Published in final edited form as:

Proteomics Clin Appl. 2010 February 3; 4(4): 416–425. doi:10.1002/prca.200900103.

Candidate biomarkers in exosome-like vesicles purified from rat and mouse urine samples

Javier Conde-Vancells¹, Eva Rodriguez-Suarez², Esperanza Gonzalez¹, Agustin Berisa³, David Gil⁴, Nieves Embade¹, Mikel Valle⁴, Zigmund Luka⁵, Felix Elortza², Conrad Wagner^{5,6}, Shelly C. Lu⁷, Jose M. Mato¹, and Juan M. Falcon-Perez¹

¹Metabolomics Unit, CICbioGUNE, CIBERehd, Bizkaia Technology Park, Derio, Bizkaia, Spain

²Proteomics Unit, CICbioGUNE, ProteoRed, CIBERehd, Bizkaia Technology Park, Derio, Bizkaia, Spain

³R&D and Innovation Department, FAES FARMA S.A., Leioa, Bizkaia, Spain

⁴Structural Biology Unit, CICbioGUNE, Bizkaia Technology Park, Derio, Bizkaia, Spain

⁵Department of Biochemistry, Vanderbilt University, Nashville, TN, USA

⁶Tennessee Valley Department of Medical Affairs, Medical Center, Nashville, TN, USA

⁷Division of Gastrointestinal and Liver Diseases, Keck School of Medicine, University Southern California, Los Angeles, CA, USA

Abstract

Purpose—There is a compelling clinical imperative to identify discerning molecular biomarkers of hepatic disease in order to inform the diagnosis, prognosis and treatment.

Experimental design—We have investigated the proteome of urinary vesicles present in urine samples obtained from experimental models for the study of liver injury, as an approach for identifying potential biomarkers for hepatic disease.

Results—The biochemical and proteomic characterization of highly purified exosome-like urinary vesicles has identified 28 proteins previously unreported in these vesicles, and many that have been previously associated with diseases, such as the prion-related protein. Furthermore, in urine samples from D-galactosamine-treated rats, a well-characterized experimental model for acute liver injury, we have detected a severe reduction in some proteins that normally are clearly detected in urinary vesicles. Finally, differential protein content on urinary vesicles from a mouse model for chronic liver injury has been also identified.

Conclusions and clinical relevance—Our results argue positively that urinary vesicles could be a source for identifying non-invasive biomarkers of liver injury. We proposed some proteins such as Cd26, Cd81, Slc3A1 and Cd10 that have been found to be differentially expressed in urinary vesicles from some of the analyzed models as potential biomarkers for liver injury.

© 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Correspondence: Dr. Juan Manuel Falcon-Perez, Metabolomics Unit, CIC bioGUNE, CIBERehd, Bizkaia Technology Park, Bldg.801-A, Derio, 48160, Bizkaia, Spain, jfalcon@cicbiogune.es, Fax: +34-944-061-301.

All raw data have been uploaded to the public data repository PRIDE (Accession nos. 9764–9767).

The authors have declared no conflict of interest.

Keywords

Biomarker; Exosomes; Subproteome; Urine; Vesicles

1 Introduction

Urine is an ideal non-invasive source of biomarkers because of the convenience of its collection in large amounts and the ability to collect it repeatedly over lengthy time periods. Because of this, its proteome has been extensively studied for the discovery of biological markers for the diagnosis and classification of diseases mainly – but not exclusively – from the urological tract, and for monitoring the efficacy of treatments. However, the presence of highly abundant proteins (*e.g.* albumin) may mask the identification of under-represented protein that has potential pathophysiologic significance, so the analysis of urinary sub-proteomes would help to overcome this problem. Regarding that, urinary vesicles including exosomes are nanometer sized particles that have been described as good sources for biomarker discovery, unveiling promising results since they allow the detection of relatively low-abundant proteins and decrease the complexity of the urinary proteome [1,2]. In the past few years, several vesicle-associated candidates of potential diagnostic value have been identified. Aquaporin-2, present in these vesicles, appears to correlate with circulating vasopressin levels, and measurements of its excretion have begun to be exploited for the study of water balance abnormalities in humans [3,4]. Exosomal Fetuin-A protein is elevated in patients with acute kidney injury, but not in prerenal azotemia [5]. Proteins such as resistin, GTPase NRas or galectin-3-binding protein has been reported to be differentially expressed in urinary vesicles of patient with bladder cancer *versus* healthy controls [6]. Recently, the analysis of these vesicles in prostate cancer patients has shown that they contain well-established prostate markers (PSA and PSMA) and the tumour-associated marker 5T4 [7]. Furthermore, urinary vesicles from patients with Bartter syndrome type I, associated with mutations in the SLC12A1 gene, which encodes for the NKCC2 sodium–potassium–chloride co-transporter protein, were shown to contain very low concentrations of this transporter, compared with normal individuals [1]. In addition, Gonzales *et al.* have recently detected up to 1132 unique proteins in human urinary vesicles, which includes 177 disease-related supporting the usefulness of these vesicles to biomarker discovery [1]. Remarkably, results obtained from many of these laboratories have revealed the profound variability observed among different specimens, even those from healthy individuals, which may delay or prevent the biomarker definition of a determined protein [6,7].

Animal models provide the necessary tools to overcome typical confounding variables, such as genetic heterogeneity, gender differences and environmental factors, including diet and lifestyle. Different rat and mouse models have been extensively used to address an array of physiological questions including: metabolism, toxicology and multiple disease processes. Our research group has similarly investigated the urinary vesicles present in rat and mouse urine samples in an effort to identify potential biomarkers for diseases prior to attempting human trials. We performed for the first time cryo-electron microscopy on these urinary vesicles, revealing the presence of repetitive “mushroom”-shape structures on their surfaces. We also performed the first proteomic analysis of highly purified exosome-like vesicles from urine samples. In total, we have detected 134 proteins, including metabolic enzymes, solute transporters, peptidases and proteins involved in cell signaling and in cytoskeleton organization. Many of these proteins have previously been associated with diseases. The presence of different vesicle populations with a size smaller than 220 nm was also demonstrated based on their protein composition. Finally, using two animal models, one for acute and other for chronic hepatic damage, we are able to detect significant changes in these vesicles that can constitute indicators of cellular damage. This further supports the hypothesis that the study of

these vesicles in animal models may provide us with potential biomarkers with respect to better disease diagnosis and prognosis.

2 Materials and methods

2.1 Reagents

All reagents were of analytical grade and primarily acquired from Sigma-Aldrich (St. Louis, MO, USA). Mouse monoclonal antibodies were purchased from the indicated vendors: anti-CD63 (clone AD1), anti-flotillin (clone 18) from BD Biosciences (Mountain View, CA, USA), anti-CD10 (nephrilysin) (clone F-4) from Santa Cruz Biotech. (Santa Cruz, CA, USA), anti-Hsp70 (clone BRM-22) from Sigma-Aldrich, and anti-TSG101 (clone 4A10) from Abcam (Cambridge, UK). Rabbit polyclonal antibodies were acquired from the indicated vendors: anti-SLC3A1 (rBAT, H-300) from Santa Cruz Biotech, anti-Caveolin from BD Biosciences, anti-CD26 (DPP4) and anti-LIMPII from Abcam, and anti-AQP1 from Sigma-Aldrich. Goat polyclonal antibody anti-Probasin (R-15) was purchased from Santa Cruz Biotech. Sheep polyclonal anti-albumin was purchased from Abcam. Hamster Armenian anti-mouse Cd81 (clone Eat2) was purchased from Serotec (Oxford, UK). Rabbit polyclonal anti-PrP (EP1802Y) was purchased from Abcam. HRP-conjugated secondary antibody was from GE Healthcare (Buckinghamshire, UK).

2.2 Animal studies

Male Wistar rats, 14 wk of age (body weight 300–400 g), were maintained in an environmentally controlled room at 22°C on 12-h light/dark cycle and provided with standard diet (Rodent Maintenance Diet, Harlan Teklad Global Diet 2014) and water *ad libitum*. Different groups were made for the study (Supporting Information Fig. S1): A group (I) of 24 animals was used to collect urine samples to perform the biochemical and proteomic characterization of rat urinary vesicles. Another group (II) including 12 rats received an intraperitoneal injection of 1 g/kg/5 mL of D(+)-galactosamine hydrochloride (GalN, Sigma-Aldrich). A final control group (III) of 12 animals received the same volume of saline solution. Six hours after injection, GalN- and saline-treated animals were housed in metabolic cages to collect urine samples. After urine sample collection, GalN-treated and saline-treated animals were sacrificed and livers were formalin-fixed, embedded in paraffin and 5- μ m thick sections were cut and stained with hematoxylin and eosin according to the standard procedure. Furthermore, wild-type (WT) and glycine *N*-methyltransferase (*GNMT*) knockout (GNMT-KO) mice were also maintained in an environmentally controlled room at 22°C on 12-h light/dark cycle and provided with standard diet and water *ad libitum*. In this case, ten animals from each of the two groups (WT and GNMT-KO) were housed in metabolic cages to collect urine at the indicated ages. In all studies, the rats and mice were fasted for 6 h prior to starting a 16-h urine collection in the absence of food and the presence of water *ad libitum*. The urine samples collected for same group were pooled together due to the small amount (23 and 5 mL as mean values in rats and mice, respectively) of individual urine samples. The pooled samples were kept at -80°C until the purification of urinary vesicles. All animal experimentation was conducted in accordance with the Spanish Guide for the Care and Use of Laboratory Animals, and protocols were approved by the CIC bioGUNE Ethical Review Committee that has been accredited by AAALAC and OLAW organizations.

2.3 Urinary vesicle isolation

Urinary vesicles from 200 mL (rat) or 50 mL (mouse) of urine were isolated as described by Thery *et al.* [8]. Briefly, collected urine samples were centrifuged for 30 min at $1500 \times g$. The resultant supernatants were subjected to filtration on 0.22 μ m pore filters, followed by ultracentrifugation at $10\,000 \times g$ and $100\,000 \times g$ for 30 and 60 min, respectively. The resulting pellets were suspended in PBS, pooled and again ultracentrifuged at $100\,000 \times g$ for 60 min.

The final pellet of urinary vesicles was suspended in 150 μ L of PBS, aliquoted and stored at -80°C .

For proteomic analysis, rat urinary exosomes isolated from group I were further purified on 30% sucrose cushion as described previously [8]. Briefly, the PBS-suspended urinary vesicle preparation was diluted in 20 mL of PBS and under-layered on top of a density cushion composed of 20 mM Tris/30% sucrose/deuterium oxide (D_2O), pH 7.4 (4 mL), forming a visible interphase. The samples were ultracentrifuged at $100\,000 \times g$ at 4°C for 75 min in a SW-32 Ti swinging bucket rotor. The ultracentrifuged tubes were pierced on the side with an 18-gauge needle and 3.5 mL were withdrawn from the bottom. Vesicles contained in the 30% sucrose/ D_2O cushion were collected, diluted a minimum of ten times with PBS and centrifuged at $100\,000 \times g$ and 4°C for 60 min. The final pellet, highly enriched in exosome-like vesicles, was resuspended in PBS to half of the initial volume, aliquoted and stored at -80°C .

2.4 Fractionation of urinary vesicles on continuous sucrose gradient

Hundred microliter of rat urinary vesicles isolated from group I were diluted in 2 mL of HEPES/sucrose stock solution (20 mM HEPES, 2.5 M sucrose) and poured at the bottom of a SW32 centrifuge tube. A continuous 0.25–2 M sucrose gradient was created on the top by means of a gradient maker device coupled to an auto densi-flow density gradient fractionator (Labconco, Kansas City, MO, USA). In the gradient maker, 6 mL of 2 M sucrose solution (20 mM HEPES, 2 M sucrose) were poured in the proximal compartment of the gradient maker, and 6 mL of 0.25 M sucrose solution (20 mM HEPES, 0.25 M sucrose) were poured in the distal compartment. The continuous sucrose gradient was ultracentrifuged overnight (≥ 14 h) at $210\,000 \times g$, 4°C , in an SW32Ti swinging-bucket rotor. Onemilliliter fractions were collected from top to bottom and 10 μ L of each fraction were used for measurement of the refractive index to density determination. Each fraction was diluted with 2 mL of 20 mM HEPES (pH 7.4) and ultra-centrifuged during 1 h at $110\,000 \times g$, 4°C , in a TLA-110 rotor. Supernatants were aspirated and the pellets were resuspended in 25 μ L PBS and frozen at -80°C .

2.5 NanoLC-MS/MS analysis and database search

Proteomic profiles were performed with rat urinary vesicles isolated from group I. The exosome-enriched preparation derived from the 30% sucrose cushion or selected urinary vesicular fractions obtained from the continuous sucrose gradient were separated on a 4–12% SDS-PAGE gel, fixed and stained with Coomassie blue. The corresponding gel lane was sliced, and each slice was subjected to trypsin digestion. Nanoflow LC-MS/MS analysis was performed using a nanoflow UPLC system coupled to a QTOF Premier mass spectrometer (Waters). Samples were loaded in 1% aqueous formic acid (FA) and analyzed by reverse phase LCMS/MS in a UPLC reverse phase chromatography system. Tryptic peptides were desalted on a Symmetry C18 trapping cartridge and further separated on an analytical column with an integrated electrospray ionization emitter tip. Peptides were eluted at a flow rate of 250 nL/min from the analytical column directly to electrospray ionization emitter tip by using a 30 min gradient from 0 to 30% solvent B (solvent A: 1% aqueous FA and solvent B: 100% ACN, 1% FA). Data was acquired in the data-dependent acquisition mode, in which a full scan mass spectrum (m/z : 300–1500) was followed by MS/MS (m/z : 50–1995) in the three most abundant multi-charged ions (+2 and +3) every 4 s. Argon was used as the collision gas. Collision energies were interpolated linearly as a function of a charge state and m/z of each peptide. Dynamic exclusion was incorporated for 30 s. A scan of the reference compound (Glufibrinopeptide B) was acquired every ten scans of the analyte through the entire run. Raw data were processed using ProteinLynx Global Server v2.2.5. The resulting pkl file was searched against v54 of Swiss-Prot sequence database (Rattus: 6719 sequences) with rat as taxonomy using an in-house MASCOT server (Version 2.2.03, Matrix Sciences, London, UK). One miss cleavage was allowed; carbamidomethyl was chosen as fixed modification and

methionine oxidation as variable modification. A peptide mass tolerance of 10 ppm and 0.1 Da of fragment mass tolerance were allowed. To make these data available for future studies, all the raw data (exosome-enriched preparation as 9764, fraction 6 as 9765, fraction 9 as 9766 and fraction 12 as 9767) have been uploaded to the public data repository PRIDE (<http://www.ebi.ac.uk/pride/>). Only proteins with at least two specific peptides and with a false discovery rate (FDR) lower than 1% were included in the study. FDR was performed according the automated tool that is included in MASCOT search engine following the instructions of Matrix Science, and the Supporting Information Table S6 shows the data obtained from this analysis.

The lists with the proteins with all the parameters (protein accession number, description, mass and score) are provided in Supporting Information as Table S1 for exosome-enriched preparation, Table S2 for fraction 6, Table S3 for fraction 9 and Table S4 for fraction 12. An additional Supporting Information Table S5 is also provided with information (number, sequence and charge state) regarding the peptides considered to identify the proteins.

2.6 Western blot analysis

The protein concentration of the preparations was determined by means of a Bradford protein assay using BSA as the standard. Samples were incubated for 5 min at 37, 65 and 95°C, and separated on 4–12% pre-casted gels from Invitrogen (Carlsbad, CA, USA). After being transferred to nitrocellulose membranes and blocked overnight (5% milk and 0.05% Tween-20 in PBS), primary antibody was added for 1 h, followed by PBS washing and the application of secondary HRP-conjugated antibody. All proteins, except for prion-related protein (PrP^C), were detected under non-reducing conditions. Chemiluminescence detection of bands was performed with ECL Plus reagent.

2.7 Electron microscopy

For cryo-electron microscopy, vesicle preparations were directly adsorbed onto glow-discharged holey carbon grids (QUANTIFOIL, Germany). Grids were blotted at 95% humidity and rapidly plunged into liquid ethane with the aid of VITROBOT (Maastricht Instruments BV, The Netherlands). For negative staining, 2.5 µL drops of purified vesicles were adsorbed onto glow-discharged carbon-coated copper grids, washed with distilled water and stained with freshly prepared 2.0% uranyl acetate in aqueous suspension. Vitrified samples were imaged at liquid nitrogen temperature using a JEM-2200FS/CR transmission cryo-electron microscope (JEOL, Japan) equipped with a field emission gun and operated at an acceleration voltage of 200 kV. In cryo-EM sessions, digital images were taken using low-dose technique by means of an ULTRASCAN 4000SP (4096 × 4096 pixels) cooled slow-scan CCD camera (GATAN, UK). An in-column energy filter (Omega Filter) was used to improve the signal-to-noise ratio (Frank, 1995) of these images, by zero-loss filtering. Negative stained samples were imaged at room temperature using a JEM-1230 transmission electron microscope (JEOL, Japan) equipped with a thermionic tungsten filament and operated at an acceleration voltage of 120 kV. Images were taken using the ORIUS SC1000 (4008 × 2672 pixels) cooled slow-scan CCD camera (GATAN, UK).

3 Results

3.1 Structural, biochemical and proteomic characterization of rat urinary vesicles

We first characterized the nanometer-sized vesicles present in rat urine samples. Membrane vesicles less than 220 nm in size were purified from pooled samples of rat urine by filtration and differential centrifugation, as described above. The analysis by cryo-electron microscopy demonstrates that the isolated material contains rounded vesicles that have their surfaces covered by mushroom-shaped structures (Fig. 1, *inset*). Quantitative analysis of cryo-electron

micro-graphs (Fig. 1B) illustrates that the mean diameter of these vesicles in two independent preparations were 95.8 ± 50.7 nm ($n = 95$) and 92.6 ± 43.9 nm ($n = 94$). Equal amounts of protein derived from urine samples, and the urinary vesicles purified from these same urine samples, were subjected to Western blot analysis using antibodies specific for different proteins. The enrichment in exosomal markers such as the proteins Cd63, Cd81, Flotillin or Tsg101, as shown in Fig. 1C, argues positively for the elevated presence of exosome-like vesicles in this material, as has been previously reported in human urine samples [1,2]. Remarkably, we have also detected the PrPc in urinary vesicles (Fig. 1C). This protein is believed to be present in exosomes from a number of different sources [9–11]. This Western blot analysis also shows the clear reduction of highly abundant proteins such as albumin (Fig. 1c), supporting the hypothesis that a better characterization of these vesicles could be a good approach to identify biomarkers that are under-represented in urine samples. At present, the proteome of the whole urinary vesicles has been reported [1]; therefore, our interest was to more specifically characterize the protein content of exosome-like vesicles. To do that, we have performed a further purification step involving a flotation on 30% sucrose cushion that have been widely described to purify these exosomes in other systems [8]. The exosome highly enriched vesicles were resolved by loading in SDS-PAGE, the gel lanes were sliced into pieces, subjected to in-gel trypsinization and analyzed by nano-spray LC-MS/MS. Overall, 134 unique proteins were identified by at least two peptides with a FDR lower than 1% (Supporting Information Table S1). These included several solute transporters, peptidases, proteins involved in redox homeostasis, cytoskeleton reorganization, cell signaling and cytosolic enzymes that were most likely engulfed during the exosome formation. Proteins identified in this study included 28 proteins reported for the first time present in urinary vesicles (Supporting Information Table S1), and many proteins that have been associated with different diseases (Table 1 and Supporting Information Table S1) that may be considered as candidate urinary exosomal biomarkers.

A more exhaustive characterization of the different vesicle populations existing in urinary vesicles was obtained using a continuous sucrose gradient. As shown in Fig. 2, at least three different sub-populations based on their size, density and protein content could be clearly identified. These populations were obtained in fractions corresponding to densities of 1.180, 1.246 and 1.252 g/mL, respectively. Their median diameter sizes determined by cryo-electron microscopy were 99.4, 74.4 and 72.4 nm, respectively. At the protein level, by Western blot analysis the population with the lowest density has enriched its content in the protein flotillin compared to the content in Cd63. On the contrary, the densest population has less amount of flotillin compared with Cd63, and the intermediate population present more or less similar amount of these two proteins. Other proteins such as Cd10 appear to be equally distributed in these three populations. To refine the proteomic profile of these three populations, a LC-MS/MS experimental approach was undertaken (Fig. 3 and Supporting Information Tables S2–S4), and from the 105 different proteins identified, 20 were widely distributed in the different vesicle populations as indicated by their identification in the three analyzed fractions. Amongst this group that could be essential constituents of these urinary vesicles were found many solute transporters such as Slc3a1 or S23a1, peptidases as Cd26, Cd10, AmpE or AmpN, and some cytoskeletal-related and anchoring proteins including Nherf, Pdzd1, ezrin or actin.

3.2 Indicators of hepatic damage detected in urinary vesicles

To obtain specific indicators of damage in urinary vesicles, we have studied them in experimental models resembling two pathological conditions. D-galactosamine (GalN) is a well-established hepatotoxin frequently used in animal experiments. Administration of GalN causes severe liver damage in rats closely resembling those seen in human viral hepatitis [12]. A group of 12 rats were intraperitoneally injected with GalN (1 g/kg/5 mL), whereas another group of 12 rats received a similar volume of saline solution. Six hours later the animals

were individually housed and urine samples were collected during a 16-h period. No significant differences were found in the amount of urine samples collected between GalN- or saline-treated rats obtaining mean values ($n = 12$) of 26.4 ± 14.8 and 20.0 ± 12.2 mL, respectively. After collecting urine samples, the animals were sacrificed and the liver of each animal was processed for histological hematoxylin–eosin staining. In all GalN-treated rats were observed liver-damaged regions similar to that one shown in the representative image of Fig. 4A, and no liver alterations were appreciated in none of the saline-treated rats, confirming the efficiency and the consistency of the GalN treatment. Urinary vesicles from GalN-treated rats were purified and compared by Western blotting to those vesicles obtained from urine samples of the control saline-treated group (Figs. 4B and C). As shown in Fig. 4C, no significant differences were found between the two urine samples regarding to the levels of albumin. As expected, after the purification of urinary vesicles the presence of albumin was undetectable (Fig. 4C) indicating the good efficacy of the vesicle isolation procedure. In contrast, the presence of several proteins normally present in urinary vesicles including Cd26 (DPP4), Slc3a1 (rBAT) or Cd81 was dramatically reduced in sample obtained from GalN-treated rats (Fig. 4B). Other proteins (*e.g.* LimpII and Cd10) were also reduced in this sample although at less extent. All these data indicate a severe effect on the urinary vesicles content in urine samples caused by this hepatotoxic compound.

We have also analyzed the urinary vesicles in mice carrying a deletion for the gene coding for GNMT, a liver-specific enzyme involved in the homeostasis of S-adenosylmethionine, which is a universal donor of methyl groups in biological methylation processes [13]. These knockout mice have been shown to develop steatosis, fibrosis and hepato-cellular carcinoma (HCC), resembling many of the features observed in human chronic liver fatty acid disease [14–16]. We have obtained urinary vesicles from these mice at different ages and compared them with those obtained from WT animals by analyzing the presence of some common vesicular proteins. As shown in Fig. 5, while no significant differences were found for the presence of proteins Cd81 and flotillin, a clear increment in the level of Cd10 protein was observed in urinary vesicles derived from the knockout mice. Remarkably, this increased concentration was detected in very early disease stages and was consistently observed over the time course of disease progression (Fig. 5), supporting the fact that these vesicles are suitable biological sources to identify biomarkers in early stages of diseases.

4 Discussion

We have performed an extensive proteomic and biochemical characterization of urinary vesicles smaller than 220 nm in diameter, which include exosome-like vesicles. In our knowledge, all of the existing electron micrographs of these urinary vesicles have been obtained using negative staining procedures involving techniques that alter the shape of these vesicles. We have used a cryo-electron microscope to study their real size and morphology, and consistently observed the presence of repetitive mushroom-shape structures on the surface of these vesicles. Interestingly, these superficial structures were not found in cryo-electron micrograph of exosome-like vesicles secreted by hepatocytes [17], suggesting that this feature could be unique to urinary vesicles; further studies of additional exosome populations by cryo-electron microscopy will help to establish this fact.

The whole proteome of urinary vesicles has already been reported [1,2]; we herein report on the sub-proteome of vesicle populations with a diameter of less than 220 nm that can be found in urine samples. First, we have obtained a preparation highly enriched in exosome-like vesicles when applying the methodology that has been well established to characterize these vesicles in cell culture supernatants [8]. In these preparations, we accurately identified 134 proteins, 20% of which are being reported for first time to be present in urinary vesicles. This strongly suggests that the analysis of sub-proteomes is a suitable methodology to detect the presence

of additional proteins. Among these newly detected exosomal proteins are the metalloproteinases Mephrin A and B, which have been associated with inflammatory bowel disease [18]. At least 40% of the identified proteins have been reported to be associated with several diseases and further characterizations of these exosome-detected proteins under normal and pathological conditions will help to define them as truly biomarkers of diseases. In another approach, by fractionating these urinary vesicles (<220 nm) in a continuous sucrose gradient, we have demonstrated the presence of at least three vesicle populations defined according to their content of proteins such as flotillin, Cd63 or Cd10. A further proteomic characterization of these populations revealed that more than half of the identified proteins were detected in the fraction with a similar density to the exosomes, supporting the view that urinary vesicles below 220 nm in diameter contain primarily, but not exclusively, exosome-like vesicles. Furthermore, these findings raise an important issue concerning those proteomes that have been obtained so far with urinary vesicles because previous studies related to these vesicles have been performed with a heterogeneous population of vesicles which probably have different cellular origins. Our work is the first study to characterize different vesicle populations that exist in urine samples, in order to obtain additional insight that may help to define the origin of the different vesicles that could be useful to obtain the localization of the damage. However, our study indicates that the proteomic approach alone is not enough to determinate the cellular origin of the different urinary vesicles because the identified proteins so far have a wide cell type distribution providing low information in terms of their origins. Further studies in this direction using well-characterized animal models with pathologies affecting specific cell types would be needed to obtain the right origin of the different vesicles.

Several laboratories have previously reported the presence of the prion-causing disease protein in exosomes derived from different, primarily neuronal cell types [9–11]. Remarkably, we have shown here that the native protein PrPc was also detected in two independent preparations of urinary vesicles. The band that we have detected corresponds to the highest glycosylated form of the protein in clear agreement with previous exosomal detections of this protein in which also just one band was observed. While in cellular extracts additional bands are detectable, in exosomal preparations just one band with the highest glycosylation state is observed [9,19]. This finding could open new avenues to investigate the metabolism and clearance mechanism of PrPc during prion infection and disease progression. It may also provide opportunities to impact diagnosis and prognosis applications.

Regarding the liver pathology, we have analyzed urinary vesicles in two animal models and even though a low number of biological replicas have been used in this study, our results support the usefulness of these vesicles to serve as biological sources to look for biomarkers of hepatic diseases. The development of acute liver injury induced by the administration of GalN is well documented [12,20], and due to the similar morphological and pathophysiological lesions, it has been widely used as model for the study of human viral hepatitis [21,22]. Our analysis of urinary vesicles in GalN-treated rats has shown severe reduction on their content in Cd26, Slc3a1 and Cd81 proteins, supporting the potential use of these three proteins as candidate urinary indicators of acute liver damage. Further studies with other hepatotoxins will be required to confirm the potential of urinary vesicles as biological sources for liver biomarker discovery.

Previously, it has been shown that the activity of Cd26 in the serum of GalN-treated rats and patients with hepatitis was elevated [23], and more recently, it has been reported that patients with chronic hepatitis C also have elevated serum activity of this enzyme [24,25] and a negative correlation with the response to interferon therapy was observed [25]. According to our results, these elevated levels of Cd26 activity in the serum could be generated by a deficiency in the urine elimination of this protein. We have also focused on the study of another animal model recently reported for the study of liver disease, the knockout mouse for the hepatic specific

GNMT gene. This genetic model develops chronic liver damage resembling many of the clinical manifestations observed in patients of non-alcoholic fatty acid disease, such as steatosis, fibrosis and HCC [14–16]. The analysis of urinary vesicles from WT and *GNMT*-KO mice have revealed that Cd10 protein is elevated in vesicles isolated from knockout samples even in 2-month-old animals that are in the first stages of the disease, supporting the notion that an increase in this protein within urinary vesicles could be considered an early indicator of chronic liver disease. Cd10 is a 100-kDa transmembrane glycoprotein, also known as neprilysin, neutral endopeptidase, membrane metallo-endopeptidase, or enkephalinase, which is involved in the cleavage and inactivation of multiple physiologically active peptides. Loss or decrease in Cd10 expression has been reported in many types of malignancies, including renal cancer [26], invasive bladder cancer [27], poorly differentiated stomach cancer [28], small cell and non-small cells lung cancer [29], endometrial cancer [30] and prostate cancer [31]. Furthermore, Cd10 has been found to be over-expressed in liver samples from HCC [32,33], and recently, its canalicular immunostaining has been shown to be useful in discriminating HCC and metastatic carcinoma of the liver [34]. These studies, combined with our results, suggest that Cd10 could be a good disease biomarker that can be analyzed in urinary samples.

In summary, this study investigates the proteome of different vesicle populations that exist in urine samples and indicates several candidate biomarkers including PrPc, Cd26, Slc3a1, Cd81 and Cd10 that are detected in urinary vesicles and may be useful for diagnostic purposes. In addition, our results emphasize the effectiveness using urinary vesicle analysis to identify potential diagnostic markers.

Clinical Relevance

Liver injury ranging from mild infection to life-threatening liver failure is a serious worldwide health issue that our society is currently facing. A major goal in liver pathology is the identification of biomarkers for early detection of the different liver alterations. Currently, differentiation of these alterations depends mainly on histological examination of liver biopsies. However, the numbers of patients with liver injury means that use of liver biopsy in their investigation to reach a reliable diagnosis is both practically and financially impractical; consequently, there is a compelling clinical imperative to identify non-invasive discerning molecular biomarkers of hepatic injury.

Recently, several reports have shown that urine samples contain vesicles that could be used in the biomarker discovery area. In this study, we have characterized urinary vesicles from normal and from two well-characterized animal models for the study of liver disease, and we have demonstrated that several proteins localized in these vesicles including Cd26 and Cd10 are sensitive to liver injury. Our findings suggest these urinary vesicles could be a biological source to identify non-invasive biomarkers of liver disease, and support that a better characterization of these vesicles in urine samples from patients with liver pathologies will provide new hepatic biomarkers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

FA	formic acid
FDR	false discovery rate
GNMT	glycine <i>N</i> -methyltransferase

HCC	hepatocellular carcinoma
PrPc	prion-related protein
WT	wild type

Acknowledgments

The authors gratefully thank Richard Finnell for his critical reading of the manuscript, and J. Rodriguez, A. Peña, V. Perez, N. Teso, B. Martínez de la Pera, C. Oveja, I. Iturriza and L. María for their technical assistance and FAES FARMA S.A. for its support with rat experimentation procedures and sample collection. This work was supported by grants from the Fondo de Investigaciones Sanitarias (Instituto de Salud Carlos III, 06/ 0621 to J. M. F. P.); Program “Ramon y Cajal” of Spanish Ministry (to J. M. F. P.); PN I1D SAF 2005-00855 (to J. M. M.); NIH grant (AT-1576 to S. C. L. and J. M. M.); DK15289 (to C. W.); HEPADIP consortium (HEPADIP-EULSHM-CT-205); BBVA foundation. CIBERehd is funded by the Instituto de Salud Carlos III. Mass spectrometry analysis was performed at CIC bioGUNE Proteomics Core Facility, member of ProteoRed.

References

- Gonzales PA, Pisitkun T, Hoffert JD, Tchapyjnikov D, et al. Large-scale proteomics and phosphoproteomics of urinary exosomes. *J. Am. Soc. Nephrol* 2009;20:363–379. [PubMed: 19056867]
- Pisitkun T, Shen R-F, Knepper MA. Identification and proteomic profiling of exosomes in human urine. *Proc. Natl. Acad. Sci. USA* 2004;101:13368–13373. [PubMed: 15326289]
- Kanno K, Sasaki S, Hirata Y, Ishikawa S, et al. Urinary excretion of aquaporin-2 in patients with diabetes insipidus. *N. Engl. J. Med* 1995;332:1540–1545. [PubMed: 7537863]
- Wen H, Frokiaer J, Kwon TH, Nielsen S. Urinary excretion of aquaporin-2 in rat is mediated by a vasopressin-dependent apical pathway. *J. Am. Soc. Nephrol* 1999;10:1416–1429. [PubMed: 10405197]
- Zhou H, Pisitkun T, Aponte A, Yuen PS, et al. Exosomal Fetuin-A identified by proteomics: a novel urinary biomarker for detecting acute kidney injury. *Kidney Int* 2006;70:1847–1857. [PubMed: 17021608]
- Smalley DM, Sheman NE, Nelson K, Theodorescu D. Isolation and identification of potential urinary micro-particle biomarkers of bladder cancer. *J. Proteome Res* 2008;7:2088–2096. [PubMed: 18373357]
- Mitchell PJ, Welton J, Staffurth J, Court J, et al. Can urinary exosomes act as treatment response markers in prostate cancer? *J. Transl. Med* 2009;7:4. [PubMed: 19138409]
- Thery C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. Chapter 3. *Curr. Protoc. Cell Biol.* 2006 Unit 3.22.
- Fevrier B, Vilette D, Archer F, Loew D, et al. Cells release prions in association with exosomes. *Proc. Natl. Acad. Sci. USA* 2004;101:9683–9688. [PubMed: 15210972]
- Vella LJ, Greenwood DL, Cappai R, Scheerlinck JP, Hill AF. Enrichment of prion protein in exosomes derived from ovine cerebral spinal fluid. *Vet. Immunol. Immunopathol* 2008;124:385–393. [PubMed: 18501435]
- Vella LJ, Sharples RA, Lawson VA, Masters CL, et al. Packaging of prions into exosomes is associated with a novel pathway of PrP processing. *J. Pathol* 2007;211:582–590. [PubMed: 17334982]
- Keppler D, Lesch R, Reutter W, Decker K. Experimental hepatitis induced by d-galactosamine. *Exp. Mol. Pathol* 1968;9:279–290. [PubMed: 4952077]
- Lu SC, Mato JM. S-adenosylmethionine in cell growth, apoptosis and liver cancer. *J. Gastroenterol. Hepatol* 2008;23:S73–S77. [PubMed: 18336669]
- Luka Z, Capdevila A, Mato JM, Wagner C. A glycine N-methyltransferase knockout mouse model for humans with deficiency of this enzyme. *Transgenic Res* 2006;15:393–397. [PubMed: 16779654]
- Martinez-Chantar ML, Vazquez-Chantada M, Ariz U, Martinez N, et al. Loss of the glycine N-methyltransferase gene leads to steatosis and hepatocellular carcinoma in mice. *Hepatology* 2008;47:1191–1199. [PubMed: 18318442]

16. Wagner C, Luka Z, Mato JM. Hepatocellular carcinoma in GNMT^{-/-} mice. *Toxicol. Appl. Pharmacol* 2009;237:246. [PubMed: 19345238]
17. Conde-Vancells J, Rodriguez-Suarez E, Embade N, Gil D, et al. Characterization and comprehensive proteome profiling of exosomes secreted by hepatocytes. *J. Proteome Res* 2008;7:5157–5166. [PubMed: 19367702]
18. Sun Q, Jin HJ, Bond JS. Disruption of the meprin alpha and beta genes in mice alters homeostasis of monocytes and natural killer cells. *Exp. Hematol* 2009;37:346–356. [PubMed: 19110362]
19. Robertson C, Booth SA, Beniac DR, Coulthart MB, et al. Cellular prion protein is released on exosomes from activated platelets. *Blood* 2006;107:3907–3911. [PubMed: 16434486]
20. Decker K, Keppler D. Galactosamine induced liver injury. *Prog. Liver Dis* 1972;4:183–199. [PubMed: 4631501]
21. El-Mofty SK, Scrutton MC, Serroni A, Nicolini C, Farber JL. Early, reversible plasma membrane injury in galactosamine-induced liver cell death. *Am. J. Pathol* 1975;79:579–596. [PubMed: 1137005]
22. Komano T, Egashira Y, Sanada H. L-Gln and L-Ser suppress the d-galactosamine-induced IL-18 expression and hepatitis. *Biochem. Biophys. Res. Commun* 2008;372:688–690. [PubMed: 18514057]
23. Eggstein S, Kreisel W, Gerok W, Eggstein M. Dipeptidyl aminopeptidase IV in hospitalized patients and in galactosamine hepatitis of the rat: activity and lectin affinity chromatography in serum and hepatic plasma membranes. *J. Clin. Chem. Clin. Biochem* 1989;27:547–554. [PubMed: 2575117]
24. Andrieu T, Thibault V, Malet I, Laporte J, et al. Similar increased serum dipeptidyl peptidase IV activity in chronic hepatitis C and other viral infections. *J. Clin. Virol* 2003;27:59–68. [PubMed: 12727530]
25. Firneisz G, Lakatos PL, Szalay F. Serum dipeptidyl peptidase IV (DPP IV, CD26) activity in chronic hepatitis C. *Scand. J. Gastroenterol* 2001;36:877–880. [PubMed: 11495085]
26. Gohring B, Holzhausen HJ, Meye A, Heynemann H, et al. Endopeptidase 24.11/CD10 is down-regulated in renal cell cancer. *Int. J. Mol. Med* 1998;2:409–414. [PubMed: 9857225]
27. Koiso K, Akaza H, Ohtani M, Miyanaga N, Aoyagi K. A new tumor marker for bladder cancer. *Int. J. Urol* 1994;1:33–36. [PubMed: 7627835]
28. Sato Y, Itoh F, Hinoda Y, Ohe Y, et al. Expression of CD10/neutral endopeptidase in normal and malignant tissues of the human stomach and colon. *J. Gastroenterol* 1996;31:12–17. [PubMed: 8808423]
29. Cohen AJ, Bunn PA, Franklin W, Magill-Solc C, et al. Neutral endopeptidase: variable expression in human lung, inactivation in lung cancer, and modulation of peptide-induced calcium flux. *Cancer Res* 1996;56:831–839. [PubMed: 8631021]
30. Pekonen F, Nyman T, Ammala M, Rutanen EM. Decreased expression of messenger RNAs encoding endothelin receptors and neutral endopeptidase 24.11 in endometrial cancer. *Br. J. Cancer* 1995;71:59–63. [PubMed: 7819049]
31. Papandreou CN, Usmani B, Geng Y, Bogenrieder T, et al. Neutral endopeptidase 24.11 loss in metastatic human prostate cancer contributes to androgen-independent progression. *Nat. Med* 1998;4:50–57. [PubMed: 9427606]
32. Borscheri N, Roessner A, Rocken C. Canalicular immunostaining of neprilysin (CD10) as a diagnostic marker for hepatocellular carcinomas. *Am. J. Surg. Pathol* 2001;25:1297–1303. [PubMed: 11688465]
33. Xiao SY, Wang HL, Hart J, Fleming D, Beard MR. cDNA arrays and immunohistochemistry identification of CD10/CALLA expression in hepatocellular carcinoma. *Am. J. Pathol* 2001;159:1415–1421. [PubMed: 11583969]
34. Ahuja A, Gupta N, Kalra N, Srinivasan R, et al. Role of CD10 immunohistochemistry in differentiating hepatocellular carcinoma from metastatic carcinoma of the liver. *Cytopathology* 2008;19:229–235. [PubMed: 18070122]

Figure 1.

Characterization of rat urinary vesicles. (A) Cryo-electron micrograph of material isolated by filtration and differential centrifugation from rat urine samples. Note the presence of rounded vesicles and repetitive structures decorating the surface of these vesicles. *Inset*, a surface area was $4\times$ magnified to underline that this structures have a mushroom-shaped appearance. *Bar*, 100 nm. (B) Quantitative analysis of vesicle diameter was performed in cryo-electron micrographs acquired and processed by using Digital Micrograph Software. Distributions of percentage of vesicles, number of vesicles analyzed (n) and mean and SD of two independent preparations are indicated. (C) Western blot analysis of two independent urine preparations.

Similar amount (5 μ g) of protein from urine and vesicles isolated from same sample were analyzed by Western blotting using antibodies against the indicated proteins.

Figure 2.

Analysis of different vesicle populations present in the urine. Rat urinary vesicles (<220 nm in diameter) were subjected to 0.25–2 M continuous sucrose gradient and equal volumes of each collected fractions were analyzed by electron microscopy and Western blotting with the indicated antibodies. Representative negative staining micrograph of fractions 6, 9 and 12 are showed along with the median diameter size of the vesicles and the density (δ , g/mL) for the corresponding fraction. *Bar*, 100 nm.

Figure 3.

Proteomic overlap of three vesicle populations existing in rat urine samples. Urinary vesicles (<220 nm in diameter) were subjected to continuous sucrose gradient and collected fractions F6, F9 and F12 corresponding to the indicated densities (δ , g/mL) and diameter size vesicles were subjected to proteomic analysis by LC-MS/MS. Only proteins identified by at least two peptides were considered in the analysis. Protein accession numbers were used to perform the comparative analysis and number of proteins found in common among the different fractions is indicated. Based on their activities, proteins (indicated by accession name) were categorized in metabolic enzymes (brown), protein processing and modification (red), transporters (blue), cytoskeleton reorganization, protein localization, membrane trafficking or cell signaling (pink), redox homeostasis (green) and miscellaneous (black).

Figure 4.

Candidate biomarkers in urinary vesicles for acute liver injury. Acute-liver injury was generated in male rats by intraperitoneal injection with α -galactosamine (+) and 6 h after injection, urine sample were collected during a 16-h period of time. In parallel, it was collected urine from animal controls (−) injected with same volume of saline solution. After urine sample collection, the animals were sacrificed and livers were processed for histological procedures. (A) Representative images of liver sections stained with hematoxylin–eosin of GalN- or saline-treated rats. Note the liver damage induced by GalN. (B, C) Same volumes (5 μ L) of urine sample from GalN- or saline-treated rats and same volumes (20 μ L) of urinary vesicles preparations isolated from those urine samples were analyzed by Western blotting with antibody against the albumin protein. (C) Briefly, 20 μ L of urinary vesicles preparations from GalN- or saline-treated rats were analyzed by Western blotting with the indicated antibodies. Note that while no significant differences were found in levels of the urine soluble protein albumin, significant changes were observed in the levels of several proteins in the urinary vesicles preparations.

Figure 5.

Candidate biomarkers in urinary vesicles for chronic liver injury. Urine from a mouse model for chronic liver disease defective in the liver-specific enzyme GNMT (KO) and from WT animals were collected at the indicated ages and urinary vesicles were purified, and same amount of proteins were analyzed by Western blotting with antibodies against Cd81, flotillin and Cd10 proteins.

Table 1Disease-associated proteins detected in exosome-like urinary vesicles^{a)}

Disease	Protein^{b)}
Polycystic kidney disease	AQP1_RAT, TMM27_RAT
Inflammatory bowel disease	MEP1A_RAT , MEP1B_RAT , MDR1_RAT
Triosephosphate isomerase deficiency	TPIS_RAT
Aminoacylase 1 deficiency	ACY1A_RAT
Fructosuria, essential	KHK_RAT
Fructose-1,6-bisphosphatase deficiency	F16P1_RAT
Fructose intolerance, hereditary	ALDOB_RAT
Phosphoglycerate kinase 1 deficiency	PGK1_RAT
Cystinuria	SLC3A1_RAT
Cystathioninuria	CGL_RAT
Glutathionuria	GGT1_RAT
Dystonia, juvenile-onset	ACTB_RAT
Obesity	PTER_RAT
Hypertension	HPCL1_RAT , GSH1_RAT
Myocardial infarction, susceptibility to	GSH0_RAT
Osteoarthritis	CALM_RAT
Neurological diseases	SODC_RAT, G3P_RAT, AADAT_RAT, 3HAO_RAT , DHPR_RAT, GLNA_RAT, PARK7_RAT
Obsessive-compulsive disorder	EAA3_RAT
Chronic liver disease	DPP4_RAT
Acute liver injury	RGN_RAT
Cancer, angiogenesis, metastasis	NEP_RAT, DPP4_RAT, CLIC4_RAT, UBIQ_RAT, GSTP1_RAT, COF1_RAT, PDZ11_RAT, ANXA2_RAT, 1433E_RAT, 1433Z_RAT, 1433T_RAT, RAPIA_RAT, PEBP1_RAT, G3P_RAT, CD82_RAT , UK114_RAT, PARK7_RAT
Pharmacogenetics	S22A5_RAT, S47A1_RAT , MDR1_RAT, GSH1_RAT, GSTA1_RAT, GSTP1_RAT

^{a)} Data mining analysis in PubMed database was performed with identified proteins. References supporting these associations are provided in Supporting Information Table S1.

^{b)} Protein accession number is provided. Bold indicator the proteins described in exosome-like urinary vesicles for first time.