Usefulness of HPLC Assay for Early Detection of Microalbuminuria in Chronic Kidney Disease

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Background: Since the degree and increase of albuminuria predict cardiovascular and renal diseases even within the range of normoalbuminuria. The high-performance liquid chromatography (HPLC) assay has been reported as a useful tool for earlier detection of microalbuminuria than turbidimetric immunoassay (TIA) in diabetes but has not been evaluated for other common diseases that caused chronic kidney disease (CKD). **Methods:** We measured albumin in spot urine by TIA and HPLC assay in 119 patients with diabetes, hypertension, IgA nephropathy in complete remission, or autosomal-dominant polycystic kidney disease whose dipstick screening tests were negative. **Results:** There were significant correlations ($r = 0.946$) between TIA and HPLC assay, and the ratios of urine albumin/creatinine (ACR) mea-

sured by HPLC assay were always higher than those measured by TIA. The value of microalbuminuria was highest in IgA nephropathy patients, and higher in diabetic patients with hypertension than in those without hypertension. Fifty-one patients were classified as having normoalbuminuria and 42 as having microalbuminuria by both TIA and HPLC assay. However, 26 patients (21.8%) were classified having as normoalbuminuria by TIA but microalbuminuria by HPLC assay. Three of these patients were reclassified as microalbuminuria by both assays within 1 year. **Conclusion:** These results suggest that ACR measurements by HPLC assay are better than TIA for early detection and monitoring of microalbuminuria in patients with diabetes and hypertension. J. Clin. Lab. Anal. 27:333–338, 2013. © 2013 Wiley Periodicals, Inc.

Key words: immuno-unreactive albumin; intact albumin; turbidimetric immunoassay; ratios of urine albumin/creatinine; normoalbuminuria

INTRODUCTION

Microalbuminuria has been identified as an independent risk factor for chronic kidney disease (CKD), cardiovascular disease, and cerebrovascular disease in the general population (1–4). Screening for microalbuminuria is recommended in patients with diabetes or hypertension to aid in risk stratification and target treatment. Several reports have indicated that higher concentration of urinary albumin, even less than the microalbuminuria cut-off value, are a risk factor for future events, suggesting the importance of precise measurement and early recognition of microalbuminuria (5–7).

Comper et al. reported that immuno-unreactive with an intact molecular weight albumin was identified in urine of patients with diabetes (8). High-performance liquid chromatography (HPLC) assay is capable of measuring both

immunoreactive intact albumin and immuno-unreactive intact albumin (9, 10). They demonstrated that HPLC assay is able to detect microalbuminuria far earlier than radioimmunoassay for both type 1 and type 2 diabetic patients (11). Recently, Toth et al. found that the albumin-to-creatinine ratio (ACR) measured by HPLC assay was correlated with urinary ortho-tyrosine, which is an indicator of oxidative stress but ACR measured by turbidimetric

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immunoassay (TIA) was not correlated in patients with acute ischemic stroke. They concluded that HPLC assay is more sensitive than TIA for measurement of ACR in these patients (12).

The precise mechanism of the presence of immuno-unreactive intact albumin in urine has not been clarified. Russo et al. reported that albumin undergoes denaturation and fragmentation during renal passage and assumed that albumin filtered from the glomeruli is biochemically modified by lysosomal proteinase before excretion from proximal tubules (13,14). This process may induce immunoreactive albumin, immuno-unreactive albumin and fragment albumin in urine. This degenerated albumin was not reactive to anti-albumin antibody and not measured by conventional immunochemical assays such as radioimmunoassay, immunoturbidimetry, and immunonephelometry because they can detect only immunoreactive albumin. Therefore, immunoassays might underestimate and underdiagnose microalbuminuria. Furthermore, it is not clear whether there is a difference in the relative amount of urinary immunoreactive and immuno-unreactive albumin among primary diseases since most reports concern albuminuria measured in diabetes (8, 11, 15).

The present study was performed to examine if there are proportional differences in urinary immunoreactive and immuno-unreactive albumin among normal subjects, and patients with type 2 diabetes, essential hypertension, IgA nephropathy in complete remission or ADPKD whose dipstick screening tests for proteinuria were all negative. We also followed-up urinary albumin in several patients who presented normoalbuminuria (ACR of less than 30 mg/g) measured by TIA but microalbuminuria (ACR of more than 30 mg/g) measured by HPLC assay.

MATERIAL AND METHODS

Subjects

One hundred nineteen outpatients of the Division of Nephrology and Hypertension in Juntendo University Hospital who were negative in the dipstick screening tests for proteinuria (less than 20 mg/dl) were enrolled in this study. Spot urine samples from the patients and 39 healthy volunteers were also examined. The study protocol was approved by the institutional review board of Juntendo University Hospital and all patients gave written informed consent. Since one patient withdrew consent, we discarded the urine sample and excluded the patient's information from our study. All urine samples were stored at -80°C before the analyses.

Primary diseases in this study were type 2 diabetes in 49 patients (41.2%), 32 of whom (65.3%) had hypertension, essential hypertension (42.9%) in 51, IgA nephropathy in

complete remission (10.1%) in 12, and ADPKD (5.9%) in seven patients. These diagnoses were based on clinical data, imaging, and renal biopsy. The following information was obtained from medical records in our hospital: body mass index (BMI), systolic and diastolic blood pressure (sBP and dBP) at the time of visiting doctor in the sitting position, hemoglobin (Hb), HbA1c, serum creatinine, uric acid, LDL cholesterol and estimated GFR (eGFR). eGFR was calculated by a modified three variable equation for eGFR in Japanese patients (16).

METHODS

Before the assay, urine samples were thawed under running water, mixed by Vortex for 10 s, and let stand for 1 min. Total albumin consisting of immunoreactive and immuno-unreactive albumin was determined in urine aliquots by HPLC assay using Accumin•TI-MAB (Reagent for Total Intact Albumin Assay, Nipro Co., Osaka, Japan). Immunoreactive albumin was measured by TIA, which is the standard method in Japan. Urine creatinine was measured in the same urine aliquots by the enzyme assay using a HITACHI 7170 auto-analyzer. ACR was calculated and compared in the study subjects.

STATISTICS

Statistical analyses were performed using JMP software (SAS Institute Inc., Cary, NC). Comparisons of the clinical characteristics between the disease subjects were made by Students' *t*-test. *P* values of less than 0.05 were considered statistically significant. Data were expressed as mean $\pm$ standard error and *n* represented the number of determinations.

RESULTS

Clinical Characteristics and Laboratory Findings

Demographic and clinical characteristics, and laboratory findings of all subjects at the time of urine sampling from 119 patients and 39 normal controls are summarized in Table 1. Age of patients ranged from 19 to 83 (63.4 ± 13.4) years and 77 patients were male (64.7%). The mean age for IgA nephropathy was significantly lower than that for the other diseases ($P < 0.001$). No sex differences were revealed. BMI in diabetes was significantly higher than that for IgA nephropathy and ADPKD ($P < 0.005$) but not that for essential hypertension. Twenty seven patients with diabetes (55.1%), 36 patients with essential hypertension (70.6%), five patients with IgA nephropathy (41.7%), and five patients with ADPKD (71.4%) were under treatment with an angiotensin-converting enzyme inhibitor (ACEI) or angiotensin II receptor blocker (ARB).

TABLE 1. Clinical Characteristics and Laboratory Findings at the Time of Urine Sampling in 119 Patients and 39 Normal Subjects

	u-Alb (mg/gCre)			M/F	Age (y.o)	s BP (mmHg)	d BP (mmHg)	BMI	Hb (g/dL)	s Cr (mg/dL)	eGFR (ml/min)	UA (mg/dL)	LDL-C (mg/dL)	HbA1c (%)	ACEI or ARB treatment (%)
	TIA	HPLC													
Healthy subjects n = 39	8.02 ± 5.72	11.86 ± 8.37	26/13	37.56 ± 10.57											
Total n = 119	33.26 ± 36.61	52.03 ± 45.77	77/42	63.41 ± 13.35	128.38 ± 16.39	75.32 ± 11.65	23.62 ± 3.22	14.00 ± 1.54	0.82 ± 0.29	74.34 ± 21.39	5.85 ± 1.29	109.27 ± 26.19			62.2
DM n = 49	24.32 ± 21.38	41.97 ± 28.59	41/8	66.65 ± 7.78	126.81 ± 13.47	74.47 ± 9.38	24.63 ± 3.16	14.25 ± 1.71	0.82 ± 0.29	76.67 ± 21.41	6.10 ± 1.15	109.37 ± 21.14	6.78 ± 1.16		55.1
DM w/ HT n = 32	25.68 ± 21.39	45.76 ± 25.92	25/7	67.31 ± 8.08	130.97 ± 14.35	72.42 ± 10.15	25.52 ± 3.05	14.22 ± 1.80	0.84 ± 0.34	75.00 ± 23.09	6.27 ± 1.17	110.48 ± 23.82	6.58 ± 1.06	75	
DM w/o HT n = 17	21.76 ± 21.77	34.85 ± 25.21	16/1	65.41 ± 7.26	118.75 ± 6.28	72.63 ± 7.65	22.71 ± 2.57	14.33 ± 1.57	0.78 ± 0.18	79.82 ± 18.05	5.79 ± 1.07	107.21 ± 15.25	7.29 ± 1.20	17.6	
HT n = 51	32.04 ± 35.05	50.66 ± 45.92	30/21	63.82 ± 14.95	129.86 ± 18.28	76.29 ± 12.74	23.50 ± 3.27	13.86 ± 1.30	0.85 ± 0.32	71.37 ± 21.87	5.78 ± 1.28	107.02 ± 28.31		70.6	
IgAN n = 12	70.25 ± 61.83	92.15 ± 72.40	6/6	44.67 ± 11.54	119.58 ± 14.56	71.33 ± 10.28	21.52 ± 2.31	14.24 ± 1.71	0.80 ± 0.10	76.73 ± 18.26	5.81 ± 0.88	110.29 ± 30.53		45.5	
ADPKD n = 7	40.17 ± 45.20	63.03 ± 57.77	1/6	61.14 ± 14.05	137.29 ± 12.84	81.57 ± 17.36	21.98 ± 2.51	13.56 ± 1.20	0.64 ± 0.19	80.07 ± 17.83	4.57 ± 1.98	129.25 ± 45.92		71.4	
TIA < 30 HPLC < 30 n = 51	8.99 ± 4.66	17.04 ± 6.67	39/12	64.57 ± 13.36	127.12 ± 17.32	75.94 ± 10.19	24.19 ± 3.17	14.26 ± 1.45	0.80 ± 0.19	76.46 ± 19.50	6.07 ± 1.07	102.72 ± 23.11		62.7	
TIA < 30 HPLC ≥ 30 n = 26	19.42 ± 7.43	47.55 ± 12.85	16/10	65.58 ± 10.02	130.76 ± 14.96	74.96 ± 13.19	23.70 ± 3.76	14.29 ± 1.56	0.78 ± 0.30	76.26 ± 21.70	5.36 ± 1.37	118.79 ± 25.18		53.8	
TIA ≥ 30 HPLC ≥ 30 n = 42	71.34 ± 33.16	97.30 ± 47.29	22/20	60.67 ± 14.88	128.51 ± 16.25	74.76 ± 12.58	22.89 ± 2.86	13.52 ± 1.53	0.88 ± 0.37	70.34 ± 23.24	5.91 ± 1.43	112.14 ± 28.79		63.6	

Abbreviations: DM, diabetes; HT, hypertension; IgAN, IgA nephropathy; sBP, systolic blood pressure; dBP, diastolic blood pressure; UA, uric acid.

Fig. 1. Scatter plots of ACRs from all subjects including normal healthy volunteers ($n = 158$) and regression line to determine the relationship between TIA and HPLC assay. Correlation coefficient of $r = 0.947$.

BP in patients with essential hypertension and diabetes with hypertension were both well controlled but sBP and dBP in essential hypertension patients were still significantly higher than those in diabetes and IgA nephropathy patients ($P < 0.05$).

Urinary Albumin Excretion

All of the ACRs derived from urine samples measured by HPLC assay (52.0 ± 45.8 mg/g) were higher than those measured by TIA (33.3 ± 36.6 mg/g), ($P < 0.005$). The correlation between TIA and HPLC assay was excellent with a correlation coefficient of $r = 0.947$ although the regression equation ($y = 1.22x + 9.29$) had a slope and intercept (Fig. 1). There were no significant differences in the scattered patterns and regressions among primary diseases (Fig. 2). Among 119 patients, 51 patients (42.9%) were classified as having normoalbuminuria (ACR of less than 30 mg/g) and 42 patients (35.3%) as having microalbuminuria (ACR of 30–300 mg/g) determined by both TIA and HPLC assay. However, 26 patients (21.8%) showed normoalbuminuria with TIA but microalbuminuria in the HPLC assay (Fig. 3). This discrepancy between TIA and HPLC assay was observed in 12 patients with diabetes (24.5%), eight patients with hypertension (16.0%), two patients with IgA nephropathy (16.7%), and four patients with ADPKD (57.1%). Three 12 patients with diabetes represented microalbuminuria by both methods within 1 year.

eGFR of 45 to 59 ml/min was observed in 31 119 patients (26.1%). Among them, ten patients had diabetes (20.4%), 19 patients had essential hypertension (37.3%),

Fig. 2. Scatter plots of ACRs from diabetes (A), essential hypertension (B), IgA nephropathy, (C) and ADPKD (D) and regression line to determine the relation between TIA and HPLC assay.

Total n=119		TIA	
		<30mg/g	≥30mg/g
HPLC	<30mg/g	n=51 42.9%	n=0 0%
	≥30mg/g	n=26 21.8%	n=42 35.3%

Fig. 3. Number and percentage of normoalbuminuria (<30mg/g) and microalbuminuria (30–300 mg/g) measured by TIA and HPLC assay. (A), diabetes; (B), essential hypertension; (C) IgA nephropathy; (D), ADPKD.

and two patients had IgA nephropathy (16.7%). Of these 31 patients, 14 patients showed microalbuminuria by both TIA and HPLC assay and seven patients showed normoalbuminuria by TIA but microalbuminuria by HPLC assay.

DISCUSSION

Microalbuminuria significantly increases the risk of overt nephropathy as well as those of cardiovascular and cerebrovascular complications (17). These risks increase proportionally with the amount of urinary albumin even within the normal range. Therefore, primary prevention or delaying the onset of microalbuminuria seems to be very important.

For an effective screening strategy to reduce end-stage kidney disease (ESKD) in Japan, screening with the urine dipstick test for proteinuria has been used for every resident over 40 years old and has resulted in major achievements (18). Konta et al. examined 2,136 subjects who were negative in the urine dipstick test for proteinuria in a community-based health check-up in Takahata, Japan. Among them, 317 subjects (10.1%) showed microalbuminuria measured by TIA (19). These results suggest that more accurate screening for microalbuminuria is needed for patients who have CVD risk factors.

It has been reported that there are two types of albumin in appreciable amounts in human urine: immunoreactive intact albumin and immuno-unreactive intact albumin (8). The latter can not be detected by TIA using anti-albumin antibody. Since HPLC assay is capable of measuring immunoactive and immuno-unreactive intact albumin, the quantitative value of ACR measured by HPLC assays is always higher ($18.8 \pm 16.2\%$) than that measured by TIA (20, 21). The result indicated that approximately 20% of immuno-unreactive albumin may include in total urinary albumin.

Before starting the study, to avoid contaminations in albumin peak, we confirmed urinary proteins that have similar molecular weight peaks to albumin such as transferrin, α 1-antitrypsin, haptoglobin, prealbumin, α 2-HS-glycoprotein, and IgG; the amounts of these proteins were very few (9).

In this study, we measured ACR by TIA and HPLC assay in 119 patients with diabetes, essential hypertension, IgA nephropathy in complete remission, or ADPKD whose dipstick screening test for proteinuria was negative. In the ACRs of all patients, 42.9% were classified as having normoalbuminuria and 35.3% as having microalbuminuria by both TIA and HPLC assay. However, 21.8% of them showed microalbuminuria in HPLC assay but normoalbuminuria in TIA. The relationship between TIA and HPLC assay was highly significant, suggesting that HPLC assay might be a more sensitive method for diagnosis of microalbuminuria than TIA. Interestingly, these differences became negligible in the range of macroalbuminuria (Fig. 1). These results also suggest that increased urinary immuno-unreactive albumin indicates early kidney injury and measurement of urinary albumin by HPLC assay may be used as a biomarker for detecting the early stage of CKD.

Comper et al. reported that measurement of urinary albumin by HPLC permitted earlier detection of microalbuminuria associated with diabetic nephropathy (11). In this study, we measured ACR not only in diabetics but also in patients with essential hypertension, IgA nephropathy in complete remission, and ADPKD. Since there were no specific differences between the rate of urinary immunoreactive and immuno-unreactive albumin among these diseases, the presence of immuno-unreactive albumin did not seem to be specific for diabetic nephropathy. During our study, we measured ACR several times in 14 patients who showed microalbuminuria by HPLC assay but normoalbuminuria by TIA in the first examination and three patients with diabetes who showed microalbuminuria by both TIA and HPLC assay within 1 year. During follow-up periods, these patients returned to the normoalbuminuria measured by both assays with reducing HbA1c after treatments with enhanced diet and exercise therapies or administration of DPP-4 inhibitor. This earlier recognition of microalbuminuria may contribute to prevent future vascular diseases although large studies are needed. Recently, Schmieder et al. analyzed two large prospective trials; ONgoingTelmisartan Alone and in combination with ramipril Global Endpoint Trial (ONTARGET) and The Telmisartan Randomized Assessment Study in ACE iNtolerant subjects with cardiovascular Disease (TRANSCEND) and reported changes in the degree of albuminuria, even in the ranges of microalbuminuria associated with cardiovascular, stroke, and renal outcomes (22). Thereafter, screening and monitoring extending beyond patients with known hypertension and/or diabetes was proposed. For this purpose, HPLC assay seems to be more useful than TIA for measuring urinary albumin since the cost for HPLC assay is almost the same as TIA by health insurance in Japan.

Hypertension is causally related to kidney outcomes and the prevalence of hypertension increases as kidney disease progresses. We observed that the value of ACR in HPLC assay in diabetics with hypertension, even well controlled by RAS inhibitors in the normal range, was higher than that in those without hypertension. This result suggests that rising blood pressure even within the normotensive range may affect microvascular complications in the future.

The causes of immuno-unreactive albumin in urine are not known. Comper et al. published a new concept of the mechanism of albumin excretion in urine (23). In their concept, relatively higher amounts of filtered albumin are retrieved by the proximal tubular cells and the small quantities of filtered albumin that are not retrieved undergo lysosomal degradation before urinary excretion. Russo et al. also observed that there were no significant changes in glomerular filtration of albumin between diabetic and normal control rats but albumin-derived urinary peptide excretion was increased in rats with early diabetic nephropathy. Filtered albumin from glomeruli may lose immune reactivity to anti-albumin antibody using TIA during the process occurring in tubules (24).

The majority of patients with CKD defined as eGFR of less than 60 ml/min are at particularly high risk for both CVD and ESKD, especially in the presence of albuminuria. Based on the results of a population-based study, Hallan et al. reported that urinary albumin and eGFR were independently associated with progression to ESKD and measuring both risk factors markedly improved diagnostic accuracy (25). They concluded that ACR and eGFR should always be complemented by more information to achieve an optimal prediction of the risk of progression to ESKD. In our study, eGFR of 31 (26.0%) patients was less than 60 ml/min and seven patients showed microalbuminuria in HPLC assay and normoalbuminuria in TIA. We may be able to have any therapeutic intervention for the high-risk patients for vascular diseases earlier using HPLC assay.

CONCLUSIONS

It appears that the use of HPLC assay for determination of urinary albumin concentration permits earlier diagnosis of microalbuminuria than TIA and could lead to a better outcome in patients with CKD. ACR measurements should be reserved for detecting microalbuminuria and monitoring patients with or without diabetes.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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