Clinical significance of autoantibodies in the assessment and treatment of idiopathic membranous nephropathy

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Abstract. The present study aimed to explore the correlation between the dynamic serum levels of phospholipase A2 receptor (PLA2R), aldose reductase (AR) and superoxide dismutase 2(SOD2) antibodies with disease activity and treatment response in patients with idiopathic membranous nephropathy (IMN). The present study included 56 patients with IMN who were diagnosed through a renal biopsy and presenting with nephrotic syndrome. The patients were divided into two treatment groups: One treated with cyclophosphamide (CTX) and one with tacrolimus (FK506). Serum was collected prior to treatment, and at 1, 3, 6, 9 and 12 months after the start of the 12-month-long therapy. Samples were tested by ELISA to measure anti-PLA2R, anti-AR and anti-SOD2 antibody titers. In addition, urinary protein excretion, serum albumin (Alb) and other blood biochemical indexes were measured. Theanti-PLA2R antibody positivity rate was 71.43% in the patients prior to treatment. After 12 months of treatment, proteinuria and PLA2R antibody levels were decreased, whereas serum Alb was increased. There was no significant difference of remission rates between the CTX and FK506 groups. In conclusion, the results of the present study indicate that the anti-PLA2R antibody level is correlated with the severity of IMN, whereas anti-AR and anti-SOD2 antibody levels are not. In addition, there was no significant difference between the CTX and FK506 groups in regards to the remission rates of patients with IMN.

Introduction

Idiopathic membranous nephropathy (IMN) is the most common cause of nephrotic syndrome in adults, accounting for ~30% of all types of renal biopsy (1). In recent years, the proportion of IMN cases out of the number of cases of primary glomerular disease has continued to rise (1). IMN is identified in adults by the presence of anti-M-type phospholipase A2 receptor (PLA2R) antibodies in the serum. M-type PLA2Ris expressed by podocytes in patients with membranous nephropathy (MN); however, patients with secondary or other glomerular diseases exhibit less anti-PLA2R antibodies in the serum, which indicates that PLA2R may be a pathogenic target antigen in adults with IMN (1). Therefore, anti-PLA2R antibodies may be an important biomarker for the diagnosis of IMN (2,3). Numerous studies have proposed that the anti-PLA2R antibody titers of patients with IMN are associated with proteinuria and response to treatment (4,5). A European research group also identified that the anti-PLA2R antibody titer was associated with serum creatinine, urine β2 protein microspheres and urinary IgG (4). In addition, in 2010 Prunotto *et al* (5) identified specific IgG4 antibodies directed against the podocyte antigensaldose reductase (AR) and superoxide dismutase (SOD) in the serum and renal tissue of patients with MN through proteomic methods.

In the present study, anti-PLA2R, anti-SOD2 and anti-AR antibodies in the serum of patients with IMN were detected, in addition to the levels of other serum biochemical factors. Then, the correlation between these factors and the response to treatment of the patients with IMN was investigated.

Materials and methods

Study population. A total of 56 patients with a histological diagnosis of IMN were recruited between April 2012 and August 2015 from Qianfoshan Hospital (Jinan, China). The inclusion criteria were as follows: i) Meet the International Classification of Diseases 10 diagnostic criteria for nephrotic syndrome (lCD-10. Geneva, World Health Organization, 1992) [clinical manifestations of nephrotic syndrome (proteinuria >3.5 g/d, serum albumin (Alb) <30 g/l, and hyperlipidemia and edema) and a renal biopsy diagnosis of MN]; ii) complete clinical data; and iii) Written informed consent of the patient was obtained prior to inclusion in the present study. The exclusion criteria were the follows: i) nephrotic syndrome due to infection, other autoimmune diseases, cancer, hepatitis, diabetes, drugs or other secondary factors; ii) a sustained creatinine level >309.4 mol, estimated

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glomerular filtration rate (eGFR)<30 ml/min/1.73 m^2, significantly reduced kidney volume (long diameter <8 cm) or the presence of a severe infection; and iii) other immunotherapy contraindications. The 2012 Kidney Disease: Improving Global Outcomes glomerulonephritis Clinical Practice Guidelines (6) outline the following IMN remission criteria: i) Complete remission, 24 h urinary protein <0.3 g and normal serum Alb (35-50 g/l); ii) partial remission, 24 h urinary protein <3.5 g ≥50% reduction in urinary protein excretion, and a normal or elevated serum Alb level; iii) no-remission, patient does not meet the above criteria. The present study was approved by the Ethics Committee of Shandong University (Jinan, China).

Treatment groups. All the patients were divided into groups according to their 24 h urinary protein excretion. Patients whose urinary protein level was <4 g/24 h were given an angiotensin converting enzyme inhibitor (Lotensin, 10-20 mg/day) and supportive treatment. Patients were given Diovan in addition to Lotensin (80-160 mg/day) if they could not tolerate coughing and other side effects. Patients who had aurinary protein excretion of 4-6 g/24 h were observed and treated ibid. Patients were observed for proteinuria during drug treatment, until a urinary protein level of <4 g/24 h was reached. Close observation was continued if the proteinuria didn't resolve within1 month. Patients with a proteinuria level >6 g/24 h were given immunosuppressive therapy. The randomly allocated cyclophosphamide (CTX) group of 36 patients received oral methylprednisolone (1 mg/kg/day) and 0.8 g CTX via intravenous injection once a month for 6 months, then once every 3 months for a total of 12 months; the total CTX dose did not exceed 11 g. The randomly allocated tacrolimus (FK506) group of 20 patients received 0.03-0.05 mg/kg FK506 orally twice a day (with an interval of 12 h between doses to maintain a drug concentration in the blood of 5-10 ng/ml) for 6-12 months, while prednisone (0.5 mg/kg/day) could be reduced following mitigation and the administration of FK506.

Serum antibody and biochemical measurements. During treatment, renal function, serum Alb, urinary protein, high-sensitivity C-reactive protein, and anti-PLA2R, anti-SOD2 and anti-AR antibody titers were measured. Serum samples were taken prior to and at 1, 3, 6, 9 and 12 months after treatment. Anti-PLA2R, anti-AR and anti-SOD2 antibodies were detected through ELISAs. The anti-PLA2R antibody ELISA kit was purchased from EUROIMMUN Medizinische Labordiagnostika AG (cat. no. FA1254; Lübeck, Germany). The anti-AR and anti-SOD2antibodyELISA kits were from CUSABIO (Wuhan China; cat. nos. CSB-EL001975MO andCSB-EL022398RA, respectively). The absorbances of the wells of the ELISA plates were measured at 450 nm using a microplate reader. The standard antibodies provided in the kits were used to make the standard curve and fit equation in order to calculate the concentration of the test antibodies.

Statistical analysis. All statistical analyses were performed using SPSS software (version 20.0;IBM Corp., Armonk, NY, USA). Normally distributed continuous variables were expressed as the mean ± standard deviation. Count data was expressed as a percentage. The statistical significance of differences among groups of normal measurement data were measured using the Student's t-test between two groups or one-way analysis of variance followed by a post hoc Tukey's range test for multiple groups. Non-normality was examined using the Shapiro-Wilk test and heterogeneity of variance between data groups was examined using Levene's test. The correlation between two normally distributed continuous variables was investigated using the Pearson correlation coefficient and further analyzed using the multiple linear regression equation. Serum antibody titers were the dependent variable and all biochemical indicators were the independent variables. The multiple linear regression equations were analyzed using the stepwise method. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Clinical characteristics of the study population. The present study included 56 patients with IMN. There were 36 males and 20 females, with a mean age of 46.85 years (range, 13-66 years). The follow-up time was 9.21 months on average (range, 6-24 months). On average, the 24 h urinary protein levels were 6.40±3.65 g, serum Alb was 24.21±6.08 g/l, the eGFR was 113.33±36.35 ml/min/1.73 m^2, total cholesterol was 9.43±6.82 mmol/l, systolic blood pressure was 131.7±16.09 mmHg and diastolic blood pressure was 81.3±12.05 mmHg. The clinical characteristics of the study population and the two treatment groups are presented in Table I. The CTX and FK506 groups exhibited no significant difference in any MN-associated serum biochemical indicators or antibodies prior to treatment. During the experiment, serum Alb levels increased (Fig. 1) and proteinuria decreased (Fig. 2) with immunosuppressive therapy.

Anti-PLA2R antibody expression in patients with IMN over the course of treatment. The level of serum anti-PLA2R antibodies were compared in 56 patients with IMN prior to treatment with normal ranges. A total of 40 patients with IMN (71.43%) tested positive for serum anti-PLA2R antibodies. Patients were separated into a CTX group and a FK506 group, which received two different treatment regimens. Among the 40 patients that tested positive for serum anti-PLA2R antibodies, 26 were from the CTX group and 14 were from the FK506 group. Of the 16 patients that tested negative for serum anti-PLA2R antibodies, 10 patients were from CTX group and the remainder was from the FK506 group. Prior to treatment, all patients were positive for anti-PLA2R antibodies. Comparing the anti-PLA2R antibody level prior to and after treatment (Table II), out of the 36 patients in the CTX group, 21 patients tested negative following treatment, while 15 patients remained positive, although the titers decreased after treatment. Out of the 20 patients in the FK506 group, 14 patients tested negative for serum anti-PLA2R antibodies following treatment, while 6 patients remained positive, although again the titers decreased following treatment.

Correlation between anti-PLA2R antibody titers and blood biochemical indexes in patients with IMN over the course of treatment. Table II lists the first and last measurements of urinary protein, serum Alb, the eGFR, and anti-PLA2R, anti-AR and anti-SOD2 antibody levels. After immune suppression treatment, the anti-PLA2R antibody titers of patients with IMN significantly

Table I. Clinical characteristics of the study population.

Characteristics	All patients (n=56)	Group		P-value (CTX vs. FK506)
		CTX (n=36)	FK506 (n=20)	
Sex, ratio (male/female)	1.8 (36/20)	2.0 (24/12)	1.5 (12/8)	-
Age (years)	46.9±13.62	48.6±10.29	43.2±17.21	0.22
Proteinuria (g/24 h)	6.40±3.65	6.02±3.54	7.13±3.83	0.28
Serum albumin (g/l)	24.21±6.08	25.31±6.11	22.24±5.64	0.07
eGFR (ml/min/1.73 m^2)	113.33±36.35	112.72±29.35	114.44±47.28	0.88
TC (mmol/l)	9.43±6.82	8.17±2.49	11.70±10.72	0.16
SBP (mmHg)	133.5±16.98	131.4±17.65	132.4±12.65	0.79
DBP (mmHg)	81.3±12.05	81.8±13.22	80.2±9.40	0.51
PLA2R antibody level (mU/ml)	1.25±0.72	1.35±0.66	1.06±0.79	0.17
AR antibody level (mU/ml)	16.88±14.10	16.15±12.77	18.34±16.79	0.65
SOD2 antibody level (mU/ml)	3.73±6.15	2.87±5.78	5.27±6.65	0.19

eGFR, estimated glomerular filtration rate; TC, T-cell; SBP, systolic blood pressure; DBP, diastolic blood pressure; PLA2R, M-type phospholipase A2 receptor; AR, aldose reductase; SOD2, superoxide dismutase.

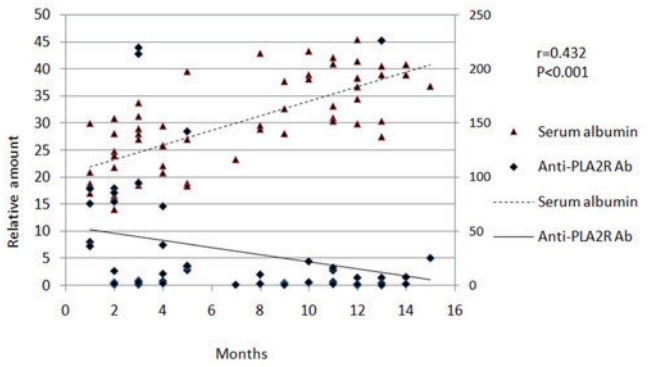

Figure 1. Serum albumin and anti-PLA2R antibody levels are significantly negatively correlated over the course of immunosuppressive treatment. PLA2R, M-type phospholipase A2 receptor.

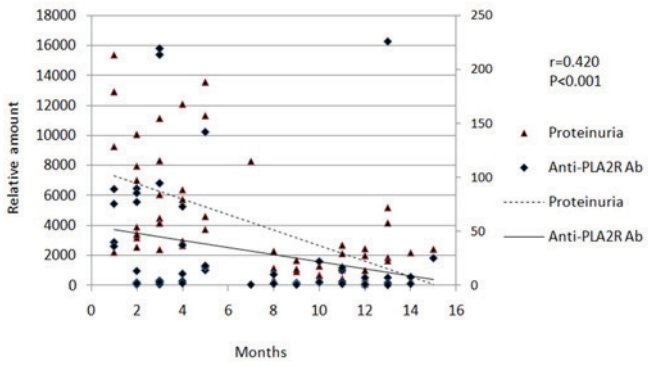

Figure 2. Proteinuria and anti-PLA2R antibody levels are significantly positively correlated over the course of immunosuppressive treatment. PLA2R, M-type phospholipase A2 receptor.

decreased compared to those prior to treatment (P<0.001). In addition, compared to levels prior to treatment, proteinuria levels significantly decreased and serum Alb levels significantly increased (P<0.001). The analysis of the correlation for these data revealed that there was a significant positive correlation between the reduction in anti-PLA2R antibody titers and the decrease in proteinuria (r=0.420; P<0.001; Fig. 2). By contrast, there was a significant negative correlation between the reduction in anti-PLA2R antibody titers and the increase in serum Alb (r=0.432; P<0.001; Fig. 1). However, the reduction of eGFR (r=-0.103, P=0.294) and serum cholesterol (r=-0.078, P=0.437) had no significant association with the reduction in anti-PLA2R antibody titers (data not shown).

Correlation between anti-AR and anti-SOD2 antibody titers and blood biochemical indexes in patients with IMN over the course of treatment. Out of the 56 patients with IMN, 26 tested positive for serum anti-AR antibodies. The number of patients who tested positive for serum anti-AR antibodies was not significantly different compared with the normal controls. For the patients who tested negative for anti-PLA2R antibodies following treatment, 54.4% tested positive for anti-AR antibodies, although this difference was not statistically significant when compared with samples taken prior to treatment (data not shown). Correlation analysis revealed that the serum anti-AR antibody titers were significantly positively correlated with urinary protein levels (r=0.204; P<0.05) and the eGFR (r=0.338; P<0.01) (data not shown). Of the 56 patients with IMN, 38 tested positive for serum anti-SOD2 antibodies. Serum anti-SOD2 antibody titers were significantly positively correlated with urinary protein (r=0.223; P<0.05) and serum Alb (r=-0.194; P<0.05) levels; however, there was no correlation between anti-SOD2 antibody titers and the eGFR or total cholesterol (data not shown).

Effect of CTX and FK506on IMN remission. The56 patients with IMN were followed up after treatment for 9.21 months on average (Table III). In the CTX group, 12 patients (33.3%)

Table II. Biochemical measurements prior to and following immunosuppressive therapy in patients with IMN.

Factor measured	Group	Time point		P-value
		Prior to treatment	After treatment	
Proteinuria (g/24 h)	All patients	6.40±3.65	1.81±1.70	<0.001
	CTX	6.02±3.54	1.54±1.34	<0.001
	FK506	7.14±3.83	2.25±2.17	<0.001
Serum albumin (g/l)	All patients	24.21±6.08	35.81±5.74	<0.001
	CTX	25.31±6.11	36.31±5.47	<0.001
	FK506	22.24±5.56	34.90±6.24	<0.001
eGFR (ml/min/1.73 m^2)	All patients	113.33±36.35	113.27±37.59	0.84
	CTX	112.72±29.35	108.53±27.97	0.44
	FK506	114.44±47.28	121.34±49.75	0.23
PLA2R antibody level (mU/ml)	All patients	1.25±0.72	0.58±0.59	<0.001
	CTX	1.35±0.66	0.82±0.54	<0.001
	FK506	1.05±0.79	0.12±0.37	<0.001
AR antibody level (mU/ml)	All patients	16.88±14.10	16.45±15.93	0.78
	CTX	16.15±12.77	14.71±13.88	0.42
	FK506	18.34±16.80	19.94±19.42	0.53
SOD2 antibody level (mU/ml)	All patients	3.73±6.15	4.82±9.63	0.92
	CTX	2.87±5.78	4.81±11.09	0.32
	FK506	5.26±6.64	4.85±6.44	0.23

CTX, cyclophosphamide; FK506, tacrolimus; eGFR, estimated glomerular filtration rate; PLA2R, M-type phospholipase A2 receptor; AR, aldose reductase; SOD, superoxide dismutase.

Table III. Remission rates of patients with IMN following immunosuppressive therapy.

Group	Remission status, no. of patients (%)		
	Complete remission	Partial remission	No remission
CTX (n=36)	12 (33.3)	20 (55.6)	4 (11.1)
FK506 (n=20)	4 (20.0)	16 (80.0)	0 (0.0)
All patients (n=56)	16 (28.6)	36 (64.3)	4 (7.1)

CTX, cyclophosphamide; FK506, tacrolimus.

achieved clinical complete remission, 20 patients (55.6%) exhibited partial remission and4 patients (11.1%) had no remission. In the FK506 group, all patients achieved complete or partial remission; 4 patients (20%) exhibited complete remission and 16 patients (80%) achieved partial remission. After treatment, serum anti-PLA2R antibody titers in the two groups were significantly decreased compared with prior to treatment (data not shown). Between the two groups, the complete and partial remission rates were not significantly different (Table III), which suggests that CTX and FK506 do not have significantly different effects on IMN remission.

Discussion

IMN is a chronic disease that can exhibit spontaneous remission and relapse; typically in the first 2 years after onset ~40% of patients go into spontaneous remission (7,8). Predictive factors for spontaneous remission include a proteinuria level<8 g/24 h at baseline, being female, an age of<50 years and good renal function at the time of the disease onset (9). In addition, 2/3 patients with IMN can exhibit persistent proteinuria but maintain good renal function long-term; although, despite receiving immunosuppressive therapy, the majority of patients progress to end-stage renal disease (ESRD) (10). MN is a common cause of primary glomerulonephritis, which leads to ESRD (10).

Currently, the diagnosis of IMN mainly relies upon renal pathology, which is invasive, as there is a lack of sensitive biomarkers to predict the disease effectively (1). A noninvasive examination or serum biomarkers would be preferable, as they can effectively reduce the risk of bleeding, infection and other side effects from invasive procedures. The present

study investigated the significance of serum autoantibodies in the diagnosis of MN, which could replace traditional biopsy diagnosis and serve a role in treatment strategies. A previous meta-analysis evaluated the diagnostic value of serum anti-PLA2R antibodies in the detection in IMN, which revealed that its sensitivity and specificity were 78 and 99%, respectively (11).

The present study examined the levels ofanti-PLA2R antibodies in the serum of 56 patients with IMN, which demonstrated that 71.43% of the patients exhibited expression of these antibodies. This is similar to the results of previous studies that reported a positive rate for PLA2R antibodies of 71.0-77.8% in patients with IMN (12,13). The present study also identified a correlation between the level of serum anti-PLA2R antibodies and serum biochemical indicators of IMN. Correlation analysis revealed that serum anti-PLA2R antibody titers were significantly positively correlated with proteinuria over the course of treatment, which was consistent with the findings of several previous studies (13-15). IMN diagnosis and staging relies on pathology, however, the pathological stage has no clear association with clinical disease activity or drug efficacy (16). Clinicians monitor the activity of IMN and determine drug efficacy primarily through monitoring proteinuria and serum Alb levels (16). The results of the current study demonstrated that the level of anti-PLA2R, anti-AR and anti-SOD2antibody titers were significantly positively correlated with proteinuria, which is an indicator of renal function. In patients with IMN who respond to drugs, achieving complete or partial remission, these serusm antibody titers may decrease or disappear (16). Previous studies have demonstrated that serum anti-PLA2R antibody titer decline occurs prior to the decrease of urinary protein and to the other laboratory parameters of remission (13-15).

Investigations into the association between serum anti-PLA2R antibody levels and the development of IMN has primarily been on small sample populations and over a short-term period; thus, the reliability of antibody titers for monitoring disease progression and treatment efficacy still requires further verification. It has been reported that the anti-PLA2R antibody titer can predict the recurrence of MN at the end of immunosuppressive therapy. After 5 years of immunosuppressive therapy, ~58% of patients with IMN who are negative for anti-PLA2R antibodies do not suffer recurrence, while patients who are positive for these antibodies at the end of treatment are more likely to relapse (12). Thus, the presence or absence of anti-PLA2R antibodies can be used as a strong predictor for the clinical remission of PLA2R-associated MN. In addition, monitoring serum antibodies regularly prior to and during treatment can help predict the efficacy of therapy (9). Certain studies have suggested that prior to starting immunosuppressive treatment, antibody titers should be measured once every 2 months in order to avoid unnecessary treatment (12,16). Another study reported that in the first 6 months of immunosuppressive therapy, antibody titers should be tested once a month to assess the effect of drug treatment (17). In the present study, certain patients continued to express anti-PLA2R antibodies during and after treatment; however, the levels decreased significantly after treatment, so the potential long-term beneficial effects of immunosuppressive therapy cannot be excluded in these patients. If a long-term period of follow-up observation was performed, these patients may stop expressing anti-PLA2R antibodies.

At present, the impact of anti-AR and anti-SOD2 antibodies on the diagnosis and treatment response of patients with IMN remains unclear. Previous studies have identified AR and SOD2 in IMN biopsy specimens (5). Anti-AR and anti-SOD2 antibodies and C5b-9 are co-localized in the electron-dense material of podocytes in patients with IMN (5). In the present study, anti-AR antibody levels in the serum were significantly positively correlated with proteinuria and the eGFR in patients with IMN, but had no significant effect on the dynamics of disease progression (as determined by urinary protein and serum Alb levels). Particularly, the anti-SOD2 antibodies are similar to the inflammatory markers for the oxidative stress reaction in the body, and there is no specific or sensitive method for monitoring the progress of IMN (5). In the clinic, anti-AR and anti-SOD2antibodies cannot be used as specific monitoring indicators.

In conclusion, the expression of anti-PLA2R antibodies in patients with IMN is associated with the efficacy of immunosuppressive therapy. Immunosuppressive therapy alone or combined with hormone therapy has been proven to relieve the symptoms of IMN effectively in clinical practice, but not to influence clinical remission (9). In the present study, two different immunosuppressive treatment regimens, CTX and FK506, were used, and it was revealed that anti-PLA2R antibody levels and progress of the disease were not significantly different between the two groups, which is in agreement with the findings of previous studies (18,19). However, the present study was limited by a small sample size and so the influence of this cannot be ruled out. The results of the present study determined that the specificity of anti-AR and anti-SOD2 antibodies for the diagnosis of MN is low, thus their use in clinical diagnosis is limited. In addition, the present study had a relatively short follow-up time and did not include the patients with secondary MN as a control. Therefore, future research with a larger sample size, an extended follow-up period and including patients with secondary MN is required to validate the results of the current study.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WWH and DMX conceived and designed the experiments. WWH, HY and XLK collected the data. WWH and XLK were

involved in the analysis of data. WWH and LJT performed the experiments. WWH edited the manuscript. DMX revised the manuscript. All authors have read and approved this article.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Shandong University (Jinan, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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