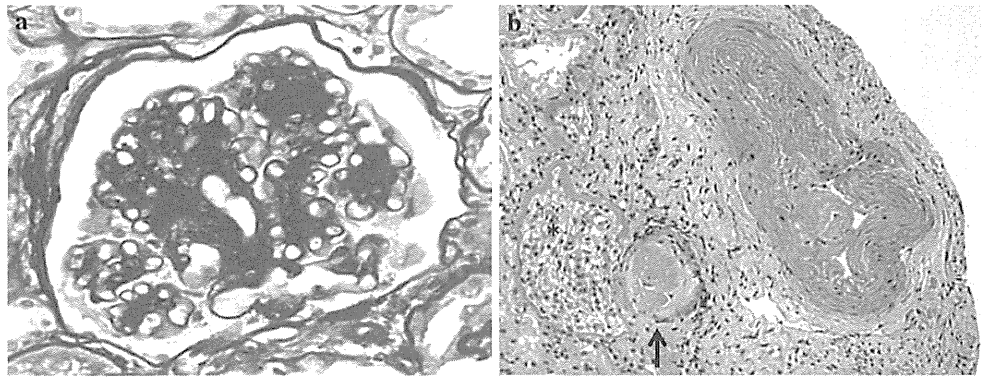

Fig. 1 Representative microscopic findings in type 2 diabetic patients with normoalbuminuria (normal proteinuria) and low eGFR (<60 ml/min/1.73 m^2). **a** Severe diffuse lesions in a patient classified as typical lesions of diabetic nephropathy (category II) (Case no. 3, Table 1; periodic acid-Schiff (PAS) stain ×200). **b** Mild diffuse lesions (*asterisk*) associated with global glomerular sclerosis (*arrow*) and disproportionately advanced arteriosclerosis, which denotes the presence of diabetic kidney lesions as well as nephrosclerosis, in a patient classified as atypical patterns (category III) (Case no.12, Table 1; PAS stain ×100)

and 10 % of patients with proteinuria); category II with typical diabetic lesions with an approximately balanced severity of glomerular, tubulointerstitial, and arteriolar changes similar to type 1 diabetes (30 % of patients with microalbuminuria and 55 % of patients with proteinuria); and category III with disproportionately advanced tubulointerstitial lesions, vascular lesions, and global glomerulosclerosis, despite minor diabetic glomerulopathy (35 % of patients with microalbuminuria and 35 % of patients with proteinuria). We categorized the renal lesions of 15 patients with normoalbuminuria (normal proteinuria) and low eGFR according to this classification (Table 1). As a result, no patients were classified as having almost normal biopsies (category I), 6 patients (40 %) were classified as having typical lesions of diabetic nephropathy (category II), and 9 patients (60 %) were classified as having atypical patterns (category III). Representative microscopic findings of renal biopsies from type 2 diabetic patients with normoalbuminuria (normal proteinuria) and low eGFR are shown in Fig. 1. Figure 1a shows severe diffuse lesions in a patient classified as category II (Case no. 3, Table 1). Figure 1b shows mild diffuse lesions associated with global glomerular sclerosis and disproportionately advanced arteriosclerosis, which denotes the presence of diabetic kidney lesions as well as nephrosclerosis, in a patient classified as category III (Case no. 12, Table 1). Another study of type 2 diabetic patients with low eGFR (<60ml/min/1.73 m^2) by research biopsy also indicated that the typical glomerular changes of diabetic nephropathy were less common in normoalbuminuric (3/8) patients than in microalbuminuric (5/6) or macroalbuminuric (17/17) patients [30].

These results suggest that multifactorial pathogenesis in addition to diabetic conditions may contribute to the kidney lesions in type 2 diabetic patients with normoalbuminuric renal insufficiency.

Outcomes of diabetic patients with normoalbuminuric renal insufficiency

Although the outcome of diabetic patients with normoalbuminuric renal insufficiency remains controversial, it is likely to be better than that of diabetic patients with albuminuria, even with preserved renal function. In a study of 89 diabetic patients with eGFR <60 ml/min/1.73 m^2 (22 type 1 diabetes, 67 type 2 diabetes), 15 (17 %), 36 (40 %), and 38 (43 %), were found to be normoalbuminuric, microalbuminuric, and macroalbuminuric at baseline, respectively. During the 38-month follow-up, none of the normoalbuminuric patients started dialysis (microalbuminuric patients 2/36, macroalbuminuric patients 10/38), and their albumin excretion rates and serum creatinine levels were stable. Furthermore, none of the normoalbuminuric patients died (microalbuminuric patients 3/36, macroalbuminuric patients 7/38) during the follow-up period [25]. In a population-based study of 1,538 people with type 2 diabetes, 51, 32, and 17 %, were found to be normoalbuminuric, microalbuminuric, and macroalbuminuric at baseline, respectively. During the 11-year follow-up, increasing trends of hazard rate ratios for all-cause mortality and cardiovascular mortality by decreasing eGFR values were observed in macroalbuminuric people only [31]. In addition, our study also showed similar results [20]. In our study, the outcomes were defined as the first occurrence of renal events (requirement of dialysis, or a 50 % decline in eGFR from baseline), cardiovascular events (cardiovascular death, nonfatal myocardial infarction, coronary interventions, or nonfatal stroke), and all-cause mortality. The mean follow-up period was 8.1 years (range 5–9,739 days), and there were 118 renal events, 62 cardiovascular events, and 45 deaths during the follow-up period. The hazard ratios for renal events, cardiovascular events, and all-cause mortality were increased by

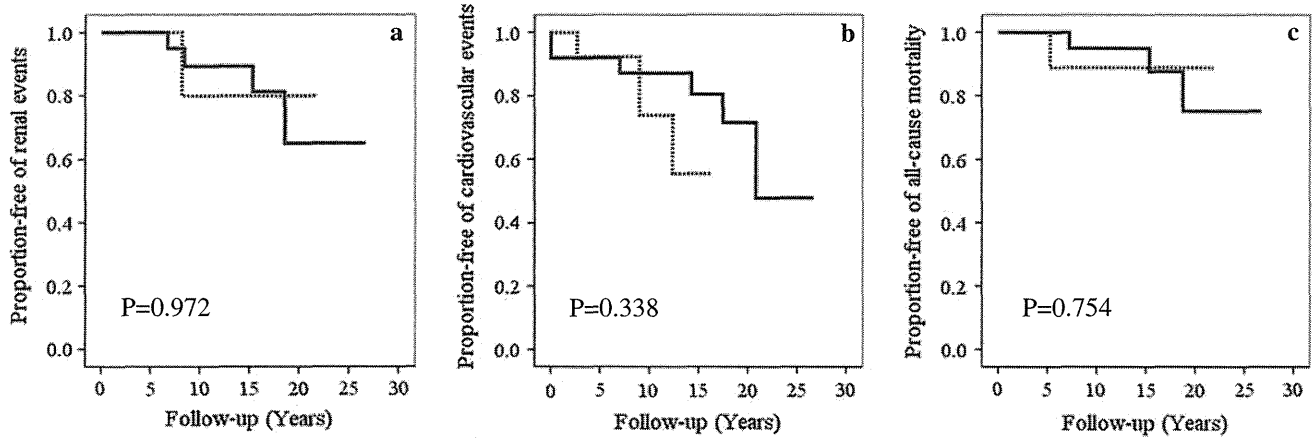

Fig. 2 Event-free rates of **a** renal events, **b** cardiovascular events, and **c** all-cause mortality in type 2 diabetic patients with normoalbuminuria (normal proteinuria) stratified by eGFR categories according to Kaplan–Meier method. Differences between groups were compared by the log-rank test. *Solid line* normoalbuminuria (normal proteinuria) and eGFR ≥60 ml/min/1.73 m^2 group, *dotted line* normoalbuminuria (normal proteinuria) and eGFR <60 ml/min/1.73 m^2 group

decreasing eGFR levels in patients with macroalbuminuria (severe proteinuria) only, and macroalbuminuria (severe proteinuria) was a major clinical determinant of renal events and all-cause mortality. Fifteen patients with normoalbuminuria (normal proteinuria) and low eGFR had 1 renal event, 3 cardiovascular events, and 1 death during the follow-up period (Table 1). One patient who developed end-stage renal disease was positive for anti-hepatitis C virus (HCV) antibody, although renal biopsy showed typical diabetic glomerular lesions, such as diffuse and exudative lesions, without HCV-related glomerulonephritis (Case no. 5, Table 1). Event-free rates of renal events, cardiovascular events, and all-cause mortality in patients with normoalbuminuria (normal proteinuria) were not significantly different between eGFR categories (Fig. 2).

In contrast to the previous results outlined above, including our report, several studies showed that higher levels of urinary albumin excretion within the normal range predict faster decline in GFR and higher incidence of cardiovascular disease in diabetic patients [32, 33]. Furthermore, some studies demonstrated that albuminuria and renal function independently or respectively predict renal events, cardiovascular events, and death in diabetic patients [13, 34–36]. Therefore, further studies on clinical impacts of low GFR without albuminuria and new biomarkers for early and definitive diagnosis of diabetic nephropathy are required in clinical settings.

In this sense, the newly established prospective registry system, the Japan Diabetic Nephropathy Cohort Study (JDNCS), may provide key insights for future perspectives [37]. The Japanese Society of Nephrology established a nationwide, web-based, and prospective registry system including two basic registries— the J-RBR and the J-KDR [18, 38]. In addition to the two basic registries, the JDNCS enrolled Japanese diabetic patients with broad-ranging albuminuria (proteinuria) stage and GFR levels. The aims of the JDNCS are to obtain clinical data and urine samples for revising the clinical staging of diabetic nephropathy, and to develop new diagnostic biomarkers for early detection or prediction of diabetic nephropathy. The prevalence of normoalbuminuria in patients with low eGFR (<60 ml/min/1.73 m^2) at baseline was 19.8 % in the JDNCS. Interestingly, the JDNCS is now prospectively collecting clinical data annually. Moreover, participants with diabetes are also enrolled in the J-RBR and the J-KDR [37]. Therefore, the combined data of the three registries will allow evaluation of the natural course and long-term outcomes of diabetic nephropathy, including patients with normoalbuminuric renal insufficiency, in the near future.

Conclusion

We focused on the structural–functional relationships and outcomes of diabetic nephropathy with normoalbuminuric renal insufficiency. Although the pathogenesis of normoalbuminuric renal insufficiency in diabetic nephropathy remains to be fully elucidated, disproportionately advanced tubulointerstitial lesions, vascular lesions, and global glomerulosclerosis, despite minor diabetic glomerular lesions, which denote the presence of diabetic kidney lesions as well as nephrosclerosis, are likely to be related to the development of normoalbuminuric renal insufficiency in some type 2 diabetic patients. However, other processes may contribute to the development of normoalbuminuric renal insufficiency with advanced diabetic glomerular lesions. Furthermore, long-term outcomes of diabetic patients with normoalbuminuric renal insufficiency remain

controversial. Further studies to gain a better understanding of the structural–functional relationships and natural history of diabetic patients with normoalbuminuric renal insufficiency may provide insights for the development of therapeutic strategies for diabetic nephropathy.

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REVIEW ARTICLE

WCN 2013 Satellite Symposium "Kidney and Lipids"

Treatment and impact of dyslipidemia in diabetic nephropathy

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Abstract Recent epidemiological research revealed that dyslipidemia is a risk factor for development and progression of diabetic nephropathy. Results from interventional studies revealed the possibility that anti-hyperlipidemic agents have a better effect on diabetic nephropathy through improvement of albuminuria and loss of renal function. In addition, dyslipidemia may be a consequence of albuminuria and renal dysfunction, thereby perpetuating kidney damage. Today, the proportion of diabetic patients receiving statins is increasing due to their beneficial effect on cardiovascular mortality. However, treatment for patients should be determined based on consideration of the risk and benefit of the treatment. More insight into the pathogenesis of diabetic nephropathy and the effects of life-style changes is required.

Keywords Diabetic nephropathy · Dyslipidemia · Cardiovascular disease · End-stage renal disease

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Introduction

In the past, epidemiological research in diabetes has found that albuminuria and renal dysfunction are dominant risk factors for the progression of diabetic nephropathy. Some interventional studies have revealed that strict glycemic control reduces the risk of development and progression of albuminuria [1, 2].

It is a crucial fact that diabetic patients are at high risk of cardiovascular events. To prevent these events, dyslipidemia should be carefully controlled because it is one of the well-known risk factors. Statins and fibrates are representative drugs for dyslipidemia. Besides reducing plasma cholesterol levels they are thought to have many pleiotropic effects including improvement of endothelial function and inflammation [3, 4]. However, treatment of patients with dyslipidemia is complicated because it is not a simple metabolic disorder but closely related to the patient's lifestyle. For this reason, lowering the level of cholesterol will not always result in a reduction of the risks.

Here, we focus on the treatment and impact of dyslipidemia on the progression of diabetic nephropathy.

Dyslipidemia as a complication of diabetic nephropathy

One cross-sectional study implied that patients with diabetic nephropathy had significant increases in triglycerides and total cholesterol levels, reduced levels of apolipoprotein A (ApoA)-I and ApoA-II, and increased levels of ApoC-II and ApoC-III [5]. Other cross-sectional studies of patients from the Diabetic Control and Complications Trial/Epidemiology of Diabetic Interventions and Complications study group revealed that high levels of triglycerides, low-density lipoprotein (LDL) cholesterol, total

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cholesterol, and ApoB are associated with albuminuria [6]. ApoB is thought to be related to cardiovascular events in some studies [7, 8]. In this way, the studies revealed the relationships between lipid profiles and diabetic nephropathy.

Cardiovascular events are also important complications in diabetic patients [9]. A meta-analysis reported the relationship between dyslipidemia and cardiovascular risk [10]; however, risks for diabetic patients are not well known.

Dyslipidemia and loss of renal function

The 'lipid nephrotoxicity' hypothesis was advocated by Moorhead et al. in 1982 as a description of the effect of dyslipidemia on renal dysfunction [11]. Under this hypothesis, mesangial proliferation caused by accumulation of lipoprotein into mesangial cells induces glomerulosclerosis. This theory has been updated recently including the concept of inflammation stress modifying lipid homeostasis and tissue lipid accumulation [12]. With regard to diabetes and lipids, Hartroft [13] discovered in 1954 that intraluminal fat was found in both preglomerular and postglomerular vessels of diabetics patients with Kimmelstiel–Wilson lesions. In addition to this study, a lot of basic research has discovered the mechanisms between dyslipidemia and diabetic nephropathy [14]. Studies revealed that transforming growth factor-β signaling [15], renin–angiotensin system [16], S100A8/TLR4 signaling [17], and oxidative stress [18] may play an important role in the progression of diabetic nephropathies. Concerning the development of albuminuria, the importance of the deterioration of glycocalyx, which is on the surface of endothelium, was highlighted [19]. These factors orchestrated each other, thereby perpetuating the progression of diabetic nephropathy. Further studies will be required for a better understanding of diabetic nephropathy.

Some epidemiological studies of general cohorts have elucidated the relationships between dyslipidemia and loss of renal function. The Framingham Offspring Study which consists of 1,916 general population subjects with a follow-up of 9.5 years, revealed that low high-density lipoprotein (HDL) cholesterol levels are one of the risk factors for incident albuminuria [20]. An analysis of 1,440 general Japanese cohorts that participated in the Hisayama study revealed that metabolic syndrome defined as the presence of components including high triglyceride levels and low HDL cholesterol levels are associated with a risk of developing chronic kidney disease (CKD) [21]. A study of 4,483 healthy males revealed that dyslipidemia including high total cholesterol levels, high non-HDL cholesterol levels, and low HDL cholesterol levels are associated with a risk of renal dysfunction [22].

According to these facts, dyslipidemia may be one of the potential risk factors for loss of renal functions in a healthy subject.

Relationships between dyslipidemia and progression or regression of diabetic nephropathy

The stages in diabetic renal disease were reported by Mogensen et al. [23] in 1983. According to their theory, elevated urinary albumin excretion and following persistent proteinuria are important manifestations of diabetic nephropathy, and many studies defined them as surrogate markers for end-stage renal disease.

Some cohort studies of diabetic patients have proven the risk factors associated with the progression or regression of the staging. Regarding the development of micro- and macroalbuminuria, a cohort study of 27,805 patients with type 1 diabetes followed up for 2.5 years revealed that, besides diabetes duration and glycosylated hemoglobin, dyslipidemia is a risk factor for developing albuminuria [24]. A cohort study of 574 patients with type 2 diabetes followed up for 7.8 years also revealed that, as well as high mean blood pressure and hyperglycemia, high plasma cholesterol levels are the main risk factors for development of dyslipidemia [25]. In this study, the participants with a combination of these three risk factors are a high-risk group for progression to diabetic nephropathy.

Associations between reduction of urinary albumin and dyslipidemia were reported in a cohort study of 386 patients with type 1 diabetes [26]. In this study, along with low levels of glycosylated hemoglobin and low systolic blood pressure, low levels of both cholesterol and triglycerides were independently associated with regression of microalbuminuria. Moreover, these factors had additive effects on regression of microalbuminuria.

A small number of studies reported an association between dyslipidemia and loss of renal functions. Regarding the rate of decline in glomerular filtration rate (GFR), a prospective study of 30 patients with type 1 diabetes revealed that high serum cholesterol, triglycerides and apolipoprotein B were correlated to a rapid decline in glomerular filtration rate [27].

As described above, evidence has been accumulated to suggest that dyslipidemia is one of the risk factors for progression and regression of diabetic nephropathy. However, as far as we knew, there have been few studies reporting the association with end-stage renal disease, or renal replacement therapy. A report of a scientific workshop sponsored by the National Kidney Foundation (NKF) and the US Food and Drug Administration (FDA) indicated that evidence was insufficient to use a change of albuminuria as a surrogate marker as a clinical endpoint [28].

Long-term follow-up studies are needed to demonstrate the causal relationships between dyslipidemia and end-stage renal disease from diabetic nephropathy.

Treatment of dyslipidemia and diabetic nephropathy

With regard to the treatment of dyslipidemia in patients with diabetes, there were some interventional trials of anti-hypercholesterolemic agents including fibrates and statins.

The Diabetes Atherosclerosis Intervention Study (DAIS) is a randomized study that assessed the effect of fenofibrate on type 2 diabetic patients [29]. In this study, fenofibrate reduced the worsening of urine albumin excretion and the effects were mainly observed in the progression from normoalbuminuria to microalbuminuria. The Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study also evaluated the effect of fenofibrate on type 2 diabetes [30]. From this study, it was proved that fenofibrate is effective in lowering the decline of the estimated glomerular filtration rate (eGFR) and reducing the progression of albuminuria. Additionally in this study, patients treated with fenofibrate had higher rates of regression of albuminuria than the placebo group. This evidence suggests that fenofibrate is effective in ameliorating diabetic nephropathy. In a meta-analysis of these two studies, the significant effect on the regression from microalbuminuria to normoalbuminuria was proved; however, progression from microalbuminuria to macroalbuminuria was not significant [31].

The effect of statins on diabetic nephropathy was examined in the Collaborative Atorvastatin Diabetes Study (CARDS) [32]. Treatment with atorvastatin was compared with a placebo in this study, and was associated with an improvement in annual changes in eGFR (0.18 mL/min/1.73 m^2/year). It is noteworthy that atorvastatin ameliorated eGFR without improving albuminuria, when comparing angiotensin-converting enzyme inhibitors which have renoprotective effects and prevent the onset of albuminuria [33].

There is still a lot of uncertainty about the effect of statins. The effect on renal protection was not demonstrated in the Study of Heart and Renal Protection (SHARP) which included 2,094 (33 %) patients with diabetes [34], and the Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial (ALLHAT) which included 3,638 (36 %) patients with diabetes [35]. A meta-analysis also showed that regression of albuminuria [31] and changes in eGFR [36] were not observed in patients with diabetes treated with statins.

There seems to be no definite answer for treatment of dyslipidemia in diabetic patients from the viewpoint of anti-hyperlipidemic agents. One of the supposed causes of inconsistency in results is that kidney diseases in patients with diabetes may not be uniform, but consist of many renal diseases [37]. In some cases, renal biopsies might be needed to assess the accurate risks [38].

Diabetic patients are at higher risk for cardiovascular mortality compared with non-diabetic patients [10, 39]. There is sufficient evidence, such as SHARP [34], to show that statins reduce the risk of cardiovascular events. Considering these facts, many diabetic patients might benefit from statin treatment. An increasing number of patients are now receiving this treatment. In the analysis of the National Health and Nutrition Examination Survey (NHANES) 2005–2006, 93.5 % of diabetic men aged 65–69 without cardiovascular disease received statins [40].

On the other hand, administration of statin may have adverse side-effects, including myopathy [41], renal toxicity [42], and incident diabetes [43]. A study comparing the risks and benefits of statins concluded that cardiovascular benefits outweigh the increased risk of new-onset diabetes [44]. It is beyond doubt that each patient's risk must be taken into account before administration of statins.

It is also important to consider changes in life-style; however, the difficulty lies in improving renal and cardiovascular events through life-style changes [45]. It remains a challenge for future research to examine the impact of life-style changes.

Concluding remarks and future directions

In considering the complexity of the problem of diabetic nephropathy, many aspects of a patient's condition and treatment should be taken into account. Further insight into the pathogenesis of dyslipidemia, and the risk and benefits of each treatment may be beneficial for each patient.

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Conflict of interest The authors have declared that no conflict of interest exists.

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Predictive Effects of Urinary Liver-Type Fatty Acid–Binding Protein for Deteriorating Renal Function and Incidence of Cardiovascular Disease in Type 2 Diabetic Patients Without Advanced Nephropathy

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OBJECTIVE—To improve prognosis, it is important to predict the incidence of renal failure and cardiovascular disease in type 2 diabetic patients before the progression to advanced nephropathy. We investigated the predictive effects of urinary liver-type fatty acid–binding protein (L-FABP), which is associated with renal tubulointerstitial damage, in renal and cardiovascular prognosis.

RESEARCH DESIGN AND METHODS—Japanese type 2 diabetic patients ($n = 618$) with serum creatinine ≤1.0 mg/dL and without overt proteinuria were enrolled between 1996 and 2000 and followed up until 2011. Baseline urinary L-FABP was measured with an enzyme-linked immunosorbent assay. The primary end points were renal and cardiovascular composites (hemodialysis, myocardial infarction, angina pectoris, stroke, cerebral hemorrhage, and peripheral vascular disease). The secondary renal outcomes were the incidence of a 50% decline in estimated glomerular filtration rate (eGFR), progression to an eGFR <30 mL/min/1.73 m^2, and the annual decline rate in eGFR.

RESULTS—During a 12-year median follow-up, 103 primary end points occurred. The incidence rate of the primary end point increased in a stepwise manner with increases in urinary L-FABP. In Cox proportional hazards analysis, the adjusted hazard ratio in patients with the highest tertile of urinary L-FBAP was 1.93 (95% CI 1.13–3.29). This relationship was observed even when analyzed separately in normoalbuminuria and microalbuminuria. Patients with the highest tertile of urinary L-FABP also demonstrated a higher incidence of the secondary renal outcomes.

CONCLUSIONS—Our results indicate that urinary L-FABP may be a predictive marker for renal and cardiovascular prognosis in type 2 diabetic patients without advanced nephropathy.

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Patients with type 2 diabetes are at a high risk for the progression to end-stage renal disease (ESRD) and incidence of cardiovascular disease (CVD), both of which are life-threatening complications (1). To improve prognosis in diabetic patients, it is clinically important to identify patients at high risk for these disorders as early as possible and to initiate disease management in a timely and appropriate manner.

ESRD and CVD share a number of clinical features and risk factors that are important therapeutic targets. Microalbuminuria is well known to be a common risk factor of ESRD and CVD, and a reduction of urinary albumin excretion (UAE) via any intervention results in a reduced future incidence of these disorders (2,3). However, many patients still develop ESRD and CVD despite improvements in their outcome resulting from recent aggressive multifactorial management (4–6). Thus, we need to explore new predictive markers for these disorders that are independent of UAE.

Renal dysfunction, also referred to as chronic kidney disease (CKD), is also an important predictive factor for ESRD and CVD that is independent of increases in UAE (7,8). There is a growing body of evidence suggesting that tubulointerstitial damage, as well as glomerular damage, contributes to a decline in renal function (9). Thus, measuring factors that relate to the risk of renal tubulointerstitial damage may be potentially useful for identifying patients at higher risk for ESRD and CVD.

Liver-type fatty acid–binding protein (L-FABP), an intracellular carrier protein of free fatty acids, is expressed in the liver and kidney. In the kidney, the expression of L-FABP is predominantly located in the proximal tubules. The high levels of urinary L-FABP were previously suggested to be associated with renal tubulointerstitial damage because excessive reabsorption of free fatty acids into the proximal tubules induces tubulointerstitial damage (10–12). Based on these findings, we conducted a long-term observational study to investigate whether urinary levels of L-FABP were predictive for the progression

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of renal dysfunction and incidence of CVD in patients with type 2 diabetes without advanced nephropathy.

RESEARCH DESIGN AND METHODS

Subject recruitment

Japanese patients with type 2 diabetes were recruited from participants that were registered in the Shiga Prospective Observational Follow-up Study between 1996 and 2000 (13). Patients with cancer, recent occurrences of CVD within the past year, infectious disease, collagen disease, and nondiabetic kidney disease, as confirmed by a renal biopsy, were excluded from the study. After obtaining written informed consent, each individual provided a 24-h urine sample and fasting blood sample at baseline. The serum and urine samples were kept at −80°C if they were not analyzed immediately. In this study, patients with normoalbuminuria/microalbuminuria and serum creatinine (Cr) ≤1.0 mg/dL were eligible. Based on the UAE rate (UAER) at baseline, patients were classified as having normoalbuminuria (UAER <20 μg/min), microalbuminuria (20≤ UAER <200 μg/min), or overt proteinuria (UAER ≥200 μg/min). Serum concentrations of Cr were measured via an enzymatic method. Finally, 618 patients with normoalbuminuria (n = 422) and microalbuminuria (n = 196) were enrolled and followed up until the end of 2011 or the first occurrence of any renal and cardiovascular composite end points. The participants annually underwent standardized clinical examinations and biochemical tests during the follow-up period. HbA_{1c} levels were presented as National Glycohemoglobin Standardization Program values, according to the recommendations of the Japanese Diabetes Society (14). The study protocol and informed consent procedure were approved by the Ethics Committee of Shiga University of Medical Science.

Measurement of urinary L-FABP

Urinary concentrations of L-FABP were measured using a two-step sandwich enzyme-linked immunosorbent assay (15), and all stored samples obtained at baseline were simultaneously measured in 2002. In this study, the baseline levels of urinary L-FABP in each individual were obtained from one urine sample, as described above. The sensitivity of this assay was >3.0 μg/L. Both of the intra- and interassay coefficients of variation were <10%, respectively. Urinary concentrations of Cr were also measured via an enzymatic method. Urinary excretion levels of L-FABP were expressed as micrograms per gram of Cr.

Follow-up evaluation

The primary end point was the first occurrence of any of the renal and cardiovascular composites, which were as follows: initiation of chronic hemodialysis and the occurrence of myocardial infarction, angina pectoris, stroke, cerebral hemorrhage, peripheral vascular disease (PAD), and death from cardiovascular causes. Myocardial infarction was defined as a clinical presentation characterized by typical symptoms, electrocardiographic changes associated with an elevation of cardiac biomarkers, and angiographic evidence of coronary thrombosis. Angina pectoris was defined as the presence of responsible lesions detected by imaging studies with a history of typical chest pain or electrocardiographic changes and invasive cardiovascular interventions. Stroke, including ischemic stroke and cerebral hemorrhage, was defined as a persistent focal neurologic symptom in which the onset was sudden and was not due to trauma or a tumor and where the responsible lesion was detected by imaging studies. PAD was defined as revascularization with typical symptoms such as cold feet or intermittent claudication. At the annual physical examination of this cohort, we directly examined patients and checked their medical records to identify the onset of primary end points. In a fatal case, the medical record was reviewed by physicians to identify the cause of death. If the cause of death was unclear, it was not counted as a death from cardiovascular cause.

In evaluating the secondary outcomes, we separately assessed CVD events and renal secondary outcomes. In regards to secondary renal outcomes, we assessed two categorical outcomes: a 50% decline in the estimated glomerular filtration rate (eGFR) from baseline and the progression to stage 4 CKD (eGFR <30 mL/min/1.73 m^2) and one outcome as a continuous variable, the annual rate of decline in eGFR over the study period. eGFR was calculated using the simplified prediction equation proposed by the Japanese Society of Nephrology (16): eGFR (mL/min/1.73 m^2) = 194 × [age (years)]$^{-0.287}$ × [serum Cr (mg/dL)]$^{-1.094}$ × 0.739 (for female). At baseline, all participants had an eGFR >60 mL/min/1.73 m^2. In the analysis of the annual rate of decline in eGFR, only patients that were observed over 3 years were used in the estimation of the rate of decline in eGFR. The annual rate of decline in eGFR over the course of the study was determined from the slope of each individual from the linear regression analysis and expressed in mL/min/1.73 m^2/year.

Statistical analysis

Data are expressed as mean ± SD or median (interquartile range [IQR]), where appropriate. Patients were divided into tertiles according to the urinary levels of L-FABP at baseline. Statistical significance of the differences among the three subgroups was determined via a χ^2 test for categorical variables, and an ANOVA followed by the Tukey-Kramer test for normally distributed variables or the Kruskal-Wallis test for nonnormally distributed continuous variables. The incidence rate per 1,000 person-years for each outcome was calculated. The cumulative incidence was estimated by using the Kaplan-Meier method and compared with the log-rank test. The follow-up time was censored if any primary end point occurred or if the patient was unavailable for follow-up. The adjusted hazard ratio (HR) for each outcome was evaluated by using a Cox proportional hazards regression model. In this analysis, the known cardiovascular risk factors were age, sex, BMI, HbA_{1c}, total cholesterol, triglycerides, HDL cholesterol, hypertension, use of renin-angiotensin system (RAS) inhibitors, systolic and diastolic blood pressure, past history of CVD, stage of nephropathy (or log UAER for log urinary L-FABP), and eGFR at baseline. The difference of the annual decline rate in eGFR after controlling for the effect of systolic BP and log albumin excretion rate (AER) was assessed with the ANCOVA model. All analyses were performed with the SPSS software package (version 11; SPSS Inc., Chicago, IL). A two-sided P value <0.05 was considered statistically significant.

RESULTS—The baseline characteristics of the 618 patients and three subgroups stratified by urinary levels of L-FABP at baseline are presented in Table 1. Age, duration of diabetes, HbA_{1c}, total cholesterol, systolic BP, hypertension, use of RAS inhibitors, urinary AER, microalbuminuria, urinary β_2-microglobulin, and

Table 1—*Baseline clinical characteristics of all patients with type 2 diabetes and the three subgroups stratified according to the levels of urinary L-FABP*

Variable	All	Urinary L-FABP (μg/g Cr)			*P* value[a]
		≤5.0	5.0–9.5	>9.5	
n	618	206	206	206	
Male (%)	54.9	49.5	58.3	56.8	NS
Age (years)	59 ± 10	58 ± 10	58 ± 10	62 ± 10	<0.01
BMI (kg/m^2)	23.4 ± 3.3	23.5 ± 3.3	23.4 ± 3.4	23.4 ± 3.3	NS
Duration (years)	11 ± 8	10 ± 7	10 ± 8	13 ± 9	<0.01
Diet/OHA/insulin (%)	25/52/23	31/52/17	26/56/18	17/50/33	<0.01
HbA_{1c} (%)	7.5 ± 1.1	7.5 ± 1.1	7.4 ± 1.0	7.7 ± 1.2	<0.01
Total cholesterol (mg/dL)	213 ± 36	220 ± 34	209 ± 34	212 ± 38	<0.01
HDL cholesterol (mg/dL)	56 (46–66)	57 (47–67)	54 (46–67)	55 (47–64)	NS
Triglycerides (mg/dL)	98 (71–143)	98 (71–148)	96 (69–141)	98 (63–143)	NS
Systolic BP (mmHg)	129 ± 14	127 ± 14	132 ± 14	134 ± 13	<0.01
Diastolic BP (mmHg)	76 ± 10	76 ± 9	77 ± 9	76 ± 11	NS
Hypertension (%)	46.9	40.7	45.1	54.9	<0.05
Using RAS inhibitors (%)	14.2	11.1	10.2	19.9	<0.05
Past history of CVD (%)	10.0	8.2	7.3	14.6	<0.05
Urinary AER (μg/min)	11 (7–27)	8 (5–15)	12 (7–28)	16 (9–43)	<0.01
Microalbuminuria (%)	31.7	18.9	32.5	43.7	<0.01
eGFR (mL/min/1.73 m^2)	88 ± 18	87 ± 18	89 ± 17	87 ± 19	NS
Urinary β_2-microglobulin (μg/g Cr)	120 (81–206)	93 (69–136)	122 (82–183)	175 (106–369)	<0.01
Urinary L-FABP (μg/g Cr)	7.2 (4.2–11.5)	3.4 (2.3–4.3)	7.2 (6.0–8.4)	14.2 (11.4–20.6)	<0.01

Data are expressed as mean ± SD for normally distributed continuous variables or median (IQR) for skewed continuous variables unless otherwise indicated. OHA, oral hypoglycemic agent. [a]Differences between the three subgroups were compared with a χ^2 test for categorical variables and ANOVA for continuous variables.

past history of CVD were significantly different between the three subgroups. Additionally, urinary levels of L-FABP in patients with microalbuminuria were higher than in those with normoalbuminuria (9.1 μg/g Cr [IQR 5.9–15.8 μg/g Cr] vs. 6.1 μg/g Cr [3.7–9.9 μg/g Cr]; $P < 0.01$, Mann-Whitney *U* test).

Incidence rates of the primary end point

During a 12-year (IQR 6–15 years) median follow-up, the primary end points occurred in 103 patients (i.e., 7 patients presented with chronic hemodialysis, 25 with myocardial infarction, 35 with angina pectoris, 24 with stroke, 5 with cerebral hemorrhage, and 7 with PAD). The incidence rate per 1,000 person-years of the primary end point was 16.5 in all participants, and increased in a stepwise fashion with increasing urinary levels of L-FABP (i.e., 9.5 in the lowest tertile of urinary L-FABP, 15.5 in the middle tertile, and 25.4 in the highest tertile) (Table 2). As shown in Fig. 1, the cumulative incidences of the primary end point were significantly different among the three subgroups ($P < 0.0001$, log-rank test). The risk for the primary end point was evaluated by using the Cox proportional hazards model (Table 2). When adjusted for known cardiovascular risk factors, the HR in the highest tertile of urinary L-FABP was 1.93 (95% CI 1.13–3.29). Using log urinary L-FABP as a continuous variable, instead of the tertiles of urinary L-FABP, the HR of log urinary L-FABP for primary end points was 2.16 (95% CI 1.23–3.79) after adjusting for age, sex, log UAER, and eGFR at baseline, and 1.79 (1.06–3.01) after adjusting for known cardiovascular risk factors.

Effects of urinary L-FABP on secondary renal outcomes

The incidence rates per 1,000 person-years for a 50% decline in eGFR from baseline in the three subgroups were 4.8 in the lowest tertile, 6.0 in the middle tertile, and 18.3 in the highest tertile. Also, the incidence rates for the progression to stage 4 CKD (eGFR <30 mL/min/1.73 m^2) were 1.8 in the lowest tertile, 2.4 in the middle tertile, and 11.1 in the highest tertile. The adjusted HRs for these secondary renal outcomes were significantly higher in the highest tertile (Table 2). The annual rate of decline in eGFR (mL/min/1.73 m^2/year) was −1.31 (95% CI −0.46 to −2.33) in the lowest tertile, −1.65 (−1.02 to −2.25) in the middle tertile, and −1.80 (−1.05 to −3.21) in the highest tertile ($P = 0.002$, Kruskal-Wallis test), and there was a significant effect of urinary L-FABP on the annual decline rate in eGFR after controlling for the effect of systolic BP and log AER ($F = 3.54$, $P = 0.03$, ANCOVA). In addition, patients in the highest tertile of urinary L-FABP showed the highest incidence of a 50% decline in eGFR, which was associated with the highest incidence of CVD. The cumulative incidence of CVD was significantly higher in patients with a 50% decrease in eGFR than those without it ($P = 0.034$, log-rank test).

Risk of urinary L-FABP according to the stage of diabetic nephropathy

We finally investigated the incidence rates and HRs for the primary end point in the subgroups stratified according to the levels of urinary L-FABP and the stages of diabetic nephropathy at baseline. As shown in Table 3, the incidence rates and HRs adjusted from known cardiovascular risk factors increased with increasing stages of nephropathy and urinary L-FABP levels. Interestingly, the adjusted HR of the subgroups, categorized according to the highest tertile of urinary L-FABP, was significantly higher even in

Table 2—*Incidence rates and HRs for primary end point and secondary outcomes of patient subgroups stratified according to the levels of urinary L-FABP*

	n	Incidence rate (1,000 person-years)	Adjusted HR (95% CI)[a]		
			Model 1	Model 2	Model 3
Primary end point (hemodialysis and CVD)					
Lowest tertile	21	9.5	1 (reference)	1 (reference)	1 (reference)
Middle tertile	33	15.5	1.60 (0.93–2.77)	1.51 (0.87–2.64)	1.64 (0.93–2.88)
Highest tertile	49	25.4	2.30 (1.37–3.86)	2.04 (1.20–2.69)	1.93 (1.13–3.29)
Secondary end points					
CVD events					
Lowest tertile	19	8.6	1 (reference)	1 (reference)	1 (reference)
Middle tertile	33	15.5	1.75 (0.99–3.09)	1.65 (0.93–2.92)	1.78 (0.99–3.20)
Highest tertile	44	23.4	2.26 (1.31–3.88)	2.00 (1.15–3.49)	1.76 (1.00–3.12)
50% decline in eGFR					
Lowest tertile	10	4.8	1 (reference)	1 (reference)	1 (reference)
Middle tertile	12	6.0	1.27 (0.55–2.94)	1.09 (0.47–2.54)	1.04 (0.44–2.46)
Highest tertile	32	18.3	3.87 (1.89–7.91)	3.09 (1.48–6.45)	2.43 (1.14–5.16)
Progression to stage 4 CKD[b]					
Lowest tertile	4	1.8	1 (reference)	1 (reference)	1 (reference)
Middle tertile	5	2.4	1.27 (0.34–4.74)	1.19 (0.32–4.47)	1.18 (0.30–4.57)
Highest tertile	21	11.1	5.92 (2.02–17.37)	5.05 (1.68–15.21)	3.53 (1.15–10.88)

[a]Adjusted HRs were calculated via the Cox proportional hazards model. Model 1, adjusted for age and sex; model 2, adjusted for age, sex, stage of nephropathy, and eGFR; model 3, adjusted for age, sex, BMI, HbA_{1c}, total cholesterol, log triglycerides, log HDL cholesterol, hypertension, use of RAS inhibitors, systolic and diastolic blood pressure, past history of CVD, stage of nephropathy, and eGFR. [b]Stage 4 CKD denotes eGFR <30 mL/min/1.73 m^2.

patients with normoalbuminuria. The effects of diabetic nephropathy and three categories of urinary L-FABP levels were independent of each other (P = 0.34 for interaction).

CONCLUSIONS—The present long-term observational study on type 2 diabetic patients without advanced nephropathy revealed that higher urinary levels of L-FABP were associated with deteriorating renal function and a higher incidence rate of CVD. These associations were observed in those with normoalbuminuria as well as those with microalbuminuria, when separately analyzed according to the stages of diabetic nephropathy. Thus, these findings suggest that urinary L-FABP can be used as a biomarker for predicting future renal dysfunction and incidence of CVD in type 2 diabetic patients with an early stage of nephropathy, in addition to albuminuria.

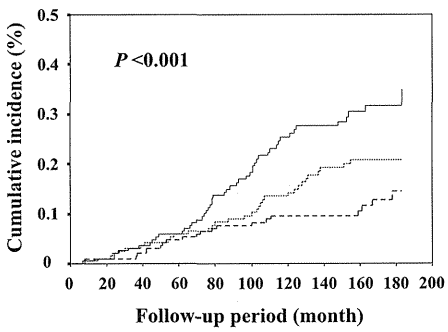

Figure 1—*Kaplan-Meier curves for cumulative incidences of primary end points of the three groups stratified by urinary L-FABP. Solid line, highest tertile group* (n = *206,* ≤*5.0 μg/g Cr); short-dashed line, middle tertile group* (n = *206, 5.0–9.5 μg/g Cr); long-dashed line, lowest tertile group* (n = *206,* >*9.5 μg/g Cr). Differences between groups were compared by a log-rank test.

Renal dysfunction is reported to correlate with the degree of tubulointerstitial damage (9). Although albuminuria per se reflects glomerular damage and subsequently induces renal tubulointerstitial damage, other factors and mechanisms, independent of albuminuria, must be involved in the development of tubulointerstitial damage under diabetic conditions. In fact, a recent study reported on cases where renal function rapidly declined without an increase in UAE (17). Urinary levels of L-FABP have been reported to be associated with the histological severity of renal tubulointerstitial lesions in human (15) and animal studies (18,19). Our study also found that urinary L-FABP correlated with urinary β_2-microglobulin, a marker of renal tubulointerstitial injury. Taken together, these findings suggest that urinary L-FABP may reflect tubulointerstitial damage and, therefore, predict the progression of deteriorating renal function. Furthermore, these results suggest the importance of tubulointerstitial damage in the development of renal dysfunction under diabetic conditions.

In the current study, we focused on the predictive effects of urinary L-FABP for deteriorating renal function and the onset of CVD in type 2 diabetic patients with early stages of nephropathy. Previously, there have been several clinical studies investigating the association between urinary L-FABP levels and the progression of diabetic nephropathy that mainly focused on the progression of nephropathy based on UAE. In a 4-year prospective cohort study on 54 patients with type 2 diabetes, Kamijo-Ikemori et al. (20) reported that higher urinary L-FABP levels were associated with the progression of eGFR to <60 mL/min/1.73 m^2. Additionally, Nielsen et al. (21) reported that higher urinary L-FABP levels predicted all-cause mortality in 165 patients with type 1 diabetes and normoalbuminuria, independent of UAE and other established risk factors. Our findings strengthen these previous results and provide further evidence that urinary L-FABP is a predictive biomarker for renal dysfunction and the onset of CVD in diabetic patients.

However, Nielsen et al. (22) recently reported that urinary L-FABP levels are

Table 3—*Incidence rates and adjusted HRs for primary end points in patient subgroups stratified according to the levels of urinary L-FABP and stages of diabetic nephropathy*

	Urinary L-FABP		
	Lowest tertile	Middle tertile	Highest tertile
Incidence rate (1,000 person-years)			
Normoalbuminuria	7.8	10.9	21.7
Microalbuminuria	17.8	25.7	31.0
Adjusted HR (95% CI)[a]			
Normoalbuminuria	1 (reference)	1.49 (0.72–3.09)	2.26 (1.15–4.45)
Microalbuminuria	1.72 (0.68–4.38)	2.70 (1.26–5.81)	2.18 (1.08–4.40)

[a]The HRs were adjusted for age, sex, BMI, HbA_{1c}, total cholesterol, log triglycerides, log HDL cholesterol, hypertension, use of RAS inhibitors, systolic and diastolic blood pressure, past history of CVD, and eGFR in the Cox proportional hazards model.

not related to a rapid decline in GFR in a 3-year intervention study on 63 type 1 diabetic patients with overt proteinuria. Massive albuminuria per se induces tubulointerstitial damage and then leads to renal dysfunction. Therefore, the effects of urinary L-FABP on tubulointerstitial lesionsand decline in GFR may disappear with an increase in albuminuria, such as overt proteinuria. Further investigation is needed to clarify this argument.

CKD, even a mild decline in renal function, is well acknowledged as an important risk factor for cardiovascular morbidity and mortality. A number of diabetic patients with renal dysfunction experience an onset of CVD before they initiate chronic hemodialysis. Also, our study demonstrated a higher incidence of CVD in patients who showed a 50% decline in eGFR during the follow-up than those who did not show a 50% decline.

There are some limitations in this study that must be addressed. In general practice, we do not perform renal biopsies in diabetic patients unless the complication of other renal diseases is suspected. Thus, we could not investigate the correlation between the urinary L-FABP levels and renal lesions in this study. Our study was designed as an observational follow-up study, and not an intervention trial. The treatment protocol for patients in this cohort was not controlled, and the influence of potential cofounders during the observation period was not analyzed. Furthermore, the time-dependent changes of urinary L-FABP levels during the follow-up period were not assessed. Urinary L-FABP may be modified by any intervention (23,24). Thus, a further study is required to answer the important question of whether the changes of urinary L-FABP levels are associated with the prognosis in diabetic patients.

In conclusion, the current study indicated that the high levels of L-FABP in urinary excretion were associated with deteriorating renal function and the high incidence of CVD in patients with type 2 diabetes. This association was markedly observed even in patients with normoalbuminuria. Thus, measurements of urinary L-FABP, in addition to albuminuria, may be clinically useful for the early identification of diabetic patients without advanced nephropathy and at a higher risk for renal disease and CVD. In addition, these results suggest the importance of tubulointerstitial damage in the development of renal dysfunction and CVD under diabetic conditions.

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S.A. designed the study protocol, researched data, and wrote the manuscript. M.H. designed the study protocol, contributed to discussion, and reviewed and edited the manuscript. D.K. researched data, contributed to discussion, and reviewed and edited the manuscript. T.S, K.I., and S.K. researched data. A.K., T.U., and H.M. contributed to discussion and reviewed and edited the manuscript. S.A. 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.

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PLOS ONE

Urinary Fetuin-A Is a Novel Marker for Diabetic Nephropathy in Type 2 Diabetes Identified by Lectin Microarray

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Abstract

We analyzed the urine samples of patients with type 2 diabetes at various stages of diabetic nephropathy by lectin microarray to identify a biomarker to predict the progression of diabetic nephropathy. Japanese patients with type 2 diabetes at various stages of nephropathy were enrolled and we performed lectin microarray analyses (n = 17) and measured urinary excretion of fetuin-A (n = 85). The increased signals of urine samples were observed in Siaα2-6Gal/GalNAc-binding lectins (SNA, SSA, TJA-I) during the progression of diabetic nephropathy. We next isolated sialylated glycoproteins by using SSA-lectin affinity chromatography and identified fetuin-A by liquid chromatography–tandem mass spectrometer. Urinary excretion of fetuin-A significantly increased during the progression of albuminuria (A1, 0.40±0.43; A2, 0.60±0.53; A3 1.57±1.13 ng/gCr; $p = 7.29 \times 10^{-8}$) and of GFR stages (G1, 0.39±0.39; G2, 0.49±0.45; G3, 1.25±1.18; G4, 1.34±0.80 ng/gCr; $p = 3.89 \times 10^{-4}$). Multivariate logistic regression analysis was employed to assess fetuin-A as a risk for diabetic nephropathy with microalbuminuria or GFR<60 mL/min. Fetuin-A is demonstrated as a risk factor for both microalbuminuria and reduction of GFR in diabetic nephropathy with the odds ratio of 4.721 (1.881–11.844) and 3.739 (1.785–7.841), respectively. Collectively, the glycan profiling analysis is useful method to identify the urine biomarkers and fetuin-A is a candidate to predict the progression of diabetic nephropathy.

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Introduction

The most critical issue in clinical nephrology is relentless and progressive increase in the patients with end-stage renal disease (ESRD) in worldwide. The impact of diabetic nephropathy on the increasing population with chronic kidney disease (CKD) and ESRD is enormous. The intensified multifactorial intervention in patients with type 2 diabetes mellitus resulted in reduced risk of microangiopathy, cardiovascular events and mortality in Steno type 2 randomized studies [1]; however, the incidence of ESRD is progressively increasing in worldwide. To predict the progression of diabetic nephropathy and cardiovascular outcome, the simultaneous evaluation of albuminuria and glomerular filtration rate (GFR) is recommended by the KDIGO: Kidney Disease Improving Global Outcomes CKD Work Group [2]. In The Action in Diabetes and Vascular Disease: Preterax and Diamicron-MR Controlled Evaluation (ADVANCE) study, the measurements of albuminuria, eGFR or their combination predicted the cardiovascular events and death, and renal outcome [3]. In addition to the albuminuria at baseline, the changes of albuminuria further well-predicted mortality and cardiovascular and renal outcomes, independent of baseline albuminuria reported by ONTARGET investigators [4]. Although the repeated measurements of albuminuria is recommended in the clinical practice in diabetes, the presence of GFR decliners in both type 1 and type 2 diabetes has been reported. In type 1 diabetes, the GFR decliners with early reduction of GFR were reported in 9% of the patients with normoalbuminuria and 31% of microalbuminuria [5]. In the patients with type 2 diabetes, the rapid GFR decliners demonstrated the reduction of GFR although they were treated with olmesartan in addition to the angiotensin converting enzyme inhibitors. In such patients, it was difficult to predict the natural course of diabetic nephropathy by the combination of albuminuria and eGFR [6].

Table 1. A list of lectins of LecChip™Ver.1 and the specificity.

Lectin No.	Lectin	Origin	Reported specificity
1	LTL	*Lotus tetragonolobus*	Fuc α1-3(Galβ1-4)GlcNAc, Fuc α1-2Galβ1-4GlcNAc
2	PSA	*Pisum sativum*	Fuc α1-6GlcNAc, α-D-Glc, α-D-Man
3	LCA	*Lens culinaris*	Fuc α1-6GlcNAc, α-D-Glc, α-D-Man
4	UEA-I	*Ulex europaeus*	Fuc α1-2Galβ1-4GlcNAc
5	AOL	*Aspergillus oryzae l-fucose-specific lectin*	Fuc α1-6GlcNAc (core fucose)
6	AAL	*Aleuria aurantia*	Fuc α1-6GlcNAc, Fuc α1-3(Galβ1-4)GlcNAc
7	MAL	*Maackia amurensis*	Siaα2-3Galβ1-4GlcNAc
8	SNA	*Sambucus nigra*	Siaα2-6Gal/GalNAc
9	SSA	*Sambucus sieboldiana*	Siaα2-6Gal/GalNAc
10	TJA-I	*Trichosanthes japonica*	Siaα2-6Gal/GalNAc
11	PHAL	*Phaseolus vulgaris*	tri/tetra-antennary complex-type N-glycan
12	ECA	*Erythrina cristagalli*	Galβ1-4GlcNAc
13	RCA120	*Ricinus communis*	Galβ1-4GlcNAc
14	PHAE	*Phaseolus vulgaris*	bi-antennary complex-type N-glycan with outer Gal and bisecting GlcNAc
15	DSA	*Datura stramonium*	(GlcNAc β1-4)n, Galβ1-4GlcNAc
16	GSL-II	*Griffonia simplicifolia*	agalactosylated tri/tetra antennary glycans, GlcNAc
17	NPA	*Narcissus pseudonarcissus*	High-Mannose, Man α1-6Man
18	ConA	*Canavalia ensiformis*	High-Mannose, Man α1-6(Manα1-3)Man
19	GNA	*Galanthus nivalis*	High-Mannose, Man α1-3Man
20	HHL	*Hippeastrum hybrid*	High-Mannose, Man α1-3Man, Man α1-6Man
21	ACG	*Agrocybe cylindracea*	Siaα2-3Galβ1-4GlcNAc
22	TxLCI	*Tulipa gesneriana*	Man α1-3(Manα1-6)Man, bi- and tri-antennary complex-type N-glycan, GalNAc
23	BPL	*Bauhinia purpurea alba*	Galβ1-3GalNAc, GalNAc
24	TJA-II	*Trichosanthes japonica*	Fuc α1-2Galβ1-> or GalNAc β1-> groups at their nonreducing terminals
25	EEL	*Euonymus europaeus*	blood group B antigen, Galα1-3Gal
26	ABA	*Agaricus bisporus*	Galβ1-3GalNAc
27	LEL	*Lycopersicon esculentum*	GlcNAc trimers/tetramers
28	STL	*Solanum tuberosum*	GlcNAc oligomers, oligosaccharide containing GlcNAc and MurNAc
29	UDA	*Urtica dioica*	GlcNAc β1-4GlcNAc, Mixture of Man5 to Man9
30	PWM	*Phytolacca americana*	(GlcNAc β1-4)n
31	Jacalin	*Artocarpus integrifolia*	Galβ1-3GalNAc, GalNAc
32	PNA	*Arachis hypogaea*	Galβ1-3GalNAc
33	WFA	*Wisteria floribunda*	GalNAc β1-4GlcNAc, Galβ1-3(-6)GalNAc
34	ACA	*Amaranthus caudatus*	Galβ1-3GalNAc
35	MPA	*Maclura pomifera*	Galβ1-3GalNAc, GalNAc
36	HPA	*Helix pomatia agglutinin*	α-linked terminal GalNAc
37	VVA	*Vicia villosa*	α-linked terminal GalNAc, GalNAc α1-3Gal
38	DBA	*Dolichos biflorus*	blood group A antigen, GalNAc α1-3GalNAc
39	SBA	*Glycine max*	α- or β-linked termincal GalNAc, GalNAcα1-3Gal
40	Calsepa	*Calystegia sepium*	Mannose, Maltose
41	PTL-I	*Psophocarpus tetragonolobus*	α-linked terminal GalNAc
42	MAH	*Maackia amurensis*	Siaα2-3Galβ1-3(Siaα2-6)GalNAc
43	WGA	*Triticum unlgaris*	chitin oligomers, Sia
44	GSL-I A4	*Griffonia simplicifolia Lectin I Isolectin A4*	α-linked GalNAc
45	GSL-I B4	*Griffonia simplicifolia Lectin I Isolectin B4*	α-linked Gal

These data were collected from lectin vendors and reports found by internet searches.
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Based upon these clinical observations, we need to search more reliable urinary biomarkers to predict both renal and cardiovascular outcome. The biomarkers of renal dysfunction such as transferrin, type IV collagen and N-acetyl-β-D-glucosaminidase,

inflammatory markers including orosomucoid, tumour necrosis factor-α, transforming growth factor-β, vascular endothelial growth factor and monocyte chemoattractant protein-1, as well as oxidative stress markers such as 8-hydroxy-2′deoxyguanosine may be more sensitive than urinary albumin, the current gold standard, in the detection of incipient nephropathy and risk assessment of cardiovascular disease; however, the sensitivity of these markers compared with albumin requires further investigation [7].

Recently, the urinary proteome analyses have been performed using 2-dementional gel electrophoresis and subsequent mass spectrometry to identify the novel urinary markers [8–10]; however, the identification of new markers may be suffered from contamination of urinary major proteins such as albumin, immunoglobulins, α1-antitrypsin, transferrin, and haptoglobin. In the line of considerations, we focused on the alterations of glycochains to identify useful urinary biomarkers. The changes in glycoproteome profile in the urine may be due to the alterations in the glycoprotein leakage into the urine by the damages of capillary selective permeability and also attributed to the high glucose-induced changes in the expression of the enzymes which are responsible to the glycochain modification. For example, increased hexosamine biosynthesis induced by high glucose conditions plays a key role in the development of insulin resistance in primary cultured adipocytes [11] and the increased flux through the hexosamine biosynthetic pathway and subsequent enhanced O-linked glycosylation (N-acetylglucosamine [O-GlcNAc]) of proteins have been implicated in insulin resistance in skeletal muscle [12]. However, the glycoproteome profile has not been well-investigated because of the technical obstacles. We employed the evanescent-field fluorescence-assisted lectin microarray: a new strategy for glycan profiling, which allows sensitive, real-time observation of multiple lectin-carbohydrate interactions under equilibrium conditions, to identify the changes in the functional glycans in a high-throughput manner [13]. We identified the increase in the biding activity to Siaα2-6-Gal/GalNAc in urine samples from the patients with diabetic nephropathy. We next identified fetuin-A, α1-microglobulin, and orosomucoid as sialylated glycoproteins and we found fetuin-A may be a useful urinary marker to predict the development of microalbuminuria and reduction of GFR in diabetic nephropathy.

Materials and Methods

Patients

Urine samples of Japanese healthy subjects without type 2 diabetes (n = 12) and Japanese patients with type 2 diabetes with various stages of normoalbuminuria (n = 7), microalbuminuria (n = 5) and macroalbuminuria (n = 5) were obtained and subjected to lectin microarray studies. Based on the lectin microarray studies, we identified sialylated glycoproteins, such as fetuin-A, α1-microglobulin, and orosomucoid as candidate markers for diabetic nephropathy and we newly recruited Japanese patients with type 2 diabetes (n = 85, 62.9±11.3 years) into this study. The patients with type 2 diabetes were treated with oral hypoglycemic agents (n = 48) and insulin treatment (n = 49). The patients with eGFR <15 ml/min/1.73 m^2 or under dialysis were excluded from the current study. All recruited patients with type 2 diabetes agreed to perform lectin microarray of urine samples and measure urinary levels of fetuin-A, α1-microglobulin, and orosomucoid. The study was conducted in accordance with the ethical principle of the Declaration of Helsinki and approved by ethical committee of

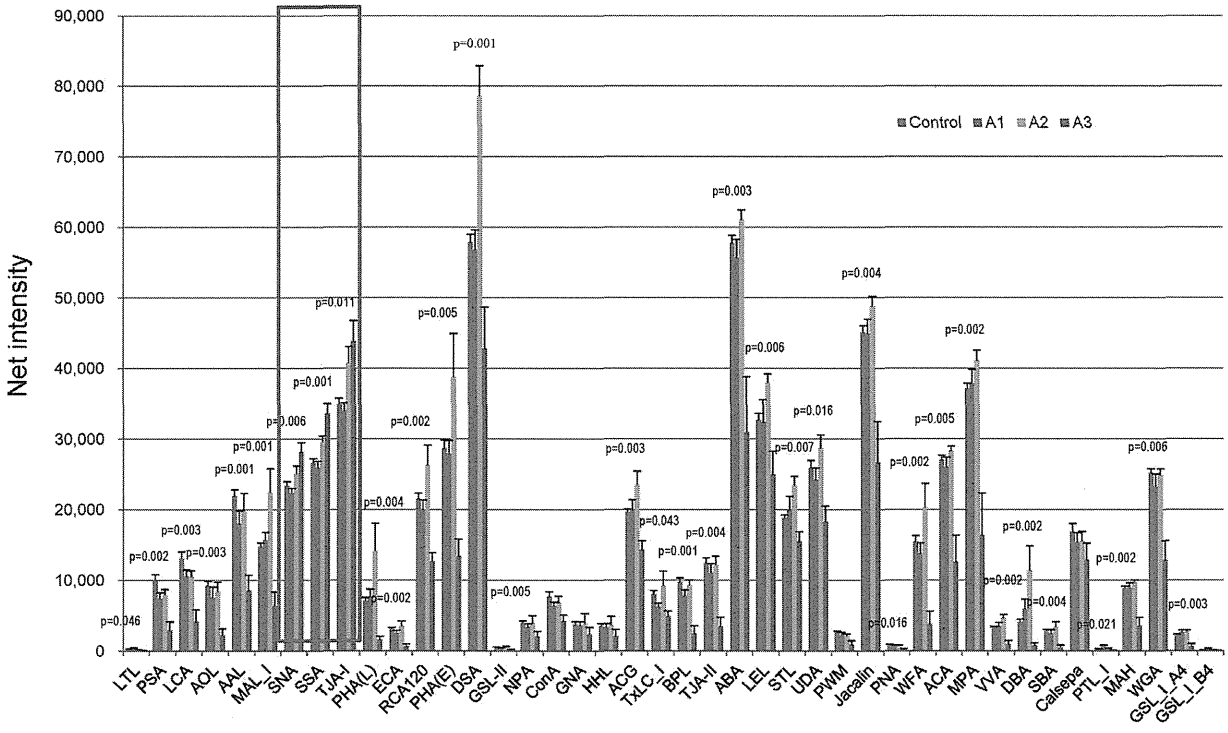

Figure 1. Lectin microarray analysis using urine samples from the patients with various albuminuria stages. Lectin microarray analysis of urine samples were performed in the healthy subjects without type 2 diabetes (Control, n = 12) and the patients with type 2 diabetes with various stages of normoalubuminuria (A1, n = 7), microalbuminuria (A2, n = 5) and macroalbuminuria (A3, n = 5). Signals to various lectins are compared by Kruskal-Wallis test.
doi:10.1371/journal.pone.0077118.g001

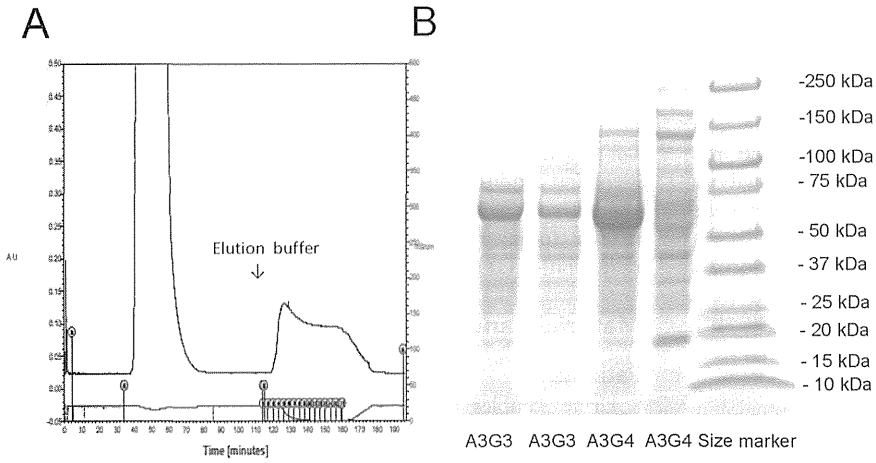

Figure 2. SSA-Agarose column choromatography performed in the 4 patients with type 2 diabetes. A. The concentrated urine samples were applied to SSA-Agarose column, washed with PBS and eluted with 0.2 M lactose. **B.** The effluents from the patients manifested with various albuminria and GFR stages, A3G3 and A3G4, were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue. The bands were visualized and they were subjected to liquid chromatography-tandem mass spectrometer (LC/MS-MS) analysis.
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Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences. We obtained written informed consent from each patient.

Lectin Microarray

Fifty mL of urine samples were concentrated by Centricon at 5,000 *g* for 40 min and further by Microcon at 14,000 *g* for 70 min to the volume of 0.5 mL (Millipore, Billerica, MA). Ten μL of concentrated urine samples were applied to Multiple Affinity Removal Spin Cartridge for Human Serum (Agilent Technologies, Santa Clara, CA) to remove major serum proteins such as albumin, IgG, α1-antitrypsin, IgA, transferrin, and haptoglobin. Five hundred μl of the effluents dialyzed against PBS were applied to ULTRAFREE 0.5 BIOMAX-5k (Millipore) and concentrated to final volume of 50 μL. Protein concentration was measured with MicroBCA Protein Assay Kit (Thermo Scientific Pierce,

Table 2. Liquid chromatography–tandem mass spectrometer (LC/MS-MS) of samples from the patients with A3G3 and the search result through NCBInr and Swiss-Prot database performed by Mascot.

Pos.	Ac. No.	Protein Name	Sequences	emPAI*[1]	Score*[2]
1	ALBU_HUMAN	Serum albumin	36	11.04	3985
2	TRFE_HUMAN	Serotransferrin	15	1.08	965
3	AMBP_HUMAN	Protein AMBP (alpha 1-microglobulin)	5	0.57	224
4	VTDB_HUMAN	Vitamin D-binding protein	3	0.14	130
5	HEMO_HUMAN	Hemopexin	3	0.23	112
6	PTGDS_HUMAN	Prostaglandin-H2 D-isomerase	1	0.18	75
7	IGKC_HUMAN	Ig kappa chain C region	1	0.34	70
8	HPT_HUMAN	Haptoglobin	3	0.17	63
9	DTX3L_HUMAN	E3 ubiquitin-protein ligase DTX3L	1	0.04	49
10	CLUS_HUMAN	Clusterin	1	0.07	39
11	SAP_HUMAN	Proactivator polypeptide	1	0.06	34
12	A1AT_HUMAN	Alpha-1-antitrypsin	2	0.08	33
13	AFAM_HUMAN	Afamin	2	0.05	32
14	FETUA_HUMAN	Alpha-2-HS-glycoprotein (Fetuin-A)	1	0.09	29
15	THRB_HUMAN	Prothrombin	1	0.05	25
16	TRPC4_HUMAN	Short transient receptor potential channel 4	1	0.03	20
17	RABE1_HUMAN	Q15276	2	0.04	19
18	MARK1_HUMAN	Serine/threonine-protein kinase MARK1	1	0.04	16

*[1]emPAI (Exponentially Modified Protein Abundance Index) is calculated for the estimation of absolute protein amount as follow; $emPAI = 10^{Nobserved/Nobservable} - 1$.
*[2]Probability Based Mowse Score. Ions score is −10*Log(P), where P is the probability that the observed match is a random event. Individual ions scores >16 indicate identity or extensive homology ($p<0.05$).
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Rockford, IL) and the final concentration was adjusted to 50 μg/mL, in which 20 μL was incubated with Cy3 at room temperature for 1 hour. Cy3-labeled samples were applied to gel filtration columns (Zeba Desalt Spin Columns 0.5 ml, Thermo Scientific Pierce) and the samples with 2, 1, 0.5, 0.25, 0.125, 0.063, 0.031 μg/mL were prepared with Probing Buffer and 100 μL/well of samples were applied to Lectin Array, LecChip (GP Biosciences, Tokyo, Japan) at 20°C for 15 hours. The lectin signals were measured with Glycostation™ Reader 1200 with exposure time (133 msec) and gain (85, 95, 105, and 115). Scanned images of 16 bit TIFF were analyzed with Array-Pro Analyzer (MEDIA CYBERNETICS, Rockville, MD) and GlycoStation Tools (GP Biosciences). The list of lectins is indicated in the **Table 1** and blood group A antigen (HPA) and group B antigen (EEL) were excluded from the analysis.

Isolation of Sialylated Urinary Proteins in the Patients with Diabetic Nephropathy

Hundred mL of urine samples were concentrated by Centricon at 5,000 *g* for 40 min and further by Microcon at 14,000 *g* for 70 min to the volume of 1 mL. Affinity chromatography was performed using SSA-Agarose (Lectin-Agarose Set-III) and BioLogic LP system II (#731-8300X2, BIO-RAD, Hercules, CA). The SSA-Agarose column was equilibrated by 6.0 mL of PBS at the flow rate of 0.2 mL/min. The concentrated urine samples of 1.0 mL were applied to the sample loop and PBS was loaded at 0.1 mL/min for 10 min. The SSA-Agarose column was washed with PBS at 0.1 mL/min for 70 min. Five mL of the elution buffer (0.2 M lactose) was applied to sample loop and eluted with PBS at 0.1 mL/min for 60 min and further washed with PBS at 0.5 mL/min for 20 min. While eluting the sialylated glycoproteins, the fractions of 0.5 ml were collected every 5 min. The eluted samples were subjected to SDS-PAGE analysis and the proteins were identified by Liquid chromatography–tandem mass spectrometer (LC/MS-MS) analyses as follows.

Cysteine bonds of the eluted glycoproteins were reduced by 10 mM dithiothreitol (DTT) at 56°C for 1 hour and alkylated with 50 mM iodoacetamide (IAA) at room temperature for 45 min in the dark. They were enzymatically digested with 0.1 μg of sequencing grade trypsin at 30°C for overnight. The digested

Table 3. Liquid chromatography–tandem mass spectrometer (LC/MS-MS) of samples from the patients with A3G4 and the search result through NCBInr and Swiss-Prot database performed by Mascot.

Pos.	Ac.No.	Protein Name	Sequences	emPAI*[1]	Score*[2]
1	ALBU_HUMAN	Serum albumin	52	21.13	3829
2	TRFE_HUMAN	Serotransferrin	23	1.61	800
3	HPT_HUMAN	Haptoglobin	17	3.1	683
4	IGHG1_HUMAN	Ig gamma-1 chain C region	10	2.56	601
5	IGHG2_HUMAN	Ig gamma-2 chain C region	8	0.99	227
6	IGKC_HUMAN	Ig kappa chain C region	6	4.73	516
7	IGHA1_HUMAN	Ig alpha-1 chain C region	10	1.54	422
8	A2MG_HUMAN	Alpha-2-macroglobulin	18	0.46	417
9	A1AT_HUMAN	Alpha-1-antitrypsin	10	1.16	392
10	APOA1_HUMAN	Apolipoprotein A-I	8	1.53	251
11	AMBP_HUMAN	Protein AMBP (alpha 1-microglobulin)	7	0.88	226
12	HEMO_HUMAN	Hemopexin	7	0.62	214
13	LAC2_HUMAN	Ig lambda-2 chain C regions	4	1.45	204
14	CO4A_HUMAN	Complement C4-A	2	0.04	147
15	CERU_HUMAN	Ceruloplasmin	2	0.06	127
16	IC1_HUMAN	Plasma protease C1 inhibitor	4	0.22	94
17	A1BG_HUMAN	Alpha-1B-glycoprotein	1	0.07	94
18	PTGDS_HUMAN	Prostaglandin-H2 D-isomerase	1	0.18	94
19	A1AG1_HUMAN	Alpha-1-acid glycoprotein 1 (orosomucoid)	3	0.56	82
20	ANGT_HUMAN	Angiotensinogen	1	0.07	74
21	ANT3_HUMAN	Antithrombin-III	2	0.07	72
22	KNG1_HUMAN	Kininogen-1	2	0.05	71
23	FETUA_HUMAN	Alpha-2-HS-glycoprotein (Fetuin-A)	1	0.09	70
24	PGRP2_HUMAN	N-acetylmuramoyl-L-alanine amidase	1	0.06	62
25	CO3_HUMAN	Complement C3	5	0.02	55
26	THRB_HUMAN	Prothrombin	1	0.05	31
27	VTDB_HUMAN	Vitamin D-binding protein	1	0.07	30
28	MTUS1_HUMAN	Microtubule-associated tumor suppressor 1	1	0.03	26

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*[2]Probability Based Mowse Score. Ions score is −10*Log(P), where P is the probability that the observed match is a random event. Individual ions scores >16 indicate identity or extensive homology ($p<0.05$).
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