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Glomerular Complement Factor H–Related Protein 5 is Associated with Histologic Injury in Immunoglobulin A Nephropathy

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Introduction: Immunoglobulin A nephrology (IgAN), characterized by co-deposition of IgA and complement components, is an activation of complement system involved disease. Factor H–related protein 5 (FHR-5) antagonized the ability of factor H to negatively regulate C3 activation, which leads to overactivation of the alternative pathway. Here we explore the relationship of intensity of glomerular FHR-5 deposition and severity of IgAN.

Methods: Renal staining of FHR-5 was detected by immunofluorescence, and plasma FHR-5 was detected by enzyme-linked immunosorbent assay in 56 patients with IgAN. The relationship of intensity of glomerular FHR-5 and clinical and pathologic features of these patients were further analyzed.

Results: Glomerular staining for FHR-5 was observed in a predominantly mesangial pattern in 32 biopsy specimens (57.1%). FHR-5 co-deposited with IgA and C3c in glomerular mesangial and capillary area in patients with IgAN. Patients with IgAN with Oxford endocapillary hypercellularity ($P = 0.007$) and segmental glomerulosclerosis ($P = 0.049$) presented with greater intensity of FHR-5 deposition. There were more cases with 2+ and 3+ FHR-5 staining in cohorts of 2+ and 3–4+ mesangial C3 deposition ($P = 0.034$) and IgA deposition ($P = 0.019$). Interestingly, the glomerular FHR-5 depositions were more abundant in male versus female in patients with IgAN ($P = 0.002$). Besides, circulating FHR-5 levels were elevated in patients with IgAN compared with healthy control subjects. Plasma FHR-5 levels were significantly higher in patients with mesangial hypercellularity at diagnosis than those with nonmesangial hypercellularity.

Conclusions: We found that glomerular intensity of FHR-5 deposition could indicate the severity of histologic lesions of IgAN.

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Immunoglobulin A nephropathy (IgAN), first described >4 decades ago, has been recognized as the most frequent form of primary glomerulopathy[1] and remains a vital cause of end-stage renal disease in China.[2] IgAN is characterized by the deposition of IgA in the glomerular mesangial area, which leads to glomerular inflammation and injury.[3] To date, the specific pathogenesis of IgAN has not been well elucidated.

Complement component C3 is observed in both circulating immune complexes and glomerular deposits accompanied with IgA in IgAN,[4,5] suggesting a role for complement activation in IgAN. The degree of mesangial C3 deposits was reported to predict renal outcomes in patients with IgAN, which suggested a role for complement activation in IgAN progression.[6] Complement activation through the alternative pathway is associated with renal injury in patients with IgAN.[7] However, the role of complement activation in IgAN remains poorly understood.

Recent genome-wide association studies in patients with IgAN have identified chromosome 1q32 as an IgAN-susceptible locus, which contained several complement regulatory proteins encoding the genes *FH* and *FHR1–5*.[8,9] Complement factor H (FH), encoded by the *FH* gene, is a relatively protein-like "brake apparatus" for the complement alternative pathway.[10,11] Unlike FH, FHR proteins lack complement regulation domains.[12,13] In vitro studies have shown that FHR-5 antagonized the ability of FH to negatively regulate C3 activation.[14,15] This FH deregulation could impair

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the ability of FH to negatively regulate C3 on surfaces such as the mesangium and potentially enhance C3 deposition.[16,17]

Interestingly, a novel disease named FHR-5 nephropathy was identified after genetic analysis of a pedigree of Cypriot ancestry with primary glomerulonephritis, which shared many similarities with IgAN.[18] Rare variants in *FHR-5* contributed to the genetic susceptibility to IgAN.[19] Recently, both Medjeral-Thomas *et al.*[20] and Zhu *et al.*[21] showed that circulation FHR-5 levels were elevated in patients with IgAN. Serum FHR-5 levels correlated with histologic markers of renal injury[20,21] and were an independent risk factor for IgAN progression.[21] In addition, glomerular staining for FHR-5 was found in patients with glomerulopathy,[22] especially those with progressive IgAN.[23] The complement activation in situ (glomerular) seemed to be more important than circulations in patients with IgAN. Renal complement activation in IgAN seems to be more obvious than systemic complement activation.

In this study, we explore the relationship between the intensity of FHR-5 deposition and IgAN severity.

METHODS

Study Population

A diagnosis of IgAN was based upon the presence of dominant IgA deposition in the mesangial area by immunofluorescence and electron-dense material deposition in the mesangial area by electron microscopy. Patients with Henoch-Schönlein purpura, liver cirrhosis, and other secondary etiologies of IgAN by detailed clinical and laboratory examinations were excluded. A total of 206 patients were underwent biopsy procedures between June 2019 and February 2020. Among them, 64 patients were biopsy-confirmed IgA-dominant glomerulonephritis. Eight patients were excluded, including 1 patient with IgA vasculitis, 1 patient with IgA-dominant infection-related glomerulonephritis, 4 patients with IgAN with diabetic kidney disease, 1 patient with IgAN caused by hepatitis, and 1 patient with IgAN with thin basement membrane disease. Fifty-six patients with idiopathic IgAN were included in this study. A flow diagram is shown in Supplementary Figure S1. Paraffin-embedded renal biopsy specimens of these patients were selected.

In the meantime, anticoagulated (ethylenediamine tetraacetic acid) peripheral venous blood was obtained from these patients with IgAN (on the day of the renal biopsy procedure) and 20 age-, gender-, and geographically matched healthy control subjects (inclusion in the study) for the detection of plasma FHR-5. Blood samples were centrifuged at 2000 *g* for 15 minutes at 4 °C. Plasmas were stored at −80 °C until use.

The research complied with the principles of the Declaration of Helsinki and was approved by the ethics committees of Beijing Anzhen Hospital. Informed consent was obtained from all enrolled individuals.

Clinical and Histologic Manifestations

Clinical manifestations at the time of renal biopsy, including age, gender, serum creatinine levels, 24-hour urine protein excretion, history of high blood pressure, prodromic infection, and gross hematuria were collected from the medical records. High blood pressure was defined as a systolic blood pressure ≥140 mm Hg or a diastolic blood pressure ≥90 mm Hg or taking an antihypertensive medication to prevent hypertension. Prodromic infection was defined as onset of IgAN ≤2 weeks after signs and symptoms of infections. The glomerular filtration rate (GFR) of patients with IgAN was calculated using the modified GFR estimating equation.[24] Histologically, the Oxford classification was used for the evaluation of pathologic lesions for those with >8 glomeruli in biopsy specimens. In addition, crescent scores of C0 (no crescents), C1 (crescents in <25% of glomeruli), and C2 (crescents in >25% of glomeruli).[25,26]

Immunofluorescence in Renal Biopsy Specimens

For immunofluorescence staining, paraffin-embedded sections were cut at 4 μm thickness, deparaffinized, and subjected to antigen retrieval (pepsase incubated for 25 minutes at 37 °C). After being washed 3 times with phosphate-buffered saline (PBS), sections were incubated in 3% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, Missouri, USA) for 30 minutes at room temperature. Primary antibodies (FHR-5, GeneTex, Inc., Irvine, Califoria, USA) with a dilution of 1:200 in PBS were incubated overnight at 4 °C. After being washed 3 times with PBS, fluorescein isothiocyanate–labeled secondary antibodies (diluted 1:200 in PBS, Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA) were added for 60 minutes at 37 °C. After being washed with PBS 3 times for 5 minutes, the sections were air-dried in the dark and mounted with mounting medium with 4′,6-diamidino-2-phenylindole.

The method to detect colocalization of FHR-5 and C3c, FHR-5 and IgA1 were the same as previously described.[27] Paraffin-embedded sections were cut at 4 μm thickness, deparaffinized, and subjected to antigen retrieval (pepsase incubated for 25 minutes at 37 °C). After being washed 3 times with PBS, sections were incubated in 3% BSA (Sigma Chemical Co., St. Louis,

MO) for 30 minutes at room temperature. Primary antibodies (FHR-5; GeneTex, Inc., Irvine, CA) with a dilution of 1:200 in PBS were incubated overnight at 4 °C. After being washed 3 times with PBS, tetramethylrhodamine isothiocyanate–labeled goat antirabbit antibodies (diluted 1:200 in PBS, Jackson Immuno Research Laboratories, Inc.) were added for 60 minutes at 37 °C. After being washed with PBS 3 times for 5 minutes, the sections were added mouse antihuman C3c antibodies (diluted 1:200 in PBS; Dako, Glostrup, Denmark) or mouse antihuman IgA antibodies (diluted 1:100 in PBS; Abcam, Cambridge, MA) and incubated for 60 minutes at 37 °C. After another wash, fluorescein isothiocyanate–labeled goat antimouse antibodies (diluted 1:200 in PBS, Jackson Immuno Research Laboratories, Inc.) were added for 60 minutes at 37 °C. After being washed, the sections were air-dried in the dark and mounted with mounting medium with 4′,6-diamidino-2-phenylindole.

As blank controls, primary antibodies were replaced by PBS. The immunofluorescence was scored using a fluorescence microscope (80i; Nikon, Tokyo, Japan). Two observers (WG and LS) blinded to clinical data graded the staining intensity from anonymized sections as 0 (absent), 1+, 2+, or 3+. Staining described as "positive" included 1+, 2+, or 3+, while "negative" included 0 independently. Sections that contained <2 glomeruli were excluded.

In the analysis of fluorescence colocalization, 2-dimensional (2D) fluorograms were plotted from red and green images using Image-Pro Plus software (Media Cybernetics, Rockville, MD). A 2D fluorogram is a dot diagram that visualizes the joint distribution of intensity values of 2 detection channels (red and green). Each dot of the scatter plot represents an intensity value pair from the 2 detection channels.

Detection of Plasma FHR-5 by Enzyme-Linked Immunosorbent Assay

Plasma FHR-5 was detected by enzyme-linked immunosorbent assay as previous described.[20,21] Briefly, rabbit antihuman FHR-5 antibody (Abnova Corp., Taipei, Taiwan) was coated onto half the wells of a microtiter plate (Thermo Fisher Scientific, Waltham, MA) as the capture antibody. After blocking with 1% BSA, the standard control (recombinant human FHR-5 protein; R&D Systems, Minneapolis, MN), plasma samples and quality controls were added and incubated for 1 hour at room temperature. Binding of FHR-5 was detected using mouse anti–FHR-5 antibodies (Abnova Corp., Taipei, Taiwan). After the addition of horseradish peroxidase-conjugated rabbit antimouse immunoglobulin G (Dako), the reaction was developed using the peroxidase chromogenic substrate 3,3,5,5-tetramethylbenzidine liquid substrate system (Sigma Chemical Co., St. Louis, MO) and stopped with 1 mol/l H_2SO_4 (sulfuric acid) before the absorbance was measured at 450 and 570 nm.

Statistical Analysis

Statistical analysis was performed using SPSS software (version 11.0; SPSS Inc., Chicago, IL). Continuous variables were expressed as the mean ± standard deviation or median and interquartile range, while categorical variables were expressed as the number and percentage. The Kolmogorov-Smirnov test was used to analyze the normality of the distribution of variables. For continuous variables, an unpaired Student t test or analysis of variance between groups was used for comparison if the data formed a normal distribution; otherwise, a Mann-Whitney U or Kruskal–Wallis H test was performed. For categorical variables, a χ^2 test was used. P values < 0.05 were considered statistically significant.

RESULTS

Demographic, Clinical, and Histologic Characteristics of Patients with IgAN

Among the 56 recruited patients with IgAN, 21 (37.5 %) were male, and the patients had a mean age at renal biopsy of 40.1 ± 14.6 years (Table 1). Prodromic infection and gross hematuria were found in 8 (14.3%) and 7 (12.5%) patients, respectively. At the time the renal biopsy specimen was obtained, the median proteinuria for patients was 1.47 (0.61–2.74) g/24 hours, while the average estimated GFR was 96.41 (56.50–119.09) ml/min/1.73 m^2. In our patients, 32 (57.1%) presented with hypertension. Histologically, mesangial hypercellularity (M1), endocapillary hypercellularity (E1), and segmental glomerulosclerosis (S1) were found in 39.3%, 48.2%, and 30.4% of patients, respectively. For tubular atrophy and interstitial fibrosis (Oxford-T), T0, T1 and T2 were found in 57.1%, 28.6%, and 14.3% of patients, respectively. For crescent lesions, C0, C1, and C2 were found in 64.3%, 28.6%, and 7.1% of patients, respectively.

Association Between the Presence of Renal FHR-5 Deposition and Clinical, Laboratory, and Pathologic Parameters in IgAN

The presence of FHR-5 was examined in 56 renal biopsy specimens from patients with IgAN. Glomerular staining for FHR-5 was observed in a predominantly mesangial pattern in 32 (57.1%) biopsy specimens (Figure 1, b to d), whereas glomeruli in 24 (42.9%) IgAN biopsy specimens stained negative for FHR-5 (Figure 1, a). Based on the intensity of FHR-5 deposition in glomeruli of patients with IgAN can be divided

Table 1. Demographic, clinical, and histologic characteristics of patients with IgAN

		Intensity of FHR-5 deposition in patients with IgAN				
Characteristic	**n = 56**	**0, n = 24**	**1+, n = 11**	**2+, n = 11**	**3+, n = 10**	***P* value[a]**
Clinical features						
Male/female, n	21/35	6/18	3/8	3/8	9/1	0.002
Age, yr, mean ± SD	40.1 ± 14.6	43.75 ± 16.37	38.09 ± 12.80	38.91 ± 13.54	41.70 ± 14.21	0.801
HBP, n (%)	32 (57.1)	13 (54.2)	5 (45.5)	7 (63.6)	7 (70.0)	0.667
SBP, mm Hg, median (IQR)	130 (119–144)	131 (113–159)	129 (128–138)	136 (121–145)	123 (118–142)	0.861
DBP, mm Hg, median (IQR)	82 (75–94)	81 (70–95)	80 (75–89)	88 (79–95)	82 (76–88)	0.785
Prodromic infection, n (%)	8 (14.3)	6 (25.0)	0 (0.0)	2 (18.2)	0 (0.0)	0.1
Gross hematuria, n (%)	7 (12.5)	4 (16.7)	1 (9.1)	2 (18.2)	0 (0.0)	0.522
CKD stages 1/2/3/4-5,[b] n (%)	34 (60.7)/7 (12.5)/13 (23.2)/2 (3.6)	16 (66.7)/2 (8.3)/5 (20.8)/1 (4.2)	6 (54.5)/1 (9.1)/4 (36.4)/0 (0.0)	6 (54.5)/1 (9.1)/3 (27.3)/1 (9.1)	6 (60.0)/3 (30.0)/1 (10.0)/0 (0.0)	0.908
Laboratory measurements						
Albumin, g/L, median (IQR)	41.45 (36.7–44.7)	39.85 (31.98–43.38)	43.00 (39.10–45.70)	41.30 (39.50–45.60)	43.95 (38.80–46.15)	0.169
Hemoglobin, g/L, mean ± SD	135.96 ± 21.46	129.92 ± 19.31	135.91 ± 24.45	134.82 ± 24.50	151.80 ± 12.37	0.036
Serum creatinine, μmol/l, median (IQR)	76.55 (58.75–102.45)	68.80 (49.05–106.30)	77.60 (53.10–102.80)	71.70 (64.10–144.80)	85.80 (68.65–102.88)	0.172
eGFR, ml/min per1.73m^2, median (IQR)	96.42 (56.50–119.09)	108.37 (53.20–124.45)	112.02 (55.87–117.72)	93.89 (55.23–95.29)	96.42 (79.23–109.68)	0.387
Initial proteinuria, g/d, median (IQR)	1.47 (0.61–2.74)	1.14 (0.62–3.87)	2.30 (0.67–3.75)	1.25 (0.55–2.29)	1.18 (0.56–2.88)	0.529
Plasma IgA, g/L, mean ± SD	3.06 ± 1.10	2.88 ± 0.91	2.87 ± 1.26	3.17 ± 1.54	3.56 ± 0.73	0.061
Plasma C3, g/L, mean ± SD	1.18 ± 0.24	1.17 ± 0.28	1.23 ± 0.20	1.19 ± 0.23	1.10 ± 0.15	0.547
Plasma C4, g/L, mean ± SD	0.29 ± 0.09	0.28 ± 0.09	0.29 ± 0.10	0.29 ± 0.10	0.30 ± 0.07	0.869
Plasma FHR-5, ng/ml, median (IQR)	5263.89 (4178.38–6203.30)	5163.57 (4286.35–6099.12)	5516.13 (4151.69–6733.76)	4324.32 (2451.52–6315.93)	5550.89 (4162.89–8737.99)	0.474
Histologic features						
Renal IgA deposits (1+, 2+, and 3+∼4+), n (%)	2 (3.6)/22 (39.3)/32 (57.2)	2 (8.3)/12 (50.0)/10 (41.7)	0 (0.0)/5 (45.5)/6 (54.6)	0 (0.0)/3 (27.3)/8 (72.7)	0 (0.0)/2 (20)/8 (80.0)	0.019
Renal C3 deposits (0, 1+, 2+, and 3+∼4+), n (%)	3 (5.4)/5 (8.9)/31 (55.4)/ 17 (30.4)	3 (12.5)/2 (8.3)/15 (62.5)/4 (16.7)	0 (0.0)/2 (18.2)/4 (36.4)/5 (45.5)	0 (0.0)/1 (9.1)/6 (54.5)/4 (36.4)	0 (0.0)/0 (0.0)/6 (60.0)/ 4 (40.0)	0.034
Oxford classification,[c] n (%)						
M1	22 (39.3)	8 (33.3)	5 (45.5)	4 (36.4)	5 (50.0)	0.444
E1	27 (48.2)	6 (25.0)	6 (54.5)	9 (81.8)	6 (60.0)	0.007
S1	17 (30.4)	4 (16.7)	3 (27.3)	6 (54.5)	4 (40.0)	0.049
T1/T2	16 (28.6)/8 (14.3)	3 (12.5)/3 (12.5)	1 (9.1)/2 (18.2)	6 (54.5)/2 (18.2)	6 (60.0)/1 (10.0)	0.040
C1/C2	16 (28.6)/4 (7.1)	5 (20.8)/2 (8.3)	2 (18.2)/1 (9.1)	5 (45.5)/1 (9.1)	4 (40.0)/0 (0.0)	0.579

CKD, chronic kidney disease; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; FHR-5, factor H–related protein 5; HBP, high blood pressure; IgAN, immunoglobulin A nephropathy; IQR, interquartile range; SBP, systolic blood pressure; SD, standard deviation.
[a]Used to indicate the difference among the 4 groups.
[b]CKD stage 1, 2, 3, and 4-5 denote eGFRs ≥90, 60–89, 30–59, and ≤29, respectively, according to the Kidney Foundation Kidney Disease Outcomes Quality Initiative.
[c]Mesangial hypercellularity score (M1 > 0.5), the presence of endocapillary proliferation (E1: present), segmental glomerulosclerosis/adhesion (S1: present), severity of tubular atrophy/interstitial fibrosis (T1: 26–50%, T2 >50%), and the presence of crescent (C1: 1-25%, C2: 26-100%). Oxford classification was developed by the working group of the International IgA Nephropathy Network and the Renal Pathology Society. Oxford scores of 10 patients were unavailable because each of the glomeruli counts were <8.

into 4 groups: group 0, negative; group 1, 1+ deposition; group 2, 2+ depositions; and group 3, 3+ depositions.

We found that patients with IgAN with a high intensity of FHR-5 deposition in glomeruli showed more severe pathologic manifestations than those with less deposition or without deposition. We found the proportion of Oxford E1 (group 0, 6 [25.0%]; group 1, 6 [54.5%]; group 2, 9 [81.8%]; and group 3, 6 [60.0%]; $P = 0.007$, Table 1), S1 (group 0, 4 [16.7%]; group 1, 3 [27.3%]; group 2, 6 [54.5%]; and group 3, 4 [40.0%]; $P = 0.049$, Table 1), and T1/T2 (group 0, 3 [12.5%]/3 [12.5%]; group 1, 1 [9.1%]/2 [18.2%]; group 2, 6 [54.5%]/2 [18.2%], and group 3, 6 [60.0%]/1 [10.0%]; $P = 0.040$, Table 1) were different among the 4 groups. The proportion of E1, S1, and T lesions were increased from group 0 to group 2, but not from group 2 to group 3. However, we found that patients with IgAN with E1 lesions (FHR-5 0/1+/2+/3+—E1, 6 [22.2%]/6 [22.2%]/9 [33.3%]/6 [22.2%]; E0, 18 [62.1%]/5 [17.2%]/2 [6.9%]/4 [13.8%]; $P = 0.007$, Figure 2e) and S1 lesions (FHR-5 0/1+/2+/3+—S1, 4 [23.5%]/3 [17.6%]/6 [35.3%]/4 [23.5%]; S0, 20 [51.3%]/8 [20.5%]/5 [12.8%]/6 [15.4%]; $P = 0.049$, Figure 2f) presented with more intensity of FHR-5 deposition. Besides, the intensity of C3 deposition (0/1+/2+/3∼4+—group 0, 3 [12.5%]/2 [8.3%]/15 [62.5%]/4 [16.7%]; group 1, 0 [0.0%]/2 [18.2%]/4 [36.4%]/5 [45.5%]; group 2, 0 [0.0%]/1 [9.1%]/6 [54.5%]/4 [36.4%]; group 3, 0 [0.0%]/0 [0.0%]/6 [60.0%]/4 [40.0%]; $P = 0.034$,

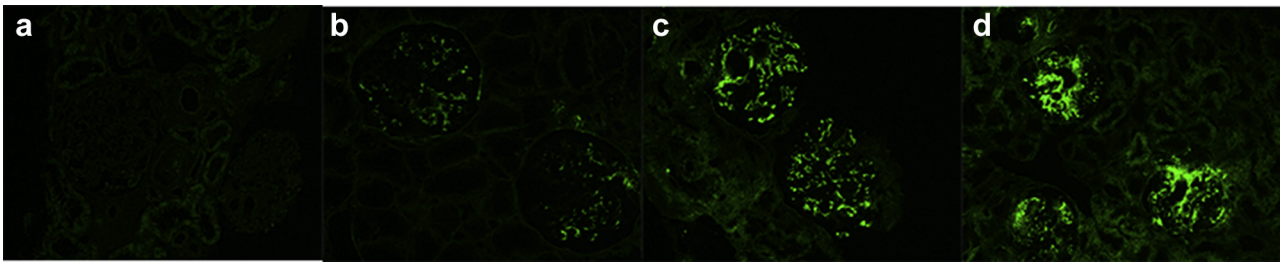

Figure 1. Representative pictures of immunofluorescence staining of mesangial complement factor H–related protein 5 (FHR-5) 0 to 3+ in patients with immunoglobulin A nephropathy (IgAN). Granular positive staining of FHR-5 by immunofluorescence along the glomerular mesangial and capillary area in patients with IgAN. (a) Negative. (b) 1+ intensity. (c) 2+ intensity. (d) 3+ intensity. (Original magnification ×200.)

Table 1) and renal IgA deposition (1+/2+/3∼4+—group 0, 2 [8.3%]/12 [50.5%]/10 [41.7%]; group 1, 0 [0.0%]/5 [45.5%]/6 [54.6%]; group 2, 0 [0.0%]/3 [27.3%]/8 [72.7%]; group 3, 0 [0.0%]/2 [20%]/8 [80.0%]; $P = 0.013$, Table 1) were also different among the 4 groups.

There were more cases with 2+ and 3+ FHR-5 staining in cohorts of 2+ and 3∼4+ mesangial C3 deposition (FHR-5 2+/3+—cohort 0 mesangial C3 deposition: 0 [0%]/0 [0%]; 1+ C3 deposition, 1 [20%]/0 [0%]; 2+ C3 deposition, 6 [19.4%]/6 [19.4]; 3∼4+ C3 deposition, 4 [23.5%]/4 [23.5%]; $P = 0.034$, Figure 2b) and in the cohorts of 2+ and 3∼4+ mesangial IgA deposition (FHR-5 2+/3+—cohort 1 mesangial IgA deposition, 0 [0%]/0 [0%]; 2+ IgA deposition, 3 [13.6%]/2 [9.1%]; 3∼4+ IgA deposition, 8 [25%]/8 [25%]; $P = 0.019$, Figure 2c). Interestingly, the glomerular FHR-5 depositions were more abundant in male versus female in patients with IgAN. We found that the proportion of male gradually increased (group 0, 6 [25.0%]; group 1, 3 [27.3%]; group 2, 3 [27.3%]; and group 3, 9 [90%]; $P = 0.002$, Table 1). Other clinical and pathologic manifestations, including gross hematuria, initial proteinuria, eGFR, hypertension, hypertension, and Oxford-M, C scores, showed no differences among the 3 groups (Table 1).

Colocalization of FHR-5 and IgA and FHR-5 and C3c on Glomeruli in Patients with IgAN

As FHR-5 were positive in 57.1% patients with IgAN, and was strongly corelated with renal local complement activation, we further investigated the colocalization of FHR-5 and IgA and FHR-5 and C3c on glomeruli in patients with IgAN by immunofluorescence. There was granular positive staining of IgA by immunofluorescence along the glomerular mesangial and capillary area in patients with IgAN (Figure 3a) and granular positive staining of FHR-5 by immunofluorescence along the glomerular mesangial and capillary area in the same section seen in part a (Figure 3b). FHR-5 and IgA colocalized along the glomerular mesangial and capillary area (Figure 3c). In addition, FHR-5 and C3c colocalized along the glomerular mesangial and capillary area in patients with IgAN (Figure 3g). To quantify the colocalization, 2D fluorograms have been included to confirm the degree of colocalization (Figure 3d, Pearson correlation = 0.9486, overlap coefficient = 0.9500; Figure 3h, Pearson correlation = 0.9351, overlap coefficient = 0.9400).

To show the results of colocalization clearly, the merge images and 2D fluorograms of 1+ to 3+ intensity of FHR-5 and IgA (Supplementary Figure S2) and C3c (Supplementary Figure S3) were presented.

Plasma FHR-5 Level Between Patient with IgAN and Healthy Control Subjects

Plasma FHR-5 levels in patients with IgAN were significantly higher than levels in healthy control subjects (5263.89 ng/ml [4178.38–6203.30 ng/ml] vs. 3711.54 ng/ml [3203.69–3886.00 ng/ml]; $P < 0.001$, Figure 4a). However, there was no significant difference in plasma FHR-5 levels between the FHR-5–positive and FHR-5–negative groups (5469.60 ng/ml [3832.30–6629.30 ng/ml] vs. 5163.57 ng/ml [4286.35–6099.02 ng/ml]; $P = 0.868$, Figure 4b). The plasma FHR-5 levels between male and female patients with IgAN showed no difference (5476.94 ng/ml [4385.92–6157.25 ng/ml] vs. 5165.59 ng/ml [4132.25–6315.93 ng/ml]; $P = 0.451$, Figure 4c). Neither eGFR ($r = -0.010$, $P = 0.945$, Figure 5a) nor initial proteinuria ($r = 0.064$, $P = 0.640$, Figure 5b) was correlated with plasma FHR-5. There was no correlation between plasma FHR-5 and circulating C3 levels ($r = 0.156$, $P = 0.251$, Figure 5c).

Relationship Between Plasma FHR-5 Levels and Histologic Markers of Renal Injury

We found that patients with IgAN in groups with high FHR-5 levels showed more severe pathologic manifestations than those with lower FHR-5 levels. Plasma FHR-5 levels were significantly higher in patients with M1 at diagnosis than those with no M0 (5731.34 ng/ml [5027.27–7091.34 ng/ml] vs. 4475.52 ng/ml [3621.61–5828.28 ng/ml]; $P = 0.013$, Figure 6a). FHR-5 levels in patients with IgAN with and without renal biopsy

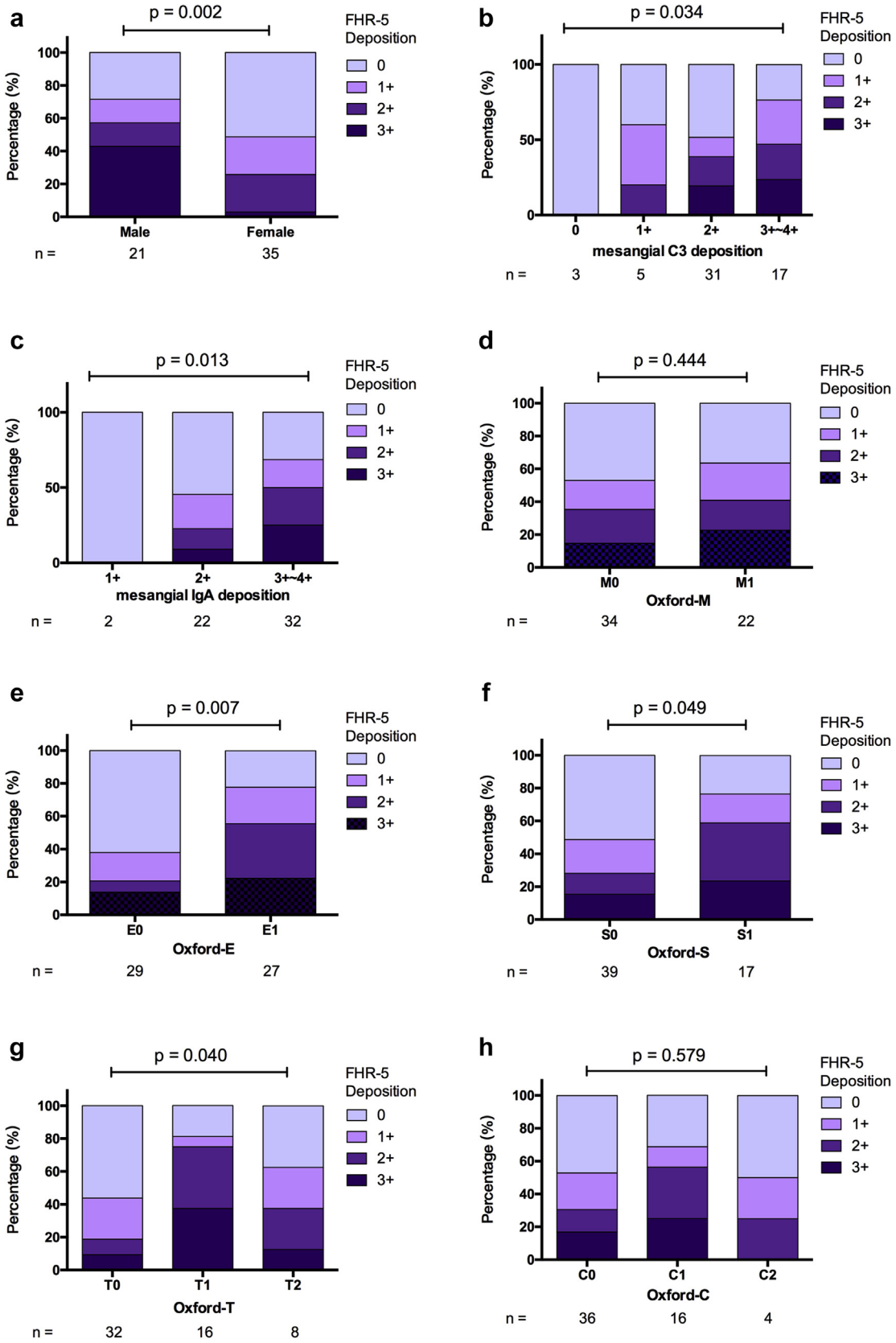

Figure 2. The intensity of glomerular factor H–related protein 5 (FHR-5) deposition was associated with renal damage. (a) Glomerular FHR-5 depositions were more abundant in male versus female patients with immunoglobulin A nephropathy (IgAN). FHR-5 intensity grades increased according to mesangial C3 deposition 1+ to 3+∼4+ (b) and mesangial IgA deposition 1+ to 3+∼4+ (c). The proportion of different glomerular FHR-5 intensity in IgAN stratified according to (d) Oxford-M, (e) Oxford-E, (f) Oxford-S, (g) Oxford-T, and (h) Oxford-C. Glomerular FHR-5 intensity was influenced by Oxford-E, Oxford-S and Oxford-T scores, but not Oxford-M and Oxford-C scores. Significance was determined by χ^2 test-Pearson χ^2 (in a) and linear-by-linear association (in b through h).

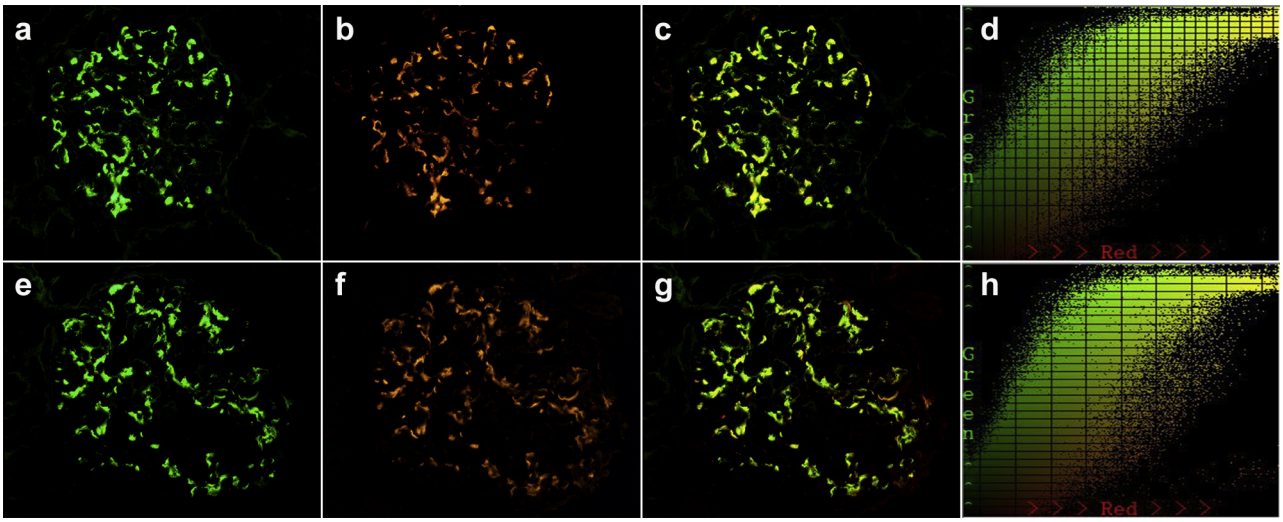

Figure 3. Paraffin-embedded sections for colocalization of factor H–related protein 5 (FHR-5) and immunoglobulin A (IgA) and FHR-5 and C3c by immunofluorescence in patients with immunoglobulin A nephropathy (IgAN). Granular positive staining of IgA (a), and C3c (e) by immunofluorescence along the glomerular mesangial and capillary area in patients with IgAN. (b and f) Granular positive staining of FHR-5 by immunofluorescence along the glomerular mesangial and capillary area in the same section with a and e. (c) FHR-5 and IgA colocalized completely along the glomerular mesangial and capillary area. (g) FHR-5 and C3c colocalized along the glomerular mesangial and capillary area. (d and h) The corresponding 2-dimensional fluorograms have been included to confirm the degree of colocalization (d, Pearson correlation = 0.9486, overlap coefficient = 0.9500; h, Pearson correlation = 0.9351, overlap coefficient = 0.9400). (a through c and e through g, original magnification ×400.)

specimen evidence of E, S, T, and C did not differ (Figure 6, b through e). Patients with IgAN with benign arteriolar nephrosclerosis had higher plasma FHR-5 levels than those without it (6218.20 ng/ml [4675.33–8030.79 ng/ml] vs. 4864.71 ng/ml [4151.69–5774.74 ng/ml]; $P = 0.020$, Figure 6f).

DISCUSSION

The high similarity of IgAN with FHR-5 nephropathy aroused our interest in exploring the role of FHR-5 in IgAN. Complement activation, especially renal local complement alternative activation, played a pivotal role in the pathogenesis and prognosis of IgAN.[7,28] In our study, we found FHR-5 deposited in the mesangial and capillary areas in 57.1% of patients with IgAN. FHR-5 staining intensity associated with histologic injury markers, including Oxford E1, S1, and T1/T2 lesions.

Before our study, glomerular staining for FHR-5 was observed in patients with membranous nephropathy, lupus nephritis, and diabetic nephropathy,[22] which suggested those depositions were not specific to IgAN. However, the glomerular FHR-5 depositions were more abundant in patients with progressive versus stable IgAN.[23] Because of a lack of prognostic data, we compared clinical and histologic data among groups with different FHR-5 deposition intensities. To further explore the clinical implication of the intensity of FHR-5

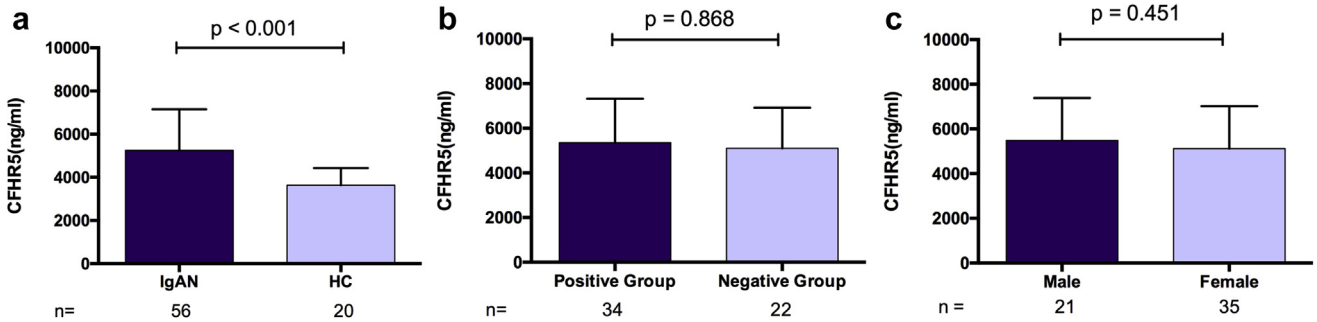

Figure 4. Plasma complement factor H–related protein 5 (FHR-5) levels in IgA nephropathy (IgAN). (a) Compared with healthy control (HC) subjects, patients with IgAN presented with significantly higher plasma FHR-5 levels (5263.89 ng/ml [4178.38–6203.30 ng/ml] vs. 3711.54 ng/ml [3203.69–3886.00 ng/ml]; $P < 0.001$). (b) There was no significant difference in plasma FHR-5 levels between FHR-5–positive and FHR-5–negative groups (5469.60 ng/ml [3832.30–6629.30 ng/ml] vs. 5163.57 ng/ml [4286.35–6099.02 ng/ml]; $P = 0.868$). (c) The plasma FHR-5 levels between male and female patients with IgAN showed no difference (5476.94 ng/ml [4385.92–6157.25 ng/ml] vs. 5165.59 ng/ml [4132.25–6315.93 ng/ml]; $P = 0.451$). Significance was determined by the Mann-Whitney *U* test.

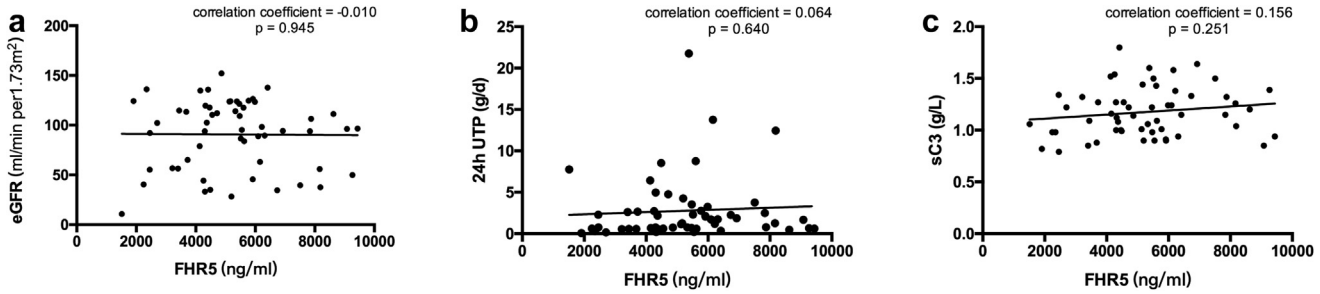

Figure 5. Correlation of complement factor H–related protein 5 (FHR-5) and markers of renal injury. There was no significant correlation of plasma FHR-5 levels with estimated glomerular filtration rate (a), initial proteinuria (b), and circulating C3 (c).

deposition in IgAN, we stratified patients with IgAN into 4 groups based on the intensity of FHR-5 deposition in glomeruli as described above. No difference among the 4 groups was found for gross hematuria, prodromic infection, initial proteinuria, and eGFR. Nevertheless, there was a high intensity of FHR-5 deposition in patients with IgAN who presented with severe E1 lesions (a histologic marker of active inflammation[29]) and S1, and T1/T2 lesions. In vitro studies have shown that FHR-5 could function as competitive antagonists of FH for the regulation of C3 activation,[14,15] which may lead endothelial cell injury as seen in atypical hemolytic uremic syndrome. Besides, FH deposition was reduced in progressive disease, which might be a role for FH deregulation by FHR-5 in renal injury in IgAN.[23] Consistent with a previous study, FHR-5 was deposited in glomeruli with sclerotic lesions,[22] while the role of FHR-5 is less clear. Oxford-T lesion was an independent risk factor for IgAN.[25] Patients with IgAN with high-intensity FHR-5 deposition presented with more serious T lesions, which may imply that FHR-5 deposition in patients with IgAN might influence the prognosis. Interestingly, we found that the glomerular FHR-5 depositions were more abundant in male versus female in patients with IgAN. In the Peking University IgAN cohort, they found circulating FHR-5 levels were significantly higher in male patients with IgAN compared with female patients with IgAN.[21] As we know, both in IgAN and FHR-5 nephropathy, males were more common than females, but the underlying mechanism is still unclear. Significant sex differences in FHR-5 deposition in IgAN need further investigation. First, it is necessary to identify whether those difference was specific to IgAN.

We found that glomerular FHR-5 deposition intensity correlated positively with the staining intensity of IgA and C3 rather than circulating IgA or C3. FHR-5 codeposited with IgA and C3c in glomerular mesangial and capillary area in patients with IgAN. Previous studies showed glomerular FHR-5 correlated with C3b/iC3b/C3c/C3d and C5b-9 staining.[22,23] As an alternative pathway regulator protein, FHR-5 may be a potential marker of the activation of alternative pathway in glomeruli. There was no correlation between circulation FHR-5 and intensity of FHR-5 deposition, which might imply that renal deposits of FHR-5 may not come from circulation. Inhibition of glomerular FHR-5 would be a potential treatment to improve complement-mediated glomerular inflammation and injury.

Consistent with previous work from Medjeral-Thomas *et al.*[20] and Zhu *et al.*,[21] we also found that circulating FHR-5 levels were elevated in patients with IgAN. As we failed to find any correlation between plasma FHR-5 and eGFR or initial proteinuria, we assessed the correlations between plasma FHR-5 and histologic markers of renal injury in IgAN. Plasma FHR-5 levels were associated with Oxford M1 lesions and benign arteriolar nephrosclerosis. However, glomerular FHR-5 depositions were not associated with M1 lesions and benign arteriolar nephrosclerosis. Perhaps circulating and glomerular deposition of FHR-5 may have different functions in patients with IgAN. This was an observation study, and further studies are needed to reveal the mechanisms that result in glomerular FHR-5 deposition and its relationship with glomerular damage.

As activation of complement plays a pivotal role in the pathogenesis and progress of IgAN, the interventions of complement system targeted treatment are increasingly used in the treatment of IgAN. Eculizumab, an anti-C5 antibody, has achieved significant results in reducing urinary protein and stabilizing renal function in cases of children with crescentic IgAN.[30] Inhibition of glomerular FHR-5 may improve glomerular complement regulation and ameliorate complement-mediated glomerular inflammation and injury. The number of patients is a main limitation of our study. When divided into subgroups depending on the intensity of FHR-5, the number of patients is low. The observations from the small sample size must be verified in a larger cohort.

To conclude, we found that glomerular intensity of FHR-5 deposition could indicate the severity of histologic lesions in patients with IgAN.

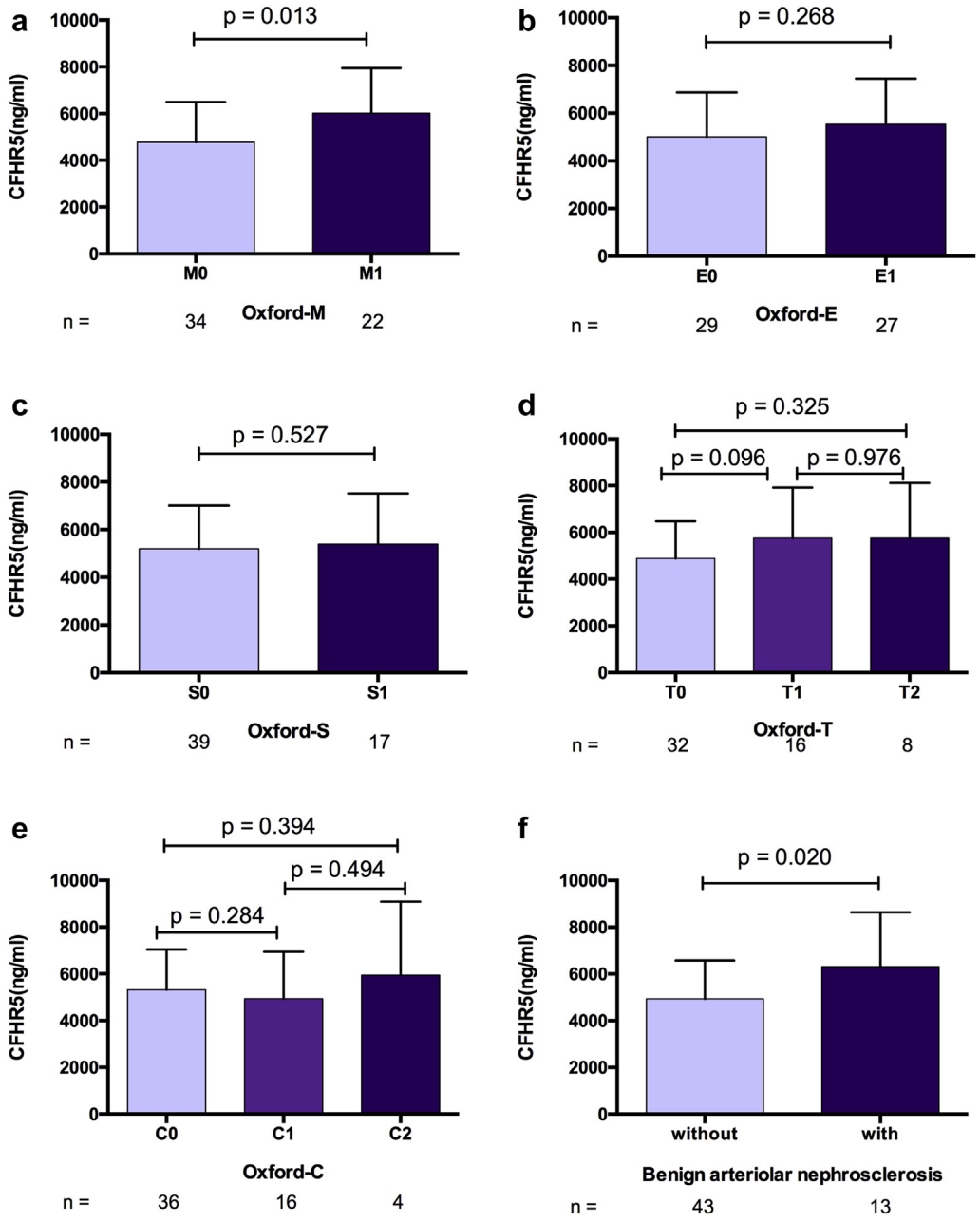

Figure 6. Relationship of plasma complement factor H–related protein 5 (FHR-5) levels with histologic markers of disease severity in immunoglobulin A nephropathy (IgAN). Plasma FHR-5 in patients with IgAN were stratified according to (a) Oxford-M, (b) Oxford-E, (c) Oxford-S, (d) Oxford-T, (e) Oxford-C, and (f) with or without benign arteriolar nephrosclerosis. Plasma FHR-5 levels of patients with IgAN were influenced by Oxford M lesion and benign arteriolar nephrosclerosis. The bar presents *P* values derived using the Mann-Whitney *U* test.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Figure S1. Flow diagram of patients of IgA nephropathy enrolled in the study

Figure S2. Colocalization of different intensity of FHR-5 and IgA by immunofluorescence in patients with IgAN

Figure S3. Colocalization of different intensity of FHR-5 and C3c by immunofluorescence in patients with IgAN

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