Urinary Cystatin C and Tubular Proteinuria Predict Progression of Diabetic Nephropathy

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OBJECTIVE—The aim of this study was to evaluate the association of urinary cystatin C, a tubular damage marker, with the progression of type 2 diabetic nephropathy.

RESERCH DESIGN AND METHODS—The baseline values of serum and urinary cystatin C were measured as primary parameters and those of urinary nonalbumin protein (NAP) were measured as secondary parameters. In this prospective observational study, a total of 237 type 2 diabetic patients were followed up for 29 months (13–44 months).

RESULTS—Both the urinary cystatin C-to-creatinine ratio (CCR) and NAP-to-creatinine ratio (NAPCR) were significantly different according to the degree of albuminuria. Both markers had strongly positive correlations at baseline. After adjusting for several clinical factors, both urinary CCR and NAPCR had significant associations with the decline of the estimated glomerular filtration rate (eGFR) ($r = 0.160$, $P = 0.021$; $r = 0.412$, $P < 0.001$, respectively). Urinary CCR had positive correlations with the decline of eGFR in the subpopulation of patients with eGFR ≥ 60 mL/min/1.73 m^2. In patients with eGFR ≥ 60 mL/min/1.73 m^2 and normoalbuminuria, only urinary NAPCR showed a significant association with the decline of eGFR; urinary CCR did not. In multivariate regression analysis, the number of patients who progressed to chronic kidney disease stage 3 or greater was higher in those in the upper tertiles of both the urinary levels of cystatin C and NAP than in those in the lower tertiles.

CONCLUSIONS—The results of this study suggest that urinary cystatin C and NAP may be predictors of the progression of type 2 diabetic nephropathy.

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D iabetic nephropathy is a complication with high morbidity and mortality as well as a major cause of end-stage renal disease. Although glomerular dysfunction is thought to be a major factor for the development and progression of diabetic nephropathy, tubulointerstitial damage may also play an important role in the pathogenesis of diabetic nephropathy (1–3). Recently, several studies have shown that some tubular damage markers have clinical implications as biomarkers for the development and progression of diabetic nephropathy (4–10).

Cystatin C is a 13-kDa cysteine proteinase inhibitor and is produced by all nucleated cells at a constant rate (11). In healthy subjects, cystatin C is almost freely filtered by the renal glomeruli and almost entirely reabsorbed in the proximal tubule like other low molecular weight proteins; there is no tubular secretion of cystatin C (12–14). Similar to the serum cystatin C, the urinary cystatin C level is not affected by age or muscle mass in healthy subjects or in proteinuric patients without renal tubular damage (15). On the other hand, increased urinary cystatin C has been recognized as a marker of renal tubular dysfunction (16,17). In addition, urinary leakage of proteins other than albumin (nonalbumin protein [NAP]) can also indicate tubular damage rather than glomerular damage (18).

The aims of this study were to evaluate the impact of urinary cystatin C on the progression of type 2 diabetic nephropathy and to determine whether urinary cystatin C has an association with the decline of the glomerular filtration rate (GFR) in type 2 diabetic patients. In addition, we also evaluated whether urinary NAP has any correlation with urinary cystatin C or has any effect on the decline in GFR.

RESEARCH DESIGN AND METHODS

Patients

This was a prospective observational study of patients attending the Department of Endocrinology at Pusan National University Hospital. The study was conducted with the approval of the Institutional Review Board of Pusan National University Hospital. A total of 264 Korean type 2 diabetic patients were consecutively enrolled at the outpatient clinics between May 2008 and December 2009. All patients fulfilled the following inclusion criteria: age ≥ 18 years and estimated GFR (eGFR) ≥ 30 mL/min/1.73 m^2. We excluded patients with thyroid disorders or who had been medicated within 6 months prior to the study because thyroid function could affect the cystatin C level (19). Additional exclusion criteria were *1*) active urinary tract infection, *2*) renal disease other than diabetic nephropathy, *3*) neoplastic disorders, *4*) severe liver dysfunction, *5*) active or chronic infection or inflammatory disorders, *6*) pregnancy, and *7*) a recent (within 6 months) history of acute myocardial infarction, stroke, or occlusive peripheral vascular disease.

A random spot urine sample and a blood sample were obtained from each

patient at the clinic visit. Medical histories and anthropometric measurements were also recorded the same day. The eGFR level was calculated using the Modification of Diet in Renal Disease (MDRD) Study formula for the Korean population: MDRD = $107.904 \times$ (serum creatinine $[mg/dL])^{-1.009} \times age^{-0.02}$ (20). A correction factor of 0.667 was used for women. The serum and urinary cystatin C levels were measured by the latex agglutination test (Modular P800; Roche Diagnostics, Mannhein, Germany). The interassay and intra-assay coefficients of variations of cystatin C in our laboratory were as follows: <4.2 and <3.4%, respectively, for serum and <7.9 and <10.1%, respectively, for urine. Urine specimens with cystatin C levels <0.01 mg/L were assumed to have a concentration of 0.01 mg/L. The data on urinary cystatin C was also expressed as ratios of urinary cystatin C to urinary creatinine in order to assess different hydration states and renal functions of the patients. The ratio between urine mass concentrations of cystatin C and creatinine in micrograms per millimole was calculated and designated as the urinary cystatin C-to-creatinine ratio (CCR). Since we obtained total proteinuria and albuminuria values from each patient at baseline, we were able to estimate the amount of nonalbumin proteinuria through the following calculation: NAP-to-creatinine ratio (NAPCR) = protein-to-creatinine ratio (PCR) – albumin-to-creatinine ratio (ACR). The lowest detectable level and the coefficient of variation in our laboratory were as follows: for total proteinuria, 0.7 mg/dL and <4.8%, respectively; for albuminuria, 0.2 mg/dL and <7.4%, respectively.

The patients were followed up at our clinic until March 2012. They were managed to give priority to the best treatment according to standard guidelines at each outpatient clinic of two endocrinologists (I.J.K. and S.S.K.). Thirteen patients were excluded during follow-up as follows: four patients died of other causes, six patients were hospitalized for acute myocardial infarction and active infections, such as pneumonia, and three patients were diagnosed with additional malignancies during the follow-up period. Finally, a total of 237 patients with type 2 diabetes were enrolled for this study. The two tubular damage markers were measured at intervals of 12 ± 1 (mean $\pm$ SD) months at the outpatient clinic during the follow-up period. Serum creatinine was routinely measured for the estimation of

GFR at intervals of 6 ± 1 (mean $\pm$ SD) months during the follow-up period using the same methods. The latest eGFR calculations were used in the assessment of the annual decline in eGFR. Chronic kidney disease (CKD) stage 3 or greater was defined as having eGFR <60 mL/min/1.73 m² in two consecutive measurements from the last follow-up visit.

Statistical analysis

All statistical analyses were performed using SPSS version 15.0 (SPSS Inc., Chicago, IL). The data are presented as mean $\pm$ SD for normally distributed variables and the medians (interquartile range) for nonnormally distributed variables. The distribution of continuous variables was examined for skewness and kurtosis, and logarithm-transformed values of nonnormally distributed variables were used for analysis. Differences between the groups were analyzed by ANOVA, followed by Bonferroni test. Categorical variables are reported as frequencies and proportions. Pearson χ^2 test was used to analyze the categorical data as appropriate. Pearson correlation coefficient was used to test the correlations between individual variables. We conducted multivariate regression analyses with the annual rates of decline in eGFR as dependent variables and urinary CCR and NAPCR as independent variables, respectively. Several models were gradually built to adjust for confounding factors. Multivariate logistic analysis, using a backward procedure on the basis of likelihood ratios, was conducted to determine whether both tubular markers would be predictive factors for CKD stage 3 or greater. The odds ratios (ORs) of urinary levels of cystatin C and NAP were calculated with reference to the lowest tertile of each variable. A P value of <0.05 derived from the two-tailed Student t test was considered statistically significant.

RESULTS

Baseline patient characteristics
The baseline characteristics of the patients are shown in Table 1. The mean age of the patients was 58.5 ± 11.1 years (range, 18–80 years), and there were 115 males and 122 females. The patients were categorized into three groups according to ACR: those with ACR <30 mg/g creatinine (normoalbuminuria group, $n = 149$), those with ACR 30–299 mg/g creatinine (microalbuminuria group, $n = 58$), and

those with ACR $\geq$300 mg/g creatinine (macroalbuminuria group, $n = 30$). Age, duration of diabetes, systolic blood pressure (SBP), HbA$_{1c}$, HDL cholesterol, and triglycerides were significantly different between the groups. Estimated GFR tended to decrease with increasing degrees of albuminuria (P value for trend <0.001). More antihypertensive and lipid-lowering agents were administered in the macroalbuminuria group than in the normo- and microalbuminuria groups.

The urinary cystatin C level and CCR were significantly higher in the macroalbuminuria group than in the normoalbuminuria and microalbuminuria groups (both $P < 0.001$), whereas they were not significantly different between the normoalbuminuria and microalbuminuria groups (Table 1). Urinary NAPCR was significantly higher in the macroalbuminuria group than in the normo- and microalbuminuria groups (both $P < 0.001$), and they were also significantly different between the normo- and microalbuminuria groups ($P < 0.001$). Urinary ACR positively correlated with urinary CCR ($r = 0.450$, $P < 0.001$) and urinary NAPCR ($r = 0.699$, $P < 0.001$). In addition, at baseline, urinary CCR and NAPCR, both markers of tubular damage, positively correlated with each other ($r = 0.597$, $P < 0.001$).

Associations of the annual eGFR decline with urinary CCR and NAPCR
The median follow-up period was 29.0 months (range, 13.0–44.0 months). There was a median annual decline in eGFR of 1.31 mL/min/1.73 m²/year during the follow-up period. The values of annual eGFR decline significantly correlated with both baseline urinary CCR ($r = 0.272$, $P < 0.001$) (Fig. 1A) and NAPCR ($r = 0.361$, $P < 0.001$) (Fig. 1B) in univariate regression analysis (Supplementary Table 2). After adjusting for age and significant clinical factors affecting the decline of eGFR, both urinary CCR and NAPCR remained significantly associated with a decline in eGFR (Table 2). After additionally adjusting for baseline eGFR and serum cystatin C (only analysis for urinary CCR), both urinary CCR and NAPCR remained significantly associated with the annual decline in eGFR in the final model ($r = 0.160$, $P = 0.021$; $r = 0.412$, $P < 0.001$, respectively).

To test whether urinary CCR and NAPCR might have prognostic values in early diabetic nephropathy, we analyzed

Table 1—*Baseline characteristics of metabolic and laboratory parameters in patients with type 2 diabetes*

	Normoalbuminuria (n = 149)	Microalbuminuria (n = 58)	Macroalbuminuria (n = 30)	P value
Sex, male/female	66/83	31/27	18/12	0.201
Age, years	57.6 ± 11.7	58.6 ± 10.8	63.1 ± 7.0†	0.043
BMI, kg/m^2	24.3 ± 2.9	24.4 ± 3.1	23.9 ± 3.3	0.770
Duration of diabetes, years	5.4 ± 3.7	7.9 ± 4.8†	9.6 ± 6.7‡||	0.002
SBP, mmHg	122 ± 13	124 ± 16	129 ± 25†	0.049
DBP, mmHg	74 ± 9	73 ± 12	74 ± 13	0.969
HbA$_{1c}$, %	7.4 ± 1.3	7.6 ± 1.3	8.1 ± 1.7†	0.028
Total cholesterol, mg/dL	174.4 ± 40.4	171.9 ± 45.4	162.6 ± 34.8	0.353
LDL cholesterol, mg/dL	94.9 ± 28.9	91.8 ± 36.0	85.7 ± 22.2	0.299
HDL cholesterol, mg/dL	47.4 ± 12.3	42.6 ± 12.1†	44.2 ± 14.2	0.035
Triglyceride, mg/dL*	122.0 (84.0–163.0)	170.0 (115.5–246.5)§	143.0 (93.8–201.5)	<0.001
C-reactive protein, mg/dL*	0.06 (0.03–0.16)	0.06 (0.03–0.12)	0.06 (0.03–0.15)	0.839
Serum creatinine, mg/dL	0.87 ± 0.21	0.98 ± 0.24†	1.41 ± 0.51§||	<0.001
eGFR, mL/min/1.73 m^2	96.5 ± 20.2	88.2 ± 17.8†	66.3 ± 19.5||	<0.001
Serum cystatin C, mg/L	0.86 ± 0.25	1.00 ± 0.29†	1.57 ± 0.55§||	<0.001
Urine albumin, mg/dL*	0.9 (0.6–1.5)	5.0 (3.0–9.6)§	88.5 (46.2–121.3)§||	<0.001
Urine ACR, mg/g*	11 (7–17)	56 (38–109)§	962 (502–1737)§||	<0.001
Urine cystatin C, mg/L*	0.05 (0.03–0.08)	0.04 (0.02–0.09)	0.13 (0.06–0.56)§||	<0.001
Urine CCR, µg/mmol*	6.9 (4.2–10.2)	6.2 (3.3–10.0)	26.8 (6.2–85.1)§||	<0.001
Urine NAP, mg/dL*	7.7 (5.3–11.4)	10.2 (6.3–14.3)	42.2 (26.7–133.7)§||	<0.001
Urine NAPCR, mg/g*	89 (66–126)	131 (92–173)†	456 (238–2173)§||	<0.001
Antihypertensive medication, n (%)	39 (26.2)	32 (55.2)§	28 (93.3)§||	<0.001
Lipid-lowering agent, n (%)	87 (58.4)	37 (63.8)	25 (83.3)†	0.035

Data are expressed as mean ± SD for parametric variables and median (interquartile range) for nonparametric variables. DBP, diastolic blood pressure. *Logarithm-transformed values were used for comparison. †*P* < 0.05 vs. normoalbuminuria. ‡*P* < 0.05 vs. microalbuminuria. §*P* < 0.001 vs. normoalbuminuria. ||*P* < 0.001 vs. microalbuminuria.

patients with baseline eGFR ≥60 mL/min/1.73 m^2 or with normoalbuminuria. In patients with eGFR ≥60 mL/min/1.73 m^2, both urinary CCR and NAPCR showed positive correlations with a decline in eGFR in the final model after adjusting for several clinical parameters (*r* = 0.144, *P* = 0.031; *r* = 0.282, *P* = 0.001,

respectively). In the normoalbuminuria group, the urinary NAPCR remained significantly associated with a decline in eGFR (*r* = 0.261, *P* = 0.002) in the final model, whereas urinary CCR did not (*r* = 0.097, *P* = 0.199). In addition, urinary NAPCR showed a significant association with a decline in eGFR (*r* = 0.201,

P = 0.018) in patients with both eGFR ≥60 mL/min/1.73 m^2 and normoalbuminuria.

Urinary CCR and NAPCR as predictive factors for the development of CKD stage 3 or greater
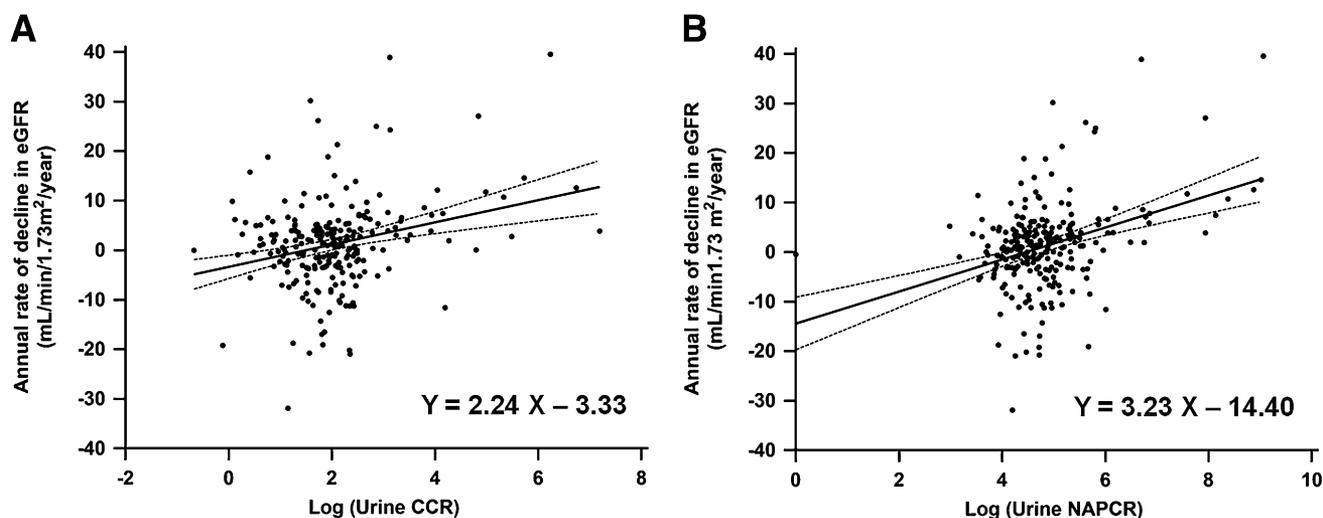In univariate logistic regression analysis, the number of patients who progressed to

Figure 1—*Single regression analysis of the annual rate of the decline in eGFR by using urinary CCR (A) and NAPCR (B). Logarithm-transformed values of urinary CCR and NAPCR were used for analysis.*

Table 2—*Multiple regression analysis of the annual rate of decline in eGFR as a dependent variable*

Urine CCR

Model	All (n = 237) Standard β	P value	eGFR ≥60 mL/min/1.73 m² (n = 218) Standard β	P value	Normoalbuminuria (n = 149) Standard β	P value	eGFR ≥60 mL/min/1.73 m² and normoalbuminuria (n = 144) Standard β	P value
1	0.272	<0.001	0.170	0.012	0.114	0.166	0.083	0.321
2	0.254	<0.001	0.169	0.014	0.137	0.087	0.083	0.310
3	0.260	<0.001	0.172	0.013	0.146	0.072	0.090	0.272
4	0.160	0.021	0.144	0.031	0.097	0.199	0.084	0.292

Urine NAPCR

Model	All (n = 237) Standard β	P value	eGFR ≥ 60 mL/min/1.73 m² (n = 218) Standard β	P value	Normoalbuminuria (n = 149) Standard β	P value	eGFR ≥60 mL/min/1.73 m² and normoalbuminuria (n = 144) Standard β	P value
1	0.361	<0.001	0.262	<0.001	0.215	0.009	0.176	0.035
2	0.413	<0.001	0.286	0.001	0.261	0.002	0.202	0.017
3	0.412	<0.001	0.282	0.001	0.261	0.002	0.201	0.018

Model 1, crude; model 2, adjusted for age and significant clinical parameters including HbA$_{1c}$, SBP, uric acid, urine ACR, and baseline eGFR; model 3, adjusted for use of RAS inhibitors and lipid-lowering agents; model 4, adjusted for serum cystatin C.

CKD stage 3 or greater (eGFR <60 mL/min/1.73 m²) was higher in those in the upper tertiles of both the urinary levels of cystatin C and NAP than in those in the lower tertiles (OR 7.26 for urinary cystatin C; 48.71 for NAPCR; P < 0.001 in both) (Table 3). The increased levels of both urinary CCR and NAPCR remained significantly associated with progression to CKD stage 3 or greater after adjusting for several clinical factors in each multivariate model.

CONCLUSIONS—In this study, urinary cystatin C and NAP, both clinical tubular damage markers, positively correlated with each other at baseline. Both markers were significantly associated with the annual decline in eGFR in type 2 diabetic nephropathy. In particular, both tubular damage makers affected a decline in eGFR at the early stage of nephropathy in type 2 diabetic patients (eGFR ≥60 mL/min/1.73 m²). Urinary NAP affected eGFR decline in patients with both eGFR ≥60 mL/min/1.73 m² and normoalbuminuria, although urinary cystatin C did not reach statistical significance. In addition, the increased levels of the two markers were also associated with the progression of CKD stage 3 or greater at the last follow-up.

In general, unlike healthy subjects, diabetic patients are continuously exposed to the various metabolic and hemodynamic risks associated with this disease (21). Recent studies have mainly focused on tubular damage, which is known to correlate with acute kidney injury in patients with diabetic nephropathy (4–10). Some cross-sectional studies have reported that several tubular markers increase more in diabetic patients than in healthy controls, and this correlated with the severity of albuminuria (4–8). In our study, NAP correlated with the severity of baseline albuminuria, which is consistent with the results of previous studies. However, urinary cystatin C mainly increased in the macroalbuminuria group and was not significantly different between the microalbuminuria and normoalbuminuria groups.

Microalbuminuria has generally been considered the earliest marker for the development of diabetic nephropathy in clinical settings (22). Microalbuminuria is diagnosed when significant glomerular damage has occurred (23) and does not necessarily lead to renal impairment because nephropathy sometimes occurs in the normoalbuminuric patients (24,25). In our study, although urinary cystatin C was not associated with a decline in eGFR in normoalbuminuric patients, urinary NAP was significantly associated with a decline in eGFR independent of baseline albuminuria and eGFR. It is necessary to assess tubular damage independent of albuminuria in patients with early development and progression of diabetic nephropathy because tubular damage may play a significant role in the normoalbuminuric renal insufficiency.

Serum cystatin C is the most valid marker to estimate the GFR, rather than serum creatinine, and to predict progression of renal dysfunction (26). An increase in urinary cystatin C, independent of serum cystatin C, is suggestive of renal tubular damage (13). In our study, urinary cystatin C was a predictor of renal impairment independent of serum cystatin C. Thus, urinary cystatin C also plays some role in predicting renal decline independent of serum cystatin C, although serum cystatin C itself, an indicator for the estimation of GFR, is very important for predicting renal decline.

NAPs include α-1 microglobulin, β-2 macroglobulin, IgG, cystatin C, transferrin nephrin, matrix metalloproteinase-9, and tissue inhibitor of metalloproteinases-1 (27). It is well known that each NAP is related to renal damage in various chronic renal diseases, including diabetic nephropathy (28). Because there is little evidence to support the role of NAP in diabetic nephropathy, it is worthwhile to investigate this issue. Interestingly, in our type 2 diabetic patients, NAP has been shown to be a stronger predictor for renal impairment than urinary cystatin C. At normal levels of protein loss, albumin is a minor component of total urinary protein, although albumin becomes the most significant single protein present as protein loss increases (29). This supports our result that indicates the clinical value of NAP in the prediction of renal impairment in normoalbuminuric patients.

A recent study on tubular damage markers in acute kidney injury has demonstrated

Table 3—*Logistic regression of development of CKD stage 3 or greater (eGFR <60 mL/min/1.73 m^2) at last follow-up*

Variable	Univariate analysis		Multivariate analysis* with urine CCR		Multivariate analysis* with urine NAPCR	
	OR	95% CI (*P* value)	OR	95% CI (*P* value)	OR	95% CI (*P* value)
Age, years	1.08	1.03–1.13 (0.002)	—	—	—	—
SBP, mmHg	1.03	1.01–1.05 (0.012)	—	—	—	—
HbA$_{1c}$, %	1.32	1.05–1.66 (0.018)	1.64	1.09–2.46 (0.018)	—	—
Uric acid, mg/dL	1.99	1.53–2.59 (<0.001)	2.70	1.51–4.82 (0.001)	1.69	1.07–2.68 (0.026)
Baseline eGFR, mL/min/1.73 m^2	0.86	0.83–0.91 (<0.001)	0.86	0.84–0.93 (<0.001)	0.87	0.82–0.93 (<0.001)
RAS inhibitor use, yes	27.22	3.64–203.38 (0.001)	15.67	0.75–327.42 (0.076)	33.68	1.92–590.97 (0.016)
Lipid-lowering agent use, yes	1.28	0.57–2.86 (0.548)	—	—	—	—
Urine ACR group						
Normoalbuminuria	1	Ref.	1	Ref.	—	—
Microalbuminuria	3.27	1.05– 10.19 (0.041)	2.46	0.42–14.36 (0.316)	—	—
Macroalbuminuria	55.61	17.97–172.15 (<0.001)	13.81	2.02–94.35 (0.007)	—	—
Urine CCR tertile						
1st tertile	1	Ref.	1	Ref.	—	—
2nd tertile	0.58	0.14–2.53 (0.473)	0.40	0.05–3.12 (0.380)	—	—
3rd tertile	7.26	2.62–20.14 (<0.001)	6.85	1.19–39.40 (0.031)	—	—
Urine NAPCR tertile						
1st tertile	1	Ref.	—	—	1	Ref.
2nd tertile	2.00	0.18–22.52 (0.575)	—	—	4.08	0.27–61.15 (0.309)
3rd tertile	48.71	6.44–368.43 (<0.001)	—	—	29.35	2.93–294.14 (0.004)

Ref., reference population group. *Multivariate models using the backward likelihood method were adjusted for age, SBP, HbA$_{1c}$, uric acid, baseline eGFR, urine ACR, RAS inhibitor use, lipid-lowering agent use, and urine CCR and NAPCR.

that unnormalized values are also useful for predicting ongoing injury (30). Thus, we analyzed unnormalized values of the two aforementioned markers. The unnormalized values had similar patterns as the normalized values in baseline characteristics and univariate analysis results (Table 1 and Supplementary Table 2). The statistical significance of unnormalized values was lost in multivariate analysis, whereas that of normalized values was not (data not shown).

Interestingly, serum uric acid was found to be associated with renal decline in the most analyzed model in this study and has recently been shown to be related to diabetic nephropathy (31,32). Uric acid might induce renal microvascular disease independent of blood pressure through stimulation of the renin-angiotensin system (RAS) and inhibition of endothelial nitric oxide (33). In addition, lowering therapy in a diabetic animal model significantly reduced albuminuria and ameliorated tubulointerstitial inflammation, suggesting a role for uric acid in diabetic nephropathy (34).

The results of this study are subject to some limitations. First, the follow-up period of this study was relatively short, even though the development and progression of diabetic nephropathy require a longer time frame. However, it is considered that the occurrence of tubular damage, as

expressed by biomarkers, could be a significant predictor for relatively rapid decline in renal function during a short period of time. Second, we measured the urinary levels of cystatin C, albumin, and protein with single random spot urine samples, although urine samples were collected at the outpatient clinic from patients without illness or renal diseases other than diabetic nephropathy. Despite these limitations, it is noteworthy that the two aforementioned tubular damage markers can easily be checked and used to assess the development and progression of diabetic nephropathy in clinical settings. Third, although there have been a few studies reporting that urinary cystatin C and NAP mainly increase due to tubular rather than glomerular damage, it is unclear whether urinary cystatin C and NAP originate from tubular damage alone in clinical conditions involving both tubular and glomerular damage, such as diabetic nephropathy. Further studies are needed to investigate whether both urinary cystatin C and NAP are biomarkers of tubular damage in clinical conditions with a massive glomerular protein load, such as diabetic nephropathy.

In conclusion, it is suggested that urinary cystatin C and NAP, along with albuminuria, may be sensitive and specific markers for predicting renal impairment in type 2 diabetic patients and may help to further elucidate the role of

tubular damage in the pathophysiological mechanisms of the development and progression of type 2 diabetic nephropathy.

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