urine volume. Increasing the starting urine volume from 25 to 200 mL increased the total spectral count, but only slightly increased the number of proteins identified. This result indicates more than 1000 proteins can be detected from urinary exosomes prepared from urine volumes as low as 25 mL using the method described in this paper. Based on the protein assay, the amount of total exosome proteins in 25 mL of urine is 11μg for this urine sample. In total, 1947 proteins were detected in four samples and 1272 proteins were detected in each of these four analyses. 65% of the identified proteins were detected in all four analyses.

Discussion

The presence of exosomes in biological fluids has begun to be exploited as a potential source of diseased-related biomarkers. Mass spectrometry-based exosome proteomic analyses are increasingly reported and advances in mass spectrometry sensitivity and bioinformatic tools should further improve results. Multidimensional Protein Identification Technology (MudPIT) uses a combination of strong cation exchange chromatography with reverse phase separation and has been developed to analyze complex protein mixtures [34]. MudPIT has previously only been used to analyze human parotid gland exosomes [6]. In this report, we used MudPIT analysis in combination with TFE in solution digestion to

extensively expand the urinary exosome protein database by using no more than 50 μg of total protein. The total analysis time on the instrument is 24 hours per sample and it is done automatically. Compared with previous urinary exosome analyses by GelC-MS [5, 24], this method requires less time for sample preparation/analysis and is expected to be sufficiently sensitive for proteomic analysis of exosomes from other sources [35].

The exosome isolation method used in this paper is the standard ultracentrifugation method with an additional 17,000g centrifugation step added after the DTT reduction process. This step was found to be essential to thoroughly remove cell debris and other particles and to obtain clean exosome samples. Proteins identified in these samples clearly indicated the multivesticular origin such as the presence of all of the members of ESCRT pathway, enriched membrane-bound vesicle, and endosomal proteins etc. Electron microscopy also confirmed a high concentration of 40–100 nm diameter vesicles; however, we cannot rule out the possibility that other non-exosome particles are present in the preparation including vesicles such as apoptotic blebs and microparticles [36–37]. Even though DTT reduction was used to reduce the amount of uromodulin in the 200,000g sediments, uromodulin was still one of the most abundant proteins in the exosome preparation probably due to association with exosome proteins or exosome membranes. The modified urinary exosome preparation was reported later by Fernandez-Llama et al. [38] to recover the entrapped exosomes in the low-speed pellets. This method was not used in our study considering majority of uromodulin in the urine was shifted from the low- to the high-speed pellets as reported by Fernandez_Llama [38] and high abundance of uromodulin in the samples will affect the identification of low abundant proteins using our analysis method. In addition to uromodulin, 2.2–3.3 μg/mL of serum albumin is also present in normal urine [39] and is abundant in our samples. The exosome preparation described here reduced the level of serum albumin, because the total exosome protein concentration in urine is below 1 μg/mL; however, serum albumin is also one of the relatively high abundance proteins in some exosome samples. It is not clear why the amount of uromodulin and serum albumin in the exosome preparation varies between urine samples; however, the presence of these two proteins in some samples may explain why the total exosomal protein concentration is much higher in some samples than the others.

The ESCRT pathway is a group of multisubunit protein complexes that play a central role in the processes of endosomal cargo sorting and multivescular bodies formation [40]. However, the function of ESCRT machinery in the formation and secretion of exosome is still unclear [41]. Recent studies support ESCRT-independent cargo sorting during exosome biogenesis such as ceramide triggered budding of exosomes [42] and protein high order oligomerization triggered budding of exosomes [43]. All of the members of the ESCRT pathway were detected in our analysis which further supports the involving of ESCRT pathway in exosome biogenesis. At the same time, previously reported proteins that may involve in the ceramide-induced budding in parotid gland exosomes such as UDP-glucose:glycoprotein glucosyltransferase 1 and Phosphatidylethanolamine-binding protein 1 were also detected. In addition, actin and actin interacting ERM (Ezrin-Radixin-Moesin) proteins were detected as highly abundant in urinary exosomes. Previous reports suggest a possible role of ERM proteins in the budding process [44–45]. Careful analysis these results may provide important information to further understand the exosome biogenesis and functions.

Besides providing information to understand exosome biogenesis and function, exosome proteomics has brought considerable research interest in developing disease-related biomarkers. Analysis of urine exosomes yielded a series of proteins in some very important biological processes including protein transport, membrane trafficking, metabolic process and signal transduction. Proteomics analysis of urinary exosomes also identified proteins

that play important roles in kidney function, such as proteins involved in water, drug, sodium, chloride, proton and glucose transport as well as some potential disease biomarkers currently under investigation. Even though all of the urine samples in this study were obtained from normal individuals, some proteins were only highly abundant in some individuals such as S100-A8, mucin-5AC, S100-A9 and serpin B4 due to unknown physiological reasons. These proteins were in very low abundance or not present in other individuals. These results indicate proteins in the exosomes are regulated and affected by specific physiological conditions.

In summary, this study utilized a TFE in-solution digestion and MudPIT strategy to thoroughly analyze urinary exosomes obtained from normal human urine. This study provides the largest set of proteins present in human urinary exosome proteomes and provides a valuable reference for future study. The method provided here is expected to be directly applicable to the analysis of exosome proteomes from other sources.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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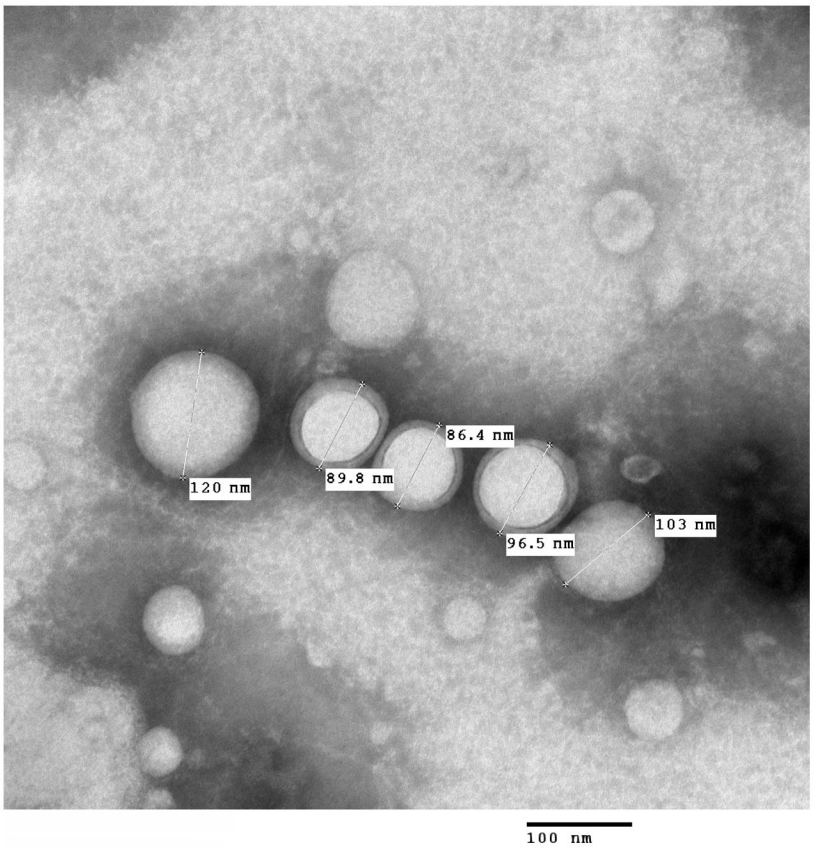

Figure 1. Electron micrograph of isolated urinary exosomes

Urinary exosomes isolated by ultracentrifugation was imaged by electron microscopy. High concentrations of 40–100 nm diameter vesicles were seen. A representative electron micrograph of urine exosomes having diameters of approximately 100 nm is shown in the figure.

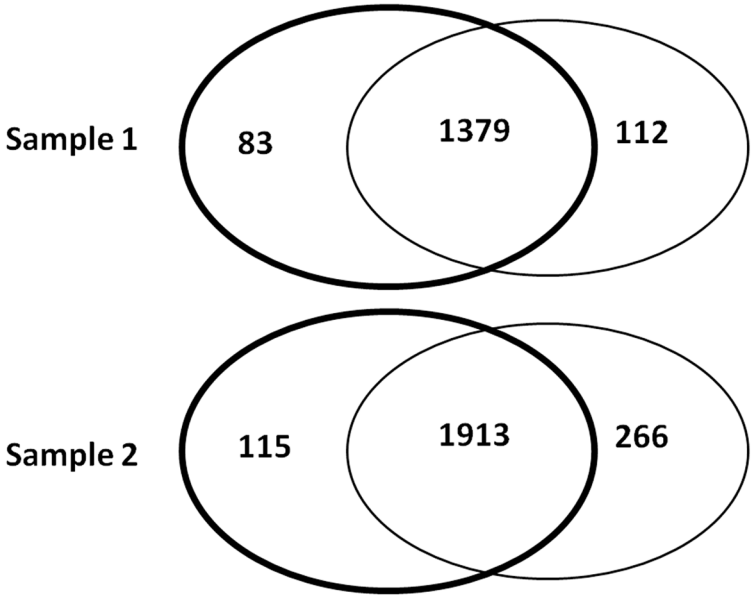

Figure 2.
Diagram of the number of proteins identified by in-gel and in-solution digestion. Dark ring is for in-gel digestion and light ring is for in-solution digestion.

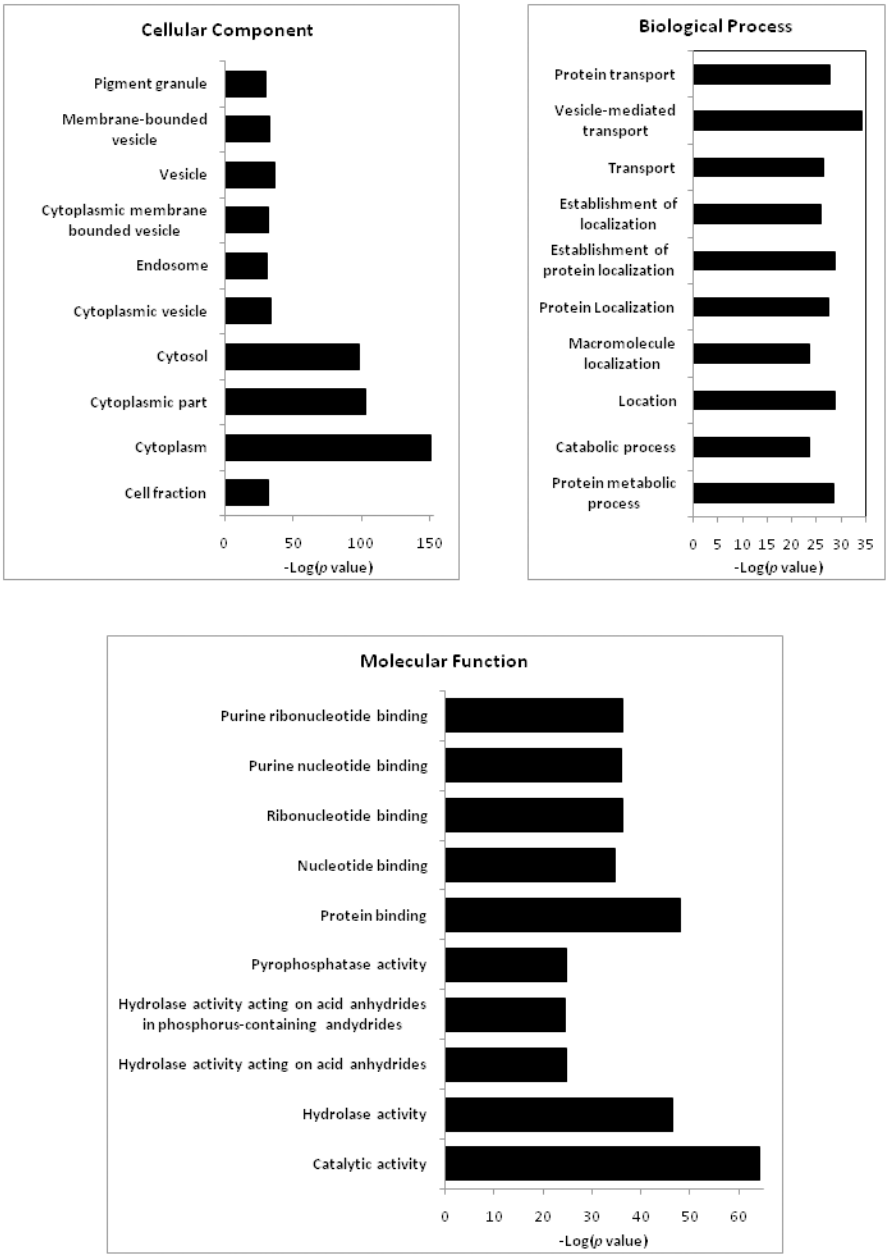

Figure 3.

Enriched GO categories.

Functional category enrichment in exosome samples was evaluated by a hypergeometric test against the entire human genome using WebGestalt. The *p*-value was adjusted by multiple test adjustment and $P < 0.05$ is considered enriched GO categories. The top 10 significantly enriched GO categories are shown.

Table 1

Protein concentrations and abundances from individual exosome preparations.

Sample	Protein concentration (μg protein/mL of urine)	Protein used for analysis (μg)	Normalized spectral counts for uromodulin and serum albumin
S12	0.347	50	0.039
S13	0.630	50	0.055
S14	0.150	33.7	0.020
S16	0.478	50	0.027
S17	0.110	23.7	0.020
S33	0.635	50	0.073
S34	0.456	50	0.062

Table 2

ESCRT pathway proteins identified in urine exosomes

Protein Name	Uniprot AC	Biological function	Sequence coverage	Total spectral count	Times detected
Hepatocyte growth factor-regulated tyrosine kinase substrate(HRS)	O14964	ESCRT-0 complex	23	41	8
Signal transducing adapter molecule 2 (STAM-2)	O75886		13	9	5
Signal transducing adapter molecule 1 (STAM1)	Q92783		16	5	5
Multivesicular body subunit 12A (MVB12A)	Q96EY5	ESCRT-I complex	60	765	9
Vacuolar protein sorting-associated protein 28 (VPS28)	Q9UK41		82	698	9
Tumor susceptibility gene 101 protein (TSG101)	Q99816		57	550	9
Vacuolar protein sorting-associated protein 37B(VPS37B)	Q9H9H4		52	498	9
Vacuolar protein sorting-associated protein 37D(VPS37D)	Q86XT2		55	190	9
Multivesicular body subunit 12B (MVB12B)	Q9H7P6		73	172	9
Vacuolar protein sorting-associated protein 37C(VPS37C)	A5D8V6		32	100	9
Vacuolar protein sorting-associated protein 37A(VPS37A)	Q8NEZ2		23	8	5
Vacuolar protein-sorting-associated protein 25 (VSP25) (EAP20)	Q9BRG1	ESCRT-II complex	78	196	9
Vacuolar protein-sorting-associated protein 36 (VPS36) (EAP45)	Q86VN1		67	159	9
Vacuolar-sorting protein SNF8 (SNF8) (EAP30)	Q96H20		48	40	8
Programmed cell death 6-interacting protein (ALIX)	Q8WUM	ESCRT-III complex	77	2812	9
IST1 homolog (IST1)	P53990		87	1784	9
Vacuolar protein sorting-associated protein 4B (VPS 4B)	O75351		79	1675	9
Charged multivesicular body protein 2a (CHMP2A)	O43633		68	886	9
Vacuolar protein sorting-associated protein 4A (VPS 4A)	Q9UN37		86	940	9
Charged multivesicular body protein 5 (CHMP5)	Q9NZZ3		86	533	9
MIT domain-containing protein 1 (MITD1)	Q8WV92		78	433	9
Vacuolar protein sorting-associated protein VTA1 homolog (LIP5)	Q9NP79		56	301	9
Charged multivesicular body protein 4b (CHMP4B)	Q9H444		64	227	9
Charged multivesicular body protein 2b (CHMP2B)	Q9UQN3		62	174	9
Charged multivesicular body protein 1b (CHMP1B)	Q7LBR1		46	132	9
Charged multivesicular body protein 4c (CHMP4C)	Q96CF2		79	108	9
Tyrosine-protein phosphatase non-receptor type 23 (HD-PTP)	Q9H3S7		24	101	9

Protein Name	Uniprot AC	Biological function	Sequence coverage	Total spectral count	Times detected
Charged multivesicular body protein 3 (CHMP3)	Q9Y3E7		54	108	9
STAM-binding protein (AMSH)	O95630		46	81	9
Charged multivesicular body protein 6 (CHMP6)	Q96FZ7		47	42	9
Charged multivesicular body protein 1a (CHMP1A)	Q9HD42		34	50	9
Charged multivesicular body protein 4a (CHMP4A)	Q9BY43		49	30	9
Ubiquitin carboxyl-terminal hydrolase 8 (UBPY)	P40818		13	18	9
Charged multivesicular body protein 7 (CHMP7)	Q8WUX9		11	7	4

Table 3

The effect of urine volume on protein identification

Urine volume (mL)	Total spectral counts	Unique Peptides	Protein groups
25	18179	8087	1734
50	16268	6356	1615
100	19034	7321	1620
200	24236	11036	1841