Urinary FBP1 as a novel biomarker for the diagnosis and progression of diabetic nephropathy

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Urinary FBP1 as a novel biomarker for the diagnosis and progression of diabetic nephropathy

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Abstract

**Background:** The pathogenesis of diabetic nephropathy (DN) is complex, and its onset is occult. It should be considered from multiple levels and angles using the concept of "system biology". New technologies, such as omics and high-throughput screening, provide more strategies for the screening of ideal markers of DN.

**Methods:** We used data-independent acquisition (DIA) mass spectrometry for urine proteomic analysis of DN patients at different pathological stages and healthy controls.

**Results:** Fructose-1,6-bisphosphatase 1 (FBP1) was specifically found in the urine of DN patients but not in that of healthy controls. Then, ELISA demonstrated that the urinary level of FBP1 in DN patients was significantly higher than that in healthy controls. We further verified the higher levels of urine FBP1 in association with DN than in association with type 2 diabetes mellitus (T2DM) and nondiabetic nephropathy. Receiver operating characteristic (ROC) curve analysis showed that FBP1 can distinguish DN and T2DM patients, and the AUC was 0.889 (sensitivity: 80%, specificity: 85%). Correlation analysis revealed that the level of urinary FBP1 was positively correlated with the progression of DN, as reflected by the fact that urinary FBP1 was negatively correlated with e-GFR and positively correlated with urea nitrogen, the interstitial inflammation score, and the interstitial fibrosis and tubular atrophy (IFTA) score.

**Conclusions:** The source of urinary FBP1 was traced, and it was revealed that it was specifically reduced in proximal renal tubules with the progression of DN, supporting the view that urinary FBP1 can be used as a marker for the progression of diabetic nephropathy. Together, our results indicate that FBP1 might not only be a novel marker for the diagnosis of DN but also be a new marker for the progression of DN.

**Keywords:** Diabetic nephropathy, FBP1, a novel marker, progression.
**Background**

Diabetic nephropathy (DN) is the leading cause of end-stage kidney disease (ESKD) worldwide [1]. It has become the leading cause of chronic kidney disease (CKD) in hospitalized individuals in China [2, 3]. At present, the main intervention methods of DN are still symptomatic treatment, such as controlling blood pressure, controlling blood glucose, reducing proteinuria, and improving renal hyperperfusion and hyperfiltration. Clinicians still lack appropriate early diagnosis, early intervention, and molecular targeted drugs to delay or terminate the occurrence and development of DN in patients [4]. Epidemiological survey data show that over the past 20 years, other important complications caused by diabetes, such as cardiovascular events and stroke, have been effectively controlled. The prevalence of ESRD caused by diabetes has not changed significantly [5]. Further interpreting the new pathogenesis of DN, revealing new molecular intervention targets, discovering new molecular diagnostic markers, and building a more accurate diagnosis and treatment model of DN have become urgent tasks and keys to sniping DN.

To date, albuminuria or proteinuria is the main index of clinical diagnosis of DN [6, 7], but their test results are easily affected by many factors, such as high fat intake [8], strenuous exercise [9], insulin resistance [10], hypertension [11], and urinary tract infection [12]. In addition, due to the occult onset of DN, although some patients have advanced renal pathological changes, the level of urinary albumin is still within the normal range [7]. It is increasingly recognized that reductions in eGFR can occur in the setting of normal urinary albumin excretion in both type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM) [13, 14]. In general, nonproteinuric CKD often points toward etiologies that are ischemic in nature or in which tubulointerstitial pathologies predominate [15]. However, nonproteinuric DN has also been described in association with the typical histopathological changes of diabetic glomerulopathy [16]. Therefore, looking for more sensitive and reliable molecular markers is of great significance for the early diagnosis and prevention of DN. In recent years, due to the rapid development of genomics [17], epigenetics [18], transcriptomics [19], proteomics [20], and metabolomics [21], high-throughput screening technology [22] has provided more ideas for the exploration of DN biomarkers. In particular, proteomics or mass spectrometry-based proteomics for DN has been used to develop noninvasive markers for DN progression based on urinary proteins [23-26]. However, proteins found based on this method often represent a suitable marker for overall renal failure but
do not specifically reflect renal injury. Therefore, there is an urgent need for urinary biomarkers that can reflect renal damage in the progression of DN [27].

In this study, we first performed urinary proteomics to explore the landscape of urinary proteins of patients with DN at different pathological stages and screened seven proteins, carbonic anhydrase II (CAII), cystatin SN (CST1), prostate stem cell antigen (PSCA), prolylcarboxypeptidase (PRCP), putative phospholipase B-like 2 (PLBD2), fructose-1,6-bisphosphatase 1 (FBP1), and thrombospondin-4 (THSP4), specifically in DN. Second, we tested the urinary levels of seven proteins in DN patients at different pathological stages and healthy controls and found that only FBP1 in the urine of patients with DN was significantly higher than that in healthy controls. Third, we compared the levels of FBP1 between DN, diabetes mellitus (DM), and nondiabetic nephropathy patients and found that FBP1 in the urine of T2DM, IgA nephropathy (IgAN), lupus nephritis (LN), and membranous nephropathy (MN) patients was significantly lower than that in DN patients. Furthermore, we not only found that urinary FBP1 can distinguish DN from T2DM but also found that the level of urinary FBP1 is related to clinical and pathological features. Finally, the source of urinary FBP1 was traced, and it was demonstrated that FBP1 was specifically reduced in the proximal renal tubules of patients with early-stage DN but not in nondiabetic nephropathy patients.

MATERIALS AND METHODS

Patients and urine sample collection

A total of 130 patients with DN who visited the National Clinical Research Center of Kidney Diseases from January 2020 to April 2022 were consecutively enrolled in this study. The diagnosis of DN was made according to the criteria of An Y et al. [28]. Briefly, patients with T2DM who had biopsy-proven DN and a follow-up time of >12 months were eligible. Those who had comorbidities of nondiabetic renal diseases or acute kidney injury at the time of renal biopsy were excluded. In the verification phase, we also formed a control group with 60 patients with T2DM who had urine samples but had no microvascular complications, were albumin-negative, and had normal renal function: 20 cases of IgAN, 20 cases of MN, and 20 cases of LN. Proteinuria and biochemical indices were measured by routine laboratory procedures. eGFR was estimated using the 2009 Chronic Kidney Disease Epidemiology Collaboration creatinine equation. Data on the baseline
clinical characteristics, which were collected within one month of renal biopsy, included age, sex, fasting blood glucose, HbA1c, 24-h urinary protein, and estimated glomerular filtration rate (e-GFR) (mL/min/1.73 m2). Twenty age- and sex-matched healthy control subjects were enrolled. Immunofluorescence colocalization studies were performed on biopsy samples of control group patients (n=2), patients with IgAN (n=2), patients with DN (n=2), patients with LN (n=2), and patients with MN (n=2). Immunohistochemical analysis was used to compare the expression of FBP1 in normal controls (N=10), patients with early DN (10 cases of stage I and IIA DN patients), and patients with late DN (10 cases of stage III and IV DN patients). All the biopsy specimens were categorized based on the pathological classification established by the Renal Pathology Society [29] and were scored by a single pathologist who was blinded to the clinical findings. The pathological score has been described previously [28]. All urine samples were immediately stored at -80 °C before use.

**Proteomic analysis**

Protein was extracted from 12 DN patients (DN I (n=3), DN II (n=3), DN III (n=3), DN IV (n=3)) and three healthy controls. The proteins were tested by SDS–PAGE electrophoresis to evaluate the consistency between samples. The same number of protein was taken from each sample to create a pooled sample for LC–MS/MS analysis and the construction of the DIA work database. Then, each sample was analyzed by LC–MS/MS, and the above database was used for qualitative and quantitative analyses. Finally, bioinformatics analysis tools were used to display the data. The experimental and analytical methods were described previously [30].

**Measurement of proteins in urine**

Enzyme-linked immunosorbent assay (ELISA) kits were purchased from Cusabio for THSP4, CA II, and CST1, from Abbexa for PLBD2 and FBP1, and from Cloud-Clone Corp for PSCA and PRCP. The urine levels of CAII, CST1, PSCA, PRCP, PLBD2, FBP1, and THSP4 in each sample were detected according to the manufacturer’s instructions.

**Immunofluorescence staining**

Immunofluorescence colocalization studies were performed on biopsy samples of controls (n=2), patients with
IgAN (n=2), patients with DN (n=2), patients with LN (n=2), and patients with MN (n=2). Cryosections of kidney biopsy specimens were fixed with 4% paraformaldehyde and incubated with primary antibodies, FBP1 (12842-1-AP, Proteintech) and SGLT2 (D-6, Santa Cruz) and secondary antibodies, FITC–conjugated rabbit anti–mouse antibody (Dako) or Cy3-conjugated secondary antibodies (Dako). Images were taken by a Leica TCS SP5 confocal laser scanning microscope (Leica, Germany).

**Immunohistochemical staining**

Immunohistochemical analysis was used to compare the expression of FBP1 in normal controls (N=10), patients with early DN (10 cases of stage I and IIA DN patients), and patients with late DN (10 cases of stage III and IV DN patients). Paraffin sections of renal biopsies were deparaffinized and rehydrated. After antigen retrieval, the sections were incubated in 10% FBS for 10 minutes. For immunohistochemistry (IHC), a Leica kit (Leica Laboratories) was used following the manufacturer’s instructions, and FBP1 was detected with FBP1 antibody. The total positive intensity of renal tubules per square millimeter was analyzed using Aprio software.

**Statistical Analysis**

GraphPad Prism 8.00 software from GraphPad Software Inc. (San Diego, CA, United States) was employed to analyze the data. The KS normality test was used to analyze whether the data fit the parametric contribution. Parametric data are expressed as the mean ± SD, and nonparametric data are expressed as the median with the interquartile range. The differences between each group were compared by the nonparametric Mann–Whitney U test. The nonparametric Spearman correlation test was performed to analyze the associations between the urinary levels of CA2, THSP4, and FBP1 and different variables. Receiver operating characteristic (ROC) curves and areas under the curve (AUCs) were determined to evaluate the sensitivity and specificity of FBP1. The two-sided principle was carried out during the analyses, and we considered differences to be significant if P < 0.05.

**RESULTS**
Experimental design

As shown in the experimental flow chart (Fig. 1A), we followed four main steps in this study. In step 1, we used DIA mass spectrometry technology to perform urinary proteomics analysis and screen differential proteins between DN at different pathological stages and healthy controls through urinary proteomics analysis. In this step, we focused on the proteins specifically expressed in the urine of DN patients but not in the urine of healthy controls. Step 2 involved verifying the urinary levels of screened proteins by enzyme-linked immunosorbent assay. In Step 3, we applied the identified protein marker to new patients to test its feasibility as a diagnostic marker of DN. At the same time, the urinary level of the identified protein in DN patients and other kidney diseases was compared to determine whether it is a specific marker for DN. Step 4 involved studying the renal distribution and expression of the identified protein in DN patients compared to other kidney diseases by confocal immunofluorescence and immunohistochemistry.

Urinary proteomics analysis revealed a unique panel of proteins distinguishing early-stage DN from healthy controls.

To systematically identify potential biomarkers of DN, we first collected urine samples of stage I DN patients (DN-I, n=3), stage II DN patients (DN-II, n=3), stage III DN patients (DN-III, n=3), stage IV DN patients (DN-IV, n=3), and healthy controls (CON, n=3). Then, DIA mass spectrometry technology was performed to analyze the protein profile of urine. A total of 15 differentially expressed proteins (fold change > 2 or <0.5; p < 0.05) were found between DN at different pathological stages and healthy controls (Fig. 1B). A total of 30 differentially expressed proteins (fold change > 2 or <0.5; p < 0.05) were found between DN-I patients and healthy controls (Fig. 1C). In addition, compared with healthy controls, patients with all stages of DN had their own specific urine protein profiles (Supplementary Table 1). Similarly, compared with patients with DN, healthy controls also had their own specific urine protein profile (Supplementary Table 2). Importantly, seven proteins, including CAII, CST1, PSCA, PRCP, PLBD2, FBP1, and THSP4, were screened as specific urine proteins in DN patients (DN-E) (Fig. 1D) but not in healthy controls, suggesting that these seven proteins might be candidate proteins for diagnostic markers of DN.

The screened proteins in the urine of DN patients and healthy controls were validated.
To confirm the urine proteomic analysis results, we next verified the urinary levels of CAII, CST1, PSCA, PRCP, PLBD2, FBP1, and THSP4 in DN patients and healthy controls. Of the 70 DN patients enrolled in the study, 51 were male (72.6%). At the time of biopsy, the mean age was 48.4±8.7 years. The mean e-GFR was 69±29 mL/min/1.73 m², and the median 24-h proteinuria was 3.4±3.7 g/day. The mean fasting blood sugar was 7.83±3.07 mmol/L. The mean HbA1c was 6.72±0.77%. The mean urea nitrogen was 31.6±14.9 mg/dL. IFTA scores of 0, 1, 2, and 3 were observed in one (1.4%), 30 (42.9%), 30 (42.9%), and nine (12.6%) patients, respectively. Interstitial inflammation scores of 0, 1, 2, and 3 were observed in one (1.4%), 38 (54.3%), 29 (41.4%), and two (2.9%) patients, respectively. Based on the glomerular classification, 11 DN-I patients, 23 DN-II patients, 22 DN-III patients, 14 DN-IV patients, and 20 healthy controls were enrolled. The clinical and laboratory characteristics of the patients are listed in Table 1. As shown in Fig. 2A, the urinary levels of FBP1 were significantly increased in patients with DN. According to pathological classifications, there was no significant difference in the urinary levels of FBP1 between DN-I patients and healthy controls. The levels of FBP1 in the urine of DN-III and DN-IV patients were significantly higher than those of healthy controls (Fig. 2A). CAII and THSP4 levels in the urine of DN patients were significantly decreased compared to those in healthy controls (Figs. 2E and G). However, there were no differences in CST1, PSCA, PLBD2, and PRCP between DN patients and healthy controls (Fig. 2B, C, D, and F).

**Urinary FBP1 allows the discrimination of DN patients from DM patients and is positively correlated with DN progression.**

We continued to apply the identified FBP1 to new subjects to test its potential as a diagnostic marker of DN. DN (n=60), DM (n=60), IgAN (n=20), MN (n=20), and LN (n=20) patients were enrolled. At the time of biopsy, the mean ages were 52.2±11.4 years, 58.4±15.5 years, 40.1±12.8 years, 46.8±12.3 years, and 37.6±11.4 years, respectively. The mean e-GFR values were 60±32 mL/min/1.73 m², 101±9 mL/min/1.73 m², 91±39 mL/min/1.73 m², 85±27 mL/min/1.73 m², and 95±30 mL/min/1.73 m². There was no significant difference between the interstitial inflammation score and IFTA score. The clinical and laboratory characteristics of the patients are listed in Table 2. As shown in Fig. 3A, the urinary levels of FBP1 in DN patients were significantly higher than those in DM, IgAN, MN, and LN patients, suggesting that FBP1 might be a urine-specific protein in patients with DN. The ROC curve analysis results showed that the area under the
ROC curve of DN and DM patients was 0.896 (p < 0.001), the sensitivity was 80%, the specificity was 85%, and the cutoff value was 1.94 (Fig. 3B). To evaluate the relationship between the urinary levels of FBP1 and disease activity, we analyzed the correlation of the levels of FBP1 with laboratory parameters, including e-GFR, urea nitrogen, interstitial inflammation score, and interstitial fibrosis and tubular atrophy (IFTA) score. The results showed that the levels of FBP1 were negatively correlated with e-GFR (r = -0.56, p <0.001; Fig. 3C) and positively correlated with urea nitrogen (r = 0.55, p <0.001; Fig. 3D). Furthermore, the urinary levels of FBP1 were also correlated with the interstitial inflammation score and IFTA score through multifactor analysis. As shown in Figs. 3E and F, increased urinary levels of FBP1 were significantly associated with a higher degree of interstitial inflammation and IFTA in the biopsy specimens of DN patients.

The renal localization and expression of FBP1 in DN patients

As the kidney is one of the main sources of urinary protein, we detected the localization and expression of FBP1 in the kidney under the condition of diseases. First, immunofluorescence staining was used to detect the renal expression distribution of FBP1 in normal control (normal, n=2), early-stage DN (DN-E, including DN-I and DN-II, n=2), late-stage DN (DN-L, including DN-III and DN-IV, n=2), IgAN (n=2), MN (n=2), and LN (n=2) subjects. Fig. 4A shows that FBP1 is colocalized with SGLT2, suggesting that it is mainly expressed in proximal renal tubules. Compared with the control group, the expression of FBP1 in patients with the early stage of DN decreased significantly, whereas the expression in MN, IgAN, and LN remained unchanged. In addition, compared with that in the early stage of DN, FBP1 expression in the late stage of DN was further decreased, indicating that the change in FBP1 expression might be related to the progression of DN. Thus, we further proved the expression changes of FBP1 during the progression of diabetes, and we used immunohistochemical staining to prove the expression changes of FBP1 during the progression of DN in expanded samples. Ten normal controls, 10 cases of early-stage DN, and 10 cases of late-stage DN were enrolled. The results also showed that FBP was expressed in renal tubules and decreased with the progression of DN (Figs. 4B and C).

DISCUSSION

Studies have shown the importance of the concept of "systems biology" in the study of DN. New technologies
based on omics data have brought a new impetus to the development of precision medicine for kidney disease. Genome-wide association studies (GWASs) provide a powerful tool for finding DN susceptibility genes [31, 32]. However, GWAS might not be able to identify causal variation and genes, and the clinical predictive value of GWAS is limited [33]. Pontillo C et al. detected the urinary proteins of CKD patients through CE-MS technology and found that the urine protein combination composed of 273 peptides can predict the decline of renal function in the early stage of the disease [26, 34]. Whereas CE-MS analysis is a high-end technology, its cost is high, and its operation is complex. Metabonomics in many patients with type 2 diabetes reports small molecule metabolites associated with DN disease progression [35, 36]. Koichi et al. found that there was a significant correlation between phenyl sulfate (PS) and proteinuria through nontarget metabonomics [37]. In this study, we identified that urinary FBP1 is a specific marker of DN and that its level is related to the progression of DN by DIA mass spectrometry screening and ELISA verification. DIA is a new and holographic data-independent acquisition quantitative technology based on Orbitrap, which is a new mass spectrometry data acquisition method developed in recent years. This technology combines the advantages of high throughput of traditional proteomes and accurate quantification of MRM/PRM by mass spectrometry with high reproducibility. They have unique views on protein screening and quantitative analysis, especially for low abundance proteins. In addition, to screen the urinary markers related to DN progression, we performed mass spectrometry analysis and subsequent ELISA verification on the urine of healthy controls and DN patients at different pathological stages. This is helpful for the discovery of early and progressive diagnostic biomarkers of DN.

FBP1 is a 35-37 kD member of the FBPase class I family of enzymes. It is the rate-limiting enzyme in gluconeogenesis[38]. FBP1 is highly conserved, heat-stable, and aldolase/phosphatase bifunctional [39]. It is widely expressed and is found in diverse cells, such as monocytes, hepatocytes, pancreatic beta-cells, and striated (skeletal plus cardiac) muscle cells [40]. Human FBP1 locus resides on chromosome 9q22, the loss of which is associated with poor prognosis for clear cell renal cell carcinoma patients [41]. In the kidney, FBP1 is localized in the cytosol of epitheliocytes of renal proximal tubules. FBP1 fulfills many criteria as a marker of the dysfunction of renal proximal tubules [42]. FBP1 antagonizes glycolytic flux in renal tubular epithelial cells and might inhibit a potential Warburg effect [43]. Measuring the enzyme activity and the protein level of
FBP1 in urine can reflect the degree of renal tubular injury. Urinary FBP1 enzyme activity is used as an indicator of proximal tubular injury in children with idiopathic nephrotic syndrome (INS) [44] and as an indicator of proximal nephron injury during nephrotoxic treatment in patients with testicular cancer [45]. We found that the urinary level of FBP1 in patients with DN was significantly higher than that in healthy controls as well as DM, IgAN, LN, and MN patients, and there was no significant difference among healthy controls and DM, IgAN, LN, and MN patients, indicating that it might be used as a specific marker of DN. The level of urinary FBP1 can distinguish DM and DN well, and the accuracy rate is 85%. In addition, urinary levels of FBP1 were positively correlated with DN prognosis, as reflected by its negative correlation with e-GFR and positive correlation with urea nitrogen. According to the pathological stages of DN, FBP1 was also positively associated with renal tubulointerstitial lesions, including chronic renal tubulointerstitial infiltration, renal tubular atrophy, and interstitial fibrosis. These results indicate that FBP1 might be used as a marker for the progression of DN.

Urinary proteins originate not only from glomerular filtration but also from tubular secretion, epithelial cells shed from the kidney and urinary tract, and secreted exosomes. Regarding the source of urinary FBP1, due to its size and expression distribution, sources other than the kidney were basically excluded from the urinary compartment in the case of DN. In our study, we not only found that FBP1 was exclusively expressed in proximal tubular cells but also found that the expression of FBP1 was decreased in the proximal tubular cells of patients with DN. Importantly, the expression of FBP1 in proximal tubules decreased with the progression of DN, suggesting that urinary FBP1 in DN patients might be derived from the release of damaged proximal tubular cells, which also supports the usefulness of urinary FBP1 as a marker for renal injury and reflects the progression of DN. Interestingly, there was no significant decrease in the expression of FBP1 in renal tubular cells of nondiabetic nephropathy, such as IgAN, LN, and MN. This suggests that there is a potential special mechanism for the decrease in FBP1 in DN, which is very worthy of further study.

Overall, for the first time, we performed a DIA mass spectrometry-based proteomic analysis to analyze the urinary protein profiles of DN patients at different pathological stages and revealed that the urinary levels of FBP1 were highly enriched in DN and correlated with disease progression. Moreover, urinary FBP1 might be
released from the injury of proximal tubular cells in the case of DN.

**Declarations**

**Ethics approval and consent to participate**

All methods were carried out according to the relevant guidelines and regulations. The study was approved by the ethics committee of the Nanjing University of Jinling Hospital, China (2017NZKY-013-01). And the need for informed consent was waived by the ethics committee of the Nanjing University of Jinling Hospital.

**Consent for publication**

Not applicable

**Availability of data and materials**

All data generated or analyzed during this study are included in this published article.

**Competing interests**

The authors claim no conflicts of interest.

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**Author Contributions**

MCZ participated in the experiments, data analysis and drafted the manuscript. JW collected patient urine samples and participated in the experiments. RL participated in the experiments and collected patient tissue samples. YYL participated in the experiments. JSS data analysis. LML and XC designed the experiments and drafted the manuscript. All authors read and approved the final manuscript.
Acknowledgments

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Reference


### Table 1. Clinical features of DN patients at different pathological stages

<table>
<thead>
<tr>
<th></th>
<th>DN-I (n = 11)</th>
<th>DN-II (n = 23)</th>
<th>DN-III (n = 22)</th>
<th>DN-IV (n = 14)</th>
<th>Total (n = 70)</th>
</tr>
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<tbody>
<tr>
<td>Age (years)</td>
<td>49.8±6.6</td>
<td>49.9±7.6</td>
<td>49.1±9.7</td>
<td>43.6±9.6</td>
<td>48.4±8.7</td>
</tr>
<tr>
<td>Male</td>
<td>5</td>
<td>19</td>
<td>15</td>
<td>12</td>
<td>51</td>
</tr>
<tr>
<td>24-h proteinuria (g/day)</td>
<td>0.73±0.6</td>
<td>1.8±1.4</td>
<td>5.3±3.6*§</td>
<td>5.2±5.2#&amp;</td>
<td>3.4±3.7*</td>
</tr>
<tr>
<td>e-GFR (mL/min/1.73 m2)</td>
<td>99±21</td>
<td>79±28</td>
<td>59±23*§</td>
<td>44±16#&amp;</td>
<td>69±29*</td>
</tr>
<tr>
<td>FBS (mmol/L)</td>
<td>8.15±1.25</td>
<td>7.70±3.59</td>
<td>8.53±3.79</td>
<td>6.75±1.93</td>
<td>7.83±3.07</td>
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<tr>
<td>HbA1c (%)</td>
<td>6.98±0.77</td>
<td>6.74±0.85</td>
<td>6.85±0.69</td>
<td>6.26±0.59*</td>
<td>6.72±0.77</td>
</tr>
<tr>
<td>Urea nitrogen (mg/dL)</td>
<td>18.6±5.3</td>
<td>22.6±11.4</td>
<td>39.3±13.1*§</td>
<td>41.5±14.7#&amp;</td>
<td>31.6±14.9</td>
</tr>
<tr>
<td>IFTA (mean ± standard)</td>
<td>0.9±0.3</td>
<td>1.4±0.5</td>
<td>2.0±0.7*§</td>
<td>2.3±0.6#&amp;</td>
<td>1.7±0.7</td>
</tr>
<tr>
<td>Interstitial inflammation (mean ± standard)</td>
<td>0.9±0.3</td>
<td>1.2±0.4</td>
<td>1.6±0.5*§</td>
<td>2.1±0.5#&amp;</td>
<td>1.5±0.6</td>
</tr>
</tbody>
</table>

e-GFR, estimated glomerular filtration rate; FBS, fasting blood sugar; IFTA, interstitial fibrosis and tubular atrophy
Data are presented as the mean ± standard deviation, the median with the range, or counts and percentages.
*P < 0.01 versus class I. #P < 0.01 versus class I. §P < 0.01 versus class II. &P < 0.01 versus class II.

### Table 2. Clinical features of patients with different diseases

<table>
<thead>
<tr>
<th></th>
<th>DM (n=60)</th>
<th>DN (n=60)</th>
<th>LN (n=20)</th>
<th>MN (n=20)</th>
<th>IgAN (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>58.4±15.5</td>
<td>52.2±11.4</td>
<td>37.6±11.4</td>
<td>46.8±12.3</td>
<td>40.1±12.8</td>
</tr>
<tr>
<td>Male</td>
<td>34</td>
<td>45</td>
<td>2</td>
<td>13</td>
<td>12</td>
</tr>
<tr>
<td>24-h proteinuria (g/day)</td>
<td>\</td>
<td>2.6±2.7</td>
<td>0.9±0.8</td>
<td>2.4±2.9</td>
<td>1.2±1.5</td>
</tr>
<tr>
<td>e-GFR (mL/min/1.73 m2)</td>
<td>101±9</td>
<td>60±32</td>
<td>95±30</td>
<td>85±27</td>
<td>91±39</td>
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<tr>
<td>FBS (mmol/L)</td>
<td>8.19±2.32</td>
<td>7.35±2.42</td>
<td>5.14±0.61</td>
<td>6.45±1.61</td>
<td>5.85±1.51</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>7.15±1.52</td>
<td>6.79±1.31</td>
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<td>\</td>
</tr>
<tr>
<td>Urea nitrogen (mg/dL)</td>
<td>\</td>
<td>29.59±15.29</td>
<td>19.34±12.79</td>
<td>19.01±9.38</td>
<td>21.1±13.2</td>
</tr>
<tr>
<td>IFTA (mean ± standard)</td>
<td>\</td>
<td>1.8±0.7</td>
<td>1.7±0.8</td>
<td>1.5±0.6</td>
<td>1.8±0.8</td>
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<tr>
<td>Interstitial inflammation (mean ± standard)</td>
<td>1.6±0.6</td>
<td>1.8±0.7</td>
<td>1.4±0.5</td>
<td>1.7±0.6</td>
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e-GFR, estimated glomerular filtration rate; FBS, fasting blood sugar; IFTA, interstitial fibrosis and tubular atrophy
Figure 1. Urinary proteomic analysis of DN patients at different pathological stages and healthy controls. (A) Overview of the experimental design. (B) Heatmap showing the differentially expressed proteins between DN patients with different pathological stages (DN-I, DN-II, DN-III, DN-IV) and healthy controls, the fold change of a protein >2, P<0.05. (C) Heatmap showing the differentially expressed proteins between patients with DN-I and healthy controls, the fold change of a protein >2, P<0.05. (D) Heatmap displaying the proteins specifically found in urine of early-stage DN (DN-E, including DN-I and DN-II) patients but not in healthy controls. DN-L, and the late stage of DN, including DN-III and DN-IV.
Figure 2. Verification of the candidate proteins screened by proteomic analysis. The urinary levels of FBP1 (A), CST1 (B), PSCA (C), PLBD2 (D), THSP4 (E), PRCP (F), and CAII (G) in DN patients at different pathological stages (n = 70, DN-I (n = 11), DN-II (n = 23), DN-III (n = 22), DN-IV (n=14)) and healthy controls (CON, n = 20) were determined by ELISA. **P < 0.01; and ***P < 0.001; ns, no significant difference.
Figure 3. The urinary levels of FBP1 were validated in patients with DM, DN, and nondiabetic nephropathy. (A) The levels of FBP1 in patients with active DN (n = 60), MN (n = 20), IgAN (n = 20), LN (n = 20), and DM (N = 60) were determined by ELISA. (B) ROC curves for FBP1 levels to distinguish DN from DM subjects. (C - F) The correlation between the levels of FBP1 and the systemic score of DN.
Figure 4. Localization and expression of FBP1 in the kidney. (A) Immunofluorescence staining showed that FBP1 was expressed in renal tubules. FBP1 decreased in the renal tubules of DN patients at the early stage and further decreased in the late stage (×200). (B) Immunohistochemical staining of FBP1 in normal controls (n=10), early-stage DN (n=10), and late-stage DN (n=10) (×200). (C) Statistical analysis of immunohistochemical staining results.
Supplementary Files

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- Supplemetarytable1.docx
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