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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P atients 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

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 ($20\leq$ 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 m²) 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 m²) = 194 $\times$ [age (years)]$^{-0.287}$ $\times$ [serum Cr (mg/dL)]$^{-1.094}$ $\times$ 0.739 (for female). At baseline, all participants had an eGFR >60 mL/min/1.73 m². 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 m²/year.

Statistical analysis

Data are expressed as mean $\pm$ 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

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.*
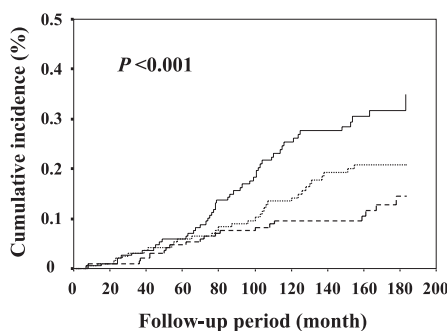
incidence rate of CVD. These associations were observed in those with normoalbuminuria as well as those with microalbuminuria, when separately analyzed according to the stages of diabetic nephropathy. Thus, these findings suggest that urinary L-FABP can be used as a biomarker for predicting future renal dysfunction and incidence of CVD in type 2 diabetic patients with an early stage of nephropathy, in addition to albuminuria.

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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