immunoglobulin G4 (IgG4). Hofstra et al. [8] reported that levels of circulating anti-PLA2R antibodies correlate strongly with the level of proteinuria. Beck et al. [9] reported that changes in the level of anti-PLA2R antibodies are associated with the level of proteinuria and precede corresponding changes in disease activity. From these observations, it would be expected that the anti-PLA2R antibody can be used as a biomarker in the differential diagnosis of iMN, monitoring of disease activity, and prediction of disease outcome. Moreover, Hoxha et al. [10] reported that the level of expression of PLA2R on the cell surface of podocytes of iMN patients with anti-PLA2R antibodies was higher than in iMN patients without the antibodies. Stanescu et al. [11] showed that polymorphisms of the PLA2R and HLA-DQA1 alleles are associated with the risk of developing iMN. They concluded that the HLA-DQA1 allele on chromosome 6p21 is most closely associated with iMN in people of white ancestry.

Beck et al. [7] also revealed that the anti-PLA2R antibody was found in 26 of 37 patients with iMN, but was not found in patients with sMN, disease controls, or healthy controls. Its prevalence in American patients with iMN was 70 %. The overall prevalence of anti-PLA2R antibodies in iMN patients with nephrotic syndrome is reported to be 66–98 % [7, 8, 12–14]. The wide range may be attributable to genetic and regional differences or to differences in the detection methods employed. The prevalence of anti-PLA2R antibodies in Japanese patients with MN has not yet been determined. Western blot analysis with chemical luminescence is the prevailing method for detecting anti-PLA2R antibodies in serum, but there are some technical problems with this method. In this study, we determined the presence of anti-PLA2R antibodies in Japanese patients with untreated MN using a highly sensitive method of Western blot analysis we developed.

Materials and methods

Patients

A total of 106 patients with iMN and 35 patients with sMN admitted to a hospital between 2005 and 2011 were enrolled in this study. Patients were diagnosed with iMN or sMN based on both a renal biopsy and screening for clinical or laboratory signs of a cause for sMN. Six patients with iMN and 4 patients with sMN were excluded from this study because they were treated with immunosuppressive therapy at the time of biopsy. Blood samples and timed urine samples were collected from a total of 100 iMN patients and 31 sMN patients for the measurement of serum albumin, creatinine, total protein total cholesterol, IgG, anti-PLA2R, and urinary protein. Patients with sMN ($n = 31$) included 11 patients with immune-associated disease, including Castleman's disease ($n = 1$) and SLE ($n = 10$); 6 patients with neoplasia, including lung cancer ($n = 4$), urinary bladder cancer ($n = 1$), and other cancer ($n = 1$); 6 patients whose disease was associated with the use of the anti-rheumatoid drug bucillamine; 2 patients with hepatitis B virus infection; and 6 patients with other primary diseases. The study protocol was approved by the ethics committees of our institution (#1135-14) and was conducted in accordance with the ethical principles stated by the Declaration of Helsinki.

Antigen

We used human glomerular extract (HGE) as a source of native glomerular PLA2R for detection of circulating anti-PLA2R antibodies in patient serum. A human kidney was obtained from a patient with renal cancer who underwent surgery at out institution. Glomeruli were collected from healthy portions of the kidney using a series of graded sieves, were rinsed with cooled phosphate buffer saline, and were dissolved with RIPA buffer with proteinase inhibitor cocktail (Roche). Insoluble debris was removed by centrifugation. To eliminate contamination with donor endogenous IgG, the HGE was treated with HiTrap Protein G HP column (GE). The prepared HGE was quality checked using Western blot to ensure there were no reactive IgG against anti-human IgG murine antibody, and a reactive PLA2R protein against both anti-human PLA2R rabbit polyclonal antibody (Atlas antibodies, product number: HPA012657) and a positive control serum containing anti-PLA2R in HGE. All reagents were obtained from Sigma Japan or Wako Pure Chemicals if not specified otherwise.

Immune reaction in Western blot analysis

The HGE was heated with 4x LDS-sample buffer (Invitrogen) at 70 °C for 10 min and then electrophoresed under non-reducing conditions on the NuPAGE 4–12 % polyacrylamide Tris-Bis gel (Invitrogen) with MOPS SDS running buffer (Invitrogen). The proteins were separated by SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked with Blocking One (Nacalai tesque) at 25 °C for 60 min. In the first immune reaction, all patient serum samples were diluted with a diluent, which was mixed with 80 % PBST (phosphate buffer saline:Tween 20 = 99.8:0.2) and 20 % of Blocking One, at a dilution of 1:25 and reacted with the blocked membrane at 37 °C for 60 min. For samples with negative results at a dilution of 1:25, we retested all samples at a dilution of 1:10 or 1 and confirmed non-reactivity based on the negative results at the higher concentration of serum. In the

second immune reaction, a horseradish peroxidase (HRP) conjugated mouse monoclonal antibody against human IgG Fc (Abcam, product number: ab99759) was diluted at a dilution of 1:7500 and reacted with a membrane at 37 °C for 60 min.

Signal detection in Western blot analysis

To obtain a high sensitivity and resolution, we attempted to pretest a signal detection method of the bound secondary antibody. In the pretest, Western blot analysis was conducted with HGE as antigen, serial diluted anti-PLA2R human antibodies known to be the same concentration as the first antibody, and the anti-human IgG Fc mouse monoclonal antibody as the secondary antibody. The serial diluted anti-PLA2R human antibodies were prepared from attached reagents as standard anti-PLA2R human antibody for concentration calibration in commercial ELISA kit (Euroimmun AG). After reacting with the secondary antibody, we weighed the chromogenic method against the chemical luminescence method. The bound secondary antibody was detected by chemical luminescence with ImmunoStar LD or chromogenic reaction with a 3, 3′, 5, 5′-tetramethylbenzidine (TMB) as a substrate of HRP. Images of reacted bands on the PVDF membrane were acquired by a CCD imager (LAS4000, GE) in the Chemical luminescence or digital camera (Canon) using the chromogenic method. We defined that the serum sample was positive for anti-PLA2R if a single reactive band appeared in the position of native PLA2R protein (approximately 180 kDa) on the PVDF membrane. The acquired figures were prepared using Photoshop CS5 (Adobe systems). In this study, all patient serum used to estimate the prevalence of anti-PLA2R antibody was analyzed using the chromogenic reaction method.

Statistical analysis

For data description, continuous variables with symmetric distribution were presented as the mean ± SD, and non-normally distributed variables were expressed as medians (25–75 % interquartile range). *T* tests were used for normally distributed data, and the Mann–Whitney *U* rank test was used for non-parametric data. The Dunn method was

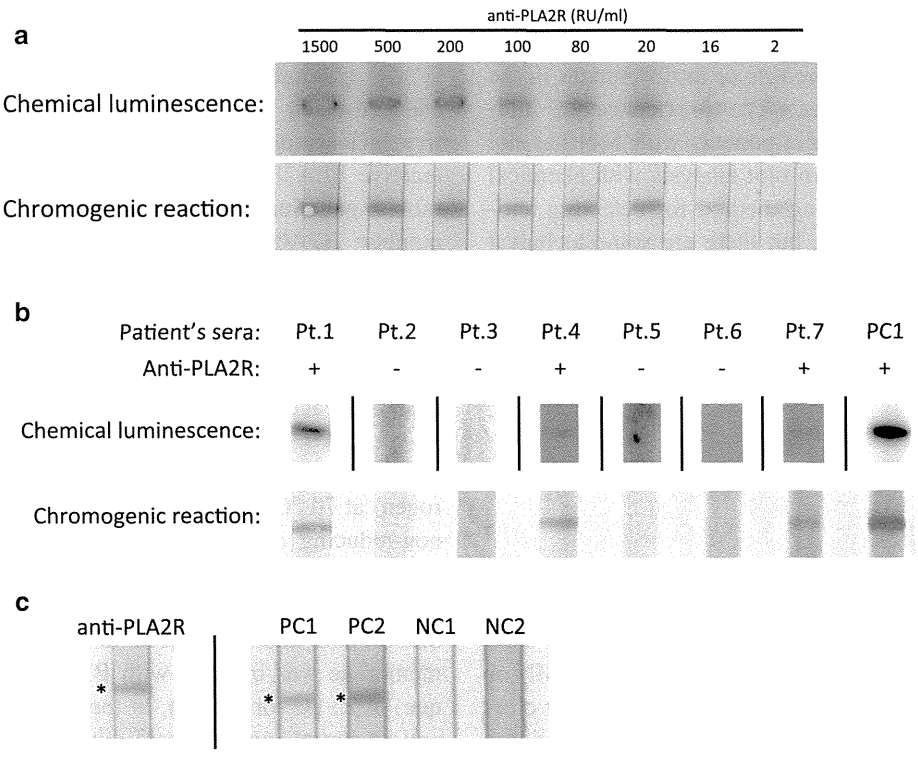

Fig. 1 **a** The results of comparative investigation between chemical luminescence and chromogenic reaction in Western blot analysis with serial concentration of human anti-PLA2R antibody. The chemical luminescence showed blurred reactive bands in all concentrations and bleached bands with high background in high concentrations of anti-PLA2R antibodies. The chromogenic reaction showed sharply defined bands with low background in all range levels of anti-PLA2R antibodies. **b** The chromogenic reaction visualized higher contrast reactive band images compared to that of chemical luminescence in Western blot analysis with HGE and serum from some of the patients with iMN who were enrolled in this study. **c** The left image shows the existence of native PLA2R protein in HGE demonstrated using commercial anti-PLA2R rabbit polyclonal antibody. The right image shows that the positive control serum reacted with native PLA2R protein in HGE, whereas the negative control serum did not. *Pt* patient, *PC* positive control, *NC* negative control

performed for multiple comparisons in non-parametric analysis. Categorical variables were described as number and percentages, and the data were analyzed with χ^2 tests. The differences were considered significant with a P value <0.05. All statistical analyses were performed with JMP version 11.0.0 (SAS Institute, USA) for Mac.

Results

Western blot analysis for the detection of anti-PLA2R antibodies

Figure 1a shows blurred reactive bands with high-background on chemical luminescence at all levels of anti-PLA2R antibodies and all exposure times. Bleached bands appeared in the lane with a high concentration of anti-PLA2R antibodies. The chromogenic reaction method showed sharply defined bands with low-background in the wide-range levels (2–1500 RU/mL) of anti-PLA2R antibody. According to an instruction manual of a commercial anti-PLA2R antibody measurement kit from Euroimmun AG, the 16 RU/mL of anti-PLA2R antibody corresponds to the threshold value for a positive determination. Timmermans et al. [15] suggested that 2 RU/mL should be the recommended cutoff. Figure 1b shows the results of a comparison test using serum from some of the patients with iMN. In chemical luminescence, most were weak bands covered by high-background signal. Therefore, we selected the chromogenic reaction as the detection method for secondary antibody in this study.

Figure 1c shows the reactive bands of a commercial anti-PLA2R rabbit polyclonal antibody and positive and negative control serum from patients with iMN in an American cohort against PLA2R in HGE. We confirmed the existence of native PLA2R in HGE based on the fact that all reactive bands were represented at approximately 180 kDa on the PVDF membrane.

Prevalence of anti-PLA2R in Japanese patients with membranous nephropathy

Table 1 shows the clinical characteristics of the patients with iMN and sMN at the time of kidney biopsy. The mean age was 67 in iMN patients and 61 in sMN patients. We found that 53 % (53 of 100) of iMN patients were positive for anti-PLA2R antibody whereas no patients with sMN (0 of 31) were positive (Fig. 2). The 53 anti-PLA2R antibody positive serum samples from iMN patients consisted of 43, 3, and 7 anti-PLA2R positive serum samples that were found at dilution levels of 1:25, 1:10 and, 1, respectively. From these results, we concluded that 43 patients had a high titer, 3 had a middle titer, and 7 had a low titer of anti-PLA2R antibodies. Anti-PLA2R antibodies were positive in 61 % (33 of 54) of the iMN patients with nephrotic syndrome (UP ≥3.5 g/day and serum albumin ≤3.0 g/dL) and in 43 % (20 of 46) of iMN without nephrotic syndrome.

Table 1 Characteristics of patients with idiopathic and secondary membranous nephropathy

	IMN ($n = 100$)	SMN ($n = 31$)
Male, *n* (%)	63 (63)	20 (65)
Age at diagnosis (years)	67 ± 9	61 ± 12
Urinary protein (g/day)	4.1 (2.6–6.4)	4.9 (1.8–6.6)
Urinary protein ≥3.5 g/day, *n* (%)	59 (59)	19 (61)
Serum albumin (g/dL)	2.4 ± 0.7	2.5 ± 0.9
Serum albumin ≤3 g/dL, *n* (%)	81 (81)	21 (68)
Both urinary protein ≥3.5 g/day and serum albumin ≤3 g/dL, *n* (%)	54 (54)	17 (55)
Serum total protein (g/dL)	5.2 ± 0.8	5.8 ± 1.1
Serum creatinine (μM)	72.1 (61.9–88.4)	64.5 (54.8–84.0)
eGFR (ml/min/1.73 m^2)	65.5 (54.3–77.8)	72.0 (58.0–97.0)
Serum IgG (mg/dL)	727 (563–1038)	1182 (767–1721)
Total cholesterol (mM)	7.6 (6.2–9.4)	6.1 (5.1–8.5)
Recognized underlying membranous nephropathy inducible disease		
Immune-associated disease [*n* (%)]	0 (0)	11 (35.5)
Neoplasia [*n* (%)]	0 (0)	6 (19.4)
Drugs [*n* (%)]	0 (0)	6 (19.4)
Infections [*n* (%)]	0 (0)	2 (6.4)
Other [*n* (%)]	0 (0)	6 (19.4)

The data are expressed as the number (%), mean ± SD or median (interquartile range)

iMN idiopathic membranous nephropathy, *sMN* secondary membranous nephropathy, *eGFR* estimated glomerular filtration rate

Relationship between patient characteristics and the anti-PLA2R antibodies in patients with iMN

We examined the patient characteristics of iMN patients negative for anti-PLA2R antibodies and positive for anti-PLA2R antibodies (Table 2). The number of patients with serum albumin ≤3.0 g/dL was significantly higher in iMN patients with anti-PLA2R antibodies (92 %, 49 of 53) than in anti-PLA2R antibody negative iMN patients (68 %, 32 of 47). Although no other significant differences were observed in patients with and without anti-PLA2R antibodies, anti-PLA2R antibody positive iMN patients had higher urinary protein levels ($p = 0.06$) and a higher rate of nephrotic syndrome (urinary protein ≥3.5 g/day and serum albumin ≤3 g/dL, $p = 0.08$) and a lower serum total

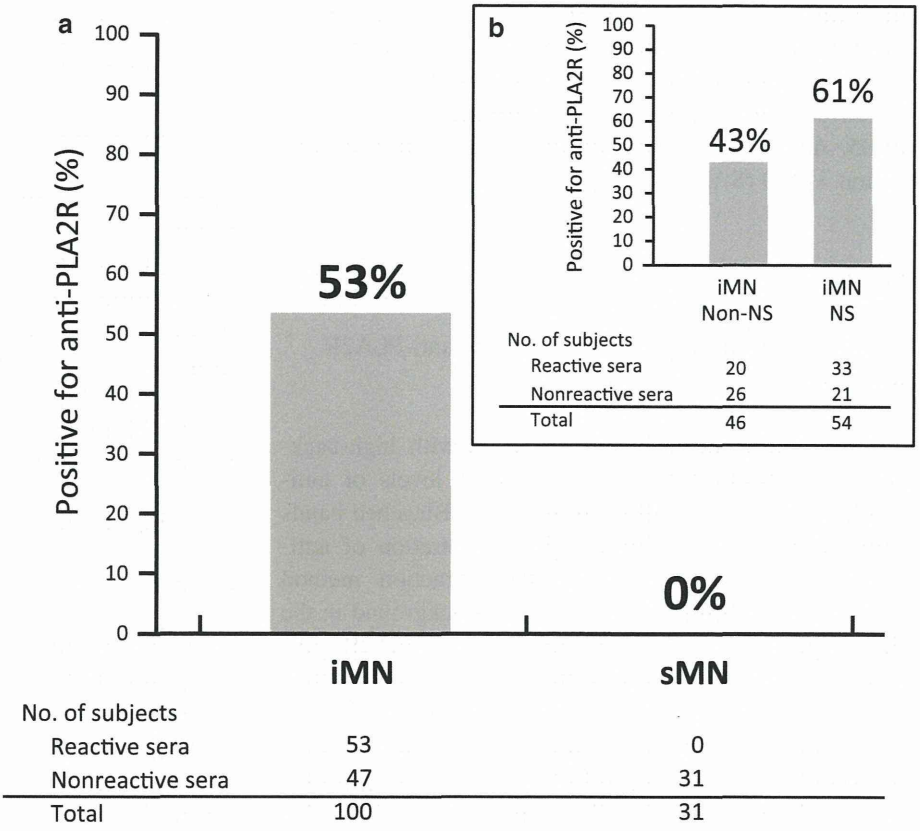

Fig. 2 The prevalence of circulating anti-PLA2R antibody in serum from Japanese patients with membranous nephropathy. **a** The anti-PLA2R antibodies are specifically detected in 53 % of patients with iMN and no patients with sMN. **b** The anti-PLA2R antibodies were positive in 61 % of the iMN patients with nephrotic syndrome (UP ≥3.5 g/day and serum albumin ≤3.0 g/dL) and in 43 % of iMN patients without nephrotic syndrome. *NS* nephrotic syndrome

protein level ($p = 0.06$) than the anti-PLA2R antibody negative iMN patients.

We then examined the relationship between the semi-quantitative value of anti-PLA2R antibodies and patient characteristics. The levels of urinary protein, serum albumin, and eGFR were not different among iMN patients with negative, low, middle, and high titer levels (Fig. 3).

Discussion

The present study revealed that the prevalence of anti-PLA2R antibodies is 53 % in Japanese patients with iMN. Although it was not significantly different, the prevalence of anti-PLA2R antibodies in iMN patients with nephrotic syndrome (61 %) was higher than that of iMN patients without nephrotic syndrome (61 % vs. 43 %, $p = 0.08$). The prevalence was lower than that found (70 %) in the original study by Beck et al. [7]. No patients with sMN were positive for anti-PLA2R antibodies.

The prevalence of anti-PLA2R antibody positive iMN patients from other countries is shown in Table 3. Previous studies have shown that the levels of urinary protein are significantly associated with the presence and titers of anti-PLA2R antibodies. Thus, the comparison of prevalence should be done among patients with nephrotic range proteinuria. The prevalence of anti-PLA2R antibodies in Chinese and Korean nephrotic patients was high, at 98 and 80 % [12, 13], respectively. In other cohorts, excluding one from Germany [14], the prevalence was 78–85 % in iMN patients with nephrotic range proteinuria (UP ≥3.5 g/day) [7, 8, 12, 13]. In contrast, 61 % of Japanese patients were positive for anti-PLA2R antibodies, even among iMN patients with nephrotic syndrome. The prevalence of anti-PLA2R antibodies in Japan was as low as that in the German cohort (66 %) [14]. The cohort from Korea showed a significant correlation between levels of urinary protein and serum albumin and the existence of circulating anti-PLA2R antibodies [12]. In our study, a low serum albumin was significantly associated with PLA2R positivity. No definitive conclusion can be made from our cohort, but the presence of anti-PLA2R antibodies may have induced more severe disease.

Why is there a low prevalence of anti-PLA2R antibody in Japanese patients with MN? There are several possible reasons for the discrepancy. First, there can be a genetic difference between people in Japan and other countries. However, it may be difficult to explain the difference in genetic background between Japanese, Korean, and Chinese cohorts. Second, Japan has a special health check-up system to detect low levels of proteinuria [16]. Japanese iMN patients may be diagnosed earlier than patients in

other countries; therefore, the anti-PLA2R antibody levels might have been under the detection limit when they underwent renal biopsy. Third, unknown environmental or dietary factors may have affected the results. The factors causing the differences need to be clarified in future studies. Fourth, in Japanese iMN patients, other antigens may also play a role in the development of iMN. Studying Japanese patients with iMN may elucidate new pathogenic antigens. Lastly, technical differences may exist between countries. Although Western blot analysis with chemical luminescence is the most common technique for the detection of anti-PLA2R antibody, there are some problems with chemical luminescence for the detection of anti-PLA2R antibody in serum. For example, the bands of anti-PLA2R antibody were covered by high background signal if serum of low dilution was used, so a high dilution was necessary to prevent the high background. Bleached reactive bands often occurred due to enzymatic over reaction. The detection range of anti-PLA2R antibody concentration per image is narrow. Therefore, it is difficult to detect a low level of circulating anti-PLA2R antibody by chemical luminescence. On the other hand, the original detection method developed by us that employs an optimized chromogenic reaction with TMB resulted in a high resolution and low background without loss of sensitivity. In our method, the serum samples can be used without dilution, leading to a clear positive signal even in serum with a very low level of anti-PLA2R antibodies. In addition, our method is easy to use because it required only a digital camera or scanner for acquiring reactive band images, and a high-priced CCD imager is not needed. In the present study, we found that 7 iMN patients, who were negative at 1:10 dilution, became positive when examined without sample dilution. We also used the positive and negative control serum from Dr. D. Salant's laboratory and confirmed that our system gave the same results as those in the original study. Therefore, it is unlikely that the low prevalence in our study is attributable to our detection system.

Western blot is considered the best method for qualitative measurement of anti-PLA2R antibody, whereas a quantitative method such as ELISA has considerable

Table 2 Comparison of the patient's characteristics between anti-PLA2R antibody positive and negative patients with idiopathic membranous nephropathy

Anti-PLA2R antibody	Negative ($n = 47$)	Positive ($n = 53$)	P value
Male, n (%)	31 (66)	32 (60)	0.56
Age at diagnosis (years)	68 ± 9	67 ± 9	0.48
Urinary protein (g/day)	3.7 (2.4–5.8)	4.6 (2.9–8.6)	0.06
Urinary protein ≥3.5 g/day, n (%)	24 (51)	35 (66)	0.13
Serum albumin (g/dL)	2.5 ± 0.9	2.3 ± 0.5	0.21
Serum albumin ≤3 g/dL, n (%)	32 (68)	49 (92)	0.02
Both urinary protein ≥3.5 g/day and serum albumin ≤3 g/dL, n (%)	21 (45)	33 (62)	0.08
Serum total protein (g/dL)	5.4 ± 0.9	5.1 ± 0.7	0.06
Serum creatinine (μM)	70.7 (61.0–91.9)	73.4 (61.9–86.2)	0.74
eGFR (ml/min/1.73 m^2)	66.0 (50.0–78.0)	65.0 (56.0–76.5)	0.69
Serum IgG (mg/dL)	791 (594–1062)	668 (536–982)	0.33
Serum total cholesterol (mM)	7.5 (6.1–9.9)	7.6 (6.4–9.2)	0.90

The data are expressed as the number (%), mean ± SD or median (interquartile range). eGFR (mL/min/1.73 m^2) = 194 × Serum creatinine $(mg/dL)^{-1.094}$ × $Age^{-0.287}$ × 0.739 (if female)

Anti-PLA2R anti-phospholipase A2 receptor autoantibody, *iMN* idiopathic membranous nephropathy, *eGFR* estimated glomerular filtration rate

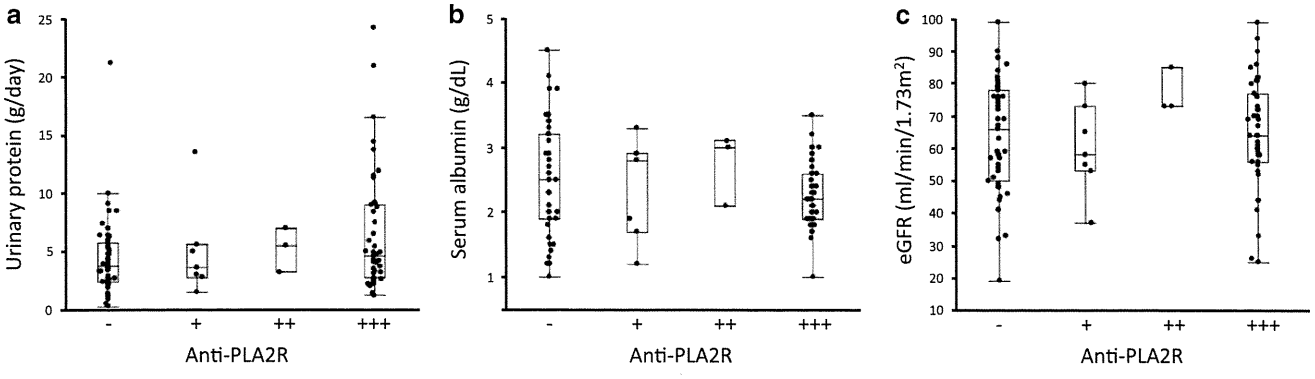

Fig. 3 The relationship between the semi-quantitative values of anti-PLA2R antibodies and patient characteristics. No significant difference was observed between the levels of anti-PLA2R antibodies (−, negative; +, low; ++, middle; +++, high) and urinary protein (**a**), serum albumin (**b**), or eGFR (**c**)

Table 3 Comparison of the prevalence of anti-PLA2R autoantibody in patients with iMN among different countries

Country	Method	Antigen	All patients	Patients with proteinuria ≥3.5 g/day	References
China	WB	rPLA2R	59/60 (98)	59/60 (98)	Qin et al. [13]
The Netherlands	WB	rPLA2R	14/18 (78)	14/18 (78)	Hofstra et al. [8]
Korea	WB	HGE	69/100 (69)	60/75 (80)	Oh et al. [12]
USA	WB	HGE	26/37 (70)	17/20 (85)	Beck et al. [7]
Germany	IIFT	rPLA2R	52/100 (52)	23/35 (66)	Hoxha et al. [14]
Japan	WB	HGE	53/100 (53)	33/54 (61)[a]	Present study

The data are expressed as the number of positive case/negative case (positive rate, %)

WB Western blot; *IIFT* indirect immunofluorescence test, *rPLA2R*, recombinant phospholipase A2 receptor, *HGE* human glomerular extract

[a] Both urinary protein ≥3.5 g/day and serum albumin ≤3 g/dl

importance in the study of MN. Recently, several types of immunoassay systems for measuring circulating anti-PLA2R antibody have been developed, such as a commercial cell-based immunofluorescence assay (CBA-IIFT, Euroimmun AG), a commercial ELISA (Euroimmun AG), and an addressable laser bead immunoassay (ALBIA).The CBA-IIFT, like the Western blot, is used only for semi-quantitative measurements, but the ELISA and the ALBIA are quantitative assays. Behnert et al. reported that these three different immunoassays showed similar efficacies in the detection of anti-PLA2R antibodies in patients with iMN [17]. We are currently trying to evaluate the performance of CBA-IIFT and ELISA using serum from Japanese patients with iMN, sMN, and other renal diseases.

Our study has several limitations. We performed the Western blot analysis using HGE, not PLA2R protein, as an antigen. It is possible that we detected other proteins with similar molecular weight at around 180 kDa. If this is the case, the real prevalence of anti-PLA2R antibody may be even lower. We studied patients only in the Tokai area, 1 limited region of Japan, and our findings may not be generalizable to iMN patients in other areas of Japan. Future studies using recombinant PLA2R as an antigen and enrolling patients in other regions of Japan will clarify these problems.

In conclusion, we determined that the prevalence of anti-PLA2R antibodies in Japanese patients with iMN is approximately 50 %, which was similar to previous reports from Germany but lower than reports from Asian countries (China and Korea). This may indicate that the presence of other pathogenic antigens plays a significant role in Japanese patients with iMN. Anti-PLA2R antibodies were not found in any patients with sMN.

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Conflict of interest The authors have declared no conflict of interest exists.

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Exploring urinary biomarkers in autosomal dominant polycystic kidney disease

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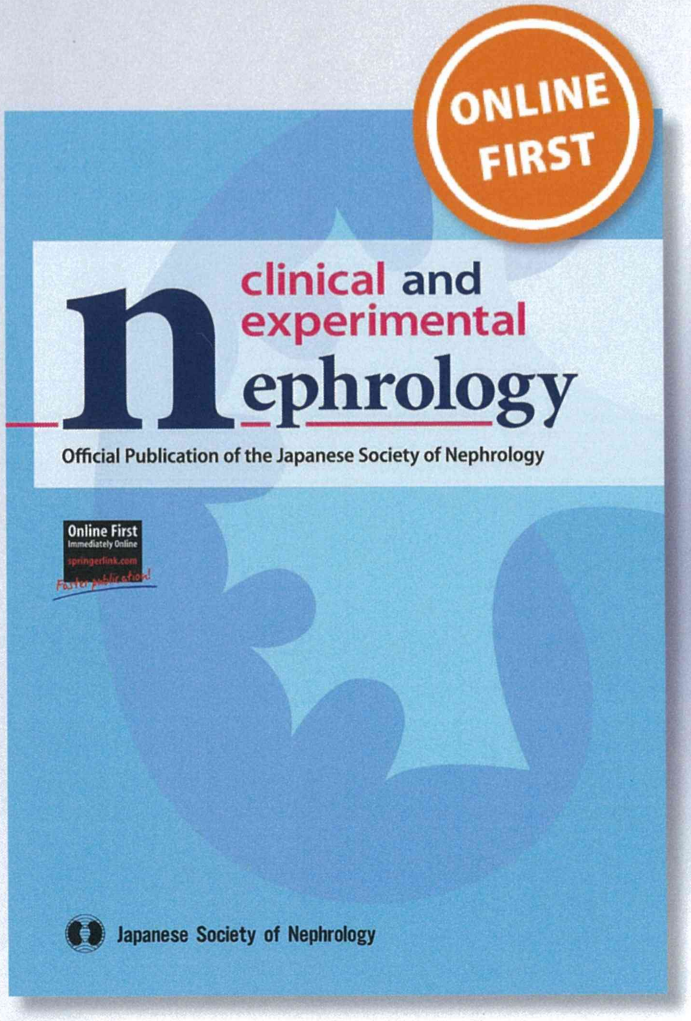

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ORIGINAL ARTICLE

Exploring urinary biomarkers in autosomal dominant polycystic kidney disease

Haruna Kawano · Satoru Muto · Yasukazu Ohmoto · Fusako Iwata · Hiroyuki Fujiki · Toyoki Mori · Lu Yan · Shigeo Horie

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Abstract
Background Autosomal dominant polycystic kidney disease (ADPKD), the most common inherited kidney disease, is a progressive disease characterized by a bilateral proliferation and enlargement of renal cysts. Recent reports have shown that tolvaptan, a vasopressin V2 receptor antagonist, has been effective in inhibiting renal cyst proliferation and enlargement in ADPKD patients, although no biomarker has identified to predict the effects of tolvaptan. We explored the effective urinary biomarkers in ADPKD in human and in an animal model.
Methods We measured 28 biomarkers in urine taken from ADPKD patients to compare with that of healthy subjects. Next, a gene expression analysis of the kidney from DBA/2FG-pcy mice (ADPKD model animals) was performed to identify prospective biomarkers. Additionally, we investigated the DBA/2FG-pcy mouse urine samples to determine the biomarkers' efficacy.
Results There were statistically significant differences in 12 of the 28 prospective urinary biomarkers between urine from ADPKD patients and that from healthy subjects. Six of these matched with highly expressed gene products of DBA/sFG-pcy mouse kidneys. Among those 6 biomarkers, NGAL, M-CSF, and MCP-1 showed significantly higher values in the urine of DBA/2FG-pcy mice than that of wild type.
Conclusions This study suggests that NGAL, M-CSF, MCP-1 are potential candidates of urinary biomarkers in ADPKD.

Keywords Autosomal dominant polycystic kidney disease · Urinary biomarker · DBA/2FG-pcy mouse

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Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is the most common inherited kidney disease, affecting approximately 1 in every 1,000 individuals [1]. Bilateral numerous renal cysts appear and proliferation of them increases with age. Kidney function gradually deteriorates concurrently. By age 70, approximately half of ADPKD patients reach end-stage renal disease and require renal replacement therapy [2]. In addition to renal cysts, this chronic-systemic disease has complications such as hepatic cysts, intracranial aneurysms, hypertension, and colon diverticula. As there is no crucial treatment for ADPKD, therapeutic focus has been on symptom management, including treatment of hypertension and regulation of water intake [3].

PKD1 and *PKD2* are the causal genes in ADPKD; mutations in *PKD1* are known to cause about 85 % of ADKPD, while mutations in *PKD2* are causal in the

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remaining 15 % [4, 5]. *PKD1* and *PKD2* encode proteins polycystin-1 (PC1) and polycystin-2 (PC2), respectively, which localize in cilia of renal tubules, forming complex proteins and functioning as calcium channels. ADPKD patients lose the function of those renal tubule calcium channels, thereby suppressing intracellular calcium uptake and suffering lowered cytoplasmic calcium concentration, which results in cyst proliferation [6, 7].

Unlike in normal kidneys, cyst production in epithelial cells of ADPKD patients is stimulated by cyclic AMP (cAMP) [8, 9]. A decrease in intracellular calcium concentration leads to cAMP-stimulated B-raf activation, causing cell proliferation [10]. Furthermore, cAMP is synthesized when the vasopressin V2 receptor antagonist in the renal collecting duct is stimulated by vasopressin, similarly promoting cyst formation. As tolvaptan, a vasopressin V2 receptor antagonist, selectively inhibits the vasopressin V2 receptor, suppressing cAMP production, it may be effective in controlling proliferation and enlargement of renal cysts [11]. The efficacy of tolvaptan is shown in the global phase III clinical trial in which tolvaptan inhibits both the increase in cyst volume and the progression of chronic kidney disease [12]. In this study, the kidney volume was evaluated as a primary endpoint since serum creatinine or eGFR is not appropriate to monitor the progression of disease [12]. However, to measure the correct kidney volume is not always achievable [13]. Those considerations led us to explore urinary biomarkers as a more convenient method of evaluating therapeutic efficacy.

Recent years have seen wider recognition of biomarkers such as NGAL (for acute kidney injury, or AKI) [14] and L-FABP (for AKI and chronic kidney disease, or CKD) [15], along with more active exploration of new biomarkers [16]. In the current study, we explored 28 urinary biomarkers to see the difference between patients with ADPKD and healthy subjects. Classification of these 28 biomarkers included the following considered to be involved in ADKPD pathology: two types of Polycystic Kidney Disease 1 (PKD1), Polycystic Kidney Disease 2 (PKD2), aquaporin2 (AQP2), cAMP, von Willebrand factor (vWF), vasopressin, Interleukin 8 (IL-8), aldosterone, vascular endothelial growth factor (VEGF), monocyte chemoattractant protein-1 (MCP-1), angiotensinogen, lactate dehydrogenase (LDH), and protein. Prospective biomarkers involved in acute nephritis included: neutrophil gelatinase-associated lipocalin, lipocalin-2 (NGAL), liver-type fatty acid-binding protein (L-FABP), and Interleukin 18 (IL-18). Biomarkers for oxidative stress included 8-hydroxydeoxyguanosine (8-OHdG) and 8-isoprostane. We also included lysozyme and ceruloplasmin, two biomarkers showing increases in our previous study, which is gene expression analysis of DBA/2FG-pcy mouse, an animal model of human type 3 nephronophthisis, which is autosomal recessive polycystic kidney disease (unpublished). Cancer-related biomarkers included: interferon receptor 2 (IFNAR2), perpetual flowering 1 (PEP1), trefoil factor family 1 (TFF1), and trefoil factor family 3 (TFF3). Biomarkers for tissue protection and regeneration included: trefoil factor family 2 (TFF2) and hepatocyte growth factor (HGF). Using existing kits or new methods of measurement which we developed and utilized on ADPKD patient urine, we assessed 28 candidate biomarkers to identify those effective in evaluating the efficacy of tolvaptan on ADPKD.

Materials and methods

Measurement of urinary biomarkers

Human urinary biomarker study

The study protocol was approved by the institutional ethical committee of the Research Division at Otsuka Pharmaceutical Co., Ltd (040823-2), Pharmaceutical Business Division, Otsuka Pharmaceutical Co., Ltd. Institutional Animal Care and Use Committee (74), and the Ethics Committee of Teikyo University. We had informed consent for all participants of this research. Single-voided urine sample was collected from 6 healthy subjects at Otsuka Pharmaceutical and 23 ADPKD patients at Teikyo University Hospital.

Measurements on the following were performed using sandwich ELISA: PKD1N, PKD1C, PKD2C, AQP2, vWF, IL-8, IL-18, M-CSF, VEGF, IFNAE2, TFF1, TFF2, TFF3, PEPI, and NGAL. Protein was measured using competitive ELISA. Meanwhile, commercial kits were used for the following: vasopressin (Assay Designs, Inc., MI, USA); aldosterone (Cayman Chemical Company, MI, USA); HGF (R&D Systems, Inc., MN, USA); cAMP (GE Healthcare, Buckinghamshire, UK); MCP-1 (R&D Systems, Inc.); 8-OHdG (NIKKEN SEIL Co., Ltd., Tokyo, Japan); 8-Isoprostane (Cayman Chemical Company); L-FABP (Immuno-Biological Laboratories Co., Ltd., Gunma, Japan); angiotensinogen (Immuno-Biological Laboratories Co., Ltd.); LDH (Roche Diagnostics International Ltd., Basel, Switzerland); lysozyme (Biomedical Technologies, MA, USA); and ceruloplasmin (Uscn Life Science Inc., Hubei, PRC). The values of urinary biomarkers were corrected by the urinary creatinine levels.

Statistical analyses were performed with SAS software version R8.1 (SAS Institute Japan Ltd., Tokyo, Japan). Values of $p < 0.05$ were considered significant.

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Mice biomarker study

15-week-old DBA/2FG-pcy mice [17] and DBA/2JJcl mice (control) were placed in metabolic cages to collect urine. Urine volume was measured and urine was centrifuged at 3000 rpm for 10 minute. The supernatant was collected and urine osmolality was measured with a Model 3400 osmometer (Advanced Instruments, Inc.). Following cryopreservation at −80 °C, we used a kit to measure NGAL (R&D Systems), M-CSF (R&D Systems), and MCP-1 (R&D Systems) Gene expression analyses. The values of urinary biomarker were corrected by urinary creatinine levels. DBA/2FG-pcy mice and DBA-2JJcl mice (wild type) were killed at 28 weeks and blood was collected from the posterior vena cava, after which kidneys were removed, frozen with liquid nitrogen, and preserved at −80 °C. RNA was extracted from these kidneys with a kit (RNeasy Mini, QIAGEN, Hilden, Germany) and analyzed with a microarray (Mouse Genome 430 2.0 Array, Affymetrix, Inc., OH, USA). TAKARA BIO INC. performed the gene expression analyses.

Results

Measurement of urinary biomarkers in ADPKD patients

Single-voided urine was collected as necessary from 6 healthy subjects (age range 36–53; median age: 40) of Otsuka Pharmaceutical and 23 ADPKD patients (age range 21–63; median age: 47) at Teikyo University Hospital. The mean serum creatinine of healthy subjects was 0.78 mg/dl. The clinical characteristics of ADPKD patients are shown in Table 1. Statistically significant differences ($p < 0.05$) were found between healthy subjects and patients with ADPKD in the following 12 biomarkers: vWF, IL-8, M-CSF, IFNAR2, PEP1, TFF3, HGF, MCO-1, 8-OHdG, NGAL, L-FABP, angiotensinogen, and ceruloplasmin (Fig. 1). These biomarkers can be independent diagnostic markers since there were no strong correlations with serum creatinine, urinary protein, or kidney volume. We further evaluate these 12 biomarkers in the mice study.

Gene expression analysis of DBA/2FG-pcy mice kidneys

The DBA/2FG-pcy mouse is a model animal for hereditary polycystic kidney disease; it is reported that gross cysts are seen at 4 weeks, kidney volume increases by 30 weeks, and, at 10 weeks, cAMP is excreted and kidney cAMP concentrations increase [18]. Using DBA/2FG-pcy mice, we conducted gene expression analyses to determine whether the 12 remaining urinary biomarkers were either expressed in or secreted from kidney (Table 2). IL-8 was eliminated from our analysis as mice have no human IL-8 ortholog, whereas 8-OHdG was eliminated as it is not a direct product of a gene.

Six of the 12 biomarkers (NGAL, M-CSF, MCP-1, vWF, ceruloplasmin, and IFNAR2) showed more than twofold expression in the kidneys of DBA/2FG-pcy mice compared to the control.

Measurement of urinary biomarkers using DBA/2FG-pcy mouse urine

We measured the urinary levels of NGAL, M-CSF, MCP-1 in DBA/2FG-pcy mouse since no commercial kits to measure urinary vWF, ceruloplasmin, and IFNAR2 were available. We found statistically significant differences ($p < 0.01$ in each case) in NGAL, M-CSF, and MCP-1 when compared to wild type (Fig. 2). The above results demonstrated that NGAL, M-CSF, and MCP-1 are common candidates of urinary biomarker for ADPKD.

Table 1 Clinical characteristics of control and ADPKD patients

	Control median (range)	Patient median (range)
Age	42.8 (36 to 53)	47 (21 to 63)
Sex male:female	3:3 (total; 6)	11:12 (total; 23)
Serum creatinine (mg/dl)	0.78 (0.6 to 1.0)	0.91 (0.57 to 3.85)
eGFR	77.5 (66.4 to 89.6)	58.4 (14.3 to 97.5)
Mean blood pressure (mmHg)	84 (70 to 103)	107.4 (80 to 152.1)
BMI (kg/m^2)	20.7 (18.7 to 24.6)	21.5 (19.4 to 26.0)
Annual serum creatinine increases (mg/dl/year)		0.18 (−0.02 to 0.61)
Annual eGFR decreases (ml/min/1.73 m^2)		2.2 (−1.6 to 6.3)
Kidney volume (ml)		1863.5 (484.4 to 4882.5)
Annual Kidney volume increases (ml/year)		51.4 (40.8 to 210.4)

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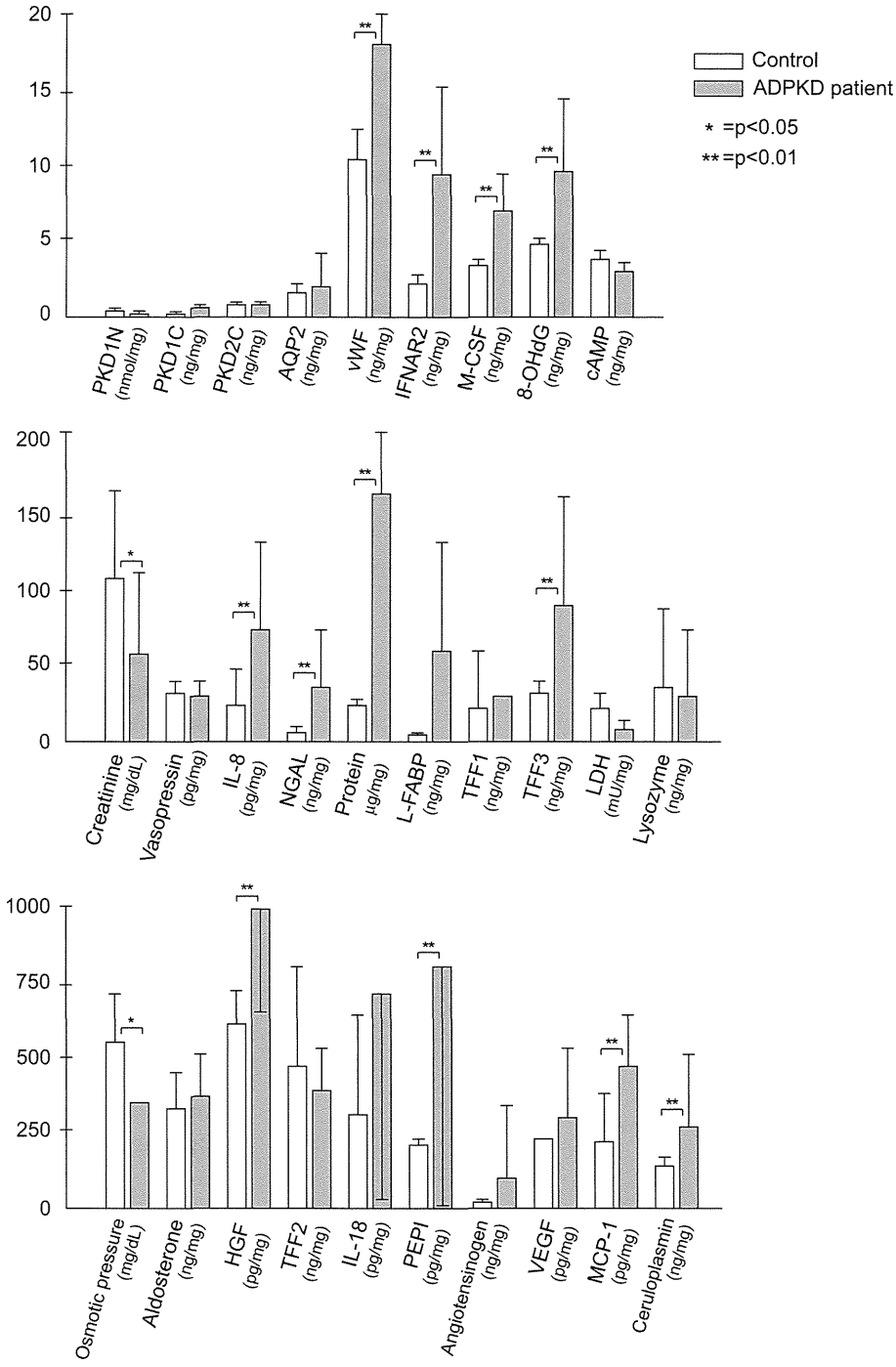

Fig. 1 Urinary biomarkers in the urine of ADPKD patients and control. Mean values of urine osmolarity, urine creatinine, and the 28 biomarkers (±SD)

Discussion

In this preliminary study, we explored potential urinary biomarkers to evaluate the therapeutic efficacy. We found that NGAL, M-CSF, and MCP-1 can be a potential common biomarker for human and murine ADPKD.

NGAL belongs to the superfamily of lipocalin proteins, and is expressed at a low level in the kidney, lung, and alimentary canal. In kidneys, NGAL helps regulate iron transport, and plays a role in epithelial differentiation pathways, inflammation, and cell proliferation [19]. Renal impairment increases its production, raising NGAL levels in serum and urine. There are some reports of NGAL as an AKI marker [14, 20]. NGAL levels are thought to increase in urine as ADPKD kidneys show constant inflammation and cyst proliferation and enlargement.

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Table 2 Gene expression analysis of DBA/2FG-pcy mice kidneys

Gene title	Gene symbol	GenBank accession no	Pcy mouse	DBA mouse	Fold increase
vWF	Vwf	Mm.22339	108.2	25.6	4.2
HGF	Hgf	Mm.267078	19.0	11.9	1.6
M-CSF	Csf1	Mm.795	316.7	61.6	5.1
IFNAR2	Ifnar2	Mm.6834	453.9	209.2	2.2
TFF3	Tff3	Mm.4641	36.2	40.8	0.9
PEPI	Grn	Mm.1568	3079.9	2539.1	1.2
MCP1	Cxcl2	Mm.4979	58.6	7.2	8.1
NGAL	Lcn2	Mm.9537	8141.1	44.6	182.5
L-FABP	Fabp1	Mm.22126	177.9	94.8	1.9
ceruloplasmin	Cp	Mm.13787	3911.1	610.1	6.4

Fold increase was obtained by calculating pcy vs. BDA

GenBank is accessible at http://www.ncbi.nih.gov

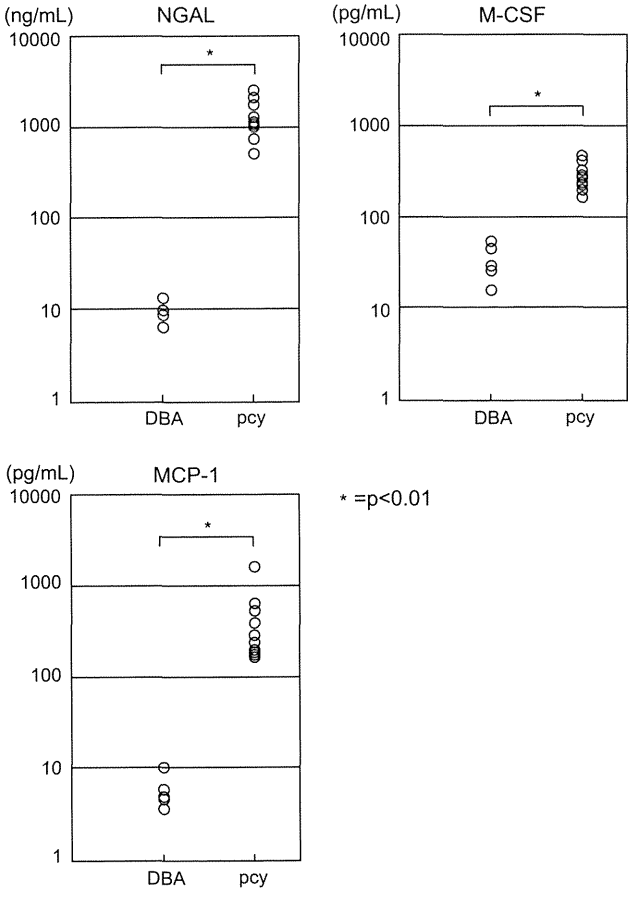

Fig. 2 Urinary biomarkers in DBA/2FG-pcy mouse urine. Urinary biomarkers in DBA/2FG-pcy mouse ($n = 10$) and that of control ($n = 5$)

M-CSF, also known as CSF-1, is a glycoprotein primarily expressed in mesenchymal tissue; it plays a role in monocyte and macrophage survival, differentiation, and proliferation. It is also involved in immune system regulation and pregnancy [21]. In kidneys, it is expressed in tubular cells, mesangial cells and podocytes, and patients with chronic renal failure are reported to show increased M-CSF levels in serum [22]. Yannick et al. reported that M-CSF is an effective marker of graft rejection in renal transplant patients [23]. High M-CSF levels in patients prior to renal transplant are reported to decrease following transplant, increasing to high levels with acute organ rejection, and improving once again following treatment. MCP-1 accelerates monocyte chemotaxis and is an activating agent, with stimulation of inflammatory cytokines causing MCP-1 production and secretion in various cells. It is thought to play a role in permeation of monocytes and macrophages in various kinds of inflammatory disease, and is known to be a marker of kidney inflammation. MCP-1 plays an important role in ADPKD and is considered to be involved in deteriorating renal function from kidney stroma fibrillization [24]. Zheng et al. reported that in ADPKD patients, MCP-1 concentration increases in urine along with cyst proliferation [25].

The limitation of this study is that this is a preliminary study to explore potential candidate biomarkers. To show if these urinary biomarkers can be a prognostic or predictive biomarker, we need a prospective study that we are now preparing for.

Conclusions

We explored the urinary biomarkers in human and murine ADPKD. NGAL, M-CSF, MCP-1 can be common urinary biomarkers between human cases and murine model.

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Conflict of interest Employment by Otsuka Pharmaceutical Co., Ltd.: Yasukazu Ohmoto, Fusako Iwata, Hiroyuki Fujiki, Toyoki Mori. Consultancies for Otsuka Pharmaceutical Co., Ltd.: Shigeo Horie.

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Association of arginine vasopressin surrogate marker urinary copeptin with severity of autosomal dominant polycystic kidney disease (ADPKD)

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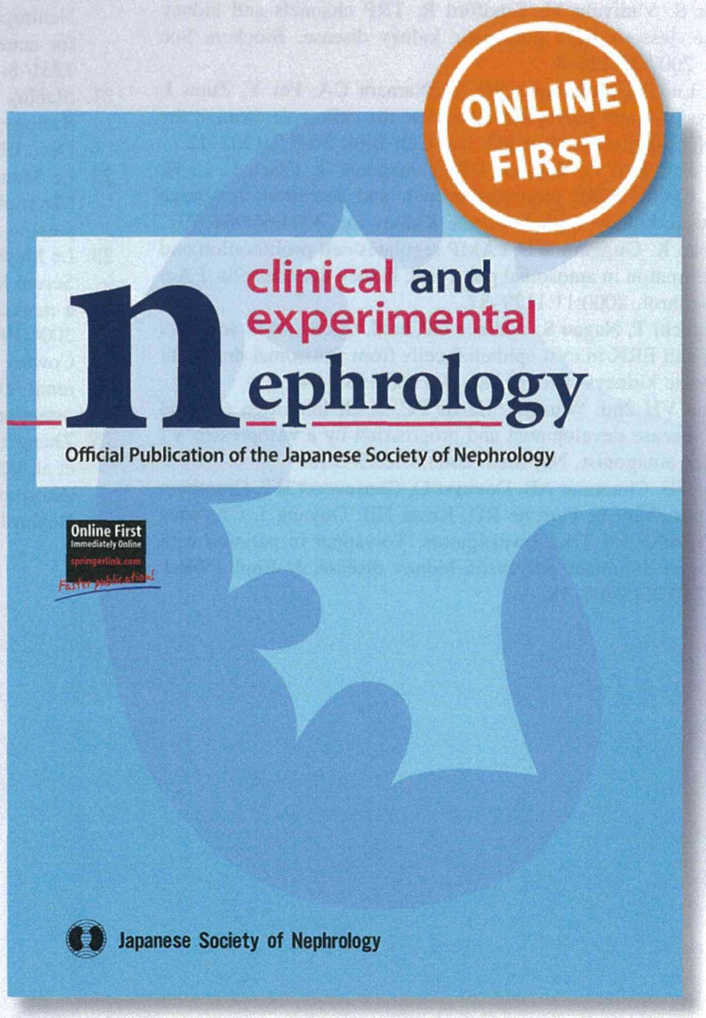

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ORIGINAL ARTICLE

Association of arginine vasopressin surrogate marker urinary copeptin with severity of autosomal dominant polycystic kidney disease (ADPKD)

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Abstract
Background Experimental studies suggest a detrimental role for cyclic adenosine monophosphate (cAMP) and vasopressin in the pathogenesis of autosomal dominant polycystic kidney disease (ADPKD). It is unknown, however, whether urinary cAMP and copeptin concentration are associated with disease severity in patients with ADPKD.
Methods Urinary cAMP (u-cAMP) and copeptin concentration (u-copeptin) were measured by immunoassay in ADPKD patients with CKD stage ≤4. We compared our measurements with clinical parameters including estimated glomerular filtration rate (eGFR), total kidney volume (TKV), and height-adjusted TKV (htTKV). Logarithmic transformation of all variables was performed to fulfill the requirement of equal distribution of the residuals.
Results We included 50 patients in this study (24 females and 26 males; mean age: 49.3 years). The median eGFR and TKV were 53.2 ml/min/1.73 m^2 (interquartile range: IQR; 29.4–68.45) and 1138.1 ml (IQR; 814.7–2065.0), respectively. The median u-copeptin level was 12.19 (IQR; 6.91–22.32) ng/ml. Although u-cAMP/u-Cr was not significantly correlated with TKV ($R = -0.006$, $p = 0.967$) and eGFR ($R = 0.077$, $p = 0.602$), urinary copeptin/u-Cr was statistically associated with the various markers of disease severity in ADPKD [positively with TKV ($R = 0.351$, $p = 0.014$), htTKV ($R = 0.383$, $p = 0.008$) and negatively with eGFR ($R = -0.304$, $p = 0.036$)].
Conclusions In ADPKD subjects, a higher u-copeptin is associated with disease progression, suggesting that u-copeptin may be a new surrogate marker to predict renal prognosis in ADPKD.

Keywords Autosomal dominant polycystic kidney disease · Vasopressin · Copeptin · GFR · Total kidney volume · Marker

Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is the most common progressive hereditary kidney disease. Since the intact kidneys can compensate for the loss of glomerular filtration in ADPKD patients, renal insufficiency usually remains undetected until close to almost the fourth decade of life, progressing rapidly once a critical expansion of renal cysts is reached [1]. Hence, reliable diagnostic and prognostic biomarkers to identify ADPKD progression are urgently needed.

When arginine vasopressin (AVP) is bound to the V2 receptor (V2R), cyclic adenosine monophosphate (cAMP) production is stimulated. In turn, cAMP leads to the proliferation of epithelial cells and stimulates cyst formation [2]. AVP measurement is often cumbersome because it is unstable with a short half-life [3] and is difficult to measure [4]. Copeptin consists of the C-terminal portion of pro-AVP and is produced in equimolar amounts as AVP during precursor processing [5]. Copeptin has been shown to be a relatively easily measured [6], reliable and stable substitute for circulating AVP concentration [7–9].

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Recently, several studies reported that plasma copeptin concentration was associated with ADPKD progression [10–13]. As far as we know, however, there are no previous reports concerning either the efficacy of urine copeptin as a surrogate marker of ADPKD progression or the association between urine cAMP levels and disease severity in ADPKD patients.

The novel treatment of V2R antagonist for ADPKD began in Japan under national health insurance in 2014. As we urgently need precise and handy biomarkers of disease progression associated with ADPKD advancement, we investigated the association between urine copeptin and urine cAMP concentration and ADPKD disease progression in this study, measured as estimated glomerular filtration rate (eGFR) and total kidney volume (TKV) in ADPKD patients.

Materials and methods

Study population and design

The present study enrolled individuals from January to October, 2014 at our outpatient clinic who had been diagnosed as having ADPKD according to Ravine's criteria [14]. We included ADPKD patients with CKD stage ≤ 4. Exclusion criteria were as follows: use of medication influencing renal concentration capacity, such as diuretics and postmenopausal hormone therapy; history of diseases influencing renal concentration capacity, such as diabetes mellitus, diabetes insipidus, hypothalamic–pituitary–adrenal deficiency, or kidney diseases other than ADPKD; other factors that can influence renal concentration capacity such as menstruation, urinary tract infection, and pregnancy; and contraindication of magnetic resonance imaging (MRI). However, patients on antihypertensive agents were eligible for inclusion in this study.

This study was approved by our institutional review board (approval number: 070475) and was performed in adherence to the Declaration of Helsinki. All participants gave written informed consent. We excluded the patients without informed consent in this study.

Weight, height, body mass index (BMI) calculated as weight (kg) divided by height (m^2), and waist circumference were measured upon admission. Blood pressure was measured in the morning before antihypertensive medication intake. Systolic and diastolic BP values were used to calculate mean arterial pressure (MAP) using the standard formula 2/3 diastolic BP + 1/3 systolic BP.

Blood and spot urine samples were collected in the morning prior to taking medications or food. Plasma osmolality (s-OSM), sodium (s-Na), creatinine (s-Cr), and cystatin C were measured in these blood samples using standard laboratory techniques. Urinary sodium (u-Na), N-acetyl-β-D-glucosaminidase (NAG), creatinine (u-Cr), and osmolality (u-OSM) were measured in these spot urine samples by freezing point depression. Fractional excretion of sodium (FENa) was calculated as [(urinary sodium concentration/plasma sodium concentration)/(u-Cr concentration/s-Cr concentration)] × 100.

The eGFR was used as a marker of renal function and was calculated using the simplified MDRD equation modified by the appropriate coefficient for Japanese populations by sex as follows: $eGFR = 194 \times Cr^{-1.094} \times Age^{-0.287}$ (female: ×0.739) (ml/min/1.73 m^2) [15].

A sandwich immunoassay was used to measure the value of urine copeptin (Copeptin, human—EIA Kit, Bachem Americas, Inc. Torrance, CA, USA), plasma copeptin (Copeptin, human—EIA Kit, Peninsula Laboratories International, Inc. San Carlos, CA, USA) and urine cAMP levels (Cyclic AMP Direct EIA kit, Arbor Assays, Inc., Ann Arbor, MI, USA), respectively.

TKV was measured by performing standard abdominal MRI without the use of intravenous contrast on T2-weighted images. TKV was obtained by calculating using a standard formula: Renal volume = $\pi/6 \times$ length × width × depth [16]. Length and width were obtained from longitudinal images acquired in planes ranging from sagittal to coronal, whereas depth was obtained from transverse images of the mid-kidney acquired in the plane perpendicular to the longitudinal plane. Because height was the best reference for TKV, we also examined with height-adjusted TKV (htTKV) (cm^3/m) using ellipsoid methods as mentioned above.

Statistical analysis

Because it is impossible to test the aliquot sample from 24-hour urine collection, we evaluated not only u-cAMP and u-copeptin but also u-cAMP divided by u-Cr (u-cAMP/u-Cr) and u-copeptin/u-Cr. To test the correlations between u-cAMP, u-cAMP/u-Cr, u-copeptin and u-copeptin/u-Cr [17], all variables were logarithmically normalized [11, 18]. Variables are expressed as median with interquartile range (IQR) for nonparametric data. Pearson's correlation coefficients were used to examine the associations among selected clinical variables and variables representing disease severity. R 2.14.0 was used to determine the outcome measure [19]. Two-tailed p values of <0.05 were considered to indicate a statistically significant difference.

Results

Patients' characteristics (Table 1)

This study involved 50 patients with ADPKD (26 men and 24 women; mean age: 49.3 years). Table 1 shows the

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Table 1 Baseline characteristics of all 50 subjects analyzed

Gender	
Male	26 (52.0 %)
Female	24 (48.0 %)
Age, mean ± SD (year)	49.3 ± 12.3
BMI, mean ± SD (kg/m^2)	22.5 ± 5.2
Waist circumference, mean ± SD (cm)	80.8 ± 10.9
MAP, median (IQR) (mmHg)	94.0 (86.7–102.8)
TKV, median (IQR) (ml)	1138.1 (814.7–2065.0)
s-Cr, median (IQR) (mg/dl)	1.04 (0.88–1.64)
u-Cr, median (IQR) (mg/dl)	62.55 (45.15–97.90)
CKD stage	
1	1 (2.0 %)
2	20 (40.0 %)
3	16 (32.0 %)
4	13 (26.0 %)
eGFR, median (IQR) (ml/min/1.73 m^2)	53.2 (29.4–68.45)
Cystatin C, median (IQR) (mg/l)	1.12 (0.95–2.01)
NAG, median (IQR) (U/l)	3.2 (2.25–5.6)
s-OSM, median (IQR) (mOsm/kg)	290 (287–294.5)
u-OSM, median (IQR) (mOsm/kg)	417 (292–551)
s-Na, median (IQR) (mEq/l)	141 (140.75–142.25)
u-Na, median (IQR) (mEq/l)	72 (43.5–102)
FENa, median (IQR) (%)	0.95 (0.64–1.82)
u-copeptin (IQR) (ng/ml)	12.19 (6.91–22.32)
u-cAMP (IQR) (pmol/ml)	1184.75 (639.50–1216.78)

subjects' baseline characteristics. The median eGFR, cystatin C and TKV were 53.2 ml/min/1.73 m^2 (interquartile range: IQR; 29.4–68.45), 1.12 mg/l (IQR; 0.95–2.01), and 1138.1 ml (IQR; 814.7–2065.0), respectively. At baseline, eGFR and TKV showed significant mutual inverse correlation ($R = -0.524$, $p < 0.001$). Of all included patients, 45 patients (90 %) take antihypertensive agents.

The efficacy of u-cAMP and u-cAMP/u-Cr in detecting ADPKD progression

The median urinary cAMP level was 1184.75 (IQR; 639.50–1216.78) pmol/L. Table 2 lists results of the associations of urine cAMP and u-cAMP/u-Cr levels with physiological parameters and measures of ADPKD disease severity. Neither u-cAMP nor u-cAMP/u-Cr showed significant correlation with TKV (u-cAMP; $R = -0.104$, $p = 0.482$, u-cAMP/u-Cr; $R = -0.006$, $p = 0.967$), htTKV (u-cAMP; $R = -0.184$, $p = 0.215$, u-cAMP/u-Cr; $R = -0.035$, $p = 0.817$), or eGFR (u-cAMP; $R = 0.224$, $p = 0.126$, u-cAMP/u-Cr; $R = 0.077$, $p = 0.602$). Although u-cAMP was significantly correlated with u-OSM ($R = 0.520$, $p < 0.001$), no significant association was found between u-cAMP and s-OSM ($R = -0.217$, $p = 0.144$). In addition, there was no significant correlation between u-cAMP/u-Cr and u-OSM ($R = -0.193$, $p = 0.254$) or s-OSM ($R = -0.094$, $p = 0.528$). Examining u-cAMP and other markers of disease severity for any association, we found that u-cAMP was positively correlated with u-Cr ($R = 0.595$, $p < 0.001$), NAG ($R = 0.465$, $p < 0.001$) and inversely correlated with FENa ($R = -0.460$, $p = 0.001$). There was no significant association between u-cAMP/u-Cr and any parameter without u-copeptin/u-Cr (Table 3).

The efficacy of u-copeptin and u-copeptin/u-Cr in detecting ADPKD progression

The median urinary copeptin level was 12.19 (IQR; 6.91–22.32) pmol/L. As with u-cAMP, we evaluated not only c-copeptin, but also u-copeptin divided by u-Cr (u-copeptin/u-Cr). We could not find any sex-based difference in u-copeptin (men: median $R = 11.076$ ng/ml and IQR 4.436–20.823; women: median 12.721 ng/ml and IQR 9.254–24.826; $p = 0.319$). However, median u-copeptin/u-Cr was significantly higher in women (median 0.219, IQR 0.188–0.387) than in men (median 0.144, IQR 0.095–0.257) ($p = 0.020$). Only in cases with preserved blood plasma ($n = 18$), we examined the plasma copeptin values. We could show the positive-correlation between u-copeptin and plasma copeptin but without significant relationship ($p = 0.198$) mainly because of a small number cases with preserved blood plasma (Fig. 1a). Although there are no significant correlations between plasma copeptin and eGFR ($R = -0.245$, $p = 0.227$), there are significant correlations between plasma copeptin and htTKV ($R = 0.458$, $p = 0.019$) and TKV ($R = 0.465$, $p = 0.017$).

Although u-copeptin was not significantly correlated with TKV ($R = 0.261$, $p = 0.073$), htTKV ($R = 0.261$, $p = 0.076$), and eGFR ($R = -0.153$, $p = 0.301$), urinary copeptin/u-Cr was positively associated with TKV ($R = 0.351$, $p = 0.014$, Fig. 1b), htTKV ($R = 0.383$, $p = 0.008$, Fig. 1c), and negatively associated with eGFR ($R = -0.304$, $p = 0.036$, Fig. 1d). With respect to osmolality, there were no significant differences between u-copeptin and s-OSM ($R = 0.198$, $p = 0.183$) or u-OSM ($R = 0.200$, $p = 0.234$). On the other hand, u-Copeptin/u-Cr was associated with s-OSM ($R = 0.306$, $p = 0.037$, Fig. 1e) and u-OSM ($R = -0.333$, $p = 0.044$, Fig. 1f).

U-copeptin was significantly correlated with u-cAMP ($R = 0.527$, $p < 0.001$) and u-cAMP/u-Cr ($R = 0.361$, $p = 0.012$). U-copeptin/u-Cr was also significantly correlated with u-cAMP/u-Cr ($R = 0.460$, $p = 0.001$).

Examining the association between u-copeptin and other markers of disease severity, we found that u-copeptin was significantly correlated with u-Cr ($R = 0.324$, $p = 0.025$)