Chapter 5
Exosomes in Urine Biomarker Discovery

Alyssa R. Huebner, Poorichaya Somparn, Thitima Benjachat, Asada Leelahavanichkul, Yingyos Avihingsanon, Robert A. Fenton and Trairak Pisitkun

Abstract Nanovesicles present in urine the so-called urinary exosomes have been found to be secreted by every epithelial cell type lining the urinary tract system in human. Urinary exosomes are an appealing source for biomarker discovery as they contain molecular constituents of their cell of origin, including proteins and genetic materials, and they can be isolated in a non-invasive manner. Following the discovery of urinary exosomes in 2004, many studies have been performed using urinary exosomes as a starting material to identify biomarkers in various renal, urogenital, and systemic diseases. Here, we describe the discovery of urinary exosomes and address the issues on the collection, isolation, and normalization of urinary exosomes as well as delineate the systems biology approach to biomarker discovery using urinary exosomes.

Keywords Exosome · Urine · Biomarker · Systems biology

5.1 Introduction

Extracellular nanovesicles called "exosomes" are small (20–100 nm) membrane vesicles that, in mammals and invertebrates, are secreted by a wide variety of cell types [1]. Exosomes are formed inside their secreting cells in endosomal compartments called multivesicular bodies (MVBs). Exosomes are released from the MVB

A.R. Huebner · R.A. Fenton · T. Pisitkun
Department of Biomedicine and Center for Interactions of Proteins in Epithelial Transport, Aarhus University, Aarhus, Denmark

P. Somparn · T. Pisitkun (✉)
Systems Biology Center, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand
e-mail: trairak@gmail.com

T. Benjachat · A. Leelahavanichkul · Y. Avihingsanon
Center of Excellence in Immunology and Immune Mediated Diseases, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

Y. Gao (ed.), *Urine Proteomics in Kidney Disease Biomarker Discovery*,
Advances in Experimental Medicine and Biology 845,
DOI 10.1007/978-94-017-9523-4_5

lumen into the extracellular environment upon fusion of the outer membrane of the MVBs with the plasma membrane. Exosomes contain various molecular constituents of their cell of origin, including proteins and genetic materials, i.e., mRNA and miRNA. The potential roles of exosomes in intercellular communications have been studied in immunology, neurobiology, stem cell, and tumor biology. In 2004, exosomes in human urine were identified and partially characterized [2]. Urinary exosomes contain proteins that are characteristic of every epithelial cell type facing the urinary space, including multiple protein products of genes responsible for renal and systemic diseases. Urinary exosomes provide a non-invasive means of acquiring unique information about the physiological or pathophysiological state of the renal cells of their origin; many studies have been performed aimed at identifying urinary biomarkers of specific diseases [3–12]. In this chapter, we discuss the discovery of urinary exosomes; collection, isolation, and normalization of urinary exosomes; and the potential of urinary exosome constituents in biomarker discovery.

5.2 Discovery of Urinary Exosomes

Over 15 years ago, integral membrane-bound proteins, such as water channel aquaporin-2 (AQP2), could be detected in low-density membrane vesicles isolated from human urine by ultracentrifugation [13, 14]. The presence of these proteins in urine allowed studies of water-balance disorders [15]. However, the mechanism by which these AQP2-containing vesicles actually got into urine was unknown. Further investigations determined that other membrane-bound transporters were detectable in urine low-density membrane fractions [16], including major Na transporters of the proximal tubule (the type 3 Na–H exchanger [NHE3]), the thick ascending limb of Henle's loop (the bumetanide-sensitive Na–K-2Cl cotransporter [NKCC2]), and the distal convoluted tubule (the thiazide-sensitive Na–Cl cotransporter [NCC]). Based upon these initial observations, researchers sought to discover the mechanisms behind the release of these so-called low-density membrane vesicles into the urine.

In 2004, a pivotal study used a variety of techniques to characterize the orientation, size, and protein contents of low-density urinary membrane vesicles isolated from normal human subjects by differential centrifugation [2]. These studies allowed the authors to hypothesize that AQP2 and other apical membrane-bound transporter proteins were being excreted into the urinary space through the process of exosome formation, i.e., a process where the internal vesicles of multivesicular bodies (MVBs) are delivered to the urinary space by fusion of the outer membrane of MVBs with the apical plasma membrane of renal tubular epithelial cells. To demonstrate the orientation of proteins within the low-density urinary vesicles, the authors used immunogold electron microscopy and antibodies against epitopes on the cytoplasmic side of the integral membrane proteins AQP2 and NCC, or antibodies against epitopes on the external side of aminopeptidase N and CD9. Analogous to exosomes found in other bodily fluids, characteristic of vesicles found

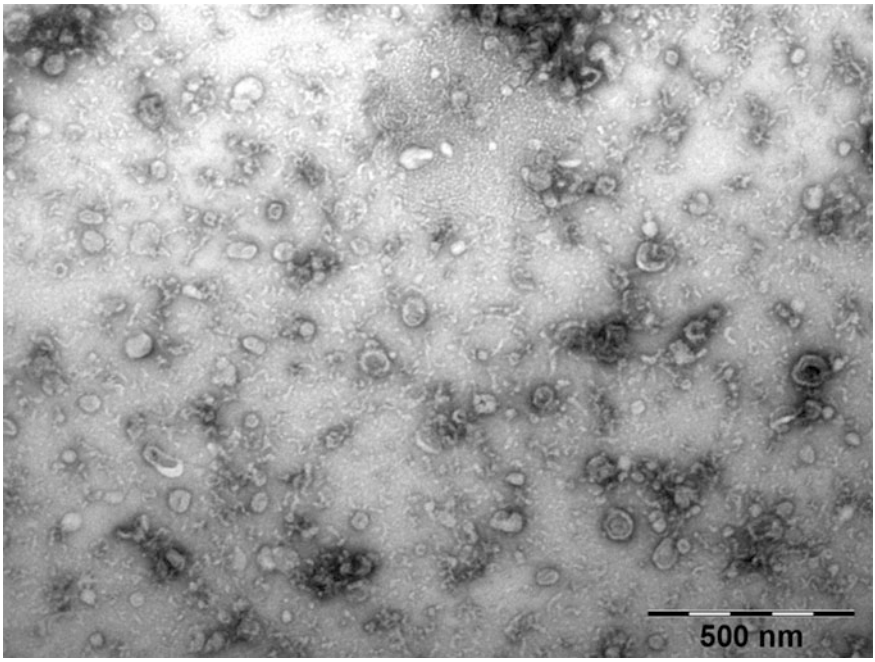

Fig. 5.1 Negative staining of urinary exosomes from normal human subjects using electron microscopy at 41,000× magnification. This magnification was used to show a variety of exosomes from a 200,000×*g* low-density pellet based on the 20–100 nm size and round, cup-like shape

in MVBs, and the membrane proteins within low-density urinary vesicles were oriented with the cytoplasmic-side inward. Negative stained electron micrographs revealed that the low-density urinary vesicles were small, cup-shaped nanovesicles between 20 and 100 nm in diameter (Fig. 5.1), and a quantitative analysis showed that the mode of the vesicle size was 35–40 nm [2]. These findings were consistent with the size criterion of exosomes proposed by Thery and colleagues and similar to that of exosomes found from other tissue types in the human body [17]. Finally, Pisitkun et al. [2] analyzed the proteome of the low-density urinary vesicles using nanospray liquid chromatography–tandem mass spectrometry (LC-MS/MS). Over 290 unique proteins were identified with a role in a wide range of cellular processes and included proteins from renal epithelia extending from the glomerular podocytes through the proximal tubule, the thick ascending limb of Henle's loop, the distal convoluted tubule, and the collecting duct as well as from the transitional epithelium of the urinary bladder. Twenty-one of the identified proteins are associated with kidney diseases or hypertension. Importantly, the proteins identified included 73 endosomal trafficking proteins and many class E vacuolar protein-sorting (VPS) proteins. These VPS proteins including members of the endosomal sorting complexes required for transport (ESCRT) are well-established proteins associated with MVB biogenesis and exosome formation (Fig. 5.2) [18].

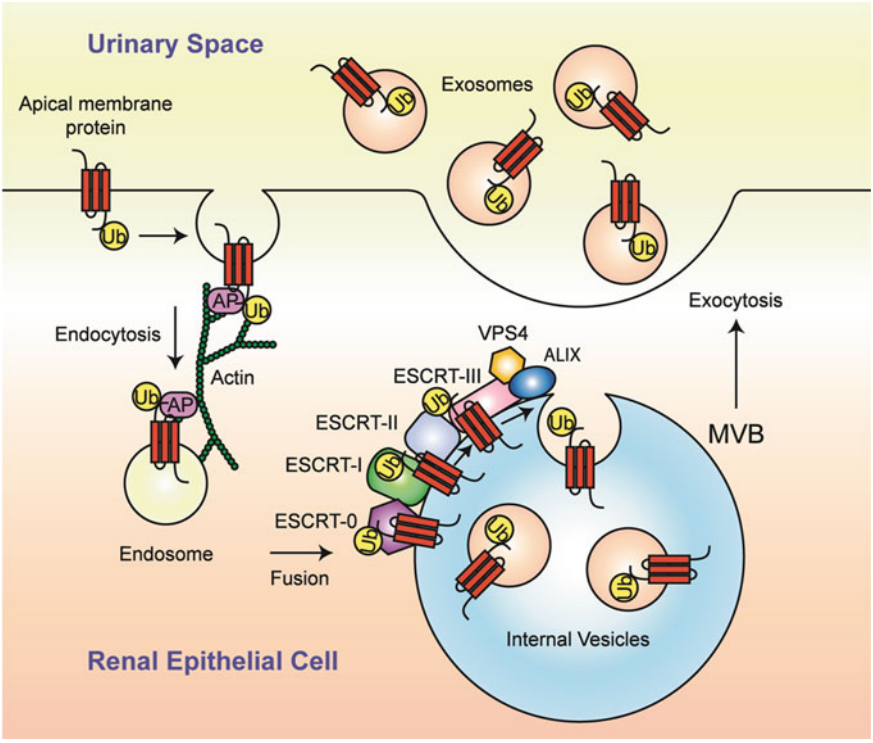

Fig. 5.2 A model for multivesicular body (MVB) formation and exosome secretion into urine. Monoubiquitination (Ub) is the signal that marks plasma-membrane proteins for incorporation into MVBs. Monoubiquitinated proteins are endocytosed by a process dependent on adaptor proteins (AP) and sorted in the endosomal pathway. Fusion with and internalization of the ubiquitinated cargo into the MVB requires assistance from endosomal sorting complexes required for transport (ESCRT)-protein machinery (ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III), VPS4, and ALIX. Eventually the outer membrane of the MVB fuses with the apical plasma membrane of the renal epithelial cell, releasing exosomes into the urinary space

Based on these data, the authors concluded that low-density urinary vesicles are largely made up of exosomes derived from the internal vesicles of MVBs of every epithelial cell type facing the urinary space (Fig. 5.2). Importantly, urinary exosomes contain proteins that potentially reflect the physiological or pathophysiological state of their cells of origin, and therefore, exosome isolation, as highlighted by the authors, may provide an efficient first step in biomarker discovery in urine.

To expand their initial findings, in 2009, the same investigators conducted a large-scale proteomic study of human urinary exosomes using a more sensitive LC-MS/MS system. Overall, 1,132 proteins were identified including 14 phosphoproteins. The Online Mendelian Inheritance in Man (OMIM) database highlighted that 177 of these proteins were disease-related proteins, 34 of which were associated with renal diseases and/or hypertension [19]. An online database of human urinary exosomal proteins can be found at http://dir.nhlbi.nih.gov/papers/lkem/exosome/.

These studies showed proof-of-principle that the proteomic analysis of urinary exosomes can serve as an excellent tool for discovering disease-related biomarkers.

In addition to a wide range of proteins [2, 19, 20], Miranda et al. [5] discovered in 2010 that nucleic acids, including mRNA and microRNA (miRNA), were contained and preserved in urinary exosomes. The identified mRNA species encoded proteins from all segments of the renal tubule. These studies demonstrated that exosomal genetic materials have potential roles for urine biomarker discovery and that exosomal RNAs may have functional roles, i.e., in cell-to-cell communication along the nephron [21].

5.3 Collection, Isolation, and Normalization of Urinary Exosomes

5.3.1 Collection and Storage of Urine Samples

For clinical and research purposes, it is important that urine destined for isolation of exosomes is collected and stored in a standardized way. There are three important steps that should be addressed to standardize the collection and storage of urine: (1) addition of protease inhibitors and preservative during urine collection, (2) storage of urine at stable temperatures, for short-term and long-term use, and (3) extensively vortexing urine samples after thawing [12, 22] (see more at http://www.niddk.nih.gov/research-funding/at-niddk/labs-branches/kidney-disease-branch/renal-diagnostics-therapeutics-unit/sample-collection-storage-exosome-analysis/Pages/default.aspx). Without the use of protease inhibitors, various key proteins often detected in urinary exosomes can degrade. The typical cocktail of protease inhibitors and preservative includes phenylmethanesulfonyl fluoride (PMSF), leupeptin, and sodium azide. From our own experiences, storage of urine at −80 °C provides a more stable condition than storing samples at −20 °C. With extensive vortexing after thawing, samples stored at −80 °C have the highest recovery of exosomes—up to 100 % when compared to freshly processed urine. In contrast, urine stored at −20 °C has only 87 % exosome recovery after extensive vortexing. Nanoparticle tracking analysis of urinary exosomes has recently confirmed a storage condition of −80 °C with the addition of protease inhibitors is the best [22]. Urine collected in the morning, both first and second urine of the day, has similar exosome contents and can be used interchangeably for experimental research purposes [12].

5.3.2 Processing Procedures for Isolation of Urinary Exosomes

After the discovery of potential renal disease-related biomarkers in urinary exosomes, researchers have worked to identify a variety of methods for faster and more

efficient ways to collect urinary exosomes for clinical applications. This exploration began because the first method of isolation, ultracentrifugation, requires long processing times and access to expensive equipment. Despite the applications of novel techniques, including membrane filtration and exosome precipitation methods, ultracentrifugation methods show the highest exosome purity. Table 5.1 shows a comparison of urinary exosome isolation techniques.

Ultracentrifugation was the original method of reproducibly isolating urinary exosomes [2]. This technique requires collecting urine in the collection solution

Table 5.1 Comparison of urinary exosome isolation techniques

	Methods	Advantages	Disadvantages	References
Ultracentrifugation	Ultracentrifugation	• Reproducible results • High yield of intact proteins and nucleic acids	• 4 to 5 h to process single sample • Some contamination of highly abundant proteins • Expensive equipment	[2]
	Double-cushion ultracentrifugation	• Less contamination of highly abundant proteins • Reproducible results	• Long processing time • Tedious separation techniques • Expensive equipment	[23]
	Sucrose gradient ultracentrifugation			[24]
	Ultracentrifugation—size exclusion chromatography			[25]
Membrane Filtration	Nanomembrane filtration	• Shorter processing time, 0.5–2 h • Many samples can be processed at one time • Relatively inexpensive • Can be used in a clinical setting	• Possible clogging of membrane • Sample loss • Contamination of highly abundant proteins	[3]
	Micromembrane filtration			[26]
Precipitation	Precipitation by ExoQuick-TC	• Shorter processing time, 0.5–2 h • Yields intact RNA • Relatively inexpensive • Can be used in a clinical setting	• Low purity of protein • Modified protocol	[27]

(protease inhibitors and sodium azide), spinning the urine at a low speed (17,000×*g*) to remove whole cells, large membrane fragments, and cellular debris, followed by spinning the supernatant at a high speed (200,000×*g*) for 1 h to pellet the exosomes (for more details please check http://www.niddk.nih.gov/research-funding/at-niddk/labs-branches/kidney-disease-branch/renal-diagnostics-therapeutics-unit/exosome-preparation/Pages/default.aspx). This initial low-density exosome pellet commonly includes some contamination of highly abundant urinary proteins, including albumin and uromodulin, also known as Tamm-Horsfall protein (THP) [2, 23]. THP forms double-helical fibrils by a disulfide cross-link zona pellucida (ZP) domain, which entraps exosomes and is co-isolated with the low-density pellet. Adding the reducing agent dithiothreitol (DTT) with a subsequent high-speed spin reduces the presence of THP in the low-density pellet [2]. However, this method does not completely decontaminate the sample of all THP and other abundant proteins. Alternatively, a more purified exosome pellet can be obtained using an additional step of double-cushion ultracentrifugation [23] or the use of heavy water and a sucrose gradient in ultracentrifugation [24] to separate the exosomes and contaminating proteins based on density, whereas ultracentrifugation followed by size exclusion chromatography (UC-SEC) can be used to separate contaminating proteins from exosomes based on molecular weight [25]. However, these methods are still time-consuming and labor intensive and require expensive equipment.

In 2007, Cheruvanky et al. [3] used commercially available nanomembrane concentrators to filter exosomes from urine of healthy volunteers and proteinuric patients with focal segmental glomerulosclerosis (FSGS). The exosome isolation time was reduced from 4 h using standard ultracentrifugation to 0.5–2 h using nanomembrane filters [3]. Using this system, the authors were able to detect various proteins typically found in urinary exosomes, even from patients who had high quantities of contaminating proteins, demonstrating that this method could be used for both clinical and routine experimental applications. Other microfiltration methods have also been shown to provide an efficient way of isolating urinary exosomes with reduced contamination of highly abundant urinary proteins as compared to nanomembrane filtration and ultracentrifugation techniques [26]. Alternatively, precipitation methods have been used to isolate urinary exosomes. In 2012, Alvarez et al. [27] showed that a commercially available exosome precipitation kit called ExoQuick-TC could be used to isolate urinary exosomes, although a modified protocol provided improved results. This modified exosome precipitation method was shown to provide a more efficient yield of miRNA and mRNA than protein compared to sucrose cushion ultracentrifugation.

5.3.3 Normalization

A challenge that researchers in the urinary exosome field face is defining the methods of exosome normalization. Without standardized normalization protocols, biomarker discovery studies from urinary exosomes and the subsequent comparisons

between patient-to-patient samples will be less reliable. There are several methods of normalization, including time normalization, creatinine normalization, and protein normalization.

The quantitative measurement of urinary biomarkers in terms of excretion rate is the optimal method for normalization and has been used for many years for analysis of classical urinary biomarkers such as total protein and albumin (e.g., expressed in g/day or μg/min). Assessments of urinary biomarkers based only on concentrations are unsatisfactory because normal physiological variations in water excretion can dilute or concentrate urinary proteins making this measurement unreliable for both intra- and interpersonal comparisons. Time normalization, the collection of urine from patients within the same bracket of time, is thus the most accurate method of measuring urine exosomal protein excretion rates and is the most reliable method of comparing patient exosome products side-by-side. An even more accurate time normalization would be the collection of all urine from the same patient over 24 h, but this approach has several practical limitations.

As there are difficulties acquiring time-normalized urine samples, and this method also rules out using urine samples from a biobank because these samples are typically spot urine samples, a more common method of normalization, creatinine normalization, is used in a clinical setting. Creatinine is typically excreted in urine at a steady rate. The average 24-h urine creatinine excretion rate for the general population is approximately 1,000 mg/day per 1.73 m^2 body surface area. Given the amount of creatinine in the spot urine, an estimated rate of exosome excretion can be assigned. However, this method has limitations; it does not take into account actual difference in individual creatinine excretion rates or renal diseases that make creatinine excretion rates unstable, such as acute kidney disease.

Normalization based on the amount of a particular protein in urine has been proposed, including the uses of THP, exosomal markers (e.g., ALIX or TSG101), or other biomarker proteins that when measured together provide a concentration ratio that correlates with disease state [6, 28]. However, further studies are needed to confirm the validity of these normalization methods. An alternative method of normalization that has recently been utilized is counting exosome number. Using a new nanoparticle tracking analysis system, urinary exosomes in whole urine were counted, sized, and analyzed [22]. This new method of analysis may ultimately be less time-consuming and provide a more standardized normalization procedure, yet comes with the disadvantage of requiring expensive equipment.

5.4 Urinary Exosomes in Biomarker Discovery

Urinary exosomes provide a non-invasive method of discovering novel biomarkers for renal diseases. A technique that has become popular over the past decade for the discovery of novel biomarkers in the renal system and exploration of the urinary exosomal proteome is LC-MS/MS [7]. A technical description of LC-MS/MS is beyond the scope of this chapter (see review by Pisitkun et al. [29]), but a brief

description is informative for the context of this section. LC-MS/MS is highly sensitive and can be used for both qualitative and quantitative analysis of peptides/proteins. Qualitative approaches involve observing the absence or presence of a particular protein in a given sample, while quantitative approaches show a comparison of the relative abundance of specific proteins. LC-MS/MS-based quantification of patient and control samples can be performed side-by-side using label-free [30], label-based (e.g., iTRAQ [31] and TMT [32]), and the more recent targeted quantification [33]. To validate and compare proteins of interest discovered by LC-MS/MS from urinary exosomes, Western blots, ELISA, or immuno-electron microscopy are commonly used.

The paradigm of utilizing a systems biology approach for clinical and translational research in the field of urinary exosome biomarker discovery is gaining momentum. The systems biology approach using mass spectrometry generally includes three phases: (1) discovery, (2) validation, and (3) implementation (Fig. 5.3) [7]. In the discovery phase, LC-MS/MS is used to identify unknown biomarkers and assist in developing hypotheses for the following phases. Typically, a limited number of well-defined patient samples are used to identify quantitative or qualitative differences in protein or mRNA/miRNA expression. Animal models are often exploited to show physiological changes within a specific experimental condition, e.g., increasing the excretion of AQP2 in urine from rats with elevated vasopressin levels by administering dDAVP or thirsting [14]. Once candidate biomarkers have been identified, the validation phase can begin.

A variety of promising biomarkers from urinary exosomes have been identified from many renal, urogenital, and systemic diseases. Some biomarkers of acute kidney injury (AKI), urogenital cancer, chronic kidney diseases, glomerular diseases, renal allograft rejection, and unique tubulopathies are summarized in Table 5.2. In addition to being a great source of protein biomarkers, exosomes also contain functional mRNA and miRNA, which markedly expands the potential repertoire of biomarkers. In recent studies, for example, mRNA and miRNA in urinary exosomes were used to identify potential markers for prostate cancer and renal fibrosis [34–36]. In many cases, early detection of a disease or injury is required for increasing the chances of successful treatment, and several of the biomarkers already discovered could provide early detection possibilities. For example, patients in the intensive care unit or patients undergoing heart surgery have a high risk of acute kidney injury that may increase morbidity and mortality.

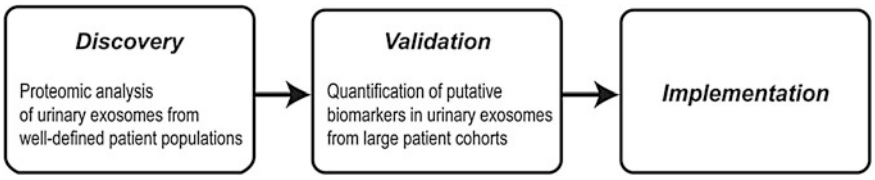

Fig. 5.3 Systems biology workflow paradigm showing the three major steps required for the development of urinary biomarker assays for routine clinical application

Table 5.2 Potential biomarkers for renal and systemic diseases from urinary exosomes

Groups	Diseases	Urinary exosomal proteins	Urinary exosomal RNAs	References
Acute kidney injury	Acute kidney injury (AKI)[a]	Fetuin-A		[11]
	Acute kidney injury (AKI)	Activating transcription factor 3		[10]
	Renal ischemia-reperfusion injury	Aquaporin-1		[37]
Cancer	Prostate cancer		PCA-3 and TMPRSS2: ERG	[36]
	Bladder cancer[a]	CD36, CD44, trophoblast glycoprotein, basigin, and CD73		[40]
	Non-small cell lung cancer (NSCLC)[a]	Leucine-rich α-2-glycoprotein		[41]
	Renal cell carcinoma[a]	Matrix metalloproteinase 9, ceruloplasmin, podocalyxin, Dickkopf-related protein 4, carbonic anhydrase IX, aquaporin-1, extracellular matrix metalloproteinase inducer, neprilysin, dipeptidase 1, and syntenin-1		[42]
	Bladder cancer[a]	Tumor-associated calcium signal transducer 2		[43]
Chronic kidney disease	Renal fibrosis		microRNA-29c	[34]
	Renal fibrosis		CD2AP	[35]
	Chronic kidney disease	Osteoprotegerin		[44]
Glomerular disease	Puromycin-treated rats or podocin-Vpr transgenic mice and from patients with focal segmental glomerulosclerosis	Wilms tumor 1		[10]
	IgA nephropathy versus thin basement membrane nephropathy[a]	Aminopeptidase N, vasorin precursor, α-1-antitrypsin, and ceruloplasmin		[45]

(continued)

Table 5.2 (continued)

Groups	Diseases	Urinary exosomal proteins	Urinary exosomal RNAs	References
	Diabetic nephropathy[a]	Xaa-Pro dipeptidase, major urinary protein 1 and neprilysin		[46]
	Diabetic nephropathy	Wilms tumor 1		[47]
	Podocyte injury in FSGS or SSNS	Wilms tumor 1		[48]
Misc.	Liver injury[a]	CD26, CD81, SLC3A1, and CD10		[49]
	Light chain amyloidosis (Al)	Immunoglobulin light chain species		[50]
Renal Transplantation	Renal allograft recipients with acute decrease in renal function[a]	Several pairs of biomarkers		[6]
Tubulopathy	Aldosteronism	Phosphorylated NCC and prostasin		[9]
	Pseudohypoaldosteronism type II (PHAII)	Total and phosphorylated NCC		[38]
	Gitelman syndrome	Total and phosphorylated NCC		[38]
	Gitelman syndrome	NCC		[39]
	Bartter syndrome type I[a]	NKCC2		[19]
	Post-obstructed kidney	Aquaporin-1 and transforming growth factor β1		[51]
	Salt sensitivity of blood pressure		45 exosomal microRNAs	[52]

[a] indicates studies that used LC-MS/MS for discovery

Proteins in urinary exosomes such as Fetuin-A [11], activating transcription factor 3 (ATF3) [10], and aquaporin-1 [37] have been shown to change significantly in the early phase of patients with AKI compared to controls and could be promising markers for early detection of AKI.

Biomarkers for genetic diseases, such as Gitelman syndrome [38, 39] and Bartter syndrome type 1 [19], which have mutations in the SLC12A3 gene (encoding NCC) or the SLC12A1 gene (encoding NKCC2), respectively, have been investigated in urinary exosomes. Little to non-detectable levels of these proteins were observed in exosomes from these patients. Early detection of the diseases could guide appropriate treatments and prognosis. This innovation may replace cumbersome functional tubular tests, i.e., 24-h urinary electrolyte excretion. Furthermore, the absence in detection of NCC or NKCC2 protein in urinary exosomes could

directly reflect the abnormalities in gene products and be more physiological relevant than genetic testing methods.

Despite many potential biomarkers having been discovered, these identifications are only the first phase in the systems biology paradigm—discovery. In the validation phase, potential biomarkers are quantified in large patient cohorts, with the sensitivity and specificity of each candidate biomarker assay evaluated. It is important to note that the assays developed in the validation phase should be used in the implementation step, which requires the development of easy, time-efficient, and affordable assays to use in a clinical protocol. Currently, there are a limited number of translational studies investigating the validity of biomarkers from urine. One example is a recent study investigating pseudohypoaldosteronism type II (PHAII) and Gitelman syndrome, where an ELISA was developed as a practical clinical tool to detect total NCC and phosphorylated NCC in urinary exosomes of patients with PHAII and Gitelman syndrome as well as a large patient pool with a variety of clinical backgrounds [38]. This study revealed that the ELISA yielded similar sensitivity as immunoblotting technique, thus providing a practical diagnostic tool for detecting changes in urinary NCC excretion. Although this study has potential, including the generation of a sensitive immunoassay, the protocol still requires laborious ultracentrifugation for the isolation of urinary exosomes. An increased commitment to validate promising biomarkers from urinary exosomes and develop a user-friendly clinical assay is needed in order to bring diagnostic tools from the bench to finally "implement" at the bedside.

5.5 Summary and Recommendations

Characteristics of exosomes Urinary exosomes have similar characteristics of exosomes secreted from other cell types in the body, including exosomes released by dendritic cells and B-lymphocytes. These defining characteristics include that proteins in the membranes of urinary exosomes have an orientation of cytoplasmic-side inward; urinary exosomes are small in size, between 20 and 100 nm in diameter; and urinary exosomes contain several class E vacuolar protein-sorting (VPS) proteins known to be involved in MVB biogenesis and exosome formation, along with many endosomal trafficking and membrane proteins. Urinary exosomes contain numerous proteins associated with renal and systemic diseases and thus are a great resource for biomarker discovery. Nucleic acids also contribute to the biological makeup of urinary exosomes and should be considered during biomarker discovery.

Collection and storage Urine, following the addition of protease inhibitors, can be stably stored at −80 °C over long periods of time. Following extensive vortexing, this storage condition provides an exosome recovery rate up to 100 %. First and second morning urine contains similar exosome content and can be used interchangeably for research purposes.

Isolation of exosomes There are a variety of methods available for isolating human urinary exosomes. Although ultracentrifugation is laborious, it is a standard method that yields intact exosomes, with reproducible results, that can be used for many research techniques. To decrease contamination of abundant urinary proteins such as albumin or Tamm-Horsfall protein, additional steps of ultracentrifugation with heavy water and a sucrose gradient or double-cushion ultracentrifugation can be used to separate proteins based on density. Ultracentrifugation followed by size exclusion chromatography can be used to separate contaminates based on molecular weight. However, for large-scale, clinical protocols that call for isolation of urinary exosomes, membrane filtration, and precipitation methods may be a better choice because many samples can be processed in a shorter amount of time. These quicker methods could increase the discovery rate of disease-related biomarkers.

Normalization To accurately compare side-by-side patient or control exosome samples, 24-h urine collection or timed urine collection yields the most accurate results based on the excretion rate of exosomal biomarkers. However, when this type of controlled collection is not available, creatinine and protein normalization can be used. The new technology of nanoparticle tracking could play an important role in developing a new normalization protocol.

Biomarker discovery and clinical implementation Urinary exosomes provide a non-invasive method of identifying potential protein and mRNA/miRNA biomarkers for renal and systemic diseases. LC-MS/MS is a common method used to discover urinary biomarkers because it is highly sensitive and can be used for both quantitative and qualitative analysis. A systems biology approach to biomarker discovery includes three phases: (1) discovery, (2) validation, and (3) implementation. Many potential biomarkers for early detection of different renal disorders, genetic disorders, and systemic diseases have been discovered. However, many of these biomarkers have not made it to the translational stage of validation or implementation. There is a need in the field to validate these biomarkers for clinical and routine use.

Acknowledgments This work was supported by the Lundbeck Foundation, Danish Medical Research Foundation, Novo Nordisk Foundation, Carlsberg Foundation, Aarhus University Research Foundation, and the Ratchadapiseksomphot Endowment Fund of Chulalongkorn University (RES560530124-HR).

References

1. Bobrie A, Colombo M, Raposo G, Thery C (2011) Exosome secretion: molecular mechanisms and roles in immune responses. Traffic 12(12):1659–1668. doi:10.1111/j.1600-0854.2011.01225.x
2. Pisitkun T, Shen RF, Knepper MA (2004) Identification and proteomic profiling of exosomes in human urine. Proc Natl Acad Sci USA 101(36):13368–13373. doi:10.1073/pnas.0403453101

3. Cheruvanky A, Zhou H, Pisitkun T, Kopp JB, Knepper MA, Yuen PS, Star RA (2007) Rapid isolation of urinary exosomal biomarkers using a nanomembrane ultrafiltration concentrator. Am J Physiol Ren Physiol 292(5):F1657–1661. doi:10.1152/ajprenal.00434.2006
4. Hoorn EJ, Pisitkun T, Zietse R, Gross P, Frokiaer J, Wang NS, Gonzales PA, Star RA, Knepper MA (2005) Prospects for urinary proteomics: exosomes as a source of urinary biomarkers. Nephrology 10(3):283–290. doi:10.1111/j.1440-1797.2005.00387.x
5. Miranda KC, Bond DT, McKee M, Skog J, Paunescu TG, Da Silva N, Brown D, Russo LM (2010) Nucleic acids within urinary exosomes/microvesicles are potential biomarkers for renal disease. Kidney Int 78(2):191–199. doi:10.1038/ki.2010.106
6. Pisitkun T, Gandolfo MT, Das S, Knepper MA, Bagnasco SM (2012) Application of systems biology principles to protein biomarker discovery: urinary exosomal proteome in renal transplantation. Proteomics Clin Appl 6(5–6):268–278. doi:10.1002/prca.201100108
7. Pisitkun T, Johnstone R, Knepper MA (2006) Discovery of urinary biomarkers. Mol Cell Proteomics 5(10):1760–1771. doi:10.1074/mcp.R600004-MCP200
8. van Balkom BW, Pisitkun T, Verhaar MC, Knepper MA (2011) Exosomes and the kidney: prospects for diagnosis and therapy of renal diseases. Kidney Int 80(11):1138–1145. doi:10.1038/ki.2011.292
9. van der Lubbe N, Jansen PM, Salih M, Fenton RA, van den Meiracker AH, Danser AH, Zietse R, Hoorn EJ (2012) The phosphorylated sodium chloride cotransporter in urinary exosomes is superior to prostasin as a marker for aldosteronism. Hypertension 60(3):741–748. doi:10.1161/HYPERTENSIONAHA.112.198135
10. Zhou H, Cheruvanky A, Hu X, Matsumoto T, Hiramatsu N, Cho ME, Berger A, Leelahavanichkul A, Doi K, Chawla K, Illei LS, Kopp GG, Balow JE, Austin HA 3rd, Yuen PS, Star RA (2008) Urinary exosomal transcription factors, a new class of biomarkers for renal disease. Kidney Int 74(5):613–621. doi:10.1038/ki.2008.206
11. Zhou H, Pisitkun T, Aponte A, Yuen PS, Hoffert JD, Yasuda H, Hu X, Chawla L, Shen RF, Knepper MA, Star RA (2006) Exosomal Fetuin-A identified by proteomics: a novel urinary biomarker for detecting acute kidney injury. Kidney Int 70(10):1847–1857. doi:10.1038/sj.ki.5001874
12. Zhou H, Yuen PS, Pisitkun T, Gonzales PA, Yasuda H, Dear JW, Gross P, Knepper MA, Star RA (2006) Collection, storage, preservation, and normalization of human urinary exosomes for biomarker discovery. Kidney Int 69(8):1471–1476. doi:10.1038/sj.ki.5000273
13. Kanno K, Sasaki S, Hirata Y, Ishikawa S, Fushimi K, Nakanishi S, Bichet DG, Marumo F (1995) Urinary excretion of aquaporin-2 in patients with diabetes insipidus. N Engl J Med 332(23):1540–1545. doi:10.1056/NEJM199506083322303
14. Wen H, Frokiaer J, Kwon TH, Nielsen S (1999) Urinary excretion of aquaporin-2 in rat is mediated by a vasopressin-dependent apical pathway. J Am Soc Nephrol 10(7):1416–1429
15. Ishikawa SE, Schrier RW (2003) Pathophysiological roles of arginine vasopressin and aquaporin-2 in impaired water excretion. Clin Endocrinol 58(1):1–17
16. McKee JA, Kumar S, Ecelbarger CA, Fernandez-Llama P, Terris J, Knepper MA (2000) Detection of Na(+) transporter proteins in urine. J Am Soc Nephrol 11(11):2128–2132
17. Thery C, Zitvogel L, Amigorena S (2002) Exosomes: composition, biogenesis and function. Nat Rev Immunol 2(8):569–579. doi:10.1038/nri855
18. Stoorvogel W, Kleijmeer MJ, Geuze HJ, Raposo G (2002) The biogenesis and functions of exosomes. Traffic 3(5):321–330
19. Gonzales PA, Pisitkun T, Hoffert JD, Tchapyjnikov D, Star RA, Kleta R, Wang NS, Knepper MA (2009) Large-scale proteomics and phosphoproteomics of urinary exosomes. J Am Soc Nephrol 20(2):363–379. doi:10.1681/ASN.2008040406
20. Wang Z, Hill S, Luther JM, Hachey DL, Schey KL (2012) Proteomic analysis of urine exosomes by multidimensional protein identification technology (MudPIT). Proteomics 12(2):329–338. doi:10.1002/pmic.201100477
21. Street JM, Birkhoff W, Menzies RI, Webb DJ, Bailey MA, Dear JW (2011) Exosomal transmission of functional aquaporin 2 in kidney cortical collecting duct cells. J Physiol 589(Pt 24):6119–6127. doi:10.1113/jphysiol.2011.220277

22. Oosthuyzen W, Sime NE, Ivy JR, Turtle EJ, Street JM, Pound J, Bath LE, Webb DJ, Gregory CD, Bailey MA, Dear JW (2013) Quantification of human urinary exosomes by nanoparticle tracking analysis. J Physiol. doi:10.1113/jphysiol.2013.264069
23. Raj DA, Fiume I, Capasso G, Pocsfalvi G (2012) A multiplex quantitative proteomics strategy for protein biomarker studies in urinary exosomes. Kidney Int 81(12):1263–1272. doi:10.1038/ki.2012.25
24. Chen CY, Hogan MC, Ward CJ (2013) Purification of exosome-like vesicles from urine. Methods Enzymol 524:225–241. doi:10.1016/B978-0-12-397945-2.00013-5
25. Rood IM, Deegens JK, Merchant ML, Tamboer WP, Wilkey DW, Wetzels JF, Klein JB (2010) Comparison of three methods for isolation of urinary microvesicles to identify biomarkers of nephrotic syndrome. Kidney Int 78(8):810–816. doi:10.1038/ki.2010.262
26. Merchant ML, Powell DW, Wilkey DW, Cummins TD, Deegens JK, Rood IM, McAfee KJ, Fleischer C, Klein E, Klein JB (2010) Microfiltration isolation of human urinary exosomes for characterization by MS. Proteomics Clin Appl 4(1):84–96. doi:10.1002/prca.200800093
27. Alvarez ML, Khosroheidari M, Kanchi Ravi R, DiStefano JK (2012) Comparison of protein, microRNA, and mRNA yields using different methods of urinary exosome isolation for the discovery of kidney disease biomarkers. Kidney Int 82(9):1024–1032. doi:10.1038/ki.2012.256
28. Fernandez-Llama P, Khositseth S, Gonzales PA, Star RA, Pisitkun T, Knepper MA (2010) Tamm-Horsfall protein and urinary exosome isolation. Kidney Int 77(8):736–742. doi:10.1038/ki.2009.550
29. Pisitkun T, Hoffert JD, Yu MJ, Knepper MA (2007) Tandem mass spectrometry in physiology. Physiology 22:390–400. doi:10.1152/physiol.00025.2007
30. Wang G, Wu WW, Zeng W, Chou CL, Shen RF (2006) Label-free protein quantification using LC-coupled ion trap or FT mass spectrometry: Reproducibility, linearity, and application with complex proteomes. J Proteome Res 5(5):1214–1223. doi:10.1021/pr050406g
31. Ross PL, Huang YN, Marchese JN, Williamson B, Parker K, Hattan S, Khainovski N, Pillai S, Dey S, Daniels S, Purkayastha S, Juhasz P, Martin S, Bartlet-Jones M, He F, Jacobson A, Pappin DJ (2004) Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. Mol Cell Proteomics 3(12):1154–1169. doi:10.1074/mcp.M400129-MCP200
32. Thompson A, Schafer J, Kuhn K, Kienle S, Schwarz J, Schmidt G, Neumann T, Johnstone R, Mohammed AK, Hamon C (2003) Tandem mass tags: a novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS. Anal Chem 75(8):1895–1904
33. Yocum AK, Chinnaiyan AM (2009) Current affairs in quantitative targeted proteomics: multiple reaction monitoring-mass spectrometry. Brief Funct Genomic Proteomic 8(2):145–157. doi:10.1093/bfgp/eln056
34. Lv LL, Cao YH, Ni HF, Xu M, Liu D, Liu H, Chen PS, Liu BC (2013) MicroRNA-29c in urinary exosome/microvesicle as a biomarker of renal fibrosis. Am J Physiol Ren Physiol 305(8):F1220–1227. doi:10.1152/ajprenal.00148.2013
35. Lv LL, Cao YH, Pan MM, Liu H, Tang RN, Ma KL, Chen PS, Liu BC (2013) CD2AP mRNA in urinary exosome as biomarker of kidney disease. Clin Chim Acta. doi:10.1016/j.cca.2013.10.003
36. Nilsson J, Skog J, Nordstrand A, Baranov V, Mincheva-Nilsson L, Breakefield XO, Widmark A (2009) Prostate cancer-derived urine exosomes: a novel approach to biomarkers for prostate cancer. Br J Cancer 100(10):1603–1607. doi:10.1038/sj.bjc.6605058
37. Sonoda H, Yokota-Ikeda N, Oshikawa S, Kanno Y, Yoshinaga K, Uchida K, Ueda Y, Kimiya K, Uezono S, Ueda A, Ito K, Ikeda M (2009) Decreased abundance of urinary exosomal aquaporin-1 in renal ischemia-reperfusion injury. Am J Physiol Ren Physiol 297(4):F1006–1016. doi:10.1152/ajprenal.00200.2009
38. Isobe K, Mori T, Asano T, Kawaguchi H, Nonoyama S, Kumagai N, Kamada F, Morimoto T, Hayashi M, Sohara E, Rai T, Sasaki S, Uchida S (2013) Development of enzyme-linked immunosorbent assays for urinary thiazide-sensitive Na-Cl cotransporter (NCC) measurement. Am J Physiol Ren Physiol. doi:10.1152/ajprenal.00208.2013

39. Joo KW, Lee JW, Jang HR, Heo NJ, Jeon US, Oh YK, Lim CS, Na KY, Kim J, Cheong HI, Han JS (2007) Reduced urinary excretion of thiazide-sensitive Na-Cl cotransporter in Gitelman syndrome: preliminary data. Am J Kidney Dis 50(5):765–773. doi:10.1053/j.ajkd.2007.07.022
40. Welton JL, Khanna S, Giles PJ, Brennan P, Brewis IA, Staffurth J, Mason MD, Clayton A (2010) Proteomics analysis of bladder cancer exosomes. Mol Cell Proteomics 9 (6):1324–1338. doi:10.1074/mcp.M000063-MCP201
41. Li Y, Zhang Y, Qiu F, Qiu Z (2011) Proteomic identification of exosomal LRG1: a potential urinary biomarker for detecting NSCLC. Electrophoresis 32(15):1976–1983. doi:10.1002/elps.201000598
42. Raimondo F, Morosi L, Corbetta S, Chinello C, Brambilla P, Della Mina P, Villa A, Albo G, Battaglia C, Bosari S, Magni F, Pitto M (2013) Differential protein profiling of renal cell carcinoma urinary exosomes. Mol BioSyst 9(6):1220–1233. doi:10.1039/c3mb25582d
43. Chen CL, Lai YF, Tang P, Chien KY, Yu JS, Tsai CH, Chen HW, Wu CC, Chung T, Hsu CW, Chen CD, Chang YS, Chang PL, Chen YT (2012) Comparative and targeted proteomic analyses of urinary microparticles from bladder cancer and hernia patients. J Proteome Res 11 (12):5611–5629. doi:10.1021/pr3008732
44. Benito-Martin A, Ucero AC, Zubiri I, Posada-Ayala M, Fernandez-Fernandez B, Cannata-Ortiz P, Sanchez-Nino MD, Ruiz-Ortega M, Egido J, Alvarez-Llamas G, Ortiz A (2013) Osteoprotegerin in exosome-like vesicles from human cultured tubular cells and urine. PLoS ONE 8(8):e72387. doi:10.1371/journal.pone.0072387
45. Moon PG, Lee JE, You S, Kim TK, Cho JH, Kim IS, Kwon TH, Kim CD, Park SH, Hwang D, Kim YL, Baek MC (2011) Proteomic analysis of urinary exosomes from patients of early IgA nephropathy and thin basement membrane nephropathy. Proteomics 11(12):2459–2475. doi:10.1002/pmic.201000443
46. Raimondo F, Corbetta S, Morosi L, Chinello C, Gianazza E, Castoldi G, Di Gioia C, Bombardi C, Stella A, Battaglia C, Bianchi C, Magni F, Pitto M (2013) Urinary exosomes and diabetic nephropathy: a proteomic approach. Mol BioSyst 9(6):1139–1146. doi:10.1039/c2mb25396h
47. Kalani A, Mohan A, Godbole MM, Bhatia E, Gupta A, Sharma RK, Tiwari S (2013) Wilm's tumor-1 protein levels in urinary exosomes from diabetic patients with or without proteinuria. PLoS ONE 8(3):e60177. doi:10.1371/journal.pone.0060177
48. Zhou H, Kajiyama H, Tsuji T, Hu X, Leelahavanichkul A, Vento S, Frank R, Kopp JB, Trachtman H, Star RA, Yuen PS (2013) Urinary exosomal Wilms' tumor-1 as a potential biomarker for podocyte injury. Am J Physiol Ren Physiol 305(4):F553–559. doi:10.1152/ajprenal.00056.2013
49. Conde-Vancells J, Rodriguez-Suarez E, Gonzalez E, Berisa A, Gil D, Embade N, Valle M, Luka Z, Elortza F, Wagner C, Lu SC, Mato JM, Falcon-Perez M (2010) Candidate biomarkers in exosome-like vesicles purified from rat and mouse urine samples. Proteomics Clin Appl 4 (4):416–425. doi:10.1002/prca.200900103
50. Ramirez-Alvarado M, Ward CJ, Huang BQ, Gong X, Hogan MC, Madden BJ, Charlesworth MC, Leung N (2012) Differences in immunoglobulin light chain species found in urinary exosomes in light chain amyloidosis (Al). PLoS ONE 7(6):e38061. doi:10.1371/journal.pone.0038061
51. Li ZZ, Zhao ZZ, Wen JG, Xing L, Zhang H, Zhang Y (2012) Early alteration of urinary exosomal aquaporin 1 and transforming growth factor beta1 after release of unilateral pelviureteral junction obstruction. J Pediatr Surg 47(8):1581–1586. doi:10.1016/j.jpedsurg.2011.12.024
52. Gildea JJ, Carlson JM, Schoeffel CD, Carey RM, Felder RA (2013) Urinary exosome miRNome analysis and its applications to salt sensitivity of blood pressure. Clin Biochem 46 (12):1131–1134. doi:10.1016/j.clinbiochem.2013.05.052