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Original Article

An ROC curve analysis of serum PLA2R antibody, glomerular PLA2R, and IgG4 in membranous nephropathy

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Abstract: Membranous nephropathy is a common pathological type of adult nephrotic syndrome. The anti-M-type phospholipase A2 receptor (PLA2R) antibody is considered to be a membranous nephropathy-specific antibody. Membrane nephropathy activity is related to the PLA2R antibody titer. IgG4 plays an important role in membranous nephropathy. This study investigated the role of the serum PLA2R antibody, glomerular PLA2R, and IgG4 in membranous nephropathy. Patients with membranous nephropathy admitted to the hospital and diagnosed by renal biopsy were selected, including 25 cases of idiopathic membranous nephropathy (IMN), 14 cases of secondary membranous nephropathy (SMN), and 25 cases of atypical membranous nephropathy (UAMN). An indirect immunofluorescence assay (IFA) was used to determine the level of serum anti-PLA2R antibodies. An immunofluorescence assay (IIF) was performed to measure the glomerular PLA2R expression and the IgG subtype. An ROC curve was drawn to evaluate the diagnostic value of the serum anti-PLA2R antibodies, glomerular PLA2R, and IgG4 in membranous nephropathy. The deposition positive rates of the serum anti-PLA2R antibodies, glomerular PLA2R, and IgG subtypes were significantly different among IMN, SMN, and UAMN ($P < 0.05$). The sensitivity and specificity of their positive expression in diagnosing IMN were 72.00%, 92.00%, 88.00%, 82.05%, 84.62%, and 89.74%, respectively. 88.00% of IMN patients exhibited positive glomerular PLA2R expression with moderately positive IgG4. No SMN or UAMN patients showed positive glomerular PLA2R expression with moderately positive IgA. An ROC curve revealed that the area under the curve of the serum anti-PLA2R antibodies, glomerular PLA2R, and the IgG subtype deposition was 0.860, 0.930, and 0.920, respectively. The sensitivity of the combined detection in IMN was improved. The positive rate of glomerular PLA2R expression in IMN was high, which was mainly IgG4 subtype deposition. UAMN kidney tissue mainly had IgG1 subtype deposition. Serum anti-PLA2R antibody and glomerular PLA2R and IgG subtype positive deposition plays an important role in differentiating diagnosis and evaluating the efficacy of membranous nephropathy.

Keywords: Membranous nephropathy, serum anti-PLA2R antibody, glomerular PLA2R, IgG4 subtype

Introduction

Membranous nephropathy is a common pathological type of adult nephrotic syndrome. The prevalence among males is higher than it is among females. Its peak onset age is 40-60 years old, and it has an increasing incidence in China [1, 2]. Depending on the etiological agent, SMN is mainly caused by infection (tuberculosis, syphilis, etc.), tumors, drugs (non-steroidal anti-inflammatory drugs, captopril, etc.), and autoimmune diseases [3, 4]. On the other hand, IMN accounts for about 66.7% of membranous nephropathy with slow progression. About 33.3% of patients heal themselves. The left may develop into refractory nephropathy. SMN patients need to be treated for primary diseases. The incidence of UAMN keeps increasing year by year. It is unclear whether UAMN is a special pathological type of IMN or a single pathological type of SMN [5, 6]. Both the clinical manifestations and the pathology are inconclusive in distinguishing membranous nephropathy types. The different treatment programs and prognosis of IMN, SMN, and UAMN make it important for early diagnosis and treatment in patients with membranous nephropathy [7, 8].

Serum PLA2R antibody is considered to be a membranous nephropathy-specific antibody. It

was shown that membranous nephropathy activity is associated with the PLA2R antibody titer. The positive rate of serum PLA2R antibody in IMN patients is 53%-82% [9, 10]. It was reported that PLA2R is the pathogenic antigen of IMN in kidney tissue. The detection positive rate of PLA2R in renal tissue is higher than it is in a blood test. The relationship of PLA2R expression in podocytes and immune deposits with membranous nephropathy is still unclear, which is due to the fact that serum anti-PLA2R antibody detection lags behind glomerular PLA2R expression, and the positive rate of serum PLA2R antibody expression is affected by immunosuppressants and other factors [11, 12]. Depending on the different IgG in heavy chains, IgG can be divided into the IgG1, IgG2, IgG3, and IgG4 subtypes. Because of different biological activities, they participate in different immune responses. Cytokines IgG1 and IgG3 secreted by helper T cells exert cytotoxicity, but IgG4 mainly participates in the immune response by the Th2 system to activate B cells. Different pathological types of nephropathy patients exhibited different IgG subtypes of glomerular deposits. SMN glomerular deposits are mainly IgG1 and IgG3. IMN glomerular capillary sputum has IgG4 deposition. IMN is closely related to IgG4 [13, 14]. In this study, we tested the serum anti-PLA2R antibody, glomerular PLA2R expression, and IgG subtype deposition in patients with different types of membranous nephropathy, and we used an ROC curve to explore their roles in the differential diagnosis of membranous nephropathy.

Materials and methods

Clinical information

Sixty-four patients with membranous nephropathy confirmed by renal biopsy who were admitted to the Department of Nephrology from May 2016 to December 2017 were selected [15], including 25 cases of IMN patients and 14 patients with SMN (6 cases of lupus membranous nephropathy and 8 cases of hepatitis B virus associated membranous nephropathy), and 25 patients with UAMN. There were 40 males and 24 females, with a mean age of 47.36 ± 5.13 (22-76) years old. Inclusion criteria [14, 15]: pathological diagnosis of membranous nephropathy, no use of hormone or immunosuppressive agents, and no history of the application of nephrotoxic drugs. Exclusion criteria [14, 15]: serious primary disease in the liver, brain, and blood, IMN excluding systemic lupus erythematosus, tumor, hepatitis B virus infection, and drug-induced membranous nephropathy. The renal pathology of UAMN is characterized by SMN, which is clinically excluded from the common causes of secondary membranous nephropathy. The selected patients underwent a renal biopsy to collect clinical data (age, gender, course of the disease, medical history, and clinical manifestations), laboratory indicators (serum creatinine, albumin, 24 h urine protein, and glomerular filtration rate), and pathological data. All the patients signed an informed consent.

Indicator detection

Renal histopathological examination: a direct immunofluorescence assay was used to test the IgG subtype deposition of glomeruli.

Indirect IFA was selected to determine the serum anti-PLA2R antibodies. An IIF was performed to detect glomerular PLA2R expression. The staining intensity was evaluated by semiquantitative assessment, and 0 to 4 + referred to a negative to the strongest positive expression. Sensitivity = true positive/(true positive + false negative), specificity = true negative/(true negative + false positive), positive predictive value = true positive/(true positive + false positive), negative predictive value = true negative/(true negative + false negative).

Detection of anti-PLA2R antibodies in serum by indirect immunofluorescence assay

A recombinant, cell-based, indirect immunofluorescence assay (RC-IFA, EUROIMMUN, Lübeck, Germany) was used to detect the anti-PLA2R antibodies. RC-IFA contained a BIOCHIP mosaic of HEK293 cells overexpressing a full-length PLA2R isoform 1 (Uniprot reference [PLA2R HUMAN]) and mock-transfected HEK-293 cells as a negative control. Fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG antibody was used for the detection of bounded IgG-antibodies. The test was performed following the manufacturer's instructions. The initial serum dilution was 1:10. All slides were evaluated by 2 independent observers using a fluorescence microscope (EUROStar III Plus, EUROIMMUN, Lübeck, Germany). In case

Table 1. Clinical information comparison ($\bar{X}$ ± S)

Group	IMN (n = 25)	SMN (n = 14)	UAMN (n = 25)	*F* value	*P* value
Age (year)	50.13±4.23	49.23±4.11	37.04±3.15*	6.03	< 0.05
Gender (male/female)	11/14	5/9	9/16	0.42	> 0.05
Globulin (g/L)	28.16±4.05	26.67±4.56	30.45±5.11	1.14	> 0.05
Urine protein (g/24 h)	3.04±1.12	5.36±1.05*	4.14±1.08#	5.74	< 0.05
Serum creatinine (μmol/L)	66.29±11.23	76.29±10.11	65.55±9.87	1.16	> 0.05
Glomerular filtration rate [ml/(min·1.73 m^2)]	112.45±16.34	98.45±16.34	122.77±14.26	1.09	> 0.05
Serum IgG (g/L)	7.34±3.12	10.11±3.49*	8.36±3.64#	6.87	< 0.05
Nephrotic syndrome (case%)	8 (32.00)	9 (64.29)	10 (40.00)	3.91	> 0.05

*P < 0.05, compared with IMN group; #P < 0.05, compared with SMN group.

of a difference of opinion about a result, a third observer decided. A result was regarded as positive when a specific cytoplasmic, partly cell membrane or cell nuclei fluorescence, occurred on the transfected cells at a dilution of 1:10 or higher. For the positive sera, the titer of anti-PLA2R antibodies was determined at the end-point dilution.

Detection of anti-PLA2R antibodies by indirect immunofluorescence assay (IIF)

PLA2R expression was examined by indirect IF using rabbit polyclonal anti-PLA2R Abs (Sigma-Aldrich, St. Louis, MO, USA) and Alexa Fluor 488-conjugated goat anti-rabbit IgG Abs (Invitrogen, Carlsbad, CA, USA). Briefly, a 3-μm-thick section was made from formalin-fixed paraffin-embedded tissue. Enzyme pretreatment with protease K was performed and the tissues were incubated for 30 min followed by rinsing. Then, rabbit polyclonal anti-PLA2R1 antibody (Sigma-Aldrich) at a dilution of 1:50 was added and incubated for 30 min. After rinsing, Alexa Fluor 488 goat anti-rabbit IgG (Life Technologies) at a dilution of 1:100 was added and incubated for 30 min followed by rinsing and covering the slip. Each case was run with a positive and negative (secondary antibody only) control. The stain was evaluated by standard immunofluorescence microscopy using a Leica L5 filtercube. It was judged to be positive if there was positive granular capillary loop staining in the glomeruli and negative if there was no staining in the glomeruli.

Statistical analysis

All data analyses were performed using SPSS 19.0 statistical software. The measurement data conforming to the normal distribution were expressed by the mean ± standard deviation ($\bar{X}$ ± S) and compared using ANOVA and LSD-t tests. The enumeration data were expressed as the ratio and compared using an X^2-test or a non-parametric test. ROC was performed for the differential diagnosis of membranous nephropathy. P < 0.05 was considered statistically significant.

Results

Clinical data comparison

The mean age of the renal biopsy in the patients was UAMN group < SMN group < IMN group (P < 0.05), total urinary protein (g/24 h) was UAMN group < SMN group (P < 0.05), The IgG level was IMN group < SMN group < UAMN group (P < 0.05). The clinical manifestations were characterized by nephrotic syndrome. Gender, glomerular filtration rate, albumin and other indicators exhibited no statistical significance (P>0.05, **Table 1**).

Comparison of immunofluorescence positive rates in patients with membranous nephropathy

The positive rate of IgA, IgM, and C1q were higher in the UAMN group than in the IMN group (P < 0.05). The positive rate of the IgG1 subtype was 40.00% in the UAMN group and 12.00% in the IgG3 subtype. The IgG4 subtype positive rate was higher, but the IgG2 and IgG3 subtype positive rates were lower in the IMN group. SMN group: The IgG1 subtype positive rate was higher, but the IgG4 subtype positive rate was lower in the SMN group. IgG1, IgG2, and IgG3 subtype positive rates were higher, but the

Table 2. Comparison of the immunofluorescence positive rates in patients with membranous nephropathy (case %)

Group	IMN (n = 25)	SMN (n = 14)	UAMN (n = 25)	*U* value	*P* value
IgA	0 (0.00)	13 (92.86)*	21 (84.00)*	9.57	< 0.05
IgG	25 (100.00)	13 (92.86)	25 (100.00)	0.42	> 0.05
IgM	3 (12.00)	12 (85.71)*	18 (72.00)*	8.14	< 0.05
C3	25 (100.00)	13 (92.86)	25 (100.00)	0.42	< 0.05
C1q	3 (12.00)	13 (92.86)*	23 (92.00)*	7.16	< 0.05
IgG1	4 (16.00)	10 (71.43)*	10 (40.00)	6.09	< 0.05
IgG2	1 (4.00)	9 (64.29)*	7 (28.00)#	6.87	< 0.05
IgG3	1 (4.00)	6 (42.86)*	3 (12.00)	4.91	< 0.05
IgG4	21 (84.00)	4 (28.57)	5 (20.00)*	9.53	< 0.05

*$P < 0.05$, compared with IMN group; #$P < 0.05$, compared with SMN group.

IgG4 subtype positive rate was lower in the SMN group than in the IMN Group (P < 0.05) (**Table 2**).

Serum anti-PLA2R antibody, glomerular PLA2R and IgG subtype deposition test results

Among the IMN patients, 88.00% (22/25) of those with the IgG4 subtype were positive, 72.00% (18/25) of those with the anti-PLA2R antibody were positive, and 92.00% (23/25) of those with the glomerular PLA2R expression were positive. Among the SMN patients, 7.14% (1/14) of those with the glomerular PLA2R expression was positive, 71.43% (10/14) of those with the serum anti-PLA2R antibody were positive, and 28.57% (4/14) of those with the IgG4 subtype deposition were positive. Among the UAMN patients, 76.00% (19/25) of those with the glomerular PLA2R expression were positive, 64.00% (16/25) of those with the serum anti-PLA2R antibody were positive, and 20.00% (5/25) of those with the IgG4 subtype deposition were positive. The positive rates of the three groups were statistically significant (P < 0.05). The sensitivity and specificity of the serum anti-PLA2R antibody, the IgG4 subtype deposition, and the glomerular PLA2R positive expressions in diagnosing IMN were 72.00%, 92.00%, 88.00%, 82.05%, 84.62%, and 89.74%, respectively.

Combined detection of serum anti-PLA2R antibodies, glomerular PLA2R, and IgG subtype deposition

88.00% (22/25) of the IMN patients with a positive glomerular PLA2R expression were positive for IgG4. None of the SMN and UAMN patients were positive for glomerular PLA2R expression with a moderate positive IgG4 expression. In the IMN group, the total positive rate was 72.00%, and the total negative rate was 8.00%. There were no patients showing a total positive in the SMN and UAMN groups (**Table 3**). The ROC curve demonstrated that the AUC of the single serum anti-PLA2R antibodies, glomerular PLA2R, and IgG subtype deposition in diagnosing IMN were 0.860, 0.930, and 0.920, respectively. Combined detection increased the diagnostic sensitivity of IMN (**Table 3**, **Figure 1**).

Discussion

Membranous nephropathy is a common cause of adult nephrotic syndrome. IMN is an immune disease. The important target antigen of adult IMN is M-type PLA2R. SMN may be related to foreign antigens [13, 14]. The PLA2R antibody can reflect the activity of IMN, and the anti-PLA2R antibody negative rate is 100% in lupus nephritis [16, 17]. UAMN is a secondary factor of nephropathy caused by viral infections but excluding non-steroidal anti-inflammatory drugs, the hepatitis B virus, or systemic lupus erythematosus. There is no basis for the diagnosis of secondary membranous nephropathy in clinical practice [18, 19]. At present, there are many studies focused on the role of glomerular PLA2R, serum anti-PLA2R antibody expression, and IgG4 deposition in membranous nephropathy [16-18]. This cohort included patients with different types of membranous nephropathy (IMN, SMN, UAMN), and tested glomerular PLA2R expression and IgG4 deposition by IIF, determined serum anti-PLA2R antibody by IFA, and explored their significances in the differential diagnosis of membranous nephropathy. The ROC curve analysis was performed in the diagnosis to provide a reference for the diagnosis and an evaluation of the clinical membranous nephropathy.

This study showed that the positive rates of IgA, IgM, and C1q fluorescence were higher in the UAMN group than in the IMN group. The positive rate of the IgG1 subtype was higher, but the

Table 3. Combined detection of serum anti-PLA2R antibodies, glomerular PLA2R, and IgG subtype deposition

Indicator	AUC	95% CI	Sensitivity/%	Specificity/%
Renal PLA2R+	0.930	0.897~0.990	92.00	84.62
Serum anti-PLA2R antibody+	0.860	0.750~0.970	72.00	82.05
IgG4 type+	0.920	0.864~0.987	88.00	89.74
Serum anti-PLA2R antibody+ and renal PLA2R+	0.940	0.864~0.997	96.64	83.85
Renal PLA2R+ and IgG4 type+	0.960	0.897~0.998	99.04	86.15
Serum anti-PLA2R antibody+ and IgG4 type+	0.952	0.898~0.994	97.76	89.43
Serum anti-PLA2R antibody+, renal PLA2R+, and IgG4 type+	0.965	0.899~0.999	99.73	92.49

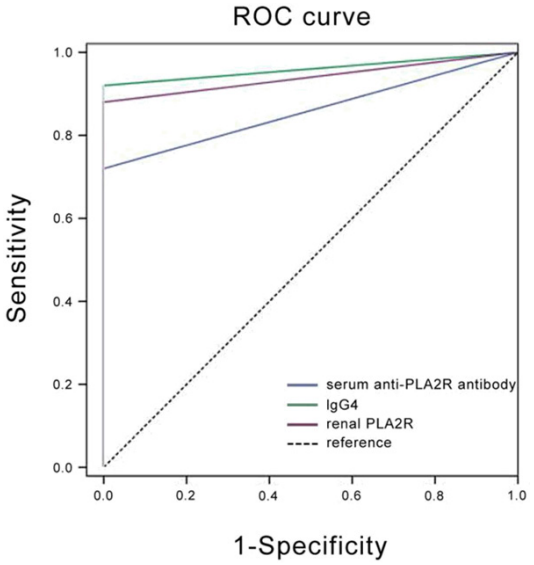

Figure 1. Comparison of serum anti-PLA2R antibodies, glomerular PLA2R, and IgG subtype deposition in diagnosing IMN.

positive rate in the IgG3 subtype was lower in the UAMN group. The positive rate of the IgG4 subtype was higher, whereas the positive rates of IgG2 and IgG3 were lower in the IMN group. The positive rate of the IgG1 subtype was higher, while the positive rate of the IgG4 subtype was lower in the SMN group. The positive rates of the IgG1, IgG2, and IgG3 subtypes were higher, and the positive rate of the IgG4 subtype was lower in the SMN group than it was in the IMN group, suggesting that the IgG4 subtype can be used to identify IMN, SMN, and UAMN. The positive rates of the serum anti-PLA2R antibodies, glomerular PLA2R, and IgG subtype deposition were statistically significant among the IMN, SMN, and UAMN groups. The positive rate of glomerular PLA2R expression was high in the IMN patients, as other similar studies found [20, 21]. 88.00% (22/25) of the IMN patients with positive glomerular PLA2R expression were positive with IgG4. None of the SMN and UAMN patients were positive for glomerular PLA2R expression with moderate positive IgG4 expression, indicating that serum anti-PLA2R antibody, glomerular PLA2R and IgG subtype deposition can be used as important indicators for the differential diagnosis of membranous nephropathy. The ROC curve demonstrated that the combined detection of serum anti-PLA2R antibody, glomerular PLA2R and IgG subtype deposition increased the diagnostic sensitivity of IMN. The diagnostic efficacy of glomerular PLA2R expression is superior to IgG subtype deposition and serum anti-PLA2R antibodies, which contradicts other findings [15-17]. SMN is mainly systemic lupus erythematosus and hepatitis B virus-associated membranous nephropathy. The positive rate of serum anti-PLA2R antibodies in SMN patients varies with secondary causes. It was revealed that the serum anti-PLA2R antibody positive rate was about 20% in patients with secondary membranous nephropathy, such as malignant tumors, HBV, and blood system diseases [19, 20]. We were limited by the sample size in this study, so the influence of the secondary factors on the positive rate still needs further exploration. Currently, the diagnostic value of serum anti-PLA2R antibodies, glomerular PLA2R and IgG subtype deposition cannot be used diagnostically in the various types of membranous nephropathy.

Conclusion

The positive rate of glomerular PLA2R expression in IMN was high, which was mainly IgG4 subtype deposition. The UAMN kidney tissue mainly had the IgG1 subtype deposition. Serum anti-PLA2R antibodies, glomerular PLA2R, and

IgG subtype positive deposition play an important role in differentiating diagnosis and evaluating the efficacy of membranous nephropathy.

Disclosure of conflict of interest

None.

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