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Podocyte Infolding Glomerulopathy: A Special Morphology of Podocyte Injury Caused by Heterogeneous Diseases

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Introduction: Podocyte infolding glomerulopathy (PIG) is a newly recognized rare glomerular injury. The clinical significance and mechanism of this injury pattern remains unclear.

Methods: We conducted a retrospective study of renal biopsies from January 2018 to December 2020 in Kingmed Diagnostics. The renal biopsy features and clinical data were reviewed. Laser scanning microdissection and mass spectrometry (LMD/MS) was conducted to analyze the potential mechanism.

Results: A total of 116 (0.092%) out of 126,086 biopsies were diagnosed as PIG during the period. Of these, 89 (76.7%) cases were found to have PIG coexisting with immune-complex associated glomerulonephritis (IC-PIG) whereas 27 (23.3%) were identified as isolated PIG without immunoglobulin or complement deposition. Systemic lupus erythematosus (SLE), especially with membranous lupus nephritis (LN), was diagnosed in most (70.8%) IC-PIG cases. Of the isolated PIG cases, 51.9% had no known underlying conditions; however, a relatively high positive rate (42.1%) of antinuclear antibody (ANA) was detected. Nearly half (47.5%) of the patients presented with nephrotic syndrome (NS). PIG grade was associated with proteinuria in isolated PIG ($P = 0.035$). LMD/MS revealed dysregulated cytoskeletal protein α-actinin4 (ACTN4) and tubulin beta-4 chain in PIG compared with normal donor kidney and minimal change disease (MCD). The displacement of ACTN4 into the glomerular basement membrane (GBM) was confirmed by the confocal microscope.

Conclusion: PIG is a rare podocyte injury that can exist alone without underlying disease or be concurrent with various diseases, especially SLE. Podocyte cytoskeletal protein ACTN4 and tubulin beta-4 chain were dysregulated, which may be involved in the mechanism of PIG.

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PIG is a newly defined and relatively rare glomerular injury that has been increasingly recognized in recent years. It is characterized by microstructures originating from the cytoplasmic invagination of podocytes in the GBM visualized under electron microscopy (EM).[1] In 1992, Sato H. first described features of PIG as diffuse thickening of the GBM with fine intramembranous electron-dense deposits, which is similar to the membranous glomerulonephritis.[2] In 2008, the Japanese Society of Nephrology discovered a total of 25 cases of microspheres or microtubule structures in GBM further describing PIG lesions as a new disease entity.[1] In a few cases from other countries, various degrees of proteinuria and renal dysfunction were reported.[3–13] Most of the reported cases are associated with connective tissue disease, such as LN,[14] Sjogren's syndrome,[6] scleroderma,[7] etc., among which membranous LN is the most common pathological finding.[1,6] To date, the mechanism and significance of this rare and unique pattern of glomerular injury remains to be clarified.

This study aimed to elucidate the prevalence, clinicopathologic features, and outcomes of PIG. Renal biopsies were reviewed from January 2018 to December

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2020 at Kingmed Medicine which is the largest renal pathology center receiving cases from most of the geographic areas in China. To our knowledge, this is the largest retrospective study providing comprehensive morphological details, clinicopathologic correlation, and outcomes of PIG.

METHODS

Diagnostic Criteria of PIG

A total of 126,086 renal biopsies were retrospectively reviewed from January 2018 to December 2020 for screening PIG. Patients fulfilling the following diagnostic criteria were included: (i) EM showing a diffuse (>75%) microsphere or microtubular structures correlated with podocytic cytoplasm infolding into the GBM and (ii) light microscopy showing diffuse GBM thickening with crater resembling membranous nephropathy (MN).[1] Microsphere was defined as uniformly-sized spherical particles with obscured membrane, measuring 50–100 nm in diameter. Microtubule refers to unbranched hollow tubular structure with clear membrane, measuring 50–150 nm in diameter. Cluster formation is aggregates of microspheres and/or microtubule structures (Supplementary Figure S1). This study was approved by the Ethics Committee of both the first affiliated hospital of Sun Yat-sen University and Guangzhou Kingmed Medicine Diagnostic.

PIG Classification, Grading, and Other Pathological Findings

PIG classification was employed according to the report of the Japanese Society of Nephrology.[1] Briefly, subtype A referred to primary podocytic infolding, subtype C indicated microstructures in the GBM, and subtype B had both features.[1] Considering that various degrees of PIG lesion were found, from a minor involvement of the outer surface of GBM to a florid lesion involving the whole thickness of GBM, PIG grading was categorized into mild, moderate, and severe based on the severity of the lesion. Mild PIG was defined as cytoplasmic processes of podocytes inverted into the GBM involving less than the outside one-third thickness of GBM. Moderate PIG represents the irregular thickening of GBM with a number of podocyte cytoplasmic fragments extending into more than one-third but less than two-thirds of GBM thickness. Severe PIG appears as florid thickened GBM with a large number of podocytes cytoplasmic protrusions involving almost the whole thickness of GBM (Supplementary Figure S2). Other ultrastructural changes, including the thickening of GBM, the existence and distribution of the deposit, and the extent of foot process effacement were analyzed as well.

Based on the positivity of immunoglobulins and complements in immunofluorescence (IF), patients were divided into immune-complex associated PIG and isolated PIG. The working flow chart is shown in Figure 1. The minimal IF intensity of IC-PIG is IgG/IgA/IgM with at least 1+ intensity along the glomerular capillary loops and/or mesangial area, with or without the deposition of complement C3 and C1q. In addition, the deposition must be confirmed by EM in order to rule out the possibility of nonspecific entrapment or adherence of plasma proteins. Light microscopy changes, including mesangial and endocapillary

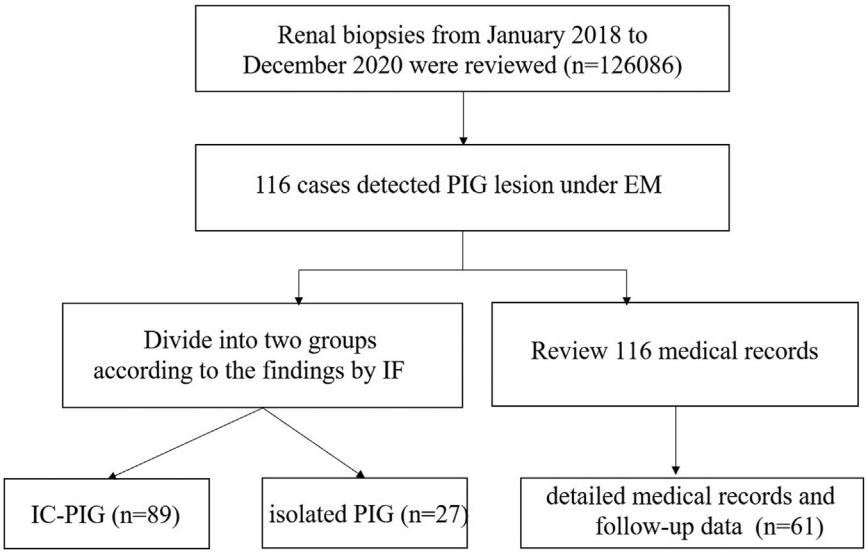

Figure 1. Screening process for podocyte infolding glomerulopathy. The 126,086 kidney biopsy specimens covered 23 of the 31 provinces, municipalities, and autonomous regions (74.2%) in China from 2018 to 2020. EM, electron microscopy; GBM, glomerular basement membrane; IF, immunofluorescence; LM, light microscopy; PIG, podocyte infolding glomerulopathy.

hypercellularity, global and segmental sclerosis, interstitial inflammation, interstitial fibrosis and tubular atrophy, arteriosclerosis, and hyalinosis were evaluated.[15] All of the pathological features were independently evaluated by 2 experienced renal pathologists.

Clinical and Laboratory Data

Serum laboratory examinations, including albumin, serum creatinine, ANA, anti-double-stranded DNA antibody, anti-Sjogren's syndrome-related antigen A autoantibodies, anti-Sjogren's syndrome-related antigen B autoantibodies, anti-Smith antibody, and complement levels were collected and analyzed. Urinalysis and 24-hour urinary protein excretions were collected as well.

Treatment Regime and Patients' Response

Treatment and following-up information were collected and analyzed. Patients were treated with glucocorticoids or glucocorticoids plus immunosuppressors for induction and maintenance therapy. The dose of glucocorticoid induction was 30–60 mg/d (0.5–1.0 mg/kg/d of oral prednisone or methylprednisolone), which lasted for 12 weeks or until 2 weeks after complete remission (CR). It was then tapered by 8 mg/d or 10 mg/d per month for 6 to 8 months in total. Glucocorticoid pulse therapy was administered at doses of 250 mg or 500 mg over 3 to 5 days per course. Other immunosuppressants were composed of cyclophosphamide, tacrolimus, mycophenolate mofetil and cyclosporine A. In addition, the other treatments included angiotensin-converting enzyme inhibitors, angiotensin II receptor antagonists, or diuretics.

Patients' response was evaluated after 12 weeks induction therapy and was categorized as CR, partial remission (PR), or no response. CR refers to the proteinuria $<$0.5 g/24h or urinary protein-to-creatinine ratio $<$500 mg/g or $<$50mg/mmol, no active urinary sediment, normal albumin level [$\geq$35 g/l], and normal serum creatinine level or an elevation $<$10% of the baseline value. PR refers to a decrease in proteinuria excretion $>$50% of the baseline and the proteinuria $<$3.0 g/24h, serum albumin $>$30g/l with normal serum creatinine level or an elevation $<$10% of the baseline value. No response refers to no CR or PR after the 12-week induction treatment.

Laser Micro-Dissection and MS

Briefly, glomeruli from 19 formalin fixed paraffin-embedded renal biopsy were dissected and collected with LMD6 laser microdissection system (Leica), including 5 isolated PIG, 5 IC-PIG, 5 MCD, and 4 donor kidneys. All donor kidney samples were from preimplantation renal biopsy of donor-after-cardiac death donors. IF results were all negative. Both light microscopy and EM examination were unremarkable. For each formalin fixed paraffin-embedded sample, tissues were sliced with a thickness of 7μm and about 300,000 μm^2 area were dissected for MS. Samples were placed in 40 μl of Tris EDTA buffer and 0.5% sodium deoxycholate detergent. After finishing the 1-minute centrifugation at 10,000g, the glomerular fragments were sonicated with the ultrasonic cleaner for 30 minutes at room temperature after being incubated for 60 minutes at 98 °C. The solubilized proteins were digested into peptides after being incubated with the sequencing-grade trypsin (Promega) overnight. The peptide was desalted with the C18 Ziptip (Millipore) and was vacuum-dried. After being redissolved in 0.1% trifluoroacetic acid, the sample was subjected to LC-MS/MS with the Ultimate 3000 RSLC nanoLC, which was coupled online with the Q Exactive MS (Thermo Scientific). The raw data obtained was transferred to the mgf file with ProteoWizard and was explored through the algorithm Mascot (Matrix Science). The Percolator was used to rescore the peptide-spectrum-match hit, and the script homemade was used to summarize the protein information. Upon recombining the outcomes, they were assigned probability scores of proteins and peptides within the Scaffold software. The false discovery rate was set at 1% for the peptide-to-spectrum matches. The relative abundance of protein was assessed by using its normalized spectral count (SC) with the formula, $NC_i = C_i / \sum^{n}_{k=1} C_k \times 1000$. NC_i serves as the normalized SC of protein i, C_i represents the absolute SC within the MS raw data, and n means the number of each identified proteins.

Double IF Staining and Laser Scanning Confocal Microscope Imaging

Double IF staining of podocyte marker α-actinin 4 and GBM marker COL4A3 was performed on the frozen sections, including 3 isolated PIG and 3 normal donor kidneys. Primary antibody COL4A3 (mouse monoclonal to COL4A3, Santa Cruz, sc-52317) and α-actinin 4 (rabbit monoclonal to ACTN4, Abcam, ab108198) were mixed in a ratio of 1:1 and applied following an hour of incubation at 37 °C. Slides were rinsed with PBS and were then incubated with a 1:1 mixture of the secondary antibody of anti-mouse IgG(H+L), F(ab')2 Fragment (CST, 8890S) and polyclonal swine anti-rabbit immunoglobulins FITC (Dako, F0205) at 37 °C for 40 minutes. The colocalization of ACTN4 and COL4A3 was examined with a Zeiss LSM 880 with Airyscan and was analyzed with the Zeiss Confocal Software (version 2.61; Zeiss, Wetzlar, Germany).

Statistical Analysis

Data were presented as the median (interquartile range) for continuous variables or frequency and percentage for categorical variables. Chi-square test or Fisher's exact test was used for the comparison of disordered variables between groups, and Mann-Whitney U test was used for the comparison of ordered variables. Statistical significance was assessed using 1-way analysis of variance; multiple comparisons were adjusted by LSD and Bonferroni methods. For proteomics statistical hypothesis testing, a 2-sample Welch's *t*-test was performed for each protein using normalized SC for the 2 groups. If a protein was only detected in 1 group, a 1-sample Welch's t-test was performed, using the smallest detected normalized SC as the null hypothesis. A *P*-value of less than 0.05 was taken to be significant. The statistical analyses and graphing were performed using SPSS (version 25.0, IBM, Chicago, IL) and GraphPad Prism (version 9.0, GraphPad Software, San Diego, CA).

RESULTS

Clinical Findings

Out of 116 PIG cases, 89 (76.7%) had positive IF results of immunoglobulin and complements, indicating the presence of immune complexes (IC-PIG), and 27 (23.3%) PIG cases were negative (isolated PIG), as shown in Figure 2a–f. Among the 89 patients with IC-PIG, 63 (70.8%) were clinically diagnosed with SLE, and the renal pathology was consistent with PIG lesion superimposed on LN. The majority of them had membranous changes, including 18 (28.1%) cases of class V and 23 (35.9%) cases of class III/IV plus V. In total, 26 (29.2%) cases in were found to have immune complexes associated glomerulonephritis other than LN, including 12 cases of MN, 3 cases of primary Sjogren's syndrome, 2 cases of IgA nephropathy, 1 case of diffuse large B-cell lymphoma, 1 case of Aicardi-Goutières syndrome type 7,[16] and 7 cases with unknown reasons. In contrast, only 6 out of 27 (22.2%) isolated PIG was clinically diagnosed as SLE, but without any IC deposit in glomeruli. More than half of the patients (51.9%) with isolated PIG had no known underlying disease. Four cases of focal segmental glomerulosclerosis, 1 case of diabetic nephropathy, 1 case of primary Sjogren's syndrome, and 1 case of undifferentiated connective tissue disease were diagnosed with isolated PIG. The spectrum of underlying diseases and the renal biopsy diagnosis of both groups are shown in Figure 3.

Renal Pathology

Renal biopsy findings are presented in Table 1. Detailed pathological and clinical data of isolated PIG are shown in Supplementary Table S1. The PIG lesion was classified into 3 grades (Figure 4a–d). Grade 3 accounted for more than half of all cases (60, 51.7%), which were 49 (55.1%) and 11 (40.7%) in the IC-PIG group and the isolated PIG group, respectively. For the classification of PIG, subtype B and subtype C accounted for more than 90% of the cases in both groups, whereas subtype A was the least. No difference in PIG subtypes and grades were found between the 2 groups. GBM was thickened in not only all of the 89 IC-PIG cases but also the vast majority of isolated PIG cases (25, 92.6%). Diffuse foot process enhancement (>75%) was seen in all of the isolated PIG cases and 79 (88.8%) of the IC-PIG cases. In the IC-PIG group, electronic dense deposits were found mainly in subepithelial, mesangial,

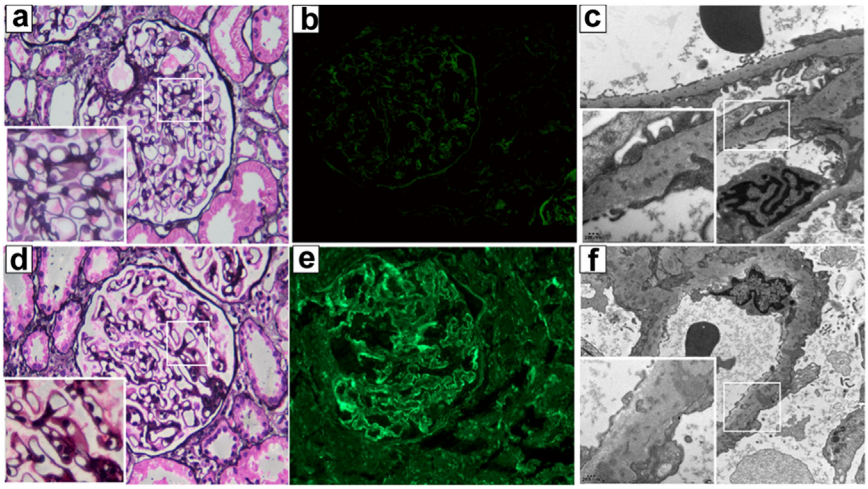

Figure 2. Morphologic features of isolated PIG (a–c) and IC-PIG (d–f). (a) Slight thickening of GBM mimicking MN (PASM, ×400); (b) Negative IgG staining in glomerular capillary wall (IF, ×400) (c) Numerous intramembranous microspheres without identifiable deposition (EM, ×20, 000); (d) Diffuse GBM thickening with subepithelial deposits (PASM, ×400); (e) Granular IgG deposition along the glomerular capillary wall (IF, ×400); (f) Thickened GBM due to a large number of intramembranous microspheres in addition to subepithelial deposition (EM, ×8000). EM, electron microscopy; GBM, glomerular basement membrane; IF, immunofluorescence; LM, light microscopy; PIG, podocyte infolding glomerulopathy.

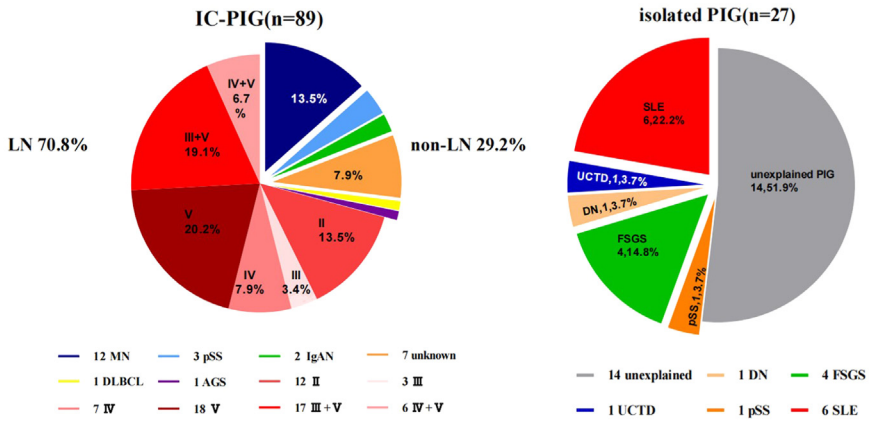

Figure 3. Underlying disease entities of IC-PIG and isolated PIG. AGS, Aicardi-Goutières syndrome type7; DLBCL, diffuse large B-cell lymphoma; DN, diabetic nephropathy; IC-GN, immune complex mediated glomerulonephritis; pSS, primary Sjogren's syndrome; UCTD, undifferentiated connective tissue disease.

or subendothelial & intra-base membranes (69, 80.2%). Only a small portion of the IC-PIG had only subepithelial deposits (8, 9.3%). No electronic dense deposits were observed in the isolated PIG group. Interestingly, it was also found that 44.8% of PIG cases had concurrent endothelial cytoplasm invaginated into GBM in addition to podocyte invagination.

Light microscopy revealed that 101 out of 116 (87.1%) patients with PIG developed mesangial proliferation. Compared to the isolated PIG group, the extent of mesangial proliferation was found to be more prominent in the IC-PIG group ($P < 0.001$). A similar result was seen with endocapillary proliferation as well ($P = 0.005$). There was no difference in global sclerosis, segmental sclerosis, interstitial fibrosis and tubular atrophy, interstitial inflammation, and vascular lesions between the 2 groups.

The IFs of the IC-PIG group are shown in the Supplementary Table S2. It was found that the immunoglobulin IgG (73, 82%), IgA (46, 51.7%), IgM (75, 84.3%), C3 (60, 67.4%), and C1q (48, 53.9%) were positive in the mesangial region and/or capillary wall. The staining positive rates of C3 and C1q in the LN group were higher than those in non-LN group ($P = 0.006$, $P = 0.001$), whereas there was no significant difference in other immunoglobulins. The results of κ and λ IFs were available in 38 PIG cases, showing no light chain restriction (Supplementary Table S3).

Repeated Renal Biopsy

Three patients underwent a second renal biopsy, whose initial biopsies showed MN in case 1 and case 2 and Renal Pathology Society LN classification IV+ V in case 3. All 3 cases presented with NS and normal renal function at the time of biopsy. Cases 1 and 2 received full-dose glucocorticoid therapy, whereas case 3 received full-dose glucocorticoids plus cyclophosphamide. Cases 1 and 3 showed CR of NS after 6 months. Case 1 had a recurrence of NS 1 year and a half later after the withdrawal of therapy. NS recurred in case 2 very soon because the patient stopped the treatment 1 month after he got remission. Case 3 was maintained with a low dose of glucocorticoids and antimalarials, but NS relapsed twice within 7 years. A second biopsy was performed and newly emerging PIG lesions overlapping with membranous changes were found in all 3 cases compared with the first biopsy (Supplementary Figure S3). Two cases developed a minor decrease of IgG IF intensity and 1 case increased from 2+ to 3+. The IF changes in 3 patients with repeated renal biopsy are shown in Supplementary Table S4. For cases 1 and 2, the treatment regimen was adjusted to a low dose of glucocorticoids plus rituximab and the patients achieved PR. Case 3 switched to glucocorticoids plus tacrolimus plus mycophenolate mofetil after the second relapse and got PR of proteinuria and stable renal function.

Clinicopathologic Correlation

Clinical data and follow-up information were retrieved from 61 patients out of the 116 cases including 42 IC-PIG cases and 19 isolated PIG cases. The general information and laboratory findings at the time of renal biopsy are shown in Table 2. There was no difference between the 2 groups in age, sex, serum creatinine, NS rate, and disease duration at the time of renal biopsy. The mean follow-up time of 61 patients was 20.1 ± 1.0 months, 18.4 ± 1.2 months in the IC-PIG group, and 24.0 ± 1.8 months in the isolated PIG group.

Patients with IC-PIG had lower albumin which was 29.88 g/l compared with 33.83 g/l in patients with isolated PIG ($P = 0.042$); however, no significant difference in 24-hour proteinuria was found ($P = 0.129$). The proportion of patients with positive ANA (37, 88.1%), anti-double-stranded DNA antibody (19, 45.2%), and

Table 1. The clinicopathologic findings of patients with PIG ($N = 116$)

Clinicopathologic changes	Total ($N = 116$)	IC-PIG ($n = 89$)	isolated PIG ($n = 27$)	P value[a]
Light microscopy				
Mesangial proliferation, *n* (%)				<0.001
absent	7 (6.0)	1 (1.1)	6 (22.2)	
mild	75 (64.7)	55 (61.8)	20 (74.1)	
moderate	26 (22.4)	26 (29.2)	0 (0.0)	
severe	8 (6.9)	7 (7.9)	1 (3.7)	
Endocapillary hypercellularity, *n* (%)	28 (24.1)	27 (30.3)	1 (3.7)	0.005
Segmental sclerosis, *n* (%)	32 (27.6)	26 (29.2)	6 (22.2)	0.476
Global sclerosis, *n* (%)	62 (53.4)	47 (52.8)	15 (55.6)	0.802
IFTA, *n* (%)				0.063
absent	28 (24.1)	17 (19.1)	11 (40.7)	
mild	71 (61.2)	58 (65.2)	13 (48.1)	
moderate	12 (10.3)	11 (12.4)	1 (3.7)	
severe	5 (4.3)	3 (3.4)	2 (7.4)	
Interstitial inflammation, *n* (%)				0.041
absent	8 (6.9)	4 (4.5)	4 (14.8)	
mild	85 (73.3)	64 (71.9)	21 (77.8)	
moderate	16 (13.8)	16 (18)	0 (0.0)	
severe	7 (6.0)	5 (5.6)	2 (7.4)	
Vascular lesion, *n* (%)				
artery intimal hyperplasia	87 (75.0)	69 (77.5)	18 (66.7)	0.254
Hyaline degeneration	11 (9.5)	7 (7.9)	4 (14.8)	0.280
Electron microscopy				
PIG grade, *n* (%)				0.246
mild	19 (16.4)	12 (13.5)	7 (25.9)	
moderate	37 (31.9)	28 (31.5)	9 (33.3)	
severe	60 (51.7)	49 (55.1)	11 (40.7)	
PIG subtype, *n* (%)				0.725
A	7 (6.0)	5 (5.6)	2 (7.4)	
B	56 (48.3)	42 (47.2)	14 (51.9)	
C	53 (45.7)	42 (47.2)	11 (40.7)	
Microsphere positive, *n* (%)	111 (95.7)	86 (96.6)	25 (92.6)	0.366
Microtubule, *n* (%)	71 (61.2)	58 (65.2)	13 (48.1)	0.112
GBM thickening, *n* (%)	114 (98.3)	89 (100.0)	25 (92.6)	0.010
Diffuse foot process effacement (>75%), *n* (%)	106 (91.4)	79 (88.8)	27 (100.0)	0.068
Cluster formation, *n* (%)	39 (33.6)	29 (32.6)	10 (37.6)	0.651
Endothelial cells invasion	52(44.8)	44 (49.4)	8 (29.6)	0.070
Electronic dense deposits, *n* (%)				
Subepithelial only	8 (9.3)	8 (9.3)	N/A	
Other location[b] & intra-GBM	61 (70.9)	61 (70.9)	N/A	
Mesangial only	17 (19.8)	17 (19.8)	N/A	
Disease entity (SLE), *n* (%)	69 (59.5)	63 (70.8)	6 (22.2)	<0.001

GBM, glomerular basement membrane; IFTA, interstitial fibrosis and tubular atrophy; PIG, podocyte infolding glomerulopathy; SLE, systemic lupus erythematosus.
[a]$P < 0.05$ is considered significant.
[b]Other location, subepithelial, mesangial, or subendothelial; both; or 3.

anti-Smith antibodies (13, 31%) was significantly higher in the IC-PIG group than in the isolated PIG group ($P = 0.001$, $P = 0.027$, and $P = 0.006$). There was no significant difference between the Sjogren's syndrome-related antigen A autoantibodies and the Sjogren's syndrome-related antigen B autoantibodies. Moreover, the rate of decreased C3 and C4 levels was significantly higher in the IC-PIG group than in the isolated PIG group ($P = 0.001$ and $P = 0.049$), and the absolute levels of C3 and C4 were much lower in the IC-PIG group ($P = 0.003$ and $P = 0.002$) as well. Despite no complement deposition within the glomeruli of isolated PIG, a considerable proportion of the patients had a persistent low serum C3 level (4, 31.6%) and C4 level (4, 21.1%) during the follow-up.

The correlation of proteinuria with PIG grades and subtypes was analyzed. In the IC-PIG group, there was no difference among different PIG subtypes in 24-hour proteinuria and the rate of NS (Table 3). However, proteinuria was associated with the grade of the PIG lesion ($P = 0.035$) in isolated PIG, but no difference was found in NS rate. This is likely due to the fact that most of the isolated PIG patients were non-NS with only very few exceptions.

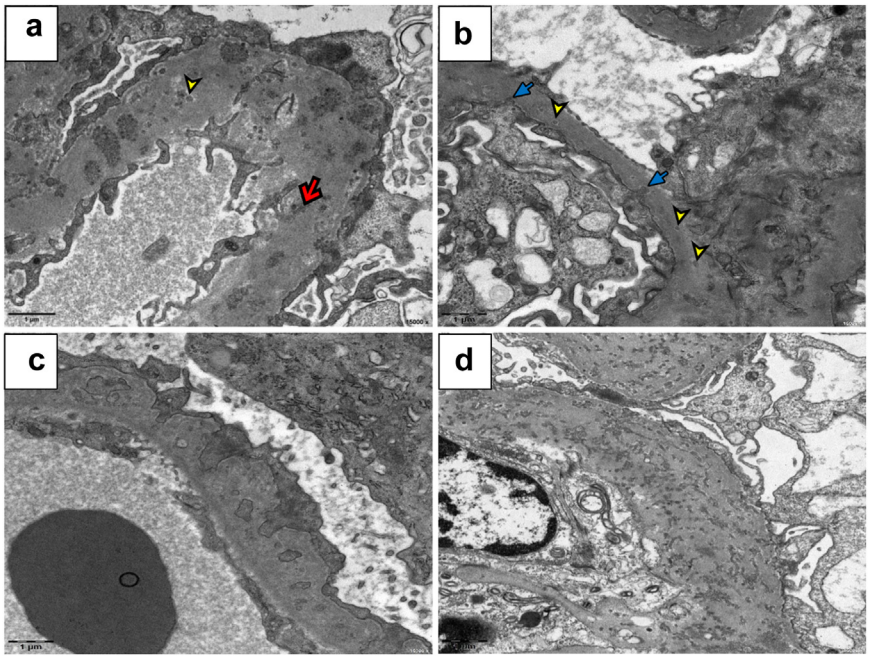

Figure 4. Ultrastructural changes and grading of PIG. (a) Podocyte invaginating into the GBM, forming numerous microspheres (yellow arrow) and microtubules (red arrow); (b) Slightly thickened GBM with mild PIG showing a few cytoplasmic processes of podocytes inverted into the superficial area of GBM(blue arrow) and a few scattered microspheres (c) Moderate PIG showing irregular GBM thickening with numerous cytoplasmic fragments and microspheres extending into about half thickness of the GBM; (d) Severely thickened GBM with florid cytoplasmic protrusions of podocytes forming both microspheres and microtubules involving the whole thickness of GBM (a–d, EM ×15000). EM, electron microscopy; GBM, glomerular basement membrane; PIG, podocyte infolding glomerulopathy.

Treatment and Outcomes

Remission was achieved in 58 (95.1%) patients after induction therapy with glucocorticoids monotherapy (27, 44.3%) or glucocorticoids plus other immunosuppressors (29, 47.5%), including 28 (45.9%) CR and 30 (49.2%) PR. The 3 patients, including 2 IC-PIG patients and 1 isolated patient, showed no response after 12 weeks of induction therapy (Supplementary Tables S5 and S6). There were significant differences in the initial treatment regimens ($P = 0.010$) though CR and

Table 2. Clinical characteristics at the time of biopsy in 61patients with PIG

Clinical and Laboratory Parameters	Total ($N = 61$)	IC-PIG ($n = 42$)	isolated PIG ($n = 19$)	P value[a]
Sex, *n* (%)				0.520
Male	13 (21.3)	8 (19.0)	5 (26.3)	
Female	48 (78.7)	34 (81.0)	14 (73.7)	
Age (years), median (IQR)	38 (29–48)	38 (26–46)	37 (30–51)	0.541
Duration of renal disease, median (IQR), months	6 (1–14)	6 (1–24)	6 (2–12)	0.758
Hematuria, *n* (%)	30 (49.2)	21 (50.0)	9 (47.4)	0.849
Proteinuria, median (IQR), g/24h	2.87 (1.09–4.56)	3.36 (1.33–4.62)	2.33 (0.74–4.32)	0.237
Albumin, median (IQR), g/l	29.90 (25.45–34.35)	29.88 (24.90–33.63)	33.83 (26.60–38.40)	0.042
Creatinine, median (IQR), mg/dl	0.74 (0.54–1.04)	0.77 (0.58–1.10)	0.64 (0.46–0.99)	0.469
Nephrotic syndrome, *n* (%)	29 (47.5)	22 (52.4)	7 (36.8)	0.260
Disease entity (SLE), *n* (%)	39 (63.9)	33 (78.6)	6 (31.6)	0.001
ANA positive, *n* (%)	46 (75.4)	37 (88.1)	9 (47.4)	0.001
A-dsDNA, *n* (%)	22 (36.1)	19 (45.2)	3 (15.8)	0.027
A-Sm, *n* (%)	13 (21.3)	13 (31.0)	0 (0.0)	0.006
A-SSA, *n* (%)	23 (37.7)	18 (42.9)	5 (26.3)	0.217
A-SSB, *n* (%)	7 (11.5)	6 (14.3)	1 (5.3)	0.306
Decreased C3, *n* (%)	40 (65.6)	34 (81.0)	6 (31.6)	0.001
C3, median (IQR), g/l	0.69 (0.46–0.83)	0.63 (0.41–0.76)	0.75 (0.63–0.97)	0.003
Decreased C4, *n* (%)	25 (41.0)	21 (50.0)	4 (21.1)	0.049
C4, median (IQR), g/l	0.14 (0.07–0.22)	0.10 (0.06–0.17)	0.17 (0.17–0.34)	0.002

ANA, antinuclear antibody; A-dsDNA, anti-double-stranded DNA antibody; A-SSA, anti-Sjogren's syndrome-related antigen A autoantibodies; A-SSB, anti-Sjogren's syndrome-related antigen B autoantibodies; A-SM, anti-Smith antibod; IQR, interquartile range; PIG, podocyte infolding glomerulopathy.
[a]$P < 0.05$ is considered significant.

Table 3. Correlation between the grade and subtype of PIG and proteinuria ($N = 61$)

Classification	IC-PIG ($n = 42$)		isolated PIG ($n = 19$)	
	NS[b]	Proteinuria	NS[b]	Proteinuria
Subtype (total), *n* (%), mean ± SD	22 (52.4)	4.17 ± 4.37	7 (36.8)	2.85 ± 2.63
A	2 (9.1)	6.43 ± 7.44	1 (14.3)	5.14
B	12 (54.5)	3.66 ± 3.12	5 (71.4)	3.05 ± 2.83
C	8 (36.4)	4.43 ± 5.40	1 (14.3)	2.08 ± 2.33
P value[a]	1.000	0.569	0.310	0.540
Grade (total), *n* (%), mean ± SD	22 (52.4)	4.15 ± 4.37	7 (36.8)	2.85 ± 2.63
mild	2 (9.1)	3.20 ± 2.60	2 (28.6)	1.77 ± 1.21
moderate	6 (27.3)	3.55 ± 4.19	3 (42.8)	5.70 ± 2.30
severe	14 (63.6)	4.71 ± 4.83	2 (28.6)	2.45 ± 3.04
P value[a]	0.556	0.658	0.286	0.035

NS, nephrotic syndrome; PIG, podocyte infolding glomerulopathy.
[a]$P < 0.05$ is considered significant. NSb, proteinuria > 3.5 g/24h.

PR rates were similar in the 2 groups. The majority of patients with isolated PIG (12, 63.2%) received a standard dose of glucocorticoids treatment, whereas most patients with IC-PIG (25, 59.5%) were treated with glucocorticoids plus other immunosuppressors, such as the cyclophosphamide, calcineurin inhibitors, mycophenolate mofetil, or rituximab. Four patients with IC-PIG were treated with glucocorticoid pulse therapy at the early stage.

No difference in treatment response among 3 subtypes of PIG was found ($P = 0.342$); however, there was a significant difference in terms of the PIG grades ($P = 0.001$). All of the mild PIG cases were found to respond well to the treatment with CR in a majority of patients and a few PRs, whereas severe patients with PIG had the lowest CR and PR rates. This indicates that the higher the grade of PIG, the less sensitive it is to the treatment with glucocorticoids and immunosuppressors (Table 4).

Laser Scanning Micro-Dissection and MS

Renal biopsy tissue from 5 isolated PIG, 5 IC-PIG, 5 MCD and 4 donor kidneys were randomly selected for the MS analysis. The top 100 proteins were identified and analyzed across the specimens respectively (Figure 5). They are composed of podocyte cytoskeletal proteins (ACTN4, ACTB, ACTC, and VIME), components of basement membranes (LAMA5, LAMB2, and PGBM), and albumin, etc. The heat map showed the normalized expression levels (relative quantitative values) of each protein among the 4 groups (Figure 5a). Volcano plots were used to find differentially expressed genes among PIG, MCD, and healthy controls (Figure 5b–f). The gene ontology (data not shown) and Kyoto Encyclopedia of Genes and Genomes pathway enrichments of differentially expressed genes were performed by DAVID online analyses[17] (Figure 5g and h). Regulation of actin cytoskeleton signaling pathway was screened out as a commonly enriched pathway. This indicated that dysregulation of actin cytoskeleton may be related to the pathogenesis of PIG.

Double staining of ACTN4 and COL4A3 showed that they were expressed separately in podocytes and the GBM, respectively in control, whereas the expression of ACTN4 was more scattered and colocalized with COL4A3 in some segments in PIG cases (Figure 6) which confirmed that ACTN4, normally expressed on the podocyte, shifted into the GBM in PIG.

Table 4. Correlations between pathological features and response in 61 patients with PIG

Classification	Remission rate				*P* value[a]
	total	CR	PR	no response	
Subtype, *n* (%)					0.342
A	5 (8.2)	1 (3.6)	3 (10.0)	1 (33.3)	
B	34 (55.7)	15 (53.6)	18 (60.0)	1 (33.3)	
C	22 (36.1)	12 (42.9)	9 (30.0)	1 (33.3)	
Grade, *n* (%)					0.001
mild	13 (21.3)	11(39.3)	2 (6.7)	0 (0.0)	
moderate	18 (29.5)	10 (35.7)	6 (20.0)	2 (66.7)	
severe	30 (49.2)	7 (25.0)	22 (73.3)	1 (33.3)	

CR, complete remission; PIG, podocyte infolding glomerulopathy; PR, partial remission.
[a]$P < 0.05$ is considered significant.

DISCUSSION

This study reported a total of 116 patients who presented with kidney injury and ultrastructurally revealed PIG lesions. PIG occurred independently or accompanied with immune complexes, and therefore the 2 groups of PIG, IC-PIG and isolated PIG were identified. The relatively higher incidence of IC-PIG is consistent with previous reports.[3–9,12–14,18–22] LMD/MS identified the protein ACTN4, which serves as a significant actin crosslinking cytoskeletal protein for maintaining normal podocyte structure, dramatically decreased than in the donor kidney group. Confocal microscope found that the ACTN4 and COL4A3 colocalized, which further verified that the GBM

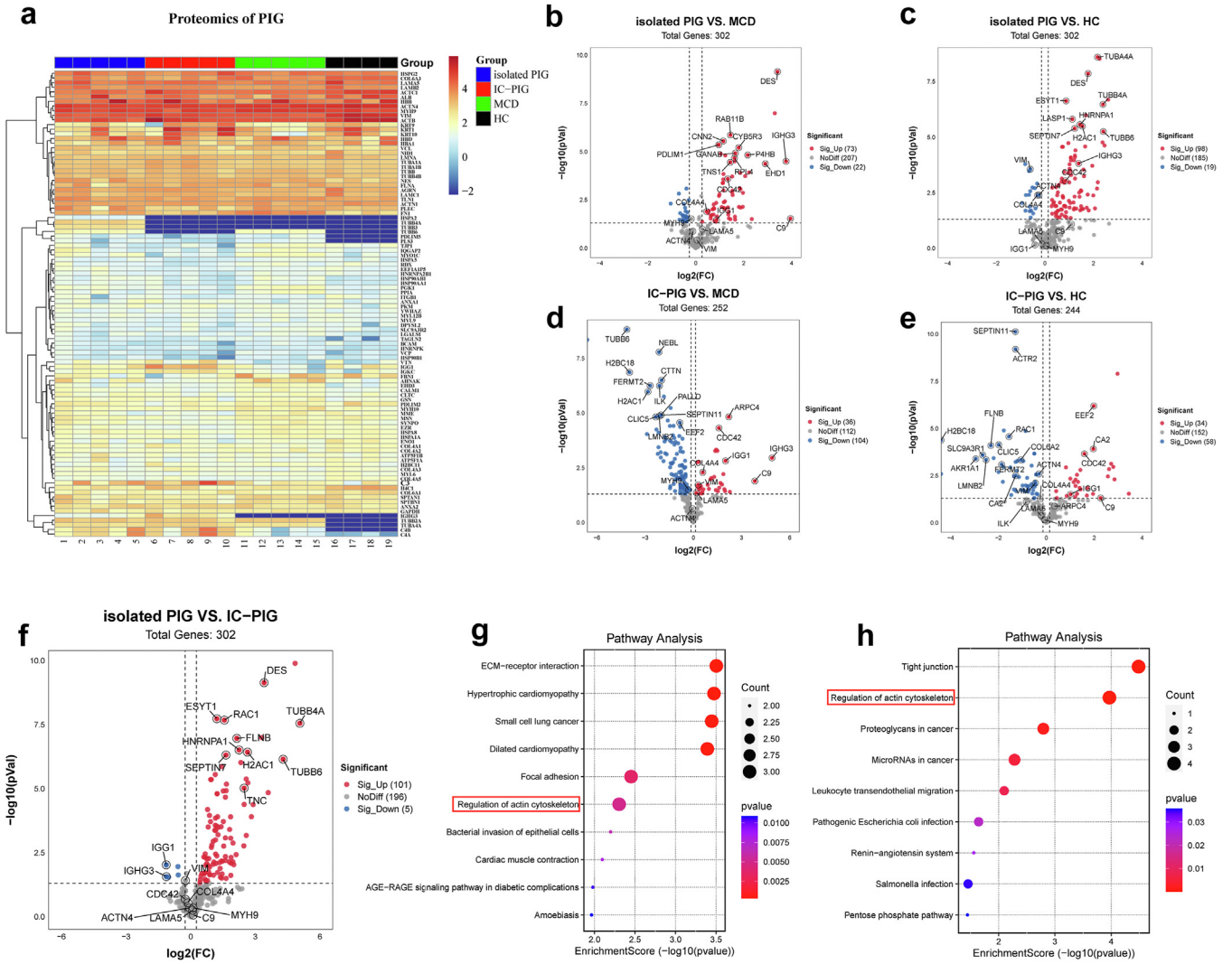

Figure 5. Laser microdissection and mass spectrometry. (a) Normalized SCs of the top 100 proteins are presented as heatmap. Protein abundance of each group is labeled by column, and protein name is labeled by row; Volcano plots showing the differentially expressed proteins between the isolated PIG vs. MCD (b) and isolated PIG vs. Healthy Control (c); IC-PIG vs. MCD (d); IC-PIG vs. Healthy Control (E); and isolated PIG vs. IC-PIG (F); The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichments of differentially expressed genes (DEGs) were performed by DAVID online analyses. Panel G showed KEGG enrichment analysis of common DEGs between the isolated PIG vs. MCD and isolated PIG vs. Healthy Control; Panel H showed KEGG enrichment analysis of common DEGs between the IC-PIG vs. MCD and IC-PIG vs. Healthy Control respectively.
IC-PIG, PIG coexisting with immune-complex associated glomerulonephritis; MCD, minimal change disease; PIG, podocyte infolding glomerulopathy; SC, spectral counts.

microstructures visualized in EM originated from the podocyte.

Our results reveal that the most frequent underlying disease of PIG was SLE, as reported in a previous study.[6] Other non-LN glomerulonephritis, such as primary MN, IgA nephropathy, etc., were also found in our case series and reported in previous studies.[1,3,4,6,8] In terms of the classification of these SLE cases, class V was the most frequent subtype and some were accompanied by various degrees of mesangial and endocapillary proliferation. This increases the possibility that PIG, as part of podocyte cytoplasm invagination in GBM, is likely to be induced by the membranous lesion due to the persistent subepithelial deposition.[23] The EM confirmed that in addition to other sites in glomeruli, 69 (77.5%) of 89 cases had subepithelial or intramembranous deposits. This possibility is even strengthened because the 3 cases with the first renal biopsy showing membranous changes demonstrated newly emerging PIG lesions in the subsequent renal biopsy when NS relapsed. However, the mechanism of the cellular fragments of podocyte invasion into GBM remains unclear.[7] It was found that the membrane attack complex was positive in GBM,[24] suggesting intra-GBM complement activation, which may attract cells to move into GBM.[7,24] These findings

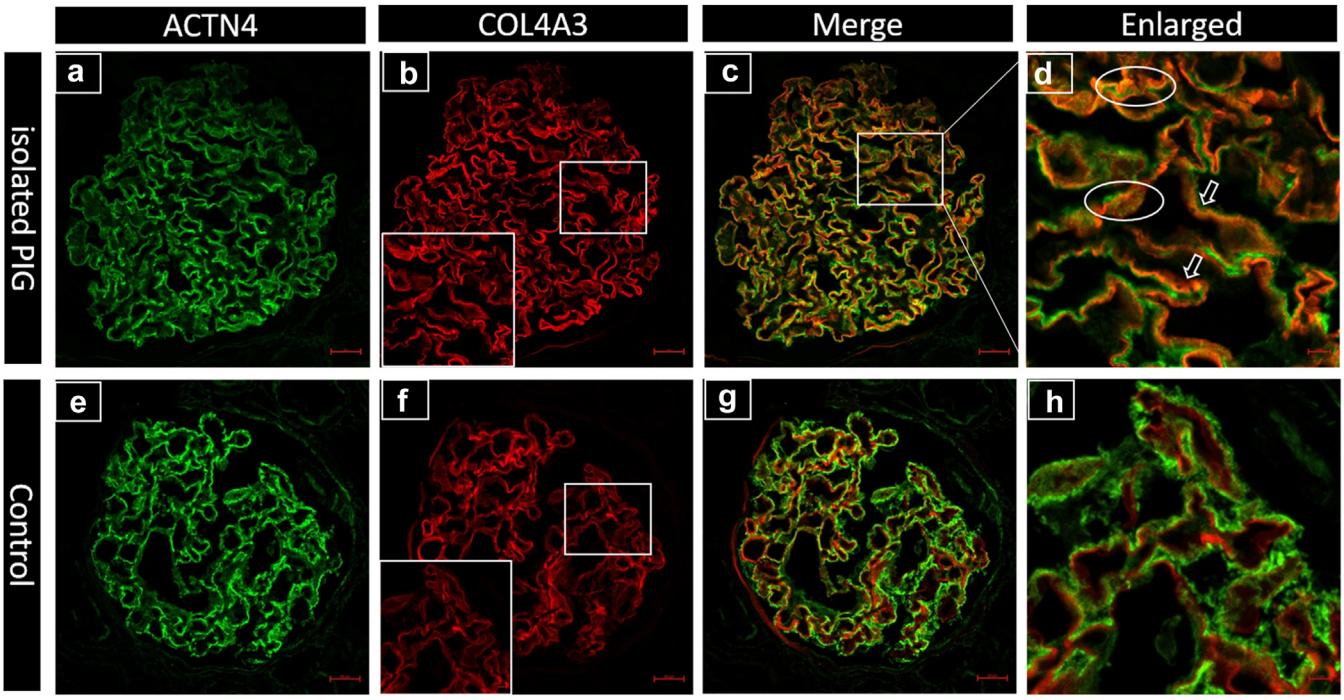

Figure 6. Double staining of ACTN4 and COL4A3 in PIG glomeruli. (a–d) isolated PIG, (e–h) control. ACTN4 (green) and COL4A3 (red) were expressed separately in normal donor kidney (h) whereas colocalization of ACTN4 and COL4A3 was found in some segments of PIG (circles in d). Craters were easily found in isolated PIG (arrows in d). ACTN4, α-actinin4; PIG, podocyte infolding glomerulopathy.

indicate that complement activation may play a role in the podocyte damage of IC-PIG.[7]

Isolated PIG had no immunoglobulin or complement deposition but is also occasionally reported to be associated with lupus.[5] In our series, SLE accounted for a small portion (22.2%) of the isolated PIG cases, and the majority of patients had no known underlying disease compared to IC-PIG. These findings indicated that isolated PIG is a rather rare and peculiar type of podocyte injury in SLE, whose morphology is quite different from so-called lupus podocytopathy characterized by diffuse foot process enhancement with no or minimal depositions.[25] Although only a minority of isolated PIG was clinically diagnosed as SLE, a considerably high rate of ANA positivity (63.2%) was found, therefore indicating that the injury is possibly associated with autoimmune. Whether these cases with isolated PIG and positive ANA will progress to SLE is unknown. Fortunately, our results revealed that isolated PIG lesion is quite sensitive to glucocorticoids, which are similar to lupus podocytopathy.[26] Previous repeat biopsies have shown that the PIG lesion is reversible to some degree because the reduction of characteristic microstructures was found.[6] The exact mechanisms of these peculiar podocytic infolding lesions are unclear, and hydronephrosis is reported to be a possible reason.[20] However, this is not the case in our case series.

Proteinuria is the most prominent manifestation in patients with PIG in both groups. Despite the similar manifestation, the contribution of PIG to proteinuria may be different. In the IC-PIG group, there was no difference in the level of proteinuria among different subtypes and grades of PIG, therefore indicating that the key mechanism of the proteinuria was the membranous change caused by deposits, and the PIG lesion may serve as an accompanied scenario. In contrast, proteinuria in isolated PIG increased with grades but had no correlation with PIG subtypes. Accordingly, the degree of GBM damage caused by PIG may be the main cause of proteinuria rather than the morphological subtypes of PIG, although the rate of NS had no significant difference among different grades of isolated PIG. To our knowledge, this is the first report to describe the degree of PIG lesion correlated with the severity of proteinuria.

The pathogenesis of PIG lesions remains to be identified. According to the results of LMD/MS, some of the cytoskeleton protein of podocyte decreased significantly compared to normal and MCD glomeruli. The possibility is that the injuries of podocytes lead to dysfunction and loss of stability of the cytoskeleton, thus causing its invasion into the GBM.[27] In addition, the invagination of endothelial cells was found in EM, which was a phenomenon previously described by Japanese researchers using serial EM.[7] This may suggest that endothelial cells could also be influenced during podocyte migration, apart from the crosstalk between podocyte and GBM.

Presently, there is no standard treatment for PIG. In our case series, treatment of IC-PIG mainly focused on the underlying diseases, most of which were SLE. Three cases whose relapsed NS were confirmed to have newly emerged PIG lesions in the subsequent biopsy only achieved PR; whether PIG related to proteinuria relapse and affected the treatment response was unknown.

Although being the largest case series of PIG to date and detailed morphological changes and clinicopathological correlation were analyzed in this study, there are limitations. First, this study was a retrospective study, 55 of the 116 patients included were lost to follow-up, therefore our results may not completely represent the full clinical profile of PIG cases. Second, the 61 patients with clinical follow-up data came from multiple centers with different treatment regimens, which compromised our clinical outcome analysis. Third, more disease controls, such as PLA2R-associated MN and lupus class V patients without PIG are beneficial in MS for the exploration of PIG pathogenesis.

In conclusion, our findings suggest that PIG is a special type of podocyte injury occurring independently or concurrently with other immune complex-mediated GN. IC-PIG and isolated PIG have different underlying diseases because most of IC-PIG was SLE, whereas the cause of majority of isolated PIG is unknown. PIG grades rather than subtypes were associated with proteinuria in isolated PIG. The dysregulated function of cytoskeletal proteins may play a role in PIG, whereas the exact mechanism remains to be discovered.

DISCLOSURE

The authors have declared no conflicting of interest.

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AUTHOR CONTRIBUTIONS

LH collected and analyzed clinical data, interpreted the results, and drafted the manuscript. HW and QW reviewed the clinical data and statistical results. LW collected and analyzed pathological data. ShuY and XH reviewed the pathological data and confirmed the pathological diagnosis. ShiY and TC helped to analyze all the data. ZZ provided the overall direction of the series of studies and supervised the study. WC conceived and designed the study, interpreted the results, and revised the manuscript.

SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Figure S1. Different substructures of PIG.

Figure S2. A working model of PIG lesion grading.

Figure S3. PIG lesion was found in repeat biopsy of 3 cases of IC medicated glomerulonephritis.

Table S1. Clinical data of isolated PIG ($n = 27$).

Table S2. The immunofluorescence findings of patients with IC-PIG ($n = 89$).

Table S3. The results of immunofluorescence in 38 PIG cases with κ and λ.

Table S4. Changes of immunofluorescence staining intensity in 3 cases of repeated renal biopsy.

Table S5. Treatment response of 2 groups of PIG ($n = 61$).

Table S6. Results of response rate at week 24 in 2 groups (induction treatment).

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