Identification of Novel Urinary Biomarkers for Predicting Renal Prognosis in Patients With Type 2 Diabetes by Glycan Profiling in a Multicenter Prospective Cohort Study: U-CARE Study 1

Koki Mise,[1] Mariko Imamura,[1] Satoshi Yamaguchi,[1] Sanae Teshigawara,[1] Atsuhito Tone,[2] Haruhito A. Uchida,[3] Jun Eguchi,[1] Atsuko Nakatsuka,[1] Daisuke Ogawa,[1] Michihiro Yoshida,[4] Masao Yamada,[5] Kenichi Shikata,[6] and Jun Wada[1]

Diabetes Care 2018;41:1765–1775 | https://doi.org/10.2337/dc18-0030

OBJECTIVE

Because quantifying glycans with complex structures is technically challenging, little is known about the association of glycosylation profiles with the renal prognosis in diabetic kidney disease (DKD).

RESEARCH DESIGN AND METHODS

In 675 patients with type 2 diabetes, we assessed the baseline urinary glycan signals binding to 45 lectins with different specificities. The end point was a decrease of estimated glomerular filtration rate (eGFR) by ≥30% from baseline or dialysis for end-stage renal disease.

RESULTS

During a median follow-up of 4.0 years, 63 patients reached the end point. Cox proportional hazards analysis revealed that urinary levels of glycans binding to six lectins were significantly associated with the outcome after adjustment for known indicators of DKD, although these urinary glycans, except that for DBA, were highly correlated with baseline albuminuria and eGFR. Hazard ratios for these lectins were (+1 SD for the glycan index) as follows: SNA (recognizing glycan Siaα2-6Gal/GalNAc), 1.42 (95% CI 1.14–1.76); RCA120 (Galβ4GlcNAc), 1.28 (1.01–1.64); DBA (GalNAcα3GalNAc), 0.80 (0.64–0.997); ABA (Galβ3GalNAc), 1.29 (1.02–1.64); Jacalin (Galβ3GalNAc), 1.30 (1.02–1.67); and ACA (Galβ3GalNAc), 1.32 (1.04–1.67). Adding these glycan indexes to a model containing known indicators of progression improved prediction of the outcome (net reclassification improvement increased by 0.51 [0.22–0.80], relative integrated discrimination improvement increased by 0.18 [0.01–0.35], and the Akaike information criterion decreased from 296 to 287).

CONCLUSIONS

The urinary glycan profile identified in this study may be useful for predicting renal prognosis in patients with type 2 diabetes. Additional investigation of glycosylation changes and urinary glycan excretion in DKD is needed.

[1]Department of Nephrology, Rheumatology, Endocrinology and Metabolism, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan
[2]Diabetes Center, Okayama University Hospital, Okayama, Japan
[3]Department of Chronic Kidney Disease and Cardiovascular Disease, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan
[4]Center for Innovative Clinical Medicine, Okayama University Hospital, Okayama, Japan
[5]GlycoTechnica Ltd., Yokohama, Japan
[6]Division of Clinical Research of New Drugs and Therapeutics, Center for Innovative Clinical Medicine, Okayama University Hospital, Okayama, Japan

Corresponding author: Koki Mise, kokims-frz@okayama-u.ac.jp, or Jun Wada, junwada@okayama-u.ac.jp.

Received 6 January 2018 and accepted 14 May 2018.

Clinical trial reg. no. UMIN000011525, www.umin.ac.jp/ctr.

This article contains Supplementary Data online at http://care.diabetesjournals.org/lookup/suppl/doi:10.2337/dc18-0030/-/DC1.

Several biomarkers have been demonstrated to predict renal prognosis in patients with diabetes. Blood levels of tumor necrosis factor receptors 1 and 2 are well-known prognostic indicators for diabetic kidney disease (DKD) (1,2). Similarly, markers of tubulointerstitial injury, inflammation, and filtration have been reported to predict the progression of DKD (3–6). These biomarkers partly allow us to predict renal prognosis at an early stage of DKD independently of established clinical factors, such as the estimated glomerular filtration rate (eGFR) and albuminuria. However, despite early detection of high-risk patients and recent new protective treatments for DKD (7–9), the management of patients with DKD and rapid deterioration of renal function remains difficult. Thus, new biomarkers are needed to identify the pathogenesis of DKD and new therapeutic targets.

The role of glycans and their enzymatic modification (glycosylation) lately have attracted much attention in relation to research on cancer and metabolic diseases, including diabetes and DKD. Ohtsubo et al. (10) reported that human pancreatic β-cell–specific GnT-4a glycosyltransferase, which generates the core β1–4 GlcNAc linkage in *N*-glycans, has a protective effect against diabetes by increasing *GLUT2* expression and maintaining insulin secretion. Differences of glycosylation, including *O*-GlcNAcylation, were reported to contribute to the progression of DKD in rats (11,12). There have been few reports about differences of glycosylation in patients with DKD because of technical obstacles to glycan analysis as a result of the complicated structures of these molecules and the time-consuming processes required for mass spectrometry (MS). However, the evanescent-field fluorescence-assisted lectin microarray that we reported previously enables high-throughput quantification of glycan binding to 45 specific lectins (13,14). In the preliminary analysis of our previous study (14), the urinary glycosylation pattern varied considerably, whereas the changes in serum glycosylation were barely detectable among patients with various kidney diseases, including DKD, associated with almost equivalent proteinuria and eGFR. Moreover, studies of the serum *N*-glycan profile have revealed that differences of *N*-glycosylation are associated with diabetic complications and glycemic control in patients with type 1 and 2 diabetes (15,16), but the association between the urinary glycosylation profile and renal prognosis has not been investigated in patients with diabetes to the best of our knowledge.

Accordingly, we investigated the relationship between urinary excretion of *O*- and *N*-glycans binding to 45 lectins and the renal prognosis in patients with type 2 diabetes. In addition, we assessed the incremental predictive value of adding promising glycans to a model that contained established clinical variables, including albuminuria and eGFR.

RESEARCH DESIGN AND METHODS

Study Design

This prospective cohort study was initiated in 2012. Among 688 patients with type 2 diabetes admitted to eight hospitals in Japan from June 2012 to March 2013, 675 patients were eligible for enrollment. Exclusion criteria were a diagnosis of slowly progressive type 1 diabetes during follow-up or a baseline eGFR <15 mL/min/1.73 m^2 (Supplementary Fig. 1). The diagnosis of diabetes was based on Japanese Diabetes Society criteria (17). In addition, 134 volunteers who underwent medical checkups at Okayama Health Foundation in March 2016 and were confirmed to have neither diabetes nor chronic kidney disease (CKD) (17,18) were enrolled as control subjects to compare differences in glycosylation. The protocol for this study was approved by the ethics committee of Okayama University Hospital in June 2012 (identification number: H24-003). This study was registered with the University Hospital Medical Information Network in June 2012. Written informed consent was obtained from all patients with diabetes, whereas comprehensive anonymous consent was obtained from control subjects.

Laboratory Parameters and Definitions

Urine samples collected in the early morning and stored at baseline (patients in 2012–2013, control subjects in 2016) were used to measure urinary glycans and urinary albumin levels in 2015–2016. All specimens were aliquoted, stored at −80°C until measurement, and thawed for the first time to perform this study. The average storage period until measurement was 2.1 ± 0.1 years for patients and 0.9 ± 0.0 years for control subjects. As shown in Supplementary Table 1, the influence of the difference in frozen storage duration on measurement of glycosylation is negligible. Urinary glycans were measured by the evanescent-field fluorescence-assisted lectin microarray, which is a new method of glycan profiling (13,19–21). In brief, we measured urinary levels of Cy3-labeled glycoproteins that bound to 45 lectins with different specificities, and urinary glycan intensity can be measured in 300 samples in 3 working days (Supplementary Fig. 2). Cy3 binds to primary amine in principle. Urinary albumin was labeled by Cy3, although it lacks glycan modification, resulting in minimal background reactivity. Urinary creatinine also was labeled by Cy3, causing background reactivity like albumin. In preliminary experiments, both urinary albumin and creatinine were significantly associated with the intensity of background reactivity (r between log[urinary albumin concentration] and log[background intensity (BG-I)] = 0.697, r between log[urinary creatinine concentration (UCr)] and log[BG-I] = 0.166) (Supplementary Fig. 3). We also observed that the intensity of glycan reactivity (glycan intensity) in 24-h urine and spot urine samples was more closely correlated with the net glycan intensity (Net-I = raw glycan intensity [Raw-I] – BG-I) than with the Net-I/UCr ratio or the Raw-I/UCr ratio (spot urine/24-h urine Net-I ratio 1.20 ± 0.74 [mean ± SD], Net-I/UCr ratio 1.77 ± 2.69, Raw-I/UCr ratio 1.68 ± 2.00) (Supplementary Fig. 4). Furthermore, comparison of glycan intensity between original urine samples and 10-fold diluted urine samples showed that all the Raw-I ratios (ratio = original urine intensity/10-fold diluted urine intensity) and most of the Net-I ratios were <10, suggesting that the urinary glycan index did not increase linearly, but quadratically (Supplementary Table 2). On the basis of these results, we performed all analyses by using the glycan index appropriately transformed from the Net-I according to its distribution.

GFR was estimated by using the Japanese coefficient–modified Chronic Kidney Disease Epidemiology Collaboration equation (22). The baseline urinary albumin/creatinine ratio (UACR) in milligrams per gram creatinine was measured in a spot urine specimen, and normoalbuminuria, microalbuminuria, and macroalbuminuria were defined as UACR <30, ≥ 30 and <300, and ≥ 300 mg/gCr, respectively (15). HbA_{1c} data are presented as National Glycohemoglobin Standardization Program values according to the recommendations of the Japanese Diabetes Society and the

International Federation of Clinical Chemistry (23). BMI was calculated as weight in kilograms divided by the square of height in meters. Mean arterial pressure (MAP) was calculated as two-thirds of diastolic pressure plus one-third of systolic pressure (mmHg). Hypertension was defined as a baseline blood pressure (BP) ≥140/90 mmHg or use of antihypertensive drugs. The average annual values of clinical parameters, including systolic BP (SBP), diastolic BP (DBP), MAP, and HbA_{1c} plus the use of an ACE inhibitor (ACE-I) or angiotensin receptor blocker (ARB) during follow-up were compared between all patients with and without outcome and those stratified according to baseline eGFR categories. The grade of diabetic retinopathy was determined by an ophthalmologist at baseline (24). In this study, cardiovascular disease (CVD), stroke, and peripheral arterial disease (PAD) were defined as events requiring admission for treatment, cerebral bleeding or infarction requiring admission for treatment, and PAD requiring admission for intervention or surgery, respectively. Cardiovascular events were defined as any CVD, stroke, or PAD event.

Study End Point

The primary study end point was defined as a decrease of eGFR by at least 30% from baseline or commencement of dialysis for end-stage renal disease (ESRD). None of the patients received a kidney transplant during follow-up.

Statistical Analysis

Data are summarized as percentages or as the mean ± SD, as appropriate. All skewed variables were subjected to log-transformation to improve normality before analysis. Correlations among glycan indexes were evaluated by Pearson correlation analysis. Univariate and multivariate linear regression analyses were used to explore the association of urinary glycan index with baseline HbA_{1c} and age. In both regression models, the dependent variable was glycan index. In each multivariate model, the β-coefficient for HbA_{1c} was adjusted for baseline age, sex, and duration of diabetes, whereas the β-coefficient for age was adjusted for sex and duration of diabetes. The cumulative incidence rate of the primary outcome was estimated by Kaplan-Meier method for urinary glycan quartiles in all patients, and incidence rates were compared with the log-rank test. In addition, we compared Kaplan-Meier curves between groups of patients with normoalbuminuria or microalbuminuria stratified according to urinary glycan quartiles (Q1–3 vs. Q4 or Q1 vs. Q2–4) and compared groups of patients with macroalbuminuria and higher or lower glycan indexes than the median value. The Cox proportional hazards model was used to calculate the hazard ratio (HR) and 95% CI for the death-censored end point. In the multivariate model, HRs were adjusted for age, sex, MAP, HbA_{1c}, eGFR, and log-transformed UACR at baseline. These covariates were selected as potential confounders on the basis of biological plausibility and metabolic memory (25,26). We also examined another multivariate model without baseline HbA_{1c} to evaluate the effect of baseline HbA_{1c} levels on the outcome. Improvement in discriminating the risk of the study outcome at the median follow-up time (4.0 years) was assessed by analysis of category-free net reclassification improvement (NRI) and absolute/relative integrated discrimination improvement (IDI) as reported elsewhere (27–29). The median follow-up time was selected as the cutoff point for analysis because it was previously used in similar biomarker studies (3,30). We also used the Akaike information criterion (AIC) to compare the fit of various models. Moreover, we compared Harrell's concordance index (C-index) between multivariate Cox proportional hazards models with or without glycan biomarkers. The 95% CIs for category-free NRI and IDI and the differences of the C-index were computed from 5,000 bootstrap samples to adjust for optimism bias. Two-tailed $P < 0.05$ was considered to indicate statistical significance. Analyses and creation of graphs were performed with Stata/SE version 14.0 (StataCorp) and Origin version 2017 (OriginLab) software.

RESULTS

Follow-up Period and Incidence of the Outcome

The median follow-up period was 4.0 years (interquartile range [IQR] 3.9–4.0 years). During follow-up, the primary end point occurred in 63 (9%) patients, and 12 (2%) patients died as a result of causes other than ESRD after refusing dialysis or transplantation.

Patient Characteristics

The baseline characteristics of the subjects are shown in Table 1. Their mean ± SD age was 63 ± 11 years, 61% were men, and the median known duration of diabetes was 11.0 years (IQR 6.2–17.7 years). The baseline SBP, DBP, and MAP was 131.0 ± 17.1, 74.8 ± 10.9, and 93.5 ± 11.7 mmHg, respectively. One-third of the patients had diabetic retinopathy (any type), and their mean baseline HbA_{1c} was 7.1 ± 1.1% (54.3 ± 12.0 mmol/mol). In addition, the mean baseline eGFR was 71.4 ± 17.1 mL/min/1.73 m^2, and median UACR was 17.3 mg/gCr (IQR 7.8–71.1 mg/gCr); 594 patients had normoalbuminuria (64%) or microalbuminuria (24%). With respect to BP and glycemic control during follow-up, the average SBP, DBP, MAP, and HbA_{1c} were not significantly different between patients with and without outcome. As well, the use of an ACE-I or ARB during follow-up was not significantly different between the patients with and without outcome in groups stratified according to baseline eGFR categories (Supplementary Table 3).

Associations of Urinary Glycan Index with HbA_{1c} and Age

Univariate and multivariate regression analysis in all patients and patients with baseline eGFR ≥60 mL/min/1.73 m^2 revealed that lower HbA_{1c} was significantly associated with glycan complex structures that have higher GlcNAc [recognized by PHA(L), PHA(E), DSA, LEL, STL, and WGA], higher Siaα3Galβ4GlcNAc (recognized by MAL_I and ACG), higher GalNAcβ4GlcNAc(recognized by WFA), and higher GalNAcα3GalNAc (recognized by DBA), whereas higher HbA_{1c} was significantly associated with higher Manα3Man (recognized by GNA). Most of these significant associations were attenuated in the patients with baseline eGFR <60 mL/min/1.73 m^2 (Supplementary Table 4). Similar regression analysis for age showed that older age was associated with higher Fucα2GalβGlcNAc (recognized by UEA_I), higher (Galβ4GlcNAc)n (polylactosamine, recognized by LEL), higher (GlcNAcβ4MurNAc)n (peptidoglycan backbone, recognized by STL), and higher Siaα3Galβ3(Siaα6)GalNAc (recognized by MAH) (Supplementary Table 5).

Relation Between the Renal Outcome and Glycan Binding to the Lectin Panel

Unadjusted and adjusted HRs for glycan binding to the panel of 45 lectins with different specificities and the reported structure of the glycan binding to each lectin are shown in Fig. 1. A number of urinary

Table 1—Baseline clinical parameters

Clinical parameter	All patients (n = 675)
Male sex	61
Age (years)	63 ± 11
BMI (kg/m^2)	25.6 ± 4.6
Duration of diabetes (years)	11.0 (6.2–17.7)
SBP (mmHg)	131.0 ± 17.1
DBP (mmHg)	74.8 ± 10.9
MAP (mmHg)	93.5 ± 11.7
Hypertension*	70
Diabetic retinopathy	
Nondiabetic	37
Simple	17
Preproliferative	6
Proliferative	10
Serum creatinine (mg/dL)	0.85 ± 0.35
eGFR (mL/min/1.73 m^2)	71.4 ± 17.1
CKD GFR category (grade)†	
1	10
2	70
3a	12
3b	6
4	3
UACR (mg/gCr)	17.3 (7.8–71.1)
Normoalbuminuria	64
Microalbuminuria	24
Macroalbuminuria	12
HbA_{1c} (%)	7.1 ± 1.1
HbA_{1c} (mmol/mol)	54.3 ± 12.0
Triglycerides (mg/dL)	116 (81–162)
Total cholesterol (mg/dL)	180.5 ± 32.1
LDL cholesterol (mg/dL)	100.0 ± 25.4
Uric acid (mg/dL)	5.3 ± 1.4
Any type of antihypertensive agents	62
ACE-I or ARB	53
Calcium channel blocker	38
Number of antihypertensive agents	1 (0–2)
Treatment for diabetes	
Diet regimen only	4
Oral hypoglycemic agent	64
Insulin	32
Drug treatment for hyperglycemia	
Sulfonylurea	32
Meglitinide analogs	10
Biguanide (metformin)	35
α-Glucosidase inhibitor	28
Thiazolidinedione	15
Dipeptidyl peptidase 4 inhibitor	49
Glucagon-like peptide 1	7
Drug treatment for dyslipidemia	65
Drug treatment for hyperuricemia	10
Prior CVD	17
Prior stroke	10
Prior PAD	2
Prior cardiovascular event	28

Data are mean ± SD, %, or median (IQR) unless otherwise indicated. *Hypertension was defined as BP ≥140/90 mmHg or any antihypertensive drug treatment. †Grade 1, ≥90 mL/min/1.73 m^2; grade 2, 60–90 mL/min/1.73 m^2; grade 3a, 45–59 mL/min/1.73 m^2; grade 3b, 30–44 mL/min/1.73 m^2; and grade 4, 15–29 mL/min/1.73 m^2.

glycans were significantly correlated with the renal outcome in the univariate Cox regression model, whereas the urinary glycans binding to six specific lectins (*Sambucus nigra* [SNA], *Ricinus communis* [RCA120], *Dolichos biflorus* [DBA], *Agaricus bisporus* [ABA], *Artocarpus integrifolia* [Jacalin], and *Amaranthus caudatus* [ACA]) were significantly correlated with renal outcome in both the univariate and multivariate models. In the multivariate model adjusted for known indicators of DKD progression, including baseline eGFR and UACR, the HR for positive glycan binding to SNA (+1 SD for the glycan index) was 1.42 (95% CI 1.14–1.76), whereas the HR for glycan binding to RCA120 was 1.28 (1.01–1.64); DBA, 0.80 !(0.64–0.997); ABA, 1.29 (1.02–1.64); Jacalin, 1.30 (1.02–1.67); and ACA, 1.32 (1.04–1.67) (Fig. 1*A*). These associations remained largely unchanged when average MAP and/or HbA_{1c} during follow-up were incorporated into the multivariate model and when baseline HbA_{1c} was eliminated from the multivariate model (Supplementary Tables 6 and 7). As shown in Fig. 1*B*, the glycans Siaα2-6Gal/GalNAc, Galβ4GlcNAc, and GalNAcα3GalNAc were reported to bind with SNA, RCA120, and DBA, respectively, whereas Galβ3GalNAc was reported to bind with ABA, Jacalin, and ACA.

Glycan Binding to SNA, RCA120, DBA, ABA, Jacalin, and ACA in the Control Group and Patients With Diabetes Stratified According to the CKD Heat Map

We stratified the patients with diabetes into four CKD heat map groups and 11 categories by using the baseline UACR and eGFR (31). Comparisons of glycan binding to SNA, RCA120, DBA, ABA, Jacalin, and ACA among the control group and patients with diabetes stratified according to the CKD heat map groups and 11 categories are shown in Supplementary Fig. 5. Overall, the glycan index values were higher in the severe categories of the CKD heat map, albuminuria, and eGFR, except for the glycan index of DBA. Of note, the glycan index of SNA was significantly higher in the green heat map group than in the control group, even though both groups were defined by normoalbuminuria and eGFR >60 mL/min/1.73 m^2. Correlations among positive binding to ABA, Jacalin, and ACA were extremely strong (r = 0.952 between ABA and Jacalin, r = 0.931 between ABA and ACA, and r = 0.942 between ACA and Jacalin) as was the

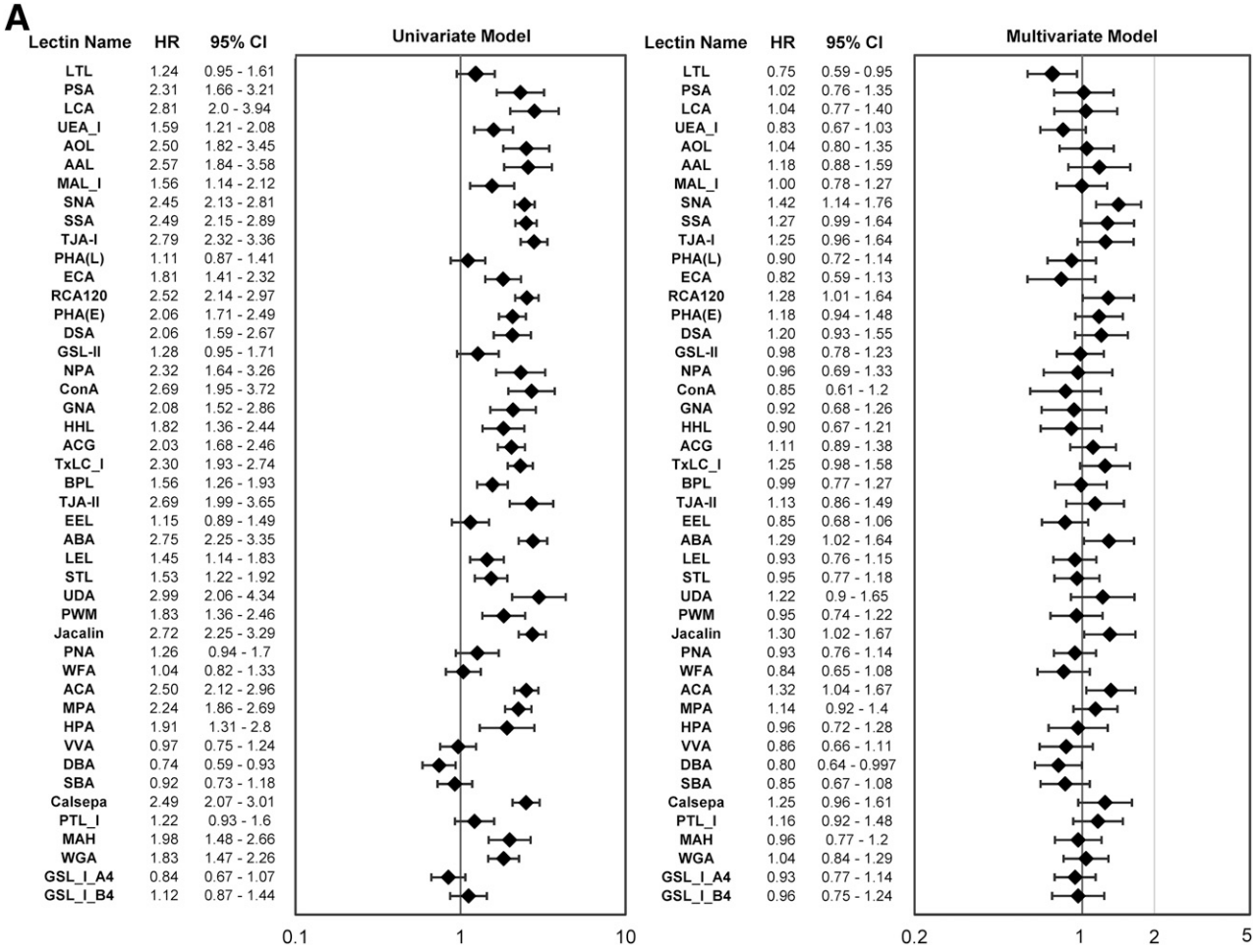

Figure 1—Univariate and multivariate Cox proportional hazard models of the renal outcome and reported glycans binding to 45 lectins. *A*: Cox proportional hazard models. In the multivariate model, HR was adjusted for age, sex, MAP, HbA_{1c}, eGFR, and log-transformed urinary albumin excretion at baseline. Renal outcome was defined as 30% eGFR decline or dialysis as a result of ESRD. *B*: Preferred glycan structures binding to 45 lectins with different specificity.

correlation between SNA and RCA120 (r = 0.921) (Supplementary Table 8).

Cumulative Incidence Rate of the Primary Outcome in Urinary Glycan Quartiles

Kaplan-Meier curves stratified according to quartiles for baseline urinary glycan binding to SNA, RCA120, DBA, ABA, Jacalin, and ACA are shown in Fig. 2. The cumulative incidence rate of the renal outcome was significantly higher in the highest quartile for urinary glycan binding to SNA, RCA120, ABA, Jacalin, or ACA than in the other quartiles, whereas it was significantly higher in the lowest quartile for glycan binding to DBA than in the other quartiles (Fig. 2*A*). Similar results for glycans binding to SNA, ABA, Jacalin, and ACA were obtained in patients with normoalbuminuria/microalbuminuria (Fig. 2*B*). Among patients with macroalbuminuria, the cumulative incidence rate was significantly higher in those with higher glycan index values than in those with lower glycan index values, except in the case of the glycan index for DBA (Fig. 2*C*).

Incremental Predictive Power of Urinary Glycan Binding to SNA, RCA120, DBA, ABA, Jacalin, and ACA

The category-free NRI, absolute/relative IDI, and AIC for predicting the primary outcome at the median follow-up time (4.0 years) obtained by adding the glycan indexes as well as the difference of the C-index between Cox regression models with or without the urinary glycan indexes are summarized in Table 2. The addition of any single glycan index to the multivariate model did not improve prediction, whereas adding all six glycan indexes significantly improved risk classification, integrated discrimination, and AIC (category-free NRI 0.51 [95% CI 0.22–0.80], relative IDI 0.18 [0.01–0.35], AIC decrease from 296 to 287). Similarly, when four glycan indexes (on the basis of the reported glycan specificities) were added, risk classification, integrated discrimination, and AIC also were significantly improved (Table 2). However, the C-index did not increase significantly when these glycan indexes were added to the multivariate Cox regression model (Table 2).

CONCLUSIONS

Glycans are involved in various biological processes, including development, immunity, infection, hormone actions, cell adhesion, and oncogenesis (19,32,33). One of the most prominent features of glycans is structural heterogeneity, and this distinguishes glycans from other major biopolymers, such as nucleic acids and proteins. Such heterogeneity is largely attributable to

B

Lectin Name	Origin	Lectin Family	Monosaccharide Specificity	Preferred glycan structure (terminal epitope)
LTL	*Lotus tetragonolobus*	Legume (L-type)	Fuc	Fucα3(Galb4)GlcNAc (Lex), Fucα2Galβ4GlcNAc (H-type 2)
PSA	*Pisum sativum*	Legume (L-type)	Fuc/Man	Fucα6GlcNAc, High-Man
LCA	*Lens culinaris*	Legume (L-type)	Fuc/Man	Fucα6GlcNAc, High -Man
UEA_I	*Ulex europaeus*	Legume (L-type)	Fuc	Fucα2Galβ4GlcNAc (H-type 2)
AOL	fungus, *Aspergillus oryzae*	Fucose lectin	Fuc	Fucα6GlcNAc (core Fuc), Fucα2Galβ4GlcNAc (H-type 2)
AAL	*Aleuria aurantia*	Fucose lectin	Fuc	Fucα6GlcNAc (core Fuc), Fucα3(Galβ4)GlcNAc (Lex)
MAL_I	*Maackia amurensis*	Legume (L-type)	Sia	Siaα3Galβ4GlcNAc
SNA	*Sambucus nigra*	Ricin B-cahin-like (R-type)	Sia	Siaα2-6Gal/GalNAc
SSA	*Sambucus sieboldiana*	Ricin B-cahin-like (R-type)	Sia	Siaα2-6Gal/GalNAc
TJA-I	*Trichosanthes japonica*	Ricin B-cahin-like (R-type)	Sia	Siaα2-6Gal/GalNAc
PHA(L)	*Phaseolus vulgaris*	Legume (L-type)	Complex	Tri/tetra-antennary complex-type *N*-glycan
ECA	*Erythrina crista-galli*	Legume (L-type)	Gal	Galβ4GlcNAc
RCA120	*Ricinus communis*	Ricin B-cahin-like (R-type)	Gal	Galβ4GlcNAc
PHA(E)	*Phaseolus vulgaris*	Legume (L-type)	Gal	*N*-glycans with outer Gal and bisecting GlcNAc
DSA	*Datura stramonium*	Hevein (Chitin-type)	GlcNAc	(GlcNAcβ4)n, triantennary, tetraantennary *N*-glycans
GSL-II	*Griffonia simplicifolia*	Legume (L-type)	GlcNAc	Agalactosylated tri/tetra antennary glycans, GlcNAc
NPA	*Narcissus pseudonarcissus*	Monocot (GNA-related)	Man	High-Man including Manα6Man
ConA	*Canavalia ensiformis*	Legume (L-type)	Man	High-Man including Manα6(Manα3)Man
GNA	*Galanthus nivalis*	Monocot (GNA-related)	Man	High-Man including Manα3Man
HHL	*Hippeastrum hybrid*	Monocot (GNA-related)	Man	High-Man including Manα3Man or Manα6Man
ACG	*Agrocybe cylindracea*	Galectin	Gal	Siaα3Galβ4GlcNAc
TxLC_I	*Tulipa gesneriana*	Monocot (GNA-related)	Man/GalNAc	Man3 core, bi- and tri-antennary *N*-glycans, GalNAc
BPL	*Bauhinia purpurea alba*	Legume (L-type)	Gal	Galβ3GalNAc, GalNAc
TJA-II	*Trichosanthes japonica*	Others	Gal	Fucα2Galβ1, GalNAcβ1
EEL	*Euonymus europaeus*	Legume (L-type)	Gal	Galα3Galβ4GlcNAc, Fucα2(Galα3)Galβ4GlcNAc
ABA	fungus, *Agaricus bisporus*	Others	Gal, GlcNAc	Galβ3GalNAc, GlcNAc
LEL	tomato, *Lycopersicon esculentum*	Hevein (Chitin-type)	GlcNAc	(GlcNAcβ4)n, (Galβ4GlcNAc)n (polylactosamine)
STL	potato, *Solanum tuberosum*	Hevein (Chitin-type)	GlcNAc	(GlcNAcβ)n, (GlcNAcβ4MurNAc)n (peptidoglycan backbone)
UDA	*Urtica dioica*	Hevein (Chitin-type)	GlcNAc	GlcNAcβ4GlcNAc, Man5～Man9
PWM	pokeweed, *Phytolacca americana*	Hevein (Chitin-type)	GlcNAc	(GlcNAcβ4)n
Jacalin	*Artocarpus integrifolia*	Jacalin	Gal	Galβ3GalNAc, αGalNAc (6O-unsubstituted)
PNA	peanut, *Arachis hypogaea*	Legume (L-type)	Gal	Galβ3GalNAc
WFA	*Wisteria floribunda*	Legume (L-type)	GalNAc	GalNAcβ4GlcNAc, Galβ3(-6)GalNAc
ACA	*Amaranthus caudatus*	Ricin B-cahin-like (R-type)	Gal	Galβ3GalNAc
MPA	*Maclura pomifera*	Jacalin	Gal	Galβ3GalNAc, GalNAc
HPA	snail, *Helix pomatia agglutinin*	Discoidin	GalNAc	αGalNAc
VVA	*Vicia villosa*	Legume (L-type)	GalNAc	αGalNAc, GalNAcα3Gal
DBA	*Dolichos biflorus*	Legume (L-type)	GalNAc	Blood group A antigen, GalNAcα3GalNAc
SBA	soybean, *Glycine max*	Legume (L-type)	GalNAc	GalNAc, GalNAcα3Gal
Calsepa	*Calystegia sepium*	Jacalin	Man	High-Man (Man2–6), *N*-glycans including bisecting GlcNAc
PTL_I	*Psophocarpus tetragonolobus*	Legume (L-type)	GalNAc	αGalNAc
MAH	*Maackia amurensis*	Legume (L-type)	Sia	Siaα3Galβ3(Siaα6)GalNAc
WGA	wheat germ, *Triticum vulgaris*	Hevein (Chitin-type)	GlcNAc	(GlcNAcβ4)n, NeuAc
GSL_I_A4	*Griffonia simplicifolia Lectin I Isolectin A4*	Legume (L-type)	Gal	αGal
GSL_I_B4	*Griffonia simplicifolia Lectin I Isolectin B4*	Legume (L-type)	GalNAc	αGalNAc

Figure 1—*Continued.*

the glycan fabrication process and depends on structural modification by glycosyltransferases, which is known as glycosylation (19).

Podocalyxin is the molecule in the kidneys for which the functional effects of changes in glycosylation have been most thoroughly investigated (34). It is a glycoprotein expressed by podocytes that plays an essential role in the maintenance of podocyte slit pore integrity and effective glomerular filtration (35). Podocalyxin is decorated by *O*-glycans, including an abundance of sialic acid, and removal of sialic acid leads to effacement of the podocyte foot processes and

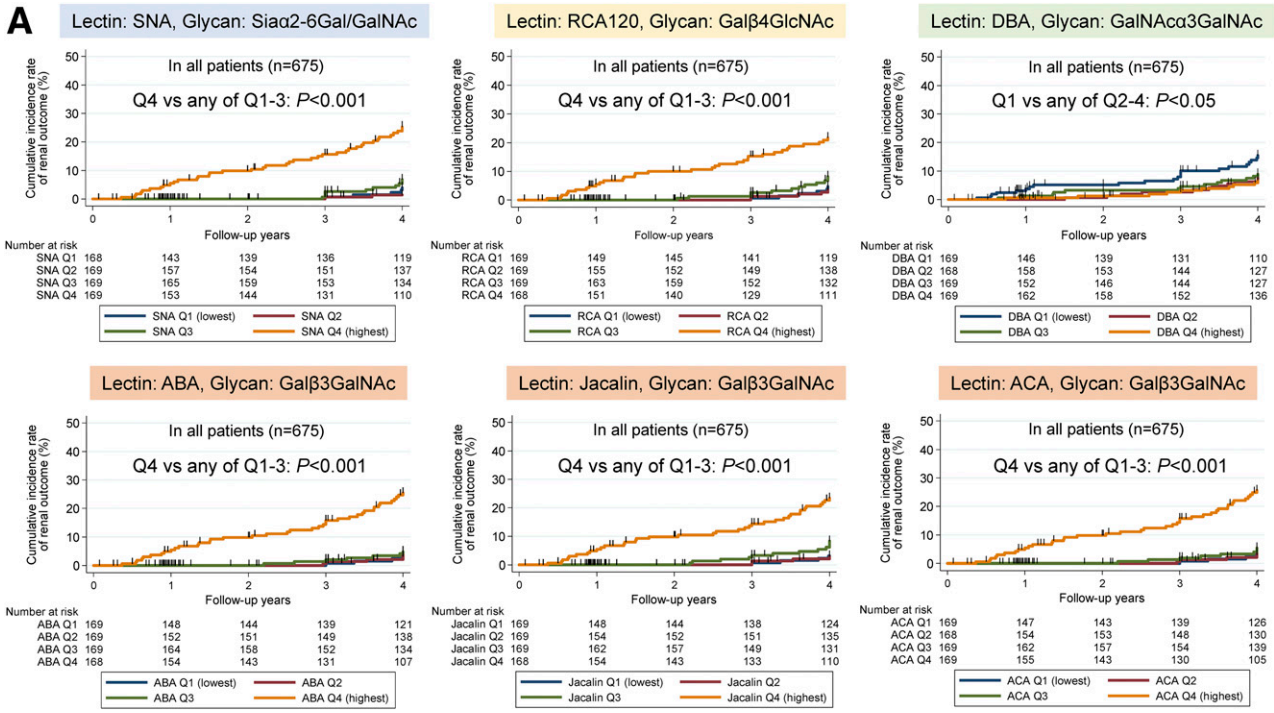

Figure 2—Cumulative incidence rate of the renal outcome. *A*: Cumulative incidence rate in all patients stratified by urinary glycan quartiles. The estimated 4-year renal failure rate was 25%, 22%, 25%, 23%, and 26% in patients from the highest glycan quartiles for SNA, RCA120, ABA, Jacalin, and ACA, respectively, whereas it was 15% in patients from the lowest glycan quartile for DBA. The cumulative incidence rate was significantly higher in the highest glycan quartiles for SNA, RCA120, ABA, Jacalin, and ACA than in the other quartiles ($P < 0.001$), although it was significantly higher in the lowest glycan quartile for DBA than in the other quartiles ($P < 0.05$). *B*: Cumulative incidence rate in the highest urinary glycan quartiles and other glycan quartiles combined in patients with normoalbuminuria or microalbuminuria. The cumulative incidence rate was significantly higher in the highest glycan quartile (Q4) for SNA, ABA, Jacalin, and ACA than in the other quartiles combined (Q1–3 vs. Q4: $P = 0.026$ for SNA, $P = 0.028$ for ABA, $P = 0.019$ for Jacalin, and $P = 0.002$ for ACA). On the other hand, the difference of the cumulative incidence rate between Q4 and Q1–3 was not significant for RCA120 and DBA (Q1–3 vs. Q4: $P = 0.433$ for RCA120, $P = 0.270$ for DBA). *C*: Cumulative incidence rate in patients with macroalbuminuria and higher or lower glycan indexes than the median. The cumulative incidence rate was significantly higher in patients with higher glycan indexes than in those with lower glycan indexes ($P < 0.001$), except for the glycan index for DBA ($P = 0.186$). Outcome: ≥30% decline of eGFR or dialysis as a result of ESRD. The log-rank test was used for failure analysis.

proteinuria in mice secondary to loss of the negative charge on the glomerular basement membrane (36). Similarly, doxycycline-induced global deficiency of glycosyltransferase core 1 synthase and glycoprotein-*N*-acetylgalactosamine 3-β-galactosyltransferase 1 (C1galt1) leads to marked reduction of SNA-binding Siaα2-6Gal/GalNAc on podocalyxin in mice followed by albuminuria, rapid progression of glomerulosclerosis, effacement of podocyte foot processes, and thickening of the glomerular basement membrane (37). These results might suggest that expression of mature *O*-glycans modified by a key glycosyltransferase in the kidneys, especially on podocytes, is crucial to the maintenance of the normal renal architecture. If abnormalities of renal *O*-glycosylation occur, parts of mature *O*-glycans could undergo reduction and be excreted in the urine. Although C1galt1 is not involved in the modification of Siaα2-6Gal/GalNAc (Supplementary Fig. 6*A*), deletion of C1galt1 induces a decrease of Siaα2-6Gal/GalNAc and an increase of Tn antigen in mouse podocalyxin (37), suggesting that impairment of one *O*-glycosyltransferase also affects other *O*-glycosylation pathways and leads to the creation of abnormal *O*-glycans. The current study findings might support these suggestions as follows.

We found that urinary levels of glycans binding to SNA, ABA, Jacalin, and ACA were significantly higher in the severe CKD heat map group and that the cumulative incidence rate of the renal outcome was significantly higher in the highest glycan quartiles than in the other glycan quartiles not only in all patients but also in patients with normoalbuminuria/microalbuminuria (Supplementary Fig. 5 and Fig. 2*A* and *B*). Of note, urinary excretion of Siaα2-6Gal/GalNAc binding to SNA was significantly higher in non-CKD patients with diabetes than in non-CKD control subjects without diabetes, suggesting that a change in the glycosylation of Siaα2-6Gal/GalNAc could occur in the early stage of DKD before the onset of albuminuria or detectable deterioration of renal function. On the basis of these results and the above-mentioned hypothesis that changes of glycosylation may lead to renal structural damage, it might be reasonable that indexes for these glycans may be significantly associated with the risk of the renal outcome independently of baseline albuminuria and eGFR (Fig. 1).

Galβ4GlcNAc binds to RCA120 and is involved in *N*- and *O*-glycosylation. During *O*-glycosylation, Galβ4GlcNAc plays a role in the extension of cores 2 and 4, which are finally modified by sialic acid (32) (Supplementary Fig. 6*A*). Therefore, if impairment of the enzyme adding sialic acid to cores 2 and 4 exists, urinary excretion of Galβ4GlcNAc could increase by the same mechanism as that which increases urinary levels of Siaα2-6Gal/GalNAc and Galβ3GalNAc. In this study,

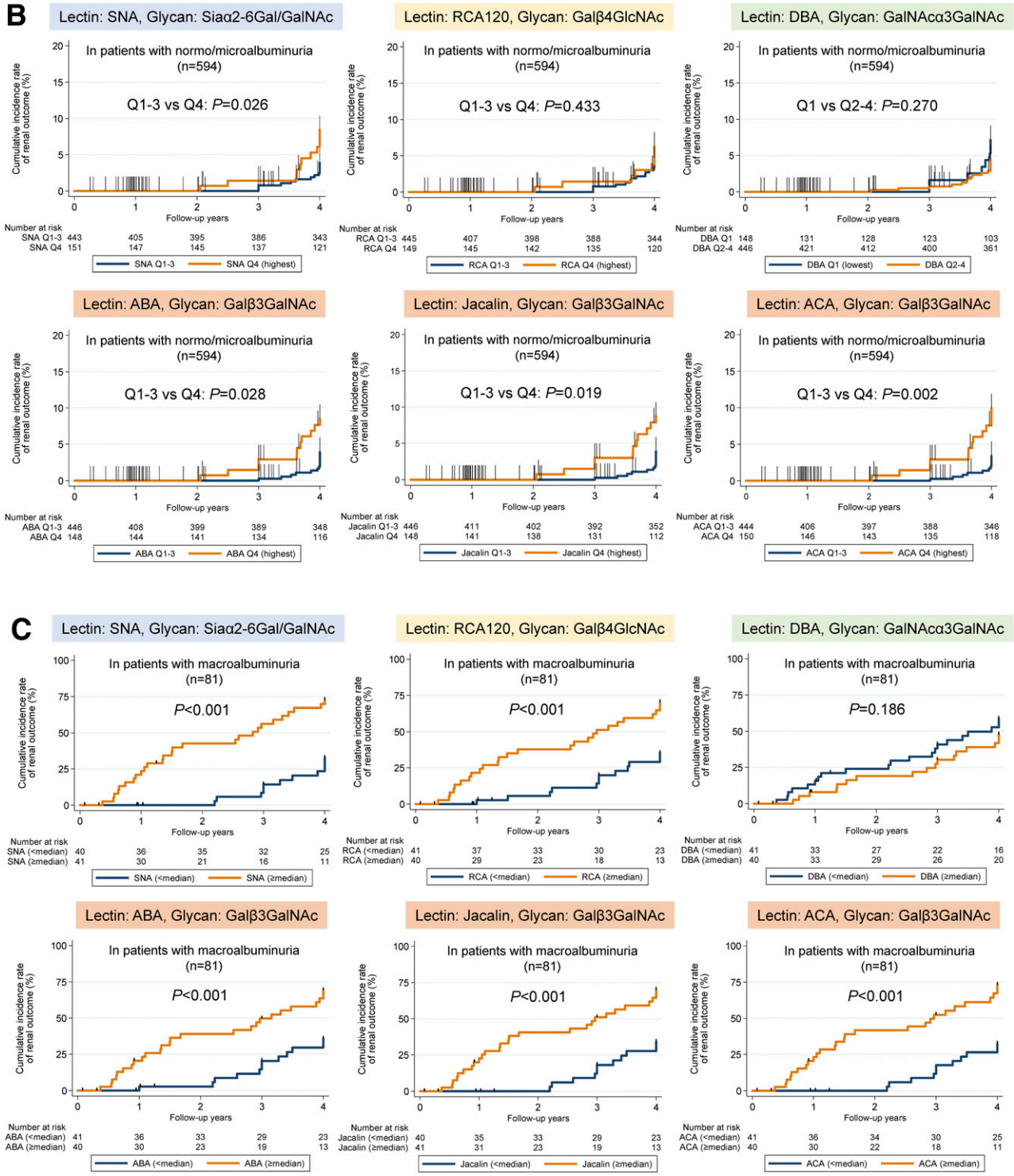

Figure 2—*Continued.*

the glycan index for RCA120 was strongly correlated with that for SNA (r = 0.921) (Supplementary Table 8), which suggests that the common underlying mechanism is impairment of sialylation. In addition, Ravidà et al. (12) demonstrated that glycans binding to RCA120 gradually show a significant decrease in the renal cortex of diabetic rats with progression of DKD, whereas these glycans increase in nondiabetic rats. Therefore, increased urinary levels of Galβ4GlcNAc might reflect abnormal *O*-glycosylation in kidney tissues.

With regard to GalNAcα3GalNAc, we could not reasonably explain why urinary excretion of this glycan binding to DBA was negatively correlated with the renal outcome. DBA is well known for recognizing the epitope of glycans on the surface of type A red blood cells (33).

Table 2—Category-free NRI, IDI, and AIC for predicting the 4-year outcome with glycan index data, and difference of C-index between Cox regression models with or without glycan index data

	Category-free NRI (95% CI)	*P* value	Absolute IDI (95% CI)	*P* value	Relative IDI (95% CI)	*P* value	AIC	C-index (95% CI)	Difference of C-index (95% CI)	*P* value
Only covariates							295.9	0.89 (0.84–0.93)		
Glycan to SNA (Siaα2-6Gal/GalNAc)	0.27 (−0.13 to 0.66)	0.184	0.02 (−0.01 to 0.05)	0.184	0.06 (−0.04 to 0.17)	0.232	292.5	0.89 (0.84–0.94)	0.00 (−0.01 to 0.01)	0.958
Glycan to RCA120 (Galβ4GlcNAc)	0.02 (−0.25 to 0.29)	0.891	0.00 (−0.01 to 0.02)	0.712	0.01 (−0.03 to 0.05)	0.725	297.6	0.89 (0.84–0.93)	−0.00 (−0.01 to 0.00)	0.491
Glycan to DBA (GalNAcα3GalNAc)	0.20 (−0.11 to 0.50)	0.203	0.01 (−0.01 to 0.02)	0.488	0.02 (−0.04 to 0.07)	0.529	295.8	0.89 (0.85–0.94)	0.00 (−0.00 to 0.01)	0.402
Glycan to ABA (Galβ3GalNAc)	0.26 (−0.10 to 0.61)	0.156	0.01 (−0.01 to 0.04)	0.309	0.04 (−0.04 to 0.12)	0.336	295.1	0.89 (0.84–0.94)	0.00 (−0.01 to 0.01)	0.801
Glycan to Jacalin (Galβ3GalNAc)	0.11 (−0.18 to 0.40)	0.439	0.01 (−0.01 to 0.04)	0.375	0.04 (−0.05 to 0.12)	0.383	295.7	0.89 (0.84–0.94)	0.00 (−0.01 to 0.01)	0.834
Glycan to ACA (Galβ3GalNAc)	0.13 (−0.17 to 0.44)	0.388	0.01 (−0.01 to 0.04)	0.286	0.04 (−0.04 to 0.13)	0.311	294.4	0.89 (0.84–0.94)	0.00 (−0.01 to 0.01)	0.975
Combination of four types of glycan (SNA, RCA120, DBA, and ABA)	0.39 (0.11–0.67)	0.006	0.06 (0.01–0.10)	0.009	0.17 (0.01–0.33)	0.037	284.9	0.90 (0.85–0.94)	0.01 (−0.01 to 0.03)	0.351
Combination of four types of glycan (SNA, RCA120, DBA, and Jacalin)	0.42 (0.15–0.69)	0.002	0.06 (0.01–0.10)	0.01	0.17 (0.01–0.33)	0.033	284.9	0.90 (0.85–0.94)	0.01 (−0.01 to 0.03)	0.441
Combination of four types of glycan (SNA, RCA120, DBA, and ACA)	0.46 (0.20–0.71)	<0.001	0.06 (0.01–0.10)	0.009	0.17 (0.02–0.33)	0.027	284.5	0.90 (0.85–0.94)	0.01 (−0.01 to 0.03)	0.402
Combination of all glycans	0.51 (0.22–0.80)	0.001	0.06 (0.02–0.10)	0.008	0.18 (0.01–0.35)	0.036	287.0	0.90 (0.85–0.94)	0.01 (−0.01 to 0.03)	0.447

Covariates were age, sex, MAP, HbA_{1c}, eGFR, and log-transformed urinary albumin excretion at baseline.

It has also been reported to bind to glomerular lesions in human DKD and to the distal tubules in human kidney tissue, but further investigations have not been performed (38,39). DBA recognizes core 5 (GalNAcα3GalNAc), which is a rare component of *O*-glycans, and the relevant glycosyltransferase has not yet been reported (32). Therefore, additional investigation of the role of this glycan in the kidneys and DKD is needed.

Taken together, changes of glycosylation in DKD seem to be strongly associated with renal prognosis because abnormal glycosylation might be involved in the progression of DKD. Supplementary Fig. 6 shows a scheme of our hypothesis regarding the mechanism by which abnormalities of glycosylation may be associated with progression of DKD. We also speculate that the urinary glycosylation difference well reflects the local glycosylation changes in kidney tissues rather than alterations of systemic glycosylation on the basis of the discrepancies of glycan profiles between urine and serum/plasma samples (15,16,40). Serum protein *N*-glycan profiling has been shown to lower levels of fucosylated biantennary glycans, and higher levels of complexes that have more GlcNAc and heavily galactosylated and sialylated glycans have been associated with higher HbA_{1c} levels in patients with diabetes (15,16), whereas those associations were not observed in the current urinary glycan profiling. Similarly, plasma IgG *N*-glycan profiling revealed that higher levels of bisecting GlcNAc and lower levels of sialylation were correlated with older age, especially >60 years (40), whereas these results were not seen in the current study with urine samples. In the preliminary data of our previous study (14), we found completely different glycosylation patterns between serum and urine samples as well as different urinary glycosylation among patients with biopsy-proven kidney diseases, including DKD, and similar degrees of proteinuria and eGFR, which might support our speculation. These hypotheses are based on in vivo and clinical studies of human DKD, so additional investigation of glycosylation changes in human kidney tissue is required.

Potential limitations of this study were that the current lectin microarray system does not allow complete determination of glycan structures as can MS, and unknown preferred glycan structure to each lectin might have biased the results. However, this technique is useful for

differentiating urinary glycan profiling in individuals, and it enabled us to measure a wide range of urinary glycan intensity in high throughput without a liberation process, although conventional methods, including MS, require both prior liberation and subsequent labeling (19). Another limitation was that this study included patients with various stages of CKD, and renal biopsy was not performed in all subjects. Therefore, we cannot exclude the possibility that the decline of eGFR in some patients resulted from kidney diseases other than DKD. However, in previous well-known cohort studies of type 2 diabetes, DKD was not always confirmed by renal biopsy (1,3). Moreover, in the patients with diabetes, renal biopsy is considered when hematuria, granular casts, sudden onset of nephrotic syndrome, and rapidly progressive glomerulonephritis are indicated, whereas such conditions were not observed during follow-up in the current study. We hope that an ongoing research biopsy study of DKD at our institution will solve this issue in the future.

In conclusion, we demonstrate that urinary excretion of glycans binding to several lectins, including SNA (recognizing Siaα2-6Gal/GalNAc), RCA120 (Galβ4GlcNAc), DBA (GalNAcα3GalNAc), ABA, Jacalin, and ACA (Galβ3GalNAc), are significantly associated with renal outcome in patients with type 2 diabetes. The addition of the combined glycan index to a model with standard risk factors significantly improves prediction of renal outcome, suggesting that these urinary glycans may be novel predictors of renal prognosis in patients with type 2 diabetes. The findings could provide new insights into changes of glycosylation related to DKD. The mechanisms that underlie differences of urinary glycan excretion and changes of glycosylation in DKD should be investigated further.

Acknowledgments. The authors thank Dr. Kazuyuki Hida (National Hospital Organization Okayama Medical Center), Dr. Tatsuaki Nakato (Okayama Saiseikai General Hospital), Dr. Takashi Matsuoka (Kurashiki Central Hospital), Dr. Ikki Shimizu (The Sakakibara Heart Institute of Okayama), Dr. Tomokazu Nunoue (Tsuyama Chuo Hospital), Dr. Katsuhiro Miyashita (Japanese Red Cross Okayama Hospital), Dr. Shinichiro Ando (Okayama City General Medical Center), and Dr. Akiho Seki (Okayama Health Foundation) for providing data and for patient treatments. The authors also thank Drs. Ryo Kodera and Satoshi Miyamoto (Okayama University Hospital), Drs. Akihiro Katayama, Chigusa Higuchi, Hidemi Takeuchi, Yusuke Shibata, Ichiro Nojima, Yuzuki Kano, Yuriko Yamamura, Nozomu Otaka, Chieko Kawakita, Keigo Hayashi, and Yasuhiro Onishi (Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama) for collecting data. The authors greatly appreciate Dr. Hirofumi Makino (Okayama University) for critical suggestions in the design of the current investigation.

Funding. This work was partly supported by the Ministry of Health, Labour and Welfare in Japan (Health Labor Sciences Research Grant 201413003), the Japan Agency for Medical Research and Development (AMED) (grant 17ek0210095h0001), Novo Nordisk Pharma Ltd. (Junior Scientist Development Grant [2016–2017]) from the Okinaka Memorial Institute for Medical Research (a grant in 2017), and The Yukiko Ishibashi Foundation (a grant in 2016).

Duality of Interest. M.Ya. was a former employee of GP BioSciences Co., Ltd., and is currently an employee of GlycoTechnica, Ltd. No other potential conflicts of interest relevant to this article were reported.

Author Contributions. K.M. contributed to designing the research, analyzing and interpreting data, measuring urinary glycan levels, collecting and summarizing clinical data, and writing the manuscript. M.I. contributed to collecting, summarizing, and assessing clinical data. S.Y. contributed to measuring urinary glycan levels and collecting and summarizing clinical data. S.T., A.T., H.A.U., J.E., A.N., and D.O., contributed to managing patients and assessing data. M.Yo. contributed to interpreting data and performing statistical analyses. M.Ya. contributed to measuring urinary glycan levels; interpreting data, especially urinary glycan data; and writing the manuscript. K.S. contributed to managing patients and assessing data. J.W. was responsible for the study design, supervised data collection and data analysis, and contributed to drafting and editing the manuscript. J.W. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Prior Presentation. Part of this work was presented at the American Society of Nephrology Scientific Oral Sessions, New Orleans, LA, 31 October–5 November 2017, and the International Society of Nephrology Frontiers Meetings, Tokyo, Japan, 22–25 February 2018.

References

1. Niewczas MA, Gohda T, Skupien J, et al. Circulating TNF receptors 1 and 2 predict ESRD in type 2 diabetes. J Am Soc Nephrol 2012;23:507–515
2. Gohda T, Niewczas MA, Ficociello LH, et al. Circulating TNF receptors 1 and 2 predict stage 3 CKD in type 1 diabetes. J Am Soc Nephrol 2012;23:516–524
3. Fufaa GD, Weil EJ, Nelson RG, et al.; Chronic Kidney Disease Biomarkers Consortium Investigators. Association of urinary KIM-1, L-FABP, NAG and NGAL with incident end-stage renal disease and mortality in American Indians with type 2 diabetes mellitus. Diabetologia 2015;58:188–198
4. Nowak N, Skupien J, Niewczas MA, et al. Increased plasma kidney injury molecule-1 suggests early progressive renal decline in non-proteinuric patients with type 1 diabetes. Kidney Int 2016;89:459–467
5. Nadkarni GN, Rao V, Ismail-Beigi F, et al. Association of urinary biomarkers of inflammation, injury, and fibrosis with renal function decline: the ACCORD trial. Clin J Am Soc Nephrol 2016;11:1343–1352
6. Foster MC, Inker LA, Hsu CY, et al.; CKD Biomarkers Consortium. Filtration markers as predictors of ESRD and mortality in Southwestern American Indians with type 2 diabetes. Am J Kidney Dis 2015;66:75–83
7. Wanner C, Inzucchi SE, Lachin JM, et al.; EMPA-REG OUTCOME Investigators. Empagliflozin and progression of kidney disease in type 2 diabetes. N Engl J Med 2016;375:323–334
8. Mann JFE, Ørsted DD, Brown-Frandsen K, et al.; LEADER Steering Committee and Investigators. Liraglutide and renal outcomes in type 2 diabetes. N Engl J Med 2017;377:839–848
9. Mise K, Hoshino J, Ueno T, et al. Clinical and pathological predictors of estimated GFR decline in patients with type 2 diabetes and overt proteinuric diabetic nephropathy. Diabetes Metab Res Rev 2015;31:572–581
10. Ohtsubo K, Chen MZ, Olefsky JM, Marth JD. Pathway to diabetes through attenuation of pancreatic beta cell glycosylation and glucose transport. Nat Med 2011;17:1067–1075
11. Gellai R, Hodrea J, Lenart L, et al. Role of O-linked N-acetylglucosamine modification in diabetic nephropathy. Am J Physiol Renal Physiol 2016;311:F1172–F1181
12. Ravidà A, Musante L, Kreivi M, et al. Glycosylation patterns of kidney proteins differ in rat diabetic nephropathy. Kidney Int 2015;87:963–974
13. Kuno A, Uchiyama N, Koseki-Kuno S, et al. Evanescent-field fluorescence-assisted lectin microarray: a new strategy for glycan profiling. Nat Methods 2005;2:851–856
14. Inoue K, Wada J, Eguchi J, et al. Urinary fetuin-A is a novel marker for diabetic nephropathy in type 2 diabetes identified by lectin microarray. PLoS One 2013;8:e77118
15. Testa R, Vanhooren V, Bonfigli AR, et al. N-glycomic changes in serum proteins in type 2 diabetes mellitus correlate with complications and with metabolic syndrome parameters. PLoS One 2015;10:e0119983
16. Bermingham ML, Colombo M, McGurnaghan SJ, et al.; SDRN Type 1 Bioresource Investigators. N-glycan profile and kidney disease in type 1 diabetes. Diabetes Care 2018;41:79–87
17. Seino Y, Nanjo K, Tajima N, et al.; Committee of the Japan Diabetes Society on the Diagnostic Criteria of Diabetes Mellitus. Report of the committee on the classification and diagnostic criteria of diabetes mellitus. J Diabetes Investig 2010;1:212–228
18. Chapter 1: definition and classification of CKD. Kidney Int Suppl (2011) 2013;3:19–62
19. Hirabayashi J, Yamada M, Kuno A, Tateno H. Lectin microarrays: concept, principle and applications. Chem Soc Rev 2013;42:4443–4458
20. Safina G. Application of surface plasmon resonance for the detection of carbohydrates, glycoconjugates, and measurement of the carbohydrate-specific interactions: a comparison with conventional analytical techniques. A critical review. Anal Chim Acta 2012;712:9–29
21. Cheng CI, Chang YP, Chu YH. Biomolecular interactions and tools for their recognition: focus on the quartz crystal microbalance and its diverse

surface chemistries and applications. Chem Soc Rev 2012;41:1947–1971
22. Horio M, Imai E, Yasuda Y, Watanabe T, Matsuo S. Modification of the CKD epidemiology collaboration (CKD-EPI) equation for Japanese: accuracy and use for population estimates. Am J Kidney Dis 2010;56:32–38
23. Kashiwagi A, Kasuga M, Araki E, et al. International clinical harmonization of glycated hemoglobin in Japan: from Japan Diabetes Society to National Glycohemoglobin Standardization Program values. J Diabetes Investig 2012;3:39–40
24. Fong DS, Aiello L, Gardner TW, et al.; American Diabetes Association. Retinopathy in diabetes. Diabetes Care 2004;27(Suppl. 1):S84–S87
25. Holman RR, Paul SK, Bethel MA, Matthews DR, Neil HA. 10-year follow-up of intensive glucose control in type 2 diabetes. N Engl J Med 2008;359:1577–1589
26. Writing Team for the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Research Group. Effect of intensive therapy on the microvascular complications of type 1 diabetes mellitus. JAMA 2002;287:2563–2569
27. Pencina MJ, D'Agostino RB Sr., D'Agostino RB Jr., Vasan RS. Evaluating the added predictive ability of a new marker: from area under the ROC curve to reclassification and beyond. Stat Med 2008;27:157–172; discussion 207–112
28. Pencina MJ, D'Agostino RB Sr, Steyerberg EW. Extensions of net reclassification improvement calculations to measure usefulness of new biomarkers. Stat Med 2011;30:11–21
29. Pencina MJ, D'Agostino RB, Pencina KM, Janssens AC, Greenland P. Interpreting incremental value of markers added to risk prediction models. Am J Epidemiol 2012;176:473–481
30. Mise K, Hoshino J, Ueno T, et al. Prognostic value of tubulointerstitial lesions, urinary N-acetyl-β-d-glucosaminidase, and urinary β2-microglobulin in patients with type 2 diabetes and biopsy-proven diabetic nephropathy. Clin J Am Soc Nephrol 2016;11:593–601
31. Levin A, Stevens PE. Summary of KDIGO 2012 CKD Guideline: behind the scenes, need for guidance, and a framework for moving forward. Kidney Int 2014;85:49–61
32. Gill DJ, Clausen H, Bard F. Location, location, location: new insights into O-GalNAc protein glycosylation. Trends Cell Biol 2011;21:149–158
33. Belický Š, Katrlík J, Tkáč J. Glycan and lectin biosensors. Essays Biochem 2016;60:37–47
34. Kerjaschki D, Sharkey DJ, Farquhar MG. Identification and characterization of podocalyxin–the major sialoprotein of the renal glomerular epithelial cell. J Cell Biol 1984;98:1591–1596
35. Dekan G, Gabel C, Farquhar MG. Sulfate contributes to the negative charge of podocalyxin, the major sialoglycoprotein of the glomerular filtration slits. Proc Natl Acad Sci U S A 1991;88:5398–5402
36. Gelberg H, Healy L, Whiteley H, Miller LA, Vimr E. In vivo enzymatic removal of alpha 2–>6-linked sialic acid from the glomerular filtration barrier results in podocyte charge alteration and glomerular injury. Lab Invest 1996;74:907–920
37. Song K, Fu J, Song J, et al. Loss of mucin-type *O*-glycans impairs the integrity of the glomerular filtration barrier in the mouse kidney. J Biol Chem 2017;292:16491–16497
38. Holthöfer H, Pettersson E, Törnroth T. Diabetes mellitus associated changes in glomerular glycocompounds: a fluorescence microscopical study. Histochem J 1987;19:351–356
39. Truong LD, Phung VT, Yoshikawa Y, Mattioli CA. Glycoconjugates in normal human kidney. A histochemical study using 13 biotinylated lectins. Histochemistry 1988;90:51–60
40. Ruhaak LR, Uh HW, Beekman M, et al. Decreased levels of bisecting GlcNAc glycoforms of IgG are associated with human longevity. PLoS One 2010;5:e12566