Diagnosing iMN (Idiopathic Membranous Nephropathy); Research Opportunities via ISN: Crossing The Geographical Boundaries

Dr. Muhammad Mohsin Riaz, Dr. Omer Sabir

Fatima Memorial Hospital

Lahore, Pakistan

Abstract:

Membranous nephropathy (MN) still remains one of the common causes of adult glomerulonephritis and leading cause of CKD (chronic kidney disease) and ESRD (end stage renal disease) in adult population. Differentiating between idiopathic (iMN) and secondary membranous nephropathy (sMN) is of key importance. The treatment and prognosis for both types vary. Phospholipase A2 receptor (PLA2R) has been shown as major auto-antigen in iMN. Its prevalence ranges from 70% – 80% in cases of iMN as described by international studies. The prevalence of PLAR in iMN from our region is not known. This study was conducted to look into prevalence of PLA2R in iMN patients from our population. While carrying out study we found some other important factors that affect the diagnostic histopathology, collectively termed as pre-analytical factors. We also found that there are no standardized protocols for performing the immunohistochemistry (IHC) studies and only set of guidelines are available. The important component of diagnostic histopathology i.e. electron microscopy is also missing in the process of making tissue diagnosis.

Key words:

Idiopathic membranous nephropathy (iMN), phospholipase A2 receptor (PLA2R), PLA2R antibody, immunohistochemistry (IHC), pre-analytical factors.

Corresponding Author:

Dr. Muhammad Mohsin Riaz
Fatima memorial Hospital
Lahore, Pakistan
Email: dr.m.mohsin@gmail.com

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Introduction:

In the last decade our understanding about etiopathogenesis of idiopathic membranous glomerulopathy (iMN) has immensely improved. The studies suggest that it is a renal limited autoimmune disease with genetic basis[1]. Membranous nephropathy is a common cause of adult nephrotic syndrome worldwide [2, 3]. It is among the leading causes of end stage renal disease[4, 5]. Variations in incidence have been reported from different regions, that may be due to specific country/physician indication for kidney biopsy and may represent real differences related to the population's socioeconomics, ethnicity, or environment[6]. Data from Pakistan reveals that MN is the second most common cause of nephrotic syndrome with prevalence of 23.5% [7] and 26.9% [8] as reported in two different studies. It can be idiopathic or secondary to various clinical conditions including systemic autoimmune disease, infections, neoplasia and drug intoxications[9]. Differentiation between these two groups of patients is of clinical importance, since therapy in the secondary membranous nephropathy must be directed at the underlying cause and some of the treatments for iMN are potentially toxic both to the patient and the kidney[10, 11]. Differentiating iMN from sMN is a difficult task [12, 13].

A major breakthrough in differentiating the two types came in 2009 with the discovery of phospholipase A2 receptors on podocytes as a target antigen in patients with iMN[14]. The association of IgG antibody with the glomerular lesions of iMN has been known since 1960s[15]. The presence of PLA2R staining, preponderance to IgG4, seropositivity for PLA2R antibody and absence of etiological factors for secondary membranous nephropathy serve as a criteria for differentiating the two types of MN[16, 17].

A genome wide association study (GWAS) has identified the most significant association within HLA-DQA1 and PLA2R1 on chromosome 2q24[18]. The combination of the susceptibility genotypes (A/A and A/G for HLA-DQ1 and A/A for PLA2R1) significantly predicted the response to treatment[19]. Despite all the progress in understanding of genetics related to iMN, the inheritance patterns could not be identified yet[1, 20].

The antigen PLA2R belongs to the mannose receptor structural family consisting of the mannose receptor, DEC205, Endo180, and FcRY. It is normally weakly expressed in the podocytes of healthy individuals[21]. It is a large transmembrane glycoproteins with short cytoplasmic tail[22]. Its exact physiological function still needs to be explored[23]. However in vitro studies suggest that it is important for podocyte adhesion to glomerular basement membrane type IV collagen[24]. The ultra-structure of PLA2R shows a N-terminal ring made up of ricin domain and a

C-terminal tail of CTLD4-8 domains. The main target epitope for anti-PLA2R is the N-terminal ricin domain. Other epitopes that have also been identified as target for anti-PLA2R include CTLD1 and CTLD7. It is interesting to note that the antibodies towards these less prominent domains appear only after saturating the primary ricin epitope. This phenomenon is described as "epitope spreading" and it indicates poor outcomes and severe disease[25].

It is an established fact that the positive staining for PLA2R in glomeruli is more sensitive than detecting PLA2R antibody in serum. It is positive in 80% to 90% cases including those showing low or absent seropositivity[26]. In a meta-analysis of 19 studies and >1100 cases focused on the diagnostic accuracy of glomerular staining in primary versus secondary MN, a sensitivity of 0.78 and a specificity of 0.84 with an area under the curve receiver operating characteristic of 0.84 was determined[27]. The glomeruli of patients with iMN showed colocalization of PLA2R with IgG4[14, 28].

We designed a study with an aim to see the glomerular expression of PLA2R in patients with iMN using immunohistochemistry (IHC) method. Immunohistochemistry (IHC) technique has gained immense popularity since 1980s due to its cost effectivity and applicability to formalin fixed and paraffin embedded (FFPE) tissue. This technique is now widely used in diagnostic histopathology[29]. Preserving tissue as FFPE tissue is a standard procedure used worldwide in all the pathology laboratories. These blocks are an important source of scientific research and have significant impact on our disease understanding and patient management[30-33]

Materials and Methods:

Data was searched from Nephrology and Pathology record from 2010 to June'18 and all the cases diagnosed as iMN were identified. The relevant clinical record was searched to rule out the possible cases of secondary membranous nephropathy. After confirming the cases for iMN based on light microscopy and clinical record, relevant formalin fixed paraffin embedded (FFPE) blocks were searched. The final inclusion in to the study was based upon and availability of adequate FFPE renal tissue. The study was approved from the institutional review board.

Routine IHC is done at our histopathology department using standard guidelines[34]. The antigen retrieval was done using citrate buffer and heating to 100^{o} C in water bath. The polyclonal rabbit antihuman antibody for PLA2R (orb31277; Biorbyt) was used.

Initially 10 cases were selected for staining, using the above-mentioned antibody. Advice was taken from the company for any alteration in the method for performing IHC and their technical team agreed with our technique. Since there was no previous study and we didn't have the positive control the antibody was diluted in various dilutions ranging from 1:50 to 1:400 in an attempt to find out the proper dilution for appropriate staining.

Technical assistance was also taken from Nephrology and Pathology departments at Peking University First Hospital, Beijing, China.

Results:

PLA2R staining was performed both at pathology department Fatima Memorial Hospital and Peking University First Hospital.

At Fatima Memorial Hospital:

Results turned out to be negative in all cases in initial attempt. Further attempts were done in which dilution was further increased and heating time for antigen retrieval was increased. All the cases again showed negative staining for PLA2R. These cases showed variable staining for IgG, however IgG subtype was not evaluated.

At Peking University First Hospital:

Considering the fact that there might be some error in our technique, nine cases in two different sessions (five and four respectively) were sent to Nephrology and Pathology department, Peking University First Hospital for evaluation. PLA2R staining is done in routine at this centre and it is included in the diagnostic criteria for iMN. All the cases were stained for PLA2R using IHC and IF along with positive controls. The PLA2R staining turned out to be negative for all of the nine cases.

For further confirmation all the cases were stained for IgG4 using IHC along with positive control. IgG4 staining also showed negative results.

To further confirm our diagnosis, we decided to perform electron microscopic studies on our renal tissue. Only one case fulfilled the eligibility criteria for EM as the rest of blocks didn't have enough renal tissue. The results of EM confirmed the diagnosis of membranous nephropathy. There were sub epithelial deposits along the basement membrane. The tissue structure was not very well preserved. It was labelled as stage one membranous nephropathy. The poor preservation of tissue structure was attributed to poor tissue fixation process.

Discussion:

This is an unusual finding to have all the cases of iMN negative for PLA2R staining. Even in the absence of PLA2R staining there is positive staining for IgG4. In our cases both showed negative results. This can be either due to some unknown variant that is involved in the disease process of patients from our region or errors in tissue handling and fixation process, which was the most likely reason in our study.

Tissue processing and fixation:

Various studies suggest that the IHC become problematic in archived FFPE tissue blocks over time. The IHC process becomes more difficult as the age of FFPE tissue sample increases[35, 36]. The antigenicity of the properly preserved FFPE tissue samples is retained for decades. The loss of antigenicity is a slow process and there are many studies found in the literature that are done on archived FFPE tissue blocks. The exact cause of antigen degradation is uncertain but suggested probabilities include oxidation process and archival conditions[37-39]. Section aging (delay between cutting sections and IHC staining) and humidity have been documented among the most important cause of decrease antigenicity. Slide mounted sections can decrease antigenicity as early as 2 weeks from sectioning[40-42]. Ideally the slides should be used within one week[43, 44]. The best suggested method for storage of FFPE blocks and sections is to keep them at cold temperature[45]. Endogenous and exogenous water content also plays a key role in loss of antigenicity[40]. Poor fixation process and use of non-standardised procedures and fixatives is major cause of increased endogenous water content. Inadequate storage conditions like damp places with environmental humidity, poor temperature control and torn plastic bags subjected to light and dust may add to the loss of antigenicity[29].

In addition to the duration of the archived tissue blocks various other factors have been identified that effect IHC process, collectively named as “pre analytical factors”[46]. As much as 15 factors have been identified that potentially effects the IHC and hence have significant impact on making final diagnosis[47]. These factors include fixation delay, fixative type, time in fixative, reagents used, conditions of dehydration, clearing, paraffin impregnation and conditions of slides drying and storing[47]. There is no standardized technique to handle these factors. Many different techniques are considered acceptable within guidelines[48].

With the passage of time the antigen retrieval becomes difficult but no exact expiry duration can be defined. Studies suggest that archived samples can be used for protein detection up to 10 years and for morphology up to 25 years[49]. Membranous and especially nuclear antigens are more subject to antigen decay[35, 50, 51]. The suggested protocol to perform IHC for such cases is to take deeper

samples i.e. sections collected after discarding first 500 – 1000 um of tissue. Lengthening the heat pre-treatment also helps in better antigen retrieval in old FFPE tissue samples[29].

Other Variants of iMN:

PLA2R staining is positive in 70 – 80% of cases with iMN. There is still a proportion of cases that do not show positivity for PLA2R. It is suggested in these cases the auto-antibodies are directed against different podocyte antigens[52]. A recent finding is detection of antibodies directed against the podocyte membrane protein thrombospondin type-1 domain-containing 7A (THSD7A). THSD7A positivity has been found in approximately 8 – 14% PLA2R-negative iMN patients[53]. THSD7A is a transmembrane protein and stimulate IgG4-predominant auto-antibody response[54]. In vitro studies show colocalization of THSD7A with focal adhesion proteins in podocytes[53]. THSD7A is located at very basal surface of podocytes in humans and rodents. These findings suggest its physiological role in adhesion of podocytes with glomerular basement membrane[55, 56].

Collectively PLA2R and THSD7A represent only 85% of iMN. Dual negative cases with IgG4 positivity have been described in some studies[57, 58]. There is still another 15% cases for which potential autoantigens are awaiting discovery[54].

Conclusion:

The pre-analytical factors play a key role in making a final diagnosis. A standardized process for tissue processing and fixation should be devised that may help in better tissue preservation. Our study also emphasises on the importance of technical staff in diagnostic histopathology. While focusing all of our attention towards clinical work we should not forget this important component. Technical staff should be encouraged to get more expertise and should be sent for advanced training in specialized centres.

Electron microscopy is now a necessity for making appropriate and correct diagnosis. Collaboration with advanced centres may help in partially overcoming this deficiency and solving mysteries.

While PLA2R and THSD7A related iMN represent the major portion of iMN, there is still a room for further discovery of auto-antigen sites. We need to promote the research culture and multi centre trials should be conducted to look into prevalence of these auto-antigens in iMN from our region.

Limitations:

The main limitations of our study include small sample size and lack of electron microscopic study on all samples. The serum samples were not available otherwise detecting anti-PLA2R in serum might have aided in making diagnosis.

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