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Role of Iron in Children With Immunoglobulin A Nephropathy and Macrohematuria-Induced Acute Kidney Injury

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Introduction: The role of iron in, and the prognosis of, pediatric Immunoglobulin A nephropathy (IgAN) with macrohematuria (MH)-induced acute kidney injury (AKI) (MH-AKI) have not been evaluated. Thirty percent of adults with MH-AKI, and especially those who are older, show progression to chronic kidney disease.

Methods: We evaluated the immunohistopathologic characteristics of renal biopsy samples from pediatric patients with MH-AKI IgAN and controls, using Berlin Blue to identify iron, CD163 (a hemoglobin-scavenging receptor), and CD68 (a total macrophage marker), then compared the findings against the clinical characteristics of the patients.

Results: We enrolled 44 children as follows: 19 with IgAN but no MH or AKI; 5 with IgAN and MH but no AKI ($MH^{(+)}AKI^{(-)}$ IgAN); 11 with MH-AKI IgAN; and 9 with no IgAN, MH, or AKI, according to a renal biopsy. Berlin Blue staining was detected predominantly at the injured tubulointerstitium, and the areas of staining in children with $MH^{(+)}AKI^{(-)}$ and MH-AKI IgAN were significantly more extensive. The areas of Berlin Blue and CD163 staining did not perfectly match; however, areas of Berlin Blue were surrounded by immunopositivity for CD163. No children with MH-AKI IgAN showed decreased renal function at their last visit.

Conclusion: Children with IgAN and MH, with or without AKI, showed considerable iron deposition in their renal tubules. CD163-positive cells might scavenge hemoglobin in patients with MH-AKI IgAN, but not their roles as macrophages. The renal prognosis of pediatric MH-AKI IgAN is good.

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Hematuria and proteinuria are common clinical findings in patients with heterogeneous glomerulonephritis. MH, a complication often experienced by patients with this type of nephritis, is derived from glomerular lesions and composed of massive numbers of red blood cells (RBCs) into the urine. Previous studies have shown that adult IgAN that features MH progresses to AKI, and in up to 25% of patients, and especially in older patients with MH-AKI, their serum creatinine (Cr) concentrations do not return to baseline.[1,2]

Tubulointerstitial injury is the main histopathological finding in MH-AKI, with mild mesangial proliferation and no obvious crescentic lesions in the

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glomeruli.[2-4] It was initially suggested that the progression of AKI in response to MH occurs because of acute tubular necrosis secondary to the obstruction of tubules by RBC casts.[1] Indeed, histologic studies have shown that lesions of acute tubular necrosis can be seen in tubules filled with RBC casts.[5,6] However, these findings are not apparent in all the tubules in renal biopsies from patients with MH-AKI, and damage to tubules can be seen in the absence of obstruction by RBCs.[7,8] Therefore, the pathophysiology of AKI associated with MH cannot be explained by RBC-mediated tubular obstruction alone.

Hemolysis, regardless of etiology, is one component of the pathophysiologic mechanism underpinning MH-AKI and involves the release of iron, hemoglobin, and other molecules that may have toxic effects in tubules, either individually or in combination.[9] Iron is toxic for a range of organs, including the kidney and liver, where it can induce oxidative stress, causing damage to hepatocytes.[10,11] In addition, renal immunostaining has demonstrated that the expression of CD163, a scavenger receptor for hemoglobin that is expressed by macrophages, is high in the sections of tubules that are occupied by RBC casts and where tubulointerstitial injury is present.[6] The binding of hemoglobin to CD163 also causes the activation of antiinflammatory pathways.[12] Although a previous study of a case of IgAN with MH-AKI showed that hepcidin, CD163, and markers of oxidative stress are expressed in areas of tubulointerstitial injury, there have been no comparative histopathologic evaluations of the kidneys of patients with and without MH and AKI. Moreover, the previous studies have been performed in adults, and there have been no evaluations of children with IgAN and MH-AKI that involved characterization of the histopathologic and clinical features of the patients.

Here, we aimed to characterize the renal deposition of iron and expression of macrophage markers in children with or without IgAN, MH, and AKI, and to compare their clinical characteristics and prognosis.

METHODS

Study Design and Participants

We conducted a retrospective multicenter study to evaluate the clinical and renal immunohistopathologic characteristics of children with biopsy-confirmed glomerulonephritis who were aged <15 years at the time of renal biopsy. We recruited patients from the following 10 hospitals: Takatsuki General Hospital, Kobe University Hospital, Kakogawa Central City Hospital, Himeji Red Cross Hospital, Hyogo Prefectural Kobe Children's Hospital, Dokkyo Medical University Saitama Medical Center, Saiseikai Takaoka Hospital, Ryukyus University Hospital, Wakayama Medical University Hospital, and National Hospital Organization Hokkaido Medical Center. We used the patients' electronic medical records to collect their clinical information. We then compared the clinical characteristics and renal immunohistopathologic findings of the following 4 groups: children with IgAN who had neither MH nor AKI at the time of renal biopsy ($MH^{(-)}AKI^{(-)}$ IgAN), those with IgAN who had MH but no AKI at this time ($MH^{(+)}AKI^{(-)}$ IgAN), those with IgAN who had MH-AKI at this time (MH-AKI IgAN), and those who showed diffuse mesangial proliferation but did not have IgAN and had neither MH nor AKI at this time ($MH^{(-)}AKI^{(-)}$ non-IgAN). Although IgAN has been demonstrated to be associated with the severity of macrophage infiltration, regardless of the presence or absence of MH or AKI, we thought it necessary to include patients without IgAN as the control group. In addition, patients with secondary IgAN were excluded from the study.

Ethics Approval

All the procedures performed in the study that involved human participants were conducted in accordance with the ethical standards set by the Ethics Board of Takatsuki General Hospital, where the study was conducted (approval no. 2020-1), and with the Declaration of Helsinki and its later amendments or comparable ethical standards.

Consent to Participate/Consent to Publish

Written informed consent was obtained from all enrolled patients and their parents prior to renal biopsy. The ethics committee waived the requirement for the patients' informed consent to publish their details. The study protocol was displayed publicly on a poster at each hospital, and all patients and their parents had the option to refuse to be included in the study.

Clinical and Pathologic Definitions

We calculated the estimated glomerular filtration rate (eGFR) of each patient using their serum Cr and height.[13] AKI was defined as an increase in serum Cr concentration to at least 2 times the baseline value, which is equivalent to stages 1 to 3 of the Kidney Disease: Improving Global Outcomes classification. We assessed the severity of hematuria by counting the number of RBCs per high-power microscopic field in a single centrifuged morning urine sample or measuring the erythrocyte excretion in uncentrifuged urine. MH was identified using red, black, or dark brown discoloration of the urine, accompanied by the presence of massive numbers of RBCs in the urine sediment. Fewer than 5 RBCs per high-power field or <10/μl at 2 consecutive measurements was regarded as indicating

the absence of hematuria. The urine protein concentration was also measured to calculate the urine protein-to-Cr (P/C) ratio or the severity of proteinuria was estimated using a dipstick in a single morning sample. A urine P/C ratio of <0.2 g/g Cr or a negative protein finding on dipstick testing on 2 consecutive occasions was defined as indicating no proteinuria. Nephrotic syndrome was defined using the presence of hypoalbuminemia (serum albumin concentration ≤2.5 g/dl) and proteinuria in the nephrotic range (urine P/C ratio >2.0 g/g Cr). Renal biopsies were performed in children with hematuria in addition to nephrotic syndrome or AKI and in those with persistent proteinuria (early morning urine P/C ratio of ≥1.0 g/g Cr for ≥1 month or ≥0.2 g/g Cr for ≥3 months). Pediatric nephrologists performed renal biopsies at each institute. Histologic diagnoses were made by the pathologists at each hospital. Children with IgAN who exhibited pathologically diffuse mesangial proliferation, nephrotic syndrome, or AKI underwent combination therapy with steroids, immunosuppressants (azathioprine, mizoribine, and/or cyclosporine), and angiotensin-converting enzyme inhibitors or angiotensin receptor blockers. Children who did not meet these criteria typically underwent treatment with an angiotensin-converting enzyme inhibitor or an angiotensin receptor blockers.

Immunohistochemistry

We performed immunohistochemical staining of paraffin-embedded or frozen sections of renal biopsy samples. The deposition of iron was evaluated by staining with Berlin Blue. Immunostaining for CD163 (Leica Biosystems, UK, Clone 10d6) was performed because it is both a hemoglobin scavenger receptor and a marker of specific macrophages, and immunostaining for CD68 (DakoCytomation, Denmark, Clone PG-M1) was used as a marker of total macrophage (Supplementary Table S1).

All types of deposits, including those of IgG, IgA, IgM, C1q, C3, C4, and fibrinogen, were scored using a scale from 0 to 3+, with 0 and trace (0.5+) being regarded as negative and ≥1+ as positive.

Quantification of Iron Deposition and the Expression of Proteins of Interest on Kidney Sections

We captured images of the Berlin Blue staining and CD68 and CD163 immunostaining of kidney sections using an Aperio AT2 scanner (Leica Biosystems, Tokyo, Japan) and calculated the areas of staining using Image Scope Positive Pixel Count v9 (Leica Biosystems).

Statistical Analysis

We performed statistical analyses using JMP version 11.0 (SAS Institute, Cary, NC). We used the Wilcoxon rank-sum test to evaluate the relationships between categorical and continuous data and Fisher exact test to evaluate the relationships between 2 sets of categorical data. All data are expressed as median (interquartile range) and statistical significance was accepted when $P < 0.05$.

RESULTS

Clinical Characteristics of the Participants at the Time of Renal Biopsy and During Therapy

We enrolled 44 children consisting of the following: 19 with $MH^{(-)}AKI^{(-)}$ IgAN, 5 with $MH^{(+)}AKI^{(-)}$ IgAN, 11 with MH-AKI IgAN, and 9 with $MH^{(-)}AKI^{(-)}$ non-IgAN (Table 1). The median age of the participants at the time of renal biopsy was 12.0 years. The age and serum IgA concentrations of the participants with $MH^{(-)}AKI^{(-)}$ non-IgAN at the time of renal biopsy were significantly lower than those of the other participants. In addition, the early morning urine P/C ratio of the participants with $MH^{(-)}AKI^{(-)}$ non-IgAN was higher than that of the other participants. Most of the participants with $MH^{(-)}AKI^{(-)}$ non-IgAN underwent renal biopsy because they had nephrotic syndrome with hematuria and were diagnosed as having pathologically diffuse mesangial proliferation that was not IgAN-related. In addition, the early morning urine P/C ratio tended to be higher in children with $MH^{(+)}AKI^{(-)}$ IgAN than in the other children (approximately 4 g/g Cr), although this difference was not statistically significant. In some of the participants with $MH^{(+)}AKI^{(-)}$ IgAN, renal biopsy was performed during a temporary exacerbation of MH and proteinuria because of viral infection. These included children whose proteinuria resolved without immunosuppressive treatment and those who were diagnosed with nephrotic syndrome at the time of renal biopsy.

The participants with MH-AKI IgAN had significantly higher serum blood urea nitrogen and Cr concentrations and significantly lower Cr-eGFR than the other participants except for those with MH-AKI IgAN. There was no significant difference in the duration of MH prior to renal biopsy between the children with $MH^{(+)}AKI^{(-)}$ and those with MH-AKI IgAN, both of whom had MH on the day of biopsy. In addition, 7 (36.8%), none (0%), 1 (9.1%), and none (0%) of the children in the $MH^{(-)}AKI^{(-)}$ IgAN, $MH^{(+)}AKI^{(-)}$ IgAN, MH-AKI IgAN, and $MH^{(-)}AKI^{(-)}$ non-IgAN groups, respectively, had experienced a previous episode of MH before undergoing the biopsy. Two of the children with MH-AKI IgAN underwent

Table 1. Clinical characteristics of the participants at the time of renal biopsy and during treatment

Characteristics	IgAN			
	$MH^{(-)}AKI^{(-)}$ IgAN $n = 19$	$MH^{(+)}AKI^{(-)}$ IgAN $n = 5$	MH-AKI $n = 11$	$MH^{(-)}AKI^{(-)}$ non-IgAN $n = 9$
Background at renal biopsy				
Boy:Girl	9:10	1:4	7:4	5:4
Age (yr)	13.0 (9.9–14.9)[a]	10.7 (7.8–12.5)[b]	12.2 (9.5–14.2)[c]	3.8 (1.9–7.0)[a,b,c]
Systemic BP (mm Hg)	104.0 (100.0–117.0)	107.0 (96.5–111.5)	110.0 (102.0–117.0)	104.0 (97.0–117.0)
Diastolic BP (mm Hg)	62.0 (55.0–68.0)	67.0 (62.5–76.0)	60.0 (59.0–70.0)	54.0 (45.0–64.0)
The number of children with hypertension, *n* (%)	1 (5.3%)	1 (20.0%)	2 (18.2%)	3 (33.3%)
Serum IgA level (mg/dl)	239 (178–279)[a]	218 (134–257)[b]	260 (177–369)[c]	76 (60–107)[a,b,c]
Serum BUN level (mg/dl)	12.1 (11.0–14.3)[d]	11.5 (9.4–14.6)[e]	24.0 (14.2–37.6)[d,e,c]	13.5 (8.2–20.6)[c]
Serum Cr level (mg/dl)	0.52 (0.45–0.55)[d,a]	0.45 (0.42–0.49)[e,b]	1.0 (0.56–1.26)[d,e,c]	0.33 (0.21–0.39)[a,b,c]
Cr-eGFR (ml/min per 1.73 m^2)	108.5 (97.3–128.7)[d]	113.0 (97.8–119.9)[e]	58.9 (22.4–73.1)[d,e,c]	126.3 (91.7–129.9)[c]
The number of children with proteinuria, *n* (%)	19 (100%)	5 (100%)	11 (100%)	9 (100.0%)
Early morning urine protein-to-Cr ratio (g/g Cr)	0.61 (0.36–1.32)[a]	4.1 (1.87–6.3)	1.07 (0.36–1.54)[c]	9.1 (4.6–41.0)[a,c]
The number of children with micro or macro hematuria, *n* (%)	19 (100%)	5 (100%)	11 (100%)	9 (100.0%)
The duration of macrohematuria (yr)[f]		7.0 (3.5–37.5)	8.0 (5.0–26.0)	
Treatment				
Methylprednisolone pulse therapy, *n* (%)	0 (0%)	0 (0%)	2 (18.2%)	0 (0%)
Oral prednisolone, *n* (%)	10 (52.6%)[a]	4 (80.0%)	9 (81.9%)	9 (100.0%)[a]
Immunosupressant (azathioprine, mizoribine), *n* (%)	10 (52.6%)	4 (80.0%)	7 (63.7%)	3 (33.3%)
Angiotensin-converting enzyme inhibitors or angiotensin receptor blockers, *n* (%)	17 (89.5%)	5 (100%)[b]	7 (63.7%)	3 (33.3%)[b]

BP, blood pressure; BUN, blood urea nitrogen; Cr, creatinine; Cr-eGFR, creatinine-estimated glomerular filtration rate; $MH^{(-)}AKI^{(-)}$ IgAN, children with IgA nephropathy who had neither macrohematuria nor acute kidney injury at the time of renal biopsy; $MH^{(+)}AKI^{(-)}$ IgAN, children with IgA nephropathy who had macrohematuria but not acute kidney injury at the time of renal biopsy; MH-AKI IgAN, children with IgA nephropathy who had macrohematuria-induced acute kidney injury at the time of renal biopsy; $MH^{(-)}AKI^{(-)}$ non-IgAN, children who demonstrated pathologically diffuse mesangial proliferation with an etiology other than IgAN and who had neither MH nor AKI at the time of renal biopsy.
[a]$P < 0.05$ for $MH^{(-)}AKI^{(-)}$ and $MH^{(-)}AKI^{(-)}$ non-IgAN.
[b]$P < 0.05$ for $MH^{(+)}AKI^{(-)}$ and $MH^{(-)}AKI^{(-)}$ non-IgAN.
[c]$P < 0.05$ for MH-AKI IgAN and $MH^{(-)}AKI^{(-)}$ non-IgAN.
[d]$P < 0.05$ for $MH^{(-)}AKI^{(-)}$ and MH-AKI IgAN.
[e]$P < 0.05$ for $MH^{(+)}AKI^{(-)}$ and MH-AKI IgAN.
[f]Evaluated $MH^{(+)}AKI^{(-)}$ and MH-AKI IgAN children with macrohematuria.

methylprednisolone pulse therapy, 9 of them were administered oral prednisolone, 7 were administered immunosuppressants (azathioprine and/or mizoribine), and 7 underwent angiotensin-converting enzyme inhibitor or angiotensin receptor blocker treatment.

Histologic Examination

In Table 2, we summarize the histologic findings obtained from the renal biopsies. All the children with MH-AKI IgAN showed desquamation of epithelial cells, indicating tubuloepithelial injury. Acute tubular necrosis was identified in 3 children with MH-AKI IgAN, but no participants in this group had crescentic IgAN. Nine children with MH-AKI IgAN showed histologic cast nephropathy, in which RBC casts occluded the renal tubules; however, these lesions were present in some but not all the tubules. The children with histologic cast nephropathy exhibited injuries to the tubules and interstitium, but no obstruction by RBCs.

None of the participants had glomerulosclerosis. The percentage of the glomeruli associated with crescents and the proportion of children with fibrocellular crescents, interstitial fibrosis, and mesangial IgA and C3 deposition were significantly higher in the $MH^{(-)}AKI^{(-)}$, $MH^{(+)}AKI^{(-)}$, and MH-AKI IgAN groups than in the $MH^{(-)}AKI^{(-)}$ non-IgAN group. The Oxford classification of the nephropathy did not significantly differ among the $MH^{(-)}AKI^{(-)}$, $MH^{(+)}AKI^{(-)}$, and MH-AKI IgAN groups. Electron microscopy showed that none of the participants had thin basement membranes.

Berlin Blue staining of kidney sections from children with MH-AKI IgAN showed no iron deposition in the tubules occluded by RBC casts, but a great deal of deposition in the injured tubules and interstitium (Figures 1 and 2). In all 4 groups, there was no Berlin Blue staining of any of the glomeruli (Figure 3). The areas of Berlin Blue staining on sections from children with MH-AKI IgAN were significantly more extensive than on those from children with $MH^{(-)}AKI^{(-)}$ IgAN and $MH^{(-)}AKI^{(-)}$ non-IgAN (Figure 4a). In addition, the area of staining in kidneys from children with $MH^{(+)}AKI^{(-)}$ IgAN was primarily in the injured tubules and interstitium, as in those from children with MH-AKI IgAN, and was significantly more extensive than that observed for children with $MH^{(-)}AKI^{(-)}$ IgAN and $MH^{(-)}AKI^{(-)}$ non-IgAN. There was no CD163 immunostaining in the glomeruli of any of the 4 groups (Figure 3). As shown on serial kidney sections (Figure 2), there was strong CD163 staining in the

Table 2. Renal histologic findings

Characteristics	IgAN			$MH^{(-)}AKI^{(-)}$ non-IgAN $n = 9$
	$MH^{(-)}$ $AKI^{(-)}$ IgAN $n = 19$	$MH^{(+)}AKI^{(-)}$ IgAN $n = 5$	MH-AKI $n = 11$	
Mesangial proliferation of IgAN				
focal:diffuse	14:5[a,b]	1:4[a,c]	9:2[c,d]	0:9[b,d]
The percentage of crescents (%)	12.5 (6.0–29.4)[b]	36.4 (6.2–46.8)[e]	7.1 (2.9–15.4)[d]	0 (0–0)[b,e,d]
Cellular crescents, *n* (%)	5 (26.3%)	0 (0%)	3 (27.3%)	0 (0%)
Fibrocellular crescents, *n* (%)	18 (94.7 %)[f,b]	5 (100.0 %)	4 (36.4 %)[f]	0 (0%) [b,e,d]
Fibrous crescents, *n* (%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Interstitial fibrosis, *n* (%)	8 (42.1%)[b]	4 (80.0%)[e]	8 (72.7%)[d]	0 (0%)[b,e,d]
Tubular necrosis, *n* (%)	0 (0%)	0 (0%)	3 (27.3%)	0 (0%)
Adhesion, *n* (%)	6 (31.6%)	1 (20.0%)	1 (9.1%)	0 (0%)
The Oxford classification[g]				
M 0/1	13/6 (68.4/31.6%)	3/2 (60.0/40.0%)	8/1 (88.9/11.1%)	
E 0/1	9/10 (47.4/52.6%)	1/4 (20.0/80.0%)	5/4 (55.6/44.4%)	
S 0/1	12/7 (63.2/36.8%)	3/2 (60.0/40.0%)	7/2 (77.8/22.2%)	
T 0/1/2	19/0/0 (100.0/0/0%)	5/0/0 (100.0/0/0%)	9/0/0 (100.0/0/0%)	
C 0/1/2	5/13/1 (26.3/68.4/5.3%)	2/3/0 (40.0/60.0/0%)	3/6/0 (33.3/66.7/0%)	
Immunohistochemistry, n (%)				
IgG positive	14 (73.7%)[b]	2 (40.0%)	7 (63.7%)	1 (10.0%)[b]
2+	1 (5.3%)	0 (0%)	0 (0%)	0 (0%)
1+	14 (73.7%)	2 (40.0%)	6 (54,5%)	1 (10.0 %)
IgA positive	19 (100.0%)[b]	5 (100.0%)[e]	11 (100.0 %)[d]	2 (20.0%)[b,e,d]
2+	16 (84.2%)	3 (60.0%)	6 (54,5%)	0 (0%)
1+	3 (15.8%)	2 (40.0%)	5 (45.5%)	2 (20.0%)
IgM positive	12 (63.2%)	5 (100.0%)	5 (45.5%)	5 (50.0%)
2+	1 (5.3%)	0 (0%)	0 (0%)	0 (0%)
1+	12 (63.2%)	5 (100.0%)	5 (45.5%)	5 (50.0%)
C1q positive	7 (36.8%)	1 (20.0%)	1 (9.1%)	4 (40.0%)
2+	0 (0%)	0 (0%)	0 (0%)	0 (0%)
1+	6 (31.6%)	1 (20.0%)	1 (9.1%)	4 (40.0%)
C3 positive	16 (84.2 %)[b]	5 (100.0 %)[e]	11 (100.0 %)[d]	1 (10.0%)[b,e,d]
2+	7 (36.8%)	1 (20.0%)	1 (9.1%)	0 (0%)
1+	9 (47.4%)	4 (80.0%)	10 (90.9%)	1 (10.0%)
C4 positive	4 (21.1 %)	0 (0 %)	3 (27.3 %)	5 (50.0%)
2+	2 (10.5%)	0 (0%)	0 (0%)	3 (30.0%)
1+	3 (15.8%)	0 (0%)	2 (18.2%)	2 (20.0%)
Fibrinogen positive	5 (26.3 %)	5 (100.0 %)[c,e]	4 (36.4 %)[c]	0 (0%)[e]
2+	2 (10.5%)	1 (20.0%)	0 (0%)	0 (0%)
1+	3 (15.8%)	4 (80.0%)	3 (27.3%)	0 (0%)
Electron dense deposit, *n* (%)	13/15 (86.7%)[b]	5 (100.0 %)[e]	9/10 (90.0 %)[d]	0 (0%)[b,e,d]

IgAN, IgA nephropathy; $MH^{(-)}AKI^{(-)}$ IgAN, children with IgA nephropathy who had neither macrohematuria nor acute kidney injury at the time of renal biopsy; $MH^{(+)}AKI^{(-)}$ IgAN, children with IgA nephropathy who had macrohematuria but not acute kidney injury at the time of renal biopsy; MH-AKI IgAN, children with IgA nephropathy who had macrohematuria-induced acute kidney injury at the time of renal biopsy; $MH^{(-)}AKI^{(-)}$ non-IgAN, children who demonstrated pathologically diffuse mesangial proliferation with an etiology other than IgAN and who had neither MH nor AKI at the time of renal biopsy.
[a] $P < 0.05$ for $MH^{(-)}AKI^{(-)}$ vs. $MH^{(+)}AKI^{(-)}$ IgAN.
[b] $P < 0.05$ for $MH^{(-)}AKI^{(-)}$ IgAN vs. $MH^{(-)}AKI^{(-)}$ non-IgAN.
[c] $P < 0.05$ for $MH^{(+)}AKI^{(-)}$ vs. MH-AKI IgAN.
[d] $P < 0.05$ for MH-AKI IgAN vs. $MH^{(-)}AKI^{(-)}$ non-IgAN.
[e] $P < 0.05$ for $MH^{(+)}AKI^{(-)}$ vs. $MH^{(-)}AKI^{(-)}$ non-IgAN.
[f] $P < 0.05$ for $MH^{(-)}AKI^{(-)}$ vs. MH-AKI IgAN.
[g] Evaluated 9 children with MH-AKI IgAN for whom Oxford classification data were available.

injured tubules and interstitium of children with MH-AKI IgAN. Whereas the areas of Berlin Blue staining and CD163 immunostaining did not perfectly overlap, the areas surrounding those stained with Berlin Blue were positive for CD163 (Figure 2e and f). The area of CD163 immunostaining in children with MH-AKI IgAN was significantly more extensive than that in children with $MH^{(-)}AKI^{(-)}$, $MH^{(+)}AKI^{(-)}$ IgAN, and $MH^{(-)}AKI^{(-)}$ non-IgAN (Figure 4b). The areas of CD68 immunostaining in all 4 groups comprised not only the injured tubules and the interstitium, but also the interstitium around the structured tubules, and did not significantly differ among the 4 groups (data not shown).

Prognosis

The median duration of monitoring was 1.9 (interquartile range: 0.4–3.9) years. At the time of last visit, proteinuria was present in 3 (15.8%), zero (0%), and 2 (18.2%) patients with $MH^{(-)}AKI^{(-)}$, $MH^{(+)}AKI^{(-)}$, and MH-AKI IgAN, respectively; and microhematuria was

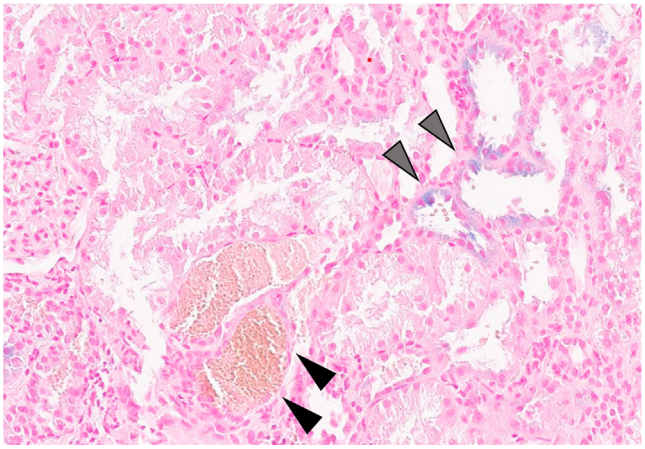

Figure 1. Results of Berlin Blue staining of kidney sections from children with IgA nephropathy who had macrohematuria-induced acute kidney injury. Magnification ×400. Berlin Blue staining was not apparent in injured tubules that were obstructed by red blood cell casts (black arrow) and was mainly at sites of desquamation of epithelial cells as tubuloepithelial injuries (gray arrow).

present in 9 (47.4%), 2 (40.0%), and 5 (45.5%) patients with $MH^{(-)}AKI^{(-)}$, $MH^{(+)}AKI^{(-)}$, and MH-AKI IgAN, respectively. None of the children had MH at their last visit.

The Cr-eGFR at the last follow-up examination did not significantly differ among the 4 groups (Table 3). Three participants in the $MH^{(-)}AKI^{(-)}$ IgAN group and 2 in the $MH^{(-)}AKI^{(-)}$ non-IgAN group had Cr-eGFRs at the last follow-up examination of <90 ml/min per 1.73 m^2. The serum Cr concentrations of all 11 children with MH-AKI IgAN returned to normal during the study period. The Cr-eGFRs of all these children at the last follow-up examination were >90 ml/min per 1.73 m^2 and the median value was 110.4 (interquartile range: 95.7–123.1) ml/min per 1.73 m^2.

DISCUSSION

In the present study, we have shown that the renal prognosis of IgAN with MH-AKI in children is good in the medium term. Children with IgAN and MH, with or without AKI, show considerable iron deposition in their renal tubules. The expression of CD163, which is not only a marker of macrophages, but also a hemoglobin scavenger receptor, has been shown to be associated with MH-AKI IgAN. To the best of our knowledge, this is the first study to characterize the deposition of iron and CD163 expression histologically and to compare the prognosis of children with MH-AKI according to the presence or absence of MH and AKI.

The present histologic analysis revealed significant iron deposits in the renal tubules of children with IgAN and MH, including those with $MH^{(+)}AKI^{(-)}$ and MH-AKI IgAN. This is significant because oxidative stress-related DNA damage is closely associated with hepatic inflammation and the hepatic expression of hepcidin, which is involved in hepatic iron storage in patients with chronic hepatitis C.[11] Several previous studies have shown that hemoglobin and iron from RBCs have toxic effects in proximal tubular cells, as well as in other organs.[10,14-16] Toxic effects in tubular cells that can lead to AKI, which is most commonly caused by IgAN, have been reported in patients with MH during anticoagulant therapy.[17,18] Moreover,

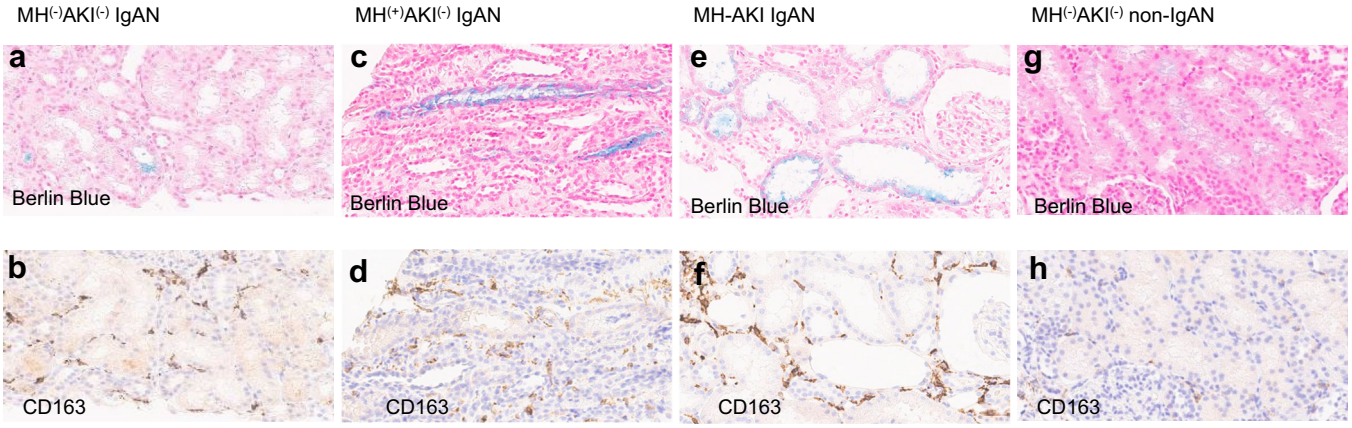

Figure 2. Tubulointerstitial findings in serial kidney sections. (a, c, e, and g) Berlin Blue staining was mainly apparent at sites of desquamation of tubuloepithelial cells in children with IgA nephropathy who had macrohematuria but no acute kidney injury ($MH^{(+)}AKI^{(-)}$ IgAN) and those who had macrohematuria-induced acute kidney injury (MH-AKI IgAN), was weak in those who had neither macrohematuria nor acute kidney injury ($MH^{(-)}AKI^{(-)}$ IgAN), and absent in those who demonstrated pathologically diffuse mesangial proliferation with an etiology other than IgAN and who had neither MH nor AKI at the time of renal biopsy ($MH^{(-)}AKI^{(-)}$ non-IgAN). In MH-AKI IgAN, the injured tubules and interstitium in which CD163 staining is positive. (f) Although the localization of Berlin Blue staining and CD163 immunostaining did not perfectly match, the areas of CD163 immunostaining were surrounded by areas of Berlin Blue staining. (c and d, and e and f) CD163 immunostaining was weak in tubuloepithelial cells and the interstitium in $MH^{(-)}AKI^{(-)}$ and $MH^{(+)}AKI^{(-)}$ IgAN children, and $MH^{(-)}AKI^{(-)}$ non-IgAN, regardless of the Berlin Blue staining status. Magnification ×400. AKI, acute kidney disease; IgAN, IgA nephropathy; MH, macrohematuria.

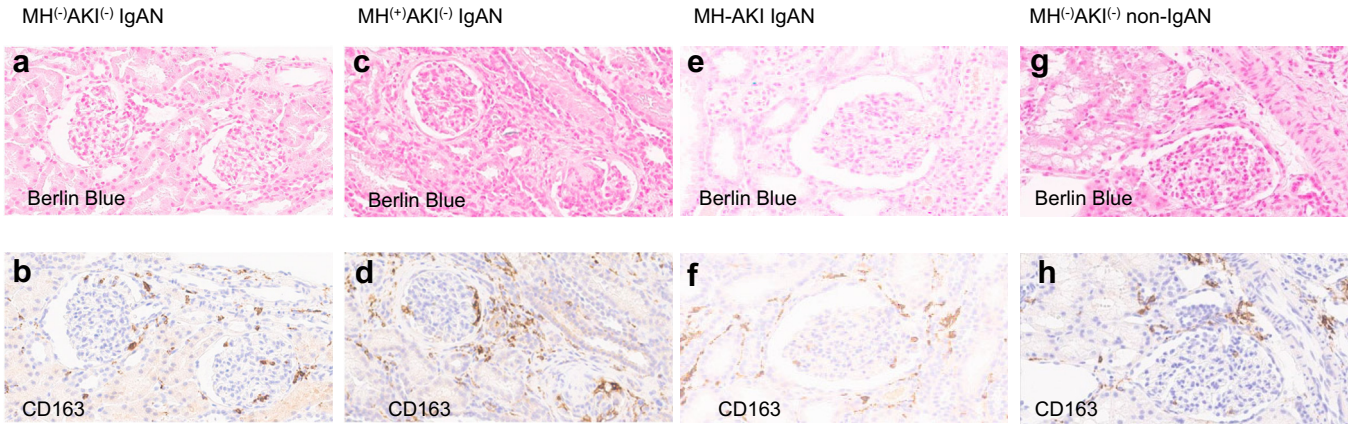

Figure 3. Glomerular findings of serial kidney section. The Berlin Blue staining are negative at any glomerulus in all 4 groups. (a, c, e, and g) the CD163 staining are negative at any glomerulus in children with IgA nephropathy who had neither macrohematuria nor acute kidney injury ($MH^{(-)}AKI^{(-)}$ IgAN), those who had macrohematuria but not acute kidney injury at renal biopsy ($MH^{(+)}AKI^{(-)}$ IgAN) and those who had macrohematuria-induced acute kidney injury (MH-AKI IgAN). (b, d, and f) in children who demonstrated pathologically diffuse mesangial proliferation other than IgAN who had neither MH nor AKI at renal biopsy ($MH^{(-)}AKI^{(-)}$ non-IgAN), the positive depositions of CD163 staining are at the glomerular cells (h). Magnification ×400. AKI, acute kidney disease; IgAN, IgA nephropathy; MH, macrohematuria.

patients with IgAN present with MH (the presence of massive numbers of RBCs in the urine), which is frequently triggered by upper respiratory infections or mucosal inflammatory processes.[19,20] The release of iron from RBCs induces oxidative stress-related toxicity, which can lead to mitochondrial dysfunction and damage to DNA and the endoplasmic reticulum.[21-24] There have been no studies of the histopathology associated with iron deposition and microhematuria or MH in any kidney diseases, even though renal

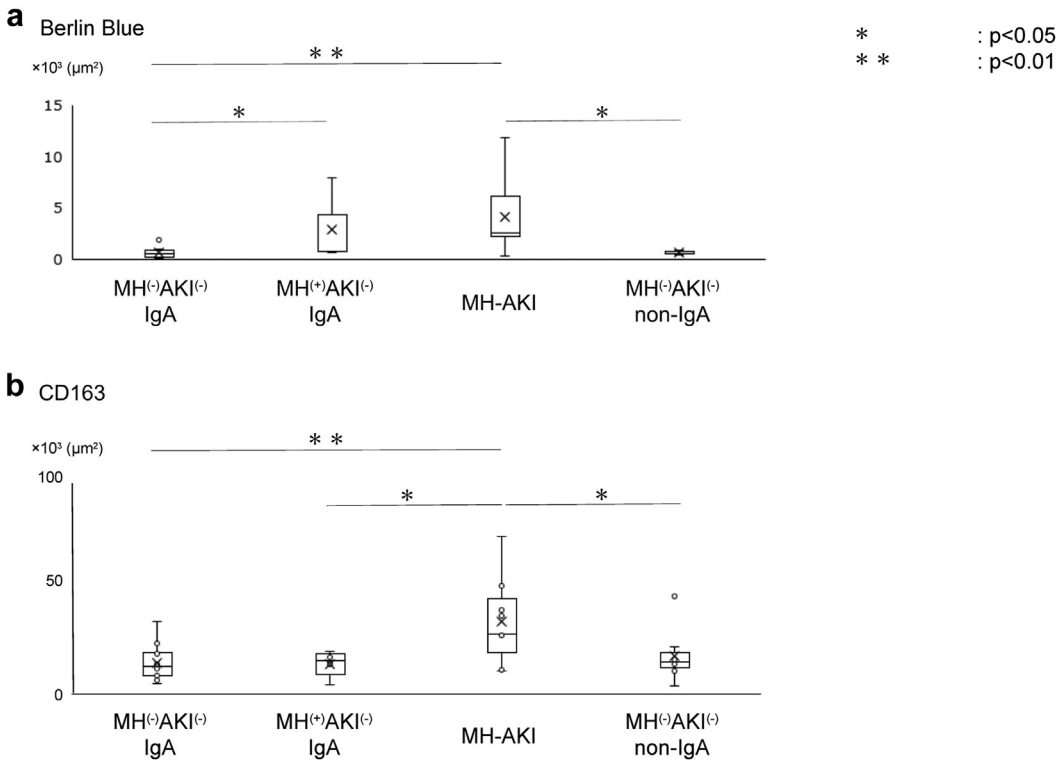

Figure 4. Quantification of the areas of immunostaining. Children with IgA nephropathy who had neither macrohematuria nor acute kidney injury: $MH^{(-)}AKI^{(-)}$ IgAN. Children with IgA nephropathy who had macrohematuria but no acute kidney injury: $MH^{(+)}AKI^{(-)}$ IgAN. Children with IgA nephropathy who had macrohematuria-induced acute kidney injury: MH-AKI IgAN. Children who demonstrated pathologically diffuse mesangial proliferation with an etiology other than IgAN and who had neither MH nor AKI: $MH^{(-)}AKI^{(-)}$ non-IgAN. (a) The areas of Berlin Blue staining in sections from $MH^{(+)}AKI^{(-)}$ and MH-AKI IgAN children were significantly more extensive than those of the other groups. (b) The areas of CD163 immunostaining in kidney sections from children with MH-AKI IgAN or $MH^{(-)}AKI^{(-)}$ non-IgAN were significantly more extensive than those of the other groups. AKI, acute kidney disease; IgAN, IgA nephropathy; MH, macrohematuria.

Table 3. Clinical characteristics of the participants at the final follow-up examination

Characteristics	IgAN			
	$MH^{(-)}AKI^{(-)}$ IgAN $n = 19$	$MH^{(+)}AKI^{(-)}$ IgAN $n = 5$	MH-AKI $n = 11$	$MH^{(-)}AKI^{(-)}$ non-IgAN $n = 9$
At the last follow up				
Age (yr)	15.5 (13.1–18.1)[a]	14.4 (10.1–15.9)[b]	14.1 (11.9–15.0)[c]	4.5 (2.0–11.4) [a,b,c]
Observation period (yr)	1.9 (1.0–3.2)	3.4 (1.8–4.1)	0.5 (0.1–4.0)	0.7 (0.1–4.7)
The persistence of proteinuria, *n* (%)	9 (47.4 %)	0 (0.0 %)	2 (18.2 %)	
The persistence of hematuria, *n* (%)	3 (15.8 %)	2 (40.0 %)	5 (45.5 %)	
Systemic BP (mm Hg)	104.0 (90.5–118.0)	103.0 (83.0–111.5)	103.0 (102.0–110.0)	100.0 (94.0–106.0)
Diastolic BP (mm Hg)	54.0 (51.5–60.5)	54.0 (57.5–67.0)	56.0 (53.0–75.0)	58.0 (51.5–88.0)
Hypertension, *n* (%)	0 (0.0 %)	0 (0.0 %)	0 (0.0 %)	1 (9.1%)
Cr-eGFR (ml/min per 1.73 m^2)	109.2 (95.1–121.8)	124.4 (102.1–139.7)	107.1 (90.7–114.1)	121.2 (86.5–124.7)
Cr-eGFR <90 ml/min per 1.73 m^2, *n* (%)	3 (15.8 %)	0 (0.0 %)	0 (0.0 %)	2 (22.2 %)

BP, blood pressure; Cr-eGFR, creatinine-estimated glomerular filtration rate; IgAN, IgA nephropathy; $MH^{(-)}AKI^{(-)}$ IgAN, children with IgA nephropathy who had neither macrohematuria nor acute kidney injury at the time of renal biopsy; $MH^{(+)}AKI^{(-)}$ IgAN, children with IgA nephropathy who had macrohematuria but not acute kidney injury at the time of renal biopsy; MH-AKI IgAN, children with IgA nephropathy who had macrohematuria-induced acute kidney injury at the time of renal biopsy; $MH^{(-)}AKI^{(-)}$ non-IgAN, children who demonstrated pathologically diffuse mesangial proliferation with an etiology other than IgAN who had neither MH nor AKI at the time of renal biopsy.
[a] $P < 0.05$ for $MH^{(-)}AKI^{(-)}$ vs. $MH^{(-)}AKI^{(-)}$ non-IgAN.
[b] $P < 0.05$ for $MH^{(+)}AKI^{(-)}$ vs. $MH^{(-)}AKI^{(-)}$ non-IgAN.
[c] $P < 0.05$ for MH-AKI IgAN vs. $MH^{(-)}AKI^{(-)}$ non-IgAN

symptoms are often related to the presence of RBCs within the renal tubules, even if they do not form casts. All 4 groups of participants in the present study had microhematuria or MH, and we focused on whether MH was associated with considerable iron deposition in the renal tubules of children with IgAN.

We found that the renal prognosis of patients with MH-AKI was good because none of the participants exhibited a deterioration of renal function at their last visit. However, 16.7% of the participants with $MH^{(-)}AKI^{(-)}$ IgAN had eGFRs of <90 ml/min per 1.73 m^2 at their last visit, although the median eGFR at the last follow-up visit did not differ significantly among the groups. We found that the renal prognosis of children with MH-AKI IgAN was better than that previously reported for adults. In general, patients with IgAN show severe mesangial proliferation,[25,26] and older patients with chronic lesions[27] experience poor renal outcomes. In contrast, adults with MH-AKI IgAN exhibit mild mesangial proliferation and have few crescents but show acute tubular necrosis.[2-5,9] These findings imply that the severity of acute tubular necrosis significantly affects the recovery of renal function in patients with MH-AKI IgAN. In the present study, all the children with MH-AKI IgAN showed mild mesangial proliferation, but no chronic lesions that were identified as fibrous crescents or glomerulosclerosis, and 27.3% of them had acute tubular necrosis. Therefore, the lower prevalence of acute tubular necrosis with mild mesangial proliferation and the lack of chronic lesions in children with MH-AKI IgAN might imply that they have a superior prognosis to that of adult patients.

It is necessary to discuss whether there were more CD163-positive cells because of AKI or whether AKI occurred because there were more CD163-positive cells. In a further analysis, we found that CD163-positive cells were more numerous in the kidneys of children with renal diseases other than IgAN who had AKI but no MH. The CD163 immunostaining in the MH-AKI group was principally in the tubulointerstitium and almost absent in the glomeruli, whereas there was weak immunostaining in the tubulointerstitium of the non-IgA group, along with a number of CD163-positive cells in the glomeruli (data not shown). Because MH was not a feature of the non-IgA group, we speculate that CD163-positive cells acted as a macrophage rather than a hemoglobin scavenger in the glomeruli. Moreover, a previous study of IgAN[28] showed a correlation between the degree of mesangial matrix proliferation, which can cause AKI, and the number of CD163-positive macrophages in the glomeruli. In addition, if CD163-positive cells had caused AKI in the MH-AKI group, it would be expected that CD163-positive cells would uniformly infiltrate the tubulointerstitium. In the MH-AKI group, the CD163 staining was predominantly in the tubulointerstitium, but this was not uniform. Therefore, CD163-positive cells may have infiltrated the areas where there were toxic effects and may have had a hemoglobin-scavenging role. Furthermore, in IgAN that did not feature MH, there was little iron deposition, whereas in IgAN that involved MH, infiltration of CD163-positive cells was observed in the tubular tissue surrounding the areas of iron deposition. This suggests that iron deposition and hemoglobin scavenging by CD163 are both features of MH-AKI.

We found that the children with IgAN and MH, with or without AKI, at the time of renal biopsy had considerable iron deposition in their renal tubules, although the extent of iron deposition did not significantly differ between children with $MH^{(+)}AKI^{(-)}$ and those with MH-AKI IgAN. These findings indicate that

iron might not directly affect the renal tubules. In addition, tubular damage is induced by factors related to iron deposition in children with MH-AKI IgAN. Further detailed investigation is warranted to identify the mechanisms of these toxic effects. Furthermore, it is clear that there are larger numbers of acute tubular necrosis lesions in adult patients with MH-AKI IgAN than in pediatric patients, which may reflect differing susceptibility to toxins, but further investigations are warranted. Finally, CD163 immunostaining was significantly more extensive in children with MH-AKI IgAN than in those with $MH^{(+)}AKI^{(-)}$ IgAN. A previous case report[6] demonstrated that CD163, which scavenges haptoglobin–hemoglobin complexes,[29] is expressed in injured tubules and the interstitium. Moreno *et al.* speculated that CD163-positive cells accumulate to reduce the toxic effects of iron on tubules.[9] Although it was not as high as that reported for adult patients with MH-AKI IgAN,[2-5,9] the proportion of pediatric patients with MH-AKI IgAN, featuring tubulointerstitial damage, including interstitial fibrosis and tubular necrosis, was higher than that of those with $MH^{(+)}AKI^{(-)}$ IgAN in the present study. Although not statistically significant, these differences were associated with differences in the area of CD163 immunostaining. Taking these findings together, we postulate that CD163-expressing cells accumulate in response to iron deposition and mitigate its toxic effects, while leading to moderate tubular damage in pediatric patients with MH-AKI IgAN. In the present study, we found that there was CD68 immunostaining in the kidneys of all 4 groups, not only in the injured tubules and the interstitium, but also the interstitium around the structured tubules. CD68 is a total-macrophage marker, whereas CD163 is expressed specifically in M2-specific macrophages,[28] and the 2 showed differing localization. Although we compared several antigen retrieval methods, this difference in localization remained. We judged that this may have been a methodological artifact and led us to conclude that satisfactory results were not obtained using the CD68 antibody.

This study had several limitations. First, there were relatively few participants with MH-AKI IgAN. However, MH-AKI IgAN is a rare form of nephritis, especially in children, making this, to some extent, inevitable. Second, some patients with MH-AKI IgAN showed little renal iron deposition. Because there was RBC cast nephropathy in the kidneys of the participants with MH-AKI, iron-mediated tubular injury could only be seen in some of the tubules. We believe that the mechanisms involved in MH-AKI are heterogeneous and that further studies are required in which the histopathologic findings are evaluated quantitatively, including parameters relating to tubules filled with RBC casts and tubules injured by iron deposition, and the expression of markers of toxicity in tubules. Third, we performed double staining with Berlin Blue and anti-CD163 antibody to evaluate the distribution of iron and CD163 in the kidney. However, we were unable to successfully stain the same sections for both. Fourth, we could not evaluate the relationship between MH-AKI and macrophage accumulation using macrophage markers other than CD163 because of methodological issues. Finally, we followed-up with the participants for a relatively short period of time, and therefore further longer-term studies should be performed.

In conclusion, we have used an immunohistopathologic approach to evaluate iron deposition in children with MH and AKI and found that there is considerable iron deposition in the renal tubules of children with IgAN and MH. CD163-positive cells might play a role as hemoglobin scavengers but not their role as macrophages, in the injured tubulointerstitium of MH-AKI IgAN. In addition, we have shown that the renal outcomes of pediatric patients with IgAN and MH-AKI are good in the short to medium term.

DISCLOSURE

All the authors declared no competing interests.

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AUTHOR CONTRIBUTIONS

SI and HK conceived the study. SI wrote the manuscript and interpreted the data. SI, TH, TY, JF, NM, HK, YT, HM, WS, YS, AK, and YA performed renal biopsies and collected the clinical data. SI and SH performed immunohistochemical staining of the renal biopsy samples and interpreted the results. YA, KNa, SH, and KNo critically reviewed the manuscript. All the authors have read and approved the final version of the manuscript.

SUPPLEMENTARY MATERIALS

Supplementary File (PDF)

Table S1. Steps involved in immunohistochemical staining.

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