ORIGINAL

Molecular composition of adiponectin in urine is a useful biomarker for detecting early stage of diabetic kidney disease

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Abstract : We previously developed two immune complex transfer enzyme immunoassays (ICT-EIA) to measure total adiponectin (T-AN) and high molecular weight adiponectin (H-AN) in urine and have verified their usefulness as biomarkers for diabetic kidney disease. In this study, we developed T-AN and H-AN assays using the sandwich EIA (Sand-EIA). The reactivities of Sand-EIAs were compared with ICT-EIAs by measuring size exclusion chromatography (SEC) fractions of urine and adiponectin standard. As a result, ICT-EIAs showed higher macromolecular specificity. We then analyzed the molecular profile of adiponectin in the urine of 5 patients with different eGFR stages by measuring SEC fractions of urine. The results showed that smaller adiponectin correlated relatively well with eGFR stage. Finally, because SEC is time-consuming, we investigated that the ratio of T-ANs by Sand-EIA and ICT-EIA could be a good indicator of the monomer adiponectin. The ratio was evaluated using 77 urine samples from patients with diabetes and showed a significant decrease at an earlier stage compared with other biomarkers. In conclusion, we demonstrated a new index to estimate monomer adiponectin in urine by using Sand-EIA and ICT-EIA, and urinary monomer adiponectin can be a good early indicator of deterioration of renal function in diabetic patients. J. Med. Invest. 70 : 464-470, August, 2023

Keywords **:** *Immune complex transfer enzyme immunoassay, Ultrasensitive immunoassay, Urinary adiponectin, Kidney disease, Biomarker*

INTRODUCTION

Chronic kidney disease (CKD) is a disease that is initially asymptomatic, but leads to a gradual decline in kidney function, causing cardiovascular disease (CVD) such as myocardial infarction, stroke, and heart failure (1) and eventually requires dialysis or a kidney transplant (2). Diabetes mellitus is one of the major contributing causes, and when CKD develops as a complication of diabetes mellitus, it is called diabetic nephropathy or diabetic kidney disease (3). Therefore, it is important to find the high-risk group of worsening renal function among diabetic patients for therapeutic intervention, and biomarkers that enable early diagnosis are considered useful. Past studies have shown that adiponectin in urine may be useful as a biomarker for CKD (4-6). In particular, unlike the Glomerular filtration rate (GFR) and urinary albumin excretion rate (UAER), which have been used as biomarkers, adiponectin can predict the onset and progression of CKD (7, 8). It is expected to be used as an early CKD biomarker which can detect the change of the renal function from earlier stages compared with the conventional biomarkers.

We have developed two assays to measure trace amounts of adiponectin in urine using an ultra-sensitive immunoassay technique called immune complex transfer enzyme immunoassay (ICT-EIA). One is a total adiponectin (T-AN) assay that measures all of the high molecular weight (HMW), medium molecular weight (MMW), low molecular weight (LMW), and monomers in urine. The other is the HMW adiponectin (H-AN) assay, which specifically measures only HMW. Previously, using these two assays, we have shown that T-AN and H-AN levels are higher with advancing eGFR stage and may be elevated earlier than the conventional biomarker, UAER (9, 10). Furthermore, we showed that baseline H-AN levels may be associated with a decline in eGFR after 2 years, which suggested that H-AN may predict CKD progression (11).

In previous studies, several urine samples were size-fractionated by SEC and measured for T-AN and H-AN using ICT-EIAs, and it was found that the molecular weight of adiponectin in urine changed with eGFR stage. Higher molecular weight adiponectin tended to be discharged into urine as CKD progressed (9, 10). The aim of this study was to analyze the molecular weight composition of adiponectin in the urine of patients with different eGFR stages, and to examine the relationship with CKD progression and the potential as a new biomarker.

In addition, the ICT-EIA method is a technique to improve the signal-to-noise ratio by decreasing the background signal of immunoassays, which results in higher sensitivity. However, it has been found that the immune complexes in the assay, once formed, dissociate during the reaction process (12). The effect of this phenomenon on T-AN and H-AN measured levels has not been investigated. In this study, we tested not only the previously developed ICT-EIAs, but also the new conventional sandwich EIAs, which were used to measure urine samples in order to evaluate the measured levels with and without dissociation.

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MATERIALS AND METHODS

Urinary adiponectin assays

Buffers, antibodies and antigen used in the present study were described previously and the assay reagents were prepared using the same protocol (10). The sandwich EIA method for

T-AN and H-AN measurement on HI-1000 was as follows : 30 μL of sample was mixed with 80 μL of capture and detection antibody conjugates and incubated for 1.0 min. 30 μL of streptavidin coated magnetic particles was added and incubated for 14 min. After washing, the bound alkaline phosphatase activity was assayed by chemiluminescence with HISCL substrate reagent (Sysmex, Hyogo, Japan) for 5.0 min at 42 ℃. The ICT-EIA method for T-AN and H-AN measurement on HI-1000 was the same as described previously (10).

Subjects and samples

Seventy-seven outpatients with type 1 or type 2 diabetes in Tokushima University Hospital were enrolled in this study. Patient backgrounds are shown in Table 1. Blood samples were drawn from an antecubital vein of the subjects. Early morning urine samples were collected after overnight fasting. The urine samples (10 mL) were mixed with 0.1 mL of 10% BSA and NaN_3 and dialyzed overnight at 4 ℃. Dialyzed urine samples were kept frozen at -30 ℃ until analysis.

Analysis of urine samples using size exclusion chromatography (SEC)

Two randomly chosen patient urine samples (1.0 mL) out of the 77 outpatients were subjected to size exclusion chromatography (SEC) using a HiLoad 16/60 Superdex 200 prep grade (1.6 x 60cm) column on an AKTA Explorer 10S FPLC chromatography system (GE Healthcare, Tokyo, Japan). An aliquot of each fraction was tested for T-AN and H-AN by both sandwich EIA and ICT-EIA methods. Urine from subjects in the 25^{th} and 75^{th} percentiles of each eGFR stage (G1, G2, G3a, G3b, G4), 10 samples in total, were selected and separated using the same chromatography method and each fraction was tested for T-AN by the sandwich EIA method. HMW, MMW, LMW and monomer adiponectin concentrations were calculated based on concentrations of fraction No. 50-52, 54-56, 62-64 and 85-89, respectively using recombinant human adiponectin (Oriental yeast, Tokyo, Japan) as calibrator.

Statistical analysis

For biomarker and index correlation analyses, Spearman's correlation coefficients were calculated. Due to the small sample size, the p-values may not be accurate, but they are provided for reference.

Ethical considerations

The study protocol was approved by the ethical committee of Tokushima Bunri University (No. H29-17) and by the ethical committee of Tokushima University Hospital (No. 2894-1). All diabetic participants gave written informed consent. All methods were performed in accordance with the relevant guidelines and regulations.

Data Availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Table 1. Patient background

	Outpatients (n = 77)
Gender (Male/Female)	38/39
Age	62 (52, 69)
BMI (kg/m^2)	24.5 (21.6, 28,3)
HbA1c	7.24 (0.82)
Type of diabetes (T1DM/T2DM)	31/46
eGFR stage (G1/G2/G3a/G3b/G4)	9/43/14/8/3
ALB stage (1/2/3)	57/13/7
Microvascular complications	
Neuropahthy	42 (54.5%)
Retinopathy (NDR/SDR/PPDR/PDR/Unknown)	38/17/9/7/6 (49.4%/22.1 %/11.7%/9.1 %/7.8%)
Medications	
Insulin	51 (66.2%)
GLP-1 receptor agonist	15 (19.5%)
Sulfonylurea	5 (6.5%)
Glinide	1 (1.3%)
DPP-4 inhibitor	22 (28.6%)
Biguanide	23 (29.9%)
Thiazolidine	2 (2.6%)
α-glucosidase inhibitor	8 (10.4%)
Sodium glucose transporter 2 inhibitor	11 (14.3%)
Antihypertensive	38 (49.4%)
Statins	35 (45.5%)

NDR : No visible diabetic retinopathy, SDR : Simple diabetic retinopathy, PPDR : Pre-proliferative diabetic retinopathy, PDR : Proliferative diabetic retinopathy

RESULTS

Different reactivity of sandwich EIA and ICT-EIA methods

Adiponectin standard material and urine samples collected from diabetic patients were size-fractionated by SEC, and each fraction was tested for T-AN and H-AN by sandwich EIA and ICT-EIA methods as shown in Fig 1.

The adiponectin standard consisted of MMW and HMW components. Both components were detected by the T-AN assays, with the ICT-EIA method generating slightly higher signals compared to the sandwich EIA method. The H-AN assays demonstrated more specificity for the HMW component with little to no detection of the MMW component. For these assays, the sandwich EIA method generated more signal than the ICT-EIA method.

In the measurement of the urine sample using the two T-AN assay methods, similar levels of HMW, MMW, and LMW were observed. However, the monomeric adiponectin was detected in greater quantities by the sandwich EIA method.

Regarding the analysis of the urine sample using the two H-AN assay methods, the SEC profile and signals generated by each assay were similar to that of the adiponectin standard material. The sandwich EIA method generated more signal than the ICT-EIA method of the H-AN assays, opposite to the T-AN assays.

Analysis of adiponectin monomer and multimers in the urine from diabetic patients

Seventy-seven outpatients were classified based on eGFR stage, and urine samples from patients in the 75th percentile of eGFR in each group (G1, G2, G3a, G3b, G4) were size-fractionated by SEC, and each fraction was assayed by the T-AN sandwich EIA method, which best detects all adiponectin forms (monomer and multimers), and the results are shown in Fig 2. Relatively high levels of monomeric and LMW adiponectin are detected in urine samples from diabetic patients with stages G1-G3a, whereas in G3b-G4, the presence of MMW and HMW adiponectin were found to be increased.

The same analysis was then performed for subjects at the 25th percentile of each eGFR stage, and the amounts of monomeric, LMW, MMW, and HMW adiponectin, as well as their percentage of total adiponectin were calculated. Correlation between the adiponectin biomarkers / index values and eGFR are shown in Table 2. The correlation coefficients (r) were relatively high between eGFR and the LMW measure, the LMW/total adiponectin ratio, and the monomer/total adiponectin ratio, indicating that the variation of low-molecular-weight adiponectin may be a strong indicator reflecting the progression of kidney disease.

The relationship between eGFR stage and the biomarkers including adiponectin indexes is shown in Fig 3. Albumin, the conventional biomarker, T-AN, H-AN, and LMW adiponectin tended to be elevated in eGFR stages G3b and G4. LMW adiponectin had a higher correlation coefficient with eGFR than the other biomarkers. On the other hand, the LMW/total adiponectin ratio and monomer/total adiponectin ratio, unlike other biomarkers, showed increasing and decreasing trends even in the early stages of eGFR, indicating that they may be biomarkers that fluctuate from earlier stages. These results suggested that they may be variable biomarkers from earlier stages.

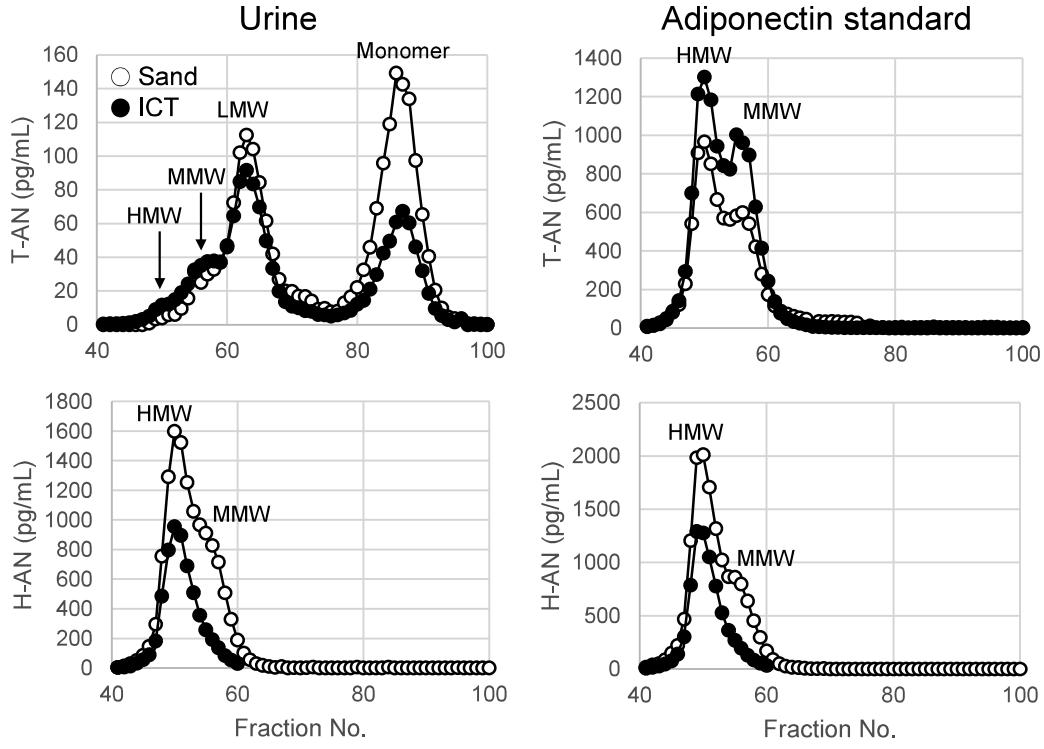

Fig 1. Specificities of T-AN and H-AN assays by sandwich EIA and ICT-EIA.
T-AN : Total adiponectin, H-AN : High molecular weight adiponectin, LMW : Low molecular weight, MMW : Middle molecular weight, HMW : High molecular weight, Sand ; Sandwich EIA, ICT ; ICT-EIA. Urine samples from patients with diabetes and adiponectin standard were separated by SEC and fractions were measured by the assays.

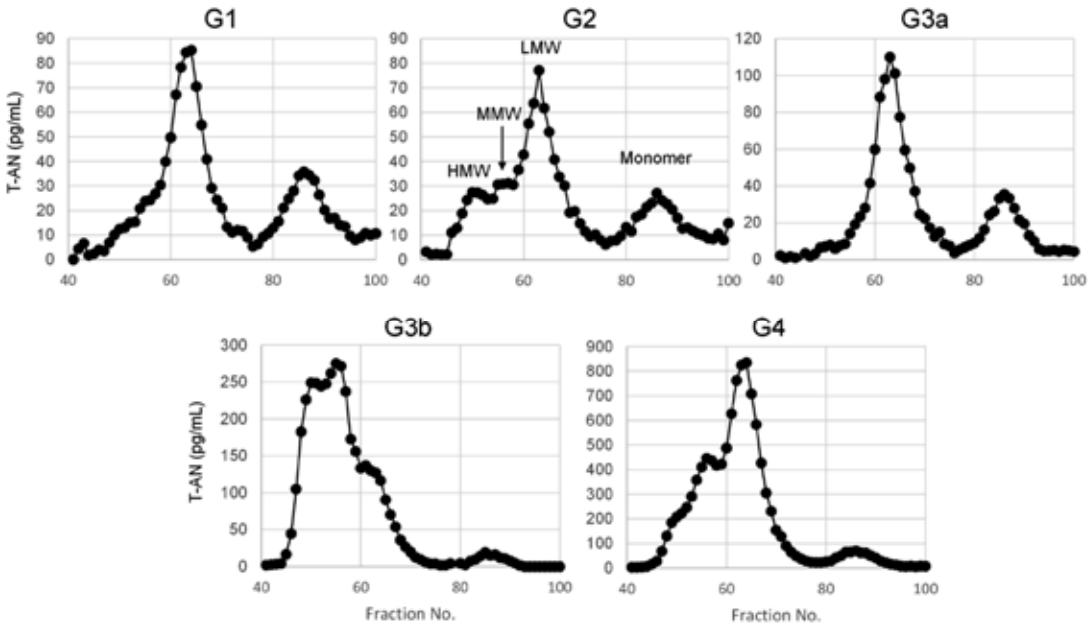

Fig 2. HMW, MMW, LMW and monomer of adiponectin in urine from patients with G1-G4 stages of diabetic kidney disease. Sixty SEC fraction samples were measured for one case from each eGFR stage.
T-AN : Total adiponectin, H-AN : High molecular weight adiponectin, LMW : Low molecular weight, MMW : Middle molecular weight, HMW : High molecular weight. Urine samples from patients with diabetes were separated by SEC and fractions were measured by sandwich T-AN assay.

Table 2. Correlation between eGFR and urinary biomarker / index values.

Biomarker / Index	r	p	
ALB	-0.58	0.082	N.S.
sT-AN	-0.54	0.11	N.S.
iT-AN	-0.54	0.11	N.S.
sH-AN	-0.47	0.17	N.S.
iH-AN	-0.47	0.17	N.S.
sMonomer	-0.09	0.80	N.S.
sLMW	-0.67	0.033	p<0.05
sMMW	-0.58	0.082	N.S.
sHMW	-0.45	0.187	N.S.
sTOTAL	-0.58	0.082	N.S.
sMonomer/sTOTAL	0.65	0.043	p<0.05
sLMW/sTOTAL	-0.67	0.033	p<0.05
sMMW/sTOTAL	-0.39	0.26	N.S.
sHMW/sTOTAL	-0.09	0.80	N.S.

s : Measured by sandwich EIA, i : Measured by ICT-EIA. ALB : Albumin, T-AN : Total adiponectin, H-AN : High molecular weight adiponectin, LMW : Low molecular weight, MMW : Middle molecular weight, HMW : High molecular weight. Spearman's correlation coefficients were calculated. Due to the small sample size, the p-values may not be accurate, but they are provided for reference.

Estimation of adiponectin indexes

Although the LMW/total adiponectin ratio and monomer/total adiponectin ratio were shown to be potentially useful biomarkers that reflect changes in diabetic kidney disease at an early stage of eGFR, sample analysis by SEC is time-consuming and labor-intensive, making it unsuitable for practical use. Therefore, we investigated the possibility of estimating the values of LMW/total adiponectin ratio and monomer/total adiponectin ratio from the results of T-AN and H-AN measurements by sandwich EIA and ICT-EIA methods, which are measurements directly obtained from urine samples.

T-AN and H-AN levels were measured by sandwich EIA and ICT-EIA methods and indexes using the measured values were calculated. The correlations between those indexes and the values of target parameters, including LMW/total adiponectin ratio and monomer/total adiponectin ratio, are shown in Table 3. It was difficult to estimate the value of LMW/total adiponectin ratio using any of the indexes as correlations were poor. On the other hand, T-AN and H-AN levels measured by the sandwich EIA and ICT-EIA methods correlated well with the monomer/total adiponectin ratio. Furthermore, the ratio of T-AN as measured by two assays (sandwich EIA method / ICT-EIA method) was newly identified as a potential index which correlates with monomer/total adiponectin ratio and reflects the progression of diabetic kidney disease.

Early biomarker for diabetic kidney disease

Urine samples collected from a total of 77 diabetic patients (G1 : 9, G2 : 43, G3a : 14, G3b : 8, G4 : 3) were tested using both sandwich EIAs and ICT-EIAs for T-AN and H-AN for a total of 4 measures. Adiponectin assay results and indexes were plotted for each eGFR stage (Fig 4) along with the levels of albumin, a conventional biomarker.

For T-AN, neither the sandwich EIA values nor the ICT-EIA values were significantly elevated compared to the G1 stage. The

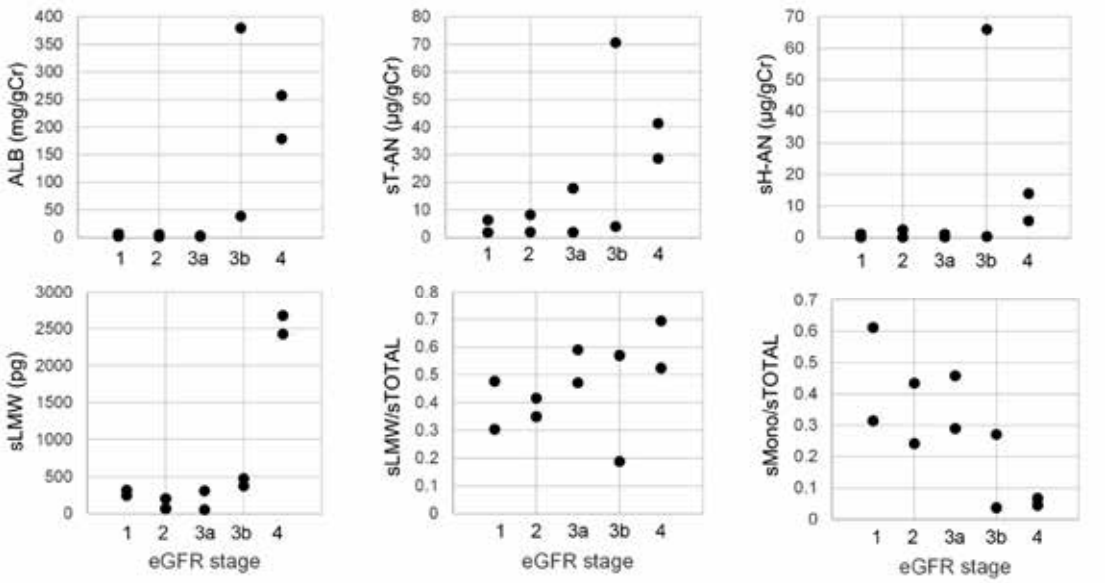

Fig 3. Trend of urinary biomarker/index values depending on eGFR stages.
s : Measured by sandwich EIA, i : Measured by ICT-EIA. ALB : Albumin, T-AN : Total adiponectin, H-AN : High molecular weight adiponectin, LMW : Low molecular weight, Mono : Monomer.

Table 3. Correlation between urinary biomarker / index values, LMWs/TOTALs and Monomers/TOTALs

Taget parameter	Biomarker / Index	r	p	
sLMW/sTOTAL	sT-AN	0.25	0.49	N.S.
	iT-AN	0.26	0.47	N.S.
	sT-AN/iT-AN	-0.18	0.63	N.S.
	sH-AN	0.16	0.65	N.S.
	iH-AN	0.16	0.65	N.S.
	sH-AN/iH-AN	0.067	0.85	N.S.
	sT-AN/sH-AN	0.055	0.88	N.S.
	sT-AN/iH-AN	0.018	0.96	N.S.
	iT-AN/sH-AN	0.14	0.70	N.S.
	iT-AN/iH-AN	0.055	0.88	N.S.
sMonomer/sTOTAL	sT-AN	-0.93	0.00010	p<0.001
	iT-AN	-0.89	0.00050	p<0.001
	sT-AN/iT-AN	0.94	<0.0001	p<0.0001
	sH-AN	-0.90	0.00030	p<0.001
	iH-AN	-0.90	0.00030	p<0.001
	sH-AN/iH-AN	0.36	0.31	N.S.
	sT-AN/sH-AN	0.81	0.0049	p<0.01
	sT-AN/iH-AN	0.79	0.0061	p<0.01
	iT-AN/sH-AN	0.73	0.016	p<0.05
	iT-AN/iH-AN	0.75	0.013	p<0.05

s : Measured by sandwich EIA, i : Measured by ICT-EIA. ALB : Albumin, T-AN : Total adiponectin, H-AN : High molecular weight adiponectin. Spearman's correlation coefficients were calculated. Due to the small sample size, the p-values may not be accurate, but they are provided for reference.

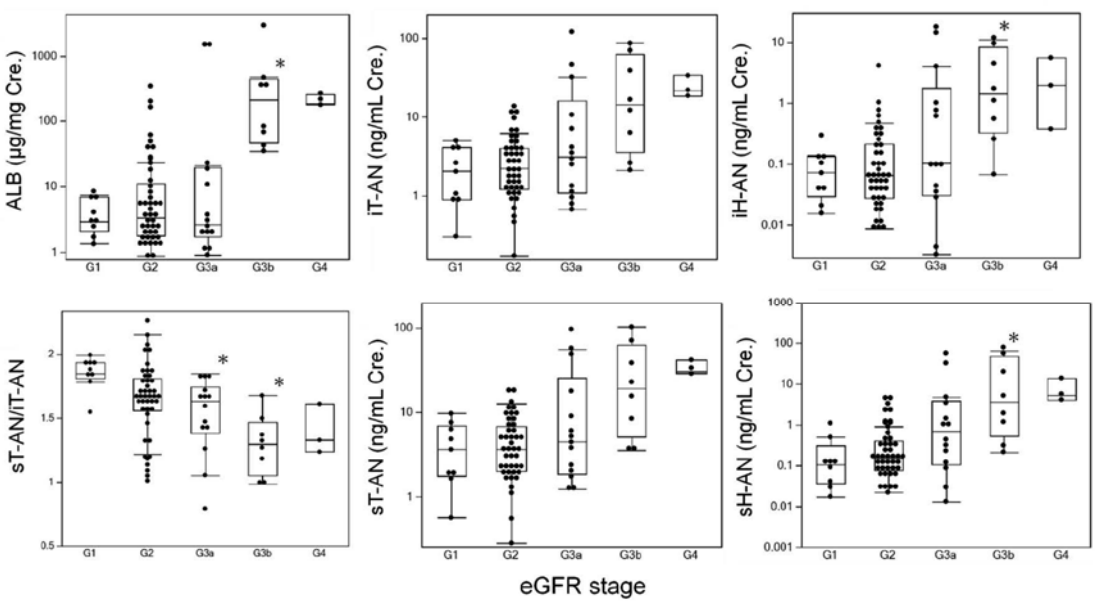

Fig 4. Levels of urinary biomarker/index values and eGFR stages.
s : Measured by sandwich EIA, i : Measured by ICT-EIA. ALB : Albumin, T-AN : Total adiponectin, H-AN : High molecular weight adiponectin.
* : $p<0.05$ vs. G1 stage by Steel-Dwass test.

H-AN levels as measured by sandwich EIA and ICT-EIA methods as well as albumin levels showed a significant upward trend in the G3b stage compared to the G1 stage.

Against these trends, the ratio of T-AN as measured by sandwich EIA / ICT-EIA methods (sT-AN/iT-AN), which was newly shown to be a potential indicator of diabetic kidney disease progression, showed a significant decrease at stage G3a compared to the G1 stage.

DISCUSSION

We previously reported that T-AN and H-AN assays by an ICT-EIA method may be useful as biomarkers for diabetic kidney disease (9-11). In this study, we also developed sandwich EIAs for T-AN and H-AN and compared their reactivity with that of the ICT-EIAs. For each of the T-AN and H-AN assays, the same antibody pair was used in both the sandwich EIA and the ICT-EIA assay, only the assay method was different.

For the T-AN assays, the ICT-EIA method proportionally detected higher molecular weight adiponectin species compared to the sandwich EIA method. This is observed more easily in the analysis of the urine sample. The adiponectin standard looked similar between sandwich-EIA and ICT-EIA methods.

For the H-AN assays, the ICT-EIA method exclusively detected higher molecular weight adiponectin species compared to the sandwich EIA method, which also detected some MMW species. This is observed in the analysis of both the urine sample and the adiponectin standard.

This difference in reactivity is likely due to the difference in assay method. In the ICT-EIA method, it has been found that after the first wash, dissociation of the antibody/adiponectin complex occurs due to the absence of excess antibody reagent in the reaction buffer (12). In this process of dissociation, it is possible that lower molecular weight forms are more prone to dissociate compared to higher molecular weight forms of adiponectin, resulting in the observed reactivity profile.

Next, we examined the abundance of different forms of adiponectin in the urine of diabetic patients using the T-AN sandwich EIA, which is capable of detecting all adiponectin forms, ranging from monomeric to HMW forms, at the highest efficiency (Fig 2). In a previous report, we performed a similar analysis using the ICT-EIA (9, 10) and observed more HMW adiponectin in patients with advanced eGFR stages. In the present study, we similarly found that urine from patients with advanced eGFR stage contained more high-molecular-weight adiponectin. For more detailed analysis, urine samples (10 samples in total) from subjects selected at the 25th and 75th percentile of each eGFR stage (G1, G2, G3a, G3b, and G4) were size-fractionated by SEC before testing of fractions by T-AN and H-AN assays. The amounts of monomeric, LMW, MMW, and HMW adiponectin were measured and the ratio of monomeric, LMW, MMW, and HMW to total adiponectin was calculated. Correlation of the calculated adiponectin indexes (ratios) between eGFR stage and the LMW measure, the LMW/total adiponectin ratio and the monomer/total adiponectin ratio showed relatively strong correlation (Table 2). Among them, the LMW/total adiponectin ratio and the monomer/total adiponectin ratio demonstrated a linear correlation with eGFR stage over the entire dataset (Fig 3). Urine collected from patients with relatively early eGFR stages contained mainly monomer and LMW adiponectin, and as diabetic kidney disease progressed, MMW and HMW adiponectin became more abundant, suggesting that the relatively low-molecular-weight adiponectin in urine may reflect changes caused during earlier stages of kidney disease.

However, the calculation of LMW/total adiponectin ratio and monomer/total adiponectin ratio requires size fractionation by SEC, which is time-consuming and labor-intensive, making it unsuitable as a clinical test. Therefore, we investigated a method to estimate these ratios. Results of the T-AN and H-AN assays by sandwich EIA and ICT-EIA methods were used to calculate adiponectin indexes. The correlation between the target parameters

(LMW/total adiponectin and monomer/total adiponectin) and the adiponectin indexes representing results measured from different assay methods were examined (Table 3). As a result, it was difficult to estimate the LMW/total adiponectin ratio using any of the assays and indexes, but several indexes showed relatively strong correlation with the monomer/total adiponectin ratio, among which the ratio of T-AN by sandwich EIA / ICT-EIA may be useful as a new index of diabetic kidney disease.

Finally, we examined the usefulness of the ratio of T-AN as measured by sandwich EIA / ICT-EIA methods as a new indicator of diabetic kidney disease. All other biomarkers showed significant changes starting at stage G3b compared to the G1 stage, while the ratio of T-AN by sandwich EIA and ICT-EIA (sT-AN/iT-AN) methods showed significant differences at stage G3a.

In the present study, we found that adiponectin in the urine of patients with diabetic kidney disease increased from monomer and LMW to high molecular weight adiponectin (MMW and HMW) as the disease progressed. Also, the LMW/total adiponectin ratio and monomer/total adiponectin ratio were found to strongly correlate with eGFR stage in a linear fashion across all stages of disease. The monomer/total adiponectin ratio can be estimated by calculating the ratio of T-AN as measured by sandwich EIA and ICT-EIA as sT-AN/iT-AN, indicating that this index may be useful as a biomarker for early diabetic kidney disease.

The limitations of this study are the lack of comparison with healthy individuals, limited clinical data, cross-sectional studies and the lack of accurate diagnosis of diabetic nephropathy that may be done by biopsies. At this time, the mechanism of adiponectin accumulation in urine is not fully understood. Further studies are needed to validate the biological significance of urinary adiponectin variability and whether it brings new significance compared to the eGFR currently used in clinical practice. In the future, the usefulness of urinary adiponectin levels as a biomarker for DKD should be tested in a larger prospective study currently underway.

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