The Proteome-Transcriptome-Combined Database of Specific Nephron Segment Proteins for Novel Urinary Biomarker Discovery

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Abstract

The aim of the study is to discover urinary biomarkers which identify the kidney injured sites as distal or proximal tubules.

The proteome-transcriptome-combined database was completed with 3 databases; the Human Protein Atlas (immunohistochemistry), the microarray database of human kidney, and the urinary proteome database. By the immunohistochemistry database, 468 proteins were determined as kidney proteins of tubule-predominant expression compared with glomerular expression. By the microarray database, 268 proteins were verified as kidney tubule-predominant gene expression. Among them, 64 proteins were confirmed in the urinary proteome database. The localizations of these 64 proteins in kidney tubules were specified visually in the immunohistochemistry database; 14 proteins were defined as distal originated urinary proteins, and 50 proteins were proximal.

The expressions of these proteins were studied in normal and diseased kidneys. In rat UUO kidneys, mRNA expressions of 8 of 14 distal proteins such as ANXA3 were up-regulated while 3 of 14 distal proteins such as CALB1 were down-regulated, and mRNA expressions of 7 of 50 proximal proteins such as FBLN5 were up-regulated while 12 of 50 proximal proteins such as ALDOB were down-regulated. By immunohistochemistry, decreased CALB1 in the injured distal tubules and ALDOB in the injured proximal tubules were observed while increased ANXA3 were observed in interstitial infiltrating cells. Urinary CALB1 were shown reduced whereas those of ANXA3 were shown increased in some patients with tubulointerstitial injury.

Our proteome-transcriptome-combined database may be a good tool to discover new urinary biomarkers which identify the injured sites as distal or proximal tubules.

ABBREVIATIONS


INTRODUCTION

Several proteins have been proposed as new urinary biomarkers of kidney injury [1]. Although the biomarkers are more useful for the early detection of acute kidney injury (AKI) than elevated serum creatinine levels, they do not always identify the injured kidney nephron segment, especially in case the biomarker may be derived from plasma [2]. For example, NGAL (Neutrophil Gelatinase-Associated Lipocatin), one of the promising urinary biomarkers of AKI, appears in urine as a result of reduced NGAL reabsorption by proximal tubule cells and/or enhanced de novo NGAL synthesis by distal tubule cells [3]. NGAL is a low molecular weight protein, which is cleared from plasma mainly through glomerular filtration and avidly reabsorbed into proximal tubule cells by endocytosis. Although increased urinary NGAL may reflect proximal tubule cells injury, it is because of reduced absorption of NGAL via the damaged proximal tubule.
cells, not because of increased production of NGAL from the proximal tubule cells. Furthermore, NGAL does not have any functions on the proximal tubule cells in the physiological (no AKI) condition and the role of increased production of NGAL in AKI kidney is not yet well clarified. From this point of view, NGAL may not fully meet the criteria for ideal biomarkers, for example, the biomarker must originate from the injured cells and should play a role in the pathophysiology of the affected organ [4].

The aim of the study is to discover urinary biomarkers which identify the kidney injured sites as distal or proximal tubules. In order to determine nephron specific segment-originated urinary proteins, the proteome-transcriptome-combined database has been completed with 3 databases; the Human Protein Atlas (immunohistochemistry, http://www.proteinatlas.org/), the microarray database of human kidney [5], and the urinary proteome database of normal human subjects (http://141.61.102.16/urine/).

MATERIALS AND METHODS
The human protein atlas

By the Human Protein Atlas database, Protein expression profiles based on immunohistochemistry can be determined using more than 20000 antibodies, in 44 different normal human tissues including kidney. Tubular proteins were defined as the antibody staining intensity level in tubular cells was strong (weak or negative in glomerular cells) or moderate (negative in glomerular cells). Among the 1038 tubular proteins, 468 proteins of highly reliable immunostaining were defined as kidney tubule-predominant proteins.

The Microarray database [5]

The microarray analysis of normal human kidney was done by preparation of Cy5-labeled cRNA from human glomeruli and Cy3-labeled cRNA from human cortices. Normal sections of kidney tissues were obtained by nephrectomy from patients with renal neoplasia. Only genes of positive Cy3/Cy5(C/G) ratio (>1.1) were defined as tubule-predominant genes. Among the 468 kidney tubule-predominant proteins, 268 proteins were verified kidney tubule-predominant gene expression.

The urinary proteome database

The urinary proteome database was completed by employing ultrafiltration unit and molecular weight cutoff 3kDa for protein concentration and desalting, and 1D SDS gel (In-gel digestion) and reverse phase HPLC (In-solution digestion, shotgun proteomics) for protein separation and fractionation, and next the digests were analyzed with the LTQ-FT and LTQ-Orbitrap [6]. In the database, 1543 urinary proteins are listed in order of the abundance of identified peptides (peptide count and number of unique peptides). Among 268 kidney tubule-predominant proteins, 64 proteins were confirmed in the urinary proteome database.

The proteome-transcriptome-combined database

The proteome-transcriptome-combined database was completed (Figure 1). The localizations of these 64 proteins in kidney tubules were specified visually in the Human Protein Atlas database [7] (http://www.proteinatlas.org/); 14 proteins were defined as distal tubule originated urinary proteins, and 50 proteins were defined as proximal tubule originated urinary proteins.

Rats [8]

By using inbred male WKY rats, two rat models of kidney disease involving tubular cell injury were investigated: unilateral ureteral obstruction (UUO) [9], anti-glomerular basement membrane glomerulonephritis (anti-GBM GN) [10]. Five rats were subjected to UUO. The obstructed left [ipsilateral] and right [contralateral] kidneys were harvested and studied. And as for anti-GBM GN, five rats each were sacrificed on days 0, 2, 4 and 7.

Humans [8]

Ten renal biopsy tissue samples were obtained from patients with chance hematuria and/or proteinuria, and these patients were divided into 2 groups; 5 kidneys were histologically normal, and 5 kidneys had been diagnosed with immune-mediated glomerulonephritis (IgA nephropathy, IgAN) involving significant tubulointerstitial injury. The kidney tissues were fixed in Methyl Carnoy solution and subjected to histological diagnosis and immunohistochemical staining.

Real-time PCR (rats) [8]

Total kidney cortex RNA was extracted from the ipsilateral and contralateral kidneys of the UUO rats. The PCR reactions were performed with the SYBR Green method using the SYBR Premix Ex Taq II Kit (Takara Bio, Otsu, Japan) on a Thermal
Cycler Dice Real-Time System (Takara Bio). In each sample, the mRNA concentration was normalized to that of GAPDH.

**Antibodies**

Used antibodies were: mAb against calbindin-D-28k (Sigma Aldrich, Tokyo, Japan) for CALB1, pAb against annexin A3 (Atlas Antibodies, Stockholm, Sweden) for ANXA3, pAb against aldolase B, fructose bisphosphonate (Atlas Antibodies) for ALDOB, and pAb against fibulin 5 (Atlas Antibodies) for FBLN5.

**Creatinine concentration (rats and humans)**

The amount of creatinine present in urine and serum samples was determined by an enzymatic assay method (Kainos CRE-EN, Kainos Laboratories, Tokyo, Japan).

**Immunohistochemistry (rats and humans)** [8]

Three µm-thick sections of kidney tissue that had been fixed in Methyl Carnoy and then embedded in paraffin were dewaxed and used for periodic acid-Schiff (PAS) staining and the immunohistochemistry, using horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit immunoglobulins (EnVision) for secondary antibody.

**Western blotting (Urine)** [8]

Spot urine samples from 10 human subjects described above, and 24-hour urine samples from anti-GBM rats were used for the Western blotting. Densitometric analysis was performed using the NIH image software package (version 1.62, NIH), and the results were corrected for urinary creatinine concentration.

**RESULTS AND DISCUSSION**

**[Real-time PCR (rats’ UUO)]**

Rats were sacrificed at day 10 of post-obstruction, since dilatation of all tubular segments including distal and proximal tubules were observed by the day [11]. The mRNA expressions of 8 of 14 distal proteins such as ANXA3 were up-regulated in the UUO kidneys (the ratio of ipsilateral hydronephrotic kidney / contralateral kidney >1.1), while 3 of 14 distal proteins such as CALB1 were down-regulated in the UUO kidneys (the ratio <0.9) (Figure 2A). And the mRNA expressions of 7 of 50 proximal proteins such as FBLN5 were up-regulated in the UUO kidneys, while 12 of 50 proximal proteins such as ALDOB were down-regulated in the UUO kidneys (Figure 2B).

**Immunohistochemistry (rats and humans)**

Four proteins of CALB1, ANXA3, ALDOB and FBLN5 were selected for immunohistochemistry, since the % reduction or % increase of these mRNA expressions were significant in UUO kidneys compared with contralateral kidneys, and the proteins could not be detected in the human plasma samples by Western blotting (http://www.proteinatlas.org/). Decreased CALB1 protein expression was confirmed in the injured dilated distal tubules of rat UUO kidneys (data not shown), which was also observed in anti-GBM GN kidneys and human IgAN kidneys with tubulointerstitial injury (Figure 3A, 3B). Since CALB1 plays a role in renal tubular Ca\(^{2+}\) reabsorption [12], hypercalciuria and/or hypocalcemia may occur in rats with distal tubule cells injury accompanied by reduced CALB1 expression. Increased ANXA3 protein expression was observed in some interstitial infiltrating cells in rats and humans (Figure 3C, 3D), contrary to the hypothesis that it may increase in the injured distal tubule cells. ANXA3 may be involved in leukocyte migration and inflammatory response [13] in kidneys with interstitial cells infiltration. Decreased ALDOB was confirmed in the injured kidneys of rats and humans (Figure 3E, 3F), probably because of destruction of proximal tubule structure. The immunostaining intensity in the healthy proximal tubule cells does not seem to be decreased. Since ALDOB plays an important role in fructose metabolism [14], persistent intake of fructose can accelerate kidney failure in kidney with proximal tubule cells injury accompanied by reduced ALDOB expression. And modestly increased FBLN5 intensity in the proximal tubule cells was observed in the injured proximal tubules (brush border) in rats and humans (Figure 3G, 3H), although the number of healthy proximal tubules were decreased. FBLN5 plays a role in tissue repair and remodeling [15] in kidneys with injured tubule cells.

**Western blotting (urine)**

Western blotting demonstrated that the anti-GBM GN rats displayed a lower urinary calbindin 1 protein level than the normal rats at day 4 (Figure 4A), which were coincided with serum creatinine levels elevation. The earlier decrease of urinary calbindin 1 protein than serum creatinine elevation could not be demonstrated. As for humans, some of the IgAN patients displayed quite lower levels of urinary CALB1 protein excretion compared with the normal human subjects (Figure 2B).
Although the overall difference between the two groups was not significant. In contrast, about ANXA3, the anti-GBM GN rats displayed an increased urinary protein level than the normal rats at day 4 (Figure 4C). And some of the IgAN patients displayed higher levels of urinary ANXA3 protein excretion compared with the normal human subjects (Figure 4D), although the overall difference between the two groups was not significant. And there were no significant correlations between the degree of tubulointerstitial injury and urinary calbindin 1 or ANXA3 protein levels. Urinary ALDOB was shown increased in some patients of IgAN, in contrast that the protein expression in the diseased kidney was decreased (data not shown). Urinary FBLN5 could not be detected in any of the human subjects (data not shown). It may be difficult to study humans due to an individual difference and/or confounding variations. Although it is sure that the phenomena observed in animals are not always observed in humans in a same way, study using animals has been enrolled in this study for comparison and validation. The proteins of same localization and regulation between rats and humans may be functionally important.

CONCLUSION

Our proteome-transcriptome-combined database may be a good tool to discover new urinary biomarkers which identify the injured sites as distal or proximal tubules. Study of other urinary proteins of kidney proximal/distal tubule origin has been continued, in order to find new biomarkers of which are altered earlier than serum creatinine levels elevation.
Figure 4  (A) (C) – rat urine samples of normal and aGBM GN. (B) (D) – human urine samples of normal histology and IgAN patients with tubulointerstitial injury.
Abbreviations: PT: Proximal tubule-predominant protein; DT: Distal tubule-predominant protein
Western blotting of urinary proteins

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