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ORIGINAL ARTICLE

IgG4 anti-phospholipase A2 receptor might activate lectin and alternative complement pathway meanwhile in idiopathic membranous nephropathy: an inspiration from a cross-sectional study

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Abstract The deposition of IgG4 of antibodies against phospholipase A2 receptor (anti-PLA_2R) is predominating in the kidneys of patients with idiopathic membranous nephropathy, while its predictive value has not been determined. It was a retrospective study, and 438 patients were included. Serum samples of two time points [before intervention (baseline) and after 1.5-year treatment (endpoint)] were detected for total and IgG4 anti-PLA_2R. IgG4 <0.26 RU/mL or total <20 RU/mL was considered as seronegativity. Bi-positivity/bi-negativity was defined when patients'antibodies were found positive or negative both at the baseline and endpoint. Completed remission (CR) was a major clinical outcome. A series of complement ingredients (MASP-1/2, MBL, C3a, C5a, Factor B, Ba, Bb and C5b-9) were measured in the patients of bi-positivity and bi-negativity: (1) meta-analysis based on six papers conducted seropositivity of anti-PLA_2R was a useful predictor for achieving CR, but there was a high heterogeneity; (2) there was significant correlation between the baseline and decrease in IgG4 subclass and the achievement of CR; (3) bi-negativity of IgG4 has a high accuracy of predicting CR compared with total antibodies; (4) in patients of bi-positivity, those achieving CR showed lower MASP-1/2, MBL, C3a, C5a, FB, Ba and Bb than patients failing to achieve CR; (5) the titers of endpoint and decrease in Ba and Bb were associated with improvement of 24 h-UP in those of bi-positivity; and (6) the decrease in Ba was a significant factor for achieving CR in those of bi-positivity. Continuous IgG4 negativity was a useful tool to predict the achievement of CR; however, in patients of continuous IgG4 positivity, those with lower activation of lectin and alternative pathways would still more probably achieve CR.

Keywords Anti-phospholipase A2 receptor · IgG4 subclass · Idiopathic membranous nephropathy · Complement · Clinical outcomes · Meta-analysis

Introduction

Idiopathic membranous nephropathy (IMN), the most frequent pathological type of nephrotic syndrome (NS) in adult, draws more and more attention of nephrologists. Though majority of patients with IMN always share a similar clinical manifestation originally, the clinical outcomes differ significantly among individuals, ranging from spontaneous remission to persistent proteinuria and even end stage of renal diseases (ESRD) [1, 2]. Besides, there was an obvious individual variation on the therapeutic effect of immunosuppressive agents in the patients with IMN. Therefore, it is urgently required to develop a dependable biomarker to predict the clinical outcomes and make immunosuppressive schedules individually. The antibodies against phospholipase A2 receptor (anti-PLA_2R) were considered to be specific in the patients with IMN and it made para-diagram of early outcome evaluation and individual clinical care of these patients to be possible [3]. Recently, researches associated with the antibodies mainly referred to two aspects: (1) its specificity and sensitivity in

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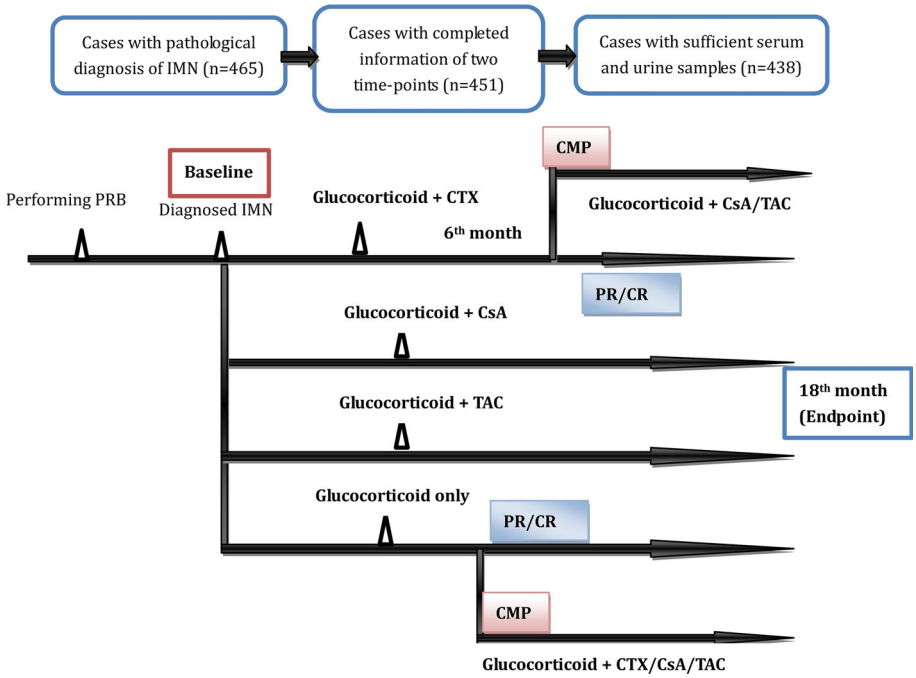

Fig. 1 Enrollment of patients and the therapeutic protocol in the study; *PRB* percutaneous renal biopsy, *CR* completed remission, *PR* partial remission, *CMP* continuous macroproteinuria, *CTX* cyclophosphamide, *CsA* ciclosporin A, *TAC* tacrolimus

identification of IMN from secondary MN, such as lupus nephrology, hepatitis B virus-associated nephrology and multiple myeloma [4–6] and (2) its prediction for different clinical outcomes in IMN [7, 8]. The predominant subclass in the circulation and renal tissue of patients with IMN is IgG4; however, it is unable to bind C1q and activate classical complement pathway [9, 10]. The definite mechanism is not much clear. More than that, predictive value of IgG4 for the clinical outcomes requires further evaluation. Therefore, in the present study, we included 438 patients with IMN, analyzed the database and would like to evaluate the predictive value of IgG4 for the achievement of therapeutic success and explore how IgG4 subclass participates in the activation of complement pathway in IMN.

Methods

Study design and participants

It was designed as a cross-sectional study. Participants were included from 2007 to 2015. The inclusion criteria were: (1) age $\geq$16 year, male or female; (2) required a proteinuria $\geq$3.5 g/24 h and serum albumin $\leq$30 g/L; (3) first episode of NS was caused by IMN and the diagnosis was confirmed by renal biopsy; (4) complete information about two time points. First point was a time before the biopsy (baseline), and a second point was a visit after 18-month therapy (endpoint). All secondary MN was excluded according to the routine clinical workup of our center. Totally, 465 patients had been diagnosed as IMN and received treatment during the period. Blood and urine samples applied in the study came from our sample resource pool (storage in liquid nitrogen or ultra-low temperature freezers). Finally, 438 patients with sufficient blood and urine samples of two points were included.

Treatment program and major clinical outcomes

Patients received glucocorticosteroid (0.8–1.0 mg/kg/day, p.o., at the beginning, gradually decreased to 0.1–0.2 mg/kg/day and sustained). A total of 117 patients only received cyclophosphamide (CTX, 0.8–1.0 g/month, i.v., CTX total dose <12.0 g) during the treatment; 97 patients only received cyclosporine A (CsA, 3.0–4.0 mg/kg/day, p.o., at the beginning, gradually decreased to 0.5–1.0 mg/kg/day) throughout the therapy. Sixty patients received tacrolimus (TAC, 0.1 mg/kg/day, initially and adjusted to the blood trough level at 5–10 ng/mL for 6 months and then reduced to 2–6 ng/mL during the subsequent 6 months). Fifty-one patients had been administrated CTX for 6 months, but no partial remission was achieved and changed to CsA. Eighty-seven patients had received CTX for 6 months, but no partial remission was observed and changed to TAC. Twenty-six patients only received glucocorticosteroid during the treatment (Fig. 1).

We evaluated the achievement of clinical remission as primary outcomes, and it was evaluated by two physicians blinded of grouping procedure. Completed remission (CR) was diagnosed when 24-h urine protein (24 h-UP) <0.3 g,

albumin (ALB) >35 g/L and estimated glomerular filtration rate (eGFR) >90 ml/(min 1.73 m^2) at the endpoint. Partial remission (PR) was diagnosed when 24 h-UP <3.5 g, ALB >30 g/L and eGFR >75 ml/(min 1.73 m^2) at the endpoint. Continuous macroproteinuria (CMP) was defined as 24 h-UP still >3.5 g (with or without renal function impairment) at the endpoint.

ELISA detection for anti-PLA_2R

Total and IgG4 anti-PLA_2R were detected by a commercial ELISA kit that was developed by EUROIMMUN Medizinische Laborordianostika AG (Lübeck, Germany) [11]. According to the manufacturer, results of detection were considered positive if the level >20 RU/ml for total antibodies and >0.26 RU/mL for IgG4 [4, 11]. If a patient both had anti-PLA_2R detected at the baseline and endpoint, he would be diagnosed as bi-positivity and vice versa, he would be diagnosed as bi-negativity.

Measurement and histological diagnosis

24 h-UP was assayed by the Randox protein Reagent (Randox® Laboratories Ltd, Antrim, UK) by pyrogallol red–molybdate colorimetric method. ALB and serum creatinine were assayed by Hitachi analyzer 7600 (Hitachi® High-Tech Corporation, Japan). GFR was estimated using the modified MDRD equation based on the Chinese population [12]. C3 was assayed by nephelometry (Siemens® Healthcare Diagnostics, Deerfield, USA). All complement ingredients were measured by commercial ELISA kits. Direct sandwich ELISA was used to measure C3a, C5a, soluble C5b-9 (sC5b-9), Factor Ba and Factor Bb (Quidel Corporation, San Diego, CA; ProGen Biologics, Saint Louis, MO). Factor B was measured by indirect sandwich ELISA (Abcam, Cambridge, MA; Hycult Biotech, Plymouth Meeting, PA). Serum mannose-binding lectin (MBL) was assayed by commercial ELISA kit (Sanquin, Amsterdam, Netherlands) according to the protocol provided by manufacturers. Plasma MBL-associated serine protease 1(MASP-1) and MASP-2 were detected by commercial ELISA kits (Hycult Biotech, Uden, Netherlands) according to the protocol provided by manufacturers. Renal tissue samples which were achieved by biopsy were processed and strained with hematoxylin–eosin (HE), periodic acid–Schiff (PAS) and Masson stain. Direct immunofluorescence was applied to detect C3, C4, C1q, IgA, IgG and IgM at the glomerulus in frozen sections of the fresh samples. To exclude Hepatitis B virus-associated MN, streptavidin–biotin peroxidase method was applied for the immunohistochemical staining with antibodies against HBsAg (Novocastra, monoclonal, mouse, clone, 1:25, UK).

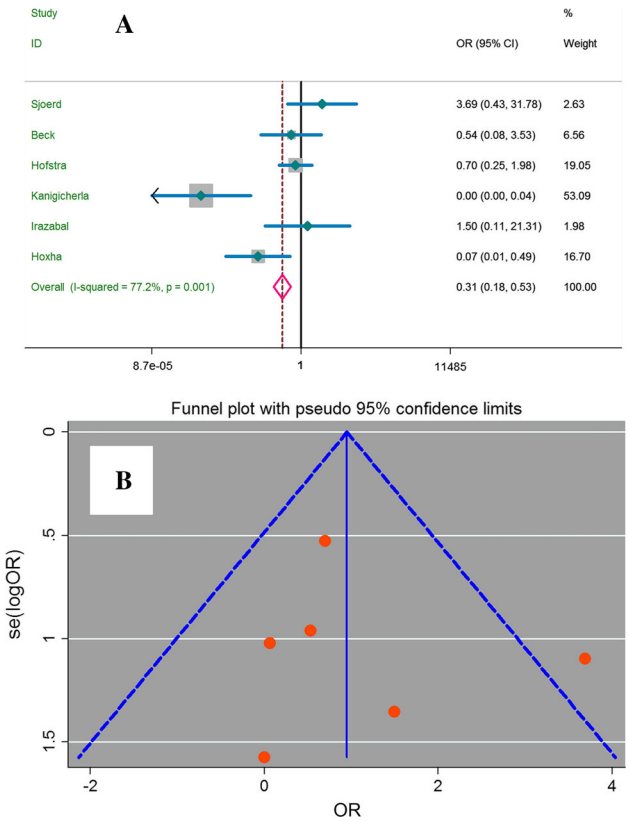

Fig. 2 Mata-analysis based on six researches: forest plot (**a**) and funnel plot (**b**) and their 95 % confidence limitations for total anti-PLA_2R in the prediction of achieving completed remission

Table 1 Characteristics of eligible studies applied in meta-analysis

	Available patients number	Anti-PLA_2R positivity at baseline	Anti-PLA_2R positivity		Anti-PLA_2R negativity	
			PR	CR	PR	CR
Timmermans et al. [26]	109	69	7	8	0	1
Beck et al. [27]	35	25	13	5	3	4
Hofstra et al. [6]	117	84	30	24	8	11
Kanigicherla et al. [5]	90	42	10	2	17	21
Irazabal et al. [28]	20	14	10	2	1	2
Hoxha et al. [4]	33	16	4	3	5	10

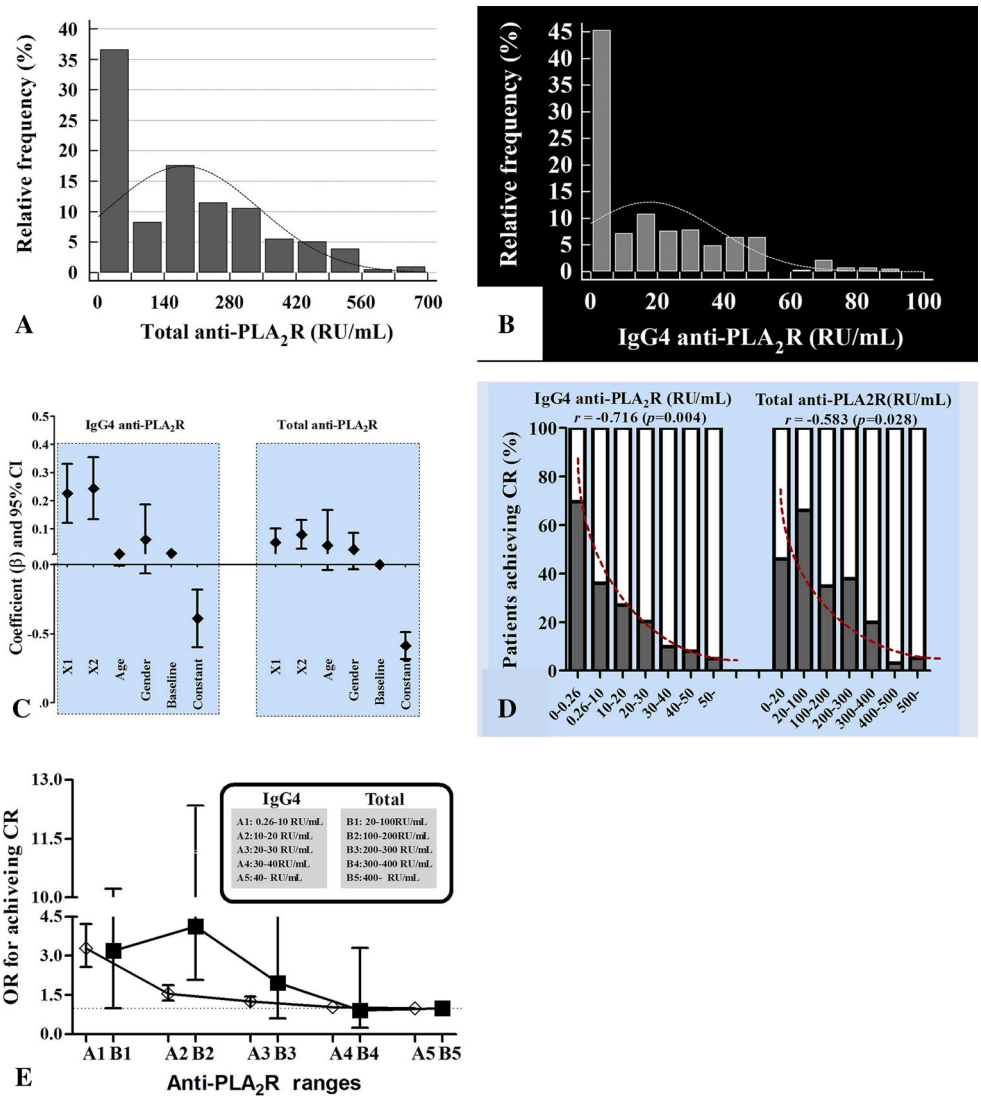

Fig. 3 Distribution of total **(a)** and IgG4 anti-PLA_2R titers (**b**); multivariable linear regression for the change of total and IgG4 anti-PLA_2R titers, CTX: $X1 = 1$, $X2 = 0$; CsA: $X1 = 0$, $X2 = 1$; TAC: $X1 = 1$, $X2 = 1$; only glucocorticoid: $X1 = 0$, $X2 = 0$ (**c**); the correlation between the mean of anti-PLA_2R titers at baseline and the percentage of patients achieving completed remission (**d**); odd ratio and its 95 % confidence interval of achieving completed remission according to the ranges of anti-PLA_2R (**e**)

Statistical analysis

Dichotomous variables were expressed as ratios, and continuous variables were expressed as means or medians. Dichotomous variables were compared using Chi-square test; continuous variables were compared with *t* test, Mann–Whitney *U* test or Wilcoxon's rank-sum test, as appropriate. The proportions were compared using Pearson's Chi-square test or Fisher's exact probability test. Multiple linear regression was applied to analyze the effective factors for the changes in anti-PLA_2R, and logistic regression was applied to analyze the contribution of clinical characteristics (including complement ingredients) to the achievement of CR. Receiver operating characteristic (ROC) curve was used to compare the accuracy of continuous IgG4 and total antibodies negativity to predict the achievement of CR. Data were analyzed using the SPSS software version 16.0 (SPSS, Inc. Chicago, IL, USA). p values <0.05 were considered to indicate significance.

Meta-analysis: prediction of anti-PLA_2R for the achievement of CR

PubMed, Embase, Cochrane database and Chinese National Knowledge Infrastructure (CNKI) database were applied to identify eligible researches until Jan 1, 2015 by terminologies "idiopathic membranous nephropathy" and "primary membranous nephropathy" combined with the terminology "phospholipase A2 receptor." Total two reviewers independently extracted data from all published research papers fulfilling the criteria; a third opinion was given to settle the disagreement. The information mainly included: journal, published year, sample size, race, methods of anti-PLA_2R measurement, antibodies positivity and how many patients achieving CR. An author

Table 2 Baseline characteristics between baseline antibody positivity and negativity

	Total anti-PLA_2R		IgG4 anti-PLA_2R	
	Negativity ($n = 113$) Mean (SD) or N (%)	Positivity ($n = 325$) Mean (SD) or N (%)	Negativity ($n = 145$) Mean (SD) or N (%)	Positivity ($n = 293$) Mean (SD) or N (%)
General information				
Age (year)	40.1 (13.0)	41.2 (16.2)	40.2 (15.5)	40.1 (15.5)
Male	73 (64.6)	280 (86.2)*	105 (72.4)	248 (84.6)#
BMI (Kg/m^2)	24.7 (4.5)	24.4 (4.8)	24.4 (4.4)	24.6 (4.6)
Symbolic BP (mmHg)	137.5 (24.4)	136.0 (21.2)	139.8 (25.3)	137.8 (20.1)
Diabolic BP (mmHg)	86.9 (18.1)	85.2 (14.6)	87.1 (17.7)	84.9 (14.4)
Administrating ACEI/ARB	70 (61.9)	214 (65.8)	98 (67.6)	186 (63.5)
Immunosuppressive therapy				
Administrating CTX only	20 (17.7)	97 (29.9)*	26 (17.9)	91 (31.1)#
Administrating CsA only	26 (23.0)	71 (21.9)	54 (37.2)	43 (14.7)#
Administrating TAC only	11 (9.7)	49 (15.1)	15 (15.1)	45 (15.3)
Administrating CTX followed by CsA	23 (20.4)	28 (8.6)*	16 (8.6)	35 (12.0)
Administrating CTX followed by TAC	28 (24.8)	59 (18.2)	23 (15.9)	64 (21.8)
Important laboratory indices				
24 h-UP (g)	7.6 (2.0)	7.3 (1.8)	7.4 (2.1)	7.3 (1.8)
ALB (g/L)	23.9 (3.5)	23.0 (3.9)	23.5 (3.8)	23.0 (3.8)
eGFR (ml/min.1.73 m^2)	95.4 (30.5)	96.8 (39.9)	95.4 (33.1)	96.5 (39.7)

* Comparing with the patients of total anti-PLA_2R negativity, $p < 0.05$

Comparing with the patients of IgG4 anti-PLA_2R negativity, $p < 0.05$

Table 3 Correlation between anti-PLA2R and clinical characteristics

	Total anti-PLA_2R $_{Baseline}$	IgG4 anti-PLA_2R $_{Baseline}$	Δ Total anti-PLA_2R	Δ IgG4 anti-PLA_2R
24 h-$UP_{Baseline}$	–	–	NA	NA
$ALB_{Baseline}$	–	–	NA	NA
$eGFR_{Baseline}$	0.202*	0.231*	NA	NA
Δ24 h-UP†	−0.225*	−0.561**	0.221*	0.599**
ΔALB	–	–	–	–
ΔeGFR	–	–	0.167*	0.217*

NA no analyzing

* $p < 0.05$; ** $p < 0.01$

† ΔData: $(Data_{baseline} - Data_{endpoint})/Data_{baseline}$

independently evaluated the quality of all included papers by the guideline of updated Quality Assessment of Diagnostic Accuracy Studies (QUADAS). Odds ratio (OR) and its 95 % confidence interval were applied to evaluate the prediction of anti-PLA_2R for the achievement of CR in patients with IMN. Both chi-square and I^2 tests were used to detect heterogeneity and evaluate statistical significances. A funnel plot was used to explore potential publication biases. All analyses were performed in *mdias* module in Stata version 11.0 (College Station, TX, USA).

Results

There were 140 potential articles found in the research, and finally, six articles were included in the present meta-analysis, which is given in Table 1. In 404 available patients from above six studies, 154 individuals were found seronegativity. There were 44 patients of seropositivity achieving CR; in contrast, 49 patients of seronegativity achieved CR. The general OR and its 95 % confidence interval of the seropositivity at the baseline for the

Table 4 Clinical outcomes according to baseline antibody detection

	Total anti-PLA_2R		IgG4 anti-PLA_2R	
	Negativity ($n = 113$)	Positivity ($n = 325$)	Negativity ($n = 145$)	Positivity ($n = 293$)
PR (%)	21 (18.6)	174 (53.5)*	14 (9.7)	181 (61.8)#
CR (%)	52 (46.0)	102 (31.4)*	101 (69.7)	53 (18.1)#
CMP (%)	40 (35.4)	49 (15.1)*	30 (20.7)	59 (20.1)

* Comparing with the patients of total anti-PLA_2R negativity, $p < 0.05$

Comparing with the patients of IgG4 anti-PLA_2R negativity, $p < 0.05$

achievement of CR were 0.31 and 95 % CI (0.18–0.53) ($I^2 = 77.2$ %, $p = 0.001$, Fig. 2a). Funnel plot and its pseudo 95 % confidence limits are shown in Fig. 2b.

Next we would introduce our results of the study:

Totally 438 patients were collected. A total of 325 samples (74.2 %) were detected for total anti-PLA_2R and 293 (66.6 %) for IgG4 subclass. The distribution of baseline total and IgG4 subclass is shown in Fig. 3a, b. Baseline characteristics were also compared between the patients of negativity and positivity, including total and IgG4 anti-PLA_2R: the gender construction significantly differed between positivity and negativity (Table 2); meanwhile, male was a significant effective factor (EF) for the generation of total [OR 3.531; 95 %CI (2.134–5.841)] and IgG4 [OR 2.007; 95 %CI (1.274–3.386)]. The correlation between total and IgG4 anti-PLA_2R titers and clinical characteristics is given in Table 3: Compared with total antibodies, the decrease and baseline of IgG4 titers both had higher correlation with the decrease in 24 h-UP. By multivariable linear regression, we found that appliance of immunosuppressive agents was a significant EF for the decrease in total and IgG4 anti-PLA_2R titers as adjusted by gender, age and baseline antibody titers (Fig. 3c).

After 18-month treatment, 154 patients (35.0 %) had achieved CR and 195 patients (44.3 %) had achieved PR but 89 patients (20.7 %) still manifested CMP. Patients of seropositivity (total and IgG4 subclass) at baseline achieved significantly less CR than those of seronegativity; besides, patients of total anti-PLA_2R seropositivity at baseline manifested more CMP than those of seronegativity (Table 4). For CR was most vital for evaluating the clinical outcomes, we analyzed the association between anti-PLA_2R and the percentage of achieving CR. With the increase in total and IgG4 anti-PLA_2R titers at baseline, less patients achieving CR were observed; the relation correlation (r) between mean of baseline anti-PLA_2R titers and the achievement of CR is shown in Fig. 3d: $r_{\text{IgG4 subclass}} > r_{\text{Total}}$. ORs for achieving CR decreased significantly along with the increase in baseline total and IgG4 titers, while the trend was obviously impaired when analyzing total antibody titers >200 RU/mL (Fig. 3e). We found that seropositivity of total and IgG4 anti-PLA_2R at baseline was both independent EFs for achieving CR (Table 5).

The predictive value of seronegativity of anti-PLA_2R including total and IgG4 at two time points (bi-negativity)

Table 5 Contribution of multivariable for the achievement of CR

	Multivariable analysis by total anti-PLA_2R			Multivariable analysis by IgG4 anti-PLA_2R		
	β	p	OR 95 % CI	β	p	OR 95 % CI
$\text{Seropositivity}_{\text{Baseline}}$	−0.545	**0.021**	0.580 0.365–0.921	−2.394	**0.001**	0.091 0.056–0.148
Age	0.003	0.718	1.003 0.989–1.017	0.005	0.504	1.005 0.990–1.022
Male	−0.322	0.222	0.724 0.432–1.25	−0.173	0.564	0.841 0.467–1.515
Mean blood pressure	0.007	0.289	1.007 0.994–1.020	0.004	0.567	1.004 0.990–1.019
Body weight index	0.016	0.489	1.016 0.971–1.064	0.032	0.228	1.032 0.980–1.087
$\text{ALB}_{\text{Baseline}}$	−0.043	0.150	0.958 0.904–1.06	−0.067	0.048	0.935 0.875–0.999
$\text{24 h-UP}_{\text{Baseline}}$	0.051	0.373	1.052 0.941–1.178	0.049	0.445	1.051 0.925–1.193
$\text{eGFR}_{\text{Baseline}}$	−0.004	0.247	0.996 0.990–1.003	−0.002	0.616	0.998 0.991–1.005
Constant	−0.175	0.899	–	0.943	0.546	–

Bold values indicate statistical significance ($p < 0.05$)

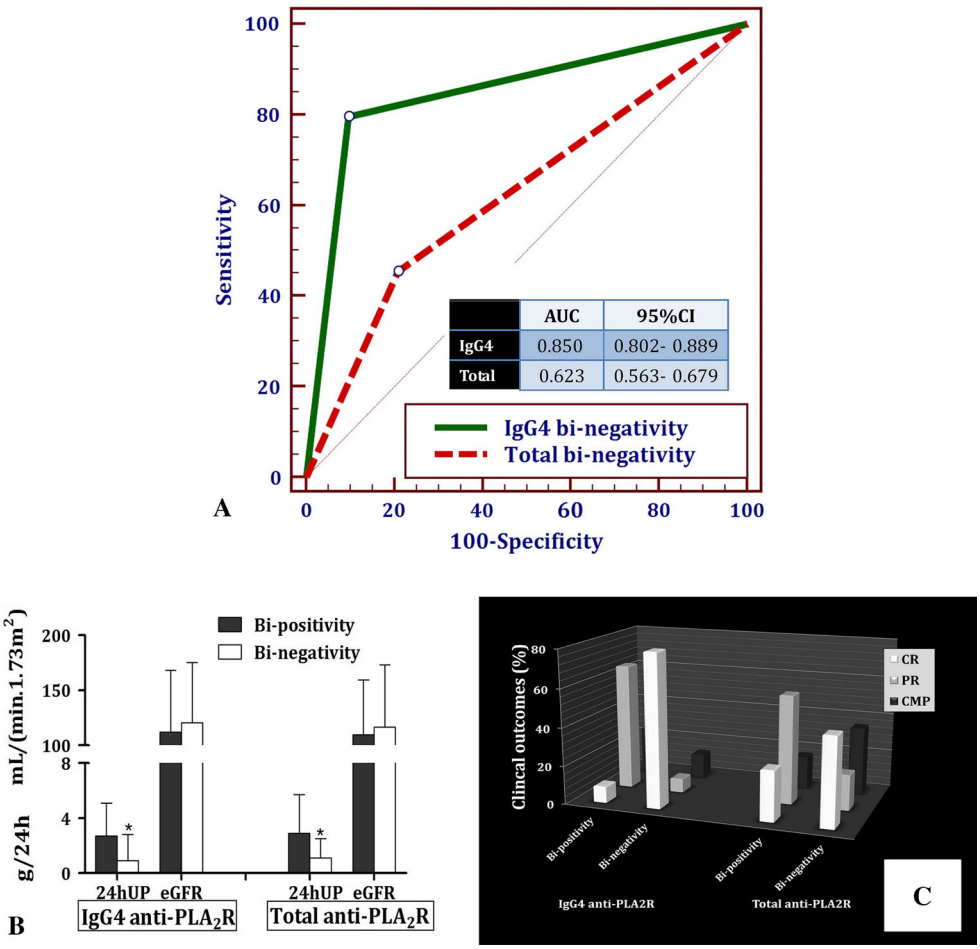

Fig. 4 Comparison of the area under ROC curves (AUC) between bi-negativity of IgG4 subclass and total antibodies (**a**); comparison of 24 h urine protein (24 h-UP) and estimated glomerular filtration rate (eGFR) between patients of bi-positivity and bi-negativity (IgG4 and total antibodies);*$p < 0.05$ (**b**); comparison of clinical outcomes between patients of bi-positivity and bi-negativity (IgG4 and total antibodies); *CR* completed remission, *PR* partial remission, *CMP* continuous macroproteinuria (**c**)

for achieving CR was compared by area under ROC curve (AUC): $AUC_{IgG4} > AUC_{Total}$ ($p = 0.001$, by Z test) (Fig. 4a). The patients of bi-negativity (total and IgG4) manifested significantly lower 24 h-UP at the endpoint than those of bi-positivity (Fig. 4b). The patients of total anti-PLA_2R bi-negativity achieved significantly more CR than the patients of bi-positivity (46.0 vs. 26.3 %, $p < 0.001$), while the difference on the percentage of achieving CR was more obvious when analyzing IgG4 (79.8 vs. 8.6 %, $p < 0.001$) (Fig. 4c).

We measured MASP-1/2, MBL, C3, C3, C3a, Factor B (FB), Factor Ba (Ba), Factor Bb (Bb), C5a and sC5b-9 at the endpoint in the patients of IgG4 bi-negativity and bi-positivity, and we found MASP-1, MASP-2, MBL, C3, C3a, C5a, Ba and Bb were significantly different between those of bi-negativity and bi-positivity (Fig. 5). In the patients of IgG4 bi-positivity, those of achieving CR manifested significantly lower MASP-1, MASP-2, MBL, C3a, C5a, FB, Ba and Bb than those of failing to achieve CR, while in the patients of IgG4 bi-negativity, those of achieving CR only showed significantly lower Bb and C5a than those of failing to achieve CR (Table 6). In the patient of IgG4 bi-positivity, the titers of MASP-1, MBL, Ba, Bb and C5a at the endpoint were significant EFs for achieving CR using the logistic regression model (Fig. 6a); however, all complement ingredients failed to be introduced into the model in those of IgG4 bi-negativity (Fig. 6b). There were significant correlations between the changes of 24 h-UP and Ba and Bb instead of the total Factor B (endpoint) (Fig. 7a–c). We also assayed the baseline blood samples of the patients of IgG4 bi-positivity for the Ba and Bb and get the titers' changes. There was a significant correlation between the decrease in Ba, Bb and decrease in 24 h-UP in these patients, $r_{\Delta Ba \text{ and } 24h\text{-}UP} > r_{\Delta Bb \text{ and } 24h\text{-}UP}$ (Fig. 7d). Further it was found that the decrease in Ba was significantly different between those who had achieved and failed to achieve CR (Fig. 7e). By logistic regression analysis, the decrease in Ba instead of Bb was a significant EF for the achievement of CR in the patients of bi-positivity (Table 7).

Discussion

PLA_2R, as an important member of the mannose receptor (MR) family, has been found to express in podocyte and act as a target antigen in human MN. Anti-PLA_2R is

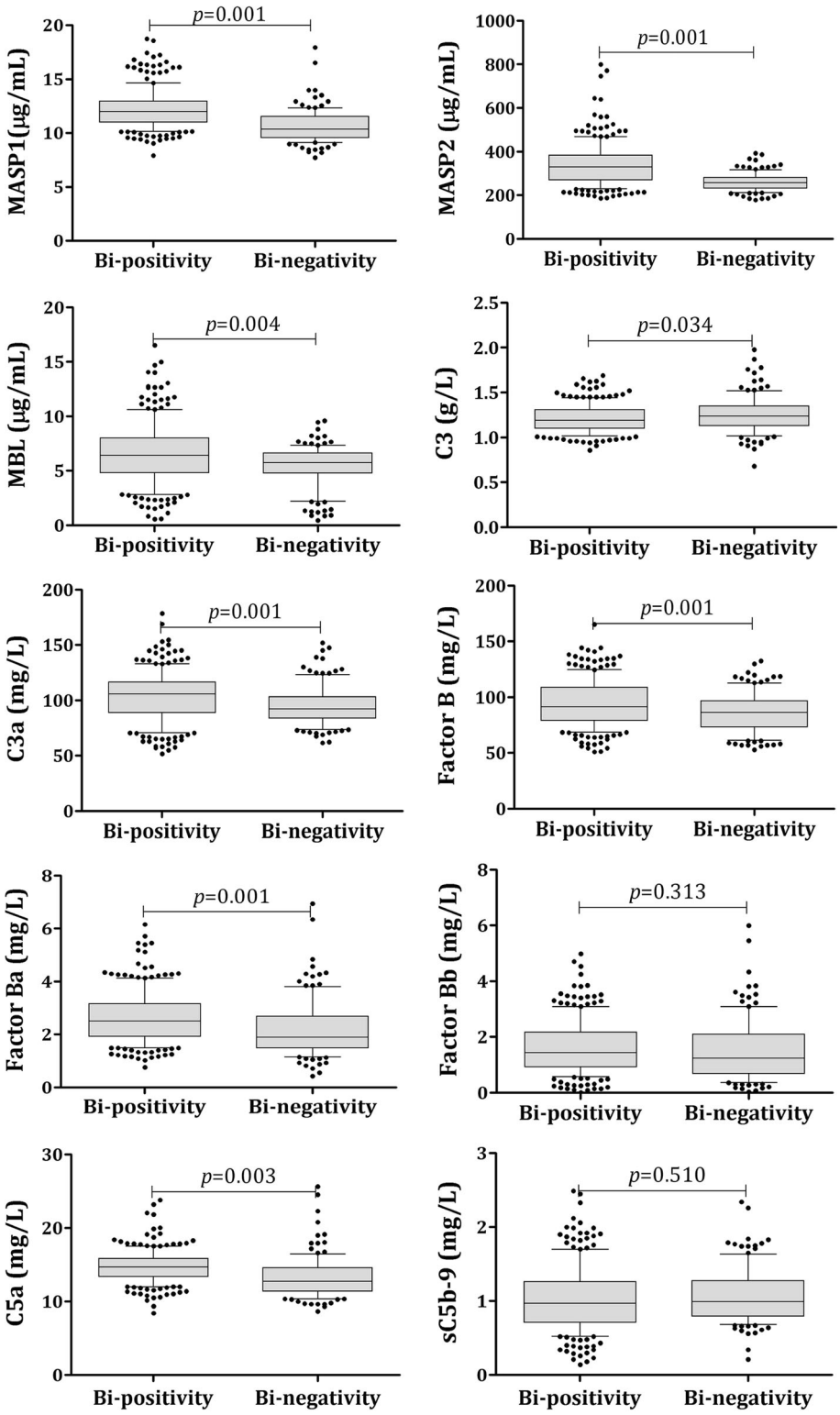

Fig. 5 Comparison of the complement ingredients including MASP-1, MASP-2, MBL, C3, C3a, BF, Ba, Bb, C5a and sC5b-9 at the endpoint between patients of IgG4 bi-positivity and bi-negativity

considered as a promising biomarker for identifying IMN, monitoring and predicting the prognosis of patients with IMN. The most vital prognosis is completed remission in patients with IMN, and our paper had focused on the prediction of anti-PLA_2R for the possibility of achieving CR in IMN. The major findings of the present study included two aspects: Continuous IgG4 seronegativity could accurately and dependably predict CR in patients with IMN, while in patients with continuous IgG4 seropositivity, the complement alternative pathway and mannan-binding

Table 6 Comparison of complements between patients achieving and not achieving CR

	IgG4 Bi-positivity		IgG4 Bi-negativity	
	Achieving CR ($n = 19$)	Failing to achieve CR ($n = 201$)	Achieving CR ($n = 99$)	Failing to achieve CR ($n = 25$)
MASP-1 (μg/mL)	10.8 (1.3)	12.4 (1.8)*	10.2 (1.2)	11.1 (1.9)
MASP-2 (μg/mL)	255.1 (30.4)	349.9 (102.2)*	260.7 (41.8)	256.9 (38.9)
MBL (μg/mL)	4.1 (0.6)	5.6 (2.3)*	3.1 (0.7)	3.5 (1.6)
C3 (g/L)	1.2 (0.2)	1.2 (0.2)	1.3 (0.2)	1.3 (0.2)
C3a (mg/L)	94.1 (17.3)	104.6 (22.9)*	95.7 (18.6)	93.4 (15.1)
FB (mg/L)	88.4 (15.7)	95.1 (21.8)*	84.2 (18.6)	82.1 (13.2)
Ba (mg/L)	1.9 (0.8)	3.4 (1.4)*	2.0 (0.8)	2.2 (1.0)
Bb (mg/L)	1.2 (0.8)	2.7 (1.4)*	1.1 (0.8)	1.4 (1.0)*
C5a (mg/L)	12.2 (1.7)	17.1 (3.4)*	11.7 (1.6)	13.1 (2.1)*
sC5b-9 (mg/L)	1.1 (0.5)	1.0 (0.5)	1.0 (0.4)	1.0 (0.4)

* Comparing with those achieving CR, $p < 0.05$

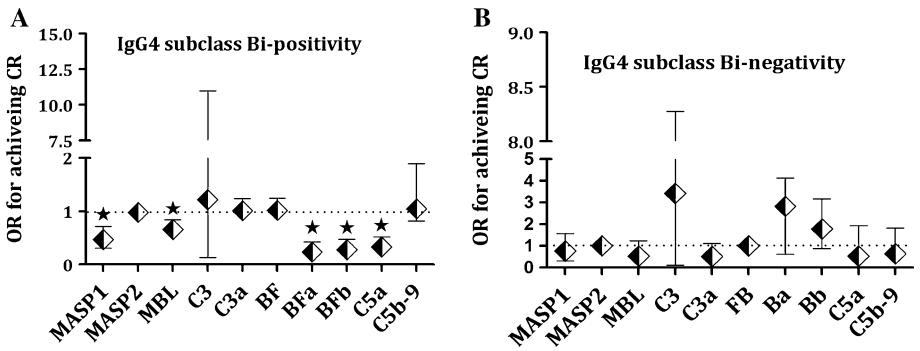

Fig. 6 Unadjusted odd ratios (OR) of the complement ingredients at the endpoint for achieving completed remission in patients of IgG4 bi-positivity (**a**) and bi-negativity (**b**) have statistical significance

lectin (MBL) pathway might be activated meanwhile and relatively low level of these two pathways might also predict CR in these patients. Prior to carrying out our work, we applied a meta-analysis to try to conduct the comprehensive effect of anti-PLA_2R seronegativity from six available papers on predicting CR in patients with IMN. Unfortunately, the result was not much dependable for their different sample sizes, measurement assays and the definition of the clinical outcomes. Based on our database, we affirmed that seronegativity of IgG4 subclass could predict CR dependably and more than that, we focused on the patients with continuous IgG4 seropositivity and found some clues for the association between the complement system and the achievement of CR. IgG4 was a dominant subclass of anti-PLA_2R in patients with IMN; however, its effects on the development of IMN is still inclusive. The constructions of IgG4 subclass had two characteristics functions: One is ability to undergo Fab arm exchange and another is non-traditional rheumatoid factor (RF)-like activity, both of which might be able to limit immune-mediated pathology and play an important anti-inflammatory role [13, 14]. Besides, IgG4 was found to be able to mimic IgG RF activity through interacting with IgG, but the effect does not create circulation immune complex and activate the classical complement pathway; besides, IgG4 might prevent inflammatory responses by shielding IgG1 or IgG3 from C1q binding; therefore, some scholars presumed that the predominance of IgG4 within subepithelial deposition might probably represent a protective response in the development of IMN [15]. The deposition of C3, C4 and their breakdown products were all observed in the major patients' kidney samples. C3c is reported to deposit in almost all IMN patients' glomerulus and its more stable product [16, 17]; C3d is found in about 70 % of patients [18]. C4d is the breakdown products of C4; it is generated with the activation of classical complement and lectin pathways; and it will bind the surface of cells and exist as a much highly stable form. Val-nernal and Espinos-Hernandez reported that C4d granularly deposited in glomerulus basement membrane (GBM) of all patients with IMN [19, 20]. Besides, Suzuki found that C4d could not be observed in normal GBM, suggesting that C4d deposition might not be intrinsic but rather indicating pathological complement activation [21]. The presence of

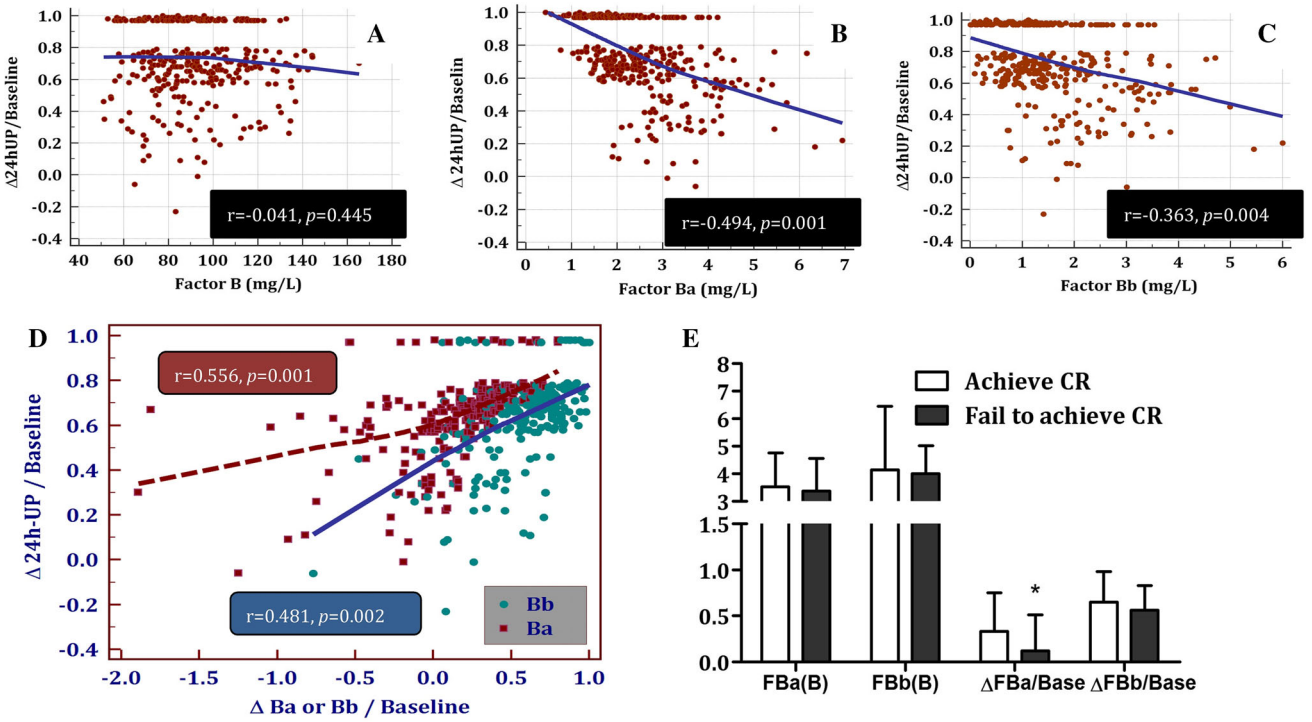

Fig. 7 Correlation between change of 24 h-UP and Factor B (**a**), Ba (**b**) and Bb (**c**) at the endpoint; and the correlation between the changes of 24 h-UP and the changes of Ba and Bb (**d**); and the comparison of baseline and changes of Ba and Bb between patients achieving completed remission and failing to achieving completed remission in those of IgG4 bi-positivity (**e**)

Table 7 Contribution of Factor Ba and Bb for the achievement of CR

	Factor Ba			Factor Bb		
	β	*p*	OR 95 % CI	*β*	*p*	OR 95 % CI
Baseline	−0.201	0.436	0.818 0.494–1.356	0.027	0.899	1.028 0.676–1.563
Δ/baseline	0.789	0.017	2.505 1.572–5.457	1.247	0.245	3.481 0.426–8.447
Constant	−0.549	0.009	– –	−3.226	0.001	– –

IgG4 and C4 in majority of IMN cases is a question itself for the typical absence of C1q in IMN, and therefore, C4d was speculated not to be the product of the activation of classical complement pathway. Besides, IgG4 is an immunoglobin that is incapable to bind C1q and launches classical complement pathway. Based on these evidences, lectin pathway has emerged as a potential explanation for the coexistence of C4d and IgG4 in IMN although the explanation was still speculative. The Second International Conference on Membranous Nephrology which held in Bergamo has provided further evidence of association of IgG4 and MBL in IMN. We found that the patients of bi-positivity showed significantly higher MASP-1, MASP-2 and MBL than those of bi-negativity, which could provide a clue for Lectin pathway activation in these patients. Factor B is one of the most vital regulators in alternative pathway and Ba is a free breakdown product and its increase had more accurate indication for alternative pathway. IgG4 subclass might bind MASP-1/MASP-2/MBL complex, activate lectin pathway and generate C3b; C3b/Bb complex further activates C3 and generates a positive feedback (Fig. 8). We found that the decrease in Ba rather than Bb as a significant effective factor for interpreting the possibility of achievement of CR; it might be explained by the reason that Ba has a free form in the circulation, but the routine form of Bb is a combination binding with C3b; therefore, Ba might be a better biomarker for representing the alternative pathway activation.

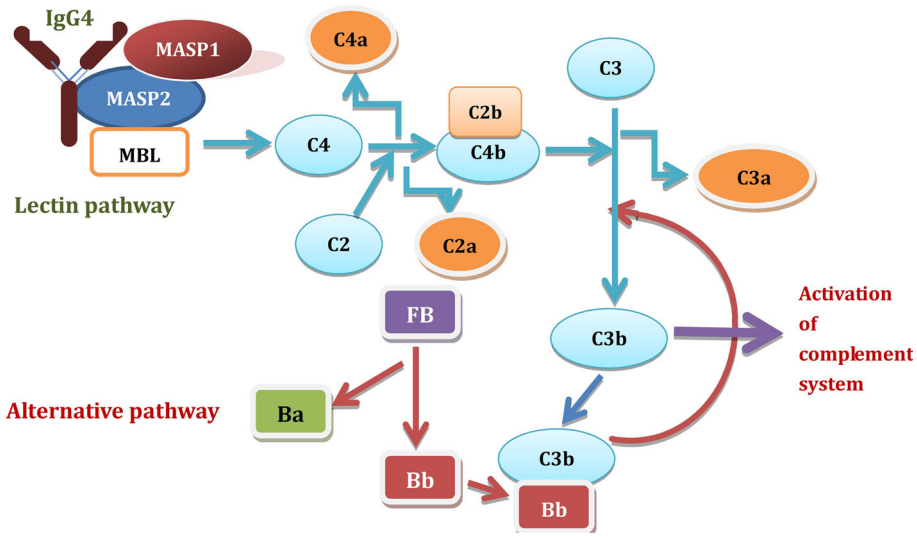

Fig. 8 Diagram of the activation of Lectin pathway and alternative complement pathway in IMN

It should be noted that the major conclusions on the complement system and the development of IMN came from the later stage of the cases. A study found IgG1 was a predominant subclass in the early stage of IMN [22]. There was an inverse relationship between IgG4 intensity within GBM of kidney and C1q straining, and it was hypothesized that IgG1 might initially activate the classical pathway at low level in early stage, with subsequent activation of lectin pathway and alternative pathway in the evolution of the disease course [22–24]. Our work could provide an evidence for the latter. Hofstra reported that 80–90 % patients with IMN have IgG4 binding with other subclasses, such as IgG1 and IgG3 [6]. It suggested one possibility that IgG4 might act as storage within GBM and transfer to different subclasses, including IgG1 or IgG3, in pathological situation. Besides, IgG4 might injure podocytes in other pathological ways rather than direct complement injury and it could not be excluded that IgG4 had other important immunomodulatory or pathophysiological functions [9, 25]. In general, many further researches for exploring the definite mechanisms and the role of IgG4 playing in IMN are required.

Three methods were applied to detect anti-PLA_2R: Beck et al. [3] had introduced a western blot (WB) method; and then, a second method was developed on the basic of a recombinant cell-based indirect immunofluorescence (RC-IFA) using human cell line HEK 293 by over-expressing PLA_2R. Recently, a new method based on ELISA assay was introduced to quantitatively assay anti-PLA_2R and a research had already confirmed its high sensitivity and specificity as well as an excellent correlation with RC-IFA [11]; besides, ELISA assays can be developed easier in the majority hospitals in China. Therefore, we introduced commercial ELISA kits for detecting anti-PLA_2R.

Our study has some limitations. First, we have to admit that it was a retrospective and observational study and immunosuppressive agents prescribed were not designed. Second, all patients had received glucocorticoids; therefore, we could not analyze the association between anti-PLA_2R and spontaneous remission. Third, we only assayed the complement ingredients in patients with bi-negativity and bi-positivity and only assayed the baseline complement ingredients in patients with bi-positivity; therefore, the data on complement ingredients were not completed and did not include those with antibodies transferring negativity; last, it was a single-center study and all conclusions we achieved might be tested in a larger cohort.

In conclusion, four highlights of our study were achieved: (1) the appliance of meta-analysis to find the predictive effect of baseline anti-PLA_2R for the possibility of achieving CR; (2) continuous IgG4 status had more predictive value than total antibodies; (3) continuous IgG4 seronegativity predicted more possibility of achieving CR; and (4) there might be alternative and lectin pathway being both activated in patients with continuous IgG4 positivity and the lower titers of Ba would be a useful indicator to predict CR in these patients.

Compliance with ethical standards

Conflicts of interest All authors declared no conflicts of interest for this article.

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