Anti-PLA2R and anti-THSD7A as Diagnostic Serological Markers of Idiopathic Membranous Nephropathy: A Single Centre Study

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Idiopathic Membranous Nephropathy (IMN) is a renal-limited autoimmune disease and accounts for approximately 80% of MNs. This study aimed to evaluate the role of circulating Anti-PLA2R and anti-THSD7A autoantibodies in the diagnosis and differentiation between primary and secondary MN. This study was conducted on 58 adult patients with biopsy-proven membranous glomerulopathy (MGN). All were subjected to measurement of Anti-PLA2R1 by an Enzyme-Linked Immunosorbent Assay and anti-THSD7A was detected by an indirect immunofluorescence assay. Among the 58 patients, 79.3% were diagnosed as IMN, and 20.7% as secondary membranous glomerulopathy (SMN). Among IMN patients, 32 patients (69.6%) showed positive anti-PLA2R1 antibodies, 2 patients (4.3%) were positive for anti-THSD7A antibodies and the remaining 12 patients (26.1%) were negative for both types of antibodies. Patients with SMN were negative for the two antibodies. The IMN patients had lower serum creatinine compared to the SMN patients (P=0.017). In conclusion, the study demonstrates that approximately 70% of patients with idiopathic membranous nephropathy have antibodies against PLA2R indicating that Anti-PLA2R may be fast, easy, relatively sensitive, and non-invasive test for diagnosis of IMN.

Membranous nephropathy (MN) is the most common cause of nephrotic syndrome among adults with an increasing incidence rates year by year [1-2]. It is the second or the third important reason of end stage renal diseases in patients with idiopathic glomerulonephritis and are the principal glomerulopathy that relapses after kidney transplantation [3].

MN is a morphological form of damage characterized by an increase in the glomerular capillary wall thickness due to the deposition of immune complexes under the epithelium and complement components with the new basement membrane formation [4].

Primary MN (PMN), also known as idiopathic membranous nephropathy (IMN), can be a renal-limited autoimmune disease and representing approximately 80% of MNs, the residual 20% are secondary membranous glomerulopathy (SMN) to different systemic diseases, autoimmune diseases, infections (hepatitis B), malignancy and drugs [5].

Differentiating PMN and SMN depend on their clinical manifestations and laboratory tests, and this is important for diagnosis, treatment and follow-up but it is very difficult [6]. Kidney biopsy is the gold standard in making the diagnosis, but a biopsy is invasive, costly, and risky [7]. So, using less or non-invasive methods such as serum or urine samples could make the diagnosis and follow up patients is less challenging.

PMN is an organ-specific autoimmune disease, in which circulating autoantibodies bind to an autoantigen on the surface of the podocyte; the M-type phospholipase A2 receptor 1 (PLA2R) and thrombospondin
Anti-PLA2R and anti-THSD7A as Diagnostic Serological Markers of IMN

PLA2R is an autoantigen of type I transmembrane receptor and one of four mammalian members of the mannose receptor family, present in glomerular podocytes, and its extracellular domain is the transformed antigen which stimulates autoimmune reactions [8]. Attached with the anti-PLA2R antibodies formed in the body are mainly IgG4, leading to the formation of in situ immune complex which stimulates the complement components causing podocyte damage, with the production of protein in urine, that is the key pathogenic feature for PMN patients [9].

Anti-PLA2R levels often mirror the disease activity. Serial anti-PLA2R measurements may provide prognostic information which help in monitoring response to therapy, baseline and post-treatment. When Anti-PLA2R levels are high, urine protein levels are typically increased while in remission its level decline or disappear [5].

THSD7A is a second autoantigen expressed by the podocyte [10]. It stimulates IgG4-predominant humoral immune responses that produce circulating autoantibodies which directly affect podocyte integrity, causing damage of cell and proteinuria. THSD7A can be used clinically for diagnostic and monitoring of adult PMN [11].

The unique antibodies against PLA2R and THSD7A were isolated from the sera of primary MN patients. These antibodies have not been detected in patients with other nephropathies or autoimmune diseases or in apparently healthy persons. [8-10]. Consequently, this work aimed to evaluate the role of circulating Anti-PLA2R and anti-THSD7A autoantibodies in the diagnosis and differentiation between primary and secondary membranous nephropathy.

Subjects and Methods

Patients and samples

The study protocol was reviewed and approved by the Research Ethics Committee, Faculty of Medicine, Benha University, Egypt (January 2018). A written consent from patients was obtained before they included in the study.

The study was conducted on 58 adult patients with biopsy-proven MGN who attended Nephrology Unit Internal Medicine Departments, Benha University Hospital, between January 2018 and April 2019. Diagnosis of IMN and SMN were based on clinical evaluation and kidney biopsy. Patients with chronic diseases (e.g., Hepatitis B or C, diabetes nephropathy, cancer, lupus nephritis type V) were included as SMN.

Clinical data were collected from all patients, including age, gender, history of hypertension. Laboratory assessment of serum creatinine and serum albumin were performed by colorimetric methods using commercial kits (for creatinine, ab204537 kit, ABCAM, USA, and for albumin Elabscience kit Catalog number: E-BC-K057-M). Urine proteins were determined in 24-hour collected urine samples by the turbidimetric method using trichloroacetic acid (TCA) precipitation. Turbidity was measured by reading the 420-nm absorbance 35 minutes after the TCA addition.

Serum samples from study participants were collected and immediately aliquoted, frozen and kept at −80°C until tested.

Measurement of Anti-PLA2R1

Anti-PLA2R antibodies were measured using a commercially available ELISA (EUROIMMUN AG, Lübeck, Germany, Order no. EA 1253-9601) that contained PLA2R1-coated microplates. The ELISA was performed according to the manufacturer’s instructions.

Briefly, patients’ serum was diluted in the ratio 1:100 then incubated in the microplate at room temperature for 30 min. After washing, the microplate was incubated with peroxidase-conjugated anti-human IgG diluted 1:1,000 in sample buffer at room temperature for 30 min. The color was developed by the addition of chromogen substrate
solution for 15 min then stopped by 0.5M H2SO4 stopping solution. Then the optical density was examined at 450 nm using an ELISA reader (Bio-Rad 550, Tokyo, Japan). All experiments were performed in duplicate and data represents mean values. Antibody positivity was defined as a level over 20 U/mL [12].

Measurement of Anti-THSD7A immunofluorescence assay

Anti-THSD7A total IgG was detected by an indirect immunofluorescence assay kit (EUROIMMUN AG, Lübeck, Germany, Order no. FA 1254-1005-1), following the manufacturer’s instructions.

Test principle: THSD7A-expressing cells are incubated with diluted patient samples. If the reaction is positive, specific antibodies attach to the antigens. Then, the attached antibodies are stained with fluorescein-labelled anti-human antibodies and become visible with a fluorescence microscope.

Briefly, patients’ plasma was diluted 1:10 then 30 µl of diluted sample incubated on the reaction fields of BIOCHIP slides at room temperature for 30 minutes. After washing the BIOCHIP slides with a flush of PBS Tween, the slides were incubated with 25 µl of fluorescein labelled anti human globulin to at room temperature for 30 minutes with protection from direct sunlight. Then the slides were examined by a fluorescence microscope (Carl Zeiss AG, Germany).

Statistical Analysis

Data were tabulated, coded and analyzed using the Statistical Package for the Social Sciences (SPSS) software version 23.0 for Windows. Normally distributed continuous variables were expressed as the mean ± standard deviation (SD) while the median (interquartile) was used for variables that were not normally distributed. Continuous data were compared using the Student’s t-test or the Mann-Whitney test, as appropriate. Categorical data were compared using the Chi2 test. All P values were 2-tailed, with P<0.05 was considered statistically significant.

Results

A total of 58 patients with biopsy proven MN were included in the study. The characteristics of these patients are shown in Table 1. A 55.2% of these patients were males. The mean age was (51.9 ± 11.6).

Among the 58 patients, 46 patients (79.3%) were diagnosed as idiopathic membranous nephropathy and 12 patients (20.7%) were secondary MN. The etiology of secondary MN (n = 12) was lupus membranous nephritis (n = 3), hepatitis B virus (n=6) and malignancy-associated MN (n = 3).

Comparison between PMN and SMN is shown in Table 1. The IMN patients had lower serum creatinine compared to the secondary MN patients (P=0.017). There were no significant differences in age, gender, proportion of hypertension, proteinuria, nor serum albumin between idiopathic and secondary membranous nephropathy patients.

Table 1. Clinical characteristics of primary and secondary MN patient groups

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>IMN (n=46)</th>
<th>SMN (n=12)</th>
<th>Total (n=58)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (median ± interquartile range)</td>
<td>53 ± 15.75</td>
<td>45.5±20.25</td>
<td>52.5 ± 18</td>
<td>NS</td>
</tr>
<tr>
<td>Gender (male %)</td>
<td>60.9%</td>
<td>33.3%</td>
<td>55.2%</td>
<td>NS</td>
</tr>
<tr>
<td>Hypertension n (%)</td>
<td>26 (56.5%)</td>
<td>5 (41.7%)</td>
<td>53.4%</td>
<td>NS</td>
</tr>
<tr>
<td>Serum Creatinine (mean ± SD)</td>
<td>0.95±0.27</td>
<td>1.17±0.32</td>
<td>0.99±0.29</td>
<td>0.017*</td>
</tr>
<tr>
<td>Urinary protein (g/24 h) (mean ± SD)</td>
<td>5.22±2.29</td>
<td>5.67 ±2.46</td>
<td>5.31±2.31</td>
<td>NS</td>
</tr>
<tr>
<td>Serum albumin (g/L) (mean ± SD)</td>
<td>27.1 ±6.47</td>
<td>26.32±6.31</td>
<td>26.94±6.39</td>
<td>NS</td>
</tr>
</tbody>
</table>

IMN – idiopathic membranous nephropathy; SMN – secondary membranous nephropathy * P>0.05 is not significant (NS).
Serological characterization

Among idiopathic MN patients (n=46), anti-PLA2R1 antibodies were detected in 32 patients (69.6%) Figure 1, and 2 patients (4.3%) were positive for anti-THSD7A antibodies Table 2. The remaining 12 patients (26.1%) were negative for both types of antibodies. All patients with secondary MN were negative for both antibodies.

According to clinical and serological results, cases can be classified into four groups: PLA2R-Associated (n=32), THSD7A-Associated (n=2), Double-Negative primary MN (n=12), and secondary MN cases (n=12). We compared the clinical differences between these groups regarding age, sex, proteinuria, serum albumin, serum creatinine and blood pressure as shown in Table 3. There was no significant difference among groups regarding gender (P=0.38), age (P=0.157) nor serum creatinine (P=0.121). The variation, however, was statically significant between groups in case of urinary protein (P=0.046), serum albumin (P=0.027) and hypertension (P=0.042).

Figure 1. Anti-PLA2R1 levels in PLA2R-Associated MN (n=32), THSD7A-Associated (n=2) and double negative idiopathic MN (n=12) and Secondary MN (n=12).

Figure 2. Detection of serum anti-THSD7A antibody by indirect immunofluorescence test. (A) Immunofluorescence pattern of a positive case for anti-THSD7A antibodies. (B) Immunofluorescence pattern of a negative case.
Table 2. The clinical characteristics of THSD7A-Associated two IMN patients.

<table>
<thead>
<tr>
<th>Case No</th>
<th>Age</th>
<th>Gender</th>
<th>Serum anti-THSD7A Ab</th>
<th>AntiPLA2R ab</th>
<th>24 h-proteinuria (g/24h)</th>
<th>Albumin (g/l)</th>
<th>Serum creatinine (mmol/L)</th>
<th>Hypertension</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>47</td>
<td>male</td>
<td>+ve</td>
<td>-ve</td>
<td>3.44</td>
<td>32.51</td>
<td>0.91</td>
<td>+ve</td>
</tr>
<tr>
<td>44</td>
<td>53</td>
<td>female</td>
<td>+ve</td>
<td>-ve</td>
<td>6.26</td>
<td>23.9</td>
<td>0.82</td>
<td>+ve</td>
</tr>
</tbody>
</table>

PLA2R – M-type phospholipase A2 receptor; THSD7A – thrombospondin type-I domain-containing 7A.

Table 3. Comparison of clinical data among different serological groups

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Idiopathic</th>
<th>Secondary (n=12)</th>
<th>*P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PLA2R-Associated (n=32)</td>
<td>THSD7A-Associated (n=2)</td>
<td>Double-Negative (n=12)</td>
</tr>
<tr>
<td>Gender (male %)</td>
<td>62.5%</td>
<td>50%</td>
<td>58.3%</td>
</tr>
<tr>
<td>Age (median ± interquartile range)</td>
<td>55 ± 17.75</td>
<td>50</td>
<td>50±21.75</td>
</tr>
<tr>
<td>Urinary protein (mean ± SD)</td>
<td>5.82±1.92</td>
<td>4.85±1.99</td>
<td>3.7±2.64</td>
</tr>
<tr>
<td>Serum albumin (mean ± SD)</td>
<td>25.32±5.21</td>
<td>28.2±6.09</td>
<td>31.67±7.67</td>
</tr>
<tr>
<td>Serum Creatinine (mean ± SD)</td>
<td>0.95±0.27</td>
<td>0.87±0.06</td>
<td>0.96±0.28</td>
</tr>
<tr>
<td>Hypertension n (%)</td>
<td>65.6%</td>
<td>100%</td>
<td>25%</td>
</tr>
</tbody>
</table>

*P value is for comparing the four serological groups: PLA2R-Associated, THSD7A-Associated; Double-Negative and Secondary membranous nephropathy. * P>0.05 is not significant (NS).

Discussion

In recent years, MN is at the attention of researchers for its increasing frequency. PMN is responsible for approximately 80% of nephrotic syndrome cases. While biopsy has been the main tool in the diagnosis, some patients refuse to do renal biopsy or it may be contraindicated in others, therefore the identification of serum biomarkers would probably make the diagnosis and treatment be easier [13].

This study was aimed at providing an alternative way for differentiation between primary and secondary MGN using simple non-invasive technique like ELISA and an indirect immunofluorescence assay for the detection of serum anti-PLA2R and anti-THSD7A antibodies in Egyptian patients with MN. The study showed that among 46 PMN patients, 32 patients (69.6%) showed positive anti-PLA2R1 antibodies. 2 patients (4.3%) were positive for anti-THSD7A antibodies and the remaining 12 patients (26.1%) were negative for both types of antibodies.

Our finding that approximately 70% of patients with PMN have auto-antibodies against PLA2R agrees with a study conducted by Beck et al., 2009 [20]. The possible explanation of why all patient with IMN do not have anti-PLA2R antibodies
Anti-PLA2R and anti-THSD7A as Diagnostic Serological Markers of IMN

...may be due to presence of other target antigen other than PLA2R or the patient are in remission state so the disease is in an inactive state at the time of sample collection [13]. Furthermore, in 2018, a meta-analysis study, analysed 35 studies, [14] showed that anti-PLA2R autoantibodies demonstrated a good diagnostic accuracy in differentiating PMN from non-PMN or SMN and the prevalence of serum anti-PLA2R reported by studies varies from 57 to 82%. In addition, our results agree with previous studies that have established that the levels of anti-PLA2R autoantibodies in PMN patients are significantly higher than in SMN patients and non-MN group [9,15,17].

Some studies reported that, using ELISA for measuring anti-PLA2R, the clinical sensitivity is usually between 72 and 82% when testing is limited to individuals with nephrotic-range proteinuria (proteinuria of ≥3.5 g/24 h [15-18-19] but it was 48% non-nephrotic patients PMN [18]. In our study, neither anti-PLA2R antibodies nor anti-THSD7A were found in secondary MN. Such observation agrees with most studies, reported that the majority of their secondary MN patients are PLA2R-negative [12, 22-24]. In 2009, a study by Beck et al., 2009 [20], reported that anti-PLA2R antibodies could not be detected in secondary MN. The specificity of these antibodies in patients with idiopathic membranous nephropathy, suggests that the antibodies are most likely the cause, rather than a consequence, of podocyte injury and proteinuria [20].

Other studies have found variable positivity of these antibodies in secondary MN. It showed 9.3% (11 patients) and 2.4% (5 patients) for anti-PLA2R and anti-THSD7A antibodies respectively in a Chinese study [21]. Only one out of 35 of secondary MN cases had anti-PLA2R in another study. The presence of these antibodies in secondary MN may present only coexistence of primary MN and other disorder [25].

In early clinical studies, the positive rate of anti-PLA2R antibody in IMN patients ranged between 70 and 80%. However, lower rates were reported in more recent studies which included different ethnic groups. One explanation could be that in early studies most patients had severe proteinuria but in recent studies patients had mild proteinuria [3].

In our study some PMN cases were anti-PLA2R antibodies negative and this may be due to several factors; (i) serum samples were withdrawn while the patient was in decreased activity or in inactive stage; (ii) all antibodies adsorbed by the kidney (sink theory); or (iii) new or cryptic auto-antigens may coexist [8]. In our study, serum anti-THSD7A antibodies were detected in two cases of 46 PMN (4.3%). Also, another study [27] reported a rate of 5% for detection of anti-THSD7A antibodies. In addition, auto-antibodies against THSD7A were detected in about 8 – 14% of patients with primary PLA2R-negative MN [23]. Such data could indicate that when PMN is not diagnosed by anti-PLA2R antibodies, few cases may be diagnosed by anti-THSD7A autoantibodies.

In 2020, a study reported that anti-THSD7A antibodies were detected in four cases with PMN and two cases of SMN, one of them had a malignancy [28], suggesting that anti-THSD7A antibodies may be occurring with malignancy.

A meta-analysis done in 2018, reported that the rate of anti-THSD7A was 3% in all patients and 10% between patients with negative anti-PLA2R. The difference was
explained due the difference in sample size or due to different studied races [10].

Regarding disease activity and prognosis of MGN with association with anti-PLA2R Ab, Anti-PLA2R levels often mirror disease activity effectively and continued measurement of its level and following up its change provided us with patient immune status, compared with using proteinuria to evaluate the severity, time and duration of treatment [9].

In our study, there were statically significant differences between studied groups (PLA2R-associated, THSD7A-associated; double-negative and secondary membranous nephropathy) regarding urinary protein and serum albumin. This observation is in agreement with many studies [3,9,20] which showed that these parameters were higher in the early stage of the disease with severe proteinuria, then decreased significantly or vanished totally in its remission and slowly increased in its reappearance. However, other studies showed that presence of serum anti-THSD7A has no relation with such laboratory parameters, including serum creatinine, albumin, and proteinuria levels, which agree with our study findings [29,30].

In conclusion, our data indicated that assessment of serum circulating autoantibodies against PLA2R may have the advantages of being rapid, simple, relatively sensitive, and non-invasive tool for diagnosis of PMN. Thus, a renal biopsy may not always be necessary for diagnosis decisions.

References


