The Intensity of PLA2R and C4d Immunoexpression in Primary Membranous Nephropathy

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Objective: Antibodies against the phospholipase A2 receptor (PLA2R) on podocyte membranes result in the formation of immune complexes that cause loss of function of the glomerular basement membrane in primary membranous nephropathy (PMN). It has also been demonstrated that there is a deposition of complement 4d (C4d) in the glomeruli in PMN. The present study aims to evaluate PLA2R and C4d immunoexpressions in PMN cases and search the correlation with the clinical parameters.

Methods: In this study, clinicopathological data and paraffin-embedded specimens were collected from 51 patients. The formalin-fixed paraffin-embedded tissues were stained using routine hematoxylin-eosin, periodic acid-Schiff, and silver methenamine stains and immunostained for anti-PLA2R and C4d. Ten normal kidney tissues and 10 focal segmental glomerulosclerosis (FSGS) cases were selected as controls for PLA2R and C4d immunoexpression.

Results: Of the PMN cases, 51 (100%) cases were positive for PLA2R, including 15 (29%) cases that scored 2+, and 36 (71%) cases that scored 3+. Forty of the 51 cases (78%) were positive for C4d. The percentages of cases staining positively for C4d, per scoring group, were as follows: 31 (61%) cases faintly (1+) positive and 9 (18%) cases moderately (2+) positive. No strong positivity was observed. All of the control cases (100%) were negative for PLA2R and C4d. There was no statistically significant difference between the intensity of the staining of PLA2R and the staining of C4d, proteinuria levels, creatinine levels, and complement 3 (C3) positivity. Similarly, there was no statistically significant difference between the intensity of the staining of C4d and proteinuria levels, creatinine levels, and C3 positivity.

Conclusion: Immunohistochemical detection of PLA2R and C4d is a safe and easy method for the diagnosis of PMN. In cases where fresh tissue is not available for the detection of IgG and C3 using the immunofluorescence method, positivity for PLA2R and C4d with immunohistochemistry may be beneficial for the diagnosis of PMN.

INTRODUCTION

Membranous nephropathy (MN) is the leading cause of nephrotic syndrome in the adult population,[1–4] The disease is characterized by immune complex deposition on the outer aspect of the glomerular basement membrane. This deposition causes loss of function of the glomerular filtration barrier, which results in proteinuria.[2–4] Traditionally, membranous nephropathy has been classified as primary or secondary membranous nephropathy. In 70–80% of the cases, there is no known etiology. If a secondary cause cannot be identified, this group is classified as “primary membranous nephropathy” (PMN). If MN is associated with a clinical condition, such as malignant tumors, autoimmune diseases, viral or bacterial infections (hepatitis B, syphilis) or drug intoxication, the disease is termed as “secondary membranous nephropathy”.[3,5,6] In situ immune complexes are formed due to the presence of antibodies against podocyte proteins. This issue has been determined to cause MN.[7] In 70% of the adult patients, PLA2R on podocytes has been demonstrated as a target antigen in PMN.[8] PLA2R appears to be a causative antigen of PMN in most of adults[3,8–17] and some pediatric populations.[18] The pathogenesis of MN is mediated by the in situ formation of immune deposits, with the resulting activation of the complement. C4d is a fragment of complement 4 (C4) that is generated during activation of the classical complement or lectin pathways. Studies of C4d have focused on...
transplant biopsies as an indicator of acute humoral rejection, and very few studies have analyzed C4d in MN. However, in these studies, it has been shown that there is C4d deposition in the glomeruli in PMN.[19–22] In addition, it has been demonstrated that there is a positive correlation between the serum levels of PLA2R autoantibodies and the degree of proteinuria, severity of the disease, and loss of kidney function. It has also been demonstrated that follow-up testing of serum levels of PLA2R autoantibodies is useful for the prediction of recurrence of the disease.[1,9,23–29]

The present study aims to evaluate PLA2R and C4d immunoexpressions in PMN cases and search the correlation with the clinical parameters.

MATERIALS AND METHODS

Clinicopathological data and paraffin-embedded specimens were collected from 51 patients (25/26 M/F; median age 50.7 years, range 19–75 years) who underwent renal biopsy in our department (Istanbul, Turkey) between 2000 and 2014. This study was approved by the local ethics committee. All cases were older than 18 years of age and clinical presentation and laboratory findings, such as proteinuria (g/24-h) and serum creatinine (mg/dl), were documented in all cases (Table 1). Patients with proteinuria (≥3.5 g/24-h) were screened for secondary conditions by obtaining a complete clinical history, physical examination, and serologic tests for systemic lupus erythematosus, hepatitis B and C, HIV, as well as screening for malignancies.

Ten normal kidney tissues and 10 focal segmental glomerulosclerosis (FSGS) cases were selected as controls for PLA2R and C4d immunoexpression. The renal biopsies were stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), periodic acid methenamine silver (Gomori PAMS), and Masson’s trichrome. Morphological findings of the cases were mainly diffuse thickening of the glomerular basement membrane in light microscopic examination (Fig. 1a-c) and subepithelial IgG and C3 deposition in immunofluorescence examination. Data on the immunofluorescence examination for IgG, C3, IgA, C1q, fibrinogen, kappa, and lambda light chain antibodies were available on the pathology report of the cases.

Immunohistochemistry

Three micron thick sections of the total 71 formalin-fixed, paraffin-embedded tissues were immunostained for anti-PLA2R (CL0474; Atlas Antibodies) and C4d (Polyclonal; CellMarque). Immunostaining was performed with Leica Bond-Max automatic immunostainer (Leica, Bannockburn, IL) following 82 minutes of incubation at room temperature in a Bond™ Polymer Refine Detection Kit (Leica Biosystems, Catalog No. DS9800).

Slides were examined blindly by two independent pathologists under a light microscope (Olympus BX53, Tokyo, Japan), and microscopic images were obtained on a light microscope linked to a digital camera (Olympus SC100, Tokyo, Japan).
The immunohistochemistry scoring results for PLA2R and C4d were based on the staining intensity of the glomerular basement membrane; no reactivity was defined as a score of 0; faint reactivity was defined as a score of 1+; moderate reactivity was defined as a score of 2+; strong reactivity was defined as a score of 3+. For PLA2R, a score of 0 or 1+ was considered negative because of the faint PLA2R immunoeexpression of the normal glomerulus.

Statistical analysis
Statistical analysis was carried out using NCSS (Number Cruncher Statistical System) 2007 Statistical Software (Utah, USA). Kruskal-Wallis and Mann-Whitney U tests were used to compare the two groups. We analyzed the correlation between the expression of PLA2R and C4d in the PMN cases and the control tissues. Statistical comparisons between the intensity of the staining of PLA2R and C4d with varying degrees of proteinuria were performed using the Kruskal-Wallis and Mann-Whitney U tests. A P-value of <0.05 was considered to be statistically significant.

RESULTS
In this study, 51 paraffin blocks of PMN, 10 normal kidney tissues, and 10 FSGS cases were examined for the expression of PLA2R and C4d. All of the control cases (10 normal kidney tissues and 10 FSGS cases) (100%) were negative for PLA2R (Fig. 2a). All 51 (100%) of the PMN cases were positive for PLA2R in a fine granular pattern, including 15 (29%) that scored 2+ (Fig. 2b) and 36 (71%) that scored 3+ (Fig. 2c).

All of the control cases (10 normal kidney tissue and 10 FSGS cases) (100%) were negative for C4d. Of the 51 cases, 40 (78%) cases were positive for C4d. The percentages of cases staining positively for C4d, per scoring group, were as follows: 31 (61%) faintly (1+) positive (Fig. 3a) and 9 (18%) moderately (2+) positive (Fig. 3b). No strong positivity was observed.

There was no statistically significant difference between the intensity of the staining of PLA2R and staining of C4d (p=0.290), proteinuria levels (p=0.462), creatinine levels (p=0.346), and C3 positivity (p=0.240). Similarly, there was no statistically significant difference between the intensity of the staining of C4d and proteinuria levels (p=0.457), creatinine levels (p=0.647), and C3 positivity (p=0.386) (Table 1).

DISCUSSION
PLA2R is a huge transmembrane glycoprotein with a molecular mass of 180–200 kDa on the podocyte membrane. Autoantibodies against PLA2R are responsible for immune complex formation and deposition on the outer aspect of the glomerular basement membrane, which results in membranous nephropathy.

The process of occurrence of autoantibodies against PLA2R, through immune complex formation and deposition on the glomerular basement membrane, can be used in different stages with different techniques for the diagnosis of PMN. Autoantibodies in serum can be detected with a Western blotting assay, enzyme-linked immunosorbent assay (ELISA), and indirect immunofluorescence assay. In fresh and paraffin-embedded tissue, a direct immunofluorescence assay and in paraffin-embedded tissue, immunohistochemistry scoring results for PLA2R and C4d were based on the staining intensity of the glomerular basement membrane; no reactivity was defined as a score of 0; faint reactivity was defined as a score of 1+; moderate reactivity was defined as a score of 2+; strong reactivity was defined as a score of 3+. For PLA2R, a score of 0 or 1+ was considered negative because of the faint PLA2R immunoeexpression of the normal glomerulus.

Figure 1. (a) Diffuse thickening of basal membranes of glomerulus (H&E, ×200). (b) Thickened basement membranes are more prominent with PAS stain (PAS, ×200). (c) “Spike”structures are seen through the basement membranes (Gomori PAMS, ×1000).

Figure 2. (a) Faint immune expression for PLA2R in normal glomeruli (Immunohistochemistry, ×200). (b) Moderate (2+) immune reactivity for PLA2R (Immunohistochemistry, ×200). (c) Strong (3+) immune reactivity for PLA2R (Immunohistochemistry, ×400).
tochemistry\textsuperscript{[3,17]} techniques can be used for the detection of glomerular immune deposits in PMN.

The sensitivity of detection of serum levels of PLA2R autoantibodies in different studies varies between 53–97\%\textsuperscript{[1,3,5,8–10,12,13,16,17,23–25,27,28,31–34]} The detection of PLA2R in tissue by immunofluorescence varies between 45–83\%\textsuperscript{[11,18,35]} There are few studies that evaluate PLA2R in PMN with immunohistochemistry. However, in these studies, sensitivity varies between 77–84\%\textsuperscript{[3,17]}

The results of our study demonstrate that the detection of PLA2R immune complexes in tissue using immunohistochemistry could be a more sensitive method than immunofluorescence and serum detection methods in the diagnosis of PMN.

In comparison to the literature, there was an increased positivity for PLA2R in our PMN cases. This result might be related to the detailed investigation of the secondary causes of MN at our university hospital. In other words, the negativity for PLA2R in the literature may be due to the overlooking of cases with secondary MN.

Immune complex formation in PMN also causes complement activation. C4d is a 44.5 kD fragment of C4, which is generated during the activation of the classical complement pathways\textsuperscript{[19–22,36]} It has been demonstrated that the presence of C4d in peritubular capillaries is valuable for distinguishing acute humoral rejection from acute cellular rejection in kidney transplantation cases. There are a small number of studies investigating the presence of C4d in PMN\textsuperscript{[19–22]} In these studies, it has been demonstrated that there is a deposition of C4d in the glomeruli in PMN. The percentage of deposition of C4d in the glomeruli was determined using the immunofluorescence method, in PMN cases are between 92–100\%\textsuperscript{[19,20]} However, the immunohistochemistry method presents this percentage as 100\%\textsuperscript{[21,22]} in these studies. Our study has revealed this percentage as 78\%, which is less than the literature.

There are some studies evaluating the relation between proteinuria and the severity of disease and the presence of PLA2R autoantibodies in PMN\textsuperscript{[1,9,23–29]} The correlation between proteinuria and the severity of disease and the presence of PLA2R autoantibodies, has been demonstrated in these studies. However, to our knowledge, there is no study investigating the relation between proteinuria and the severity of disease and the presence of C4d in the glomeruli. Also, there is no study in the English literature that compares the levels of proteinuria and the severity of disease with the intensity of immunohistochemical staining of PLA2R and C4d.

In our study, we have not demonstrated a significant relation with the immunohistochemical method between the intensity of the staining of PLA2R and C4d, and proteinuria at the time of biopsy. Longer follow-up periods for proteinuria levels (3–6 months) may provide more accurate information about this relationship. In conclusion, immunohistochemical detection of PLA2R and C4d is a safe and easy method for the diagnosis of PMN. In cases where fresh tissue is not available for the detection of IgG and C3 by the immunofluorescence method, positivity for PLA2R and C4d using immunohistochemistry may be beneficial for the diagnosis of the PMN.

To detect the significance of staining intensity in disease severity and prognosis, further studies with long-term follow-up data should be designed.

**Funding**

This study was funded by “Marmara Üniversitesi Bilimsel Araştırma Projeleri Birimi”.

**Ethics Committee Approval**

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. This study was approved by the local ethics committee (date: 23.02.2015, no: 2).

**Informed Consent**

Retrospective study.

**Peer-review**

Internally peer-reviewed.
Authorship Contributions
Concept: D.F., H.A., M.K., H.K., I.Ç.Ö., G.A.; Design: D.F., H.A., M.K., H.K., I.Ç.Ö., G.A.; Supervision: D.F., H.K.; Fundings: D.F., H.K.; Materials: D.F., G.A., M.K.; Data: D.F., G.A., H.A.; Analysis: D.F., H.A., M.K.; Literature search: D.F., H.A., H.K., I.Ç.Ö.; Writing: D.F.; Critical revision: I.Ç.Ö., H.K., H.A.

Conflict of Interest
None declared.

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Amaç: Primer membranöz nefropatide (PMN), podosit membranlarındaki fosfolipaz A2 reseptörüne (PLA2R) karşı gelişen antikorlar, immün komplekslerin oluşumuna neden olarak glomerular bazal membranda fonksiyon kaybına sebep olur. Ayrıca PMN’de glomerüllerde kompleman 4d (C4d) birikimi de gösterilmiştir. Bu çalışmanın amacı, PMN olgularında PLA2R ve C4d ekspresyonlarını değerlendirmek ve klinik parametrelerle bağlantıların araştırılmasıdır.

Gereç ve Yöntem: Elli bir hastaya ait klinikopatolojik bilgiler ve parafine gömülmüş örnekleri toplandı. Formalin fikse ve parafine gömülmüş dokular rutin hematoksilen-eozin, periyodik asit schiff ve gümüş methenamin boyalarıyla ve anti-PLA2R (CL0474; Atlas Antikorları) ve C4d (Poliklonal; CellMarque) immün boyalarıyla boyandı. On adet normal böbrek dokusu ve 10 adet fokal segmental glomeruloskleroz (FSGS) olgusu PLA2R ve C4d ekspresyonu için kontrol olarak seçildi.

Bulgular: PMN olgularının hepsi (%100) PLA2R ile pozitifti, 15’i (%29) 2+, 36’sı (%71) 3+ olarak skorlandı. Olguların 40’ı (%78) C4d pozitifti. C4d boyanma oranları: 31 olguda zayıf pozitif (1+) (%61), dokuz olguda orta şiddette pozitif (2+) (%18) olarak gözlandı. Kuvvetli boyanma gözlenmedi. Tüm kontrol olgular PLA2R ve C4d ile negatifti. PLA2R ve C4d boyama yoğunluğu ile proteinüri seviyeleri, kreatinin seviyeleri ve kompleman 3 (C3) pozitifliği arasında istatistiksel olarak anlamlı bir fark saptanmadı. Benzer olarak C4d boyama yoğunluğu ile proteinüri seviyeleri, kreatinin seviyeleri ve C3 pozitifliği arasında istatistiksel anlamlı bir fark saptanmadı.

Sonuç: PMN tanısında PLA2R ve C4d’nin immunohistokimyasal olarak sırtlanmasını güvenli ve kolay bir metoddur. İmmünfloresan yöntemiyle IgG ve C3 sırtlanması sağlayacak yeterli dokunun olmaydı durumlarında, immunohistokimyasal olarak PLA2R ve C4d pozitifliği PMN tanısında faydalı olabilir.

Anahtar Sözcükler: C4d: membranöz; PLA2R; proteinüri.