Methods Article

Phospholipase A2 Receptor Antibody IgG4 Subclass Improves Sensitivity and Specificity in the Diagnosis of Idiopathic Membranous Nephropathy

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Keywords

Phospholipase A2 receptor antibody · IgG4 · Idiopathic membranous nephropathy · Membranous nephropathy · Time-resolved fluoroimmunoassay

Abstract

Aims: The aim of this study was to develop a new method for detecting anti-phospholipase A2 receptor-IgG4 to improve the sensitivity and specificity in the diagnosis of idiopathic membranous nephropathy (IMN). **Methods:** A highly sensitive quantitative assay was developed for the detection of serum anti-phospholipase A2 receptor-IgG4 with europium chelation by time-resolved fluoroimmunoassay (TRFIA), and a mouse anti-human IgG4 tracer was prepared using europium chelation for detection. The specificity and sensitivity of anti-phospholipase A2 receptor-IgG4 in the diagnosis of IMN were further assessed in patients with different kidney diseases. **Results:** The detection limit of anti-PLA2R-IgG4 was 0.69 ng/mL. The measurement range of anti-PLA2R-IgG4 TRFIA was 0.69–2,500 ng/mL. Mean serum anti-PLA2R-IgG4 was 21.27 ± 15.15 ng/mL in 45 healthy volunteers, 31.08 ± 18.17 ng/mL in 29 IgA nephropathy patients, 49.10 ± 34.32 ng/mL in 8 lupus nephropathy patients, and $10,324.11 \pm 17,030.40$ ng/mL in 30 IMN patients. The anti-PLA2R-IgG4 cutoff concentration was > 161.2

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ng/mL with the sensitivity of 90.0% and specificity of 100% in the diagnosis of IMN. However, the cutoff for other kidney diseases was lower than 161.2 ng/mL. **Conclusion:** The serum anti-phospholipase A2 receptor IgG4 detected with the method developed in this study has higher sensitivity and higher specificity than total IgG in the diagnosis of IMN.

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Introduction

In adults, membranous nephropathy (MN) is the most common cause of nephrotic syndrome. MN mostly affects people aged 40–50 years, with a male to female ratio of 2:1. Whereas spontaneous remission occurs in one-third of patients, 40% of patients will develop renal failure [1]. In 80% of MN patients, the disease is characterized as idiopathic membranous nephropathy (IMN); in the remaining 20% of patients, it is secondary to infectious diseases, autoimmune diseases, and tumors [2, 3]. Currently, the diagnosis of MN essentially relies on the clinical symptoms and pathological findings after renal biopsy. While the pathological findings are the gold standard in the diagnosis of MN, they have several disadvantages such as bleeding and secondary infection. Since MN is a chronic disease, renal biopsy is not considered a good choice for the dynamic monitoring of therapeutic efficacy due to its invasiveness, risk of bleeding, secondary infection, and cost. Thus, it is imperative to identify a sensitive and specific serum biomarker for the MN diagnosis.

In 2009, Beck et al. [4] found M-type phospholipase A2 receptor (PLA2R) IgG in the sera of 70% of IMN patients and proposed that PLA2R IgG could serve as a specific biomarker for IMN, which is consistent with that in immune complexes in IMN patients [5–7]. Anti-PLA2R is a promising serum biomarker for IMN, even though the positive rate of PLA2R IgG varies between 52 and 81% because of the interference of secondary MN. Considering these issues, more sensitive and specific methods are required, and the method based on IgG subclass assay could be a good candidate. Anti-PLA2R IgG4 antibodies are found to be predominant IgG subclass in MN [4–6]. Moreover, in secondary MN such as lupus MN, all four subclasses of IgG are expressed [5, 6], and in malignancy-associated MN, IgG1 and IgG2 are the predominant ones [8]. Thus, anti-PLA2R subclass IgG4 is better in distinguishing IMN from secondary MN than total IgG, and may yield improved specificity. However, more sensitive immunoassay should be developed due to the trace amount of IgG4 in the serum.

In this study, an ultrasensitive quantitative assay was developed for the detection of anti-PLA2R-IgG4 using time-resolved fluoroimmunoassay (TRFIA). This novel assay was further evaluated in the patients with IMN, SMN, or IgA nephropathy serum to confirm its clinical value.

Materials and Methods

Reagents and Instrumentation

Mouse anti-human IgG4 antibodies were obtained from Hytest (Finland). The human IgG4 quantitative diagnostic kit was purchased from Siemens Healthcare Diagnostics (Germany). A europium labeling kit (1244-302), including N1-(p-isothiocyanatobenzyl)-diethylenetriamine-N1,N2,N3,N4-tetraacetic acid (DTTA), was purchased from Perkin Elmer (USA). Diethylenetriaminepentaacetate (DTPA), bovine serum albumin (BSA), Tris Base, and

Triton X-100 were purchased from Sigma (USA). Sephadex-G50 columns were purchased from Pharmacia (UK). Centricon YM-50 ultrafiltration tube was purchased from Millipore (USA). The 96-well polystyrene microtiter plates were obtained from Nunc International (Denmark). Pure water was produced with Barnstead equipment. All other reagents were of analytical grade and obtained from domestic manufacturers.

A DU-650 spectrometer was purchased from Beckman (Germany). An AutoDELFIA1235 was obtained from Perkin Elmer (USA).

Blood Samples

Serum samples were collected from 45 healthy volunteers in the Affiliated Jiangyuan Hospital of Jiangsu Institute of Nuclear Medicine. Subjects without a history of any nephropathy, gastroduodenal disorder, or liver disease were recruited into this study. Serum samples from 77 patients were collected in the Affiliated Wuxi People's Hospital of Nanjing Medical University, China, after renal biopsy and subsequent pathological examination. There were 30 patients with IMN, 29 with IgA nephropathy, and 18 with secondary MN (lupus nephropathy: $n = 8$; hepatitis B-associated glomerulonephritis: $n = 4$; diabetic nephropathy: $n = 3$; Sjögren nephrotic syndrome: $n = 1$; purpura nephritis: $n = 2$). Serum creatinine, albumin, and blood urea nitrogen were measured, and the estimated glomerular filtration rate (eGFR) was calculated according to the CKI-EPI Creatinine Equation.

Establishment of Anti-PLA2R-IgG4 TRFIA

Preparation of Solid-Phase Antigen

Recombinant PLA2R was prepared and coated on 96-microtiter plates as previously reported [9].

Preparation of the Eu³⁺-Mouse Anti-Human IgG4 Antibody

Mouse anti-human IgG4 antibody labeling was performed using the Eu³⁺ labeling kit according to the manufacturer's instructions. After purification on the Sephadex-G50 column, aliquots of the labeled antibody were stored at -20°C after dilution with elution buffer containing 0.2% BSA as a stabilizer.

Preparation of Human Anti-PLA2R-IgG4 Standard

Currently, there is no standard quantitative method for human anti-PLA2R-IgG4. Thus, in order to make each experiment comparable, a laboratory standard was prepared. First, human anti-PLA2R-IgG was purified: PLA2R coupled to CNBr-activated Sepharose 4B was extracted by affinity column chromatography. On the basis of preliminary experiment, serum with a high concentration of anti-PLA2R-IgG4 was processed for the separation of anti-PLA2R-IgG by chromatography. The eluent was ultrafiltered using a YM-50 ultrafiltration tube (Millipore, USA) and then transferred to the buffer (50 mmol/L Tris-HCl, pH 7.8, containing 8 mmol/L NaCl). The anti-PLA2R-IgG4 was detected using the Siemens Human IgG4 quantitative diagnostic kit. This solution was used for the preparation of human anti-PLA2R-IgG4 standards via dilution to different concentrations (4, 20, 100, 500, and 2,500 ng/mL) using assay buffer; the assay buffer was set as 0 ng/mL.

Anti-PLA2R-IgG4 Detection Procedure

Anti-PLA2R-IgG4 standard (100 μL) or a sample (1:20 in assay buffer) was added to each well coated with PLA2R followed by incubation at 25°C for 1 h with agitation. Each sample or standard was detected in duplicate. After three rinses, Eu³⁺-mouse anti-human IgG4 antibody diluted in assay buffer was added (100 μL /well), and the plate was incubated at 25°C for 1 h with agitation. The plate was rinsed 6 times, followed by the addition of

Fig. 1. Specific and nonspecific binding (NSB) curves of Eu^{3+} -mouse anti-human IgG4 at different concentrations. CPS, counts per second.

enhancement solution (200 μL /well; 15 μmol 2-naphthoyltrifluoroacetone, 50 μmol tri-*n*-octylphosphine oxide, and 0.1% Triton X-100, pH 3.2). The plate was agitated for 5 min before measurement. All the procedures were auto-controlled by autoDELFI₁₂₃₅ with a software designed in our laboratory. The calibration curve and calculation of the concentrations in the unknown samples were performed automatically using the Multicalc software program, where a spline algorithm on logarithmically transformed data was employed.

Assessment of the Anti-PLA2R-IgG4 TRFIA Method

Sensitivity

Mean and standard deviation (SD) were calculated for count values at zero concentration point based on the standard curves of 10 groups. The sensitivity of each assay was calculated as the concentration corresponding to the mean $\pm$ 2 SD determined via the standard curve.

Precision

The intra- and inter-batch coefficients of variation were determined from measurements obtained from quality-controlled samples at three different concentrations.

Recovery Rate

After the nonspecific binding was determined for the TRFIA, anti-PLA2R-IgG4 at three standard concentrations was added to the samples. The ratio of each measured value to its theoretical value was then calculated.

Statistical Analysis

Each sample was tested in duplicate ($n = 2$), and data were presented as mean ($\pm$ SD) where appropriate. The *t* test was used for the comparison between groups. A *p* value of <0.01 was considered statistically significant. Statistical analysis was performed using SPSS version 19 (IBM, USA).

Table 1. Clinical features of patients in five groups

Group	n	Serum albumin, g/L	Blood nitrogen, mmol/L	Serum creatinine, μ mol/L	eGFR, mL/min/1.73 m ²	Anti-PLA2R-IgG, mg/L	Anti-PLA2R-IgG4, μ g/L	Positive rates of anti-PLA2R-IgG	Positive rates of anti-PLA2R-IgG4
Negative controls	45	44.1 $\pm$ 3.7	4.6 $\pm$ 1.1	74.2 $\pm$ 12.2	104.4 $\pm$ 14.7	0.53 $\pm$ 0.18	21.27 $\pm$ 15.15	0	0
IMN	30	23.4 $\pm$ 8.0*	5.2 $\pm$ 1.7	75.4 $\pm$ 22.9	95.0 $\pm$ 21.3 [#]	10.2 $\pm$ 12.56*	10,324.11 $\pm$ 17,030.40*	83.3%	90.0%
IgA nephropathy	29	35.0 $\pm$ 7.9*	7.4 $\pm$ 5.4 [#]	154.8 $\pm$ 192.4 [#]	71.4 $\pm$ 34.0	0.60 $\pm$ 0.34	31.08 $\pm$ 18.17 [#]	31.0%	0
Lupus nephropathy	8	26.6 $\pm$ 14.0*	5.9 $\pm$ 3.1	70.7 $\pm$ 25.8	96.1 $\pm$ 23.1	0.99 $\pm$ 0.64	49.10 $\pm$ 34.32	50%	0
Other kidney disease	10	30.9 $\pm$ 6.0*	7.0 $\pm$ 2.7 [#]	110.1 $\pm$ 59.9	73.1 $\pm$ 30.7	0.78 $\pm$ 0.44	24.39 $\pm$ 11.17	0	0

Data are mean $\pm$ SD. eGFR was calculated using the CKD-EPI formula. * $p < 0.01$, [#] $p < 0.05$.

Fig. 2. Calibration curve for the anti-PLA2R-IgG4 TRFIA. CPS, counts per second.

Results

Optimal Dilution of Labelled Antibody

To obtain high fluorescence with low background, the Eu³⁺-mouse anti-human IgG4 was diluted at 1:100, 1:200, 1:400, 1:800, and 1:1,600; the optimal dilution with low background and high combination rate was found to be 1:800 (Fig. 1).

Assay Evaluation

The calibration graph of the anti-PLA2R-IgG4 TRFIA is shown in Figure 2. The limit of detection, defined by the concentration of anti-PLA2R-IgG4 corresponding to the fluorescence of zero calibrators plus 3 SDs, was 0.69 ng/mL; the measurement range was 0.69–2,500 ng/mL; and the intra- and inter-assay coefficients of variation were 4.7 and 7.3%, respectively. The assay recovery rate was 98.57%.

Clinical Characteristics of IgG4 of Anti-PLA2R

Serum was collected from different subjects: healthy volunteers, IMN patients, IgA nephropathy patients, lupus nephropathy patients, and patients with other kidney diseases. Clinical characteristics of these subjects are shown in Table 1. The concentrations of serum anti-PLA2R-IgG4 are shown in Figure 3. Results showed anti-PLA2R-IgG and -IgG4 had signif-

Fig. 3. Serum anti-PLA2R-IgG4 concentrations in different groups.

Fig. 4. Receiver operating characteristic curve of anti-PLA2R-IgG4.

icant differences in the diagnosis of IMN as compared to traditional markers such as serum albumin, urea nitrogen, and eGFR ($p < 0.01$).

Receiver operating characteristic (ROC) curve analysis was performed based on the serum anti-PLA2R-IgG4 concentrations of IMN and non-IMN patients (Fig. 4). The area under ROC was 0.956, greater than 0.500, indicating that it was useful in the clinical diagnosis and differentiation of IMN from non-IMN. According to the ROC curve, the concentration of serum anti-PLA2R-IgG4 corresponding to the first specificity of 100% was 161.2 ng/mL. The serum anti-PLA2R-IgG4 and anti-PLA2R-IgG concentrations and positive rates in patients with different kidney diseases are shown in Table 1. The mean serum anti-PLA2R-IgG4 in 45 healthy volunteers was 21.27 ± 15.15 ng/mL. When the cutoff of anti-PLA2R-IgG4 concentration was 161.2 ng/mL, the positive rate was 90.0% for IMN. However, patients with IgA nephropathy, lupus nephropathy, hepatitis B-associated glomerulonephritis, diabetic nephropathy, arteriolar nephrosclerosis, and purpura nephritis were negative for anti-PLA2R-IgG4 with the specificity of 100%. It indicates that anti-PLA2R-IgG4 has higher sensitivity and higher specificity than anti-PLA2R-IgG in the diagnosis of IMN.

Table 2. Comparison of serum anti-PLA2R-IgG4 with anti-PLA2R-IgG for IMN

Anti-PLA2R-IgG4	Anti-PLA2R-IgG	
	+	–
+	23	4
–	2	1

With regard to sensitivity, serum anti-PLA2R-IgG4 showed better performance than serum anti-PLA2R-IgG in the diagnosis of IMN (Table 2). Serum anti-PLA2R-IgG4 not only has a higher positive rate (27/30 vs. 25/30) but also shows differential data (4 vs. 2) as compared to serum anti-PLA2R-IgG.

Discussion

Currently, the diagnosis of MN essentially relies on the clinical symptoms and pathological findings after renal biopsy. Thus, there is an urgent need for the identification of a sensitive and specific serum biomarker for MN diagnosis.

Anti-PLA2R-IgG is a promising serum biomarker for IMN. PLA2R is heavily glycosylated and, after treatment with peptide N-glycosidase F, undergoes a shift in its mobility to approximately 145 kDa. The epitope-dependent PLA2R antibody is detectable in most of IMN patients, suggesting PLA2R is a major antigen in case of IMN [10]. It has been suggested that the serum PLA2R antibody can be used for the diagnosis of IMN and monitoring of clinical response to treatments [4, 11, 12]. Western blotting has been applied in the detection of PLA2R antibody, and in IMN patients, the positive rate of PLA2R antibody can exceed 70% [4], but it is still detectable in some secondary MN, such as lupus nephritis V type, hepatitis B virus (HBV)-related MN, and tumor-associated MN [13]. The unsatisfactory sensitivity and specificity might be partially attributed to the limited sensitivity of Western blotting per se because it is used mainly for qualitative or semiquantitative analysis. In recent years, indirect immunofluorescence cell-based assays and ELISAs based on recombinant human PLA2R have been developed for the precise detection of anti-PLA2R antibodies [14–20]. Hoxha represents an indirect immunofluorescence test that enables the easy and specific detection of serum anti-PLA2R antibodies. By using the indirect immunofluorescence test, anti-PLA2R antibodies were found in 52% of patients with biopsy-proven IMN, whereas they were undetectable in those with secondary MN [19]. Additionally, other studies reported no PLA2R antibody was undetectable in IMN patients, although PLA2R antigen was found in the immune deposits [21]. In summary, based on detection of serum anti-PLA2R antibodies, the positive rate varies inconsistently from 52% to 81% in IMN patients, and this range is larger in secondary MN patients. This causes poor sensitivity and specificity in the diagnosis of IMN.

Thus, in the present study, the method used for the detection of serum anti-PLA2R antibodies was improved. Initially, TRFIA was employed in this study. TRFIA is a novel nonisotopic labeling technology, and offers wide detection range, high sensitivity (10^{-18} mol/L), and less susceptibility to matrix interference. TRFIA has been well accepted as an ideal quantitative detection method in the analyses of clinical samples, and it can yield a better sensitivity for anti-PLA2R antibodies. Secondly, among four subclasses IgG4 anti-PLA2R is the major form in IMN, while other nephropathies are featured by IgG1-IgG3. Thus, anti-PLA2R IgG4 subclass will enhance the specificity. In the present study, TRFIA was used to detect the

anti-PLA2R IgG4 subclass. Using this technique with improved sensitivity and specificity, IMN can be differentiated from secondary MN based on the threshold. Additionally, the anti-PLA2R antibody concentration varied with therapy consistently [22], and thus a quantitative method is critical for the monitoring of IMN treatment. Therefore, anti-PLA2R IgG4 was prepared and identified by affinity purification and then used as the standard in this quantitative TRFIA.

To the best of our knowledge, this is the first time that serum anti-PLA2R-IgG4 TRFIA, as a highly sensitive and specific immunofluorometric method, is developed as a diagnostic assay using the dissociation-enhancement immunofluorometric principle. The TRFIA method yielded a higher sensitivity of 0.69 ng/mL. Our results showed the anti-PLA2R-IgG4 assay enhanced the detection rate up to 90% in IMN, higher than that of anti-PLA2R-IgG detected by TRFIA or other available methods [13, 23, 24]. Furthermore, the sensitivity and specificity were compared between anti-PLA2R-IgG and IgG4 subclass in the same patients. Results indicated that the more sensitive TRFIA method yielded a significantly better positive rate for serum anti-PLA2R-IgG in IMN, whereas it also amplified the noise, resulting in the positive rate of 31–50% in the secondary MN, such as IgA nephropathy and lupus nephropathy, which may confound the diagnosis of IMN. Considering the specificity of IgG4 subclass for IMN rather than secondary MN, the concentration of anti-PLA2R-IgG4 showed only a slight increase in IgA nephropathy, lupus nephropathy, and other kidney diseases. Importantly, the slight increase had no effect on the differential diagnosis of IMN, and the specificity could even reach 100% after adjusting the cutoff value to 161.2 ng/mL.

Moreover, some patients who were negative for anti-PLA2R-IgG were found to be positive for anti-PLA2R-IgG4. It indicates that serum anti-PLA2R-IgG4 may substantially increase the diagnostic accuracy of IMN when anti-PLA2R-IgG remains at a low level. Depending on the ultrasensitive method, this small increase in serum anti-PLA2R-IgG4 may be detected.

Conclusion

The anti-PLA2R-IgG4 TRFIA is more useful for the diagnosis, classification, and monitoring of IMN. Anti-PLA2R-IgG4 is promising to replace IgG as a new biomarker of IMN.

Statement of Ethics

The study was approved by the Institutional Research Ethics Committee of Jiangsu Institute of Nuclear Medicine (YYLL201502) and the Institutional Review Board of the Affiliated Wuxi People's Hospital of Nanjing Medical University (KYL2016001). All enrolled subjects provided their written informed consent for study participation, and all methods were performed in accordance with the relevant guidelines and regulations. The study was carried out in accordance to the ethical conduct of the World Medical Association Declaration of Helsinki.

Disclosure Statement

The authors declare that there are no conflicts of interest.

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Author Contributions

B.H., Y.Z., L.W., and W.X. conducted the detections and drafted the manuscript. J.Z. and Q.Z. collected data. H.S. and Z.H. designed the study, purchased reagents, and revised the manuscript.

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