Chapter 13
Effects of Diuretics on Urinary Proteins

Xundou Li

Abstract Biomarker is the measurable change associated with a physiological or pathophysiological process. Unlike blood which has mechanisms to keep the internal environment homeostatic, urine is more likely to reflect changes of the body. As a result, urine is likely to be a better biomarker source than blood. However, since the urinary proteome is affected by many factors, including diuretics, careful evaluation of those effects is necessary if urinary proteomics is used for biomarker discovery. The human orthologs of most of these 14 proteins affected are stable in the healthy human urinary proteome, and 10 of them are reported as disease biomarkers. Thus, our results suggest that the effects of diuretics deserve more attention in future urinary protein biomarker studies. Moreover, the distinct effects of diuretics on the urinary proteome may provide clues to the mechanisms of diuretics.

Keywords Diuretics · Urine proteome

Biomarker is the measurable change associated with a physiological or pathophysiological process. Unlike blood is homeostatic, urine is more likely to reflect changes of the body. In other words, urine is likely to be a better biomarker source than blood [1]. Saving more urinary protein samples on the membrane can help to speed up the biomarker research in urine proteome [2]. Furthermore, compared to plasma, urine can be collected continuously and noninvasively. Second, the urinary proteome directly reflects the conditions of the urinary system. Third, it can also reflect the physiological status of the whole human body [3]. These advantages make the urinary proteome a suitable source for disease biomarker discovery.

To date, many urinary biomarkers have been reported in a variety of diseases [3], such as various chronic and acute renal injuries [4], bladder cancer [5], prostate cancer [6] and coronary artery disease [7]. However, studies focusing on the urinary

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protein biomarker discovery still face certain challenges. A major issue is that the urinary proteomic pattern of an individual may be affected by multiple factors, such as gender, age, diet [8], medication, daily activities, exercises [9, 10], smoking [11], stress, menstrual cycle, and other physiological variations. Environmental factors including temperature and humidity may also affect the urinary proteome. Therefore, these factors should be taken into consideration in the urinary biomarker research.

Effects of some factors, such as gender, age, daily activity, and environmental conditions, have been investigated previously [12–14]. However, effects of some other factors, especially medication, are difficult to examine, since the regular therapeutic process of patients should not be disturbed during the collection of urine. Therefore, influences of medications on the urinary proteome should be taken into account during data analysis and interpretation.

Diuretics are among the most commonly used medications. They are used to induce negative fluid and sodium balances in a variety of clinical situations, including hypertension, heart failure, renal failure, nephritic syndrome, and cirrhosis [15]. However, it remains unclear whether and how diuretics affect the urinary proteome, which hampers the urinary biomarker discovery for those diseases.

The effects of furosemide, hydrochlorothiazide, and spirolactone on the urinary proteome were examined using label-free quantitative proteomics [16]. These drugs represent thiazide diuretics, loop diuretics, and potassium-sparing diuretics, respectively, which are the three types of commonly used diuretics with different modes of action [17]. The rat urine samples were collected before and after the diuretics were administered, digested using the filter aided proteome preparation (FASP) method [18], and analyzed with a high-speed TripleTOF 5600 system. Progenesis LC-MS was used to quantify the urinary proteins.

Urine samples from 15 rats were collected after each rat was given 1 ml saline by intragastric administration for 24 h using metabolic cages, and these samples were used as controls. Then, the rats were randomly divided into three groups with five rats in each group. Each group of rats was given either 20 mg/(kg days) of furosemide, 20 mg/(kg days) of spirolactone, or 25 mg/(kg days) of hydrochlorothiazide, respectively. The dosing volumes of diuretics were adjusted to 1 ml. All rats were given diuretics by intragastric administration for 5 days, and the rat urine samples were collected on 1, 3, and 5 days after diuretics administration as described above.

As shown in Table 13.1, the rat urinary volumes increased significantly ($\sim$2–3, $P < 0.05$) after the administration of furosemide (F) and hydrochlorothiazide (H), especially within the first 8 h after lavage. This period is the effective time of the diuretics. However, there is no significant increase in urine output ($P > 0.05$) after the rats were administered with spirolactone (S), probably due to the fact that spirolactone is not an efficient diuretic on its own and usually is applied in combination with other diuretics.

As a first step of the sample analysis, the urine samples collected on different days were separated by SDS-PAGE. As shown in Fig. 13.1a, the protein patterns of

Table 13.1 The urine volumes of rats before and after diuretics were administered [16]

Time	Rat 1		Rat 2		Rat 3		Rat 4		Rat 5		Mean; SD (8 h)
	Urine volume (8 h) ml	Urine volume (24 h) ml	Urine volume (8 h) ml	Urine volume (24 h) ml	Urine volume (8 h) ml	Urine volume (24 h) ml	Urine volume (8 h) ml	Urine volume (24 h) ml	Urine volume (8 h) ml	Urine volume (24 h) ml	
Furosemide											
Before	3.6	37.7	2.8	11.4	3.7	10.0	3.0	11.5	3.3	8.5	3.2; 0.3
Day 1*	7.7	38.7	3.6	15.6	9.2	22.2	6.5	27.5	5.0	13.0	6.4; 2.2
Day 3*	7.2	42.2	2.5	10.0	8.0	20.6	6.2	30.0	7.2	14.5	6.2; 2.2
Day 5*	10.0	21.5	8.0	13.5	9.0	14.0	13.3	21.3	5.5	8.5	9.2; 2.8
Hydrochlorothiazide											
Before	5.5	12.5	6.1	30.0	5.3	11.0	4.9	15.5	4.7	12.5	5.3; 0.5
Day 1*	12.0	27.5	13.4	34.9	12.0	28.2	9.5	19.7	10.5	23.0	11.5; 1.5
Day 3*	9.3	19.3	12.0	38.0	11.3	25.0	5.3	15.3	15.5	23.5	10.7; 3.7
Day 5*	16.0	27.0	12.5	21.0	10.5	20.5	11.0	17.0	12.0	18.0	12.4; 2.2

(continued)

Table 13.1 (continued)

Time	Rat 1		Rat 2		Rat 3		Rat 4		Rat 5		Mean; SD (8 h)
	Urine volume (8 h) ml	Urine volume (24 h) ml	Urine volume (8 h) ml	Urine volume (24 h) ml	Urine volume (8 h) ml	Urine volume (24 h) ml	Urine volume (8 h) ml	Urine volume (24 h) ml	Urine volume (8 h) ml	Urine volume (24 h) ml	
Spirolactone											
Before	–	27.5	–	15.5	–	27.5	–	17.5	–	14.2	–
Day 1**	–	36.0	–	18.5	–	28.0	–	18.0	–	16.0	–
Day 3**	–	23.0	–	15.5	–	27.0	–	17.4	–	21.0	–
Day 5**	–	25.0	–	17.0	–	29.5	–	19.0	–	20.5	–

Note *P values <0.05; **P values >0.05 (T-test)

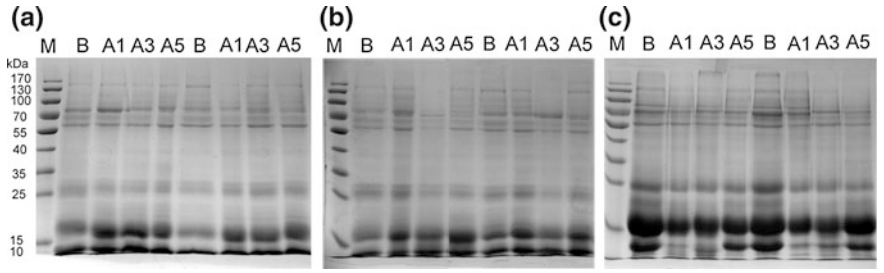

Fig. 13.1 SDS-PAGE of the urine samples from rats treated by different diuretics [16]. Urine protein samples were separated by SDS-PAGE and stained using Commassie *blue* brilliant for the hydrochlorothiazide group (H, **a**), the furosemide group (F, **b**), and the spirolactone group (S, **c**), *M* markers; *B* normal rat urine samples; *A1*, *A3,* and *A5* urine samples obtained 1, 3, and 5 days after the diuretics were administered

the urine samples in the H group changed only modestly among those obtained before and 1, 3, and 5 days after the diuretics administration. However, for the F and S groups, there were some significant changes among samples obtained at different time points, especially those on Day 3 after gavage for the F group (Fig. 13.1b) and Day 1 for the S group (Fig. 13.1c). Therefore, normal urine samples, Day 3 for the F and H groups and Day 1 for the S group, were further analyzed using 1D-LC-MS/MS.

To investigate the changes of the urine proteome after diuretics administration, a total of 18 LC-MS/MS runs of urine samples from three different rats in each diuretic group were analyzed. The 18 datasets were analyzed using Progenesis LC-MS, and Mascot Daemon. The false discovery rate (FDR) was adjusted to be less than 1 %. As a result, there were 331, 302, and 325 proteins identified in the F, S, and H group, respectively.

The coefficients of variation (CVs) for each of the three levels of sample variation before gavage, after gavage, and between these two conditions were calculated. As shown in Fig. 13.2, the CV values of the samples after gavage were slightly higher than those before gavage (median CV values: F group 0.25 vs. 0.34; S group 0.35 vs. 0.39; H group 0.28 vs. 0.31), possibly maybe because rats respond differentially to the diuretics. In contrast, the CV values of the samples for between before and after gavage and for after gavage (median CV of F group is 0.45; median CV of S group is 0.55) are significantly higher ($P < 0.05$), suggesting that furosemide and spirolactone can change the urine proteome. However, the CV values of H-diuretics (median CV is 0.33) were not changed significantly, indicating that hydrochlorothiazide has no discernible effects on the rat urine proteome at this dosage.

The CV values of proteins identified in each group before diuretics administration, after and between these two states, were calculated using SPSS 13.0. Before indicates the CV values of urine samples before diuretics administration in the F, S, and H group, respectively; after indicates the CV values of urine samples after diuretics administration in each group; between indicates the CV values of urine

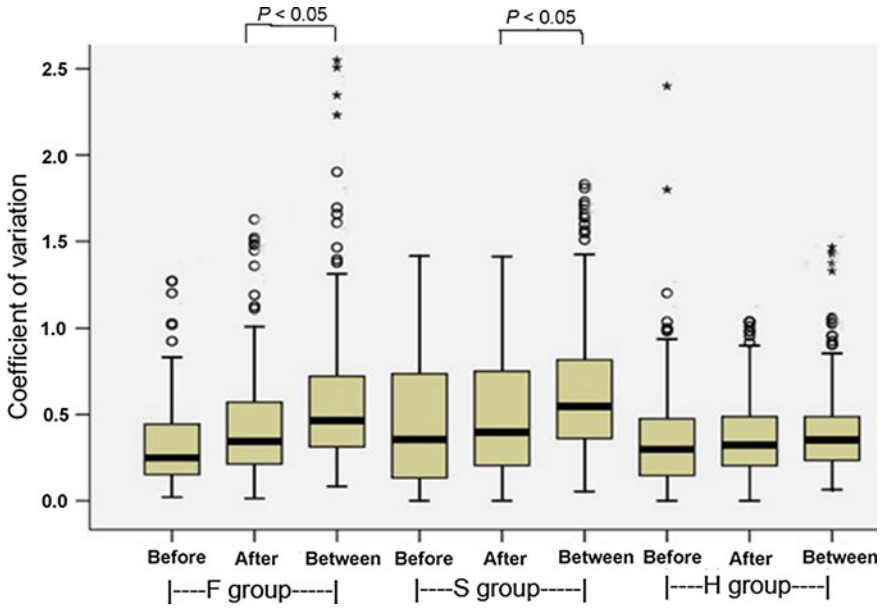

Fig. 13.2 The CV values for each of the three levels of sample variation [16]

samples between before and after diuretics administration in each group ($n = 3$; in F and S group, $P < 0.05$).

Using the label-free quantification by the Progenesis LC-MS software, we identified seven (five upregulated and two downregulated), five (one upregulated and four downregulated), and two (one upregulated and one downregulated) proteins which significantly changed in all three rats in the F, S, and H group, respectively, according to the criteria: $P \leq 0.05$, a fold change ≥2 and a spectral count ≥5. As shown in Tables 13.2 and 13.3, five of the seven proteins in the F group and all of the five proteins in the S group have been reported to be disease biomarkers. For example, haptoglobin is a candidate biomarker for patients with bladder cancers, acute kidney injury, or diabetic nephropathy. However, neither of the two significantly changed proteins (beta-microseminoprotein and EGF-containing fibulin-like extracellular matrix protein 1) has been reported as biomarkers in hydrochlorothiazide group. Moreover, hydrochlorothiazide appears to have a lower impact than furosemide and spirolactone at the dosages tested. Interestingly, no significantly changed proteins are shared by any two groups, indicating the distinct effects of the diuretics on the urinary proteome.

We next evaluated the relevance of our findings to the human disease biomarkers. As it is typically assumed that orthologs (co-orthologs) retain similar functions between species [19, 20], therefore we transformed the significantly changed proteins after intragastric administration of diuretics to human orthologs. Based on the 122.R_norvegicus.orthologues database and Ensembl Compare

Table 13.2 Urinary proteins significantly changed after intragastric administration of furosemide [16]

Accession	Protein name	Fold change			Candidate biomarkers	References
		Rat 1	Rat 2	Rat 3		
P02781	Prostatic steroid-binding protein C2	8.2↑	6.3↑	4.3↑	No	
P07647	Submandibular glandular kallikrein-9	3.5↑	6.2↑	5.2↑	Yes	[24]
P02782	Prostatic steroid-binding protein C1	7.6↑	5.7↑	5.6↑	No	
P02780	Secretoglobin family 2A member 2	9.6↑	5.0↑	6.2↑	Yes	[24]
P22283	Cystatin-related protein 2	4.7↑	3.7↑	4.3↑	Yes	[24]
P08721	Osteopontin	7.3↓	7.4↓	5.9↓	Yes	[25–28]
Q01177	Plasminogen	2.1↓	2.1↓	3.0↓	Yes	[29]

Table 13.3 Urinary proteins significantly changed after intragastric administration of spirolactone [16]

Accession	Protein name	Fold change			Candidate biomarkers	References
		Rat 1	Rat 2	Rat 3		
P06866	Haptoglobin	5.0↑	2.1↑	2.2↑	Yes	[30–35]
P81828	Urinary protein 2	3.6↓	3.3↓	3.9↓	Yes	[24]
P81827	Urinary protein 1	7.3↓	4.3↓	4.4↓	Yes	[24, 36]
P10960	Sulfated glycoprotein 1	4.0↓	3.1↓	2.4↓	Yes	[24]
Q09030	Trefoil factor 2	8.5↓	4.7↓	4.2↓	Yes	[37]

database [21], eight of the 14 rat urinary proteins have human orthologs (Table 13.4). By comparing the proteins with the human core urinary proteome, we further found that seven human orthologs are relatively stable proteins in the normal human urinary proteome [22, 23]. Therefore, such proteins could serve as potential urinary biomarkers, since significant qualitative or quantitative changes of these stable proteins may suggest some pathophysiological conditions [23].

However, some limitations of this study should be noted. First, the results need to be verified on humans before we can generalize the conclusions. Second, it would be ideal to validate our results in studies with a higher statistical power. Furthermore, the effects of doses and durations of diuretics on the urinary proteome should also be studied in the future.

In summary, we have shown for the first time through a proteomic approach that some candidate urinary biomarkers may be affected by diuretics, suggesting that the

Table 13.4 Human orthologs of rat proteins significantly changed after administration of diuretics [16]

Rat protein ID	Rat protein name	Human protein ID	Human protein name	Human core urinary proteome
Q01177	Plasminogen	P00747[a]	Plasminogen	Yes
Q09030	Trefoil factor 2	Q03403[a]	Trefoil factor 2	Yes
P08721	Osteopontin	P10451[a]	Osteopontin	Yes
O35568	EGF-containing fibulin-like extracellular matrix protein 1	Q12805[a]	EGF-containing fibulin-like extracellular matrix protein 1	Yes
P10960	Sulfated glycoprotein 1	P07602[a]	Sulfated glycoprotein 1	No
P06866	Haptoglobin	P00738[a]	Haptoglobin	Yes
P02781	Prostatic steroid-binding protein C2	P11684[b]	Secretoglobin family 1A member 1	Yes
P07647	Submandibular glandular kallikrein-9	P06870[b]	Kallikrein-1	Yes

Note [a] Present in the 122.R_norvegicus.orthologues database
[b] Present in the Ensembl Compare database

effects of diuretics should be carefully evaluated in the future urinary protein biomarker studies. The results obtained here could help minimize the interference of diuretics with biomarker discovery using the urinary proteomics. In addition, the significantly changed proteins may help the investigation of mechanisms of diuretics as well as renal clearance of proteins. Other commonly used medications, such as glucocorticoids and angiotensin-converting enzyme inhibitors (ACEIs), may likewise affect the urinary proteome and should also be further studied.

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