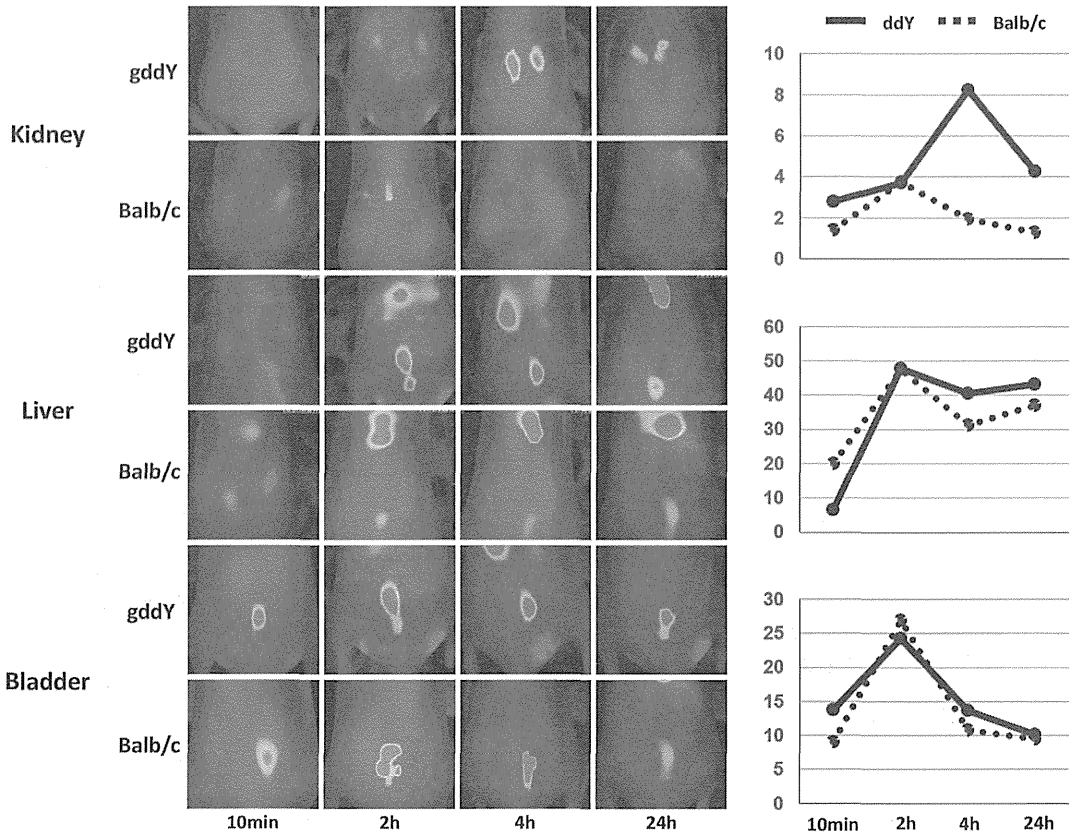

Figure 2. Kinetics of injected fluorescently labeled IgA in a fluorescence molecular tomography system. A fluorescence molecular tomography system (FMT) is capable of resolving size and concentration of fluorochromes in deep tissue *in vivo*. Fluorescein-labeled IgA samples from gddY and Balb/c mice were injected into nude mice and monitored from 10 min to 24 h postinjection by FMT. After 2 h, IgA signals in the liver and bladder were found in a similar manner in both the groups of nude mice. However, IgA signals in the kidneys clearly differed between them. Mice injected with gddY IgA showed strong signals in the kidneys, with a peak at 4 h.
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components, losing nephritogenicity. However, because these deposits were present in old ddY recipients after BMT, such organized IgA may still have epitopes that the fluorescence-labeled anti-IgA antibody recognizes. Therefore, present BMT models indicate that glomerular IgA in IgAN patients may be mixed with freshly-delivered nephritogenic IgA and non-inflammatory organized IgA, particularly in those with a longer disease history. This finding may also partly explain the discrepancy between the amount of IgA deposition and severity of glomerular lesions in human IgAN.

Although most glomeruli in mice injected with gddY IgA showed mesangial IgA deposition, deposits along capillaries were detected in a focal and segmental manner. *In vivo* serial imaging showed that such subendothelial/subepithelial IgA deposits seemed to be formed as a result of an accumulation on the focal/segmental initial aggregates of IgA, suggesting that the initial aggregates (microdeposits) may change the local physiological conditions, thereby leading to the increased glomerular affinity of IgA. These physiological changes may include the local deceleration of the glomerular capillary flow or increased permeability of the glomerular basement membrane interposed between the endothelial and epithelial layers [41–43]. These deposits along glomerular capillaries were indeed found along with morphological changes in glomerular endothelial cells and podocytes, even after 2 h. This phenomenon is suggestive of a rapid activation of glomerular resident cells by IgA deposition. We can speculate that such rapid activation (particularly in the endothelial cells, presumably in combination with slow blood flow) may facilitate leukocyte adhesion to the deposits and their subsequent clearance and/or inflammatory responses [44], [45], leading to hematuria in IgAN. This real-time imaging showed increased passage of IgA into glomerular mesangial lesions and the relevant glomerular pole after 2 h (data not shown), suggesting that subendothelial/subepithelial deposits may induce permeability factors, such as vascular endothelial growth factor (VEGF), and increase the flow of plasma into the mesangium and subsequent interstitium/lymph via the glomerular pole. Thus, nephritogenic IgA deposition may induce dynamic alterations in the glomerulus and subsequent glomerular and interstitial injury.

This study revealed the kinetics of glomerular deposition over the course of IgA-induced IgAN. These IgA molecules have a strong affinity for focal and segmental subendothelial, subepithelial, and glomerular mesangial lesions. Rapid cellular activation of endothelial cells and podocytes by IgA deposition may precede the events of hematuria in IgAN. The significant differences between human and murine IgAN limit the translation of these data to the human disease. Further, similar study using human GdIgA1 is needed. However, the present findings regarding the kinetics of IgA deposition may indicate that not only glomerular mesangial cells but also endothelial cells and podocytes are plausible targets of glomerular injury in IgAN.

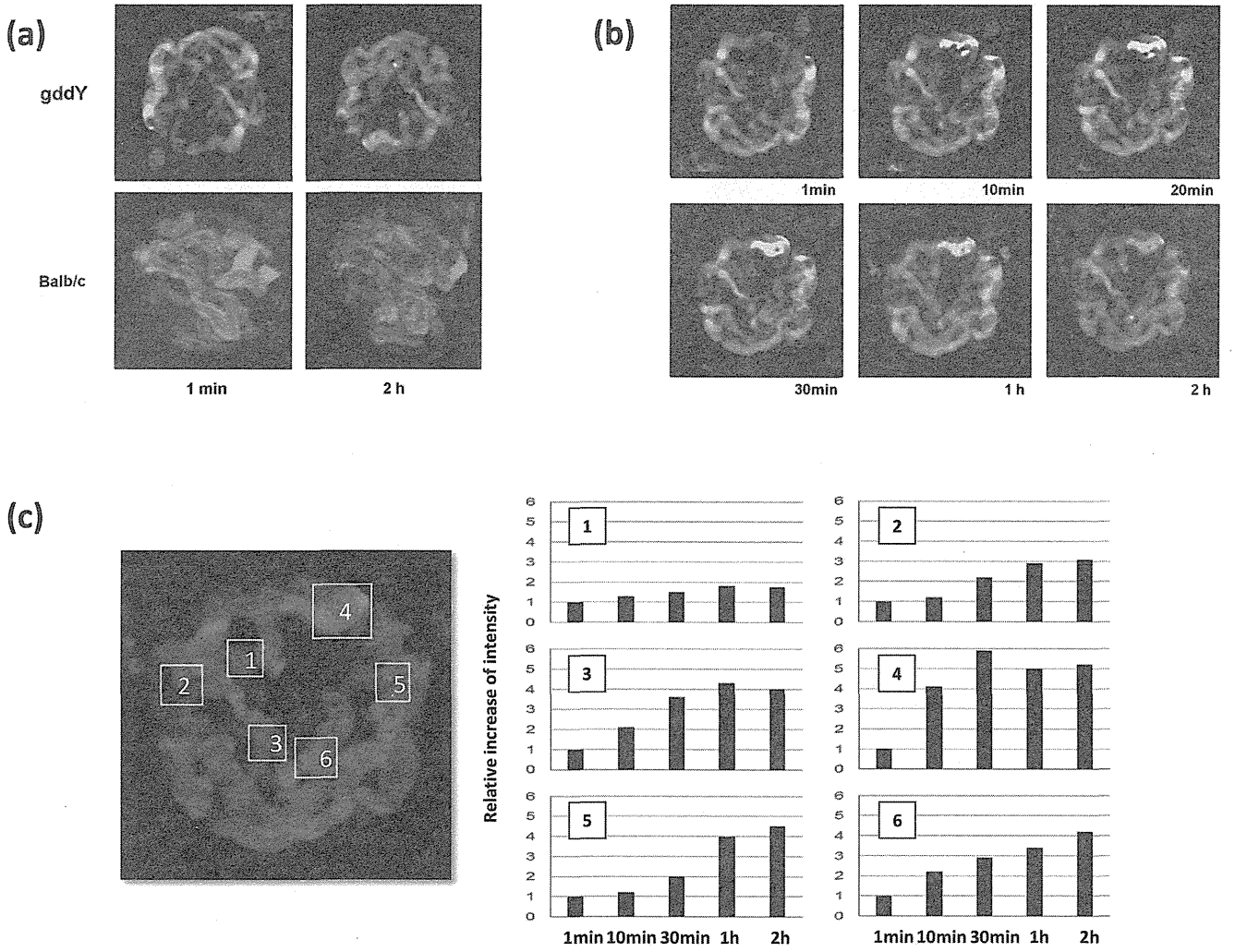

Figure 3. IgA from gddY mice is deposited along the glomerular capillary wall in a focal and segmental manner. Detailed kinetics of IgA deposition analyzed from 1 min to 2 h postinjection using confocal laser microscopy. Alexa Fluor 633-labeled IgA from gddY and Balb/c mice (red) and 500-kDa fluorescein-labeled dextran (green) were injected for analyzing kinetics of IgA deposition and visualizing blood vessel wall integrity, respectively. (a) IgA signals were detectable even after 1 min and accumulated up to 2 h in a focal and segmental manner in mice with IgA from gddY mice. In contrast, mice who received Balb/c IgA did not show a signal even after 2 h. (b)(c) Serial images of a glomerulus in mice with IgA from gddY mice showed that these IgA molecules accumulated on top of the initial aggregates along the glomerular capillaries. These aggregates were found in a focal and segmental manner but not in a diffuse and global manner.
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Supporting Information

Video S1 ***In vivo*** **3D imaging of glomerulus after injection of nephritogenic IgA.** 3D images of glomeruli at 2 hours after single injection of purified IgA from gddY mice were evaluated by a confocal laser microscopy. Alexa Fluor 633-labeled IgA from gddY (red) and 500-kDa fluorescein-labeled dextran (green) were injected for analyzing kinetics of IgA deposition and visualizing blood vessel wall integrity, respectively. IgA signals were detectable after 2 h in a focal and segmental manner in mice with IgA from gddY mice.
(MOV)

Video S2 ***In vivo*** **3D imaging of glomerulus after injection of IgA from Balb/c mice.** 3D images of glomeruli at 2 hours after single injection of purified IgA from Balb/c mice were evaluated by a confocal laser microscopy. Alexa Fluor 633-labeled IgA from and Balb/c mice (red) and 500-kDa fluorescein-labeled dextran (green) were injected for analyzing kinetics of IgA deposition and visualizing blood vessel wall integrity, respectively. IgA signals were detectable after 2 h in a focal and segmental manner in mice with IgA from gddY mice (Video images S1 and S3). In contrast, mice who received Balb/c IgA did not show clear signals after 2 h.
(MOV)

Video S3 ***In vivo*** **real-time imaging of glomerulus after injection of nephritogenic IgA.** Real-time images of glomeruli at 2 hours after single injection of purified IgA from gddY mice were evaluated by a confocal laser microscopy. Alexa Fluor 633-labeled IgA from gddY mice (red) and 500-kDa fluorescein-labeled dextran (green) were injected for analyzing kinetics of IgA deposition and visualizing blood vessel wall integrity, respectively. IgA signals were detectable after 2 h in a focal and segmental manner in mice with IgA from gddY mice.
(MOV)

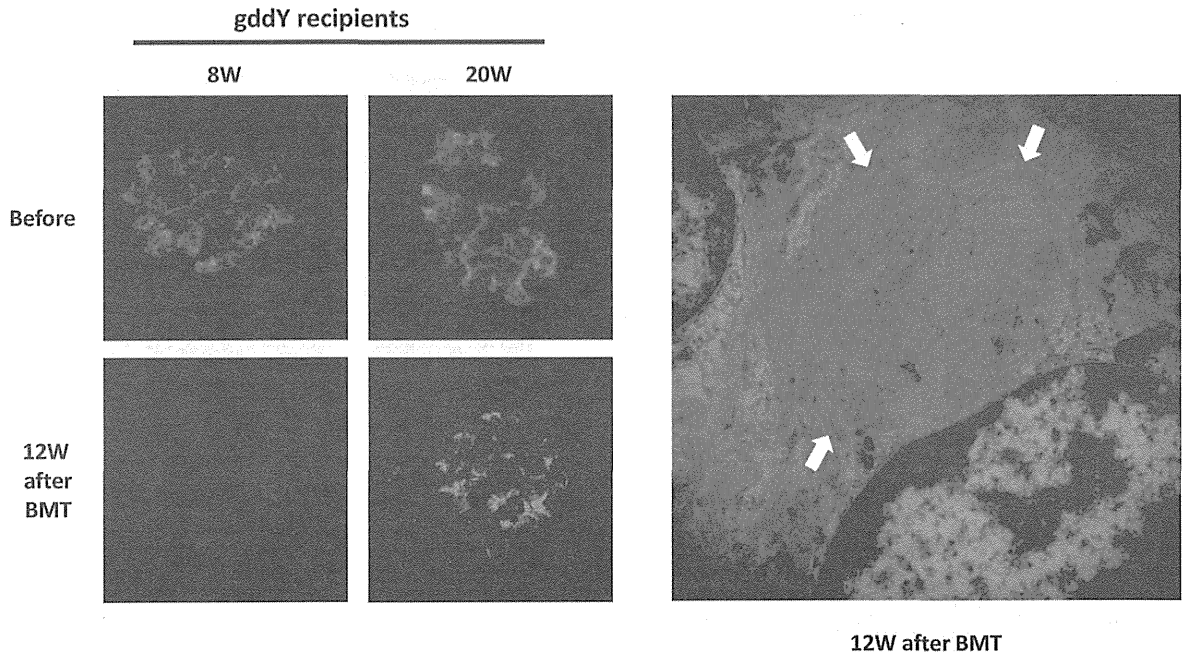

Figure 4. Glomerular IgA deposits in old gddY mice did not disappear after bone marrow transplantation (BMT) despite improvement in proteinuria. BM cells from healthy Balb/c mice were transplanted into young (8 weeks) and old (20 weeks) gddY mice at an early stage of disease. Although proteinuria was present in the young and old recipients 12 weeks after BMT, fluorescence analysis still detected glomerular IgA deposition in old gddY recipients but not in young gddY recipients (left panels). Electron microscopy still detected paramesangial dense deposits showing fibrous and lattice structures in the old recipients (right panel).
doi:10.1371/journal.pone.0113005.g004

Video S4 ***In vivo*** **real-time imaging of glomerulus after injection of IgA from Balb/c mice.** Real-time images of glomeruli at 2 hours after single injection of purified IgA from Balb/c mice were evaluated by a confocal laser microscopy. Alexa Fluor 633-labeled IgA from Balb/c mice (red) and 500-kDa fluorescein-labeled dextran (green) were injected for analyzing kinetics of IgA deposition and visualizing blood vessel wall integrity, respectively. IgA signals were detectable after 2 h in a focal and segmental manner in mice with IgA from gddY mice (Video images S1 and S3). In contrast, mice who received Balb/c IgA did not show clear signals after 2 h.
(MOV)

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Author Contributions

Conceived and designed the experiments: YT YS KY HS SH JN. Performed the experiments: KY YS HS KS. Analyzed the data: KY YS. Contributed reagents/materials/analysis tools: KY YS HS KS. Wrote the paper: KY YS.

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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) measured 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 microalbumnuria 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.

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 lisosomal 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 immunonephelemetry 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 ± 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)														ACEI or ARB treatment (%)
	TIA	HPLC	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 (%)		
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%),

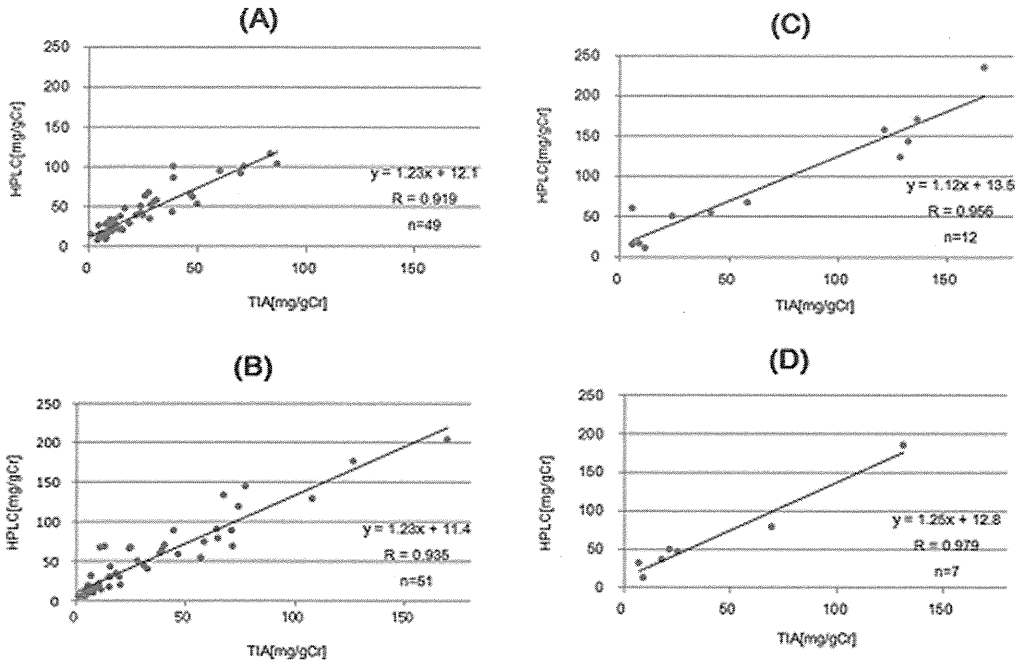

Fig. 2. Scatter plots of ACRs from diabetes (A), essential hypertension (B), IgA nephropathy, (C) and ADPKD (D) and regression line to determine the relation between TIA and HPLC assay.

Total n=119		TIA	
		<30mg/g	≧30mg/g
HPLC	<30mg/g	n=51	n=0
		42.9%	0%
	≧30mg/g	n=26	n=42
		21.8%	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 ± 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, α1-antitrypsin, haptoglobin, prealbumin, α2-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 maybe 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 (TRANCENT) 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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3. 病因に基づくバイオマーカーを用いたIgA腎症の早期発見・診断・治療の試み

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key words　galactose-deficient IgA1, anti-glycan antibody, immune complex, surrogate marker

動　向

IgA腎症は未治療の場合，約4割は15～20年の経過で末期腎不全に至る予後不良の慢性疾患である．血尿を初発とする患者が大部分を占め，進行に伴い蛋白尿を呈し進行する．Szetoら[1]が示したように，腎生検診断時に血尿のみあるいは尿蛋白が少量のいわゆる「軽症IgA腎症」といえども，その約30%の症例は腎不全に進行する．つまり，ある時点で血尿のみで蛋白尿を呈していなくともその症例の予後を約束するものではないため，確実に継続的外来観察をするか根治させるしか腎不全回避を確約できない．

IgA腎症の根治を目指した特異的治療法の開発が望まれているが，病因が不明なため未だ開発されていない．しかし，仮にそういった治療法が開発されても，20年の臨床経過で所謂「common pathway」による不可逆的な腎障害が混在するような進行した段階になってから介入しては，根治を望めないことは容易に想像される．したがって，根治治療を開発し正当に治療効果を評価するためには，早期発見・早期診断の環境を整備し，IgA腎症に特異的かつ客観的な活動性評価の方法を確立することが重要である．本邦では，年間約37,000人いる新規透析導入患者中，IgA腎症患者が3,000名以上を占めている可能性がある．また約30万人の透析患者のうち，IgA腎症を原因とする患者は5万人以上いる可能性があり，年間2500億円以上の医療費がIgA腎症由来の透析患者に使われていることになる．検尿システムが発達している日本においてさえ，血尿などの初期の尿所見異常を指摘されていたにもかかわらず，診断・治療時期を逸したIgA腎症患者がいかに多いのかが窺われ，早期診断・早期治療介入にむけてより効果的な方策が必要である．IgA腎症は糖尿病腎症などとは異なり，20年という長い経過で緩徐に推移する症例が主体である．したがって，治療法の評価は，そのoutcomeを慎重に設定する必要がある．厚労労働省進行性腎障害調査研究班IgA腎症研究会では，これまでばらばらであった寛解基準を一本化するため，全国アンケートをもとにIgA腎症の完全寛解を意味する「臨床的寛解」と，部分的寛解を意味する「血尿の寛解」「蛋白尿の寛解」を盛り込んだ「IgA腎症の寛解基準」を提唱し[2]，現在その基準の妥当性についての検証が進められている．こういった統一基準は，治療効果に関するサロゲートとなり，IgA腎症の治

療法開発には有用である．しかし，簡便かつリアルタイムな客観的活動性評価法の確立は，治療効果判定さらには治療の適応を決めるうえで重要である．こういったニーズから，IgA腎症に特異的バイオマーカーを用いた活動性評価の試みや，さらにはそれらを用いた早期診断などに向けた臨床応用が始まっている．

A. 糖鎖異常IgAおよび関連IgA免疫複合体の基礎的背景

IgA腎症患者の血清中には多量体IgA1が増加し，糸球体に沈着するIgAはIgA1が主体であることは知られていた[3,4]．この血清・糸球体IgA1の一部の糖鎖修飾異常が病因と深く関わることが明らかとなり，近年議論されている[5,6]．ヒトは２種類のIgAサブタイプ，IgA1とIgA2を有するが，両分子の違いの一つはヒンジ部位のアミノ酸組成で，特にIgA1のヒンジ部位にはO-結合型糖鎖が結合している．O-結合型糖鎖は，糖鎖修飾酵素の働きによって個々のO-結合型糖鎖構造には多様性がみられるが，内側よりN-アセチルガラクトサミン（GalNAc），ガラクトース（Gal），シアル酸（NeuAc）により構成される．HikiやAllenらの報告で，IgA腎症患者の糸球体IgA1はO型糖鎖修飾が減少していることが指摘されていた[7,8]ため，以前から流血中の糖鎖修飾異常を起こしたIgA1が，IgA腎症の発症に関わるであろうことが推測されていた．最近になりMoldoveanuらによって，IgA腎症患者の血清中にはGal修飾が減少したIgA1（GdIgA1）が増加していることが初めて示された[9]．彼女らは，（Galが欠失してむき出しになった）GalNAcを認識できるhelix aspersa agglutinin（HAA）というレクチンをELISAに用い，血清GdIgA1を定量化することに成功した．Suzukiらは，IgA腎症患者と健常人の末梢血由来IgA1産生細胞株を樹立し，糖鎖異常IgA産生機序について検証した[10]．各々の細胞株より産生されるIgA1の分子量や糖鎖構造を解析し，これまでの報告同様IgA腎症患者由来のIgA1は多量体が優位であり，Galが欠損したGal欠損型IgA1や，Galが欠損しかつNeuAcが結合した構造をもつIgA1が増加していることを見出した[10]．さらに，患者由来のIgA1産生細胞株における特異的糖鎖修飾酵素の発現や酵素活性を検証したところ，糖鎖構造解析の結果を裏付けるように，GalをGalNAcに結合するβ1,3-galactosyltransferaseは発現・活性が低下し，逆にNeuAcをGalNAcに結合するα2,6-sialyltransferaseは発現・活性が亢進していることが確認された[10]．このような糖鎖修飾酵素の発現調節には，サイトカイン環境が深く関わっていると考えられている．主にTh2サイトカインなどが，B細胞におけるβ1,3-galactosyltransferaseと，その分子シャペロンであるCosmcの発現低下を誘導し，その結果IgA1の糖鎖異常に関与していることが報告されている[11]．Gharaviらは，IgA腎症患者の複数の家系を調査し，IgA腎症患者のみならず，発症していない血縁においても血清中の糖鎖異常IgA1が増加していることより，糖鎖異常IgA1の産生は，サイトカイン産生を含む糖鎖修飾活性が遺伝的に規定されている可能性を報告した[12]．

一方GdIgA1の産生場所として，口蓋扁桃を示唆する報告が蓄積している．口蓋扁桃由来のリンパ球が糖鎖修飾異常，特にO型糖鎖異常を有するIgAの産生に関わるとする日本からの複数の報告[13,14]の他に，中国からは扁桃においてβ1,3-galactosyltransferaseの発現低下やα2,6-sialyltransferaseの発現と活性亢進を誘導する一群のサイトカインの発現異常があることも報告されている[15,16]．これに関連して，糖鎖修飾に関わる酵素活性と，粘膜免疫異常との関連性も示唆されている[17]．

我々の最近の検討も，GdIgA1産生部位としての口蓋扁桃の役割を示唆している．IgA腎症患者

では，扁摘により血清IgA値が低下することがよく知られており，我々の検討では扁摘後約4週後に平均10%程度の低下が確認された[18]．扁摘後に血清IgA値の減少が大きい群と小さい群を比較すると，前者では扁桃における自然免疫系の活性化が亢進し，特にIgA腎症との関連が示唆されるToll-like receptor（TLR）9の発現が高く，さらにその患者群では扁摘パルスの治療効果が高いことが示された[18]．腎炎惹起性IgAの一部は扁桃由来である可能性が考えられたことから，HAA-ELISAを用いて扁摘前後のGdIgA1の変化を直接解析した[19]．扁摘後にGdIgA1が低下する患者では，低下しない患者に比べ扁摘直後に有意に血尿が改善し，やはり扁桃におけるTLR9の発現量が有意に高値を示した[19]．以上より，扁桃は腎炎に関わるGdIgA1産生部位の一つであることが強く示唆された．

先述のGharaviらは，IgA腎症患者ではGdIgA1が正常者に比し血中に上昇している一方で，患者の血縁者は腎症がないにもかかわらず血縁ではない正常者に比しやはり上昇していることを示した[12]．つまり，GdIgA1だけでは病態は説明できず，IgA腎症がfull progressionするには，別な病態が関与していることが考えられた．IgA腎症患者の血中・尿中には，IgAを含む免疫複合体（IC）も増加している[5,9,20,21]．ICを形成するIgAはGdIgA1であり，患者血中には，GdIgA1-IgG ICおよびGdIgA1-IgA ICが増加している．Suzukiらは，IgA腎症患者血中にGdIgA1のO-結合型糖鎖のGalNAc残基を特異的に認識するIgG抗体を同定し，このGdIgA1特異的IgGは免疫グロブリン重鎖遺伝子の可変領域のアミノ酸配列が変化していることを明らかにした[22]．以上のことから，IgA腎症の発症・進展にはGdIgA1（1st hit）だけではなく，GdIgA1 IC形成（2nd hit）が必要と現在考えられる[23]．ヒトIgA腎症と病態および遺伝子制御が極めて類似する自然発症モデルマウス系[24]においても，血中IgA濃度ではなく血中IgAICレベルと腎炎重症度が相関し，IC形成が病態進展に重要であることが示されている[25]．糖鎖異常によるIgAの自己凝集化あるいは内因性自己抗体とのIC形成による高分子化することは，糸球体への親和性亢進や補体活性増強[26]などを介して起炎性を獲得し，2nd hitにつながっている可能性が考えられる．

B．糖鎖異常IgAおよび関連IgA免疫複合体を用いた診断・活動性評価の試み

現在，血清GdIgA1や関連ICの臨床応用にむけた検討が進められている．Zhaoらは，275名の中国人のIgA腎症患者に関して前向きに平均4年間GdIgA1を測定・観察した[27]．GdIgA1レベルが，蛋白尿，高血圧，eGFRなどで補正してもより強く腎機能低下と関連したと報告している．Camillaらは，血清GdIgA1と血中蛋白質過酸化物がeGFR低下速度と相関することを示し，GdIgA1がIgA腎症における酸化ストレス誘導因子であると同時に，両者の組み合わせによる障害マーカーとしての有用性を報告している[28]．一方Berthouxらは[29]，97名のフランス人のIgA腎症患者を対象に平均13.8年の観察期間で，診断時血清IgGとIgAタイプのanti-glycan抗体レベルと予後との関連を検討した．診断時の各内因性抗体の血清レベルは，死亡や透析導入に相関していた．Kaplan-Meier 生存曲線でもIgGタイプの内因性抗体が高い群では，死亡および透析移行が多いことが明らかにされた．また，高抗体価群では，5年，10年のsurvival rateがそれぞれ76%，56%と，低抗体価群の94%，80%に比べて明らかに低いことが示された．つまり，GdIgA1と内因性IgGあるいはIgA自己抗体とのIC形成は，予後と相関することが示された．これは，最近報告された糸球体IgG沈着量[30]が，

組織学的予後不良因子である事実と関連しており興味深い.

我々は，これらバイオマーカーが予後予測ばかりではなく，IgA腎症のリアルタイムな疾患活動性評価に用いることができないかを検討した[2]. 扁摘パルス療法施行後1年以上臨床経過を追跡できた50症例を対象として，治療前後の血清GdIgA1および関連ICを解析し，これらの値と疾患活動性との相関を解析した. 横断解析にて血尿・蛋白尿の程度とこれらバイオマーカーは有意に相関していた. 縦断解析では，特に血尿の寛解とこれらバイオマーカーの減少率はよく相関していた. このことは，これらバイオマーカーがIgA腎症に対する治療効果判定における，現実的なサロゲートマーカーになりうることを示唆している.

群間比較をするとIgA腎症患者では，健常人やIgA腎症以外の糸球体腎炎患者に比し，確かにこれらバイオマーカーが有意に上昇している[9,12,22,31]. しかし，健常人やその他腎炎患者でもこれらバイオマーカーがIgA腎症と同様に上昇している場合や，逆にこれらのいずれかが正常人と変わらない範囲で推移しているIgA腎症患者も存在する. つまり，単一のマーカーではIgA腎症の診断や全ての病態の説明は困難であり，疾患に関連する他の臨床所見，検査値と組み合わせで解析することが必要と考えられる. そこで我々は，当院で腎生検が施行されたIgA腎症患者，およびその他の腎炎患者と健診でこれまで尿検査異常を指摘されたことがない健常者の血清中のIgA，IgG，GdIgA1，GdIgA1特異的IgGおよびGdIgA1特異的IgAなどをELISA法により測定し，これら複数の血中バイオマーカーデータを多変量解析にて解析し，IgA腎症の診断への有用性を検証した[32]. 少なくともこの研究では，41%のIgA腎症患者では血清GdIgA1が上昇し，91%のIgA腎症患者は健常者よりも内因性IgA自己抗体が有意に上昇していた.

特に，内因性自己抗体はIgA腎症患者と正常者やその他腎炎を区別するのに有用であったが，予想通り単独での鑑別は難しく，GdIgA1ならびに尿所見を含む他の臨床パラメータも変数に用い重みづけをすることで，鑑別に関する特異度・感度がともに上昇することが確認された[32]. 現在，厚生労働省進行性腎障害調査研究班IgA腎症分科会において多施設共同研究にて症例数を増やし，この診断法の検証を進めている. 今後，尿中GdIgA1やIC値なども変数に加えることで，さらなる改善が期待される.

C. 糖鎖異常IgAおよび関連IgA免疫複合体による血尿の2次スクリーニングの試み

IgA腎症の初発症状は血尿が主体で，健診および検尿システムが発達している日本における発見機転は，健診時の血尿（尿潜血反応）が約70%と大半を占める. そういった本邦においても，年間腎生検により確定診断されるIgA腎症患者は5,000～6,000人程度である. 一方本邦では年間5,000万人以上が健診を受け，その大部分に検尿が施行されている. 日本の検尿における血尿（尿潜血反応陽性）の頻度は約3～5%とされ，1次スクリーニングで（少なく見積っても）年間150～200万人以上の尿潜血反応陽性者がいる可能性がある. 続く2次スクリーニングで陽性を呈しても，血尿単独の場合急性症状がある場合などを除いて経過観察として放置されることが多い. これは，合併症の危険があり，入院を要する腎生検以外に，糸球体腎炎を簡便に診断する手段がないことに起因している. 尿沈渣などによる腎炎の有無の判断は，非専門医には容易ではなく，蛋白尿を合併しない限り3次スクリーニングとして専門医に紹介する判断も難しく，経過観察となる. つまり腎炎の早期発見は，2次スクリーニングをどこで施行し，判断されるかに依存し，腎臓専門医の少ない

地域での格差は大きくなる．その経過観察される中には相当数のIgA腎症患者が含まれており，結果的に腎症が進行し尿蛋白も陽性になった時点で初めて専門医に紹介されるケースが多くなる．IgA腎症分科会の行った疫学調査によると日本のIgA腎症発症のピークは2峰性で，15～20歳と40～45歳とされる．いずれも状況によっては精査の機会を逸し易い年齢である．もし，今後IgA腎症の病因解明に伴い特異的な根治治療が開発された場合，特にその治療が上述の病因に関わるeffector moleculeの量的な調整が可能なのであれば，早期治療介入することで侵襲性の少ない短期治療が期待できる．その点で，200万人の尿潜血反応陽性者のなかに存在する確定診断がついていない潜在的なIgA腎症患者の規模を把握する臨床的意義は高く，健診が発達している国だからこそ検証可能な課題である．現在，厚生労働科学研究費腎疾患対策 研究事業「IgA腎症新規バイオマーカーを用いた血尿の2次スクリーニングの試み（主任研究者：鈴木祐介）」として，都内，宮崎，沖縄，山形県の健診施設などとの共同研究で1次スクリーニング（健診）の段階で尿潜血反応陽性を呈した被験者に対して，上記GdIgA1および内因性自己抗体・関連ICなどのバイオマーカーを測定し，潜在的IgA腎症患者の比率を検証する研究が進行しており，今後のIgA腎症診療や血尿の標準化にむけ有用な情報が提供されることが期待される．

むすび

近年活発化する病因に基づくバイオマーカーを用いたIgA腎症の診断・活動性評価の試みは，早期診断・治療適応・早期治療介入に重要ばかりでなく，予後分類や治療効果判定の際に問題であった国際間の対象患者の病期の不一致を是正するための新たな病期分類の確立，さらには今後の根治治療法の開発・普及にも極めて重要である．

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資　　料

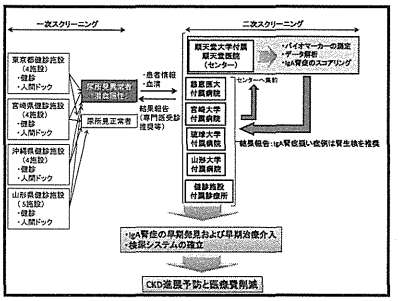
1. 平成 24 年度～26 度における最終解析報告　（都内・宮崎・山形・沖縄）
2. 平成 26 年度厚生労働科学特別研究事業　進捗管理班
   （難治性疾患実用化研究・腎疾患実用化研究・慢性の痛め解明研究）成果報告会
   （2015 年 3 月 13 日）
3. 2 年目検証研究
   ａ．検証研究実施計画書
   ｂ．検証研究　説明文・同意書・同意撤回書
4. 2 年目以降フォローアップ研究登録状況
5. IgA 腎症バイオマーカー研究取込システム
   a 取り込みシステムマニュアル
   b システム改修・機能追加
6. 巻末：別表及び別図　（表 1・表 2・図 1）
7. 健診受診者用 IgA 腎症ポスター
8. 協力施設写真
   a. 東京都
   b. 宮崎県
   c. 山形県
   d. 沖縄県

厚労省難治性疾患等克服研究事業（腎疾患対策研究事業）

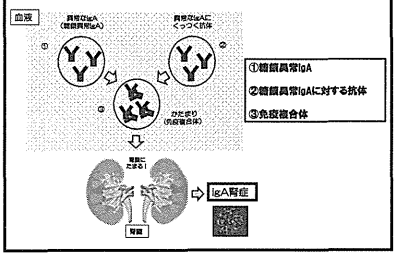
IgA腎症関連バイオマーカーを用いた
血清2次スクリーニングの試み

都内・宮崎・山形・沖縄
最終解析報告用資料

バイオマーカーの測定
および結果報告



バイオマーカー測定の理論的背景



バイオマーカー測定方法
およびスコアリング方法

バイオマーカー測定の実際

①糖鎖異常IgA1（Gd-IgA1）
当初はHAAレクチンにて測定していた→測定系の複雑さ
→モノクローナル抗体の開発
→協和メデックス測定会社へ委託（ELISA, 血清約300uL）

②糖鎖異常IgA1に対する抗体（IgA）
③免疫複合体（IgG-IgA IC）
→当科研究室にて測定（ELISA, 血清約200uL）

④血中IgA, C3, IgG, クレアチニン
→SRL検査会社へ委託（血清約700uL ）

スコアリング方法

主成分分析

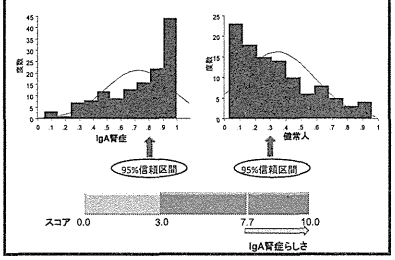
選択された変数をロジスティックモデルで解析

各バイオマーカーデータおよび臨床データを組み合わせ、ロジスティックモデルを用いて多変量解析することにより、IgA腎症の診断への有用性を検証した

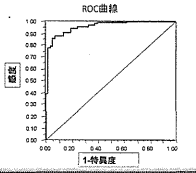
解析に用いた変数

血清バイオマーカー	臨床データ
• IgA	• 尿中蛋白量
• 糖鎖異常IgA1	• 尿中赤血球数
• IgA-IgG 免疫複合体	• 血清クレアチニン
• 糖鎖異常IgA1特異的IgA	• 年齢
	• 性別

ヒストグラムよりカットオフポイントを決定



Logistic model 解析結果



-IgA腎症の診断率-
IgA腎症 vs その他の腎症患者
感度 91%, 特異度 81%

尿潜血陽性者を対象とした本研究における問題点

- IgA腎症患者とその他の腎炎との比較検証においては、ロジスティックモデルの各成分において、血尿が強い要素の一つであった。
- 当初腎生検患者を対象としたスコアリングシステムでは、過剰にIgA腎症と診断してしまう恐れがあった。

→ そこで、新たに本研究で一貫したスコアリングシステムを確立した。

結果報告のパターン

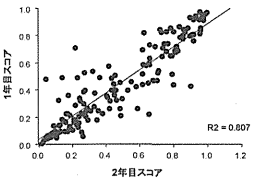
A	本研究のスコアが高値の方		
	血清クレアチニン	尿蛋白	結果報告
A1	正常	-	現時点では、IgA腎症の可能性は低いと考えられます。尿潜血(血尿)は認められましたので、今後も定期的な検診の受診をお勧めします。
A2	正常 or 高値	+	現時点では、IgA腎症の可能性は低いと考えられますし、しかし、尿潜血が陽性ですので、なんらかの腎疾患の疑いがあります。腎臓内科専門外来への受診および精査をお勧めします。
A3	高値	+/- or -	現時点では、IgA腎症の可能性は低いと考えられますし、しかし、血中クレアチニン値が高値ですので、なんらかの腎疾患の疑いがあります。腎臓内科専門外来への受診および精査をお勧めします。
B	本研究のスコアが高値の方		
	血清クレアチニン	尿蛋白	結果報告
B	正常 or 高値	+ or +/- or -	本研究結果よりIgA腎症の可能性が高いと考えられます。今後なるべく早期に腎臓内科専門外来への受診および精査をお勧めします。
C	本研究のスコアが中間値の方		
	血清クレアチニン	尿蛋白	結果報告
C1	正常	-	現時点では、IgA腎症の可能性は低いと考えられますが、経常者よりもやや高値を示しています。次年度の検診でも経過をみることをお勧めいたします。本用紙を持参していただき、昨年と同じ検診施設を受診してください。
C2	正常 or 高値	+	現時点では、IgA腎症の可能性は低いと考えられますし、しかし、尿潜血が陽性ですので、なんらかの腎疾患の疑いがあります。腎臓内科専門外来への受診および精査をお勧めします。
C3	高値	+/- or -	現時点では、IgA腎症の可能性は低いと考えられますが、経常者よりもやや高値を示しています。また、血中クレアチニン値が高値ですので、なんらかの腎疾患の疑いがあります。腎臓内科専門外来への受診および精査をお勧めします。

各地域・施設毎の結果解析

	総数	年齢	男女比(率) 男性	女性	尿蛋白陽性者(率)	血清Cr増加者(率)	診断スコア判定 A判定	B判定	C判定
宮崎県	1142	40.0	28.3%	71.7%	10.2%	4.5%	42.4%	10.7%	46.9%
京都府	289	41.1	32.9%	67.1%	6.6%	9.0%	36.0%	17.6%	46.4%
沖縄県	1131	42.0	26.3%	73.7%	5.5%	7.3%	57.0%	13.0%	30.0%
山形県	185	40.9	24.3%	75.7%	9.7%	15.7%	54.6%	11.9%	33.5%
計	2747								

（平成28年12月末までの解析結果）

1年目スコアと2年目スコアの相関解析



今後の課題

➢生理を含めた尿潜血の再現性の評価

➢本研究で用いている暫定的スコアリング方法のvalidation

A判定・B判定・C判定 の転帰の確認

研究総括