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Patients With Combined Membranous Nephropathy and Focal Segmental Glomerulosclerosis Have Comparable Clinical and Autoantibody Profiles With Primary Membranous Nephropathy

A Retrospective Observational Study

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Abstract: Patients with combined membranous nephropathy (MN) and focal segmental glomerulosclerosis (FSGS) have been reported with different clinical significance. Investigations on the possible mechanisms of the combined glomerular lesions are necessary but scarce. Twenty patients with both MN and FSGS lesions were enrolled in the study. Sixty-five patients with primary MN and 56 patients with primary FSGS were used as disease controls. Clinical data on renal biopsy and during follow-up were collected. Circulating anti-phospholipase A2 receptor (PLA2R) antibody, glomerular PLA2R expression, IgG4 deposition, and soluble urokinase receptor (suPAR) levels were detected. We found that patients with combined lesions presented with older age, less proteinuria, higher albumin, and better renal function on biopsy. These were comparable to the patients with primary MN, but differed from the patients with primary FSGS. Patients with combined lesions showed higher stages of MN, no cellular variant on FSGS classification, and more common (100.0%) tubulointerstitial injury than both primary MN and primary FSGS patients. In the patients with combined lesions, 80.0% had circulating anti-PLA2R antibody and 68.4% had IgG4 predominant deposition in glomeruli, which were comparable to primary MN. The patients with combined lesions had significantly lower urinary suPAR concentrations, than the primary FSGS patients (315.6 ± 151.0 vs 752.1 ± 633.9 pg/μmol; $P = 0.002$), but similar to the primary MN patients (267.9 ± 147.5 pg/μmol). We conclude that patients with combined MN and FSGS may share the same underlying pathogenesis with primary MN. The FSGS lesion might be secondary to primary MN.

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Abbreviations: eGFR = estimated glomerular filtration rate, ESRD = end-stage renal disease, FSGS = focal segmental glomerulosclerosis, GBM = glomerular basement membrane, KDIGO = the Kidney Disease Improving Global Outcomes, MN = membranous nephropathy, NOS = not otherwise specified, PLA2R = phospholipase A2 receptor, suPAR = soluble urokinase receptor.

INTRODUCTION

One of the most common causes of nephrotic syndrome in adults, membranous nephropathy (MN), is characterized by the presence of subepithelial immune complexes and basement membrane damage. Focal segmental glomerulosclerosis (FSGS) also presents with proteinuria, often leading to end-stage renal disease. The association of FSGS with MN has been previously reported.[1–6] Some patients with combined lesions had more severe proteinuria and worse kidney dysfunction.[1–3] FSGS lesions are associated with progressive MN, especially in Churg's stages III and IV.[1,2,5,6] Therefore, the existence of FSGS lesions in MN is clinically considered as a predictor of poor prognosis.[4,5] However, recent studies and meta-analyses did not support this statement.[6–8] Patients with combined lesions do not always present with higher serum creatinine or proteinuria.[5,6] In addition, cases of FSGS lesions have been reported in early stage I or II MN and are considered as the coexistence of primary FSGS and MN.[9] Thus, the clinical significance of combined MN and FSGS is not elucidated. Whether FSGS lesions contribute to the severer histopathological damage in MN and whether more aggressive treatments should be considered for these patients need to be answered.

Investigations on the possible mechanisms of these double glomerulopathy may provide some explanations for the clinical controversy, and be informative for therapeutic choice. Primary MN has been considered as an autoimmune glomerular disease. The M-type phospholipase A2 receptor (PLA2R) on podocytes has been recently identified as a major target antigen for autoantibodies in primary MN. Although serum anti-PLA2R antibody could be detected in approximately 70.0% of patients with primary MN,[10] this autoantibody possesses a high specificity for the diagnosis of primary MN, high consistency between antibody level and disease activity, and good prognostic value in evaluating proteinuria and treatment responses.[11–16] For primary FSGS, it has been suggested that

circulating permeability factors may be involved in the pathogenesis.[17,18] In 2011, the soluble urokinase receptor (suPAR) was considered as one of such factors.[19] Some studies indicated that both plasma and urinary suPAR levels were significantly higher in primary FSGS than in other glomerular diseases or healthy controls,[20,21] but still with controversies.[22–24]

By examining the circulating anti-PLA2R antibodies, as well as plasma and urinary suPAR levels in patients with combined MN and FSGS lesions, in the current study, we aimed to investigate the clinical and immunological features of these patients, and to provide insight into the pathogenesis of this entity.

METHODS

Patients and Samples

Seven hundred ninety-one consecutive patients with biopsy-proven primary MN and 174 consecutive patients with biopsy-proven primary FSGS diagnosed between 2004 and 2013 were reviewed retrospectively. Among them, 20 patients with MN in combination with FSGS lesions were enrolled in this study. Sixty-five patients with primary MN and 56 patients with primary FSGS were selected randomly with age and gender matching (Figure 1). Patients with known secondary MN or FSGS, such as those occur due to the hepatitis B/C virus, parvovirus B19-, human immunodeficiency virus, systemic lupus erythematosus, obesity, malignancy, medications, and heavy metal poisoning, were excluded. The latest clinical data before renal biopsy were collected from the medical records. Patients received treatments according to the Kidney Disease: Improving Global Outcomes (KDIGO) guidelines for glomerulonephritis.[25] During follow-up, complete remission was defined as urinary protein excretion of $\leq$0.3 g/24 h with normal renal function. Kidney function insufficiency was defined as serum creatinine $>$133.0 μmol/L.[7] This research study was in compliance with the Declaration of Helsinki and approved by the ethics committee of our hospital. Informed consent was obtained for sampling tissue and blood.

Plasma and the first spot urine samples were collected at the day of renal biopsy for the detection of anti-PLA2R antibody and suPAR levels. Disodium-EDTA was used as an anticoagulant for plasma collection. After collection, the plasma and urine samples were immediately centrifuged at 2000*g* for 15 minutes at 4°C, and then stored in aliquots at −80°C until used. Repeated freeze/thaw cycles were avoided.

Renal Histopathology

Renal biopsy was performed on all patients at the time of diagnosis. Renal specimens were evaluated with light microscopy, electron microscopy, and direct immunofluorescence, according to the standard procedure in our hospital.[20,21] Pathologic findings in the glomeruli, tubules, interstitium, and vessels were described in detail. The tubular atrophy and interstitial fibrosis were graded semi-quantitatively from 0 to 3 (0, normal; 1, 5.0–25.0% of interstitium affected; 2, 25.0–50.0% of interstitium affected; 3, >50.0% of interstitium affected). FSGS lesion was defined as focal segmental obliteration of glomerular capillaries presenting with extracellular matrix expansion on light microscopy, diffuse foot processes effacement on electron microscopy, and segmental staining for IgM and C3 entrapped in the areas of hyalinosis by immunofluorescence. Pathologic classification of FSGS was further clarified according to the Columbia classification of FSGS.[26]

Detection of Circulating anti-PLA2R Autoantibodies by Indirect Immunofluorescence and ELISA

Circulating anti-PLA2R autoantibodies in plasma were detected by commercially available direct immunofluorescence assay (FA1254-1005-50; EUROIMMUN AG, Lübeck, Germany), following the standard instructions as previously reported.[27] Antibody positivity was defined as green fluorescence as evaluated by the fluorescence microscope at a dilution of 1 : 10.

Plasma anti-PLA2R antibody level was detected by a commercial ELISA assay (EA1254; EUROIMMUN AG, Lübeck, Germany), according to the manufacture's instruction. Briefly, polystyrene microplates were pre-coated with PLA2R in advance. Plasma was diluted to 1 : 100, added to each well,

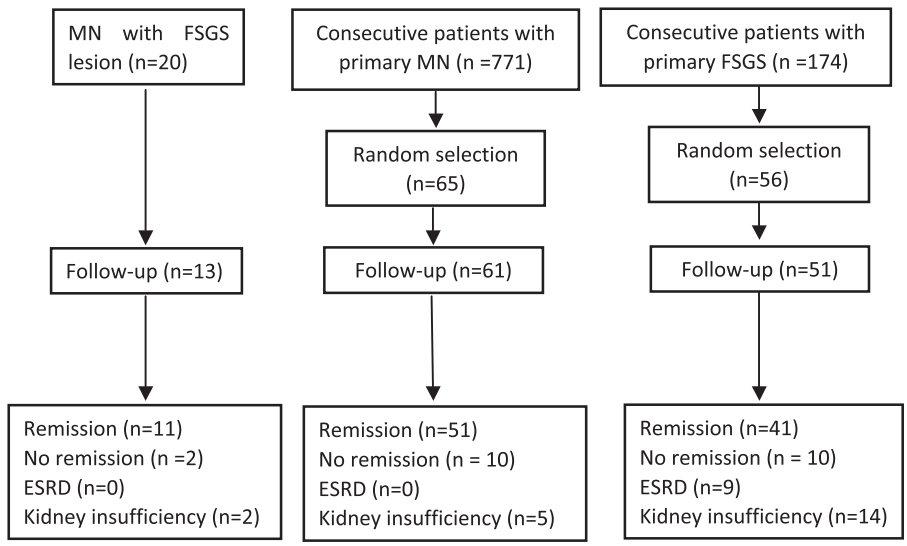

FIGURE 1. Schematic illustration of the study design.

and incubated for 30 minutes at room temperature. After incubation and washing, peroxidase-labeled anti-human IgG (rabbit) were added and incubated for 30 minutes at room temperature. After washing, a substrate solution was added to each well and incubated for 15 minutes at room temperature. Stop solution was added and the absorbance was recorded using an enzyme-linked immunosorbent assay reader at 450 nm. The anti-PLA2R antibody level of each sample was calculated using the Curve expert 1.3.

Detection of Glomerular PLA2R Expression

Renal biopsy sections were formalin-fixed, paraffin-embedded, and cut into 4 μm for immunohistochemical staining. The detection of glomerular PLA2R expression was performed with the method described previously.[13,27] Phosphate buffer saline (PBS) replaced the primary antibodies as negative controls and normal kidney tissues far from the renal carcinoma were used as healthy controls. Positivity of glomerular PLA2R expression was defined as linear or granular diffuse staining on glomeruli.

Detection of IgG Subclasses Deposition on Glomeruli

Paraffin-embedded sections of formalin-fixed renal tissue were utilized for immunohistochemistry with mouse monoclonal antibodies to human IgG1, IgG2, IgG3, and IgG4 (clone no. 4E3, HP6014, HP6050, HP6025; Southern Biotech, Birmingham, AL), as reported previously.[28] PBS replacement of primary antibodies was used as a negative control. Normal renal tissues far from renal carcinoma were used as healthy controls. All specimens were evaluated semi-quantitatively from 0 to 2 (0, no staining; 1, weak and segmental staining; 2, moderate or strong granular staining).

Quantification of Plasma and Urinary suPAR

The concentration of plasma and urinary suPAR was detected with Quantikine Human uPAR Immunoassay (R&D Systems, Minneapolis, MN), according to the manufacturer's instruction as previously reported.[20,21]

Statistical Analysis

Statistical analysis was performed using the SPSS statistical software package, version 13.0 (SPSS Inc., Chicago, IL). Differences in quantitative parameters were assessed using the Student *t* or 1-way analysis of variance (ANOVA) tests for data that were normally distributed, and nonparametric tests for data that were not normally distributed. Differences in semi-quantitative data were tested using the Kruskal–Wallis or Mann–Whitney *U* tests. Differences in qualitative data were compared using the Chi-square or the Fisher exact tests. Spearman correlation test was used to analyze the correlation between 2 non-normally distributed variables or 1 normally with 1 non-normally distributed variable. All statistical analyses were 2-tailed and a P value <0.05 was considered as significant. Statistical significance was assessed with a Bonferroni-adjusted threshold of 0.05/3 to correct the multiple comparisons among the 3 groups of patients.

RESULTS

Demographic and Clinical Parameters of Patients

Twenty patients with combined MN and FSGS were enrolled in this study. A comparison of demographic and clinical data among patients with primary MN, primary FSGS, and combined lesions are summarized in Table 1. Patients with combined MN and FSGS presented with comparable clinical features with those patients having MN only, but remarkable differences compared with those patients with only FSGS.

Compared with patients with primary FSGS, those with combined lesions were significantly older (52.4 ± 11.4 vs 35.8 ± 20.1 years, $P < 0.001$), presented with less urinary protein excretion (5.7, 2.8–11.1 vs 7.4, 5.3–12.2 g/24 h, $P = 0.088$), higher level of serum albumin (27.1 ± 6.7 vs 21.2 ± 7.1 g/L, $P = 0.001$), lower level of blood urea nitrogen (4.9, 3.8–5.9 vs 7.5, 5.3–15.4 mmol/L, $P < 0.001$), and less frequency of declined estimated glomerular filtration rate (eGFR) lower than 60.0 mL/min/1.73 m^2 (5.0% vs 35.7%, $P = 0.008$), which were all similar to those with primary MN (Table 1). The time from onset to biopsy in patients with combined MN and FSGS was 4.0 (range, 2.0–34.5) months, which was not significantly longer than in patients with primary MN (2.0, 1.0–8.0 months, $P = 0.063$), but similar to the patients with primary FSGS (4.0, 0.7–21.8 months) (Table 1).

During the follow-up of 38.8 (range, 21.2–65.7) months, treatments were comparable among the 3 groups ($P = 0.742$). Remission was achieved in 84.6% of the patients with combined lesions, which was similar to that in patients with primary MN (83.6%) and patients with primary FSGS (80.4%). During follow-up, kidney insufficiency was observed in 14 of 51 (27.5%) patients with primary FSGS, while only 2 of 13 (15.4%) patients with combined lesions and 5 of 61 (8.2%) patients with primary MN presented with kidney insufficiency ($P = 0.024$). Nine of 51 (17.6%) patients with primary FSGS went into end-stage renal disease (ESRD), but none of the patients with combined lesions or primary MN reached this end-point ($P = 0.005$) (Table 1).

Pathological Parameters

Renal biopsies were performed in all patients by light microscopy, electron microscopy, and immunofluorescence. Fifteen (75.0%) patients with combined lesions presented with advanced Churg stages (II or III) of MN, with 8 patients of stage II and 7 patients of stage III. In the patients with primary MN, only 30 (46.2%) patients were of the advanced stages of MN ($P = 0.013$) (Figure 2A; Table 1). For the patients with combined MN and FSGS, we found that the MN Churg stages were correlated with the percentage of FSGS lesions ($r = 0.430$, $P = 0.058$), tubular atrophy ($r = 0.597$, $P = 0.005$), interstitial infiltration ($r = 0.598$, $P = 0.005$), interstitial fibrosis ($r = 0.598$, $P = 0.005$), and glomeruli with global sclerosis ($r = 0.391$, $P = 0.088$). These correlations were also found in patients with primary MN. Compared with the primary MN patients with the same Churg stage I, the patients with combined MN and FSGS presented with severer tubular atrophy (100.0% vs 65.7%), interstitial infiltration (100.0% vs 68.6%), and interstitial fibrosis (100.0% vs 65.7%), though without statistical significance for small sample size. For MN Churg stage II and stage III, the patients with combined MN and FSGS presented with severer tubular atrophy (100.0% vs 83.3%, $P = 0.017$), interstitial infiltration (100.0% vs 83.3%, $P = 0.008$), and interstitial fibrosis (100.0% vs 86.7%, $P = 0.024$), than primary MN patients (Table 2).

The percentage of sclerosis lesions in the glomeruli was similar between the patients with combined lesions and those with primary FSGS (19.8, 10.9–25.9 vs 14.0, 8.0–26.8%, $P = 0.342$) (Figure 2B; Table 1). The FSGS pathological classification of patients with combined MN included not

TABLE 1. Comparison of the Clinical and Pathological Parameters of Patients With Combined MN and FSGS, Primary MN, and Primary FSGS

	Combined MN and FSGS (n = 20)	Primary MN (n = 65)	Primary FSGS (n = 56)	*P*
Gender (male/female)	16/4	44/21	39/17	0.571
Age, y	52.4 ± 11.4	53.1 ± 11.9	35.8 ± 20.1†	<0.001
Urinary protein, g/24 h	5.7, 2.8–11.1	5.9, 4.1–8.3	7.4, 5.3–12.2	0.010
Serum albumin, g/L	27.1 ± 6.7	25.8 ± 5.4	21.2 ± 7.1†	<0.001
Nephrotic syndrome, n (%)	18 (90.0)	55 (84.6)	54 (96.4)	0.076
Serum cholesterol, mmol/L	7.2 ± 1.4	7.3 ± 2.3	9.0 ± 3.5†	0.002
Serum creatinine, μmol/L	74.5,67.8–91.0	71.8, 64.0–88.0	92.0, 63.8–161.3	0.014
Declined eGFR, n (%)	1 (5.0)	3 (4.6)	20 (35.7)†	<0.001
Blood urea nitrogen, mmol/L	4.9, 3.8–5.9	4.5, 3.5–6.1	7.5, 5.3–15.4†	<0.001
Hypertension, n (%)	11 (55.0)	36 (55.4)	21 (37.5)	0.118
Time from onset to biopsy, mo	4.0, 2.0–34.5	2.0, 1.0–8.0	4.0, 0.7–21.8	0.188
Follow-up, mo	37.8, 21.9–70.5	36.5, 21.3–55.0	47.4, 21.0–70.0	0.366
Immunosuppressive treatment, n (%)	12/13 (92.3)	52/61 (85.2)	45/51 (88.2)	0.742
Remission, n (%)	11/13 (84.6)	51/61 (83.6)	41/51 (80.4)	0.884
Kidney insufficiency, n (%)	2/13 (15.4)	5/61 (8.2)	14/51 (27.5)	0.024
ESRD, n (%)	0/13 (0.0)	0/65 (0.0)	9/51 (17.6)	0.005
MN-stage, n (%)		*		0.013
I	5 (25.0)	35 (53.8)	—	
II	8 (40.0)	24 (36.9)	—	
III	7 (35.0)	6 (9.2)	—	
Glomeruli with sclerosis (%)	19.8, 10.9–25.9		14.0, 8.0–26.8	0.342
Tubular atrophy, n (%)		*	†	0.011
Grade 0	0 (0.0)	17 (26.2)	17 (30.4)	
Grade 1	16 (80.0)	46 (70.8)	31 (55.4)	
Grade 2	2 (10.0)	2 (3.1)	5 (8.9)	
Grade 3	2 (10.0)	0 (0.0)	3 (5.4)	
Interstitial infiltration, n (%)		*		0.006
Grade 0	0 (0.0)	16 (24.6)	12 (21.4)	
Grade 1	16 (80.0)	48 (73.8)	37 (66.1)	
Grade 2	3 (15.0)	1 (1.5)	4 (7.1)	
Grade 3	1 (5.0)	0 (0.0)	3 (5.4)	
Interstitial fibrosis, n (%)		*	†	0.013
Grade 0	0 (0.0)	16 (24.6)	19 (33.9)	
Grade 1	16 (80.0)	47 (72.3)	29 (51.8)	
Grade 2	3 (15.0)	2 (3.1)	4 (7.1)	
Grade 3	1 (5.0)	0 (0.0)	4 (7.1)	
Immunofluorescence				
IgG deposit, n (%)	18 (90.0)	65 (100.0)	1 (1.8)†	<0.001
IgA deposit, n (%)	3 (15.0)	10 (15.4)	4 (7.1)	0.324
IgM deposit, n (%)	11 (55.0)	26 (40.0)	33 (58.9)	0.101
C3 deposit, n (%)	18 (90.0)	57 (87.7)	12 (21.4)†	<0.001
Anti-PLA2R antibody positivity, n (%)	16 (80.0)	46 (70.8)	0 (0.0)†	<0.001
Plasma anti-PLA2R antibody, RU/mL	138.7 ± 110.3	137.3 ± 104.3		0.969
Glomerular PLA2R expression, n (%)	15/20 (75.0)	23/29 (79.3)	0/3 (0.0)†	0.021
IgG4 predominant deposit, n (%)	13/19 (68.4)	19/29 (65.5)	0/3 (0.0)	0.094
Plasma suPAR, pg/mL	2957.4 ± 765.8	2410.9 ± 989.9	3727.1 ± 2272.7	<0.001
Urinary suPAR/creatinine, pg/μmol	315.6 ± 151.0	267.9 ± 147.5	752.1 ± 633.9†	<0.001

eGFR = estimated glomerular filtration rate, FSGS = focal segmental glomerulosclerosis, MN = membranous nephropathy, PLA2R = M-type phospholipase A2receptor, suPAR = soluble urokinase receptor.

*$P < 0.05$ between combined MN and FSGS and primary MN.

†$P < 0.05$ between combined MN and FSGS and primary FSGS.

otherwise specified (NOS) variants (n = 12), tip variants (n = 6), perihilar variant (n = 1), and collapsing variant (n = 1). There was no cell variant. Compared with the primary FSGS patients, the prevalence of cell variants was significantly rare in the patients with combined lesions (0.0% vs 26.8%, $P = 0.024$).

Tubular atrophy, interstitial infiltration, and fibrosis were much more common and severe in patients with combined

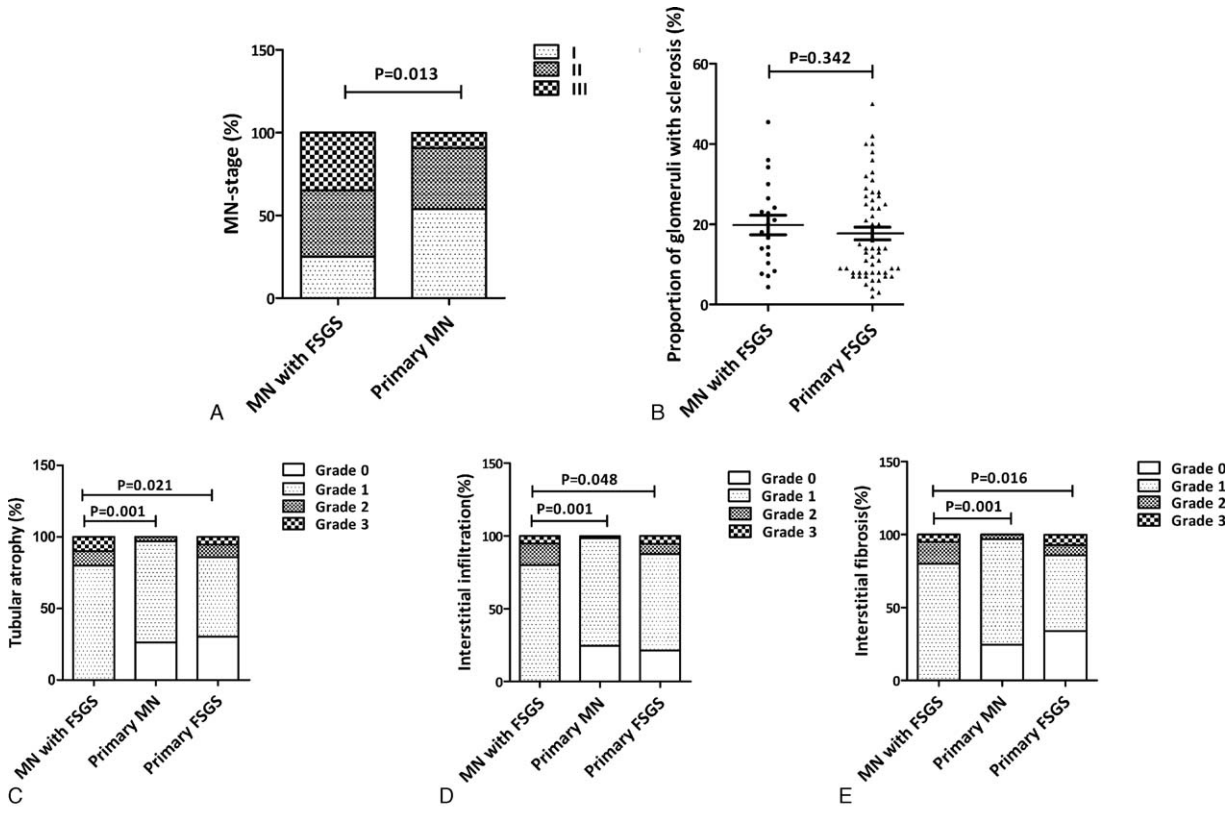

FIGURE 2. Kidney histopathological parameters of patients with combined MN and FSGS, primary MN, and primary FSGS. A, Comparison of histopathology stages of MN between MN patients with and without FSGS lesions. B, Comparison of the proportion of glomerular sclerosis between patients with combined MN and FSGS and patients with primary FSGS. C, Comparison of tubular atrophy lesions among patients with combined MN and FSGS, primary MN, and primary FSGS. D, Comparison of the interstitial infiltration lesions among the patients with combined MN and FSGS, primary MN, and primary FSGS. E, Comparison of interstitial fibrosis lesions among the patients with combined MN and FSGS, primary MN, and primary FSGS.

lesions. Tubular atrophy, interstitial infiltration, and interstitial fibrosis were shown in all the patients with combined MN and FSGS (100.0%, 100.0%, 100.0%), while they were revealed in 73.8% ($P = 0.001$), 75.4% ($P = 0.001$), and 75.4% ($P = 0.001$) of the patients with primary MN, respectively, and in 69.6% ($P = 0.021$), 78.6% ($P = 0.048$), and 66.1% ($P = 0.016$) of the patients with primary FSGS (Figure 2C–E; Table 1), respectively.

Circulating anti-PLA2R Antibodies and PLA2R Expression in Glomeruli

Circulating anti-PLA2R antibodies were detectable in 80.0% of the patients with combined MN and FSGS (Figure 3A; Table 1), which was similar to that of the patients with primary MN (70.8%) (Figure 3B; Table 1). No patient with primary FSGS was positive for anti-PLA2R antibody (Figure 3C; Table 1).

The level of anti-PLA2R antibody was comparable between patients with combined lesions and patients with primary MN (138.7 ± 110.3 vs 137.3 ± 104.3, $P = 0.969$). Among the patients with combined MN and FSGS lesions, the circulating anti-PLA2R antibody level was associated with urinary protein ($r = 0.617$, $P = 0.004$) and serum albumin ($r = -0.594$, $P = 0.006$) (Figure 4). The patients who achieved remission had significantly lower level of anti-PLA2R antibodies, than those who did not achieve remission (45.2 ± 75.3 vs $234.1 + 87.8$, $P = 0.008$). These were consistent with the patients with primary MN.

We examined the glomerular PLA2R expression in all the patients with combined lesions, 29 of 65 patients with primary MN, and 3 patients with primary FSGS. Enhanced linear or granular staining of PLA2R in glomeruli was shown in 75.0% of patients with combined MN and FSGS (Figure 5A, Table 1), and 79.3% of the patients with primary MN (Figure 5B, Table 1). The patients with primary FSGS only had faint PLA2R staining in the glomeruli (Figure 5C; Table 1), which was similar to healthy controls (Figure 5D).

Among the 20 patients with combined MN and FSGS, 14 patients (87.5%) had glomerular PLA2R expression in 16 patients with circulating anti-PLA2R antibody; in 4 patients who were negative for circulating anti-PLA2R antibody, 1 patient had enhanced glomerular expression of PLA2R. Among the 29 patients with primary MN, 17 (89.5%) patients had glomerular PLA2R expression in 19 patients with circulating anti-PLA2R antibody; in 10 patients who were negative for anti-PLA2R antibody, 6 patients had enhanced glomerular

TABLE 2. Comparison Between Combined MN and FSGS Patients and Primary MN Patients With the same Churg Stages

	Combined MN and FSGS (n = 20)	Primary MN (n = 65)	*P*
Stage I	n = 5	n = 35	
Tubular atrophy, n (%)			0.122
Grade 0	0/5 (0.0)	12/35 (34.3)	
Grade 1	5/5 (100.0)	23/35 (65.7)	
Grade 2	0/5 (0.0)	0/35 (0.0)	
Grade 3	0/5 (0.0)	0/35 (0.0)	
Interstitial infiltration, n (%)			0.146
Grade 0	0/5 (0.0)	11/35 (31.4)	
Grade 1	5/5 (100.0)	24/35 (68.6)	
Grade 2	0/5 (0.0)	0/35 (0.0)	
Grade 3	0/5 (0.0)	0/35 (0.0)	
Interstitial fibrosis, n (%)			0.122
Grade 0	0/5 (0.0)	12/35 (34.3)	
Grade 1	5/5 (100.0)	23/35 (65.7)	
Grade 2	0/5 (0.0)	0/35 (0.0)	
Grade 3	0/5 (0.0)	0/35 (0.0)	
Glomeruli with global sclerosis, (%)	0.0, (0.0, 3.1)	0.0 (0.0, 0.0)	0.253
Anti-PLA2R antibody positivity, n (%)	3/5 (60.0)	21/35 (60.0)	1.000
Plasma anti-PLA2R antibody, RU/mL	134.6 ± 137.0	128.4 ± 95.8	0.934
Glomerular PLA2R expression, n (%)	3/5 (60.0)	14/20 (70.0)	1.000
Stage II and Stage III	n = 15	n = 30	
Tubular atrophy, n (%)			0.017
Grade 0	0/15 (0.0)	5/30 (16.7)	
Grade 1	11/15 (73.3)	23/30 (76.7)	
Grade 2	2/15 (13.3)	2/30 (6.7)	
Grade 3	2/15 (13.3)	0/30 (0.0)	
Interstitial infiltration, n (%)			0.008
Grade 0	0/15 (0.0)	5/30 (16.7)	
Grade 1	11/15 (73.3)	24/30 (80.0)	
Grade 2	3/15 (20.0)	1/30 (3.3)	
Grade 3	1 (6.7)	0/30 (0.0)	
Interstitial fibrosis, n (%)			0.024
Grade 0	0/15 (0.0)	4 (13.3)	
Grade 1	11/15 (73.3)	24 (80.0)	
Grade 2	3/15 (20.0)	2 (6.7)	
Grade 3	1 (6.7)	0 (0.0)	
Glomeruli with global sclerosis, (%)	0.0 (0.0, 7.6)	0.0 (0.0, 5.5)	0.362
Anti-PLA2R antibody positivity, n (%)	13/15 (86.7)	25/30 (83.3)	1.000
Plasma anti-PLA2R antibody, RU/mL	155.5 ± 109.5	139.9 ± 112.4	0.730
Glomerular PLA2R expression, n (%)	12/15 (80.0)	9/9 (100.0)	0.426

FSGS = focal segmental glomerulosclerosis, MN = membranous nephropathy, PLA2R = M-type phospholipase A2receptor.

expression of PLA2R. These were comparable between the 2 groups ($P = 0.855$).

For the patients with combined MN and FSGS, the MN Churg stages were correlated with the level of anti-PLA2R antibody, though without statistical significance ($r = 0.571$, $P = 0.135$). For the patients with primary MN, MN stages were significantly correlated with the level of anti-PLA2R antibody ($r = 0.263$, $P = 0.035$). No correlation was observed between MN stages and glomerular PLA2R expression, no matter in patients with MN + FSGS or primary MN. Compared with the primary MN patients with the same Churg stages, the patients with MN + FSGS presented with similar prevalence of positive anti-PLA2R antibody, comparable antibody levels, and similar prevalence of glomerular PLA2R expression (Table 2).

The Deposition of IgG Subclasses in Glomeruli

We detected IgG subclass deposition in the glomeruli from 19 of 20 patients with combined MN and FSGS and 29of 65patients with primary MN, who had sufficient renal biopsy sections. IgG subclasses were detected in all the patients. Among the patients with combined MN and FSGS, IgG4, both IgG4 and IgG1, and IgG1 deposition were predominant in 68.4%, 5.3%, and 26.3% of patients (Figure 6), respectively. Among the patients with primary MN, IgG4, both IgG4 and IgG1, and IgG1 deposition were predominant in 65.5%, 6.9%, and 27.6% of patients, respectively. IgG2 and IgG3 staining were either weak or negative in almost all the patients (Figure 6B, C). The findings in the patients with combined MN and FSGS were similar to those with primary MN

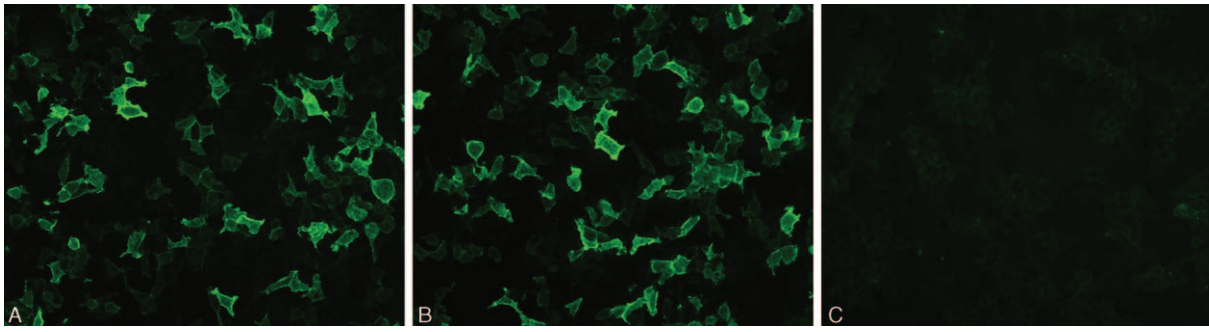

FIGURE 3. The detection of circulating anti-PLA2R antibody. A, Detection of positive circulating anti-PLA2R antibody in a patient with combined MN and FSGS. B, Detection of positive circulating anti-PLA2R antibody in a patient with primary MN. C, Negative result in a patient with primary FSGS.

(Figure 6; Table 1). Among the 3 patients with primary FSGS, only 1 patient had weak IgG1 staining, while the remaining 2 were negative for the 4 IgG subclasses deposition.

Among the 4 patients with combined MN and FSGS who were negative for anti-PLA2R antibody, an IgG4 predominant deposit was detected in 1 patient. Among the 10 patients with primary MN who were negative for anti-PLA2R antibody, IgG4 predominant deposits were detected in 4 patients.

Plasma and Urinary suPAR Levels

The plasma suPAR level of the patients with combined MN and FSGS was 2957.4 ± 765.8 pg/mL. This was higher and lower than in patients with primary MN (2410.9 ± 989.9 pg/mL) and primary FSGS (3727.1 ± 2272.7 pg/mL), respectively, but without statistical significance. Significant differences were shown between the patients with primary MN and primary FSGS ($P < 0.001$) (Figure 7A, Table 1).The urinary suPAR level was adjusted by the urinary creatinine. The urinary suPAR level of patients with combined MN and FSGS was 315.6 ± 151.0 pg/μmol, which was similar to those in patients with primary MN (267.9 ± 147.5 pg/μmol), but significantly lower than those with primary FSGS (752.1 ± 633.9 pg/μmol, $P = 0.002$). The urinary suPAR levels of the patients with primary FSGS were also significantly higher than those with primary MN ($P < 0.001$) (Figure 7B; Table 1).

DISCUSSION

Patients with combined MN and FSGS have been reported since 1977.[29] The impact of FSGS lesions on the clinical features and renal prognosis of patients with MN has been previously studied, but with different conclusions. More importantly, the underlying mechanism for the FSGS lesion in MN is not well elucidated.

In this study, we found that the patients with combined MN and FSGS lesions presented with clinical features, including elder age on diagnosis, less proteinuria, higher serum albumin, and less prevalence of renal dysfunction on biopsy. All of them were comparable with those of the patients with primary MN, but significantly different from patients with primary FSGS. These findings indicate that the clinical characters of MN and FSGS combination lesions are similar to those of primary MN. The combined FSGS lesion does not show the typical clinical features induced by primary FSGS.

The findings on proteinuria are consistent with recent studies.[2,5,6] Although Wakai and Magil[4] previously found that the patients with combined MN and FSGS had greater 24-hour urinary protein excretion, their serum albumin and cholesterol levels were similar to patients with primary MN. For renal function, our results were in agreement with the data presented by Dumoulin et al[5] and Wakai and Magil.[4] However, other studies found raised serum creatinine in the patients with combined lesions.[3,6] We speculated that the discrepancy in clinical findings might be due to the time of data collection. In this study, all the data were collected at the time of renal biopsy and the duration from disease onset to renal biopsy was comparable among the 3 groups. However, in the studies mentioned above, some data were collected at the first patient contact,[5] while others were on renal biopsy.[3,4,6] The later the time of data collection, the more severe the clinical presentation. The addition of FSGS on MN did not present with obstacles to immunosuppressive treatments or poor kidney outcomes. Our results were in agreement with the data from Heeringa et al[6] and others.[8] Although the FSGS lesion was once

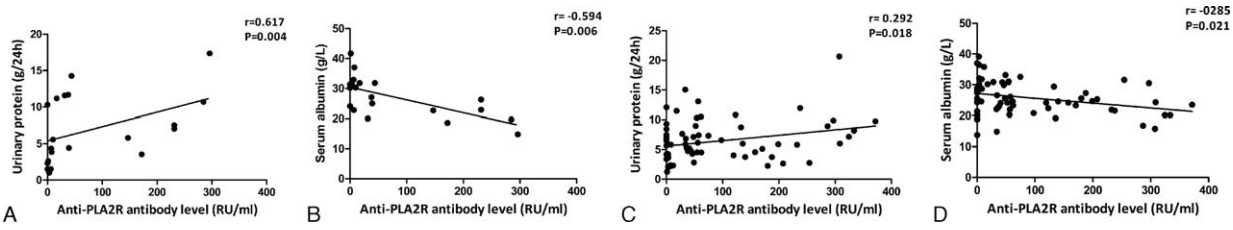

FIGURE 4. The correlations between plasma anti-PLA2R antibody level and clinical parameters. A, The correlation between plasma anti-PLA2R antibody level and urinary protein in patients with combined MN and FSGS. B, The correlation between plasma anti-PLA2R antibody level and serum albumin in patients with combined MN and FSGS. C, The correlation between plasma anti-PLA2R antibody level and urinary protein in patients with primary MN. D, The correlation between plasma anti-PLA2R antibody level and serum albumin in patients with primary MN.

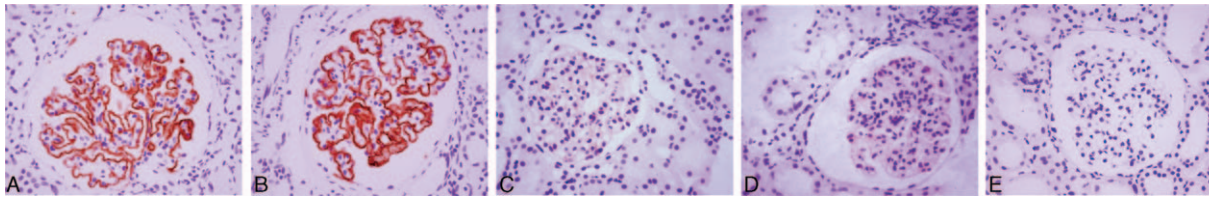

FIGURE 5. The detection of glomerular PLA2R expression (x400). A, Enhanced glomerular staining of PLA2R in a patient with combined MN and FSGS. B, Enhanced glomerular staining of PLA2R in a patient with primary MN. C, Faint glomerular staining of PLA2R in a patient with primary FSGS. D, Faint glomerular staining of PLA2R in a healthy control. E, Negative control (PBS).

found as a predictor of renal failure in MN patients,[4,5] recent studies failed to identify it as an independent risk factor. [6–8] Longer follow-up studies are needed to solve this controversy.

On histopathological examinations, the patients with combined lesions showed more commonly with advanced stages of MN, such as stage III. In these patients, the stage of MN was positively correlated with the percentage of FSGS lesion. These findings were reported in other studies[1–6] and indicate a possible secondary FSGS induced by the advanced stages of MN. Tubular atrophy, interstitial infiltration, and fibrosis were more common and severer in the patients with combined lesions. Compared with primary MN patients with the same Churg stages, the patients with combined lesions presented with severer tubular atrophy, interstitial infiltration, and interstitial fibrosis. These findings indicate that the secondary FSGS lesion may contribute to the tubulointerstitial injury independently from that accompanied by higher stages of MN. Although the percentage of glomerular sclerosis was comparable between the patients with combination lesions and primary FSGS, the FSGS classification was different. Cellular variants were commonly shown in the patients with primary FSGS, but were not seen in any patient with combination lesions. This finding is consistent with the results from the study by Gupta et al.[3] It is known that cellular variants are common in primary FSGS, but are less observed in secondary FSGS.[30] Thus, we speculated that the FSGS lesions in combination with MN may be secondary lesions.

Circulating anti-PLA2R antibodies and glomerular PLA2R expression were further investigated to explore their underlying mechanism. The antibody detection rates and antibody level were comparable between patients with combined lesions and those with primary MN. The antibody level was also associated with clinical features and treatment response. Nearly all the patients with circulating anti-PLA2R antibodies had glomerular PLA2R expression regardless of the presence of FSGS lesions. Among the 4 patients with combined lesions who were negative for the circulating antibody, 1 patient showed enhanced glomerular PLA2R expression. Among the patients with primary FSGS, the circulating anti-PLA2R antibodies were undetectable and the glomerular PLA2R expression was as faint as in healthy controls. These findings support the same pathogenesis of combined lesions and primary MN. Anti-PLA2R antibody is specific for primary MN, as the IgG eluted from the kidney deposits in the patients with primary MN, but not in those with secondary MN, recognizes the PLA2R.[10] Up to 50.0% of patients with primary MN without circulating anti-PLA2R antibody had glomerular PLA2R expression on kidney biopsy tissue.[13,31] This was also found in our current study. The staining of PLA2R on glomeruli may be a good supplement to antibody detection, but the specificity for primary MN is as low as 83.0%.[32] PLA2R is expressed on the surface of podocytes localizes with the IgG4 in the immune deposit of primary MN patients.[10] In the current study, we demonstrated IgG4-predominant deposits in 68.4% of patients with combined MN and FSGS, which supports the belief that the combination of the MN and FSGS lesions is a natural variant of primary MN.[33]

Recently, the suPAR level was suggested as a possible circulating permeability factor in the pathogenesis of primary FSGS.[19–21,34] By activating the integrin β3, the membrane-binding form of suPAR affects the migration of podocytes, contributes to its detachment from GBM, and the final proteinuria.[19,21] The plasma suPAR level is elevated in the patients with primary FSGS, but it is influenced by renal dysfunction and not used for differentiating between the primary and secondary FSGS.[35] However, the elevation of urinary suPAR levels after urinary creatinine adjustment is more specific for primary FSGS but not commonly seen in secondary FSGS.[21] In this study, we found that the urinary suPAR levels in the patients with combined MN and FSGS were comparable to those in patients with primary MN, but significantly lower than those in patients with primary FSGS. Thus, we speculated that the superimposed FSGS in MN might be a secondary lesion.

Secondary causes for FSGS had been excluded at the time of patient enrollment. Thus, the FSGS lesion in combination with MN was believed to occur secondary to MN. First, although hypertension in the course of MN is highly predictive of an association with FSGS, it is evident that the hypertension itself is not due to the FSGS lesions.[5,36] In the current study, hypertension was found in more than half of the patients with combined MN and FSGS, but the proportion was comparable

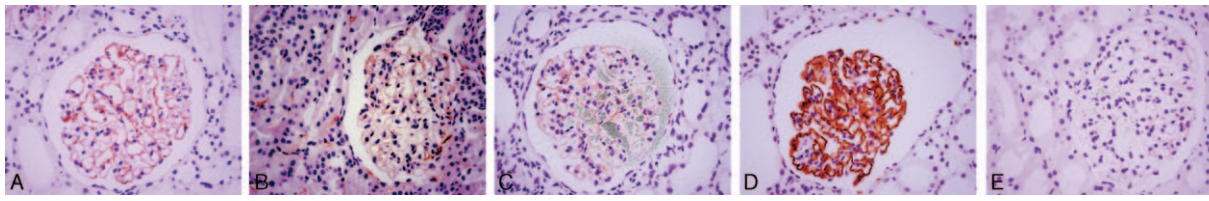

FIGURE 6. The deposition of the IgG subclasses on the glomeruli of a patient with combined MN and FSGS (x400). A, Weak IgG1 staining. B, Weak IgG2 staining. C, Weak IgG3 staining. D, Strong IgG4 staining. E, Negative control (PBS).

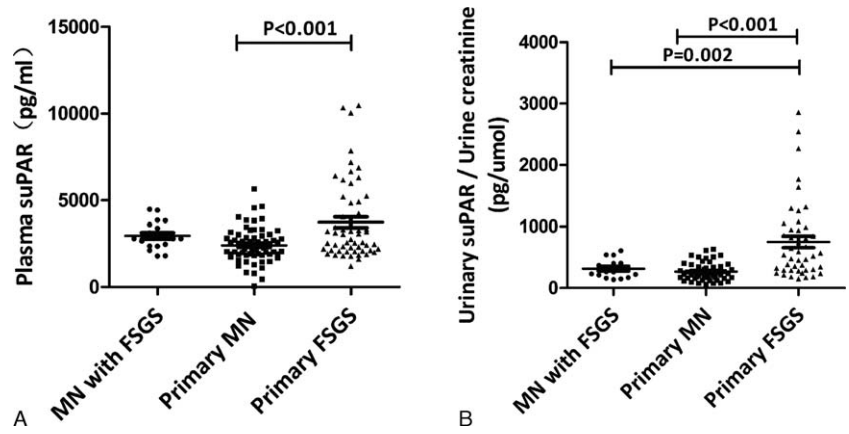

FIGURE 7. The suPAR levels of patients with combined MN and FSGS, primary MN, and primary FSGS. A, Comparison of plasma suPAR levels. Although not statistically significant, the mean plasma suPAR levels of patients with combined lesions were lower than those with primary FSGS. Significant differences were shown between patients with primary MN and primary FSGS. B, Comparison of urinary suPAR levels. The mean urinary suPAR level in patients with combined lesions was significantly lower than those with primary FSGS, but comparable to those with primary MN.

among the 3 groups. Second, FSGS lesions are often found in the advanced stages of MN and imply a possible later biopsy or nephron loss. However, the time from disease onset to renal biopsy and the renal function on diagnosis were comparable between the MN patients with and without FSGS. Third, in advanced MN lesions, it has been suggested that the subepithelial immune deposits contribute to the podocyte detachment from GBM through $\alpha 3\beta 1$ integrins, and the redistribution of podocyte adhesion molecules in FSGS,[36,37] which we suspected to be one of the causes for the secondary FSGS lesions on MN. This needs further investigation.

The limitation of this study was a retrospectively observational study; thus, a cause–effect relationship could not be established. Some of the patients were lost during follow-up period, inducing bias in the results of outcome analysis. PLA2R glomerular expression was not detected in all patients, which leads to the underestimation for PLA2R-related MN. Furthermore, the findings from this single-center study with limited number of patients require validation from multicenter studies with large cohort.

In conclusion, patients with combined MN and FSGS presented with similar clinical characteristics, but advanced stages of MN and more severe tubulointerstitial damage, than the patients with primary MN. The prevalent detection of the anti-PLA2R autoantibody and the normal urinary suPAR level indicate that the mechanism of FSGS lesion might be secondary to MN.

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