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A multicenter cross-sectional study of circulating soluble urokinase receptor in Japanese patients with glomerular disease

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Elevated serum-soluble urokinase receptor (suPAR) levels have been described in patients with focal segmental glomerulosclerosis (FSGS) in several different cohorts. However, it remains unclear whether this is the case for Japanese patients and whether circulating suPAR can be clinically useful as a diagnostic marker. To determine this, we measured serum suPAR levels in 69 Japanese patients with biopsy-proven glomerular diseases in a cross-sectional manner. The serum suPAR levels showed a significant inverse correlation with renal function by univariate (R^2 of 0.242) and multivariate ($\beta = 0.226$) analyses. Even after excluding patients with renal dysfunction, no significant difference in the suPAR levels was detected among the groups. Receiver operating characteristic analysis and measures of the diagnostic test performance showed that suPAR was not a useful parameter for differentiating FSGS from the other glomerular diseases (AUC-ROC: 0.621), although a small subgroup analysis showed that patients with FSGS, treated with steroids and/or immunosuppressants, had significantly lower suPAR levels. Patients with ANCA-associated glomerulonephritis had significantly higher levels of suPAR compared with the other disease groups, which may be owing to their lower renal function and systemic inflammation. Thus, suPAR levels are significantly affected by renal function and have little diagnostic value even in patients with normal renal function.

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Focal segmental glomerulosclerosis (FSGS) is one of the leading causes of steroid-resistant nephrotic syndrome. Research on the pathogenesis of primary FSGS has been intensified because of identification of the podocyte as the major cellular target;[1] however, the disease mechanism has not been fully elucidated. One of the best-recognized notions is that some FSGS cases may be associated with a circulating factor. This concept is supported by the recurrence of FSGS soon after renal transplantation,[2,3] by the response of proteinuria to plasmapheresis[4,5] or immunoabsorption,[6,7] and by a case of nephrotic syndrome in a newborn whose mother had FSGS.[8] Recently, Gallon *et al.*[9] reported that reimplantation of a kidney allograft with FSGS recurrence into another patient resulted in proteinuria remission, which strongly suggested involvement of a circulating factor.

Wei *et al.*[10] have recently reported that soluble urokinase receptor (suPAR) was a promising candidate for circulating permeability factor by evaluating the circulating suPAR levels in sera from primary and recurrent FSGS patients. They proposed that suPAR has a causal role in the development of FSGS based on their finding that urokinase receptor (uPAR) can cause foot process effacement through the activation of β3-integrin signaling.[11] The researchers also evaluated the suPAR levels in two large cohorts of children and adults with biopsy-proven primary FSGS.[12] Although they demonstrated that the suPAR levels were significantly elevated in primary FSGS patients from both cohorts, there has been an intense

debate over suPAR as a diagnostic marker.[13–16] Recently, Huang *et al.*[17] reported that the suPAR levels in primary FSGS patients have a considerable overlap with other glomerular diseases such as minimal change disease (MCD) and membranous nephropathy (MN), although these differences are significant. In a study of pediatric patients, Bock *et al.*[18] demonstrated that the suPAR levels in FSGS patients are even lower than those in non-glomerular kidney diseases.[18] In Japan, it is estimated that approximately 3800–4500 adults develop nephrotic syndrome annually, and primary FSGS accounts for approximately 10% of the new-onset nephrotic syndrome cases.[19] However, no data on the serum suPAR levels in Japanese FSGS patients are available yet.

In the present investigation, we performed a multicenter cross-sectional study using sera from patients with primary glomerular diseases (including FSGS) to determine whether the serum suPAR levels in Japanese patients are useful as a diagnostic marker.

RESULTS

Demographic and clinical characteristics of patients with glomerular diseases

We studied 69 serum samples from patients with biopsy-proven glomerular diseases and 17 serum samples from healthy volunteers. The subjects' demographic and clinical data are listed in Table 1. The median age of the patients was 54 years, ranging from 17 to 82 years. The patients were categorized according to the histopathological diagnoses (FSGS, MCD, IgA nephropathy (IgAN), and MN) performed by pathologists in each facility, and no overlaps were reported.

In our cohort, the MN patients (67.9 ± 10.3 years of age) were significantly older than the MCD patients (41.2 ± 18.1 years of age; $P = 0.005$), the IgAN patients (42.2 ± 20.8 years of age; $P = 0.007$), and the healthy control subjects (45.3 ± 15.5 years of age; $P = 0.0115$). All of the disease groups showed significantly lower serum albumin levels compared with healthy control group; however, no significant difference was observed between any two disease groups. As for serum total cholesterol levels, we found that the MCD patients (422.1 ± 139.3 mg/dl) had significantly higher levels compared with the FSGS patients (324.3 ± 102.0 mg/dl; $P = 0.0461$), the IgAN patients (265.5 ± 101.0 mg/dl; $P = 0.0029$), MN patients (284.3 ± 95.0 mg/dl; $P = 0.0192$), and control subjects (206.1 ± 35.7 mg/dl; $P < 0.0001$). The FSGS patients' serum total cholesterol levels were significantly higher than those of control subjects ($P = 0.0016$). We measured C-reactive protein (CRP) in the patients with these renal diseases, because it has been reported that the serum suPAR concentration rises with nonspecific inflammation.[20–22] No significant difference in CRP was detected among the disease groups and the control group. The renal function represented by the estimated glomerular filtration rate (eGFR) was significantly higher in the control group (79.9 ± 15.8 ml/min per 1.73 m^2) compared with that in the FSGS group (54.4 ± 25.6 ml/min per 1.73 m^2; $P = 0.0066$). The urinary protein excretion was compared within the disease groups. The MCD patients (9138.1 ± 3874.7 mg per day or mg/gCre) excreted significantly more urinary protein than did the IgAN patients (3874.4 ± 2476.2 mg per day or mg/gCre; $P = 0.02$) or the FSGS patients (5753.2 ± 4772.3 mg per day or mg/gCre; $P = 0.04$). The amount of proteinuria in MN patients (7538.3 ± 2711.9 mg per day or mg/gCre) was also larger than in IgAN patients ($P = 0.048$).

Serum suPAR levels in primary glomerular diseases

We analyzed the unadjusted data on the serum suPAR levels according to the histological diagnosis of the glomerular diseases (Table 1). The serum suPAR levels significantly differed among the five groups, including the control group (one-way analysis of variance (ANOVA), $P < 0.0001$, effect size $f = 0.716$, power $(1 - \beta) = 0.999$). In our cohort, however, the suPAR levels in the FSGS group (3119.0 ± 1036.6 pg/ml) did not significantly differ from any other disease groups. Moreover, no significant difference was observed between any disease groups. The serum suPAR concentrations in the

Table 1 | Demographic/clinical characteristics

	All patients, $n = 69$	FSGS, $n = 38$	MCD, $n = 11$	IgAN, $n = 11$	MN, $n = 9$	Control, $n = 17$	*P*-value
Age (years)	52.8 ± 18.5	55.6 ± 16.3	41.2 ± 18.1	42.2 ± 20.8	67.9 ± 10.3^{A,B,C}	45.3 ± 15.5	0.0007
Gender (male)	41 (59.4%)	26 (68.4%)	6 (54.5%)	5 (45.5%)	4 (44.4%)	9 (52.9%)	0.5060
Alb (mg/dl)	2.58 ± 1.00	2.56 ± 0.98	2.04 ± 0.96	3.33 ± 1.11	2.41 ± 0.37	4.63 ± 0.33^{D,E,F,G}	<0.0001
TC (mg/dl)	325.5 ± 117.9	324.3 ± 102.0^{H}	422.1 ± 139.3^{I,J,K,L}	265.5 ± 101.0	284.3 ± 95.0	206.1 ± 35.7	<0.0001
CRP (mg/dl)	0.29 ± 0.43	0.30 ± 0.47	0.23 ± 0.34	0.37 ± 0.58	0.26 ± 0.27	0.09 ± 0.13	0.4187
Steroids/immunosuppressants (yes)	19 (27.9%)	11 (29.7%)	5/6 (45.5%)	1/10 (9.1%)	2/7 (22.2%)	0 (0%)	0.0321
eGFR (ml/min per 1.73 m^2)	61.9 ± 27.6	54.4 ± 25.6	76.5 ± 29.6	68.1 ± 22.0	68.4 ± 33.1	79.9 ± 15.8^{M}	0.0062
suPAR (μg/ml)	2896.8 ± 961.7	3119.0 ± 1036.6^{N}	2374.9 ± 588.8	2311.3 ± 777.1	3311.9 ± 655.3^{O}	1745.1 ± 395.4	<0.0001
UP (mg per day or mg/g Cre)	6226.2 ± 4357.3	5753.2 ± 4772.3	9138.1 ± 3874.7^{P,Q}	3874.4 ± 2476.2	7538.3 ± 2711.9^{R}	N/A	0.0211[a]

Abbreviations: Alb, albumin; ANOVA, analysis of variance; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; FSGS, focal segmental glomerulosclerosis; HSD, honest significant difference; IgAN, IgA nephropathy; MCD, minimal change disease; MN, membranous nephropathy; NA, not available; suPAR, soluble urokinase receptor; TC, total cholesterol; UP, urinary protein.

The data are presented as the mean ± s.d. Differences among the groups were analyzed by a one-way ANOVA. The multiple comparisons for age, TC, and eGFR were performed by Tukey's HSD mean separation tests. A nonparametric Steel–Dwass test was used for Alb and suPAR. Differences between the disease groups in gender and steroids/immunosuppressants administration were determined by χ^2 tests. A: $P = 0.0048$ vs. MCD; B: $P = 0.0074$ vs. IgAN; C: $P = 0.0115$ vs. control; D: $P < 0.0001$ vs. FSGS; E: $P = 0.0002$ vs. MCD; F: $P = 0.0146$ vs. IgAN; G: $P = 0.0004$ vs. MN; H: $P = 0.0016$ vs. control; I: $P = 0.0461$ vs. FSGS; J: $P = 0.0029$ vs. IgAN; K: $P = 0.0192$ vs. MN; L: $P < 0.0001$ vs. control; M: $P = 0.0066$ vs. FSGS; N: $P < 0.0001$ vs. control; O: $P = 0.0006$ vs. control; P: $P = 0.04$ vs. FSGS; Q: $P = 0.02$ vs. IgAN; R: $P = 0.048$ vs. IgAN.

[a]Because quantitative data of urinary protein for healthy controls are not available, UP in the only disease groups were analyzed by one-way ANOVA.

patients with FSGS and MN were significantly higher than in the healthy controls ($P<0.0001$ and $P=0.0006$, respectively).

Serum suPAR levels and demographic/clinical patient characteristics

Next, we assessed the association between the patients' serum suPAR levels and their demographic characteristics and clinical parameters. Although age was significantly correlated with the serum suPAR concentrations in the overall patient cohort ($R^2=0.1496$, $P=0.001$, $\beta \pm$ s.e.m. $=20.11 \pm 5.86$, effect size $\rho=0.387$, power $(1-\beta)=0.930$; Figure 1), no significant difference was detected between male and female patients in the overall patient cohort (male: 2948.1 ± 884.8 pg/ml; female: 2821.5 ± 1076.9; $P=0.60$; Figure 2). The suPAR levels in patients with nephrotic-range proteinuria (2904.6 ± 897.4 pg/ml), independent of the underlying glomerular diseases, were not significantly higher than those in patients with non-nephrotic-range proteinuria (2872.6 ± 1167.4 pg/ml, $P=0.91$; Figure 3a). In addition, the serum suPAR levels were not correlated with urinary protein ($R^2=0.0020$, $P=0.72$; Figure 3b). We did not detect significant correlations of suPAR with CRP ($R^2=0.053$, $P=0.10$), serum albumin ($R^2=0.00048$, $P=0.86$), or total cholesterol ($R^2=0.024$, $P=0.24$). Our subgroup analysis of FSGS patients also revealed no significant correlation between suPAR and CRP ($R^2=0.0037$, $P=0.82$), serum albumin ($R^2=0.0013$, $P=0.83$), or total cholesterol ($R^2=0.0013$, $P=0.85$).

Serum suPAR levels are inversely correlated with the eGFR

Previous studies have revealed that the serum suPAR concentration has an inverse correlation with the renal function.[13,23,24] In the current investigation, as shown in Figure 4, suPAR was correlated inversely with the eGFR ($R^2=0.242$, $P<0.0001$, $\beta \pm$ s.e.m. $=-17.13 \pm 3.71$, effect size $\rho=0.492$, power $(1-\beta)=0.996$) in the total patient population. Moreover, our subgroup analysis of the FSGS patient group demonstrated that there was still a significant correlation between suPAR and the eGFR ($R^2=0.227$, $P=0.0025$, $\beta \pm$ s.e.m. $=-19.35 \pm 5.94$, effect size $\rho=0.476$, power $(1-\beta)=0.893$; Figure 4).

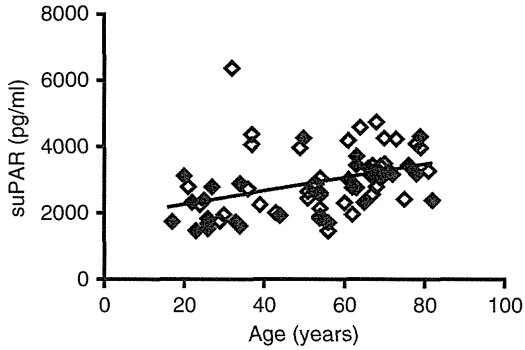


Figure 1 | The correlation between age and the serum-soluble urokinase receptor (suPAR). In all the patients with primary glomerular diseases, the serum suPAR concentration was significantly correlated with age (Pearson's correlation coefficient test, $R^2=0.1496$, $P=0.001$). Open diamonds indicate focal segmental glomerulosclerosis (FSGS) patients; filled diamonds indicate patients with the other glomerular diseases.

Serum suPAR and patient characteristics: multiple regression analysis

Next, we performed a multiple regression analysis to evaluate the association between the patients' demographic or clinical

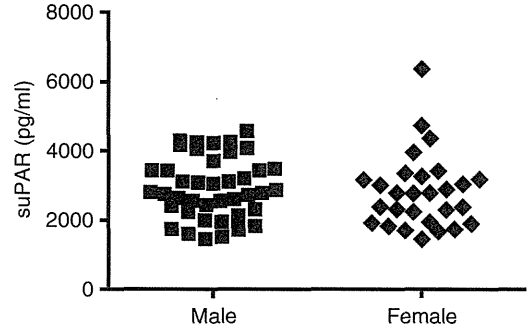


Figure 2 | Soluble urokinase receptor (suPAR) levels and gender. In the patients with primary glomerular diseases, the serum suPAR concentration did not significantly differ between male and female (unpaired *t*-test, $P=0.60$).

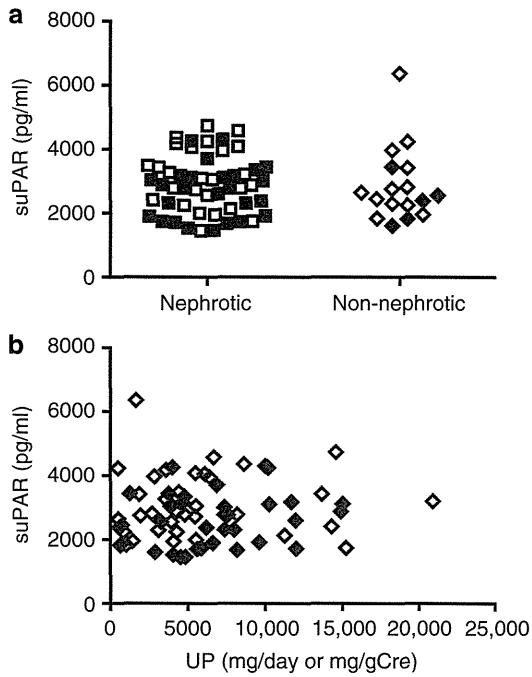


Figure 3 | Urinary protein and serum-soluble urokinase receptor (suPAR) in patients with glomerular diseases. (**a**) Serum suPAR levels in the patients with nephrotic-range proteinuria and non-nephrotic-range proteinuria. No significant difference was detected (unpaired *t*-test, 2904.6 ± 897.4 vs. 2872.6 ± 1167.4 pg/ml, $P=0.91$). (**b**) Correlation between the urinary protein excretion (UP; mg/day or mg/gCre) and the serum suPAR levels (pg/ml). No significant correlation was observed (Pearson's correlation coefficient test, $R^2=0.0020$, $P=0.72$). Open diamonds indicate focal segmental glomerulosclerosis (FSGS) patients; filled diamonds indicate patients with the other glomerular diseases.

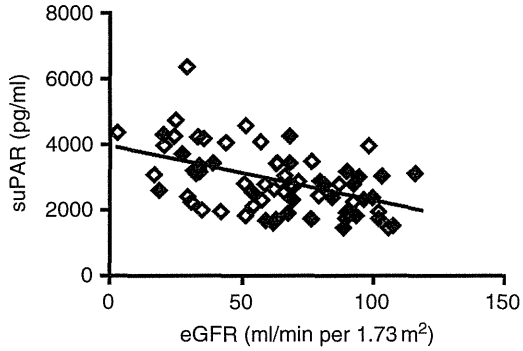
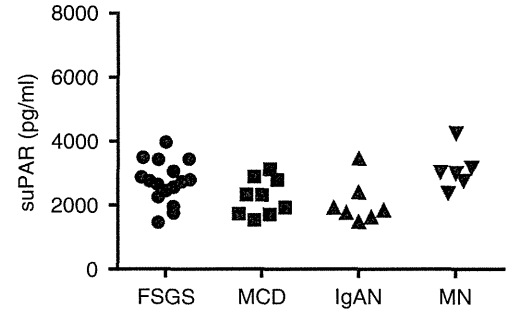


Figure 4 | The correlation between soluble urokinase receptor (suPAR) and estimated glomerular filtration rate (eGFR). Correlation between serum suPAR levels and the eGFR in all patients with primary glomerular diseases. The serum suPAR levels were significantly and inversely correlated with the eGFR (Pearson's correlation coefficient test, $R^2 = 0.242$, $P < 0.0001$). Open diamonds indicate focal segmental glomerulosclerosis (FSGS) patients; filled diamonds indicate patients with the other glomerular diseases.



suPAR	FSGS	MCD	IgAN	MN
Mean	2723.0	2260.6	2055.0	3110.2
s.d.	671.2	577.2	678.1	630.9

Figure 5 | Serum levels of the soluble urokinase receptor (suPAR) in patients with primary glomerular disease whose estimated glomerular filtration rate (eGFR) was 60 ml/min per 1.73 m^2 or higher. Patients whose eGFR was 60 ml/min per 1.73 m^2 or higher were distinguished and analyzed for their serum suPAR levels. There was significant difference in suPAR levels among the disease groups (one-way analysis of variance (ANOVA), $R^2 = 0.254$, $P = 0.018$, effect size $f = 0.584$, power $(1 - \beta) = 0.824$); however, no significant difference was detected in a multiple comparison (Steel–Dwass test).

Table 2 | Multiple regression analysis of the serum levels of the suPAR

	β	s.e.m.	*P*-value
Age (years)	0.230	0.230	0.068
Gender (male)	−0.032	0.111	0.773
Urinary protein (mg per day or mg/gCre)	0.067	0.119	0.580
Serum creatinine (mg/dl)	0.226	0.226	0.047
Disease (FSGS)	0.214	0.124	0.090
Disease (IgAN)	−0.213	0.135	0.121
Disease (MCD)	−0.123	0.134	0.365
Disease (MN)	0.121	0.102	0.241
Steroids/immunosuppressants administration (yes)	0.208	0.115	0.076

Abbreviations: FSGS, focal segmental glomerulosclerosis; IgAN, IgA nephropathy; MCD, minimal change disease; MN, membranous nephropathy; suPAR, soluble urokinase receptor.

parameters and their suPAR levels. Because the eGFR depends upon the age, gender, and serum creatinine concentration, we used serum creatinine instead of eGFR as a predictor variable. The other predictor variables were age, gender, urinary protein, disease groups, and steroid/immunosuppressant administration. The analysis ($R^2 = 0.323$, $P = 0.002$) demonstrated that serum creatinine ($\beta \pm$ s.e.m. $= 0.226 \pm 0.226$, $P = 0.0472$) was significantly correlated with the serum suPAR (Table 2). The effect size f^2 and power $(1 - \beta)$ of the analysis were 0.300 and 0.918, respectively. These findings suggest that the renal function significantly affects the suPAR levels, which is consistent with the results from the above-described univariate analyses.

Serum suPAR levels in patients with normal renal function

Because renal impairment is closely associated with the suPAR levels, we decided to thereafter limit the objectives to patients with normal renal function. In accordance with the definition of chronic kidney disease, we excluded patients whose eGFR was below 60 ml/min per 1.73 m^2. For this subgroup, the difference in suPAR levels between any disease groups was not significant by multiple comparisons (Figure 5).

Diagnostic value of suPAR: differentiating FSGS from the other glomerular diseases

Because primary FSGS and MCD often exhibit similar clinical presentations and because there are difficulties in differentiating these two diseases even with a renal biopsy, a potent diagnostic biomarker has long been awaited. In our overall cohort, which included patients with low GFR, the distribution of serum suPAR levels in the FSGS patients demonstrated that there was apparently a subpopulation that exhibited higher suPAR levels than did the other disease groups, although the difference in suPAR between the disease groups did not reach statistical significance (Table 1). This finding motivated us to test the diagnostic value of suPAR for differentiating primary FSGS from MCD in patients without renal dysfunction. We performed a receiver operating characteristic (ROC) curve analysis on the FSGS patients ($n = 16$) and the MCD patients ($n = 9$) without renal dysfunction. By identifying the point at which the difference between sensitivity and 1-specificity is maximal, we determined that the optimal cutoff value should be 2442.5 pg/ml of the suPAR concentration for these FSGS or MCD patients (Figure 6a; solid line). For this cutoff value, the sensitivity and specificity of this test were 0.750 and 0.666, respectively. The positive likelihood ratio (LR) was 2.25, which means that the suPAR levels higher than 2442.5 pg/ml do not usefully increase the probability that FSGS exists (the post-test odds). Moreover, the negative LR was 0.625, suggesting that the suPAR levels lower than 2442.5 pg/ml do not sufficiently change the probability that a patient does not have FSGS. No

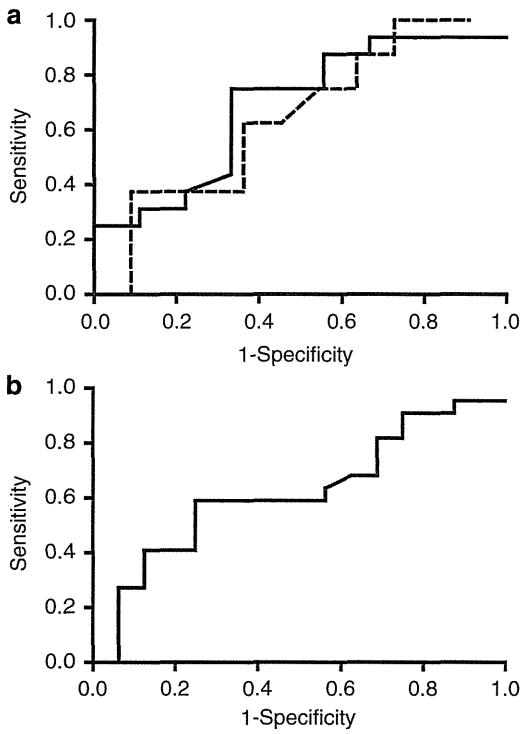


Figure 6 | Receiver operating characteristic (ROC) analysis of the serum soluble urokinase receptor (suPAR) in the patients with focal segmental glomerulosclerosis (FSGS). (**a**) ROC analysis in the patients with FSGS or minimal change disease (MCD). Solid line, ROC curve for patients with FSGS or MCD whose estimated glomerular filtration rate (eGFR) was 60 ml/min per 1.73 m^2 or higher. The area under the ROC curve (AUC-ROC) was 0.684 ± 0.114 (95% confidence interval (CI): 0.461–0.907, $P = 0.13$); dotted line, ROC curve for the nephrotic patients with FSGS or MCD whose eGFR was 60 ml/min per 1.73 m^2 or higher. The AUC-ROC was 0.642 ± 0.130 (95% CI: 0.388–0.896, $P = 0.30$). (**b**) ROC analysis in the patients with FSGS or the other glomerular diseases whose eGFR was 60 ml/min per 1.73 m^2 or higher. The AUC-ROC was 0.621 ± 0.093 (95% CI: 0.438–0.803, $P = 0.21$).

significant difference between the FSGS and MCD patients was detected regarding the probability to exhibit suPAR levels higher than the cutoff value ($P = 0.06$). The area under the ROC curve (AUC-ROC) for this patient population was 0.684 ± 0.114 (95% confidence interval (CI): 0.461–0.907, $P = 0.13$). These data suggest that the suPAR level is not useful to differentiate FSGS from MCD. By contrast, when we set 3000 pg/ml as a cutoff value, as Wei *et al.*[10,12] did in the previous studies, the sensitivity and specificity of this test were 0.313 and 0.889, respectively, suggesting that this cutoff value would yield an extremely low sensitivity.

Both primary FSGS and MCD are typically characterized by nephrotic syndrome with an abrupt onset; however, the prognosis and the response to therapy differ from each other. When we limited the objectives to nephrotic (serum albumin < 3.0 g/dl and urinary protein $\geqslant 3.5$ g per day or g/g Cre) FSGS ($n = 11$) or MCD ($n = 8$) patients, the ROC curve analysis suggested that the optimal cutoff value under this condition should be 1748.75 pg/ml (Figure 6a; dotted line). The sensitivity, specificity, positive LR, and negative LR were 0.909, 0.375, 1.45, and 0.24, respectively. The AUC-ROC for the nephrotic patient group was limited to 0.642 ± 0.130 (95% CI: 0.388–0.896, $P = 0.30$). When we tested whether the serum suPAR levels are useful for differentiation between FSGS ($n = 16$) and the other glomerular diseases ($n = 22$), the optimal cutoff value was 2442.5 pg/ml. The sensitivity, specificity, positive LR, and negative LR were 0.750, 0.591, 1.83, and 0.423, respectively. The AUC-ROC was 0.621 ± 0.093 (95% CI: 0.438–0.803, $P = 0.21$), suggesting that serum suPAR levels are not useful to discriminate FSGS from the other glomerular diseases (Figure 6b). Taken together, these data suggest that the serum suPAR concentration is not a potent diagnostic marker for clinical use.

suPAR levels in the patients taking steroids and/or immunosuppressants

Steroids and immunosuppressants are often used to treat primary glomerular diseases, including FSGS. To evaluate the association between the use of steroids/immunosuppressants and the suPAR levels, we compared the serum suPAR levels in patients with and without those medications. As shown in Figure 7a, all the patients with primary glomerular diseases who took steroids and/or immunosuppressants (IS(+)) tended to have lower serum suPAR levels; however, this difference did not reach statistical significance (IS(−) vs. IS(+) = 3039 ± 1004 vs. 2559 ± 789.2 pg/ml, $P = 0.06$, effect size $d = 0.532$, power $(1 - \beta) = 0.489$). By contrast, the FSGS patients without renal dysfunction who took steroids and/or immunosuppressants had significantly lower levels of suPAR compared with the FSGS patients not taking these medications (Figure 7b: IS(−) vs. IS(+) = 3076.88 ± 498.52 vs. 2170.00 ± 533.68 pg/ml, $P = 0.009$, effect size $d = 1.756$, power $(1 - \beta) = 0.842$). However, urinary protein excretion, which is associated with disease activity, did not differ significantly between the untreated and treated FSGS groups (IS(−) vs. IS(+) = 5453.8 ± 3261.9 vs. 4986.5 ± 6015.8 mg per day or mg/gCre, $P = 0.365$). Furthermore, the use of steroids/immunosuppressants was not a significant predictive variable for the suPAR levels in the multiple regression equation as shown above (Table 2).

The suPAR levels of MCD patients with and without steroids/immunosuppressants were 2488.8 ± 628.9 and 1975.3 ± 407.0 pg/ml, respectively, and the difference was not significant ($P = 0.270$), whereas urinary protein excretion did not differ between the two MCD groups (IS(−) vs. IS(+) = 7857.8 ± 3257.9 vs. 10495.6 ± 4308.9 mg per day or mg/gCre, $P = 0.5403$). Among MN patients, only one patient was treated with steroid. The patient's serum suPAR concentration was 3017.5 pg/ml, which was almost the same level as MN patients without steroids/immunosuppressants (3128.8 ± 703.6 pg/ml). Urinary protein of the MN patient who took steroid was 7360 mg per day, which was largely the same level as the untreated MN patients' urinary protein (6670.9 ± 3207.0 mg per day or mg/gCre). In our cohort, none of IgAN patients with normal renal function took

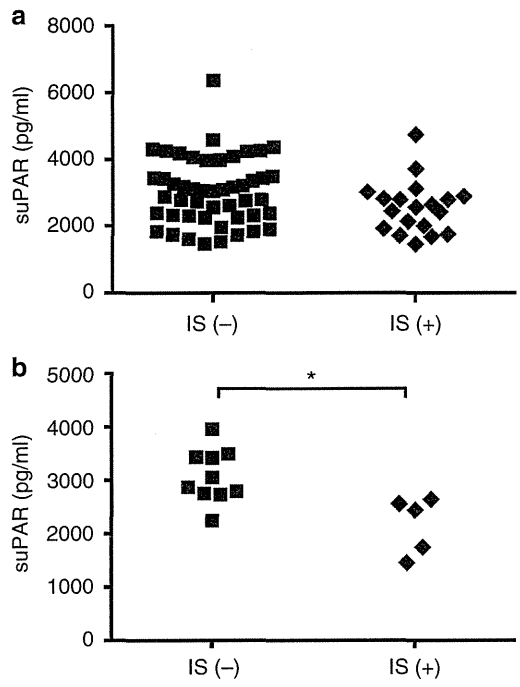


Figure 7 | Treatment with steroids/immunosuppressants and the serum soluble urokinase receptor (suPAR) levels. (**a**) Comparison of the suPAR levels between glomerular disease patients with normal glomerular function who were treated with steroids or immunosuppressants (IS(+)) and those who were not (IS(−)). No significant difference was detected (unpaired *t*-test, 2559.3 ± 789.2 vs. 3047.0 ± 994.9 pg/ml, $P = 0.06$). (**b**) Comparison of the suPAR levels between the focal segmental glomerulosclerosis (FSGS) patients with normal renal function who were treated with steroids or immunosuppressants (IS(+)) and those who were not (IS(−)). The IS(+) patients presented significantly lower suPAR levels (Mann–Whitney *U*-test, 2170.0 ± 533.7 vs. 3076.9 ± 498.5 pg/ml, $^*P = 0.009$).

steroids or immunosuppressants. Taken together, although treatment with steroids and/or immunosuppressants was associated with lower suPAR levels in the FSGS group, it is still inconclusive whether lower suPAR levels are associated with disease activity. The relationships between suPAR, steroid/immunosuppressants, and disease activities in the other disease groups are also obscure. A longitudinal study would be necessary to draw the conclusion.

High suPAR levels in ANCA-associated glomerulonephritis

As a separate cohort, we evaluated the serum suPAR concentrations in the five patients with antineutrophil cytoplasmic antibody (ANCA)-associated glomerulonephritis (ANCA-GN). Their characteristics are as follows (average ± s.d.): age, 67.8 ± 7.9 years; gender, male/female = 1/4; eGFR, 13.0 ± 5.2 ml/min per 1.73 m^2; urinary protein, 912 ± 140 mg per day or mg/gCre; serum albumin, 3.01 ± 0.78 g/dl; serum total cholesterol, 189.4 ± 65.3 mg/dl; and CRP, 5.14 ± 5.6 mg/dl. The suPAR concentration in this patient group was 6791.3 ± 1513.0 pg/ml, which was substantially higher than that in the patients with the primary glomerular diseases described above. When we compared this cohort with the subgroup of non-ANCA-GN patients matched for age and eGFR (3 FSGS, 1 MCD, and 1 MN; age, 61.4 ± 16.3 years; eGFR, 16.5 ± 8.1 ml/min per 1.73 m^2; and suPAR, 3727.5 ± 818.2 pg/ml), the suPAR levels in the ANCA-GN patients were significantly higher than in the non-ANCA-GN patients (data not shown, Mann–Whitney *U*-test, $P = 0.01$, effect size $d = 2.519$, power $(1 - \beta) = 0.935$). Although the sample size was small, it suggested that inflammation might affect the suPAR concentration. Given the inverse correlation between suPAR and the eGFR, these data suggest that the high suPAR concentration in ANCA-GN patients might be attributable to impaired renal function and inflammation.

DISCUSSION

In the current study, we performed a multicenter cross-sectional study of suPAR levels in Japanese patients with glomerular diseases to explore the usefulness of suPAR as a diagnostic marker. The first finding in this study was that the suPAR levels were inversely correlated with the eGFR. Previous investigations have demonstrated an inverse correlation between suPAR and eGFR;[13,23,24] thus, the present finding is consistent with those data. Wei *et al.*[12] also demonstrated an inverse correlation between suPAR and eGFR in their study of two large cohorts. Because the molecular weight of the major fragment of suPAR is 22 kDa, which should be small enough to be filtered through the glomerular filtration barrier, it seems reasonable that suPAR levels are inversely correlated with renal function. Although a kinetic study will be necessary to confirm this speculation, it is likely that suPAR accumulates in patients with renal impairment. On the basis of this finding, we performed an analysis on the patients whose eGFR levels were 60 ml/min per 1.73 m^2 or more. The second finding was that the suPAR levels did not have diagnostic value for differentiating FSGS from MCD or the other glomerular diseases. Moreover, we evaluated the suPAR levels only in nephrotic patients with FSGS and MCD, and the result was equivalent. Because primary FSGS often exhibits a clinical presentation similar to MCD, it is sometimes not easy to distinguish FSGS from MCD, even with a renal biopsy. Therefore, a reliable diagnostic biomarker would not only be useful to distinguish these two diseases but may also elucidate the pathogenesis of these disorders causing nephrotic syndrome. Garin *et al.*[25] reported a significant increase in the urinary excretion of CD80 (also known as B7-1) from MCD patients, but not from FSGS patients. Thus, the development of a biomarker that distinguishes FSGS from MCD has been at the center of attention in this field. However, our data suggest that serum suPAR cannot serve in this role.

The third finding was that FSGS patients without renal impairment who took steroids/immunosuppressants present significantly lower levels of suPAR. We cannot exclude the possibility that suPAR is associated with the pathogenesis; however, the association is still inconclusive because of the small sample size and inconsistent result with multiple

regression analysis. Moreover, urinary protein excretion, a clinical parameter for disease activity, was not different between untreated and treated FSGS patients in our cohort. Given that suPAR levels are increased along with nonspecific inflammation, a decrease in suPAR may be simply associated with direct effects of these medications. A longitudinal study of a larger cohort will be necessary to clarify the association between suPAR levels and the clinical outcomes of FSGS.

The fourth finding was that the ANCA-GN patients presented remarkably higher levels of serum suPAR than were observed in patients with other primary glomerular diseases. Several factors should be considered for ANCA-GN. The first factor is renal function, as the average eGFR in our ANCA-GN patients was 13.0 ml/min per 1.73 m^2. Given the previous findings and our data in this study, impaired renal function may cause increased suPAR levels. The second factor that potentially affected the suPAR levels was age. Five patients with ANCA-GN were older than the patients in the other disease groups. Given our data demonstrating the correlation of age and suPAR, aging may have a causal role in suPAR accumulation. Further studies will be necessary to define the precise relationship between age and suPAR. The other factor that may potentially affect the suPAR level is inflammation. Previous studies have suggested an association between increased suPAR levels and nonspecific inflammation,[20–22] and the CRP levels for the five patients evaluated in this study were 5.14 mg/dl, on average. The comparison of the suPAR levels between the ANCA-GN patients and the age- and eGFR-matched subgroup from our cohort revealed that the suPAR levels in the ANCA-GN patients were still significantly higher, suggesting that inflammation might have a role.

Several limitations might affect the results obtained in this study. First, although most of the analyses in the current investigation had sufficient power, the sample size of this study was still relatively small. A pathologic diversity of glomerular lesions in FSGS is evident; however, the relatively small power did not allow us to analyze the suPAR levels in each FSGS subtype. A detailed classification of FSGS patients (requiring a larger number of subjects) might give us more precise information on the association of suPAR and the development of FSGS. Second, the histological diagnosis was performed by the pathologists at each facility and was not standardized. Third, we could not evaluate the relationship between the change in the suPAR levels and the clinical outcomes because of the cross-sectional nature of this investigation. Longitudinal follow-up studies might give us a new insight into the pathological roles of suPAR. Finally, the ELISA system we used measures only the complete form of uPAR, whereas some splicing forms do exist, and there might be an FSGS-specific form of suPAR.

In conclusion, this study suggests that the serum suPAR level is affected by age, renal function, or inflammation. Furthermore, in this investigation, serum suPAR levels had little value for differentiating FSGS from the other glomerular diseases, especially MCD.

MATERIALS AND METHODS

Patient population

We conducted a multicenter cross-sectional study for serum suPAR levels in Japanese patients with primary glomerular diseases who underwent renal biopsy in the participating nephrology divisions. This study was planned and conducted by the Japanese Refractory Nephrotic Syndrome Study Group of the Ministry of Health, Labor and Welfare of Japan. We studied 70 patients with biopsy-proven primary glomerular diseases from eight different hospitals in Japan. All patients had undergone renal biopsy, and pathologists in each hospital performed the pathohistological diagnoses. After one sample collected from a patient with MCD was excluded as an outlier for the suPAR concentration based on the Mahalanobis distance, we studied 69 patients: 38 with FSGS, 11 with MCD, 11 with IgAN, and 9 with MN. Patients with a clinically identifiable cause of these diseases had been excluded. As for FSGS, patients with potential causes of secondary FSGS (i.e. obesity, family history of FSGS, drug, viral infection, and structural maladaptation) were not included. We also measured the serum suPAR concentrations in 17 healthy volunteers. In addition, the serum suPAR levels in five patients with ANCA-GN were evaluated. For one-way ANOVA analysis among four groups for glomerular diseases and a group for the healthy control, 80 samples were required when we set the effect size at 0.40, the α-value at 0.05, and the power $(1-\beta)$ at 0.8. The study protocol was approved by the Institutional Review Boards of the University of Tokyo and of each participating hospital. Informed consent was obtained from each participant.

The recorded clinical parameters included the clinical diagnosis; age; gender; urine protein/creatinine ratio or 24-h urinary protein; steroid and immunosuppressant therapy status; and serum levels of creatinine, albumin, and total cholesterol. The eGFR was calculated using the Japanese eGFR equation.[26] Histological information was also derived from each patient's medical record. The patients were categorized according to their histological diagnoses.

Serum suPAR measurement

Serum samples were collected from the patients on the day of renal biopsy. The sera were separated by centrifugation and frozen at $-80\,^{\circ}C$ until the measurement. During the investigation, refreezing the thawed samples was avoided. We performed duplicate measurements of the serum suPAR levels, using the Quantikine Human suPAR Immunoassay (R&D Systems, Minneapolis, MN), which is a solid-phase sandwich enzyme-linked immunosorbent assay kit, according to the manufacturer's protocol. This enzyme-linked immunosorbent assay kit has been used in most of the previous studies[10,12,13,17,18] of suPAR levels and FSGS, including the original investigations by Wei *et al.*[10,12]

Statistical analyses

The data are expressed as the mean ± s.d., for the continuous variables, whereas standard errors of the mean are shown for AUC-ROC and regression coefficients. We evaluated the differences in each biochemical parameter (including suPAR) among the glomerular diseases by a one-way ANOVA followed by multiple-comparison analyses. Each multiple-comparison analysis was performed with Tukey's HSD (honest significant difference) mean separation test (parametic) or the Steel–Dwass test (nonparametric), depending on the normality of the data distribution determined by the D'Agostino–Pearson omnibus normality test. Correlations between the suPAR level and the demographic or clinical parameters

were evaluated using Pearson's correlation coefficient test. For the comparison between the patients with steroids/immunosuppressants and those without, the unpaired *t*-test (for all patients) or the nonparametric Mann–Whitney *U*-test (for the FSGS group and the MCD group) was used. For the comparison between the ANCA-GN patient group and the subgroup matched for age and eGFR, the nonparametric Mann–Whitney *U*-test was used. We used the χ^2 test to evaluate the differences in positive ratios at the selected cutoff values between FSGS and MCD. Statistical analyses were performed using the JMP 9.0 statistical software (SAS Institute, Cary, NC) and GraphPad Prism version 5.04 software (GraphPad Software, San Diego, CA). Power analyses were performed using the G*Power 3 software (Heinrich-Heüine Universität Düsseldorf, Düsseldorf, Germany). *P*-values <0.05 were considered significant.

DISCLOSURE

All the authors declared no competing interests.

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ORIGINAL ARTICLE

Significance of combined cyclosporine–prednisolone therapy and cyclosporine blood concentration monitoring for idiopathic membranous nephropathy with steroid-resistant nephrotic syndrome: a randomized controlled multicenter trial

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Abstract
Background Combined treatment with cyclosporine microemulsion preconcentrate (CyA MEPC) and steroids has been widely used for idiopathic membranous nephropathy (IMN) associated with steroid-resistant nephrotic syndrome (SRNS). Recent studies have shown that once-a-day and preprandial administration of CyA MEPC is more advantageous than the conventional twice-a-day administration in achieving the target blood CyA concentration at 2 h post dose (C2). We designed a randomized trial to compare these administrations.
Methods IMN patients with SRNS (age 16–75 years) were divided prospectively and randomly into 2 groups. In group 1 ($n = 23$), 2–3 mg/kg body weight (BW) CyA MEPC was given orally once a day before breakfast. In group 2 ($n = 25$), 1.5 mg/kg BW CyA MEPC was given twice a day before meals. CyA + prednisolone was continued for 48 weeks.
Results Group 1 showed a significantly higher cumulative complete remission (CR) rate ($p = 0.0282$), but not when incomplete remission 1 (ICR1; urine protein 0.3–1.0 g/day) was added ($p = 0.314$). Because a C2 of 600 ng/mL was determined as the best cut-off point, groups 1 and 2 were

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further divided into subgroups A (C2 ≥600 ng/mL) and B (C2 <600 ng/mL). Groups 1A and 2A revealed significantly higher cumulative remission (CR + ICR1) ($p = 0.0069$) and CR-alone ($p = 0.0028$) rates. On the other hand, 3 patients with high CyA levels (C2 >900 ng/mL) in Group 1A were withdrawn from the study because of complications.

Conclusion CyA + prednisolone treatment is effective for IMN with associated SRNS at a C2 of ≥600 ng/mL. To achieve remission, preprandial once-a-day administration of CyA at 2–3 mg/kg BW may be the most appropriate option. However, we should adjust the dosage of CyA by therapeutic drug monitoring to avoid complications.

Keywords Cyclosporine · Idiopathic membranous nephropathy · Steroid-resistant nephrotic syndrome · Once-a-day administration · Preprandial administration · Therapeutic drug monitoring

Introduction

Idiopathic membranous nephropathy (IMN) is the most representative disease associated with steroid-resistant nephrotic syndrome (SRNS) in adults. Although the combination of steroids and immunosuppressants, e.g., cyclophosphamide (CPA) and chlorambucil, has been reported to induce and maintain remission in randomized controlled studies [1, 2], the beneficial effects remain controversial because of the harmful side-effects of the alkylating agents. Moreover, in our cohort study of 1,000 cases in Japan, combined treatment with steroids and CPA was not superior to steroid monotherapy [3]. Recently, cyclosporine (CyA), a calcineurin inhibitor, has been introduced as an effective agent for SRNS, and several randomized controlled trials (RCTs) on the combination of steroids and CyA showed significant remission rates [4–6].

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However, it has been recognized that clinical response does not correlate well with the administration dose. Accordingly, careful attention to the CyA concentration in blood is essential for the optimization of therapy [7]. For this reason, the blood concentration of the drug was previously monitored at the trough level before administration (C0) because the absorption of CyA is highly affected by bile acid and other factors of absorption when the original CyA formulation was used orally [8]. The introduction of CyA microemulsion preconcentrate (MEPC) minimized the influence of bile acid and stabilized the absorption profile (AP) of CyA [9]. In a transplantation study, the area under the blood concentration–time curve up to 4 h after administration of CyA (AUC0–4) was believed to accurately express CyA absorption and sensitively predict the effect of CyA [10]. Moreover, the CyA blood concentration at 2 h post dose (C2) was recommended as the best surrogate single-sample marker for routine monitoring [10].

Recent studies have shown that once-a-day administration is more advantageous than the conventional twice-a-day administration, because the former provides an AP showing the peak blood concentration of CyA, which may facilitate the remission of SRNS and prevent chronic CyA nephrotoxicity [11, 12]. In addition, preprandial administration of CyA may be favorable for achieving a stable blood concentration because CyA is absorbed without the influence of food ingestion [12, 13]. However, there is no evidence that such therapeutic strategies contribute to the remission of SRNS.

In this study, we designed a prospective, open-label randomized trial to compare the effect of preprandial once-a-day administration of CyA with that of conventional twice-a-day administration for IMN with associated SRNS. Blood CyA concentrations at C0 and C2 were also evaluated during treatment.

Methods

This study was registered at the University Hospital Medical Information Network-Clinical Trials Registry (UMIN-CTR) under trial identification no. UMIN C000000369 and was approved by the Clinical Study Review Board at Fukuoka University Hospital (approval no. 03-129). The study was conducted in accordance with the principles of the declaration of Helsinki. Written informed consent was obtained before patient enrollment and after a thorough explanation of the trial's objectives, duration, and structure. The availability of alternative drugs, the possibility of adverse reactions, privacy measures, and the voluntary nature of the trial, including the right to withdraw without repercussions, were all carefully explained. The institutional review boards at the

collaborating institutions also approved the protocol when requested.

Patients

SRNS patients (age 16–75 years) with IMN diagnosed by renal biopsy were enrolled through computerized registration from kidney centers in Japan between 2004 and 2007. Membranous nephropathy secondary to systemic diseases, e.g., diabetic nephropathy and collagen diseases, were excluded at registration. Nephrotic syndrome (NS) was defined according to the standard criteria in Japan [3]—(1) urine protein (UP) excretion >3.5 g/day; (2) serum albumin <3.0 g/dL or serum total protein <6.0 g/dL; (3) presence of edema; and (4) total cholesterol >250 mg/dL. At least the first and second criteria were necessary for the diagnosis. SRNS was determined when patients did not achieve complete remission (CR) or incomplete remission (ICR) 1 (as described in 'Clinical assessment' section) after 4 weeks of prednisolone (PSL) therapy at 40–60 mg/day. The inclusion and exclusion criteria are listed in Table 1.

Renal histology was assessed according to the following 5 parameters—presence of global sclerosis and segmental sclerosis in glomeruli, severity of tubulointerstitial changes, occurrence of vascular lesions, and ultrastructural stage of glomerular lesions according to the criteria of Ehrenreich and Churg [14]. These changes were estimated semiquantitatively as we previously reported [3], and compared between groups.

Table 1 Inclusion and exclusion criteria

Inclusion criteria
1. Age between 16 and 75 years
2. UP >3.5 g/day and serum albumin level <3.0 g/dL
3. PSLalone treatment for >4 weeks did not decrease UP into <1 g/day
4. Membranous nephropathy was diagnosed by renal biopsy.
5. No history of treatment with CyA-MEPC before registration
6. Informed consent form voluntarily signed by the participant
Exclusion criteria
1. Patients with creatinine clearance <50 mL/min or serum creatinine >2 mg/dL
2. Patients that received other immunosuppressants within 1 month before the study commencement
3. Patients treated with nephrotoxic and hyperkalemic agents during the study period
4. Patients with a malignant tumor or a history of a recurrent malignant tumor
5. Patients with hypertension uncontrolled with antihypertensive drugs
6. Patients with malabsorption syndrome, cerebral dysfunction, or epilepsy
7. Patients with hyperkalemia or hyperuricemia
8. Patients with a severe cardiac, hepatic, or pancreatic disease
9. Patients currently pregnant, suspected to be pregnant, or nursing
10. Patients with an infectious complication and not eligible for treatment with immunosuppressants
11. Patients with a history of hypersensitivity to CyA-MEPC
12. Patients determined to be inappropriate for participation in the study by an investigator

UP urine protein, *PSL* prednisolone, *CyA-MEPC* cyclosporine microemulsion preconcentrate

Study design

Patients were divided prospectively and randomly into 2 groups (groups 1 and 2). Combined administration of PSL and CyA MEPC was continued for 48 weeks. PSL was initially prescribed at 40 mg/day and tapered gradually to <10 mg/day by 48 weeks. In group 1, CyA MEPC was given orally once a day before breakfast at 2–3 mg/kg body weight (BW). In group 2, CyA MEPC was given twice a day before meals at 1.5 mg/kg BW each. Other agents, including antihypertensive, antidyslipidemic, and anticoagulant drugs, were allowed unless their combination with CyA was contraindicated. Biochemical data, including total protein, albumin, urea nitrogen, creatinine, and total cholesterol in serum, and 24-h UP, were assayed at 0, 4, 8, 12, 24, 36, and 48 weeks.

CyA treatment and monitoring

To determine the AP of CyA in each patient, blood CyA concentrations from 0 to 4 h (C0–C4) were assayed within 1 month of treatment, and the AUC0–4 (ng h/mL) was calculated. The linear trapezoid formula was used with C0 to C4. Then, C0 and C2 were repeatedly assayed during the treatment period.

In group 1, CyA was started at 2 mg/day and dose adjustments were made to achieve a C0 of 80–120 ng/mL and C2 of 800–1,000 ng/mL. The CyA dose was increased to a maximum of 3 mg/day when the target C0 and C2 were not achieved. In contrast, the dose was reduced when C0 and C2 exceeded the target levels. In group 2, adjustments were also made so as not to exceed C0 and C2 by 120 and 1,000 mg/dL, respectively. In the maintenance phase after remission, the dose was adjusted so as not to exceed C0 and C2 by 80 and 800 mg/dL, respectively. The whole blood concentration of CyA was measured by radioimmunoassay or by the fluorescence polarization immunoassay methods of SRL Co., Japan, or the biochemical laboratory of each kidney center. The average C0 and C2 during the treatment period before remission were used for the comparison of outcomes.

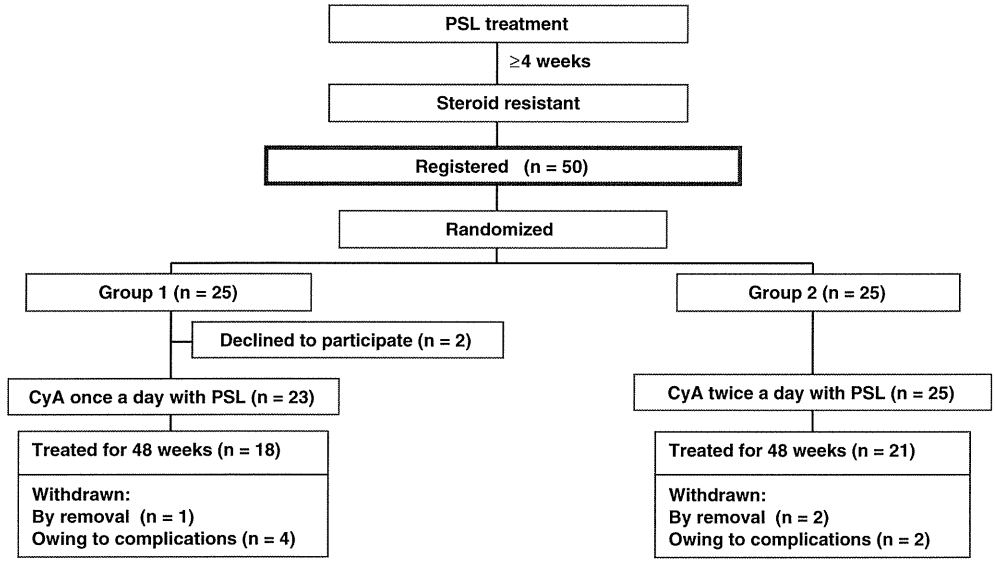

Fig. 1 Flowchart of the study design: enrollment of patients and treatment assignment

Clinical assessment

Clinical assessment of treatment outcomes was performed on the basis of changes in proteinuria and renal function, partly modified from the previous criteria in Japan [3]. Briefly, CR was defined when the UP was <0.3 g/day. ICR was defined as the resolution of NS but with continuing overt proteinuria, and was divided into 2 grades—ICR1 and ICR2 for UP of 0.3–1.0 and 1.0–3.5 g/day, respectively. No response (NR) was defined as the persistence of NS. Since patients with ICR1 showed a favorable prognosis almost equal to CR in a previous study [3], we considered CR + ICR1 as remission. For renal function, 3 categories were defined according to serum creatinine concentration—(1) normal renal function <1.5 mg/dL; (2) renal insufficiency 1.5–3.0 mg/dL; and (3) end-stage renal disease >3.0 mg/dL.

Statistical analysis

Values were given as mean ± SE or median (interquartile range). Differences in clinical characteristics between the 2 groups were evaluated with Student's t test and Mann–Whitney U test for continuous variables and Fisher's exact test for categorical variables. The incidence of remission (CR + ICR1) or CR was compared using Fisher's exact test. Time to remission or CR curves for the therapy groups were estimated using the Kaplan–Meier technique, and the curves were compared using the log-rank test.

The effects of blood CyA concentrations and clinical variants for the incidence of remission were examined using logistic regression analysis. The variants that affected serum CyA concentrations were examined using multiple regression analysis.

Receiver operating characteristic (ROC) curve analysis was used to test the prognostic value of serum CyA concentrations (average C0 and C2) and to determine the best cut-off for the prediction of CR.

All statistical analyses were performed using SPSS for Windows version 18.0 (SPSS Japan Inc., Tokyo, Japan).

Results

The flowchart of the study design regarding enrollment of patients and treatment assignment is shown in Fig. 1.

Patients

Fifty patients in 30 kidney centers in Japan were registered according to the inclusion criteria, from April 2004 to December 2007, and 25 patients each were randomly enrolled in the once-a-day (group 1) and twice-a-day (group 2) administration groups. However, 2 patients in group 1 declined to participate in this study before CyA treatment. Consequently, 23 and 25 patients were treated with PSL and CyA in groups 1 and 2, respectively. The baseline clinical characteristics of all patients are summarized in Table 2. There was no significant difference in each item between the 2 groups. Five parameters of renal histology estimated semiquantitatively did not show significant differences between groups (data not shown).

A previous study on IMN treated with a combination of PSL and CyA (2–3 mg/kg/day, twice-a-day) showed a 35 % CR ratio at the 12-month course [6]. However, there were no data for once-a-day administration. Nevertheless, the sample size (groups 1 and 2: $n = 23$ and $n = 25$, respectively) was sufficient to detect a significant difference ($\alpha = 0.05$,

Table 2 Baseline characteristics of patients with idiopathic membranous nephropathy

Characteristic	Group 1 ($n = 23$)	Group 2 ($n = 25$)	p
Sex (male/female)	16:7	17:8	0.91
Age	56 (19–70)	57 (39–70)	0.48
Urine protein (g/day)	3.5 (1.8–10)	3.8 (1.0–6.5)	0.63
Serum levels			
Urea nitrogen (mg/dL)	14 (8–24)	15 (9–33)	0.54
Creatinine (mg/dL)	0.8 (0.5–1.2)	0.8 (0.6–1.6)	0.84
Total protein (g/dL)	4.7 (3.9–6.2)	4.7 (3.6–5.6)	0.15
Albumin (g/dL)	2.7 (2.2–3.5)	2.6 (1.5–3.3)	0.09
Total cholesterol (mg/dL)	314 (229–617)	298 (213–853)	0.52

Age and laboratory data are shown as median (interquartile range)

The p values were evaluated by Fisher's exact test for sex and Mann–Whitney U test for the others

Table 3 Withdrawn patients

Group	Withdrawal period (weeks)	Reason	Average C2 (ng/mL)
Group 1 ($n = 5$)	9	Nausea	1042
	10	Uncontrolled CyA level	1200
	12	Liver dysfunction	750
	12	Pneumonia	936
	40	Removal	
Group 2 ($n = 4$)	8	Brain tumor[a]	693
	36	Noncompliance	813
	10	Removal	
	12	Removal	

[a] May not be related to CyA administration

2-sided) on the basis of 0.8 power according to Fisher's exact test when once-a-day administration is twice as effective (CR ratio 70 %) than twice-a-day administration. Therefore, we stopped the registration at the end of 2007.

As shown in Table 3, during the treatment, 1 patient in group 1 and 2 patients in group 2 were transferred to another hospital and could therefore not further participate in the study. Four patients in group 1 and 2 patients in group 2 were withdrawn because of complications and noncompliance. Finally, 18 and 21 patients in groups 1 and 2 completed the study for 48 weeks.

Responses in the once-a-day and twice-a-day administration groups

The response around 6 months is important to determine the initial effect of CyA treatment as shown in RCTs and

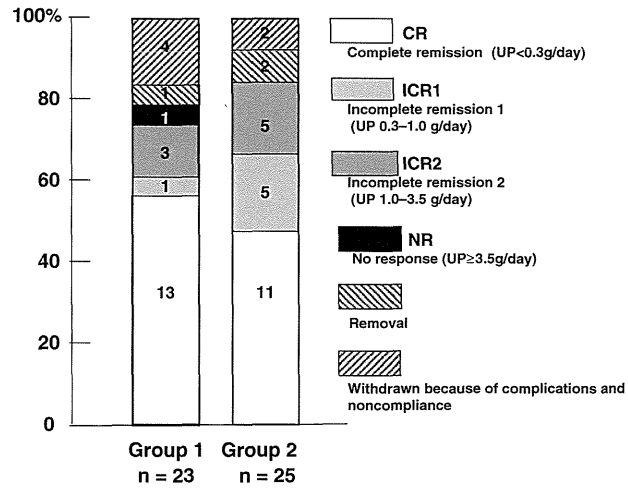

Fig. 2 Remission and withdrawal rates of groups 1 and 2 at 48 weeks. Patients were divided according to CyA administration frequency—once a day (group 1) or twice a day (group 2). In each therapeutic response, there was no significant difference

guidelines [4, 5, 15–17]. In the intention-to-treat analysis, 10 of 23 patients (43.5 %) in group 1 and 2 of 25 patients (8.0 %) in group 2 achieved CR at 24 weeks. This yielded a significant difference between groups in Fisher's exact test ($p = 0.0078$). In group 1, two other patients achieved CR at 8 and 12 weeks, respectively; however, the first patient relapsed into ICR2 by 24 weeks and the second was withdrawn thereafter because of liver dysfunction. ICR1 occurred in 1 and 10 patients in groups 1 and 2, respectively. In total, 11 (47.8 %) patients in group 1 and 12 (48.0 %) in group 2 achieved remission (CR + ICR1) ($p = 1.000$).

Between 24 and 48 weeks, more patients achieved CR in both groups, but a few patients with CR relapsed conversely. At 48 weeks, 13 of 23 patients (56.5 %) in group 1 and 11 of 25 patients (44.0 %) in group 2 were in CR, and 14 of 23 (60.9 %) in group 1 and 16 of 25 (64.0 %) in group 2 were in CR + ICR1 (Fig. 2). For each therapeutic response, there was no significant difference between groups. In the per-protocol analysis, similar results were statistically obtained at 24 and 48 weeks.

However, the time-to-remission curve analyzed using the Kaplan–Meier technique revealed a significant deference in cumulative CR rate ($p = 0.0282$; Fig. 3a) but not in cumulative CR + ICR1 rate ($p = 0.314$, Fig. 3b).

Assessment of clinical parameters

After CyA + PSL treatment, the levels of UP, serum albumin, and serum total cholesterol significantly improved in both groups; however, there were no significant differences in each parameter between the 2 groups. Serum creatinine level slightly increased in both groups but was

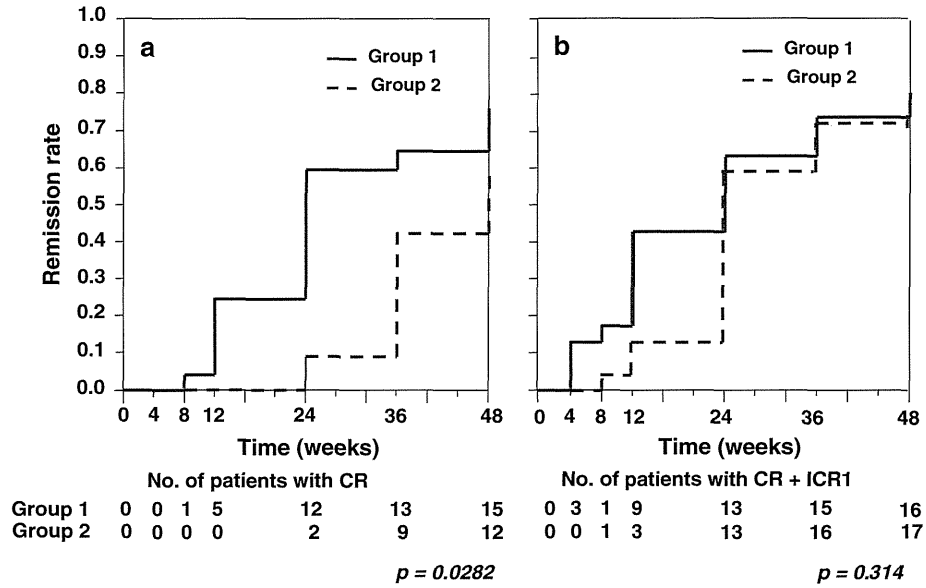

Fig. 3 Probability of cumulative complete remission (CR) (**a**) and CR + incomplete remission 1 (ICRI) (**b**) for patients treated with PSL and CyA. Group 1 showed a significantly higher rate of CR (**a**) but not of CR + ICRI (**b**) compared with group 2

not significant. Two patients in each group exhibited a doubling of serum creatinine, around 2 mg/dL, at 48 weeks, although the levels were within the reference range at the start of treatment.

At baseline, only 1 patient had mild hypertension in group 2 (155/89 mmHg), but the blood pressure normalized later. At the final observation, another patient in group 2 showed mild hypertension (150/88 mmHg). No patient had CyA-induced hypertension in either group. As the supportive therapy for MN, angiotensin II receptor blockers (4 and 2 patients in groups 1 and 2, respectively) and angiotensin-converting enzyme inhibitors (one in group 1) and a combination of both (one in each group) were administered. However, these drugs did not produce any adverse effects including hyperkalemia.

Although four patients in groups 1 and 2 showed mild hyperglycemia by steroids treatment, respectively, this did not have any serious influences on the results.

Blood CyA concentrations

The flowchart of the study design regarding assignment by blood CyA concentrations at 2 h post dose (C2) is shown in Fig. 4.

Absorption profiles of CyA in groups 1 and 2

There were significant differences in AUC0–4 between groups (group 1 vs group 2: 3678 ± 181 vs 2506 ± 164 ng h/mL, $p < 0.0001$). In comparisons between AUC0–4 and CyA concentrations at each time point (C0–C4), C2 was most strongly correlated with AUC0–4 in the total patients ($r = 0.032$, 0.609, 0.780, 0.654, 0.579 for C0, C1, C2, C3, C4, respectively).

Average C0 and C2 and the cut-off level for CR

The average C0 and C2 during treatment were significantly correlated with the C0 and C2 at the AP, respectively (C0: $r = 0.516$, $p = 0.0036$; C2: $r = 0.638$, $p = 0.0001$). The average C2 in group 1 was significantly higher than in group 2; however, the average C0 in group 1 was significantly lower than in group 2. Only C2 significantly predicted CR in logistic regression analysis based on C0, C2, age and baseline laboratory factors related to renal function and NS. Moreover, a multiple regression model showed that C2 was not significantly related to other variants as above. ROC curves were drawn to detect the optimum cut-off level of the average C2 or C0 for CR (Fig. 5). Using all data of the cases treated for 48 weeks in groups 1 and 2 ($N = 37$), the area under ROC curves were 0.731 ± 0.089 (95 % CI 0.557–0.905, $p = 0.022$) for C2 and 0.373 ± 0.109 (95 % CI 0.156–0.587, not significant) for C0. From these results, the optimum cut-off point for C2 was determined to be 615 ng/mL (sensitivity 75.0 %, specificity 76.9 %); however, C0 was inappropriate to predict remission. Using the data of group 2 alone ($N = 19$), similar results were obtained. Namely, the AUCs were 0.802 ± 0.101 (95 % CI 0.604–1.000, $p = 0.025$) for C2 and 0.444 ± 0.158 (95 % CI 0.135–0.754, not significant) for C0, and the cut-off point for C2 was determined to be 598 ng/mL (sensitivity 66.7 %, specificity 100 %). When the data of C2 were limited to the cases <340 mg/dL of total cholesterol ($N = 25$), the AUCs were greater (0.868 ± 0.072, 95 % CI 0.712–1.000, $p = 0.003$) and the cut-off point 598 ng/mL was more accurately provided (sensitivity 81.3 %, specificity 88.9 %).

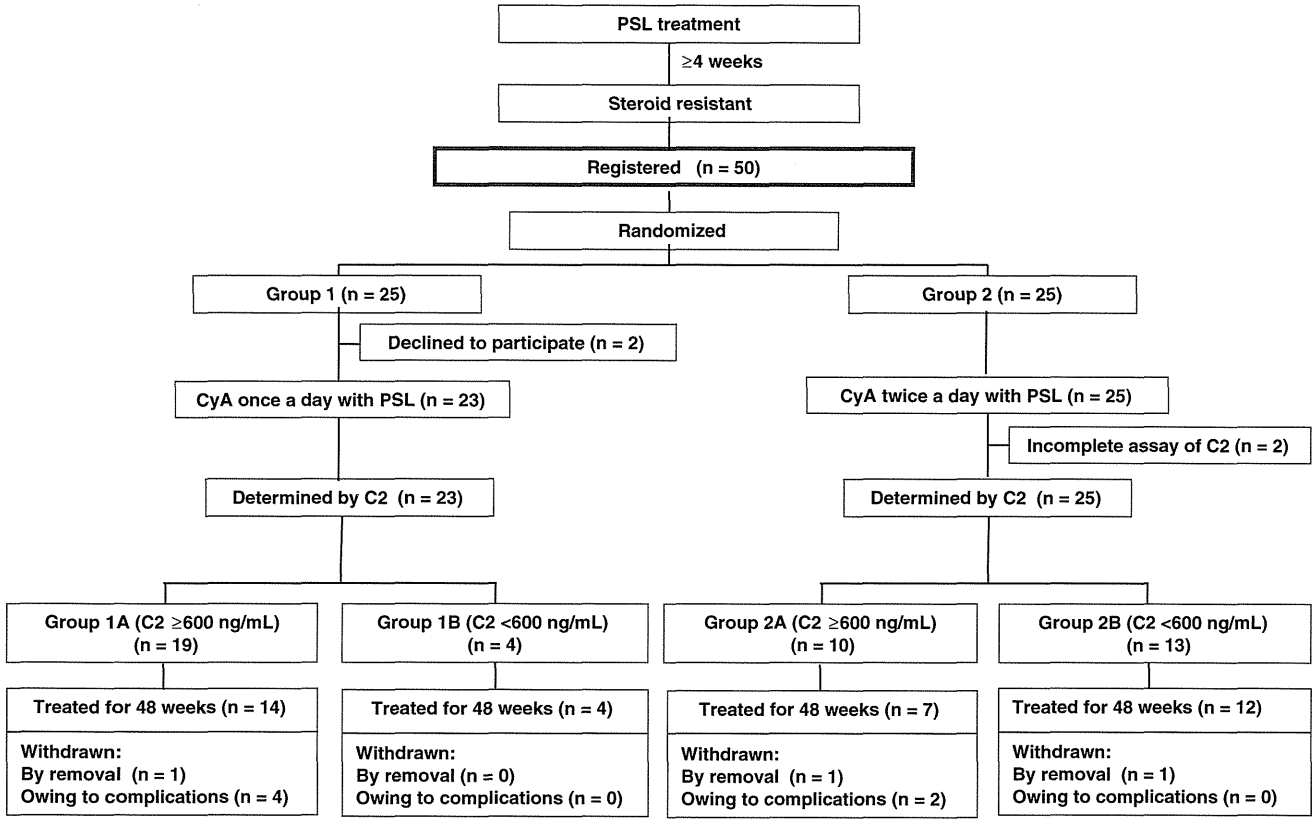

Fig. 4 Flowchart of the study design: assignment by CyA blood concentrations at 2 h post dose (C2)

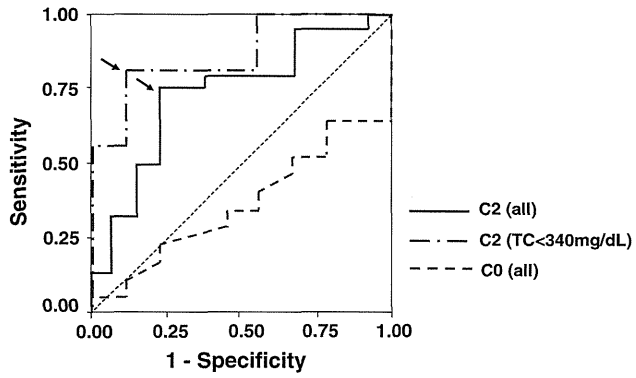

Fig. 5 Receiver operator characteristic (ROC) curves for serum CyA concentration. The optimal cut-off level of C2 for CR was determined to be 615 ng/mL (sensitivity 75.6 %, specificity 76.9 %) and 598 ng/mL (sensitivity 81.3 %, specificity 88.9 %) (*arrows*), using the ROC curve drawn from the average C2 of all cases and the cases <340 mg/dL of total cholesterol treated for 48 weeks in groups 1 and 2, respectively

Relationship between blood CyA concentration and treatment responses

Patients in groups 1 and 2 were further divided into subgroups A (C2 ≥600 ng/mL) and B (C2 <600 ng/mL) because the ROC showed that the optimal cut-off point of C2 was approximately 600 ng/mL. The number of patients in groups 1A, 1B, 2A, and 2B was 19, 4, 10, and 13, respectively (Fig. 6). Most of the patients in groups 1A and 2A achieved CR. Among these 4 groups, groups 1A and 2A showed significantly higher cumulative CR ratios than group 2B for 48 weeks; group 1B was excluded because of the statistically insufficient number of patients (Fig. 7). Meanwhile, there was no significant difference between groups 1A and 2A. Groups 1A and 2A, consisting of all patients with C2 ≥ 600 ng/mL, also showed a significantly higher cumulative ratio of not only CR ($p = 0.0028$, Fig. 8a) but also CR + ICRI ($p = 0.0069$, Fig. 8b) than groups 1B and 2B (C2 <600 ng/mL).

Four patients in group 1A were withdrawn from the study because of complications that may be related to CyA administration (Table 3). In 3 of these 4 patients, C2 was >900 ng/mL, although there was no significant difference in C2 between these 4 patients and the other 21 patients in group 1A.

Discussion

The combined administration of CyA with steroids has been reported to be useful for the treatment of IMN with

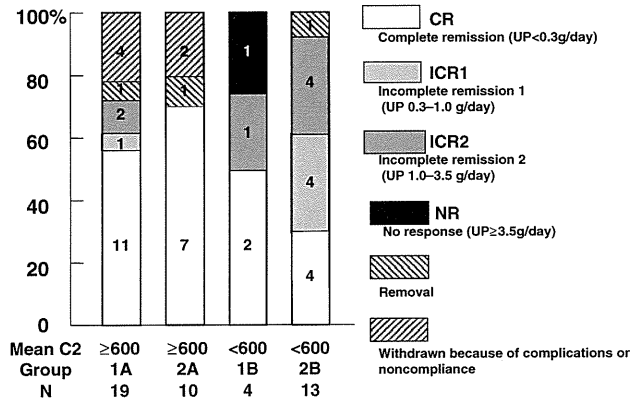

Fig. 6 Remission and withdrawal rates of groups 1A, 1B, 2A, and 2B at 48 weeks. Patients were divided into groups 1 and 2 according to administration frequency and then subdivided into subgroups A (C2 ≥600 ng/mL) and B (C2 <600 ng/mL). There was a significant difference in CR between groups A and B ($p = 0.018$, per-protocol analysis)

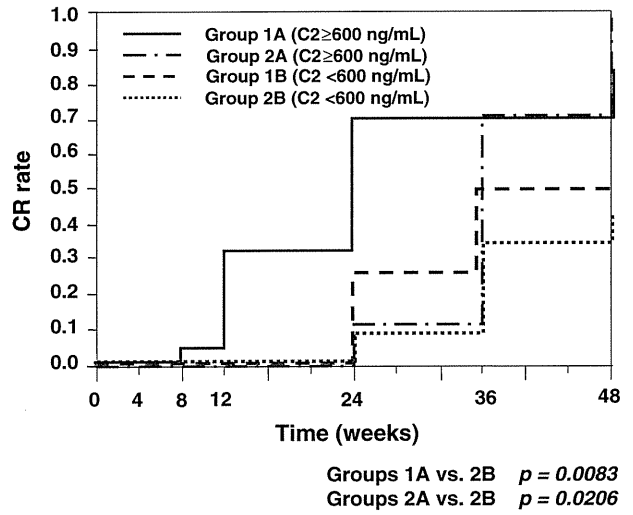

Fig. 7 Probability of cumulative CR for patients treated with PSL and CyA. Groups 1A and 2A showed significantly higher remissions compared with group 2B

associated SRNS [5, 6, 18–20]. However, only a few randomized controlled trials have succeeded in clarifying this benefit [5, 6]. In the current randomized trial, we attempted to develop a more efficient strategy for CyA treatment by preprandial once-a-day administration. The effect of this method was significant for cumulative CR rate during 48 weeks using the Kaplan–Meier technique when compared with twice-a-day administration, but not for CR incidences at 48 weeks in the Fisher's exact test. The discrepancy of the results might be influenced by the relapsing cases because these were included in cumulative CR cases in the Kaplan–Meier technique. On the other hand, it was possible that scattered distribution of blood CyA concentrations in both groups might obscure the effect, although C2 in group 1 was significantly higher than group 2.

ROC curve analysis was performed to assess the predictive value of blood CyA concentration for the outcome of NS. In comparison with C0, only C2 was available for predicting CR (Fig. 5). Interestingly, the predictive value of C2 was more enhanced when the hypercholesterolemic cases were excluded (Fig. 5). This study may demonstrate for the first time that hyperlipidemia in NS prevents CyA treatment, although the affinity of CyA to lipoproteins has been studied in transplantation [21, 22].

The optimal cut-off points for C2 were calculated as 615 and 598 ng/mL in all patients and in group 2, respectively. As these results suggest that CyA might be effective for IMN when C2 is approximately >600 ng/mL, we divided each group into subgroups A (C2 ≥600 ng/mL) and B (C2 <600 ng/mL).

Among these 4 subgroups, groups 1A and 2A showed significantly higher cumulative CR and CR + ICRI rates. Accordingly, regardless of whether the administration is once or twice a day, CyA blood concentration is a highly sensitive marker for the remission of NS. However, once-a-day administration seems to be more favorable because most of group 1 patients showed higher C2 concentrations. On the other hand, 3 patients in group 1A withdrawn from the study owing to complications showed an average C2 of >900 mg/dL, although there was no significant difference in C2 between the withdrawn patients and the remaining 21 patients in group 1A. Therefore, we think that the optimal strategy of CyA treatment is to maintain C2 between 600 and 900 ng/mL by preprandial once-a-day administration.

CyA is known to have a narrow therapeutic range of blood concentration. However, there is no study showing the relationship between drug monitoring and long-term outcomes in IMN, and C0 has been used as a standard parameter to determine the optimal dose of CyA without any evidence. Recently, transplantation studies [10, 23, 24] have shown that the AP of CyA-MEPC is stable and C2 is more reliable for 1-spot monitoring than C0 in correlation with AUC0–4. From this viewpoint, Levy et al. [28], according to the international consensus, suggested 1,400–1,600 ng/mL as the effective C2 in the early phase of renal transplantation. However, some authors have reported [26, 27] that the optimal C2 for Asian recipients is approximately 1,000 ng/mL. In NS, to achieve such an effective level of C2, a few studies have confirmed that preprandial and/or once-a-day administration was superior to the conventional twice-a-day administration [11–13].

To date, it has been assumed that the immunosuppressive effect of CyA results from the inhibition of the nuclear factor of activated T-cell signaling [28]. However, the remission of NS related to the CyA blood concentration could not be completely explained by the

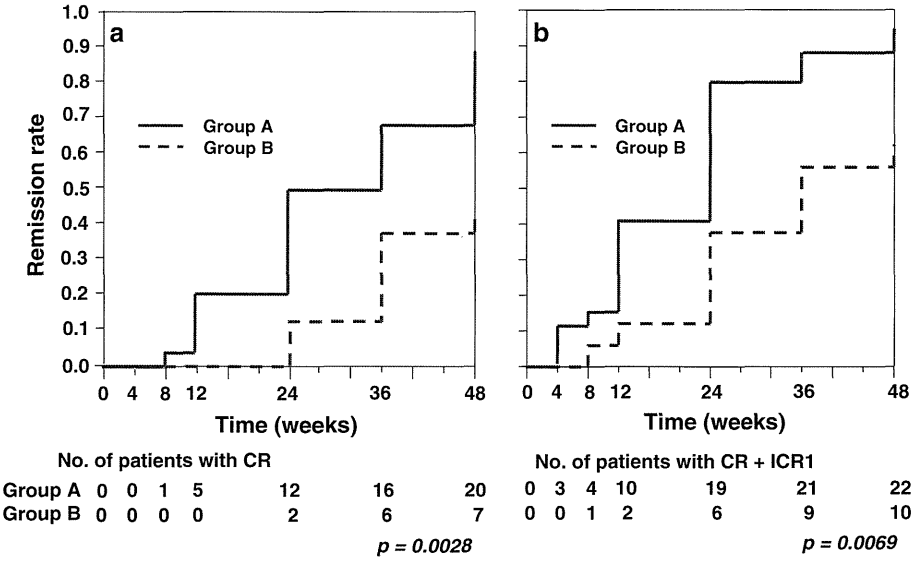

Fig. 8 Probability of cumulative CR (**a**) and CR + ICRI (**b**) for patients treated with PSL and CyA. Group A (1A + 2A) showed a significantly higher remission rate compared with group B (1B + 2B) in both analyses

immunosuppressive mechanism. Faul et al. [29] demonstrated that CyA blocks the calcineurin-mediated dephosphorylation of synaptopodin in podocytes, thereby preserving the phosphorylation-dependent synaptopodin–14-3-3beta interaction. As a result, this direct effect of CyA on podocytes may contribute to the prompt reduction of UP, and prove the significance of CyA blood concentration monitoring on the therapeutic effect for NS. As it has been reported that steroids also directly preserve the function of podocytes [30, 31], the interaction between PSL and CyA in podocytes may play a pivotal role in the induction of remission in NS, when these agents are combined.

In the KDIGO (Kidney Disease: Improving Global Outcomes) clinical and practice guideline published in 2012 [15], the initial use of CPA with steroids was preferably recommended on the basis of evidence which was accumulated from many RCTs for over several decades. As mentioned above, however, the combined use of CyA with steroids has been recognized worldwide and was recently recommended by the Cyclosporin in Idiopathic Nephrotic Syndrome working group [16]. Moreover, the guidelines for the treatment of nephrotic syndrome in Japan [17] recommend combination treatment with steroids and CyA as the first choice for IMN because of at least 2 reasons. One is, as mentioned above, that our cohort study of 1,000 cases did not show the superiority of steroids + CPA over steroid monotherapy [3]; the other reason is that the risks of CPA use, e.g., neoplasia, agranulocytosis, and viral hepatitis, seem to be more fatal than those of CyA use, e.g., nephrotoxicity and hypertension. The current study shows that improved administration and drug monitoring are useful for increasing the benefits and decreasing the risks of CyA treatment, and may support the recommendations in the Japanese guidelines [17].

In our study, blood CyA concentration was measured by radioimmunoassay or monoclonal fluorescence polarization immunoassay. These methods are known to show 10–20 % higher levels of CyA than high-performance liquid chromatography (HPLC) as the gold standard [7] because nonspecific metabolites influence the assays [32]. On the other hand, affinity column-mediated immunoassay (ACMIA) was recognized to be comparable to HPLC [32–34] and has been widely used. Accordingly, our data should be corrected to lower values if the CyA concentration is measured by a new method such as ACMIA.

In conclusion, CyA combined with PSL is effective for the treatment of IMN associated with NS when the average C2 is >600 ng/mL. To achieve this concentration and induce remission, preprandial once-a-day administration of CyA at 2–3 mg/kg with PSL may be the most appropriate option. However, high blood CyA concentrations >900 ng/mL may frequently cause adverse effects and prevent the administration continuing. To avoid this, we should adjust the dosage of CyA by therapeutic drug monitoring.

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Conflict of interest T Saito, H Yokoyama and S Nishi have received lecture's fees from Novartis Co. Y Kataoka and Y Tomino have received research funds from Novartis Co. Other authors have declared that no conflict of interest exists.

Appendix

The following members organized the trial:

Organizer: Takao Saito.

Protocol Committee: Hiroshi Sato, Shinichi Nishi, Tetsuya Mitarai, Koichi Matsumoto, Ashio Yoshimura, Hitoshi Yokoyama, Masayuki Iwano, Noriaki Yorioka, and Takao Saito.

Assessment Committee: Yasuhiko Tomino, Akio Koyama, and Shiro Ueda.

Statistics Committee: Yasufumi Kataoka, Hideki Shuto, and Satoru Ogahara.

Advisory Committee: Seiichi Matsuo and Enyu Imai, Masaomi Nangaku, and Shoichi Maruyama.

The following investigators participated in the trial:

Asahikawa Red Cross Hospital: Toshiya Ishiguro; Tohoku University: Hiroshi Sato; Fukushima Medical University: Masaaki Nagamichi; Saitama Medical University: Tetsuya Mitarai, Osamu Matsumura and Masaru Yoshikawa; Nihon University: Koichi Matsumoto and Takayuki Fujita; Showa University Fujigaoka Hospital: Ashio Yoshimura; Juntendo University: Yasuhiko Tomino and Yukihiko Takeda; The Jikei University: Yoichi Miyazaki; The Jikei University Kashiwa Hospital: Makoto Ogura and Akihiko Hamaguchi; Tokyo Women's Medical University: Minako Koike; Tokyo Women's Medical University Medical Center East: Masami Yoneda; Toho University: Sonoo Mizuiri; Teikyo University: Shunya Uchida; Hamamatsu University School of Medicine: Taro Misaki, Takehiko Miyaji, and Hideo Yasuda; Fujita Health University: Satoshi Sugiyama; Yokkaichi Municipal Hospital: Isao Ito; Mie University: Shinsuke Nomura; Osaka University: Enyu Imai; Nara Medical University: Hideo Shiiki and Masayuki Iwano; Kitano Hospital: Eri Muso and Toshiyuki Komiya; Niigata University: Shinichi Nishi; Kanazawa University: Hitoshi Yokoyama, Kiyoki Kitagawa and Takashi Wada; Kouseiren Takaoka Hospital: Miho Shimizu; Okayama University: Yohei Maeshima; Hiroshima University: Noriaki Yorioka and Takao Masaki; Kawasaki Medical School: Takehiko Tokura; Fukuoka University: Takao Saito and Satoru Ogahara; Miyazaki University: Seiichiro Hara; Kurume University: Keisuke Kono; Kyushu University: Kazuhiko Tsuruya.

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ORIGINAL ARTICLE

Kidney volume and function in autosomal dominant polycystic kidney disease

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Abstract
Background The significance of total kidney volume (TKV) as a biomarker of kidney function in autosomal dominant polycystic kidney disease (ADPKD) is controversial and has been reappraised.
Methods Between 2007 and 2012, 64 patients were followed with a mean 39.7-month observation period. TKV measurements by magnetic resonance imaging and estimation of renal function with estimated glomerular filtration rate (eGFR) using the Modification of Diet in Renal Disease equation and 24-h urine creatinine clearance were repeated annually.
Results TKV and its adjusted parameters (height-adjusted, body surface area-adjusted and log-converted TKV [log-TKV]) correlated with eGFR significantly. Among them, the correlation coefficient of log-TKV was most significant ($r = -0.6688$, $p < 0.001$). The eGFR slope correlated negatively with TKV slope ($p < 0.05$). TKV increased faster and became larger as chronic kidney disease (CKD) stage advanced. As age advanced, eGFR declined significantly ($p < 0.001$), but the eGFR slope remained constant. There was no significant correlation between TKV and age, but the log-TKV slope became smaller as age advanced. If baseline TKV was large, the eGFR slope was steeper ($p < 0.05$), which suggests that eGFR declines faster in patients with larger kidney volume.
Conclusions TKV is confirmed as a clinically meaningful surrogate marker in ADPKD. Log-TKV correlates with eGFR most significantly. Higher rates of kidney enlargement and larger kidney volume are associated with a more rapid decrease in kidney function. Kidney function decreased faster as CKD stage advanced, but its declining slope did not change significantly by age, at least after ~30 years of age.

Keywords Autosomal dominant polycystic kidney disease · Glomerular filtration rate · Kidney volume

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Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is the most common hereditary kidney disease characterized by the progressive enlargement of innumerable renal cysts that lead to the deterioration of kidney function [1–3]. The Consortium for Radiologic Imaging Studies of Polycystic Kidney Disease (CRISP) study showed that baseline total kidney volume (TKV) predicted the subsequent rate of an increase in volume, independently of age [4]. Higher rates of kidney enlargement are associated with a more rapid decrease in renal function.

In a more recent study on CRISP participants, height-adjusted TKV (ht-TKV) predicted the risk of developing renal insufficiency in ADPKD patients within 8 years of follow-up [5]. The reason for adopting ht-TKV as an adjusted TKV marker in this study was to minimize the differences in adjusted TKV values between men and women. Other adjusted TKV markers, such as body