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Plasma angiotensin-converting enzyme 2 (ACE2) is a marker for renal outcome of diabetic kidney disease (DKD) (U-CARE study 3)

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ABSTRACT

Introduction ACE cleaves angiotensin I (Ang I) to angiotensin II (Ang II) inducing vasoconstriction via Ang II type 1 (AT1) receptor, while ACE2 cleaves Ang II to Ang (1–7) causing vasodilatation by acting on the Mas receptor. In diabetic kidney disease (DKD), it is still unclear whether plasma or urine ACE2 levels predict renal outcomes or not.

Research design and methods Among 777 participants with diabetes enrolled in the Urinary biomarker for Continuous And Rapid progression of diabetic nEphropathy study, the 296 patients followed up for 9 years were investigated. Plasma and urinary ACE2 levels were measured by the ELISA. The primary end point was a composite of a decrease of estimated glomerular filtration rate (eGFR) by at least 30% from baseline or initiation of hemodialysis or peritoneal dialysis. The secondary end points were a 30% increase or a 30% decrease in albumin-to-creatinine ratio from baseline to 1 year.

Results The cumulative incidence of the renal composite outcome was significantly higher in group 1 with lowest tertile of plasma ACE2 (p=0.040). Group 2 with middle and highest tertile was associated with better renal outcomes in the crude Cox regression model adjusted by age and sex (HR 0.56, 95% CI 0.31 to 0.99, p=0.047). Plasma ACE2 levels demonstrated a significant association with 30% decrease in ACR (OR 1.46, 95% CI 1.044 to 2.035, p=0.027) after adjusting for age, sex, systolic blood pressure, hemoglobin A1c, and eGFR.

Conclusions Higher baseline plasma ACE2 levels in DKD were protective for development and progression of albuminuria and associated with fewer renal end points, suggesting plasma ACE2 may be used as a prognosis marker of DKD.

Trial registration number UMIN000011525.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Urinary albumin-to-creatinine ratio (UACR) is known biomarkers to predict the prognosis of diabetic kidney disease (DKD).

WHAT THIS STUDY ADDS

⇒ Plasma ACE2 may be used as a prognosis marker of DKD and higher baseline plasma ACE2 levels in DKD are protective for development and progression of UACR and renal function decline.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ In renin-angiotensin system, ACE2 cleaves angiotensin (Ang) II into the heptapeptide Ang (1–7) and this study sheds light on the importance of ACE2 in pathobiology of DKD.

INTRODUCTION

Diabetes is a chronic progressive disease with ever-increasing prevalence worldwide.[1] Its complications have a significant impact on morbidity and mortality, imposing a global burden.[2] Diabetic kidney disease (DKD) is one of the most common microvascular complications in diabetes. The natural history of DKD includes initial glomerular hyperfiltration, appearance and progression in albuminuria, declining glomerular filtration rate (GFR), and ultimate end-stage kidney disease (ESKD). Since DKD significantly reduces the quality of life and leads to increased mortality,[3] prevention of DKD through early detection and treatment is an urgent issue. Estimated glomerular filtration rate (eGFR) and urinary albumin-to-creatinine ratio (UACR) are well-established clinical indicators for predicting prognosis in DKD. Numerous attempts were made to identify new biomarkers, for example, blood levels

of tumor necrosis factor receptors 1 and 2, markers of tubulointerstitial injury, inflammation, and filtration have been reported to predict the progression of DKD.[4 5] Furthermore, in the Urinary biomarker for Continuous And Rapid progression of diabetic nEphropathy (U-CARE) study,[6–10] we found that the urinary glycan binding signals to several lectins improved the prediction of renal outcome in the models employing the known risk factors.[7] However, despite these indicators and new protective therapies for DKD,[11 12] it remains difficult to adequately assess the risk of DKD and prevent its progression. The identification of new biomarkers is an important challenge for the early detection and management of DKD, and ultimately for the discovery of new therapeutic targets.

The renin-angiotensin system (RAS) is one of the key regulatory mechanisms in the regulation of blood pressure (BP). Angiotensin II (Ang II) plays a central role in the RAS, and it has been shown to be an important factor in increased intraglomerular pressure, hyperfiltration, and tubulointerstitial fibrosis.[13] It is deeply implicated in the progression of renal failure as well as hypertension. ACE cleaves angiotensin I (Ang I) to Ang II, which has vasoconstrictive effects via angiotensin II type 1 (AT1) receptors (ACE/Ang II/AT1 receptor axis). Over the years, research has focused on this classical arm of the RAS. Recent evidence has also shed light on a counterbalancing component to this cascade through the action of ACE2. ACE2 is a homolog of ACE and it cleaves Ang II into the heptapeptide angiotensin 1–7 (Ang (1–7)), which acts on the Mas receptor (ACE2/Ang (1–7)/Mas receptor axis). It has been widely reported to act in an organ-protective manner with a variety of effects, including vasodilation and inhibition of fibrosis.[14] In the kidney, ACE2 is widely expressed in the proximal tubular cells, vascular endothelial and smooth muscle cells and podocytes.[15] It has been shown in experimental models and humans that renal ACE2 protein and mRNA levels are decreased in DKD,[16 17] which may accelerate kidney injury by enhancing Ang II effects.[18] However, few studies have evaluated blood and urinary ACE2 protein levels in DKD. While some reports demonstrated no correlation of blood and urinary ACE2 levels with renal function,[19] others have reported that the patients with DKD had significantly higher levels of urinary ACE2.[20] Furthermore, an observation period of around 10 years is required to evaluate renal complications in diabetes, but no studies have been reported with long-term follow-up in a large-scale clinical study.

In the line of evidence, it has been hypothesized that blood and urinary ACE2 may be important as a potential participant in the onset and progression of DKD. In this study, we aim to investigate the association of plasma and urinary ACE2 protein levels with the progression of DKD in the participants of U-CARE study.

RESEARCH DESIGN AND METHODS

Study design and participants

This is the third report of U-CARE study, a prospective cohort study which started in 2012. The precise study design was described previously.[7] In the current study, among 777 patients with diabetes admitted to multi-institutions in Japan, 296 patients who were followed up for 9 years were enrolled with full set of the data (online supplemental figure 1). The diagnosis of diabetes was based on the Japanese Diabetes Society criteria.[21] This study was registered with the University Hospital Medical Information Network in June 2012 (UMIN000011525).

Laboratory parameters and definitions

GFR was estimated by using the Japanese coefficient-modified Chronic Kidney Disease Epidemiology Collaboration equation.[22] The baseline UACR (mg/gCr) was measured in a spot urine specimen. Normoalbuminuria, microalbuminuria and macroalbuminuria were defined as UACR <30, UACR ≥30 and UACR <300, and UACR ≥300 mg/gCr, respectively.[23] Hemoglobin A1c (HbA1c) 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.[24] Body mass index was calculated as weight divided by the square of height (kg/m^2). Hypertension was defined as a baseline BP ≥140/90 mmHg or use of antihypertensive drugs. Mean arterial pressure (mmHg) was calculated as two-thirds of diastolic blood pressure (DBP) plus one-third of systolic blood pressure (SBP). The grade of diabetic retinopathy was determined by an ophthalmologist at baseline.[25] Cardiovascular disease (CVD) was defined as events requiring admission for treatment. Stroke was defined as cerebral bleeding and infarction requiring admission for treatment, while peripheral arterial disease as an event requiring admission for intervention or surgery. The medications for diabetes, hypertension, dyslipidemia, and hyperuricemia at baseline and during follow-up were recorded.

Study end point

The primary end point was a composite of a decrease of eGFR by at least 30% from baseline or initiation of hemodialysis or peritoneal dialysis. None of the patients received a kidney transplant during follow-up. The secondary end points were a 30% increase or a 30% decrease in UACR from baseline to 1 year.[26]

ACE2 protein measurements by ELISA

All plasma and urine samples collected at baseline were used to measure plasma and urinary ACE2 protein concentration. All specimens were aliquoted and stored at −80°C until measurements. Plasma and urinary ACE2 were measured by the ELISA kit, according to the manufacturer's instructions (AdipoGen, Seoul, Korea). The assay range of the kit was set at 0.0625 ng/mL to 4 ng/mL in manufacturer's instruction. A standard curve

was generated by performing 1:2 serial dilutions of human recombinant ACE2 (1 µg/mL), provided with the kit, to calculate the concentrations of the samples. For the samples below the assay ranges, the values were calculated from the standard curve. The absorbance of zero or below zero after the subtraction by the average blank values was considered as 0 ng/mL. The samples were measures by replication, and average values were used. The coefficient of variation (CV)% values were CV%<10% (88.8%), 10%≤CV%<20% (10.8%), and 20%≤CV% (0.3%) for plasma, while CV%<10% (59.8%), 10%≤CV%<20% (16.6%), and 20%≤CV% (22.3%) for urine samples. In 18.9% of urine samples ACE2 levels were below detection range, that is, 0.0625 ng/mL. After the measurements, urinary ACE2 values were adjusted by urinary creatinine levels.

Statistical analysis

Data were expressed as the percentages, mean±SD or the median and IQR, as appropriate. Skewed variables, including plasma and urinary ACE2 concentration, were subjected to natural logarithmic transformation to improve normality before analysis. Correlations among continuous variables of patient characteristics at baseline and ACE2 concentrations were evaluated by Pearson's correlation analysis. Differences in ACE2 concentrations, by baseline categorical variables, were compared using t-test. The cumulative incidence rate of the primary outcome was estimated by Kaplan-Meier curves for ACE2 concentrations, and incidence rates were compared with the log-rank test. HRs and 95% CIs for the primary end point were estimated with the use of Cox proportional hazards models. In the adjusted model, HRs were adjusted for age, sex, UACR, HbA1c, SBP, and eGFR at baseline. The logistic regression analysis was used to calculate the OR with 95% CI for the secondary end point. The multivariate analysis was performed with adjustments for confounding factors at baseline age, sex, HbA1c, SBP, and eGFR. Two-tailed p values <0.05 were considered statistically significant. All statistical analyses were performed using Stata software (V.17.0; StataCorp, College Station, Texas, USA).

RESULTS

Incidence of the outcome

During follow-up, the primary end point occurred in 47 patients (16%). As the secondary end points, a 30% increase and a 30% decrease in UACR from baseline to 1 year occurred in 115 patients (41%) and 69 patients (24%), respectively.

Clinical characteristics of the participants

The clinical characteristics of all participants at baseline are shown in table 1. Their age was 59±11 years (mean±SD), and 54% of the patients were men. The median duration of diabetes was 10.0 years (IQR 5.6–16.8), 85% of them were type 2 diabetes, and baseline HbA1c was 7.3±1.2% (56.2±12.9 mmol/mol). Approximately

Table 1 Clinical characteristics at baseline

Characteristics	All patients (n=296)
Male sex, n (%)	160 (54)
Age, years	59±11
BMI, kg/m^2	25.7±4.6
Duration of diabetes, years	10.0 (5.6–16.8)
Type of diabetes, n (%)	
Type 1	38 (13)
Type 2	251 (85)
Others	7 (2)
SBP, mmHg	129.4±16.2
DBP, mmHg	74.8±10.3
MAP, mmHg	93.0±11.0
Hypertension, n (%)	191 (65)
Diabetic retinopathy, n (%)	
Non-diabetic	202 (68)
Simple	47 (16)
Preproliferative	17 (6)
Proliferative	23 (8)
Unidentified	7 (2)
Serum creatinine, mg/dL	0.77±0.25
eGFR, mL/min/1.73 m^2	76.5±14.7
CKD GFR category, n (%)	
1	43 (15)
2	219 (74)
3a	24 (8)
3b	8 (3)
4	1 (0.3)
5	1 (0.3)
UACR, mg/gCr	10.9 (5.5–34.8)
Normoalbuminuria, n (%)	219 (74)
Microalbuminuria, n (%)	58 (20)
Macroalbuminuria, n (%)	19 (6)
HbA1c, %	7.3±1.2
Total cholesterol, mg/dL	182.7±32.5
Triglycerides, mg/dL	109 (76–155)
LDL cholesterol, mg/dL	101.8±24.7
Uric acid, mg/dL	5.2±1.4
Any type of antihypertensive agents, n (%)	170 (57)
ACE-I or ARB, n (%)	144 (49)
Calcium channel blocker, n (%)	97 (33)
Treatments for diabetes, n (%)	
Lifestyle modification only	10 (3)
Oral hypoglycemic agents	176 (60)
Insulin	110 (37)
Drug treatment for hyperglycemia, n (%)	
Sulfonylureas	85 (29)
Glinides	17 (6)

Continued

Table 1 Continued

Characteristics	All patients (n=296)
Biguanides (metformin)	107 (36)
α-Glucosidase inhibitors	75 (25)
Thiazolidinediones	45 (15)
Dipeptidyl peptidase 4 inhibitors	130 (44)
GLP-1 receptor agonists	29 (10)
Medications for dyslipidemia, n (%)	181 (61)
Medications for hyperuricemia, n (%)	23 (8)
Prior CVD, n (%)	50 (17)
Prior stroke, n (%)	29 (10)
Prior PAD, n (%)	7 (2)

Data are presented as mean±SD, n (%), or median (IQR).
CKD GFR category, 1: ≥90 mL/min/1.73 m^2, 2: 60–90 mL/min/1.73 m^2, 3a: 45–59 mL/min/1.73 m^2, 3b: 30–44 mL/min/1.73 m^2, 4: 15–29 mL/min/1.73 m^2, 5: <15 mL/min/1.73 m^2.
Insulin, treatment with insulin (including basal-supported oral therapy). Stroke, cerebral bleeding or infarction requiring admission for treatment.
ACE-I, ACE inhibitor; ARB, angiotensin II type I receptor blocker; BMI, body mass index; CKD, chronic kidney disease; CVD, cardiovascular disease; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; GLP-1, glucagon-like peptide 1 receptor agonist; HbA1c, hemoglobin A1c; LDL, low-density lipoprotein; MAP, mean arterial pressure; PAD, peripheral arterial disease; SBP, systolic blood pressure; UACR, urinary albumin-to-creatinine ratio.

two-thirds of the patients had hypertension, with baseline SBP 129.4±16.2 mmHg and DBP 74.8±10.3 mmHg. Antihypertensive medications were on 57% of the patients, ACE inhibitor (ACE-I) or angiotensin II type I receptor blocker (ARB) on 49%, and calcium channel blocker on 33%. The mean baseline eGFR was 76.5±14.7 mL/min/1.73 m^2, and median UACR was 10.9 mg/gCr (IQR 5.5–34.8), with 74% of patients having normoalbuminuria, 20% having microalbuminuria, and 6% having macroalbuminuria.

ACE2 protein measurements and relation between baseline variables

Plasma and urinary ACE2 protein levels were 1.022 ng/mL (IQR 0.623–1.825) and 0.137 ng/mg Cr (IQR 0.0219–0.455), respectively. There was a weak negative correlation between them (r=−0.2921, p=4.170×10^{-6}). In the analysis of continuous variables, plasma ACE2 demonstrated weak positive correlations with triglycerides (r=0.2028, p=4.554×10^{-4}), aspartate aminotransferase (r=0.3783, p=1.793×10^{-11}), alanine aminotransferase (r=0.3868, p=5.807×10^{-12}), and γ-glutamyl transpeptidase (r=0.3745, p=3.469×10^{-11}), while weak negative correlation was found with high-density lipoprotein cholesterol (r=−0.2073, p=3.310×10^{-4}). Blood glucose (r=0.2154, p=7.825×10^{-4}) and HbA1c (r=0.2161, p=7.489×10^{-4}) demonstrated a weak positive correlation with urinary ACE2. There was no correlation between plasma or urine ACE2 and baseline renal function or UACR (online supplemental table 1).

In the analysis of categorical variables, the use of glucagon-like peptide 1 receptor agonists (GLP-1RA) was significantly associated with plasma and urinary ACE2. Patients who received GLP-1RA were more likely to show higher level of plasma ACE2 (p=0.004) and lower level of urinary ACE2 (p=0.008). There was no significant difference in either plasma or urinary ACE2 levels between the patients who received ACE-I or ARB and those who did not (p=0.069 and p=0.848, respectively, online supplemental table 2).

Cumulative incidence rate of the primary outcome in ACE2 measurements

To evaluate plasma and urinary ACE2 levels as a risk of the primary outcome, the patients were classified according to tertiles of concentrations of ACE2 (T1: lowest tertile, T2: middle tertile, T3: highest tertile, figure 1). Analysis of plasma ACE2 revealed that the cumulative incidence of the outcome was significantly higher in T1, the lowest ACE2 concentration group (p=0.040, figure 2A). Next, we divided the patients into two groups according to tertiles of concentrations of ACE2; group 1 includes the lowest tertile and group 2 includes the middle and highest tertile. Re-evaluation showed that the occurrence of event risk in group 1, the lowest tertile group, was even more pronounced (p=0.011, figure 2B). Urinary ACE2 was classified and evaluated in the same manner as described, but no significant difference was found (p for trend: p=0.982 for tertiles of urinary ACE2, figure 2C, and p=0.864 for two groups of urinary ACE2, figure 2D).

Associations of ACE2 with primary and secondary outcomes

Unadjusted and adjusted HR for renal composite outcomes is shown in table 2. The model was adjusted for known indicators of DKD progression; model 1 was adjusted for baseline age and sex and model 2 for baseline age, sex, UACR, HbA1c, SBP, and eGFR. Group 2 significantly associated with better renal composite outcomes in the crude Cox regression model (HR 0.49, 95% CI 0.28 to 0.87, p=0.014) and adjusted model 1 (HR 0.56, 95% CI 0.31 to 0.99, p=0.047), but not in adjusted model 2 (HR 0.80, 95% CI 0.43 to 1.50, p=0.493) compared with group 1 as a reference. In the patients treated with ACE-I or ARB (n=144), group 2 demonstrated better renal composite outcomes in the crude Cox regression model (HR 0.40, 95% CI 0.20 to 0.79, p=0.009), but not in adjusted models in table 2. In the patients with normoalbuminuria (n=219), group 2 demonstrated better renal composite outcomes, but it did not reach statistically significant levels in multivariate Cox regression model 2 adjusted by age, sex, UACR, HbA1c, SBP, and eGFR (HR 0.43, 95% CI 0.16 to 1.12, p=0.084). However, in microalbuminuria and macroalbuminuria (n=77), such prediction was lost in adjusted model 2 (HR 1.66, 95% CI 0.67 to 4.15, p=0.275). In contrast, no significant difference was found in urinary ACE2 with the renal outcomes.

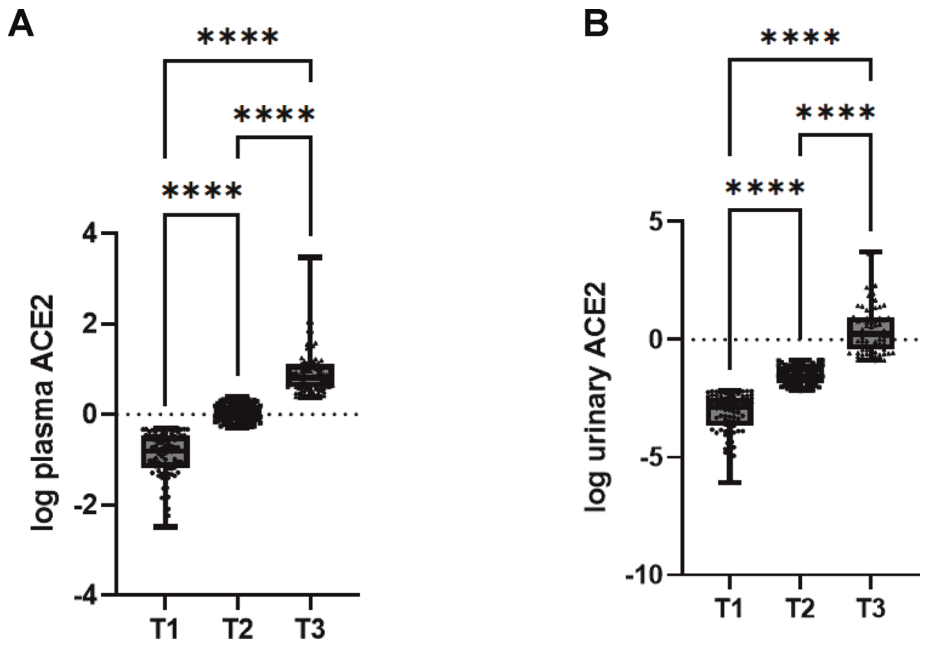

Figure 1 Plasma and urinary concentrations of ACE2 in the participants. The participants were divided into groups according to tertiles of concentrations of plasma (A) and urinary (B) ACE2. T1: lowest tertile, T2: middle tertile, T3: highest tertile. ****P<0.0001.

The association of ACE2 levels and changes in UACR was investigated using logistic regression analysis (table 3). In univariate analysis, plasma ACE2 levels demonstrated significant association with both 30% increase (OR 0.75, 95% CI 0.57 to 1.00, p=0.046) and 30% decrease (OR 1.53, 95% CI 1.11 to 2.10, p=0.010) in ACR. After adjusting for age, sex, SBP, HbA1c, and eGFR, the association between 30% increase in ACR in plasma ACE2 was not significant (OR 0.78, 95% CI 0.587 to 1.034, p=0.084), whereas 30% decrease in ACR in plasma ACE2 remained significant (OR 1.46, 95% CI 1.04 to 2.04, p=0.027). In the patients with normoalbuminuria (n=219), plasma ACE2 levels demonstrated significant association with 30% increase in both univariate (OR 0.69, 95% CI 0.49 to 0.97, p=0.033) and multivariate (OR 0.68, 95% CI 0.48 to 0.97, p=0.033) analyses. In contrast, no significant association was found in the analysis of urinary ACE2 with 30% increase in ACR (OR 1.03, 95% CI 0.88 to 1.21, p=0.697). However, in the patients with normoalbuminuria (n=219), urinary ACE2 levels were significantly associated with 30% decrease in ACR (OR 0.76, 95% CI 0.61 to 0.97, p=0.024) but not with 30% increase in ACR (OR 1.08, 95% CI 0.89 to 1.31, p=0.444) by multivariate analyses. In other subgroups, the patients with ACE-I or ARB, without ACE-I or ARB, and with microalbuminuria and macroalbuminuria, there were no significant associations between ACE2 levels and changes in ACR (table 3).

DISCUSSION

In the current study, we found that the cumulative incidence rate of the renal outcomes was significantly higher in the lowest plasma ACE2 group when classified by the tertiles of concentrations of ACE2. In addition, higher plasma ACE2 levels were associated with reduction of albuminuria. This study suggests that plasma ACE2 prevents the onset and progression of albuminuria and the deterioration of renal function and can be used as an independent marker of predicting the renal outcomes of DKD. The RAS plays a pivotal role in the pathophysiology of cardiovascular and renal diseases and has been extensively studied in animal models and clinical trials until now. In the classical RAS axis (ACE/Ang II/AT1 receptor axis), Ang II plays a major role as a multifunctional hormone or cytokine. It promotes water-sodium retention, vasoconstriction, elevation of BP, inflammation, apoptosis, and ultimately progressive renal injury.[27] Recent studies have also focused on the protective arm of the RAS (ACE2/Ang(1–7)/Mas receptor axis), which counteracts the classical axis,[14] and a role for ACE2 in renal protection is expected. In rat models, studies have shown that vascular ACE2 overexpression protects the kidneys against aging-induced renal dysfunction,[28] and the activation of ACE2 has been reported to have a renoprotective effect.[29] Recently, a large-scale study on the association between plasma ACE2 and the risk of CVD has been published.[30] However, there are still few clinical reports on the association between ACE2 and the prognosis of CKD, especially DKD, and our current report provides an important perspective. In the present study, we reported that higher baseline plasma ACE2 levels were associated with fewer renal outcomes as well as less albuminuria from the baseline to 1 year. The results suggests that higher initial plasma ACE2 levels may have a protective effect against future renal injury. In contrast, we found no correlation between serum ACE2 levels and baseline serum Cr, eGFR, or UACR, which is consistent

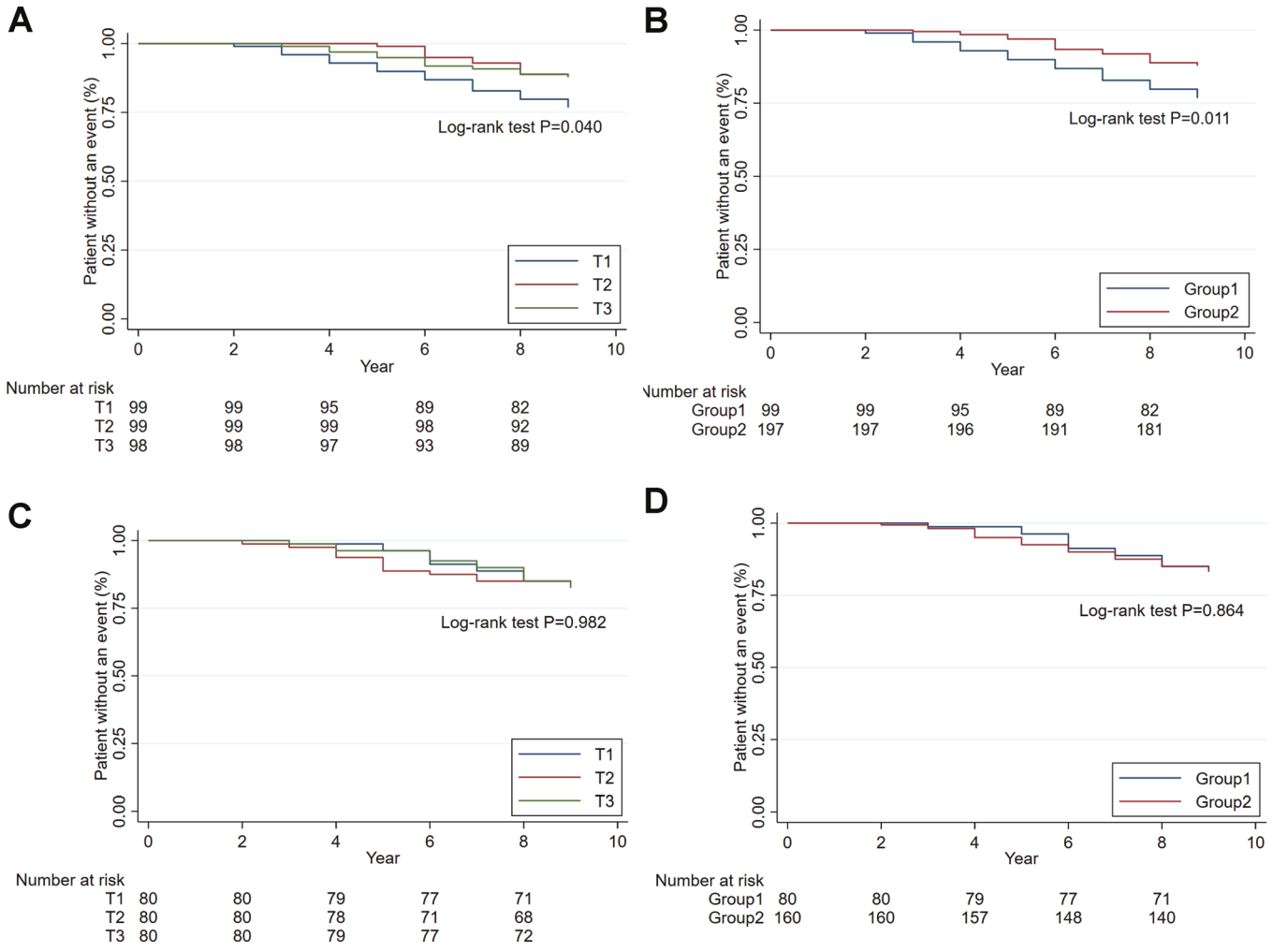

Figure 2 Kaplan-Meier survival curve for renal composite end point. The participants were divided into groups according to tertiles of concentrations of plasma (A and B) and urinary (C and D). (A and C) T1: lowest tertile, T2: middle tertile, T3: highest tertile. (B and D) Group 1: lowest tertile, group 2: middle and highest tertiles.

with previous report.[19] There is a study which has reported higher serum ACE2 levels in patients with advanced CKD stage (eGFR <30 mL/min/1.73m^2 or ESKD),[31] but the present cohort includes few such patients with advanced CKD, requiring additional future study.

Since 49% of the participants received the ACE-I or ARB, it is interesting to know the relation between ACE2 levels and the administration of RAS inhibitors. In the current investigation, there were no significant differences in plasma ACE2 levels between the ACE-I-treated or ARB-treated and non-treated groups. This is also consistent with previous observations that soluble ACE2 levels are not affected by ACE-I or ARB.[31] Although previous studies have reported higher concentrations of soluble ACE2 in males than in females,[32] we found no difference in plasma ACE2 according to sex. The present results may be explained by the high average age of the study population. Soluble ACE2 was reported to be elevated in postmenopausal women compared with premenopausal women,[33] which could explain the lack of a sex difference in plasma ACE2 in this study. Another important issue is that ACE2 serves as a receptor for SARS-CoV-2. After binding of coronavirus surface spike (S) protein to ACE2, the entry of SARS-CoV-2 depends on proteolytic cleavage between S1 and S2 subunits by type II transmembrane serine protease.[34] The cleavage of ACE2 facilitates the viral uptake, converts membrane form to soluble form of ACE2, enhances the classical RAS axis, and harmful effects with excessive RAS activation. After the internalization of SARS-CoV-ACE2, desintegrin and metalloproteinase domain 17 (ADAM17) activity is upregulated, and it also leads to ectodomain proteolytic cleavage of ACE2. It further enhances Ang II-AT1R axis and again activates ADAM17, which releases the mature form of EGFR, the soluble form of IL-6Ra, and tumor necrosis factor-α.[34] The tubular secretion of ACE2 could be mediated by ADAM17.

ACE2 is expressed at high levels in the kidney and is known to localize primarily to the proximal tubules.[35] It has been reported that urinary ACE2 protein is likely to be shed into the urine due to proteolysis from renal cells.[20 35] Studies in animal models of diabetes and in patients with DKD have shown decreased ACE2 expression in renal tissues.[16] On the other hand, urinary ACE2

Table 2 Risk of renal end point according to ACE2 levels (group 1, the lowest tertile vs group 2, middle and highest tertiles of ACE2)

	Crude			Adjusted model 1			Adjusted model 2		
	HR	95% CI	P value	HR	95% CI	P value	HR	95% CI	P value
Plasma ACE2 (n=296)	0.49	0.28 to 0.87	0.014	0.56	0.31 to 0.99	0.047	0.80	0.43 to 1.50	0.493
Plasma ACE2 (with ACE-I or ARB, n=144)	0.40	0.20 to 0.79	0.009	0.50	0.25 to 1.01	0.055	0.65	0.30 to 1.41	0.275
Plasma ACE2 (without ACE-I or ARB, n=152)	0.46	0.16 to 1.34	0.155	0.47	0.16 to 1.36	0.164	0.47	0.15 to 1.48	0.197
Plasma ACE2 (normo, n=219)	0.43	0.17 to 1.05	0.064	0.44	0.18 to 1.10	0.080	0.43	0.16 to 1.12	0.084
Plasma ACE2 (micro and macro, n=77)	0.51	0.24 to 1.08	0.080	0.85	0.38 to 1.90	0.692	1.66	0.67 to 4.15	0.275
Urinary ACE2 (n=296)	1.06	0.55 to 2.05	0.865	1.00	0.52 to 1.94	0.999	0.76	0.38 to 1.53	0.443
Urinary ACE2 (with ACE-I or ARB, n=144)	0.91	0.42 to 1.96	0.801	0.86	0.39 to 1.87	0.700	0.79	0.34 to 1.83	0.590
Urinary ACE2 (without ACE-I or ARB, n=152)	1.55	0.42 to 5.74	0.508	1.53	0.41 to 5.70	0.521	1.44	0.36 to 5.80	0.605
Urinary ACE2 (normo, n=219)	0.82	0.30 to 2.26	0.706	0.77	0.28 to 2.12	0.609	0.80	0.27 to 2.35	0.680
Urinary ACE2 (micro and macro, n=77)	1.34	0.55 to 3.22	0.519	1.23	0.51 to 2.99	0.643	0.99	0.38 to 2.63	0.990

The associations between the renal outcomes and the plasma or urinary ACE2 levels at baseline are shown. Based on ACE2 concentrations, the participants are divided into two groups, group 1 and group 2. Group 1 includes the lowest tertile of ACE2 and serves as reference. Group 2 includes middle and highest tertiles of ACE2. The renal outcome is a composite of a 30% decline in the estimated GFR or initiation of renal replacement therapy. HRs and 95% CIs are estimated with the use of Cox proportional-hazards models. The model is adjusted for confounding factors at baseline. Model 1: age and sex, model 2: age, sex, UACR, HbA1c, SBP, and eGFR.
ACE-I, ACE inhibitor; ARB, angiotensin II type I receptor blocker; eGFR, estimated glomerular filtration rate; HbA1c, hemoglobin A1c; macro, macroalbuminuria; micro, microalbuminuria; normo, normoalbuminuria;SBP, systolic blood pressure; UACR, urinary albumin-to-creatinine ratio;

Table 3 The changes in urinary ACR from baseline to 1 year and prediction by ACE2 levels

	Univariate analysis			Multivariate analysis		
	OR	95%CI	P value	OR	95%CI	P value
ACR ≥30% increase						
Plasma ACE2 (n=296)	0.75	0.57 to 1.00	0.046	0.78	0.59 to 1.03	0.084
Plasma ACE2 (normo, n=219)	0.69	0.49 to 0.97	0.033	0.68	0.48 to 0.97	0.033
Urinary ACE2 (n=296)	1.04	0.89 to 1.21	0.620	1.03	0.88 to 1.21	0.697
Urinary ACE2 (normo, n=219)	1.04	0.87 to 1.24	0.671	1.08	0.89 to 1.31	0.444
ACR ≥30% decrease						
Plasma ACE2	1.53	1.11 to 2.10	0.010	1.46	1.04 to 2.04	0.027
Plasma ACE2 (normo, n=219)	1.69	1.08 to 2.64	0.021	1.57	1.00 to 2.48	0.051
Urinary ACE2	0.88	0.74 to 1.05	0.164	0.86	0.72 to 1.04	0.115
Urinary ACE2 (normo, n=219)	0.79	0.63 to 1.00	0.051	0.76	0.61 to 0.97	0.024

The associations between the ACR outcomes and the plasma and urinary ACE2 at baseline are shown. The ACR outcomes are defined as a 30% increase or a 30% decrease in ACR from baseline to 1 year. The logistic regression model is used to calculate the OR with 95% CI. The multivariate analysis is performed and adjusted by confounding factors at baseline age, sex, HbA1c, SBP, and eGFR.
ACR, albumin-to-creatinine ratio; eGFR, estimated glomerular filtration rate; HbA1c, hemoglobin A1c; normo, normoalbuminuria; SBP, systolic blood pressure.

levels have been reported to be significantly higher in patients with CKD and those who have undergone renal transplant compared with healthy controls.[20] Increased urinary ACE2 excretion is also reported in type 2 diabetes.[36] In our study, correlation analysis showed a weak positive correlation of urinary ACE2 with blood glucose and HbA1c levels. This is consistent with previous reports showing that urinary ACE2 may be associated with the severity of glucose intolerance.[36] Previous cross-sectional study on urinary ACE2 and renal dysfunction have reported an association between urinary ACE2 and microalbuminuria excretion.[37] In contrast, others reported that there was no difference in urinary ACE2 levels depending on the degree of albuminuria in type 2 diabetes,[36] and the views remain conflicting. In the present study, we found no correlation between urinary ACE2 and baseline Cr, eGFR, or UACR, which are indicators of renal dysfunction. No association with renal outcomes was also demonstrated, either in short or long term. Although this study is a longitudinal cohort study with long-term follow-up, most of the participants were characterized by preserved renal function, which may bias the results. Further studies are needed to evaluate the association between urinary ACE2 and renal impairment.

Glucagon-like peptide 1 (GLP-1) is an incretin, a hormone produced by L-cells of the distal ileum in response to food intake. By interacting with its receptor, GLP-1 receptor (GLP-1R), it increases insulin secretion in a glucose-dependent manner and decreases glucagon release, contributing to glycemic control. It also exerts extrapancreatic effects such as suppression of appetite and the activation of lipolysis, and GLP-1RA has been developed as a novel treatment for diabetes and obesity. GLP-1R is expressed in the pancreas and in several other organs, including the heart, blood vessels, lungs, and kidneys.[38] In the present study, we reported that plasma ACE2 was significantly elevated under the treatment with GLP-1RA. Previous studies have shown that liraglutide, a GLP-1RA, strongly increased ACE2 mRNA expression in type 1 diabetes and control rats, as well as in hypertensive rat models.[39 40] The effect was shown to be independent of glycemic control and the levels of insulin.[39] As mentioned, ACE2 promotes vasodilation and apoptosis. The organ protective actions of GLP-1RA, such as cardioprotective and liver protective effects, have been extensively investigated, and part of the effects may be the result of activation of the protective arm of the RAS (ACE2/Ang (1–7)/MasR axis) via enhanced ACE2 expression.[39–41] Our results suggest that plasma ACE2 elevation may also be involved in the renoprotective effects of GLP-1RA. In contrast, lower urinary ACE2 was detected with the use of GLP-1RA. Given the possibility of urinary ACE2 shedding from renal cells into the urine, one possibility is that this result reflects a decrease in ACE2 shedding. Since this hypothesis is not confirmed, further investigation is needed.

Our study has some limitations. First, most of the participants had preserved eGFR (76.5±14.7 mL/min/1.73 m^2) and normoalbuminuria (74%), while there was limiting number of the patients with microalbuminuria and macroalbuminuria (26%). We need to follow-up the patients for 9 years to observe enough number of renal outcomes and it may bias the results. In the future, the study enrolling the patients with diabetes and CKD is required to confirm the results. Second, in the current ELISA measurement of urinary ACE2, the concentrations of ACE2 were below detection limits in some cases and a more sensitive method should be developed to further confirm the clinical importance of urinary ACE2. Third, this study was started in 2012 and the effects of SGLT2

inhibitors on ACE2 concentrations and outcomes have not been determined. Since SGLT2 inhibitors are known to activate RAS,[42] further investigations are required to determine whether SGLT2 inhibitors have the effect on plasma and urinary ACE2 levels and demonstrate beneficial impacts on renal outcomes. Fourth, the sample size of the current study was 296 participants, which is relatively small to draw definitive conclusions in DKD, a complex condition. Since current investigation was conducted in a specific population—patients with diabetes in Japan—a larger sample size encompassing populations with different demographic and clinical characteristics would enhance generalizability.

In conclusion, the present study identified that higher baseline plasma ACE2 levels in the patients with diabetes were protective against the development and progression of albuminuria and were associated with fewer renal end points. The result suggests that plasma ACE2 may be used as a prognostic marker of DKD.

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Contributors AU, YO, and KMis conceived the study, formulated the analysis plan, performed the statistical analyses, collected the clinical data, and wrote the manuscript. SY, AKan, IN, CH, HAU, KS, SM, AN, JE, KH, AKat, MW, TNa, AT, ST, TM, SK, KMu, IS, KMiy, SA, TNu, and JW recruited the patients and assessed the data. JW conceived the study, supervised the data collection, analyzed the data, and edited the manuscript. JW is responsible for the overall content as guarantor. The guarantor accepts full responsibility for the finished work and/or the conduct of the study, had access to the data, and controlled the decision to publish.

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