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Research Article

Urinary glycated uromodulin in diabetic kidney disease

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Advanced glycation end-products (AGEs) form during oxidative stress, which is increased in diabetes mellitus (DM). Uromodulin is a protein with a renal protective effect, and may be subject to glycation. The implications of uromodulin glycation and AGEs in the urine are not understood. Here, immunoprecipitation and liquid chromatography–mass spectrometry identified glycated uromodulin (glcUMOD) in the urine of 62.5% of patients with diabetic kidney disease (DKD), 20.0% of patients with non-diabetic chronic kidney disease (CKD), and no DM patients with normal renal function or healthy control participants; a finding replicated in a larger cohort of 84 patients with CKD in a case–control study (35 with DM, 49 without). Uromodulin forms high molecular weight polymers that associate with microvesicles and exosomes. Differential centrifugation identified uromodulin in the supernatant, microvesicles, and exosomes of the urine of healthy participants, but only in the supernatant of samples from patients with DKD, suggesting that glycation influences uromodulin function. Finally, the diagnostic and prognostic utility of measuring urinary glcUMOD concentration was examined. Urinary glcUMOD concentration was substantially higher in DKD patients than non-diabetic CKD patients. Urinary glcUMOD concentration predicted DKD status, particularly in patients with CKD stages 1–3a aged <65 years and with urine glcUMOD concentration ⩾9,000 arbitrary units (AU). Urinary uromodulin is apparently glycated in DKD and forms AGEs, and glcUMOD may serve as a biomarker for DKD.

Introduction

Diabetic kidney disease (DKD) is a major complication of diabetes mellitus (DM), and a leading cause of end-stage renal disease (ESRD). The number of patients with DM and ESRD who require renal replacement therapy (RRT) has been increasing, despite efforts to prevent the renal complications of diabetes [1]. Unfavorable outcomes in DKD reflect our incomplete understanding of its pathophysiologic mechanisms and its detection in its late stages [1]. Detection of microalbuminuria is the standard means of diagnosing DKD in its early stages; however, some patients with microalbuminuria have advanced renal disease [2]. Detection of microalbuminuria is not as sensitive as more invasive techniques, such as renal biopsy. There is an urgent need to identify non-invasive biomarkers of DKD in its early stages [2-6].

The microvascular complications of DM are triggered by advanced glycation end-products (AGEs), and the prevention and management of DKD must therefore focus on reducing oxidative stress and intake of exogenous AGEs [7]. During the past decade, proteomics has been a powerful means of discovering

biomarkers for a variety of diseases [8]. The presence of glycated proteins in the urine may serve as a biomarker for DM and DKD [9]. Oxidative stress and inflammation increase as part of normal aging, and decrease in humans and animals with chronic kidney disease (CKD) when dietary intake of AGEs is restricted [10,11]. AGEs are produced when reducing sugars such as glucose react with amino groups in proteins, lipids, and nucleic acids through a series of Maillard reactions [12] to form Schiff bases. The Schiff bases are slowly rearranged to form Amadori products, which are intermediates in the production of AGEs following glycation that then undergo further rearrangements, oxidation, dehydration, and condensation resulting in AGEs [12]. We recently reported that AGEs activate nicotinamide adenine dinucleotide phosphate oxidase and produce superoxide in 3T3-L1 cells, leading to activation of *Src* and *Akt* gene expression [13]. In humans, AGEs are formed continuously, even in euglycemia, and formation is accelerated in DM [14,15]. Diet is a major environmental source of pro-oxidative and pro-inflammatory AGEs *in vivo* [15]. Food- or smoking-derived AGEs have also been shown to play a pathologic role in numerous aging- or diabetes-related disorders, such as cardiovascular disease, CKD, insulin resistance, Alzheimer's disease, and osteoporosis [16]. Serum and tissue AGE concentrations are markedly elevated in patients with ESRD, and are twice as high in patients with ESRD and DM [17]. The kidney is one of the major sites for AGE accumulation and AGE-associated damage [18]. Most AGEs are removed and filtered across the glomerular membrane and reabsorbed or catabolized in the renal proximal tubules, while the rest are excreted in the urine [19-22].

Uromodulin, also known as Tamm–Horsfall protein, is the most abundant urinary protein in healthy individuals, and is normally secreted by epithelial cells lining the thick ascending limb (TAL) of the loop of Henle and the early distal tubule [23]. Uromodulin has diverse functions [23,24], including prevention of ascending urinary tract infections (UTIs) by binding type I-fimbriated *Escherichia coli*, facilitation of salt and water retention and mediation of salt-sensitive hypertension, prevention of calcium oxalate aggregation and kidney stone formation, modulation of immune response during and in the course of recovery from acute kidney injury, and up-regulation of the inflammatory response and tubular transport function. Normal and genetically determined variants of uromodulin may also actively participate in the pathogenesis of CKD [25]. Loss of protective uromodulin can impair tubular recovery after injury and cause chronic interstitial fibrosis and irreversible nephron loss [25]. Many studies have reported an association between urinary uromodulin concentration and estimated glomerular filtration rate (eGFR) in DKD [26-29]. In an acute kidney injury model, uromodulin produced in the TAL reportedly influenced the susceptibility of surrounding tubules to injury, suggesting that uromodulin-dependent TAL-S3 cross-talk in the proximal tubule is important for renal protection [30].

In a previous unpublished study, we established that uromodulin was metabolized into AGEs in the urine of some patients with DKD. Here, we tested our hypotheses that uromodulin is glycated in patients with CKD or DKD, and that glycated uromodulin (glcUMOD) could be a useful biomarker of nephropathy. The secondary objective of our study was to investigate whether glcUMOD levels are predictive of CKD or DKD stage.

Materials and methods
Patients
All experimental protocols were approved by the Institutional Review Board of Changhua Christian Hospital (approval number 140306) and all of the participants provided written informed consent to participate in the study. The CKD care program is regulated by the Clinical Care Program Certification and Joint Commission International. If patients could not be contacted, their data were categorized as incomplete. All patients join our nationwide preventive multidisciplinary early CKD or pre-ESRD program. We investigated those patients enrolled in our CKD care program between January, 2010 and September, 2016. We used the KDOGI guidelines to determine the goals for ideal blood pressure, glucose, and lipid control [31].

Eighty-four patients with CKD (35 with DM and 49 without), ten diabetic patients without CKD, and seven healthy volunteers were recruited from the nephrology clinic at Changhua Christian Hospital, a tertiary referral hospital in Taiwan. The duration of follow-up after the diagnosis of CKD was more than 6 months in all patients. Those with acute fever or infection, hepatic or cardiac disease, endocrinopathy, surgery, trauma, missing data at baseline, prior kidney transplant, acute kidney injury, or a history of RRT or hospital admission for any cause in the past 3 months were excluded. The diagnosis of DKD was based on a confirmed diagnosis of Type 2 DM for more than 12 months, a glycated hemoglobin concentration (HbA1c) between 6.5% and 7.0% for more than 6 months, and elevated urinary albumin concentration or evidence of impaired renal function [32]. The amount of urinary albumin excretion was defined by urinary ACR [31]. Microalbuminuria was established when two out of three ACR determinations were found to be within the range of 30–300 mg/g in a 6-month period [31]. Serum creatinine (Scr) concentration was used to calculate an eGFR according to the CKD Epidemiology Collaboration equation (eGFR $_{CKD-EPI}$). In women:

for Scr $\leqslant$ 0.7 mg/dl, eGFR = 144 × (Scr/0.7)$^{-0.329}$ × 0.993^{Age}. For Scr > 0.7 mg/dl, eGFR = 144 × (Scr/0.7)$^{-1.209}$ × 0.993^{Age}. In men: for Scr $\leqslant$ 0.9 mg/dl, eGFR = 144 × (Scr /0.9)$^{-0.411}$ × 0.993^{Age}. For Scr > 0.9mg/dl, eGFR = 144 × (Scr /0.9)$^{-1.209}$ × 0.993^{Age} [33]. The stages of CKD form a continuum classified as follows: stage 1, renal damage with normal or increased GFR (>90 ml/min/1.73 m^2); stage 2, mild reduction in GFR (60–89 ml/min/1.73 m^2); stage 3a, moderate reduction in GFR (45–59 ml/min/1.73 m^2); stage 3b, moderate reduction in GFR (30–44 ml/min/1.73 m^2); stage 4, severe reduction in GFR (15–29 ml/min/1.73 m^2); and stage 5, kidney failure (GFR < 15 ml/min/1.73 m^2 or recipient of RRT) [34]. As per our national policy, we divided the study population to early CKD (stages 1–3a) and advanced CKD (stages 3b–5).

After participants fasted for 8-h overnight, venous blood samples were obtained and first morning urine samples were collected from each individual for each attendance. Aliquots of urine were immediately frozen at −80°C until further analysis, but for no longer than 1 month. All assays were undertaken in duplicate. The intra-assay variation coefficient was <5%. Urinary albumin concentration was measured by an immunoturbidimetric method (Roche Diagnostics GmbH, Mannheim, Germany), and ACR was expressed as mg/g creatinine. To examine unidentified urinary proteins, we immunoprecipitated AGEs from subjects with and without DM and subjected the immuno-precipitants to LC–MS/MS and confirmed the results by Western blot analysis. Glycemic control was evaluated by assessing fasting plasma glucose and HbA1c concentrations.

Materials
Antibodies against uromodulin used for immunoprecipitation and immunoblotting were purchased from Abnova (Taipei, Taiwan) and Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) respectively. Protein G and protein A Mag Sepharose Xtra beads were obtained from GE Healthcare (Uppsala, Sweden). All other reagents were purchased from Sigma (St. Louis, MO, U.S.A.) unless otherwise specified.

Preparation of advanced glycated end-products and antibodies
Glyceraldehyde can react non-enzymatically with proteins to accelerate formation of glyceraldehyde-derived AGEs [35]. We prepared AGEs as previously described [13,36], by incubating them in bovine serum albumin (10 mg/ml) with 33 mM glyceraldehyde and 100 U/ml penicillin/streptomycin in 0.02 M sodium phosphate buffer (pH 7.4) at 37°C for 3 days in the dark. Generated AGEs were subjected to dialysis against 0.02 M phosphate buffer (pH 7.4) at 4°C for 48 h, then sterilized by being passed through a 0.22 μm filter [20,37]. Preparations were tested for endotoxin using an endotoxin assay kit (Genscript, Piscataway, NJ, U.S.A.), and no endotoxin was detectable. Rabbit polyclonal anti-AGE antibody, procured from Abcam (Cambridge, MA, U.S.A.), was raised against AGEs in BSA [38].

Immunoprecipitation and immunoblotting
We centrifuged 50 μl of urine at 18000 × *g* (4°C) for 3 h. Supernatants were diluted with TEA buffer containing 0.5% Triton X-100 and 20 mM EDTA at pH 7.5, and subjected to immunoprecipitation. Equal amounts (20 μl) of protein A and protein G sepharose beads were washed with PBST (10 mM Na$_2$HPO$_4$, 0.156 M NaCl, 2 mM KH$_2$PO$_4$, 0.02% Tween-20) and resuspended with 100 μl of PBS. One microgram of uromodulin antibody was mixed with re-suspended protein A/G beads and rotated at 4°C for 1 h. Urine supernatants were incubated with antibody–protein A/G sepharose beads with gentle rotation at 4°C overnight; immunoprecipitation complexes were washed three times with PBST. Immunoprecipitants were eluted with 25 μl of 0.1 M glycine (pH 2.8) and mixed with 6× Laemmli sample buffer and neutralizing Tris/HCl buffer. Proteins were separated with SDS/PAGE and immunoblotted with an anti-body against AGEs or uromodulin. Specific proteins were detected with an enhanced chemiluminescence blotting detection system. Bands were quantified by densitometry analysis. Urine from an individual with DM was used as an inter-assay control for normalization [13, 39].

In-gel digestion and liquid chromatography–mass spectrometry analysis
Urine samples were subjected to immunoprecipitation by incubation with antibody against AGEs and protein A sepharose beads. The immunoprecipitants were then separated by SDS/PAGE. The SDS/PAGE gels were stained with Coomassie Brilliant Blue G-250, and destained in 45% ethanol and 5% acetic acid. Protein bands were excised, washed, and subjected to reduction/alkylation. Gel cubes were digested with trypsin at 37°C overnight, and trypsin digests were extracted with 50% acetonitrile/1% formic acid (1:1, v/v) and dried completely in a Speed-Vac (Tokyo Rikakikai, Tokyo, Japan). Peptides were purified with C$_{18}$ Zip-Tip columns and reconstituted with 0.1% formic acid. Samples were separated with a NanoAcquity system (Waters, Milford, U.S.A.) by loading into the bridged ethyl hybrid C$_{18}$ column (1.7 μm, 25 cm × 75 μm analytical reversed phase column, Waters, Milford, U.S.A.). Analyses of peptides

Table 1 Identification of AGE-modified uromodulin proteins

Swiss Prot code	Peptides matched	Mascot score	Molecular weight (Da)	Sequence coverage
P07911	5	160	72451	7%
P07911	9	245	72451	14%

Criteria used for identification were significant homology scores achived in Mascot (30 for 95% confidence interval).

were performed using a Synapt G2 quadrupole time-of-flight mass spectrometer (Waters) equipped with a nanolock-spray source (Waters) fitted with a pico-tip emitter operated at a capillary voltage of 2.8 kV. Peptide identification was performed by database searching against the Swiss-Prot database using Mascot software (version 2.3.02, Matrix Science, London, U.K.) with the following parameters: taxonomy was set as *Homo sapiens*, one trypsin missed cleavage was allowed, the peptide mass tolerance was set at ± 100 ppm, and the fragment mass tolerance was set at ± 0.2 Da. Carbamidomethylation was chosen as a fixed modification and oxidation was used as a variable modification [40].

Statistical analysis

Results are presented as the median (interquartile range) or number (proportion, %). The chi-squared or Fisher's exact test was used to compare the proportion of patients with or without DM with urinary glcUMOD concentration >9,000 arbitrary units (AU). The non-parametric Wilcoxon rank-sum test was employed to compare urinary glcUMOD concentration between patients with or without DM. The predicted probability of DM at various glcUMOD concentrations was calculated from a logistic regression model. Statistical analyses were performed with SPSS Statistics software (version 19.0.0, IBM Corporation, Somers, NY, U.S.A.). A P value <0.05 was considered statistically significant. Post-hoc statistical power analyses were performed using G*Power version 3.1.9.2.

Results
Uromodulin is glycated in the urine of most patients with DKD, but is not glycated in that of DM patients with normal kidney function (diabetic patients without CKD)

We immunoprecipitated AGEs from the urine of two healthy control participants and two patients with DKD. The immunoprecipitants were subjected to Western blotting analysis with an antibody against AGEs. Glycation of several proteins was evident in the urine of patients with DKD, but not that of the healthy control participants (Figure 1A). The bands of the protein with the highest molecular mass in the urine of the two patients with DKD were excised from the gel, subjected to liquid chromatography–mass spectrometry (LC–MS/MS), and identified as uromodulin (Table 1 and Figure 1B). To confirm our findings, we immunoprecipitated AGEs using antibodies against AGEs or uromodulin from the urine of five patients with non-diabetic CKD, eight patients with DKD, and ten diabetic patients without CKD. Western blotting identified glcUMOD proteins in the urine of five of the eight patients with DKD (62.5%, Figure 2), but glcUMOD was only detected in the urine of one patient with non-diabetic CKD (20.0%). Urinary uromodulin from the control group comprising ten diabetic patients without CKD was not glycated (Supplementary Figure S1).

Glycation affects the properties of uromodulin in the urine of patients with DKD

Uromodulin is released from the luminal side of the tubular cell membrane by a specific protease and excreted into the urine. Uromodulin proteins are present in the urine as high molecular weight polymers [41] and can be readily isolated by centrifugation. We examined whether urinary uromodulin and glcUMOD proteins were present in the supernatant and/or pellet fractions by centrifuging urine samples from two healthy control participants and two patients with DKD at $18000 \times \boldsymbol{g}$. Uromodulin in the supernatant and pellet fractions were then immunoprecipitated with an anti-uromodulin antibody, and the immunoprecipitants were subjected to Western blotting with anti-AGE or anti-uromodulin antibodies. Uromodulin was identified in the supernatant and pellet fractions of centrifuged urine from healthy control participants and DKD patients (Figure 3A); however, glcUMOD proteins were detected in only the supernatant fraction of patients with DKD. To determine whether glcUMOD was associated with microvesicles and exosomes, we separated urinary proteins using differential centrifugation ($18000 \times \boldsymbol{g}$ and $110000 \times \boldsymbol{g}$) to isolate urinary microvesicles and exosomes respectively (Figure 3B). Uromodulin proteins in each fraction were immunoprecipitated with the anti-uromodulin antibody followed by Western blotting with anti-AGE or anti-uromodulin
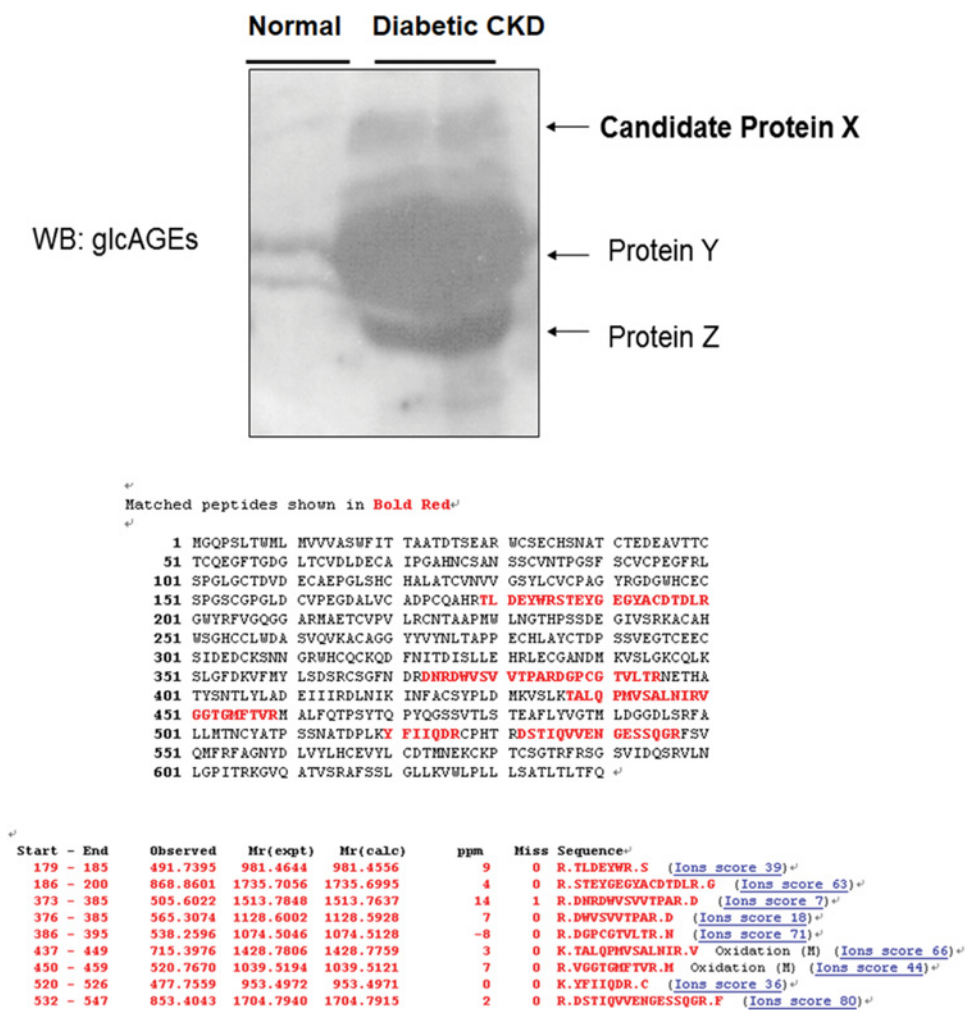
Figure 1. Identification of glcUMOD in the urine of DKD patients

(**A**) A variety of proteins are glycated in the urine of patients with DKD. Urine samples from healthy control participants and patients with DKD were subjected to SDS/PAGE and Western blot analysis using antibodies specific to AGEs. (**B**) Identification of glcUMOD proteins by liquid chromatography–mass spectroscopy (LC–MS/MS). Urine samples were subjected to immunoprecipitation with anti-AGE antibodies, and the immunoprecipitants were separated on SDS/PAGE. Protein bands were excised and digested in gels with trypsin for protein identification by ultra-performance liquid chromatography–electrospray ionization–tandem mass spectrometry. Output of the LC–MS/MS database using the Mascot program.
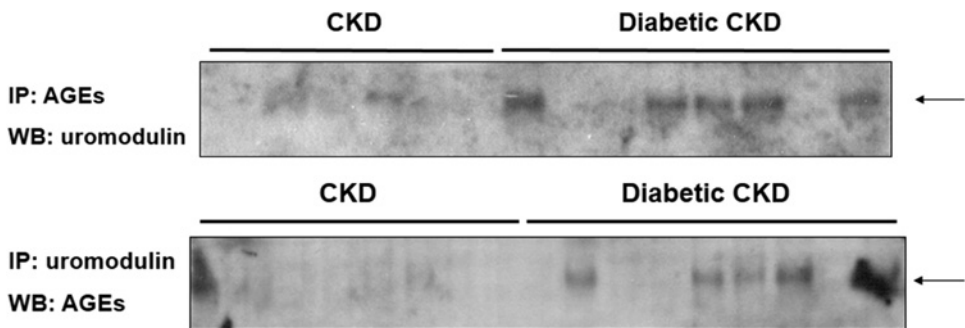

Figure 2. Glycation of uromodulin proteins in urine samples from patients with CKD without diabetes, and patients with DKD

Urine samples were subjected to immunoprecipitation with antibodies against (**A**) AGEs or (**B**) uromodulin, and the immunoprecipitants were separated by SDS/PAGE and analyzed by Western blot using antibodies specific to uromodulin (A) or AGEs (B).
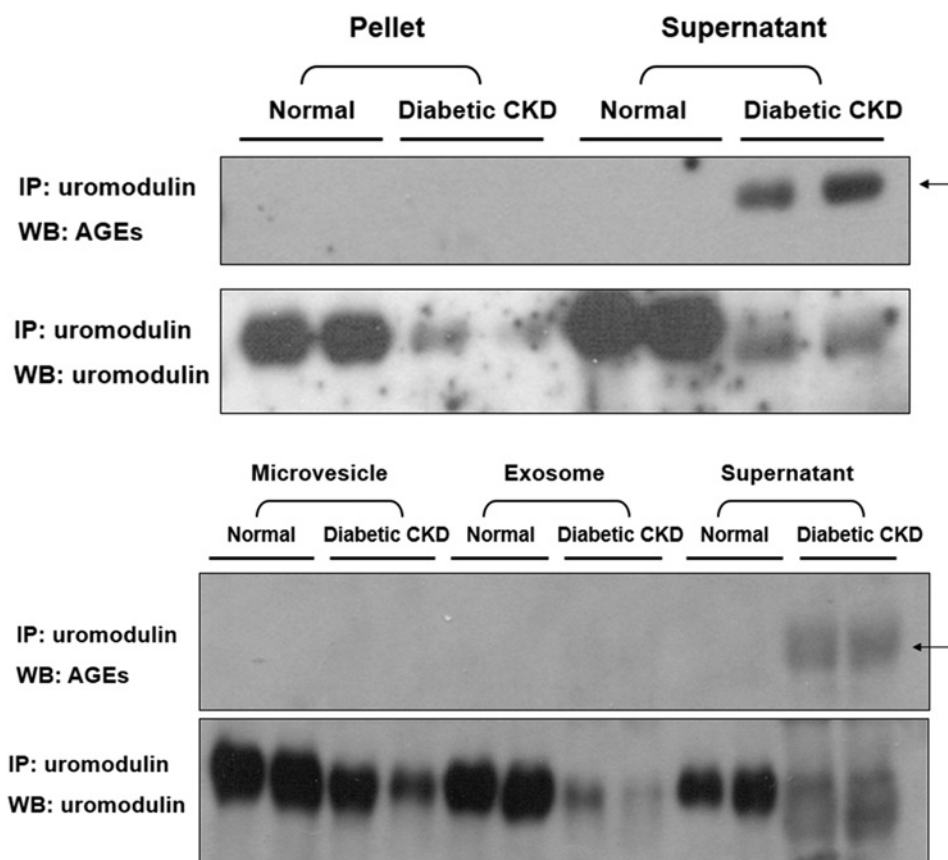
Figure 3. Differences in the distribution of urinary glcUMOD proteins in healthy control participants and patients with DKD
(**A**) Urine samples were subjected to centrifugation at $18000 \times g$ ($4°C$) for 3 h. The supernatant and pellet fractions were collected and diluted with Tris-EDTA-alkaline buffer. Fractional urine samples were subjected to immunoprecipitation with anti-uromodulin antibody, and the immunoprecipitants were separated by SDS/PAGE and analyzed by Western blot using antibodies specific to AGEs or uromodulin. (**B**) Urine samples were subjected to sequential centrifugations ($18000 \times g$ for 3 h then $110000 \times g$ for 3 h). Fractional urine samples were subjected to immunoprecipitation with an anti-uromodulin antibody, and the immunoprecipitants were separated on SDS/PAGE and analyzed by Western blot using antibodies specific to AGEs or uromodulin. The arrow indicates glcUMOD proteins.

antibodies; glcUMOD was detected in only the supernatant fraction of the urine of patients with DKD (Figure 3B), and it represented a relatively small proportion of total urinary uromodulin in these patients. Taken together, these findings suggest that glcUMOD protein may not form high molecular weight polymers in the urine of patients with DKD, and thus cannot be pelleted by centrifugation. Glycation probably alters the conformation and function of uromodulin.

Case–control study in patients with different severities of non-diabetic CKD and DKD

We next screened the supernatant fraction (from centrifugation at $110000 \times g$) of urine obtained from seven healthy control participants and 84 patients with different stages of non-diabetic CKD and DKD. The clinical features of the study participants are summarized in Table 2, and their urinary glcUMOD concentrations are shown in Figure 4(A). Uromodulin was not glycated in any of the seven healthy control participants. Uromodulin was glycated in a small proportion (16.3%) of non-diabetic CKD patients, and the extent of glycation was generally weak (Figure 4B). Conversely, glcUMOD was detected in more than half (54.3%) of patients with DKD, and the extent of glcUMOD was markedly higher than in the control participants (Figure 4A). There was a significant relationship between urinary glcUMOD concentration and CKD stage in patients with DM; a high proportion of patients with DKD had highly glcUMOD ($\geqslant 9,000$ AU; Table 3a). Age also appeared to influence glycation in patients with DKD; there was significant correlation between urinary glcUMOD in those aged < 65 years, but not in those aged $\geqslant 65$ years (Table 3b). When

Table 2 Participants' demographic and clinical characteristics

		Total	DM No	DM Yes	P
Sex	Female	29	17 (34.7%)	12 (34.3%)	0.969
	Male	55	32 (65.3%)	23 (65.7%)	
Stage of CKD	I	4	2 (4.1%)	2 (5.7%)	0.262
	2	13	9 (18.4%)	4 (11.4%)	
	3a	11	8 (16.3%)	3 (8.6%)	
	3b	18	13 (26.5%)	5 (14.3%)	
	4	20	10 (20.4%)	10 (28.6%)	
	5	18	7 (14.3%)	11 (31.4%)	
Age*	Years	84	57 (45, 61)	60 (55, 65)	0.048
BMI*	kg/m^2	84	23 (20, 26)	27 (25, 30)	0.015
glcUMOD		84	0 (0, 0)	6,027 (0, 16821)	<0.001
eGFR	ml/min/1.73 m^2	84	38.6 (26.7, 55.6)	23.3 (10.6, 48.2)	0.055

Abbreviation: BMI, body mass index.
Data are presented as number (proportion, %) or median (intertertile range).
*There was no significant correlation between glcUMOD, BMI, and age on Pearson correlation analysis.
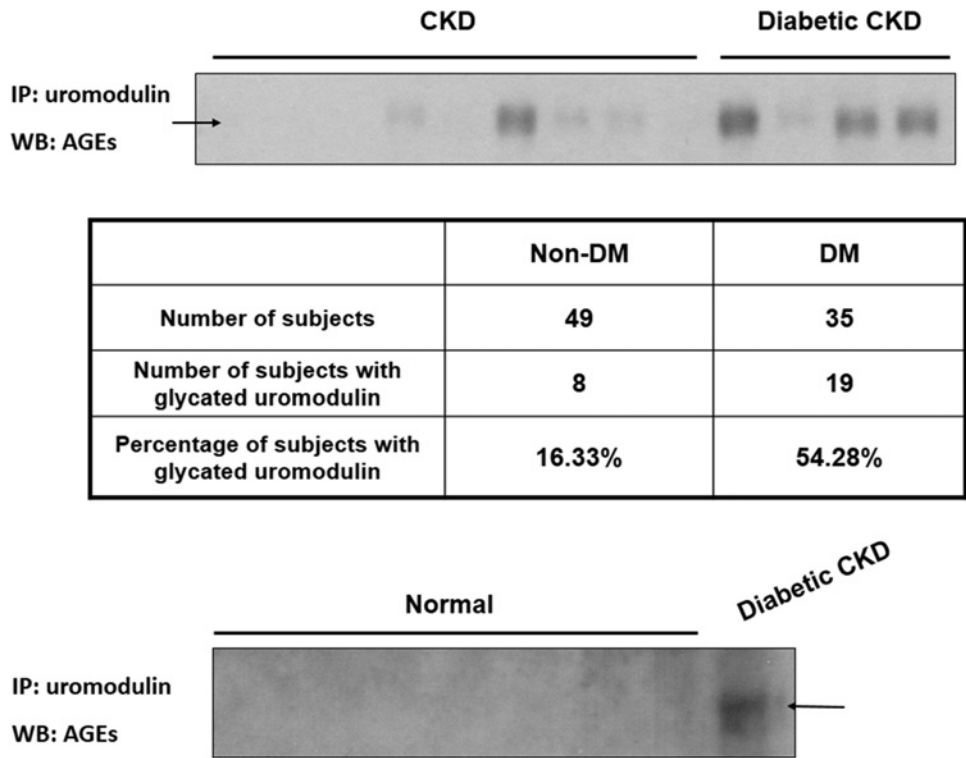

Figure 4. Glycation of uromodulin proteins in urine samples from patients with CKD but not diabetes, patients with DKD, and healthy control participants

(**A**) Urine samples were subjected to centrifugation at 4°C, 18000 × *g* for 3 h. The supernatant fraction was collected and diluted with Tris-EDTA-alkaline buffer. Fractional urine samples from patients with CKD but not diabetes, and patients with DKD were subjected to immunoprecipitation with an anti-uromodulin antibody. The immunoprecipitants were separated by SDS/PAGE and analyzed by Western blot using an antibody specific to AGEs. (**B**) Urine samples from seven healthy control participants were subjected to centrifugation at 4°C, 18000 × *g* for 3 h. The supernatant fraction was collected and diluted with Tris-EDTA-alkaline buffer. Fractional urine samples were subjected to immunoprecipitation with an anti-uromodulin antibody, and the immunoprecipitants were separated on SDS/PAGE and analyzed by Western blot using an antibody specific to AGEs.

Table 3a Relationship between urinary glcUMOD concentration in patients with CKD with or without DM

Stage	glcUMOD (AU)	Total number of patients	Patients without DM	Patients with DM	P
All stages of CKD	⩾9,000	19	3 (6.1%)	16 (45.7%)	<0.001
	<9,000	65	46 (93.9%)	19 (54.3%)	
Early stage	⩾9,000	3	0 (0%)	3 (33.3%)	0.026
(Stages 1–3a)	<9,000	25	19 (100%)	6 (66.7%)	
Advanced stage (Stages 3b–5)	⩾9,000	16	3 (10.0%)	13 (50.0%)	0.003
	<9,000	40	27 (90.0%)	13 (50.0%)	

Table 3b Relationship between urinary glcUMOD concentration in patients with CKD with or without DM aged ⩾65 and <65 years

Age	glcUMOD (AU)	Total number of patients	Patients without DM	Patients with DM	P value
Age <65 years	⩾9,000	15	2 (5.0%)	13 (50.0%)	<0.001
	<9,000	51	38 (95.0%)	13 (50.0%)	
Age ⩾65 years	⩾9,000	4	1 (11.1%)	3 (33.3%)	0.571
	<9,000	14	8 (88.9%)	6 (66.7%)	

Table 4 Risk prediction model assessed using multivariable logistic regression for urinary glcUMOD and current urine biomarkers

Markers[*]	Model 1a		Model 1b		Model 2a		Model 2b	
	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
ACR	1.48 (1.25, 1.74)	<0.0001	1.45 (1.20, 1.75)	<0.0001				
glcUMOD			1.14 (1.01, 1.29)	0.028			1.23(1.11,1.38)	<0.0001
PCR					0.94 (0.83, 1.06)	0.325	0.88 (0.77, 1.02)	0.092
VIF			1.105				1.022	

Correlation analysis using Spearman's rho between glcUMOD and ACR was 0.388 (*P*<0.001), and between glcUMOD and PCR was 0.266 (*P*=0.014). Model 1a was adjusted for ACR; Model 1b was adjusted for ACR and glcUMOD; Model 2a was adjusted for PCR; and Model 2b was adjusted for PCR and glcUMOD.
[*]The markers were transformed by log (marker + 0.5) from [0, 1,000,000] to real line.

median urinary glcUMOD concentrations were compared, those of patients with DKD were substantially higher than those of patients with non-diabetic CKD (Figure 5A). In patients with early and advanced stage CKD, urinary glcUMOD concentration was significantly higher in patients with DKD compared with non-diabetic CKD patients (Figure 5B). There was a positive correlation between urinary glcUMOD concentration and the probability of DKD (for participants with a glcUMOD of 9,000 AU the probability of DKD was 57%, and for those with a glcUMOD of 16,821 AU the probability of DKD was 79%; Figure 6).

Quantifying the improvement in risk prediction offered by glycated uromodulin

We used area under the curve–receiver operator characteristic curve (AUC–ROC) analysis to compare the ability of glcUMOD to predict DKD with protein–creatinine ratio (PCR) and albumin–creatinine ratio (ACR). The AUC–ROC for the urinary glcUMOD was 0.715 (95% confidence interval [CI]: 0.597–0.834; *P*=0.001), compared with 0.799 (95% CI: 0.696–0.903; *P* = 0.001) for ACR and 0.480 (95% CI: 0.341–0.619; *P* = 0.754) for PCR (Figure 7).

The risk prediction model was assessed using multivariable logistic regression and its predictive ability assessed using c-statistics, category-free net reclassification improvement (cfNRI) and integrated discrimination improvement (IDI) for the urinary biomarkers model. Following adjustment for ACR (Model 1a, Table 4) or ACR and glcUMOD (Model 1b, Table 4), we found that glcUMOD predicted DKD (odds ratio [OR]: 1.14, 95% CI: 1.01–1.29; *P*=0.028). With adjustment for PCR (Model 2a, Table 4) or PCR and glcUMOD (Model 2b, Table 4), we found that glcUMOD was an effective predictor of DKD (OR: 1.23, 95% CI: 1.11–1.38; *P*<0.0001). Correlation analysis showed statistically
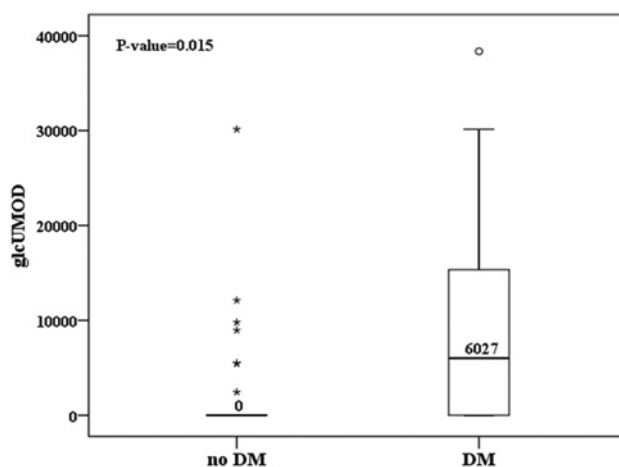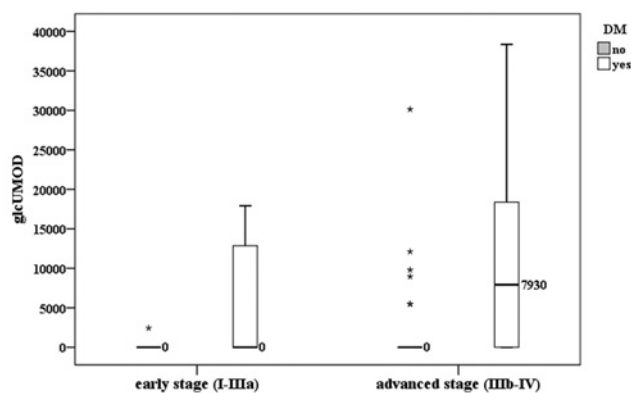
Figure 5. Comparison of glycation levels of uromodulin in urine samples from patients with CKD but not diabetes, and patients with DKD

(**A**) The extent of glycation of urinary uromodulin in samples from patients with CKD but not diabetes, and patients with DKD was determined by a scanner. Glycation levels of urinary uromodulin in patients with DKD were significantly higher than those in patients with CKD without diabetes ($P=0.015$). (**B**) Glycation levels of urinary uromodulin in the early stages (1–3a) CKD ($P=0.039$) and advanced stages (3b–5) CKD ($P=0.001$) were significantly higher in patients with diabetes than those without diabetes. * denotes significantly, $P<0.05$
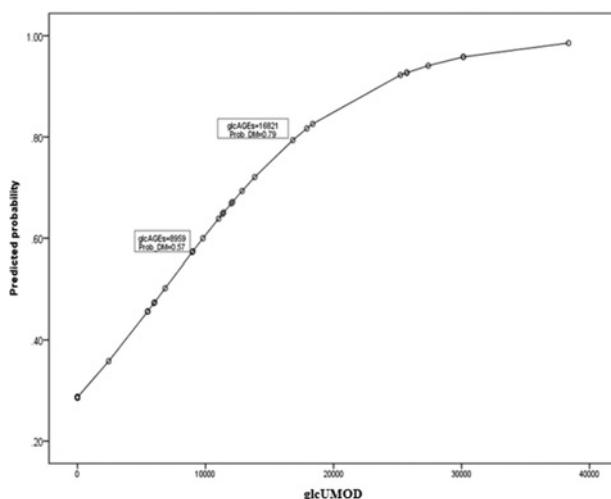

Figure 6. The correlation between urinary glcUMOD concentration and the probability of DKD.
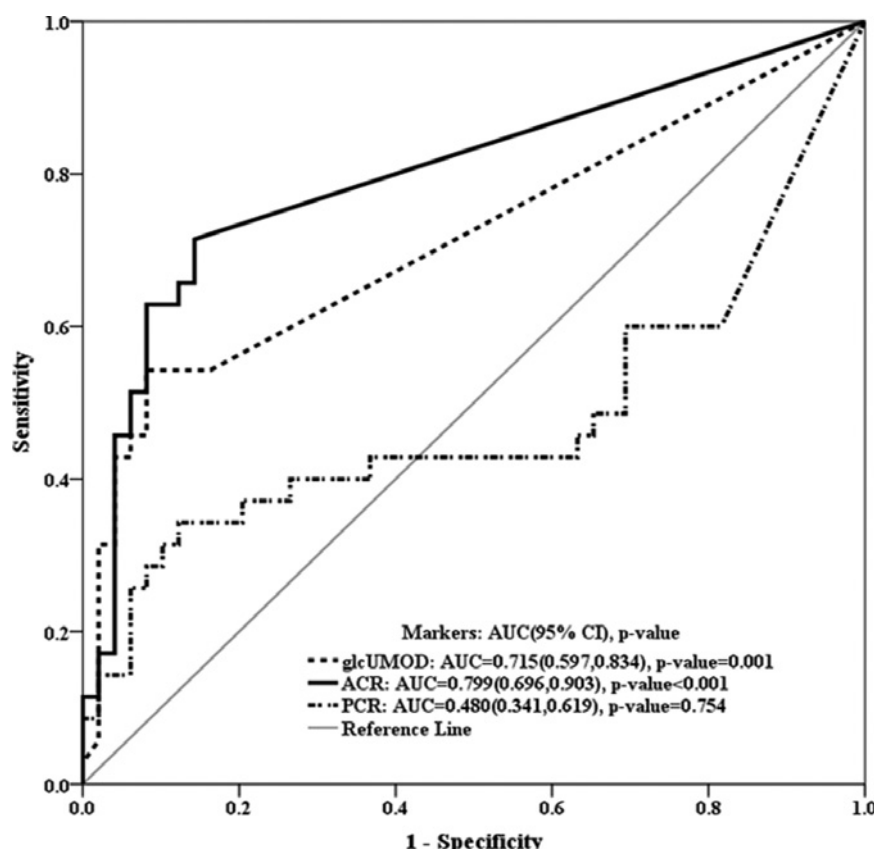
Figure 7. AUC–ROC analysis

Two other well-known early biomarkers of kidney diseases, PCR and ACR, were compared with urinary glcUMOD concentration for their ability to discriminate between patients with DKD and patients with CKD without diabetes.

Table 5 Risk for the urine biomarkers predictive ability: using cfNRI and IDI

Markers*	c statistic[†] (95% confidence interval)	Change of c statistic (95% confidence interval)	*P* value[‡]	cfNRI(%) (95% CI)	*P* value[§]	IDI(95% CI)	*P* value[‖]
Proteinuria ACR	0.799(0.70,0.90)	—	Referent	—	Referent	—	Referent
+ glcUMOD	0.867 (0.78,0.95)	0.068(-0.06,0.20)	0.311	75.92(36.96,114.88)	<0.0001	0.046(0.002,0.09)	0.048
Proteinuria PCR	0.520 (0.39,0.65)	—	Referent	—	Referent	—	Referent
+ glcUMOD	0.746 (0.64,0.86)	0.226(0.06, 0.39)	0.008	75.92(36.96,114.88)	<0.0001	0.190(0.103,0.277)	<0.0001

Abbreviation: SE, standard error.

*The markers was transformed by log (marker + 0.5) from [0, 1000000] to real line.

[†]Risk prediction was assessed by the c statistic. Each newer marker was stepwise added to the model of proteinuria (ACR or PCR) to assess the c statistic for predicting the presence of DM.

[‡§‖]The *P* value for increase in c statistic, cfNRI, and IDI in a model with proteinuria and glcUMOD, compared with proteinuria alone.

significant differences between glcUMOD, and ACR or PCR (Table 4). The variance inflation factors (VIFs) of the logistic regression were 1.105 and 1.022 respectively, indicating that collinearity between ACR and glcUMOD, and PCR and glcUMOD, was absent.

We also calculated c-statistics, cfNRI, and IDI values to describe further the ability of these markers to risk stratify diabetic patients beyond our clinical classic risk prediction model (Table 5). Category-free net reclassification improvement provides a measure of the direction of change in estimated risk that a biomarker adds to the clinical model

without considering existing cut offs [42], with results reported as proportions. Because it is possible for all events and non-events to increase the risk, the maximum value of the total cfNRI (events + non-events) is 200% [43]. In contrast, IDI assesses the direction and magnitude of the mean change in the predicted probabilities for events and non-events when additional variables or biomarkers are added [44]. The cfNRI for urinary glcUMOD concentration combined with ACR was 75.9% (95% CI: 37.0–114.9%; $P < 0.0001$) and the IDI was 0.046 (95% CI: 0.002–0.090; $P = 0.048$). The cfNRI for glcUMOD combined with PCR was also 75.9% (95% CI: 37.0–114.9%; $P < 0.0001$), but the IDI was 0.190 (95% CI: 0.103–0.277; $P < 0.0001$).

Discussion

To the best of our knowledge, ours is the first study to have examined glcUMOD in the urine of patients with non-diabetic CKD and with DKD, and to show that uromodulin was not glycated in that of diabetic patients without CKD. We found that there was significant correlation between urinary glcUMOD concentration and DKD, particularly in CKD stages 1–3a. Urinary glcUMOD concentration did not appear to be influenced by DM in older patients. It appears that urinary glcUMOD concentration may increase with increasing age. The probability of DKD was higher in patients with urinary glcUMOD concentration ⩾9,000 AU, and uromodulin was more extensively glycated in patients with DKD. Consequently, glcUMOD may have a role to play as a biomarker for CKD, especially for patients with DKD.

Human urine is an attractive fluid for proteomic study as it is simple and straightforward to collect. Compared with serum or plasma, urine is also stable and contains fewer proteins. Alterations in the constituents of urine may directly reflect changes in the functions of the kidney and urogenital tract [45], and novel biomarkers are needed to detect and monitor the progression of early disease [46]. Specific urinary proteins or microRNAs may serve as diagnostic or prognostic biomarkers, or as therapeutic targets for various kidney diseases, including glomerulonephritis, acute and chronic kidney injury, tubular disorders, and polycystic kidney disease. We found that glcUMOD levels were elevated in the urine of non-diabetic CKD patients, but were more substantially elevated in DKD patients compared with non-diabetic CKD patients. We did not detect glcUMOD in the urine of healthy control participants and diabetic patients without CKD.

It is well recognized that proteins in tissues or biofluids may be glycated in elderly subjects and patients with DM [17], but the identity of these proteins remains largely unknown. We first identified glcUMOD in the urine of two patients with DKD after immunoprecipitation with anti-AGE antibodies followed by LC–MS/MS and Western blotting (Figures 1–3 and 5). Screening more individuals revealed that glcUMOD was not present in the urine of healthy control participants (Figure 5B). Interestingly, glcUMOD was detected in approximately 55% of DKD patients, and the extent of glycation of uromodulin was generally high, while glcUMOD was detected in approximately 16% of non-diabetic CKD patients and the extent of glycation was generally low. Thus, glcUMOD may serve as a biomarker for DKD.

Uromodulin is the most abundant urinary protein in healthy individuals. It is excreted from the apical plasma membrane into the tubular lumen [19], but a small proportion is secreted basolaterally into the intersitium and interacts with immune cells, causing severe inflammation. Prajczer et al. [23] reported that significant transport of soluble uromodulin from the lumen to the intersitium could accelerate the progression of CKD by causing severe inflammation and destruction of the TAL. In this study, we found that there was significant correlation between urinary glcUMOD concentration and DKD status, especially when glcUMOD exceeded 9,000 AU. Adjustment for urinary glcUMOD concentration also reclassified patients to a more appropriate level of DKD risk using NRI and IDI. Our data suggest that urinary glcUMOD, reflecting a post-translational modification of uromodulin, should be considered as an etiologic factor for DKD.

Although there was significant correlation between urinary glcUMOD concentration and DKD, we found no significant difference in urinary glcUMOD concentration between elderly patients with DKD and those with non-diabetic CKD. It is well recognized that glycoxidant stress is elevated in DM and old age [47], and urinary AGEs and glcUMOD concentration would be expected to be elevated in the elderly and patients with DKD. This may account for there being no significant difference in urinary glcUMOD concentration between elderly patients with DKD and those with non-diabetic CKD.

There is a body of evidence that indicates that mutations, promoter variations, and expression levels of uromodulin influence renal function and the onset of renal disease. For example, mutations of uromodulin have been shown to cause congenital hyperuricemia and cystic kidney disease [41]. These mutations result in misfolding of uromodulin, with misfolded proteins retained in the endoplasmic reticulum and not secreted in the urine. Genome-wide association studies have identified single-nucleotide polymorphisms in the promoter region of the uromodulin gene that are

associated with the risk of CKD and renal function [48]. Urinary uromodulin concentration is reportedly associated with renal function and risk for incident CKD [49]. Post-translational modifications of uromodulin have, however, never been reported to be associated with kidney disease. We have shown for the first time that urinary uromodulin is glycated in approximately 54% of patients with DKD and 16% of patients with non-diabetic CKD.

Uromodulin is found in the urine as a high molecular weight polymer assembled into filaments, matrices, or gel-like structures [41]. Polymeric uromodulin associates with exosomes and can be pelleted by high speed centrifugation ($200000 \times \boldsymbol{g}$) [32]. We found that uromodulin was non-glycated and located within microvesicles and exosomes in healthy control participants and most non-diabetic CKD patients (Figure 4A and 4B), whereas it was glycated in the urine of about half of patients with DKD and glcUMOD proteins were identified in the supernatant fraction after high- or low-speed centrifugation. These findings suggest that glycation may impair the ability of uromodulin to form high molecular weight polymers, meaning that glcUMOD does not associate with microvesicles and exosomes. Recent research has suggested a role of extracellular vesicles (EVs), especially exosomes, in the maintenance of tissue homeostasis in the kidney. Exosomes have been reported to mediate cell–cell communication by transferring proteins, mRNAs, and microRNAs to mediate cross-talk between various kidney cell types [50]. Moreover, exosomes have been reported to be involved in the pathogenesis of both acute kidney injury and CKD, including renal fibrosis, glomerular diseases, and DKD [51].

Glucosuria is the main driver of UTIs in DKD patients. However, because glycation of uromodulin in the urine of patients with DKD impairs its ability to form high molecular weight polymers or matrix-like structures, glcUMOD may contribute to this increased susceptibility to UTIs in patients with DKD. High molecular weight uromodulin polymers may bind pathogenic bacteria and prevent their adherence to glycoproteins and glycolipids on luminal plasma membranes, thus reducing or preventing UTIs [18], which are common in patients with Type 2 DM [52]. Bates et al. [53] revealed that UMOD serves as a soluble receptor for type 1 fimbriated *E. coli* and helps eliminate bacteria from the urinary tract, and showed that UMOD protein knockout mice are more prone to urinary tract infection. Multiple genome-wide association studies have shown that mutation of UMOD delays intracellular protein trafficking and disturbs biological mechanisms, causing independent susceptibility to CKD and hypertension [54]. Another recent study showed that UMOD may have a physiological function related to its inhibitory effect on the NF-κB pathway, but that this function may be lost in mutant UMOD [55].

The major limitations of proteomic studies are related to the type of biological material to be analyzed and the sensitivity of the methods available for the analyses. Greater numbers of samples must be assayed to define the differences between patients with and without DM, and the potential differences in glcUMOD that may arise between patients with shorter or longer-standing DM and between those with Type 1 or Type 2 DM. Clinical studies may therefore be limited by their relatively small sample sizes. To examine whether our sample size was sufficient to assess the difference in urinary glcUMOD levels between CKD patients with DM and those without DM, we conducted a post-hoc power analysis. With a power of 80%, a sample size of 38 would be sufficient to detect the observed difference in urinary glcUMOD levels between the two groups with a two-sided α-error of 0.05. Given the inclusion of 84 patients in the present study, the probability of the detected difference with a two-sided α-error of 0.05 was 95%. In our case–control study, we were not able to illuminate the pathogenic role of glcUMOD in non-diabetic CKD or DKD patients. To clarify whether the appearance of glcUMOD precedes or follows the onset of hyperfiltration, a long-term cohort study will be necessary in the future.

In summary, we found that uromodulin is glycated in the urine of the majority of patients with DKD, and that urinary uromodulin is not glycated in healthy control participants. We also found a significant correlation between urinary glcUMOD concentration and DKD status, particularly in patients with CKD stages 1–3a, aged <65 years, and with a urinary glcUMOD concentration in excess of 9,000 AU. These findings suggest that glcUMOD, reflecting a post-translational modification of uromodulin, could be a novel biomarker for DKD. Determination of the clinical value of glcUMOD requires further study with a larger number of patients.

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Clinical perspectives

- Unfavorable outcomes in diabetic kidney disease (DKD) reflect our incomplete understanding of its pathophysiologic mechanisms and its detection in its late stages.

- In a cohort of 84 patients with chronic kidney disease (CKD) in a case–control study (35 with DM, 49 without DM), immunoprecipitation and liquid chromatography–mass spectrometry identified glycated uromodulin (glcUMOD) in the urine of 62.5% of patients with DKD, 20.0% of patients with non-diabetic CKD, and no DM patients with normal renal function or healthy control participants. Urinary glcUMOD concentration predicted DKD status, particularly in patients with CKD stages 1–3a aged <65 years and with urine glcUMOD concentration ≥9,000 AU.

- These findings suggest that glcUMOD, a post-translational modification of uromodulin, could be an innovative biomarker for DKD.

Competing Interests
The authors declare that there are no competing interests associated with the manuscript.

Author Contribution
C.-H.H., C.-C.C., and C.-S.L. designed research; C.-H.H., C.-L.K., C.-S.H., and W.-M.T. conducted research; C.-H.H. and I.-B.L. analyzed data; C.-H.H., C.-C.C. wrote the paper; C.-C.C. and C.-S.L. had primary responsibility for final content. All authors read and approved the final manuscript.

Abbreviations
ACR, albumin–creatinine ratio; AGE, advanced glycation end-product; AU, arbitrary units; AUC–ROC, area under the curve–receiver operator characteristic curve; cfNRI, c-statistics, category-free net reclassification improvement; CKD, chronic kidney disease; DKD, diabetic kidney disease; DM, diabetes mellitus; eGFR, estimated glomerular filtration rate; ESRD, end-stage renal disease; glcUMOD, glycated uromodulin; HbA1C, glycated hemoglobin concentration; IDI, integrated discrimination improvement; PCR, protein–creatinine ratio; RRT, renal replacement therapy; TAL, thick ascending limb; UTI, urinary tract infection; VIF, variance inflation factor.

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