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Original Article

Anti-M-type phospholipase A2 receptor antibody diagnosing and monitoring disease activity in idiopathic membranous nephropathy: a single center and retrospective cohort study

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Abstract: Objectives: This study was to investigate the effects of anti-M-type phospholipase A2 receptor (PLA2R) antibody in diagnosing and monitoring the progresssion in idiopathic membranous nephropathy (iMN). Methods: Fifty-six cases with iMN were chosen from October 2009 to November 2015. The control cohorts were patients with hepatitis B virus associated MN (n=17), lupus nephritis related MN (n=19) and non-MN nephrotic syndrome (n=39). The level of serum a-PLA2R antibody (a-PLA2R) was detected by enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence (IIF). The expression of renal PLA2R and IgG subclasses was examined by indirect immunofluorescence. Results: IMN patients with a-PLA2R negativity more often entered remission (68.4%), whereas positive patients with immunosuppressive therapy achieved remission more frequently than patients with conservative therapy. In a-PLA2R positive patients, antibody titers correlated with nephropathy activity. A-PLA2R combining with renal IgG4-dominant subclasses strength the determination of iMN, and absence of a-PLA2R and renal non-IgG4-dominant subclasses excluded the diagnosis of iMN. Conclusions: A-PLA2R titers are helpful in diagnosing iMN and monitoring the activity of iMN. The multiple-assays of serum a-PLA2R antibody, renal PLA2R and renal IgG4 would enhance the diagnosis of iMN. For MN patients with negative a-PLA2R or negative renal PLA2R, secondary MN is more evidently certain.

Keywords: Membranous nephropathy, nephrotic syndrome, M-type phospholipase A2 receptor, IgG subclass

Introduction

Idiopathic membranous nephropathy (iMN) is a kind of auto-immune diseases with glomerulus specifically damaged, which results in forming subepithelial immune complex deposits along or in the glomerular basement membrane (GBM) [1]. In western countries, iMN is the predominant cause of nephrotic syndrome within Caucasian adults, and possesses about 70% MN patients. The incidence of iMN increases comparatively highly in recent years, while the incidence of secondary membranous nephropathy (sMN) decreases for more primary diseases are identified with utility of advanced laboratory techniques, e.g. systemic lupus erythematosus, hepatitis B/C virus infection, toxics or drug exposure and cancer [2].

Meanwhile, various biomarkers in iMN patients have been discovered, such as M-type phospholipase A2 receptor (PLA2R), α-enolase, aldose reductase, superoxide dismutase (SOD) and cationic bovine serum albumin (BSA). All these biomarkers could lead to glomerular IgG4-dominat deposits, which is the classic symbol in auto-immune disease. Compared with the other biomarkers, PLA2R and its antibodies have been proven to be the major antigen and antibodies in iMN patients by various clinical trials [3-6]. Beck et al. [7] initially detected circulating serum a-PLA2R antibodies in about 70% adult iMN patients while no a-PLA2R positivity in sMN patients, which indicated the potential clinical significance of identifying iMN patients from sMN patients. A great deal of studies have also demonstrated the high speci-

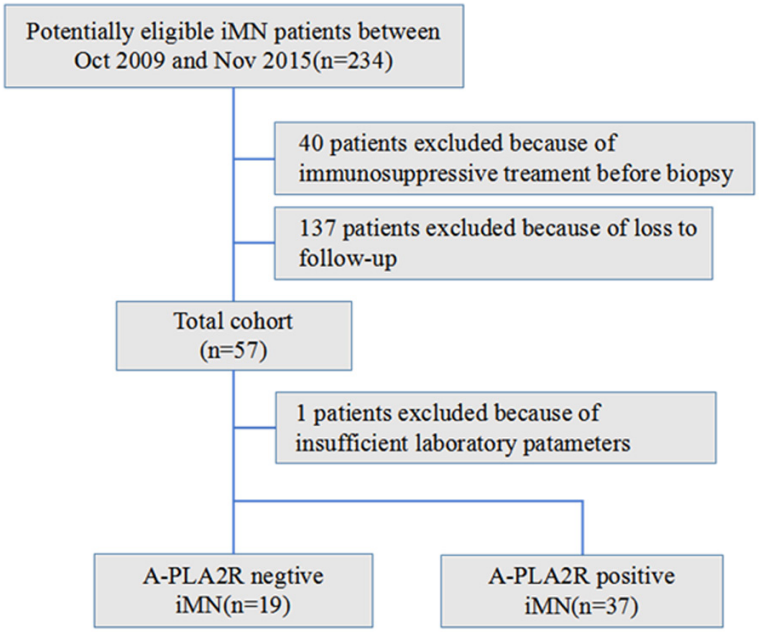

Figure 1. Flowchart for idiopathic membranous nephropathy (iMN) patients inclusion/exclusion process

ficity of a-PLA2R in Caucasia iMN patients and the correlation between a-PLA2R titers with nephropathy activity [8]. Some of them followed up patients in cohorts and confirmed the predictive role of a-PLA2R titers for clinical outcomes [9, 10].

However, a new question arose, that the absence of circulating a-PLA2R is probably not sufficient to exclude the diagnosis of iMN. Svobodova et al. [11] have proposed that joint detection of renal PLA2R should be introduced into diagnosis, but the combination with IgG subclasses is still not conducted. In this study, we collected comprehensive data about a-PLA2R and relative laboratory parameters, as well as renal PLA2R in a well-defined cohort, to investigate the presence of renal PLA-2R, prognostic flux and clinical outcomes in a-PLA2R titers. Moreover, we analyzed the intensity of renal IgG subclasses, and determined the proportion of IgG4-dominant/co-dominant immunophenotype of both a-PLA2R positive and negative iMN, for the final goal to determine iMN with comprehensive multiple-assays.

Materials and methods

Patients

In this retrospective study, a total amount of 234 cases were proved iMN after renal biopsy in Peking University Shenzhen Hospital from October 2009 to November 2015 (**Figure 1**). 40 cases were excluded for immunosuppressive therapy before biopsy; 137 cases were excluded for loss to follow-up; 1 case was not obtained sufficient laboratory parameters. Finally, 56 iMN cases finished follow-up with the median duration 14 months interquartile range (12-25). Actually, we tried to terminate the follow-up at a second sampling after 1.5 year' treatment, however, the second sampling time for every case could not be achieved at a unified time.

17 patients with hepatitis B virus associated membranous nephropathy (HBV-MN) were chosen after they had biopsy-proven MN and demonstrated serum and renal tissue hepatitis B surface antigen or hepatitis B core antigen, along with symptoms of glomerulonephtritis and without other secondary diseases. 19 patients with lupus nephritis associated membranous nephropathy (LN-MN) were enlisted if they fulfilled at one time at least four of the American Rheumatism Association 1982 revised criteria and were biopsy-proven MN, including III+V (n=5), IV+V (n=5) and V (n=9).

In addition, patients with other primary and secondary nephropathy were selected, as nephrotic controls, including cases with IgA nephropathy (n=8), minimal change disease (MCD, n=8), mesangial proliferation glomerulonephritis (MsPGN, n=8), membranous proliferation glomerulonephritis (MPGN, n=2), lupus nephritis type II (LN-II, n=2), LN-III (n=3), LN-IV (n=5), benign hypertensive nephroscelosis (BHN, n=2), and chronic interstitial nephritis (CIN, n=1).

For all patients, initial clinical data were prospectively collected, including detailed medical history and laboratory parameters on disease activity i.e. 24-hour urine protein excretion, serum albumin, serum creatinine, cholesterol, triglycerides. The estimated glomerular filtration rate (eGFR) was calculated by using the Modification of Diet in Renal Disease (MDRD)

equation for adults and the Schwartz equation for children.

Before the inclusion, no patient was allowed for immunosuppressive therapy, while every patient provided their informed consent before inclusion. The study had the approval from the ethics committee of Peking University Shenzhen Hospital.

Detection a-PLA2R by indirect immunoinfluorescence

Semi-quantitative circulating a-PLA2R was determined by using a commercially available recombinant cell-based IIF kit (EUROIMMUN, Germany), containing Biochip mosaic of formalin-fixed HEK293 cells over expressing PLA2R1. Biochip mosaic was incubated with human serum samples (1:10 in dilution buffer) for 30 minutes at room temperature. The bound IgGs of a-PLA2R were detected by FITC labeled goat anti-human IgG polyclonal antibody for 30 minutes at room temperature. Two independent observers without knowledge of the clinical details evaluated all slides with animmunofluorescence microscopy (Nikon, Apidrag, Romania).

Detection a-PLA2R by enzyme-linked immunosorbent assay

Quantitative circulating a-PLA2R was determined using a commercially available ELISA kit (EUROIMMUN, Germany) containing whole-length PLA2R1-coated microplate. Microplates were incubated with human serum samples (1:101 indilution buffer) for 30 minutes at room temperature. The bound IgGs of a-PLA2R were detected with HRP conjugated rabbit anti-human polyclonalIgG for 30 minutes. Chromogen substrate solution was added for 15-minute-reaction stopped by stopping solution. The optical density was read using an automated microplate absorbance reader (Rayto, USA). A value higher than 20 RU/ml indicated a positive result. All samples were measured in duplicate tubes.

Detection renal PLA2R and renal IgG subclasses deposition

Indirect immunofluorescence (IIF): human fresh kidney tissues from biopsy were fixed in 4% paraformaldehyde and embedded in paraffin, and sliced in 4 μm slides. Slides were deparaffinized in xylene and ehydrated sequentially in ethanol, followed by antigen retrieval in citrate buffer (0.01 M, pH 6.0) for 30 minutes in microwave. Quench endogenous peroxidasein 0.3% H_2O_2 for 10 minutes, and block slides with 10% fetal bovine serum in PBS. Slides were incubated in rabbit anti-human PLA2R1 (1:100) (Sigma-Aldrich, USA) in PBS overnight at 4°C followed by FITC labeled goat anti-rabbit IgG (1:250) (Abcam, UK) at 37°C in the dark for half hour.

Direct Immunoflourescent Assay (DFA): frozen kidney tissues from biopsy were stained with IgG subclasses. 4 μm slides were incubated in IgG1 (1:50), IgG2 (1:50), IgG3 (1:50), IgG4 (1:50) (Sigma-Aldrich, USA) in PBS at 37°C for half hour.

For IgG subclasses depositsin glomeruli, a slide was judged to be positive with positive granular deposits staining and negative withoutany staining. Everyslide was given an average score on a scale of 0 to 3 (0 negative, 1 weak staining, 2 moderate staining, 3 strong staining) from two independent observers with an immunofluorescence microscopy (Nikon, Romania). The final median score was calculated.

Medical history

Cases with proteinuria <8 g/d and stable kidney function were initially treated with conservative therapy (CT). After 6-month-treatment, if no improvement was achieved, immunosuppressive therapy (IST) would began. For 6/56 cases with proteinuria >8 g/d initially treated with IST.

CT contained symptomatic drugs, including angiotensin-converting enzyme inhibitors (ACEIs), angiotensin II receptor blockers (ARBs) anddiuretics.

IST included the use of corticosteroid (prednison and metacortandralone), cyclophosphamide (CTX), or leflunomide and/or cyclosporine A.

Clinical evaluation

Complete remission (CR) was defined as proteinuria, <0.3 g/d with stable kidney function; partial remission (PR) was defined as protein-

Table 1. Clinical parameters and a-PLA2R in MNs and non-MNs

	iMN (potential eligible)	iMN (follow-up baseline)	HBV-MN	LN-MN	Nephrotic Controls
N (Male/Female)	234 (124/110)	56 (31/25)	17 (12/5)	19 (1/18)	39 (19/20)
Age (years)	42.5±16.1	41.1±11.9	32.0±9.7	32.0±9.7	33.9±13.1
Proteinuria, g/d	5.0±2.9	4.9±2.7	5.4±2.9	3.5±3.5	4.2±2.9
SAlb, g/L	24.7±7.5	24.9±8.1	22.4±9.4	27.5±7.0	28.6±9.1
SCr, μmol/L	73.0±24.7	67.0±20.3	89.0±29.7	67.9±23.5	146.3±166.0
eGFR, ml/min/1.73m^2	117.6±49.8	132.0±68.8	98.7±33.4	419.8±193.5	294.3±153.1
A-PLA2R, % (Posivte/Negtive)	50.9% (119/115)	66.1% (37/19)	70.6% (12/5)	5.3% (1/18)	0
A-PLA2R, RU/ml	21.2 (0-1198.4)	49.7 (0.0-913.6)	77.1 (0.8-415.1)	1.0 (0.6-28.8)	0 (0-2.2)

Abbreviation: iMN, idiopathic membranous nephropathy; HBV-MN, hepatitis B virus associated membranous nephropathy; LN-MN, lupus nephritis related membranous nephropathy; SAlb, serum albumin; SCr, serum creatinine; A-PLA2R%, a-PLA2R for ELISA; A-PLA2R, RU/ml, a-PLA2R for ELISA, medium (range). Nephrotic controls including: IgA nephropathy (n=8), minimal change disease (n=8), mesangial proliferation glomerulonephritis (n=8), membranous proliferation glomerulonephritis (n=2), lupus nephritis type II (LN-II, n=2), LN-III (n=3), LN-IV (n=5), benign hypertensive nephroscelosis (n=2), and chronic interstitial nephritis (n=1). eGFR was calculated by using the modified MDRD (Modification of Diet in Renal Disease) equation for adults and the Schwartz equation for children.

uria, <3.5 g/d with a reduction of >50% from baseline and stable kidney function. Achieving remission included both CR and PR. Follow-up time was calculated from the time of biopsy until the second blood sampling.

Statistical analysis

SPSS 21.0 (IBM, USA) was applied in statistical analysis. Datadescription with mean ± stand variable was for normal distributed data and median (range) was for nonparametric data. Comparison between groups was tested by t test, Mann-Whitney, Kruskal-Wallis, Wilcoxon and Fisher's exact test. Correlation was assessed by Spearman's rank coefficient. Statistical significance was defined as $P<0.05$.

Results

Baseline clinical characteristics

At baseline, for iMN cases, there was no obvious difference in age, proteinuria, serum albumin, serum creatinine and eGFR between 234 cases with serum available and 56 cases for follow-up. Besides, the a-PLA2R titers in 37/56 a-PLA2R positive cases could reflect the prevalent baseline a-PLA2R titers in 119/234a-PLA2R positive cases. However, the positivity of a-PLA2R and the median a-PLA2R titer in 56 cases was significantly higher than all 234 cases (**Table 1**). For results in this study, we could not exclude the bias of a-PLA2R positivity and titers resulted from baseline discrepancy.

Laboratory parameters and a-PLA2R in MNs and nephrotic syndrome

The circulating a-PLA2R positivity of IIF was 71.4% (40/56) in iMN group, while the positivity of ELISA was 66.1% (37/56). 2 samples with ELISA values between 2 and 20 RU/ml demonstrated a positive signal on IIF, and the discrepancy rate was 3.6% (2/56). To detect correlations with laboratory parameters, we chose quantitative ELISA data. All the other MNs and nephritic controls showed concordant results of two assays, and none of nephrotic controls was a-PLA2R positive (**Table 1**).

In the first ELISA, the positivity of iMN group was slightly lower than HBV-MN (70.6%, 12/17). However, the positivity of both two groups was significantly higher than LN-MN group (5.3%, 1/19). Only one LN-III+V case appeared positive in 19 LN-MN cases while negative in LN-IV case and LN-V case.

For 37 a-PLA2R positive cases in iMN group, a-PLA2R titers moderately correlated with proteinuria (Spearman, r=0.361, P=0.028), and with serum albumin (Spearman, r=-0.330, P=0.046), but not with serum creatinine and eGFR respectively. For 12 a-PLA2R positive HBV-MN cases, there was no any correlation between a-PLA2R titers with above laboratory-parameters.

Table 2. Laboratory parameters and a-PLA2R at the inception and end of follow-up in iMNs

A-PLA2R		A-PLA2R Negative	A-PLA2R Positive	A-PLA2R Positive	
				CT	IST
N (Male/Female)		19 (12/7)	37 (19/18)	23 (11/12)	14 (8/6)
Age (years)		40.0±11.2	41.7±12.3	40.4±13.1	43.7±10.9
TR, % (n/N)		68.4% (13/19)	64.9% (24/37)	60.8% (14/23)	71.4% (10/14)
PR, % (n)		21.1% (4)	48.7% (18)	47.8% (11)	50.0% (7)
CR, % (n)		47.4% (9)	16.2 (6)	13% (3)	21.4% (3)
Proteinuria, g/d	1st	4.6±2.8	5.0±2.6	4.4±2.1	6.0±3.1
	2nd	1.3±1.8	2.2±1.9	2.3±1.7	2.1±2.1
SAlb, g/L	1st	26.2±8.3	24.4±8.2	24.9±7.2	23.3±9.5
	2nd	38.1±9.2	32.2±9.5	31.0±8.9	34.3±10.2
SCr, μmol/L	1st	69.7±19.1	65.9±21.2	66.9±17.0	63.4±26.8
	2nd	77.4±31.9	73.1±27.9	72.2±27.1	81.1±37.3
eGFR, ml/min/1.73 m^2	1st	120.4±28.7	138.4±83.0	122.1±35.00	164.0±123.8
	2nd	113.7±37.3	122.3±54.7	119.4±47.6	120.6±69.5
A-PLA2R, % (n)	1st	0	100.0% (37)	100.0% (23)	100.0% (14)
	2nd	15.3% (1)	56.8% (21)	73.9% (17)	28.6% (4)
A-PLA2R, RU/ml	1st	2.73 (0.0-16.4)	98.3 (22.1-913.6)	105.5 (22.1-242.1)	98.7 (29.9-913.6)
	2nd	1.74 (1.4-42.1)	32.3 (0.0-438.7)	42.2 (0.0-264.3)	2.2 (1.5-438.7)

Abbreviation: iMN, idiopathic membranous nephropathy; TR, total remission; PR, partial remission; CR, complete remission; SAlb, serum albumin; SCr, serum creatinine; A-PLA2R%, a-PLA2R for ELISA; A-PLA2R, RU/ml, a-PLA2R for ELISA, medium (range). CT: conservative therapy; IST: immunosuppressive therapy; 1st: first assay; 2nd: second assay.

Clinical evaluation of laboratory parameters and a-PLA2R in iMNs

For iMN cases, 19 initially a-PLA2R negative cases and 37 initially a-PLA2R positivecases were followed up at clinic.For a-PLA2R positive iMNcases, 7 in 19 a-PLA2R negative cases received corticosteroids,and 12 in19 cases received ACEIs or ARBs. For a-PLA2R positive iMN cases, 23/37 cases merely received ACEIs and/or ARBs, while 14/37 cases received IST plus ACEIs and/or ARBs. More specifically with IST, 5 cases were treated with corticosteroids only, 7 cases were administrated with corticosteroids plus CTX, 1 case was treated with metacortandralone plus leflunomide and 1 case with cyclosporine A alone. The characteristics of the whole cases are reported in **Table 2**.

The total proteinuria remission rate for a-PLA-2R negative group was68.4% (13/19). 2 cases with CT and 2 cases with IST achieved PR; 5 cases with CT and 4 cases with IST cases achieved CR. One a-PLA2R negative case turned to be positive at the second assay.

The total proteinuria remission rate for a-PLA2R positive group was 64.9% (24/37), being lower than the negative group. 11 cases with CT and 7 cases with IST achieved PR, and 3CT cases and 3 IST cases achieved CR. 60.8% (14/23) of cases with CT achieved remission, which was lower than 71.4% (10/14) of IST group. Correspondingly, 26.1% (6/23) of cases with CT turned to be a-PLA2R negative, which was lower than 71.4% (10/14) of cases with IST.

Within a-PLA2R positive group, the median a-PLA2R titers in prevalent 37 cases decreased dramatically from median 98.3 to 32.3 RU/mL, along with the rise of serum albumin and the release of proteinuria, as well as the decrease of serum creatinine. For CT group, there was no statistical significance for the decrease of a-PLA2R titers, even though median proteinuria released and median serum albumin increased significantly. As to IST group, a-PLA2R titers decreased dramatically from median 98.7 to 2.2 RU/mL, with much sharper slope than the decrease of prevalent 37 cases with a-PLA2R positivity. Paralleling with antibody change, the improvements of parameters (proteinuria, albumin) related nephrotic activity and eGFR improved much more significant than the prevalent 37 cases.

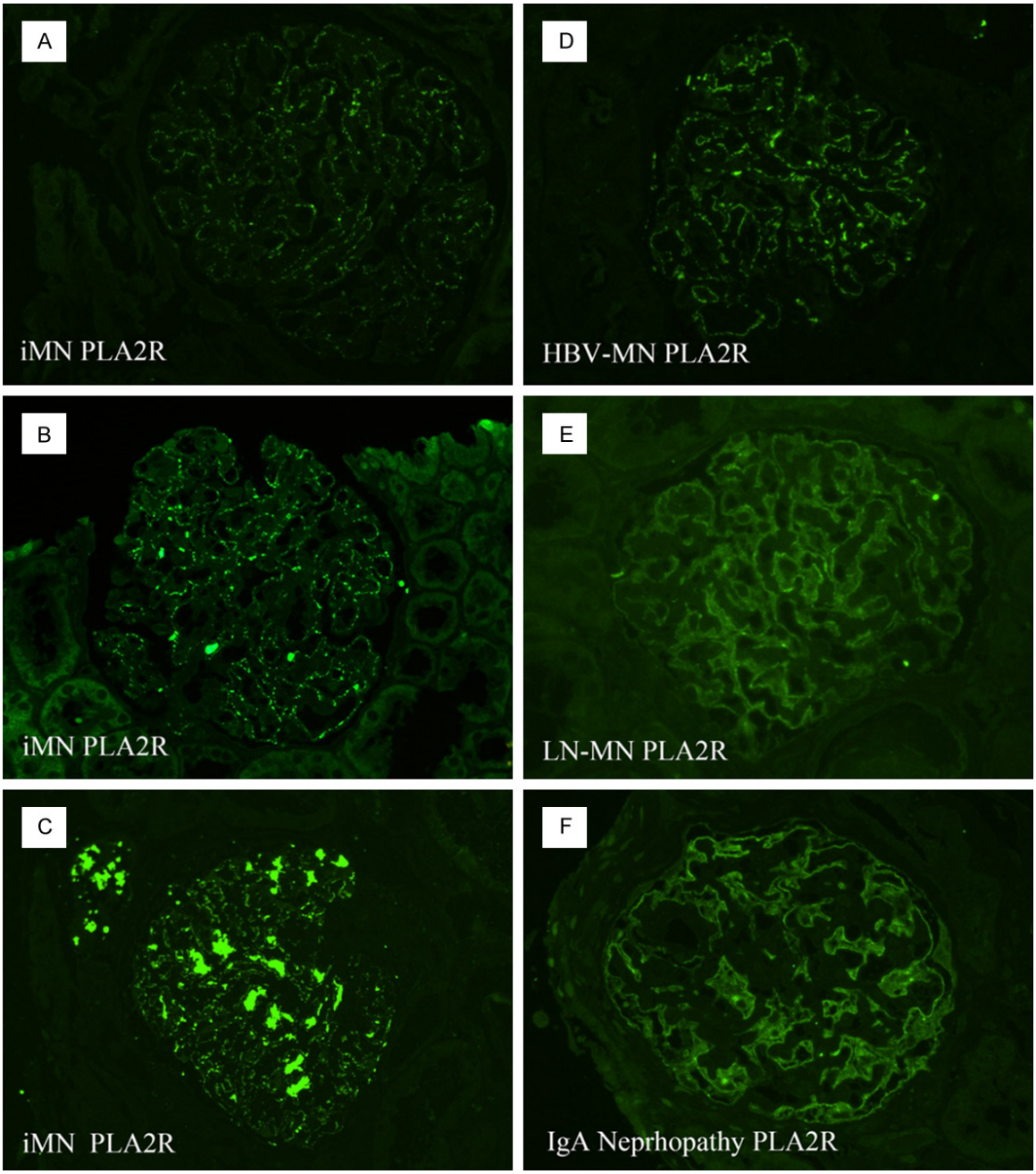

Figure 2. Positive renal PLA2R staining in idiopathic membranous nephropathy (iMN), hepatitis B virus associated membranous nephropathy (HBV-MN) and lupus nephritis related membranous nephropathy (LN-MN). Most iMN cases showed specific morphology: PLA2R deposited in weakly bright granules (A)/moderately bright granules (B)/strongly bright granules (C), and lined along with capillary loop in the glomeruli, being defined as positive staining for PLA2R. The moderately bright granules of PLA2R was also observed in HBV-MN patients (D), and distinct from the faint staining of PLA2R in LN-MN patients (E) and nephrotic controls (IgA nephropathy, F).

We also detected correlations between the last-minus-first observation differences. For 37 a-PLA2R positive cases, there was a significant correlation between the decrease in a-PLA2R titer and the decrease in proteinuria (Spearman correlation coefficient =0.510). Within CT group, however, the correlation was between the decrease in a-PLA2R titer and the decrease in proteinuria (Spearman correlation coefficient =0.487). Nevertheless, within IST group, the correlation existed between a-PLA2R and the increase of eGFR instead.

Table 3. Renal PLA2R deposits and IgG subclasses in iMNs and HBV-MNs

	iMN		HBV-MN	
A-PLA2R	Positive	Negative	Positive	Negative
Tested Cases/Total Cases	14/37	9/19	6-12	4/5
PLA2R Positivity, % (Positive/Tested)	100.0% (14/14)	77.8% (7/9)	100.0% (6/6)	0.0% (0/4)
IgG1 Score	2.5 (0.0-4.0)	1.0 (0.0-3.0)	0.3 (0.0-3.0)	2.5 (0.0-3.0)
IgG2 Score	0.8 (0.0-3.0)	0.8 (0.0-2.0)	0.3 (0.0-1.5)	0.8 (0.3-2.0)
IgG3 Score	2.0 (0.0-4.0)	0.5 (0.0-3.5)	2.0 (0.0-2.5)	1.0 (0.5-2.0)
IgG4 Score	4.0 (0.0-4.0)	4.0 (0.0-4.0)	3.0 (0.0-4.0)	0.8 (0.0-4.0)

91.3% of iMN patients were renal PLA2R positive, higher than 60% in HBV-MN patients. A-PLA2R positive patients had predominant IgG4 intensity, no matter patients with iMN or HBV-MN. HBV-MN cases with a-PLA2R negativity and PLA2R negativity were IgG1 predominant, followed by IgG3 and IgG4.

In addition, 2 a-PLA2R positive HBV-MN cases were detected twice, for the rest 17 were lost to follow up. One positive HBV-MN case turned to be negative at the second ELISA. In this case, proteinuria declined from 6.9 g/d to 0.8 g/d and renal insufficiency back to normal after the treatment of steroid plus lamivudine. The other case was treated with conservative benazepril and entecavir, for protienuria maintained about 2 g/d and renal function parameters retained stable far from renal insufficiency. The a-PLA2R titers, however, increased from 415.1 RU/ml to 1254.1 RU/ml during the next 4 months.

Renal PLA2R and IgG subclasses

Kidney samples were available in 23 of the 56 iMN cases, 10 of the 17 HBV-MN cases, 4 of the 19 LN-MN cases and 31 of the 39 nephrotic controls. All kidney samples were collected while operating renal biopsy. Cases with LN-MN and nephrotic controls shared the same staining of renal PLA2R, which faintly spread over glomerular mesangial region like thin fog (**Figure 2**). The other nephropathy shared the same staining, which was defined as the negative result for renal PLA2R in this research. Meanwhile, most iMN cases showed distinct staining: PLA2R deposited in brightly granules and lined along with capillary loop in the glomeruli, being defined as positive staining for renal PLA2R (**Figure 2**).

23 iMN cases and 10 HBV-MN cases with both serum a-PLA2R and renal PLA2R data available were analyzed. The positivity (91.3%, 21/23) of renal PLA2R in iMN group was higher than HBV-MN group (60%, 6/10) but there was no significance between iMN and HBV-MN. All a-PLA2R positive iMN and HBV-MN cases showed positive PLA2R in glomeruli; 2 a-PLA2R negative iMN cases and all the a-PLA2R negative HBV-MN cases were renal PLA2R negative. One LN-MN was renal PLA2R positive and was the same one with positive a-PLA2R in above serum assays.

Both a-PLA2R positive iMN cases and a-PLA2R negative iMN cases had predominant IgG4 intensity, so did a-PLA2R positive HBV-MN cases (**Table 3**, **Figure 3**). Interestingly, HBV-MN cases which showed negative in both a-PLA2R and renal PLA2R were IgG1 predominant, followed by IgG3 and IgG4.

Discussion

Although the exact pathogenesis of PLA2R in iMN is currently unknown, PLA2R is potentially one of the primarypathogenic antigens in glomeruli. In this study, we combined initial diagnosis and clinical progress with evaluation in a-PLA2R positive patients in order to detect relations between clinical course and a-PLA2R titers. Moreover, we assayed glomerular PLA2R and IgG subclasses immunophenotype of both a-PLA2R positive and negative iMN in order to determine iMN with comprehensive multiple-assays.

Previous studies in other Asian countries demonstrated that the positivity of a-PLA2R in iMN scoped from 69% in Korean [12] to 82% in Chinese [13]. In this study within Guangdong province, we detected serum a-PLA2R antibodies in 66.1% of iMN patients. Combined with results from a-PLA2R IIF, the positivity of PLA2R-related MN could rise to 71.4%. For a-PLA2R positive iMN cases, the baseline anti-PLAR titers moderately significantly correlated

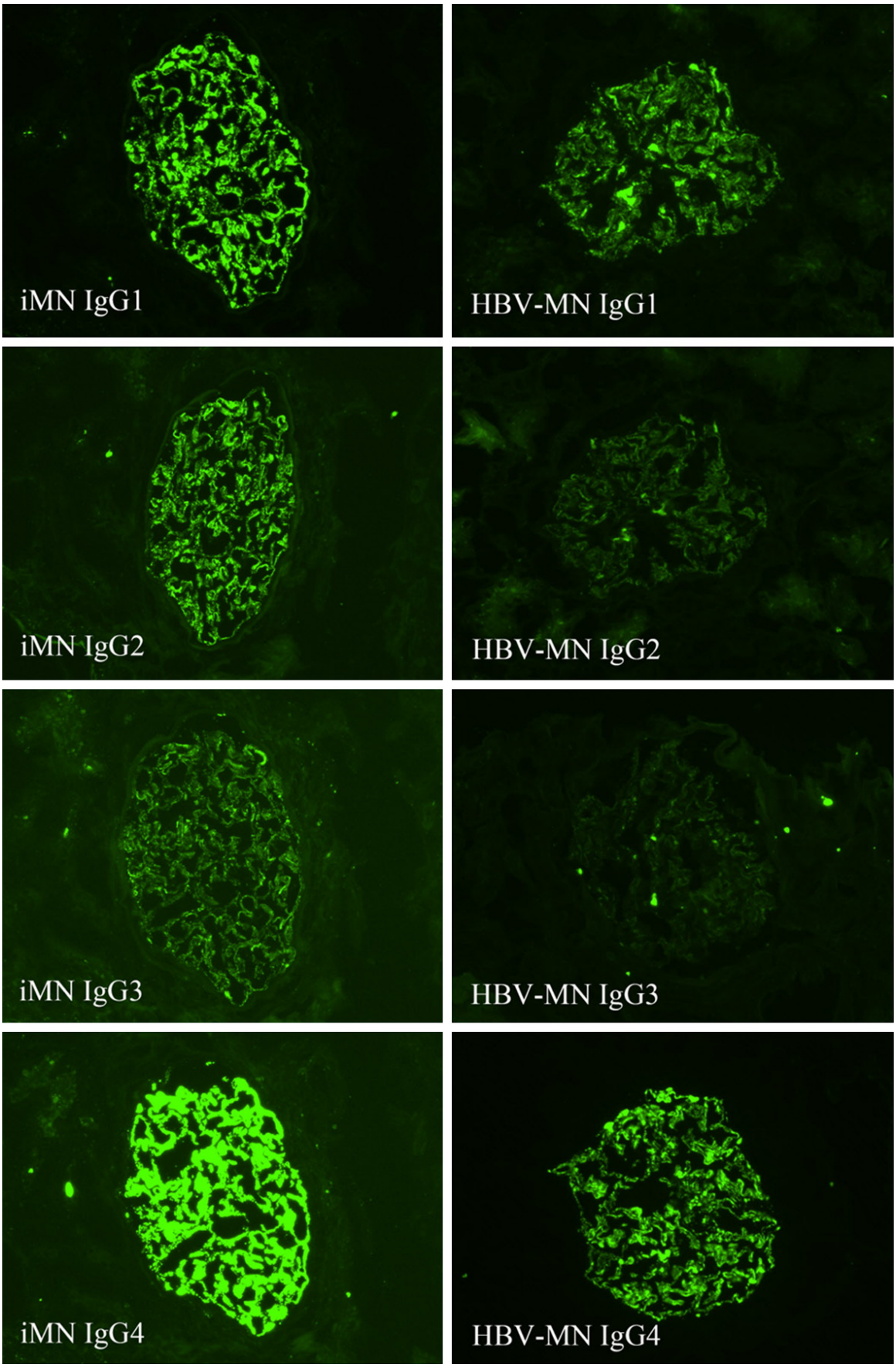
iMN IgG1
HBV-MN IgG1
iMN IgG2
HBV-MN IgG2
iMN IgG3
HBV-MN IgG3
iMN IgG4
HBV-MN IgG4

Figure 3. Renal IgG subclasses deposition in idiopathic membranous nephropathy (iMN) patients and hepatitis B virus associated membranous nephropathy (HBV-MN) patients. Both a-PLA2R positive iMN cases and a-PLA2R negative iMN cases had predominant IgG4 intensity, so did a-PLA2R positive HBV-MN cases.

with proteinuria, which indicates that a-PLA2R titers could monitor the activity of nephropathy.

During the clinical process of a-PLA2R positive iMN cases, for both prevalent cases and IST cases, the parallel change between nephropathy activity and a-PLA2R titers was quite overt to be observed. In addition, in the progression of antibody positive cases with CT, the decrease of a-PLA2R correlated with the decrease of proteinuria, being concordant with other longitudinal study [14]. These results strengthen the role of a-PLA2R titers to monitor the activity of nephropathy. Importantly, a-PLA2R titers in cases with IST declined more sharply than prevalent iMN, indicating that immunological activity was suppressed by immunosuppressive treatment while lymphatic T-helper cells and lymphatic B cells were restrained to generate circulating antibodies. Considering that a-PLA2R negative cases achieved higher remission rate, we identified the presence of a-PLA2R should be a predictor forproteinuria remission, and Hoxha et al. [10] concluded similarly that high a-PLA2R titers was an independent risk factor for not achieving remission.

7 iMN cases wereserum a-PLA2R negative, even they proved glomerular PLA2R positive. For such cases of serum negativity with glomerular positivity, we conceive three reasons: 1) the quickfiltration of serum antibody, 2) the delayed sampling from patients when proteinuria persists due to irreversible ultrastructural changes, 3) the delayed biopsy after disease oneset [15]. In addition, 2 iMN cases were found with negative serum a-PLA2R in and negative PLA2R in glomeruli. For above 2 cases, there probably exist as yet undetected causes of MN or other newly detectedpathogenic antigens, e.g. thrombospondin type-1 domain-containing 7A, α-enolase, aldose reductase, BSA and SOD2 [16, 17].

Renal IgG subclass distribution varies according to underlying disease. IgG4 is the predominant subclass in iMN, while IgG1, IgG2 and IgG3 are major subclasses in sMN [18-22]. Serum auto-antibodies to PLA2R are mainly IgG4, which were co-localized with PLA2R in glomeruli of iMN, excluding some cases with monoclonal IgG3-kappa targeting PLA2R [23]. Consistent with above results, for iMN cases with both a-PLA2R positivity and renal PLA2R positivity, renal IgG4-dominant subclasses were more common than HBV-MN cases. As to cases with a-PLA2R negativity but with renal PLA2R positivity, IgG4 was still predominant, meaning the presence of PLA2R priors the occurrence of PLA2R antibodies. Moreover, IgG4 dominancy is an unfavorable predictor of PR and CR for iMN [24]. Hofstra et al. [4] found that spontaneous remissions were less likely to occur in patients with high serum IgG4 antibody titers to PLA2R. Therefore, it is necessary to keep in close contact with these IgG4-dominant cases for sake of the possible deterioration in the future.

In previous studies, serum a-PLA2R was largely negative in patients with lupus nephritis [25, 26]. However, we detected a-PLA2R in one LN-III+V patient. Retrospectively reviewing the LN-MN's clinical data, we found severe edema, hyperlipoidemia and striking massive proteinuria of 13.8 g/d, which was the maximum in LN group and rarely observed in common LN patients. Considering these extraordinary symptoms, the possibility of coexistence with iMN shouldn't be completely excluded as complications tend to result severe deterioration. Also, recent studies have discovered a low prevalence of a-PLA2R in sMN associated with SLE, graft-versus-host disease and sarcoidosis [27-29].

70.6% of serum a-PLA2R and 60% of renal PLA2R were detected in patients with HBV-MN. The serum a-PLA2R positivity is much higher than previous studies and renal PLA2R positivity is consistent with only one study about Caucasian hepatitis-related MN, in which Larsen et al. [30] also reported that 6 in 11 of HCV-MN patients were serum a-PLA2R positive. Even though previous studies have identified that no positive a-PLA2R and no positive renal PLA2R existed in HBV-MN patients, there have appeared inverse findings in China. Xie et al. [31] detected 6 Chinese HBV-MN patients

with a-PLA2R positivity and renal PLA2R positivity at the same time. Similarly in our study, 6 in 10 HBV-MN cases showed a-PLA2R positive and renal PLA2R positive. These 6 cases were also IgG4-dominent, just like iMN cases. Reconsidering that a-PLA2R titers of a-PLA2R positive HBV-MN cases didn't correlate with proteinuria like iMN, we infer that some HBV-MN might be a PLA2R-associated disease with the pathogenesis that is different from classic iMN. Studies on how the virus leads to the production of serum a-PLA2R would be helpful for understanding the mechanism of iMN.

Moreover, in our study, the other 4 HBV-MN cases with neither a-PLA2R positivity nor renal PLA2R positivity were IgG1 predominant, followed by IgG3 and IgG4, like other classic secondary MN [32, 33]. We speculate that the pathogenesis of these HBV-MN cases is not associated with a-PLA2R reactivity and is distinct from that of iMN. Results above indicate that if there is positive a-PLA2R or positive renal PLA2R, iMN shouldn't be excluded. If there is negative a-PLA2R or negative renal PLA2R, HBV-MN is more evidently certain.

In conclusion, detecting serum a-PLA2R with glomerular PLA2R simultaneously is a more dependable method for the diagnosis of iMN at present. In case thatthe discrepancies-between the presence of serum a-PLA2R and the renal PLA2R- appears, the detection of renal IgG4 should be the supplement. In further research, based on more samples, firstly we need to sort MN patients into at least three groups in terms of serum a-PLA2R positivity/negativity and glomerular PLA2R positivity/negativity. Secondly, we would compare clinicopathological characteristics and clinical outcomes between groups. Besides, renal IgG4 deposits are valuable clues to clarify the pathogenesis of PLA2R-associated HBV-MN. In further studies, we also aim to explore the pathogenesis of a-PLA2R positive HBV-MN patients.

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Disclosure of conflict of interest

None.

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