Original Research

Anti-phospholipase A2 Receptor Antibody Measurement in Patients with Idiopathic Membranous Nephropathy Diagnosed by Renal Biopsy

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ABSTRACT

Objective: Our study is a cross-sectional study that aims to evaluate the presence and levels of anti-phospholipase A2 receptor (PLA2R) antibodies in healthy volunteers and idiopathic membranous nephropathy (IMN) patients and to assess the relationship between these levels and clinical parameters.

Methods: Serum anti-PLA2R antibody levels, complete blood count, urea, creatinine (Kre), total protein, albumin, low-density lipoprotein (LDL)-cholesterol, triglycerides (TG), high-density lipoprotein (HDL)-cholesterol, total cholesterol, C-reactive protein (crp), sedimentation, proteinuria were measured from 71 IMN patients and 48 healthy volunteers.

Results: Of the values compared between the two groups, the urea, creatinine, and modified diet renal disease (MDRD) were similar, total protein, albumin, LDL-cholesterol, TG, total cholesterol, HDL-cholesterol, and complete urinalysis protein values were statistically significantly high in the patient group, as expected in nephrotic syndrome (p<0.01). The anti-PLA2Rantibody levels measured using enzyme-linked immunosorbent assay (ELISA) in patient and control groups were found to be negative. The anti-PLA2R level was found to be 0.104 (0.093-0.129) ng/ml in the IMN group, while it was 0.141 (0.117-0.177) ng/ml in the control group (P=0,001). Although the P value was significant, the anti-PLA2R antibody level was found to be high in the control group and was outside the reference range of the kit.

Conclusion: There is a need to conduct more sensitive studies with a higher number of patients in order to distinguish between primary and secondary nature and to investigate the presence of anti-PLA2R in IMNpatients, which constitute the majority of nephrotic syndromes in adults. Antibody titer levels were observed to be low and it was revealed that the measurement range of the antibody kit used in the study should be more sensitive.

Keywords: anti-phospholipase A2 receptor (anti-PLA2R) antibody, idiopathicmembranous nephropathy (IMN), proteinuria

INTRODUCTION

Membranous nephropathy is the most common cause of nephrotic syndrome in adults [1]. There are primary or idiopathic and secondary forms. Secondary membranous nephropathy may develop due to systemic autoimmune diseases, chronic hepatitis B, non-steroidal anti-inflammatory drugs, and malignant tumors [2]. Idiopathic membranous nephropathy (IMN) is an antibody-associated autoimmune glomerular disease characterized by subepithelial immune deposition. The target antigen for IMN disease has been investigated in a wide range clinically and experimentally. Of these, podocyte antigenic targets defined as megalin in Heyman's nephritis have been shown to be responsible for in situ subepithelial immune complexes [3]. However, this antigen was not detected in human kidney podocytes. In 2002, neutral endopeptidase was found to be the antigen responsible for alloimmune antenatal membranous nephropathy [4]. In 2009, M-type phospholipase A2 (PLA2R) receptor, a membrane glycoprotein located on podocytes, was found to be the target

antigen in IMN patients [5]. Circulating antibodies against this antigen combine with PLA2R antigen on podocytes in the glomeruli, causing in situ immune complex formation and secondary complement activation. As a result of these, proteinuria begins and kidney damage occurs [6]. In some studies, serum anti-PLA2R antibody levels were found to be associated with diagnosis, prognosis and treatment response in patients with IMN. While the antibody was positive in IMN patients, low titer was positive or negative in patients with secondary membranous nephropathy, low titer positivity and low disease activity were correlated in IMN patients. Antibody negativity was also detected in patients who developed membranous nephropathy due to causes such as malignancy and heavy metals [7-9].

We aimed to evaluate the role of anti-PLA2R antibody level in the diagnosis and treatment of IMN patients diagnosed with follow-up biopsy in our nephrology clinic and secondary causes excluded.

METHODS

Our study included 71 IMN patients followed up in our nephrology clinic between January 2015-November 2017 and 48 healthy control groups. Healthy volunteers group consists of healthy individuals between the ages of 18-65 who have no previous history of disease, especially renal disease and drug use. Ethics committee approval for our study was obtained by Gaziantep University Clinical Research Ethics Committee with the date of 22.05.2017 and decision number 2017/200. Anti-nuclear antibody (ANA), perinuclear anti-neutrophil cytoplasmic antibody (p-anca), anti-neutrophil cytoplasmic antibody (c-anca), anti-double stranded deoxy ribonucleic acid (ds dna), complement 3 and 4, hepatitis B, C and human immunodeficiency virus (HIV) in terms of excluding secondary mebranous nephropathy of patients whose renal biopsies are compatible with membranous nephropathy that tests were performed and found to be negative. The study was divided into two groups as patient and control group. In addition to anti-PLA2R antibody levels, complete blood count, 24-hour urine micro total proteinuria (MTP) level, total protein, creatinine, blood urea nitrogen (BUN), albumin, LDL-cholesterol, TG, total cholesterol, HDL-cholesterol, crp, sedimentation, urea, creatinine, MDRD levels were examined. All patients who received or did not receive immunosuppressive therapy were included, but those who had undergone a change in immunosuppressive therapy within the last 1 month were

excluded. All parameters except anti-PLA2R antibody levels were obtained from the hospital registration system. Patients between the ages of 18-65, glomerular filtration rate (GFR) >60 ml/min, no more than 10% change in GFR values in the last 3 months, using angiotensin receptor inhibitors/blockers were included in the study.

Routine biochemical parameters are determined on a Roche/Hitachi Modular (Tokyo, Japan) analyzer system using solutions, controls and calibrators from the same manufacturer (Roche Diagnostics, Mannheim, Germany). Complete blood count parameters are measured in Beckman Coulter (CA, USA) complete blood count device using solutions, controls and calibrators from the same manufacturer. Creatinine is studied according to the compensated Jaffe method. The creatinine method was standardized by isotope dilution-mass spectrometry (ID MS). Albumin is measured colorimetrically by the bromocresol green method. Cholesterol is measured enzymatic colorimetrically by the cholesterol oxidase method. HDL; It is measured enzymatic colorimetrically using a polyethylene glycol modified enzyme. LDL; selective detergent, cholesterol esterase/cholesterol oxidase and homogenous enzymatic colorimetric method. From the 24-hour urine MTP of the participants; the amount of protein was evaluated in the Backmancoulter Unicel DxC800 device at University Medical Faculty Central Laboratory. GFR was calculated with the 4-parameter MDRD Formula [10].

GFR = 186 X ([Scr] -1.154) X ([Age] -0.203) X (0.742 if female) X (1.21 if black)

The level of anti-PLA2R antibody (mybiousource, USA) in serum samples was measured using the ELISA method. This kit measures human anti-PLA2R antibody level by quantitative double sandwich enzyme immunoassay method in 6 hours. Antigen and solid phase carriers were connected to form the immobilized antibodies. Uncombined antigens, foreign substances were washed off. Immobilized antigens for contact rectification participated in the test. After a while, it was expected that the antibodies and antigens in the carriers would combine with antibody complexes. Uncombined antigens and foreign substances were washed off again. Antigens were added to combine with antibodies on immune complexes. The uncombined antigens were thoroughly washed. The amount of enzyme on the transporter has now been positively assessed by the amount of the tested substance in the samples. Anti-PLA2R antibody standards (20.0; 10.0; 5.0; 2.5; 1.25; 0.625; 0.312 ng/ml) and the samples were sandwiched between immobilized antibody and PLA2R antibody-specific antibody. All non-binding materials were removed by washing again and the peroxidase enzyme substrate was added. The reaction was stopped by leaving it in the dark to form a color and adding an acid solution. The color intensity was read spectrophotometrically at 450 nm with an ELISA reader (Biotek Instruments, USA). The anti-PLA2R antibody level of the patients and controls was calculated using a standard graph.

Statistical Analysis

The suitability of the numerical data to the normal distribution was tested with the Shapiro Wilk test. The independent samples t-test and Mann–Whitney U-test were used to compare two

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Main points

- Idiopathic membranous nephropathy is a common cause of proteinuria in adults

- The primary-secondary distinction of membranous nephropathy is of vital importance in terms of treatment and prognosis

- Renal biopsy couldn't make this distinction

- Numerous studies have been conducted on the anti-PLA2R antibody in terms of a marker that can make this distinction

independent groups of variables. Relationships between non-normally distributed numerical variables were tested with the Spearman correlation coefficient. The diagnostic test effectiveness for numerical variables was revealed with the help of the ROC curve. SPSS 22.0 package program was used in the analyses. P<0.05 was considered significant.

RESULTS

In our study, the data were evaluated between the patient group and the healthy group. There was no statistically significant difference between the two groups in terms of demographic and laboratory findings, age, gender, urea, creatinine, GFR, HDL-cholesterol, hemoglobin, sodium, potassium, aspartate aminotransferase, alanine aminotransferase values. While total protein and albumin values were found to be lower in the patient group, total cholesterol, TG, LDL-cholesterol, 24-hour urine protein, and MTP values were found to be statistically significantly higher than the control group. While protein positivity was observed in the IMN group in the complete urine test, it was found to be negative in the healthy group. In addition, white blood cell countand neutrophil count, which are among the parameters of the complete blood count, were found to be statistically high in the patient group, while there was no difference in hemoglobin, platelet, mean corpuscular volume (MCV) (Table 1).

Table 1. Demographic characteristics and laboratory values of the IMN and control groups

	IMN(n=71)	Control (48)	P
Age†	38±13	36±11	P=0.867
Gender, female/male (n)	41/30	27/21	P=0.745
Anti-PLA2R ab(ng/ml)#	0.104 (0.093-0.129)	0.141(0.117-0.177)	P=0.001*
Ure(mg/dl)#	24(18-42)	22.5(19-25.5)	P=0.789
Cre(mg/dl)#	0.75(0.60-1.10)	0.69(0.62-6.84)	P=0.216
MDRD(ml/min/1.73 m2)#	109(75-123)	117.5(101.5-130.5)	P=0.674
Total Protein(g/dl)#	7(6.5-7.5)	7.6(7.4-7.8)	P=0.001*
Albumin(g/dl)#	4.1(3.8-4.3)	4.55(4.3-4.8)	P=0.001*
LDL(mg/dl)#	129(110-170)	97(75-117)	P=0.001*
TG(mg/dl)#	195(119-277)	71.5(57-111)	P=0.001*
Total cholesterol(mg/dl)#	221(178-270)	165(65.5-178.5)	P=0.005
HDL-C(mg/dl)#	48.5(42.5-64.5)	49(35-66)	P=0.512
Leucosyte(mcl)#	9430(7590-11810)	4420(3265-5280)	P=0.001*
Neutrophil(mcl)#	5630(4180-7370)	4420(3265-5280)	P=0.001*
HGB(g/dl)#	14.1(12.4-15.5)	14(13.35-15.1)	P=0.697
Urine-protein#	1(0-3)	0(0-0)	P=0.001*
Urine-erithyrocyte#	0(0-1)	0(0-0)	P=0.001*
MTP(mg/day)#	462(155-1105)	96(74-115)	P=0.001*

* p<0.05; †Mean±.standart deviasion,# Median(minimum-maximum), **MDRD**: Modification of Diet in Renal Disease, **Cre:** creatinin, **TG:** triglyceride, **HGB**: hemoglobin, **LDL-C**: Low density lipoprotein-cholesterol, **HDL-C** : high density lipoprotein-cholesterol, **MTP:** micro total protein

Table 2. Correlation analysis results between the numerical variables

		Anti-PLA2R ab
Anti-PLA2R ab	r	1.000
	p	
Age	r	0.118
	p	0.325
MDRD	r	0.084
	p	0.487
Albumin	r	-0.044
	p	0.715
Proteinuria	r	-0.166
	p	0.292

r: Spearman rank correlation coefficient.

The anti-PLA2R level was found to be 0.104 (0.093-0.129) ng/ml in the IMN group, while it was 0.141 (0.117-0.177) ng/ml in the control group (P=0.001). Although the P value was significant, the anti-PLA2R antibody level was found to be high in the control group and was outside the reference range of the kit. Also, the correlation analysis is shown in Table 2.

Although the test was below the reference values and the determined values, and the control features of the finger test to the range of curve (ROC) extension seem to have high values, the highest antibody titer was 0.308 ng/ml when all the obtained values were examined, and the smallest value to which the test was both sensitive was 0.5. A clear evaluation cannot be made if it does not match the ng/ml value and is not between 1.56-100 ng/ml values, which is the reference range of the test. For these reasons, it has been accepted that these ROC movements were taken in such a way that they could not give clear accurate data.

DISCUSSION

The patient group in our study consists mainly of women and individuals under the age of 40. In the data of a study, IMN and anti-PLA2R antibody positivity were found to be more common in men aged 40-70 years [11]. The fact that our patients were under the age of 40 and the female gender was higher may have caused meaningless results. Although this is not the only factor in negative test results, it was found to be significant in terms of playing an active role.

The sensitivity of the ELISA kit used in anti-PLA2R antibody measurement is important in detecting the disease. In a study conducted by Dou et al., the low threshold value for the diagnosis of anti-PLA2R antibody levels increased the probability of detecting the disease (12). The high threshold value of the test we used in our study led to the thought that the test was effective in producing meaningless results.

Although a small number of patients were studied in a research, low levels of anti-PLA2R antibodies can show spontaneous remission in patients with a diagnosis of IMN. When these patients are followed without treatment, a complete cure can be achieved proteinuria level < 8 g/day and no clinical findings), and it showed that low antibody titer could be a biomarker that could be used to predict this condition [13]. The protein level in the 24-hour urine of the patients in the IMN group was 462 mg/day; low proteinuria may have played a role in negative anti-PLA2R antibody results.

Contrary to the studies in the literature, there was no statistically significant difference in our study when we compared the patients diagnosed with IMN and the control group in terms of anti-PLA2R levels. Anti-PLA2R antibody level was associated with disease activity. It has been found in some studies that during the active periods of the disease, high proteinuria levels and increased periods were correlated with antibody titers [14]. In our study, it was found to be negative in both the patient groups that we accepted in remission and the patient groups in which the disease progressed actively.

Although the total protein and albumin measurements in the IMN group were significantly lower than the healthy group, these values were consistent with the values in the normal healthy population. In a study by Hofstra et al., low anti-PLA2R antibody level was found to be associated with high serum albumin level and low proteinuria [15]. Anti-PLA2R antibody negativity in our patients was evaluated to be associated with high total protein and albumin levels.

When we look at the positivity rate of anti-PLA2R antibody levels in patients with IMN diagnosis and the studies comparing these antibody titers with values such as proteinuria and serum albumin values, we saw that the test measures as RU/ml and our measurements are ng/ml. However, when we looked at the literature, we could not find a formula for this antibody in terms of converting from ng/ml to RU/ml. With the kit we studied, antibody levels were found to be negative in both the patient group and the control group, below the reference range; it has emerged that antibody kits sensitive to smaller antigen levels are needed for the detection of the antibody level. For example, in a study conducted by Qin et al., anti-PLA2R antibodies were found to be positive at a rate of 69% at the time of first diagnosis in patients diagnosed with IMN, and the prevalence of autoantibodies was found to be lower in patients who went into remission after treatment (15.8%) compared to patients at the time of initial diagnosis. This study also shows that factors such as the time of first diagnosis and the state of being in remission after treatment affect the test positivity rate [16]. Although all of our patient group was outside the reference range of the test, 50 of our patients (which corresponds to 70% of our patient group) had a MTP level below 1 g/day. This shows that if most of our patients are accepted in the remission stage, it can be shown among the possible reasons for the test to be negative.

Nanogram/milliliter was given as the unit of measurement for the kit we used in our study. When we look at the kits used to measure this antibody detection, we see that some of them give the unit of measurement as microgram/milliliter. This shows that the sensitivity of the test has increased even more. In addition, the test measures with the double sandwich technique, and in this case, it can be interpreted that the test is better in terms of technique and precision. 71 patients diagnosed with IMN in our study were diagnosed with kidney biopsy, and at the same time, all patients were diagnosed by considering the negativity of autoimmune markers and hepatitis markers in order to exclude secondary causes. Although some studies say that the antibody level may be negative if the disease is in remission, when we look at the patients with IMN, we see that the antibody titers of the patients who are not in remission are also outside the reference range. The values read were also evaluated by different biochemists, and the same values were sent to the company and similar results were obtained.

Even if we consider 70% of the patients in our study to be in remission in terms of proteinuria level, even the other 30% of the patients can be evaluated in terms of the possibility of being negative when proteinuria regresses. In a study, it was observed that the level of anti-PLA2R antibody could regress with immunosuppressive treatment, or the antibody level may decrease and become negative after the proteinuria level

decreases over time. At the same time, if it is taken into account that the antibody becomes negative or its level decreases some time before proteinuria decreases, it may occur that it is possible for the antibody to be negative even in patients we accept as active [17]. Antibody levels were measured in all IMN patients, regardless of whether our patients were in remission or in the active period. In another study, remission of the disease was found to be associated with low or negative antibody levels [18]. For this reason, it was thought that the low proteinuria level of our patients in remission or close to remission may play an active role in the negative antibody level.

We determined our group consisting of healthy volunteers as the comparison group for anti-PLA2R antibody and aimed to determine the positivity rate and antibody levels in these individuals. However, as mentioned in the material and method section above, antibody positivity and antibody levels were measured in serum samples from the collected blood in accordance with the technique. As a result of the measurements, very low values were measured in nanogram/ml in all patient groups, but the determined values were outside the reference range of the test and a significant antibody titer could not be reached. Since the values found were below the reference range and optic density (od) values, the correct values could not be accepted for comparison and evaluation of these detected antibody levels. The PLA2R antibody values of the samples we used for the patient and control groups could not be read because they were outside the measurement ranges of the kit used. It has been reported in studies that anti-PLA2R antibody measurements made with the ELISA method may differ depending on whether the disease is in remission or under treatment at the time of diagnosis. It has been reported in studies that anti-PLA2R antibody measurements made with the ELISA method may differ depending on whether the disease is in remission or under treatment at the time of diagnosis [19,20]. For this reason, it has come to the fore that different titration values can be used for anti-PLA2R antibody kits. It has been emphasized that algorithms will also be needed in this regard [21].

In addition, some studies have suggested the use of specific measurement kits for immunoglobulin G subtypes and antigen epitopes to increase the probability of detecting IMN patients [22,23]. The negative results of the kits we used in our patients suggested that the use of more sensitive kits may be beneficial.

Anti-PLA2R antibody values obtained in our study were below the reference range of the test, it was accepted that this ROC curve could not give clear accurate data and was considered meaningless. For this reason, the low threshold value for the test is also important in terms of increasing the probability of detecting the disease. In a study, the low threshold value supported that the measurement kit used in the diagnosis of the disease increased the sensitivity [24].

Our study is among the first studies in our country to evaluate anti-PLA2R antibody in IMN patients. Anti-PLA2R antibody has been widely researched all over the world since it was detected in 2009, and many countries provide their own population study data. Many countries have conducted population-based studies. This study is one of the population-based studies in our country in which patients with a diagnosis of IMN are evaluated in terms of this antibody positivity. In addition, we also considered which measurement kit could contribute to the use of anti-PLA2R antibodies in our study.

Limitations

This study had some limitations. We did not evaluate the immunosuppressive treatments received by the patients, and mixed the patients with continuing or negative proteinuria into a mixed evaluation, and not checking the antibody level in a group consisting of other nephropathies, including secondary membranous nephropathy in the comparison group, and not checking the antibody level with a kit containing a different measurement method from the same samples. It was considered to be checked with a kit with a lower anti-PLA2R measurement sensitivity, but could not be done due to the cost factor.

CONCLUSION

There is a need for patients with a diagnosis of IMN to have the test for the determination of anti-PLA2R antibody level and to measure the measurement with which unit. In our institutions, ng/ml unit has been determined for the determination of anti-PLA2R antibody, but the level of the indicator of the values found has not been increased.

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Conflict of interest: The authors have no conflicts of interest to declare**.**

Ethics Committee Approval: The study described in this article was conducted within the framework of the Declaration of Helsinki. Gaziantep University Faculty of Medicine Clinical Research Ethics Committee approved the study (approval number: 2017-200, approval date: May 22, 2017).

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