Urinary A- and C-megalin predict progression of diabetic kidney disease: a retrospective cohort study

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Abstract

Background

Urinary excretion of megalin, a proximal tubular endocytic receptor, may be associated with the development and progression of diabetic kidney disease (DKD). However, no studies have assessed whether the levels of the urinary ectodomain (A-megalin) and full-length (C-megalin) forms of megalin can predict DKD progression.

Methods

We evaluated the correlation of urinary A-megalin levels of 34 patients with type 2 diabetes as measured by novel reducing and previous methods. Then, we retrospectively analyzed 188 type 2 diabetes patients not taking sodium glucose cotransporter 2 (SGLT2) inhibitors in order to investigate whether urinary A- and C-megalin might be used as predictors of kidney outcomes. The median observation period was 3.96 years. The associations between the baseline urinary A-megalin measured by the novel method and/or C-megalin levels and the subsequent estimated glomerular filtration rate (eGFR) slope were analyzed using a generalized estimating equation. Patients were categorized into higher or lower groups based on the optimal cutoff values, obtained from a receiver operating characteristic (ROC) curve, of the two forms of urinary megalin.

Results

Urinary A-megalin levels measured by the two methods were strongly correlated. The eGFR slopes of the higher A-megalin and C-megalin groups were -0.904 (95% confidence interval [CI] $-1.584, -0.224$) and -0.749 (95% CI $-1.312, -0.186$) ml/min/1.73 m² per year steeper than those of the lower groups, respectively. Moreover, the eGFR slope was -1.888 (95% CI $-2.764, -1.011$) ml/min/1.73 m² per year steeper in the group with both higher A- and higher C-megalin than in the other groups. These results remained significant when adjusted for albuminuria or known tubular injury markers.

Conclusions

Our novel method allows urinary A-megalin measurements to be performed more easily. Baseline urinary megalin levels were associated with the subsequent eGFR slope independently of known biomarkers in type 2 diabetes patients not receiving SGLT2 inhibitors. These two forms of megalin may be distinct urinary biomarkers of the progression of DKD.

Background

Diabetic kidney disease (DKD) is a leading cause of end-stage kidney disease worldwide [1, 2]. Although albuminuria is a widely recognized biomarker of the clinical development of DKD [1], it was recently recognized that progressive renal decline can develop in many patients with type 2 diabetes independently of albuminuria [3]. The precise mechanism underlying the development and progression of DKD is also largely unknown. However, “proximal tubulopathy” has been recognized as a prime trigger of the disease [4]. Because the proximal tubule is the major site of renal metabolism, metabolic load and resultant derangements in proximal tubular epithelial cells (PTECs) are likely to be associated with the pathogenesis of DKD [5–8]. Thus, the identification of biomarkers that reflect proximal tubulopathy would help to predict DKD progression [9].

Megalin, a large (~600 kDa) glycoprotein member of the low-density lipoprotein receptor family [10], plays a pivotal role in the endocytosis of diverse glomerular-filtered substances into PTECs [11]. In a high-fat-diet-induced, obesity-related murine model of DKD, megalin internalizes pathologic proteins such as free fatty acid-enriched albumin into PTECs, leading to qualitative and quantitative protein metabolic overload in the endo-lysosomal system and to increased production of platelet-derived growth factor-B and monocyte chemoattractant protein-1 in cells. Subsequent interstitial fibrosis, peritubular capillary rarefaction, and tubular constriction appear to cause retrograde glomerular damage [7]. Advanced glycation end products are also filtered by glomeruli and reabsorbed *via* megalin by PTECs, causing glycototoxicity in diabetes [12]. Thus, megalin appears to constitute a “gateway” for internalizing lipotoxic and glycotoxic substances into the kidney for the development and progression of DKD. Furthermore, several urinary biomarkers filtered by glomeruli (e.g., α_1 -microglobulin [α_1 -MG], β_2 -microglobulin [β_2 -MG], liver-type fatty acid-binding protein, neutrophil gelatinase-associated lipocalin, as well as albumin) are known endocytic ligands of megalin [13, 14].

We previously established a sandwich enzyme-linked immunosorbent assay (ELISA) to measure the ectodomain (A-megalin) and full-length (C-megalin) forms of urinary megalin using monoclonal antibodies against the amino- and carboxyl-terminals of megalin, respectively [15]. Compared with normal controls, urinary C-megalin excretion was found to be elevated even in the normoalbuminuric stage of patients with type 2 diabetes and to increase in conjunction with DKD progression in a cross-sectional analysis [15]. We also found that urinary C-megalin is increased *via* exocytosis in association with megalin-mediated quantitative or qualitative protein metabolic load to the endo-lysosomal system of PTECs in residual nephrons in type 2 diabetes [16]. In contrast, urinary A-megalin was found to be increased in normo- and microalbuminuric patients with type 2 diabetes but not in those with macroalbuminuria [15], and its urinary excretion appears to be regulated by intracellular recycling [17] and extracellular cleavage [15] of megalin. Thus, the two forms of urinary megalin excretion may play discriminative roles as biomarkers for DKD. However, it is currently unknown whether urinary megalin can longitudinally predict the prognosis of DKD. Furthermore, the our previous method for measuring A-megalin in urine is complicated and time-consuming [15]. Therefore, we developed and validated a novel method for measuring urinary A-megalin.

To evaluate the progression of DKD, clinical studies have been designed using endpoints of end-stage kidney disease, renal death, or treatment with renal replacement therapies. However, these endpoints need a very long follow-up period and are costly. Recently, the estimated glomerular filtration rate (eGFR) slope has been recognized as a surrogate endpoint of chronic kidney disease (CKD) in clinical trials [18]. Several studies reported that the eGFR slope of cohorts could predict the progression of CKD, including DKD [19–22]. However, sodium glucose cotransporter 2 (SGLT2) inhibitors cause an initial drop in eGFR and may affect the annual assessment of the eGFR slope [23]. Hence, we examined the relationship of urinary A- and C-megalin excretion and the eGFR slope in this longitudinal study of patients with type 2 diabetes not taking SGLT2 inhibitors.

Methods

Participants and study design

This retrospective study involved 191 Japanese adults (aged 20 years or older) with type 2 diabetes who were outpatients at the Division of Clinical Nephrology and Rheumatology, Niigata University Medical and Dental Hospital. Urine samples were collected from 2007 to 2011 with their consent, which was approved by the Niigata University Ethical Committee (approval no. 191 in 2003). Patients with severe active infectious disease, severe trauma, or pregnancy were excluded. Patients in the perioperative period or with a short observation period (less than 6 months) were also excluded. Standard clinical examinations and biochemical tests were performed regularly in the patients, and urine samples were stocked annually at -80°C during the follow-up period. The oldest stocked urine sample of all participants collected during this period was defined as the baseline sample. We measured urine A-megalin, C-megalin, α_1 -MG, β_2 -MG, N-acetyl- β -D-glucosaminidase (NAG), albumin, and creatinine (Cr) levels. Serum Cr, blood urea nitrogen, uric acid, and hemoglobin A1c of blood samples collected on the same date were measured as baseline data. The eGFR slope was calculated using eGFR values derived from serum Cr levels, which were recorded about once a year in the electronic medical records and collected retrospectively. The permissible ranges for data collection at 1, 2, 3, and 4 years after the baseline day were 6–17 months, 18–29 months, 30–41 months, and 42–53 months, respectively.

This study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines and was approved by the Niigata University Ethical Committee (approval no. 2591 in 2016). In addition, to validate our novel method for measuring urinary A-megalin, we enrolled 34 patients with type 2 diabetes treated at the Division of Nephrology and Rheumatology in Niigata University Medical and Dental Hospital; this was approved by the Niigata University Ethical Committee (approval no. 2590 in 2016). All participants provided informed consent and their anonymity was preserved.

Measurement of human megalin in urine by sandwich ELISA

Urinary C-megalin was measured as reported previously [15]. A novel sandwich ELISA for measuring urinary A-megalin was developed as follows. The capture monoclonal antibody A12 (7 $\mu\text{g/ml}$) was

immobilized on ELISA plates (F16 Black Maxisorp FluoroNunc Cert; Thermo Fisher Scientific, Waltham, MA) (100 μ l/well in 50 mmol/l carbonate buffer, pH 9.5) at 4°C overnight. The plates were washed with Tris-buffered saline (25 mmol/l Tris-HCl, 137 mmol/l NaCl, 2.68 mmol/l KCl, pH 7.4), blocked with 0.2% casein and 0.05% Tween 20 (200 μ l/well) in Tris-buffered saline containing 0.1% NaN₃ at 4°C overnight, and stored at 2°C–8°C. The tracer monoclonal antibody A5 was digested by pepsin to prepare secondary fragment antibodies [F(ab')₂] that were reduced to F(ab') by 2-mercaptoethylamine. The reduced F(ab') were conjugated to alkaline phosphatase (Roche Diagnostics). Urine samples (50 μ l) were mixed with 50 μ l of solution B (400 mmol/l Tris-HCl, 40 mmol/l ethylenediaminetetraacetic acid, 2% Triton X-100, 0.05% reduced glutathione, pH 8.0) and incubated for 1 min at room temperature. Urine sample mixtures were then reacted with the alkaline phosphatase-labeled tracer monoclonal antibody in the ELISA plates. A chemiluminescent immunoassay detection system with CDP-Star substrate with Emerald-II enhancer (Applied Biosystems, Carlsbad, CA) was used according to the manufacturer's instructions (ELISA-Light System; Thermo Fisher Scientific). Urinary megalin concentrations were standardized by adjustment to urinary Cr concentrations. The novel method for urinary A-megalin measurement was validated using urine samples of 34 patients with type 2 diabetes by comparison with the previously reported method [15].

Measurement of other urinary markers

Serum Cr concentrations were measured by an enzymatic method. The eGFR was calculated with an equation validated for the Japanese population [24]. Urinary concentrations of Cr, albumin, NAG, α_1 -MG, and β_2 -MG were measured as reported previously [15]. The concentrations of each urinary marker were normalized to those of urinary Cr.

Statistical analysis

We compared our novel method for urinary A-megalin measurement with our previous method using Spearman's correlation analysis. The correlations of baseline urinary markers, including urinary megalin, and clinical parameters were analyzed using Pearson's or Spearman's correlation analysis.

A receiver operating characteristic (ROC) curve was plotted, and the area under the ROC curve was calculated to evaluate the optimal cutoff values of urinary A- or C-megalin/Cr for the median of the eGFR slopes from baseline to visit 4. After patients were categorized into two groups according to the optimal cutoff or median values of the urinary A- or C-megalin/Cr levels, their baseline characteristics were compared using Welch's *t*-test, Mann-Whitney's U test, or chi-square test. The eGFR slope of each patient was calculated with a generalized estimating equation (GEE). A generalized linear model developed by using the GEE was used to evaluate the time, prognostic factors (A- or C-megalin/Cr), and interaction between the time variables and the prognostic factors as independent variables with changes from the baseline of the target variable (eGFR) as dependent variables, adjusted for within-subject repeated variables at each time. In addition, multivariate analysis using the stepwise method was performed to compare the slope of the higher urinary A- and C-megalin/Cr group (higher megalin/Cr group) with that of

the higher A-megalin/Cr alone group or with that of the higher C-megalin/Cr alone group by using the GEE.

In addition to the crude model (model 1), models were adjusted with the urinary albumin creatine ratio (ACR) (model 2), urinary α_1 -MG/Cr (model 3), urinary β_2 -MG/Cr (model 4), and urinary NAG/Cr (model 5). The baseline characteristics of the groups were then compared, and changes from baseline were similarly evaluated with a generalized linear model by using a GEE.

Furthermore, we divided some baseline parameters, including urinary biomarkers, with a correlation with urinary A- and C-megalin/Cr into two groups by the evaluated optimal cutoff or median values, as above. Using these baseline parameters, we applied a linear estimating equation to evaluate the three-way interactions among the time, binary urinary A- or C-megalin/Cr variables, and baseline parameters.

All statistical analyses were performed using SAS Statistics version 9.4 (SAS Institute Inc, Cary, NC), R version 3.6.3 (The R Foundation for Statistical Computing, Vienna, Austria), or IBM SPSS Statistics version 27 (IBM Corp., Amonk, NY). The level of significance was $P < 0.05$.

Results

Validation of the novel sandwich ELISA to measure urinary A-megalin

The urine samples of 34 patients with type 2 diabetes were used to validate our novel deoxidization method for measuring urinary A-megalin levels. The profiles of these patients are shown in Supplemental Table 1. Supplemental Figure 1 illustrates the scatter plot of urinary A-megalin measured by our previous heating method and our novel deoxidization method. The two methods were strongly correlated ($r = 0.91$, $P < 0.001$). Therefore, we adopted the novel method for this study.

Recruitment Of Patients With Type 2 Diabetes

In this study, we measured urinary A- and C-megalin using urine samples stocked from 2007 to 2011 from 191 patients. Three patients were excluded according to the exclusion criteria. Therefore, 188 patients were analyzed in this study (Table 1). The median observation period was 1456 (interquartile range, 793–1505) days. The drugs administered to the patients are listed in Supplemental Table 2. No patient had been treated with SGLT2 inhibitors.

Table 1
Baseline parameters and other data of the 188 patients analyzed in this study

Number (male/female)	104/84
Age (years)	68 (58, 75)
BMI	25.36 ± 4.95
SBP (mmHg)	130 (120, 140)
eGFR (ml/min/1.73 m ²)	67.6 ± 21.4
BUN (mg/dl)	16 (13, 19)
S-Cr (mg/dl)	0.78 (0.64, 0.96)
S-UA (mg/dl)	5.4 (4.5, 6.3)
HbA1c (%)	7.3 ± 0.9
U-A-megalin/Cr (pmol/g Cr)	155.07 (101.60, 222.67)
U-C-megalin/Cr (pmol/g Cr)	0.478 (0.237, 0.929)
U-ACR (mg/g Cr)	38.53 (18.93, 17.13)
U- α_1 -MG/Cr (mg/g Cr)	9.06 (5.23, 17.79)
U- β_2 -MG/Cr (μ g/g Cr)	12.87 (73.74, 383.17)
U-NAG/Cr (IU/g Cr)	6.77 (4.43, 11.16)
RAS inhibitors	126
Data are number, mean ± SD, or median (25th, 75th percentile). BMI, body mass index; SBP, systolic blood pressure; eGFR, estimated glomerular filtration rate; BUN, blood urea nitrogen; S-Cr, serum creatinine; S-UA, serum uric acid; HbA1c, hemoglobin A1c; U-A-megalin, urinary A-megalin; U-C-megalin, urinary C-megalin; U-ACR, urinary albumin creatinine ratio; U- α_1 -MG, urinary α_1 -microglobulin; U- β_2 -MG, urinary β_2 -microglobulin; U-NAG, urinary N-acetyl- β -D-glucosaminidase; RAS, renin-angiotensin-aldosterone system	

Cutoff points of urinary A- and C-megalin excretion levels from ROC analysis

ROC curves were analyzed to predict the eGFR slope (-7.615 ml/min/1.73 m²/year). With a cutoff value of 222.52 pmol/g Cr, the area under the ROC curve showed that urinary A-megalin had specificity and sensitivity of 46% and 68%, respectively, for an eGFR slope > -7.615 ml/min/1.73 m²/year. With a cutoff value of 0.62 pmol/g Cr, the area under the ROC curve also showed that urinary C-megalin had specificity and sensitivity of 48% and 70%, respectively, for an eGFR slope > -7.615 ml/min/1.73 m²/year (data not shown). Patients with urinary A- or C-megalin greater than or equal to these cutoff points were respectively classified as the higher urinary A- or C-megalin groups, whereas those with values less than these cutoff points were classified as the lower urinary A- or C-megalin groups.

Longitudinal analysis of the association of urinary A- or C-megalin excretion with eGFR slopes

The baseline clinical characteristics of the higher and lower urinary A- or C-megalin groups are shown in Tables 2 and 3, respectively. Each eGFR slope analyzed from the generalized linear model is shown in Table 4, Table 5, and Figure 1. The eGFR slope was -0.904 (95% CI $-1.584, -0.224$) ml/min/1.73 m²/year steeper in the higher A-megalin/Cr group than in the lower A-megalin/Cr group in model 1. Moreover, in the models adjusted with other urinary biomarkers, the slopes showed a similar trend. The eGFR slope was -0.749 (95% CI $-1.312, -0.186$) ml/min/1.73 m²/year steeper in the higher C-megalin/Cr group than in the lower C-megalin/Cr group in model 1. Furthermore, in models adjusted with other urinary biomarkers, the slopes showed a similar trend. According to the median of each urinary megalin level at baseline, these 188 patients were categorized into higher and lower urinary megalin groups. The characteristics of these groups are shown in Supplemental Tables 3 and 4. Each eGFR slope analyzed from the generalized linear model is shown in Supplemental Tables 5 and 6. The eGFR slope was -0.749 (95% CI $-1.297, -0.202$) ml/min/1.73 m²/visit steeper in the higher A-megalin/Cr group than in the lower A-megalin/Cr group in model 1. Moreover, in models adjusted with other urinary biomarkers, the slopes showed a similar trend. The eGFR slope was -0.574 (95% CI $-1.122, -0.027$) ml/min/1.73 m²/year steeper in the higher C-megalin/Cr group than in the lower C-megalin/Cr group in model 1. In models adjusted with other urinary biomarkers, the slopes also showed a similar trend.

Table 2

Baseline characteristics of patients, divided into two groups by the cutoff point of urinary A-megalin

	Lower A-megalin/Cr	Higher A-megalin/Cr	<i>P</i> -value
Number (male/female)	87/54	17/30	0.002
Age (years)	67 (57, 75)	69 (60, 74)	0.556
BMI	25.8 ± 4.9	24.2 ± 4.9	0.062
SBP (mmHg)	130 (120, 138)	134 (122, 144)	0.076
eGFR (ml/min/1.73 m ²)	69.2 (52.2, 79.3)	72.9 (57.5, 88.0)	0.032
BUN (mg/dl)	16 (13, 20)	15 (13, 17)	0.091
S-Cr (mg/dl)	0.80 (0.67, 1.02)	0.67 (0.54, 0.82)	0.001
S-UA (mg/dl)	5.5 (4.5, 6.5)	5.1 (4.4, 6.0)	0.133
HbA1c (%)	7.2 (6.7, 7.8)	7.0 (6.5, 7.8)	0.716
U-A-megalin/Cr (pmol/g Cr)	133.08 (81.43, 165.36)	281.22 (241.96, 351.76)	<0.001
U-C-megalin/Cr (pmol/g Cr)	0.470 (0.215, 0.890)	0.570 (0.270, 1.120)	0.072
U-ACR (mg/g Cr)	38.00 (19.00, 144.00)	46.00 (22.00, 282.00)	0.182
U-α ₁ -MG/Cr (mg/g Cr)	9.5 (5.3, 17.4)	8.3 (5.2, 18.9)	0.650
U-β ₂ -MG/Cr (μg/g Cr)	161.0 (77.5, 585.0)	117.0 (60.0, 195.0)	0.025
U-NAG/Cr (IU/g Cr)	6.0 (3.9, 9.0)	10.6 (5.8, 14.3)	<0.001
Data are number, mean ± SD, or median (25th, 75th percentile). Welch's <i>t</i> -test was performed to compare BMI and eGFR. Chi-square test was performed to compare sex. Mann-Whitney's U test was performed for the other baseline parameters.			
BMI, body mass index; SBP, systolic blood pressure; eGFR, estimated glomerular filtration rate; BUN, blood urea nitrogen; S-Cr, serum creatinine; S-UA, serum uric acid; HbA1c, hemoglobin A1c; U-A-megalin, urinary A-megalin; U-C-megalin, urinary C-megalin; U-ACR, urinary albumin creatinine ratio; U-α ₁ -MG, urinary α ₁ -microglobulin; U-β ₂ -MG, urinary β ₂ -microglobulin; U-NAG, urinary N-acetyl-β-D-glucosaminidase.			

Table 3

Baseline characteristics of patients, divided into two groups by the cutoff point of urinary C-megalin

	Lower C-megalin/Cr	Higher C-megalin/Cr	P-value
Number (male/female)	63/52	41/32	0.853
Age (years)	66 (58, 74)	70 (59, 76)	0.077
BMI	25.8 ± 4.9	24.7 ± 4.9	0.090
SBP (mmHg)	130 ± 14	133 ± 16	0.135
eGFR (ml/min/1.73 m ²)	69.2 (57.4, 81.9)	70.2 (46.9, 84.6)	0.645
BUN (mg/dl)	15 (13, 18)	16 (13, 21)	0.116
S-Cr (mg/dl)	0.78 (0.65, 0.91)	0.80 (0.63, 1.15)	0.654
S-UA (mg/dl)	5.5 (4.5, 6.3)	5.2 (4.4, 6.3)	0.613
HbA1c (%)	7.4 ± 1.0	7.1 ± 0.9	0.133
U-A-megalin/Cr (pmol/g Cr)	145.29 (96.52, 209.60)	162.54 (106.90, 258.23)	0.048
U-C-megalin/Cr (pmol/g Cr)	0.300 (0.160, 0.430)	1.030 (0.835, 1.575)	<0.001
U-ACR (mg/g Cr)	30.20 (13.00, 81.00)	68.0 (28.50, 526.00)	<0.001
U-α ₁ -MG/Cr (mg/g Cr)	8.0 (3.8, 12.9)	12.7 (6.7, 22.4)	0.001
U-β ₂ -MG/Cr (μg/g Cr)	118.0 (69.0, 291.0)	198.0 (86.0, 783.5)	0.006
U-NAG/Cr (IU/g Cr)	6.0 (3.9, 8.8)	8.5 (5.0, 14.5)	<0.001
Data are number, mean ± SD, or median (25th, 75th percentile). Welch's <i>t</i> -test was performed to compare BMI, SBP, and HbA1c. Chi-square test was performed to compare sex. Mann-Whitney's U test was performed for the other baseline parameters.			
BMI, body mass index; SBP, systolic blood pressure; eGFR, estimated glomerular filtration rate; BUN, blood urea nitrogen; S-Cr, serum creatinine; S-UA, serum uric acid; HbA1c, hemoglobin A1c; U-A-megalin, urinary A-megalin; U-C-megalin, urinary C-megalin; U-ACR, urinary albumin creatinine ratio; U-α ₁ -MG, urinary α ₁ -microglobulin; U-β ₂ -MG, urinary β ₂ -microglobulin; U-NAG, urinary N-acetyl-β-D-glucosaminidase			

Table 4
Longitudinal analysis of the association of urinary A-megalin excretion with the eGFR slope

Model		β	P-value
Model 1	Year	-1.165 (-1.443, -0.886)	<0.001
	U–A-meg/Cr	-0.496 (-2.215, 1.223)	0.572
	Year* U–A-meg/Cr	-0.904 (-1.584, -0.224)	0.009
Model 2	Year	-1.076 (-1.379, -0.773)	<0.001
	U–A-meg/Cr	0.060 (-1.809, 1.930)	0.950
	Year* U–A-meg/Cr	-1.005 (-1.698, -0.312)	0.004
	ACR	-0.810 (-1.902, -0.282)	0.146
Model 3	Year	-1.091 (-1.384, -0.799)	<0.001
	U–A-meg/Cr	-0.096 (-1.880, 1.689)	0.916
	Year* U–A-meg/Cr	-0.990 (-1.678, -0.303)	0.005
	U– α_1 -MG/Cr	-0.030 (-0.067, 0.007)	0.114
Model 4	Year	-1.150 (-1.429, -0.870)	<0.001
	U–A-meg/Cr	-0.462 (-2.179, 1.254)	0.598
	Year* U–A-meg/Cr	-0.922 (-1.603, -0.242)	0.008
	U– β_2 -MG/Cr	0.000 (0.000, 0.000)	0.206
Model 5	Year	-1.091 (-1.395, -0.788)	<0.001
	U–A-meg/Cr	0.125 (-1.873, 2.123)	0.903
	Year* U–A-meg/Cr	-0.989 (-1.682, -0.295)	0.005
	U-NAG/Cr	-0.051 (-0.136, -0.033)	0.233
Model 1, crude; model 2, model 1 + U-ACR; model 3, model 1 + U– α_1 -MG; model 4, model 1 + U– β_2 -MG; model 5, model 1 + U–NAG			
U–A-meg, urinary A-megalin; Cr, U-ACR, urinary albumin creatinine ratio; creatinine; U– α_1 -MG, urinary α_1 -microglobulin; U– β_2 -MG, urinary β_2 -microglobulin; U–NAG, urinary N-acetyl- β -D-glucosaminidase; β , standard partial regression coefficient			

Table 5
Longitudinal analysis of the association of urinary C-megalin excretion with the eGFR slope

Model		β	P-value
Model 1	Year	-1.041 (-1.364, -0.718)	<0.001
	U-C-meg/Cr	-0.442 (-1.783, 0.899)	0.518
	Year* U-C-meg/Cr	-0.749 (-1.312, -0.186)	0.009
Model 2	Year	-0.970 (-1.312, -0.627)	<0.001
	U-C-meg/Cr	0.109 (-1.494, 1.712)	0.894
	Year* U-C-meg/Cr	-0.827 (-1.403, -0.250)	0.005
	ACR	-0.753 (-1.958, -0.452)	0.221
Model 3	Year	-0.988 (-1.321, -0.655)	<0.001
	U-C-meg/Cr	0.078 (-1.489, 1.645)	0.922
	Year* U-C-meg/Cr	-0.826 (-1.401, -0.251)	0.005
	U- α_1 -MG/Cr	-0.027 (-0.068, 0.015)	0.211
Model 4	Year	-1.037 (-1.360, -0.714)	<0.001
	U-C-meg/Cr	-0.293 (-1.673, 1.088)	0.678
	Year* U-C-meg/Cr	-0.768 (-1.332, -0.203)	0.008
	U- β_2 -MG/Cr	0.000 (0.000, 0.000)	0.377
Model 5	Year	-0.950 (-1.298, -0.602)	<0.001
	U-C-meg/Cr	0.237 (-1.418, 1.892)	0.779
	Year* U-C-meg/Cr	-0.860 (-1.444, -0.276)	0.004
	U-NAG/Cr	-0.062 (-0.151, 0.027)	0.171
Model 1, crude; model 2, model 1 + U-ACR; model 3, model 1 + U- α_1 -MG; model 4, model 1 + U- β_2 -MG; model 5, model 1 + U-NAG			
U-A-meg, urinary A-megalin; Cr, U-ACR, urinary albumin creatinine ratio; creatinine; U- α_1 -MG, urinary α_1 -microglobulin; U- β_2 -MG, urinary β_2 -microglobulin; U-NAG, urinary N-acetyl- β -D-glucosaminidase; β , standard partial regression coefficient			

Longitudinal analysis of the association of combined urinary megalin excretion with eGFR slopes

According to the calculated cutoff points, the 188 patients with type 2 diabetes were categorized into a higher urinary A- and C-megalin/Cr group (higher megalin/Cr group) and a group for the remaining

patients (lower megalin/Cr group). The baseline clinical characteristics of each group are shown in Table 6. Each eGFR slope analyzed from the generalized linear model is shown in Table 7 and Figure 1. The eGFR slope was -1.888 (95% CI $-2.764, -1.011$) ml/min/1.73 m²/year steeper in the higher megalin/Cr group than in the lower megalin/Cr group in model 1. In addition, the difference in the eGFR slope of the higher and lower megalin/Cr groups was greater than the differences between the higher and lower urinary A-megalin groups and between the higher and lower urinary C-megalin groups ($P = 0.008$). Moreover, in models adjusted with other urinary biomarkers, the slopes showed a similar trend. The 188 patients were also categorized into two groups according to the median urinary A- and C-megalin/Cr levels at baseline: a higher urinary A- and C-megalin/Cr group (higher megalin/Cr group) and a group for the remaining patients (lower megalin/Cr group). The characteristics of these groups are shown in Supplemental Table 7. Each eGFR slope analyzed from the generalized linear model is shown in Supplemental Table 8. The eGFR slope was -1.152 (95% CI $-1.756, -0.549$) ml/min/1.73 m²/year steeper in the higher megalin/Cr group than in the lower megalin/Cr group in model 1. Moreover, in models adjusted with other urinary biomarkers, the slopes showed a similar trend.

Table 6

Baseline characteristics of patients, divided into two groups by the cutoff point of urinary megalin

	Lower megalin/Cr	Higher megalin/Cr	<i>P</i> -value
Number (male/female)	87/54	17/30	0.002
Age (years)	67 (57, 75)	69 (60, 74)	0.556
BMI	25.8 ± 4.9	24.2 ± 4.9	0.062
SBP (mmHg)	130 (120, 138)	134 (122, 144)	0.076
eGFR (ml/min/1.73 m ²)	69.2 (52.2, 79.3)	72.9 (57.5, 88.0)	0.032
BUN (mg/dl)	16 (13, 20)	15 (13, 17)	0.091
S-Cr (mg/dl)	0.80 (0.67, 1.02)	0.67 (0.54, 0.82)	0.001
S-UA (mg/dl)	5.5 (4.5, 6.5)	5.1 (4.4, 6.0)	0.133
HbA1c (%)	7.2 (6.7, 7.8)	7.0 (6.5, 7.8)	0.716
U-A-megalin/Cr (pmol/g Cr)	133.08 (81.43, 165.36)	281.22 (241.96, 351.76)	<0.001
U-C-megalin/Cr (pmol/g Cr)	0.470 (0.215, 0.890)	0.570 (0.270, 1.120)	0.072
U-ACR (mg/g Cr)	38.00 (19.00, 144.00)	46.00 (22.00, 282.00)	0.182
U-α ₁ -MG/Cr (mg/g Cr)	9.5 (5.3, 17.4)	8.3 (5.2, 18.9)	0.650
U-β ₂ -MG/Cr (μg/g Cr)	161.0 (77.5, 585.0)	117.0 (60.0, 195.0)	0.025
U-NAG/Cr (IU/g Cr)	6.0 (3.9, 9.0)	10.6 (5.8, 14.3)	<0.001
Data are number, mean ± SD, or median (25th, 75th percentile). Welch's <i>t</i> -test was performed to compare BMI and eGFR. Chi-square test was performed to compare sex. Mann-Whitney's U test was performed for the other baseline parameters.			
BMI, body mass index; SBP, systolic blood pressure; eGFR, estimated glomerular filtration rate; BUN, blood urea nitrogen; S-Cr, serum creatinine; S-UA, serum uric acid; HbA1c, hemoglobin A1c; U-A-megalin, urinary A-megalin; U-C-megalin, urinary C-megalin; U-ACR, urinary albumin creatinine ratio; U-α ₁ -MG, urinary α ₁ -microglobulin; U-β ₂ -MG, urinary β ₂ -microglobulin; U-NAG, urinary N-acetyl-β-D-glucosaminidase			

Table 7

Longitudinal analysis of the association of urinary A- and C-megalin excretion with the eGFR slope

Model		β	P-value
Model 1	Year	-1.135 (-1.391, -0.878)	<0.001
	U-meg/Cr	-1.128 (-3.5, 1.243)	0.351
	Year* U-meg/Cr	-1.888 (-2.764, -1.011)	<0.001
Model 2	Year	-1.077 (-1.357, -0.796)	<0.001
	U-meg/Cr	-0.667 (-3.205, 1.871)	0.606
	Year* U-meg/Cr	-1.951 (-2.836, -1.065)	<0.001
	ACR	-0.531 (-1.581, -0.519)	0.321
Model 3	Year	-1.078 (-1.348, -0.808)	<0.001
	U-meg/Cr	-0.672 (-3.141, 1.797)	0.594
	Year* U-meg/Cr	-1.963 (-2.847, -1.080)	<0.001
	U- α_1 -MG/Cr	-0.024 (-0.060, 0.013)	0.201
Model 4	Year	-1.121 (-1.378, -0.864)	<0.001
	U-meg/Cr	-1.076 (-3.444, 1.292)	0.373
	Year* U-meg/Cr	-1.905 (-2.782, -1.028)	<0.001
	U- β_2 -MG/Cr	0.000 (0.000, 0.000)	0.203
Model 5	Year	-1.094 (-1.378, -0.81)	<0.001
	U-meg/Cr	-0.706 (-3.389, 1.978)	0.606
	Year* U-meg/Cr	-1.940 (-2.829, -1.05)	<0.001
	U-NAG/Cr	-0.027 (-0.107, 0.053)	0.508
Model 1, crude; model 2, model 1 + U-ACR; model 3, model 1 + U- α_1 -MG; model 4, model 1 + U- β_2 -MG; model 5, model 1 + U-NAG			
U-meg, urinary megalin; Cr, U-ACR, urinary albumin creatinine ratio; creatinine; U- α_1 -MG, urinary α_1 -microglobulin; U- β_2 -MG, urinary β_2 -microglobulin; U-NAG, urinary N-acetyl- β -D-glucosaminidase; β , standard partial regression coefficient			

Cross-sectional analysis of the association of urinary A- or C-megalin excretion with other parameters

Supplemental Table 9 shows the correlations of urinary A- and C-megalin excretion with urinary biomarkers and clinical parameters at baseline in the 188 patients with type 2 diabetes. Urinary A-megalin/Cr was correlated with eGFR ($r = 0.26$, $P = 0.001$) and U-NAG/Cr ($r = 0.19$, $P = 0.01$). Also, urinary

C-megalin/Cr was correlated with U-ACR ($r = 0.37$, $P < 0.001$), U- α_1 -MG/Cr ($r = 0.26$, $P < 0.001$), U- β_2 -MG/Cr ($r = 0.20$, $P = 0.005$), and U-NAG/Cr ($r = 0.29$, $P < 0.001$). Correlations between urinary biomarkers and other clinical parameters, excluding urinary megalin, are also shown in Supplemental Table 10.

Analysis of interactions between eGFR slopes of binary urinary A- or C- megalin excretion and other parameters

As shown in Supplemental Table 9, urinary A- and C-megalin excretion were correlated with some baseline parameters. Using these parameters, we applied a linear estimating equation to evaluate the interactions of eGFR slopes with binary urinary A- or C- megalin values. However, there were no three-way interactions among them, except urinary C-megalin and U- β_2 -MG/Cr, urinary C-megalin, and U-NAG/Cr (data not shown).

Discussion

In this study, we established a novel reducing method to measure urinary A-megalin. Furthermore, in longitudinal analysis, we demonstrated that the eGFR slope of patients with a higher urinary A-megalin level was steeper than that of patients with a lower urinary A-megalin level. Similar results were obtained with urinary C-megalin. In models adjusted with other urinary biomarkers (U-ACR, U- α_1 -MG/Cr, U- β_2 -MG/Cr, and U-NAG/Cr), the eGFR slopes also showed a similar trend. Therefore, these two forms of urinary megalin can predict a rapid eGFR decline independently of other urinary markers. The eGFR slope was also steeper in the higher urinary A- and C-megalin/Cr group (higher megalin/Cr group) than in the group consisting of the remaining patients (lower megalin/Cr group). Furthermore, the difference in the eGFR slope was greater than the differences between the higher and lower urinary A-megalin groups and between the higher and lower urinary C-megalin groups.

The mechanism underlying the excretion of urinary A-megalin has not been completely elucidated. However, we previously reported that the urinary excretion of A-megalin is decreased in children with oculo-cerebro-renal syndrome of Lowe (*OCRL*) gene mutations[17]. *OCRL*-1 is localized to clathrin-coated pits, early endosomes, and recycling endosomes in PTECs and has been implicated in endosome trafficking[25]. In Lowe syndrome and Dent-2 disease with *OCRL* gene mutations, *OCRL*-1-mediated megalin trafficking to the cell surface is impaired. As a result of the aberrant accumulation of actin at the endosomal membrane, megalin is retained in endosomes and missorted to lysosomes instead of being recycled to the brush border *via* recycling endosomes[17]. Subsequent dislocation of megalin at the cell surface may result in decreased urinary A-megalin excretion. However, in this study, the higher urinary A-megalin/Cr group had a steeper eGFR slope than the lower urinary A-megalin/Cr group. We hypothesize that metabolic overload to proximal tubules would lead to upregulation of intracellular recycling and extracellular cleavage of megalin. Increased urinary A-megalin may be used as a biomarker to predict the rapid progression of DKD.

We previously established that urinary C-megalin was elevated in DKD patients compared with normal controls[15]. In DKD patients, urinary megalin levels were also associated with serum vitamin D3 levels, which have been linked to CKD progression[26]. The clinical usefulness of urinary C-megalin is not limited to DKD because it is also associated with the severity of IgA nephropathy[27] and pediatric renal scarring[28], reflecting the metabolic load to residual nephrons in these diseases. Thus, cross-sectional analyses have revealed that urinary megalin is related to various kidney diseases. In CKD patients, urinary C-megalin/Cr is associated with urinary iron and 8-hydroxydeoxyguanosine, an oxidative stress marker[29]. Moreover, we reported that urinary C-megalin is excreted *via* exosomes, and its urinary excretion is positively related to the increase in urinary albumin excretion[30]. In addition, cultured immortalized rat proximal tubule cells exhibited an increase in excretion of exosomes with C-megalin from multivesicular bodies upon treatment with albumin and a further increase upon treatment with advanced glycation end product-modified albumin, both of which are endocytic ligands of megalin. These results indicate that increased urinary C-megalin excretion may be due to megalin-mediated quantitative or qualitative protein metabolic load to the endo-lysosomal system of PTECs, triggering the development of DKD[30]. We hypothesize that increased urinary C-megalin, which is excreted in response to metabolic overload, may also predict a rapid decline in DKD. Combined data on both urinary A- and C-megalin can therefore be expected to serve as a more powerful predictor of DKD progression.

In this study, cross-sectional analysis revealed a significant correlation between the baseline levels of urinary A-megalin and eGFR in our 188 patients with type 2 diabetes. However, there were no significant correlations of the baseline levels of urinary A-megalin with albumin, α_1 -MG, β_2 -MG, or NAG. The baseline levels of urinary C-megalin were significantly correlated with other urinary biomarkers, as we reported previously[15]. Although some studies reported urinary megalin without distinguishing between A-megalin and C-megalin[26, 31], our results suggest that A- and C-megalin are excreted in urine *via* different mechanisms. Furthermore, our study showed that the eGFR slope of the group with higher urinary A- and C-megalin/Cr was steeper than those of the groups with higher urinary A- or C-megalin, as shown in Figure 1. Therefore, it might be important to measure urinary A-megalin and C-megalin independently to detect the risk of a more rapid progression of DKD.

As mentioned above, none of our study patients was treated with SGLT2 inhibitors at baseline or during the follow-up period. Because SGLT2 is specifically located in proximal tubules, the inhibitors may also affect the function of other molecules expressed in the tubules. Indeed, β_2 -MG, an endocytic ligand of megalin, was reported to be increased in the urine of type 2 diabetes patients who were administered ipragliflozin, an SGLT2 inhibitor[32]. In addition, luseogliflozin was found to ameliorate renal pathology but increase proteinuria in a rat model of diabetic nephropathy[33]. Indeed, SGLT2 inhibitors may alter the endocytic function of megalin[34]. Therefore, we may need to carefully evaluate urinary biomarkers in patients treated with SGLT2 inhibitors. Furthermore, use of SGLT2 inhibitors led to an initial drop in eGFR but eventually ameliorated the eGFR slope in type 2 diabetes patients[23]. Thus, in the present study, which investigated the eGFR slope in a rather short period, we analyzed the clinical data of patients who

were not treated with SGLT2 inhibitors. However, in an ongoing trial, we are investigating the relationship between the SGLT2 inhibitor-induced changes in eGFR and urinary megalin excretion (unpublished).

There are some limitations to this study. First, this was a retrospective study of a small cohort from a single hospital. Moreover, the duration of the study was relatively short, and no patients progressed to end-stage renal disease and required renal replacement therapy. A confirmed 30% or 40% decline in eGFR has been an acceptable surrogate endpoint in clinical trials under some circumstances. However, these endpoints may not be practical in the relatively early stages of CKD[18]. Hence, we used eGFR slopes as outcome parameters. To solve these limitations, we plan to use a prospective, large, multicenter and longer-term cohort including patients with a lower eGFR to clarify the usefulness of urinary megalin in predicting DKD progression using an outcome defined as progression to end-stage renal disease. Second, we could not analyze the cutoff point of urinary megalin with a validation cohort but used a development cohort. Furthermore, this cohort was retrospectively analyzed using urine samples collected between 2007 and 2011 in order to analyze clinical data from patients who were not taking SGLT2 inhibitors and to exclude the effects of these drugs on urinary megalin excretion and eGFR decline.

Conclusion

Urinary A-megalin was measured by a novel and simpler method than the one that we reported previously. Urinary A-megalin and C-megalin could be novel biomarkers that predict the eGFR decline in type 2 diabetes patients independently of other urinary markers, including albumin, α_1 -MG, β_2 -MG, and NAG.

Abbreviations

DKD, diabetic kidney disease; SGLT2, sodium glucose cotransporter 2; eGFR, estimated glomerular filtration rate; ROC, receiver operating characteristic; CI, confidence interval; PTECs, proximal tubular epithelial cells; α_1 -MG, α_1 -microglobulin; β_2 -MG, β_2 -microglobulin; NAG, N-acetyl- β -D-glucosaminidase; GEE, generalized estimating equation; ACR, albumin creatine ratio; OCRL, oculo-cerebro-renal syndrome of Lowe

Declarations

Ethics approval and consent to participate

This study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines and was approved by the Niigata University Ethical Committee (approval nos. 2590 and 2591 in 2016).

Consent for publication

The participants provided informed consent and their anonymity was preserved.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

A.S., and S.G. received research grants from Denka Co., Ltd.

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Author contributions

T.I., M.H., R.K., Y.S., I.N., and A.S. were responsible for the conception and design of the study. T.I. was the chief investigator and was responsible for data analysis. M.H., K.K., S.G., H.K., T.T., and N.K. were responsible for data analysis. T.I., M.H., Y.S., and A.S. were responsible for data acquisition. M.N., S.I., and S.O. were responsible for data measurement. T.I., M.H., H.K., T.T., N.K., and A.S. were responsible for data interpretation. T.I. and M.H. were responsible for drafting the manuscript. All authors contributed to the writing of the final manuscript.

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Figures

Figure 1

Estimated glomerular filtration rate (eGFR) decline at each urinary megalin/Cr level Each plot shows the mean eGFR of each visit. The error bars show the standard error. The table below each graph shows the participant numbers for each visit. (a) eGFR transition plotted by urinary A-megalin/Cr level divided by the cutoff point. The blue line shows the lower urinary A-megalin/Cr group. The red line shows the higher urinary A-megalin/Cr group. (b) eGFR transition plotted by urinary C-megalin/Cr level divided by the cutoff point. The blue line shows the lower urinary C-megalin/Cr group. The red line shows the higher urinary C-megalin/Cr group. (c) eGFR transition plotted by urinary megalin/Cr level divided by the cutoff point. The blue line shows the lower urinary megalin/Cr group. The red line shows the higher urinary megalin/Cr group. Cr, creatinine; H, higher urinary A-megalin/Cr group or C-megalin/Cr group or urinary megalin group; L, lower urinary A-megalin/Cr group or C-megalin/Cr group or urinary megalin group.

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