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Anti-Phospholipase A2 Receptor Antibodies as Diagnostic Modality in Primary Idiopathic Membranous Nephropathy: Experience of Two Tertiary Care Centers

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ABSTRACT

Background: Phospholipase A2 receptor (PLA2R), an IgG4 subclass antibody, is being extensively studied for diagnosis, monitoring and prognostication of cases of primary membranous nephropathy (MN). There is significant heterogeneity within the studies with most of the work targeted towards circulating PLA2R antibody. The present study evaluates PLA2R glomerular staining in primary MN as a diagnostic test in conjunction with the serology.

Methods: Diagnostic study. We studied paraffin-embedded kidney biopsies of 60 patients with biopsy-proven MN, for the presence of PLA2R in glomerular immune deposits utilising immunohistochemistry. 60 cases of non MN cases along with autopsy renal tissue were used as controls. A subset of patients (n=40) were tested for circulating PLA2R antibody using quantitative sandwich enzyme immunoassay.

Results: Positive PLA2R expression was present in 37/39 (94.8%) cases of primary MN, 5/21 (23.8%) of secondary MN and 4/60 (6.6%) of non MN group. The expression was detected along glomerular basement membrane in granular pattern of varying intensities. Circulating anti-PLA2R antibodies were detected in 71.42% of primary MN cases, 8.3% of secondary MN cases and non MN group. PLA2R immunostaining exhibited higher sensitivity and modest specificity for differentiating primary from secondary MN (ROC-AUC :0.885 of IHC vs ROC-AUC: 0.776 of serology). The immunostaining however required precision and expertise to read the results.

Conclusion: Tissue staining of PLA2R antibody in renal biopsies will be complementary to serology and two done in conjunction will be better discriminator between primary and secondary MN

Keywords: *Glomerulous, Immunohistochemistry, Membranous Nephropathy, Phospholipase A2 Receptor Antibody*

Introduction

Membranous nephropathy (MN) is a common cause of adult onset nephrotic syndrome. Management of the disease is challenging since it is heterogeneous with important management differences between the categories of primary and secondary MN. [1] Beck *et al* in his seminal study found M-type Phospholipase A2 receptor (PLA2R) as major target podocyte antigen in primary MN.[2] Approximately 80% of patients with primary MN are found to be seropositive for anti-PLA2R antibodies at diagnosis and the antibody disappears following treatment only to reappear with relapse. [1,3-6] Antibodies to PLA2R have come out as a promising biomarker for the diagnosis and monitoring of disease activity in primary MN.[7-10] Moreover anti-PLA2R antibodies also help in predicting disease recurrence after renal transplantation. [11,12] Serological detection of the antibody, which is the most commonly used method, can be complemented by tissue staining of PLA2R in renal biopsies. The data pertaining to tissue staining of PLA2R is small with even fewer studies employing immunohistochemistry (IHC) over indirect immunofluorescence (IIF) for staining of renal biopsies for PLA2R.[13-16] In this study we intend to share experience of two tertiary care centers of India for evaluation of anti-PLA2R antibodies as a diagnostic test for detection of primary MN in our cohort using immunohistochemistry and enzyme-linked immunosorbent assay (ELISA).

Materials and Methods

Study Design: Diagnostic study. Sixty cases of membranous nephropathy diagnosed over a period of three years at departments of pathology and nephrology of two tertiary care hospitals were studied. All cases that underwent renal biopsy as a part of their diagnostic workup were included in the study. Patients with inadequate follow up data, and quantitatively inadequate material were excluded from the

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study. Any history of prior immunosuppression was also recorded and such cases were removed from the study. The relevant demographic and clinical data was accrued from the data maintained in the ward and OPD register. Sixty cases with renal biopsies/autopsy tissue carried out for cases other than MN were also evaluated. Ethical clearance was obtained from the institutional ethical committee of both the institutions.

Histopathological Evaluation: Informed consent was obtained from all patients. A minimum of two cores of renal biopsy were obtained. One core, received in 10% formalin, was processed for paraffin embedding and stained as per standard institutional protocol for evaluation of renal biopsy. The second renal biopsy core was processed for direct immunofluorescence (DIF) utilizing fluorescein isothiocyanate (FITC) tagged immunoglobulin (Ig)G, IgM, IgA, C3c and C1q. The biopsies were processed for electron microscopy wherever indicated. MN was defined as presence of diffuse thickening of glomerular basement membrane along with presence of subepithelial argyrophilic spikes on histopathology, granular membranous lgG deposits on DIF and subepithelial electron dense deposits on electron microscopy. Cases were designated as secondary MN if they had an active disease that is a known secondary etiology of MN and designated as primary after exclusion of known secondary etiologies.

Immunohistochemistry: The primary antibody used was anti-Phospholipase A2 receptor antibody with a dilution of 1:150 (Sigma, catalogue number: HPA012657-100UL). Secondary detection system used was poly-horse radish peroxidase reagent conjugated to a biotinylated anti-rabbit secondary antibody with Diaminobenzidine (DAB) as chromogen (DAKO- Code K5007).

Evaluation of Immunostaining:

1. Positive glomerular basement membrane (GBM) staining or negative GBM staining.
2. Pattern: granular and linear
3. Extent:
    (a) Negative staining or linear GBM stain:0
    (b) Granular weak interrupted staining: 1
    (c) Diffuse and global granular staining:2

Serological Analysis: Serum samples were collected for serological studies before institution of any treatment. These samples were tested for antiPLA2R1 antibodies by using quantitative sandwich enzyme immunoassay (MyBiosource, Catalogue number-MBS705318) with detection range of 0.312 ng/ml-20 ng/ml.

Elucidation of Diagnosis and Statistical Analysis: An excel data sheet was generated to analyze the data on SPSS version 21. The assessment of diagnostic test (PLA2R) was carried out by calculating sensitivity, specificity, positive- and negative predictive values. Receiver operating curves with area under curve (ROC-AUC) was also calculated. For all, p value of 0.05 or less was considered significant.

Result

Demographic and Clinical Profile (n=60): Mean age of the patients was 44.3 years (20-83 years). There were 50 males (83.3%) and 10 females (16.7%) with patients in secondary MN group being younger than primary MN (48 years +/-14.6 SD vs 38 years +/- 8.3 SD, p=0.001). Female preponderance was seen in secondary MN group (p=0.002, x^2=10.6). Fifty patients had nephrotic range proteinuria while 10 patients exhibited subnephrotic proteinuria. Mean 24 hour proteinuria was 5.6 grams (gm) +/- 2.6 SD (2.5-10.5 gm). Hepatitis B virus (HBV) DNA and HBsAg were positive in two cases, Hepatitis C virus (HCV) RNA was detected in 04 cases, rheumatoid factor was positive in one case. Antinuclear antibody (ANA) and dsDNA were positive in 06 cases. One case each was diagnosed to have filariasis, myasthenia gravis, mixed connective tissue disorder and graft versus host disease. Two cases exhibited history of nonsteroidal anti-inflammatory drugs (NSAID) intake and two of associated thyroiditis.

Histopathological Diagnosis and PLA2R Expression: The cases (n=60) were categorized into primary MN (39/60 cases, 65%) and secondary MN (21/60 cases, 35%). PLA2R expression was negative in 10/60 (16.7%) and preserved in 50/60 (83.3%) cases. (Figure 1-4). The distribution of cases with respect to PLA2R expression in cases is depicted in Table 1. PLA2R expression in control cases (n=60) was positive in 4/60 cases. Cases that exhibited positive granular GBM staining were two cases of IgA nephropathy, cryoglobulinaemic membranoproliferative glomerulonephritis (MPGN) and one case of light chain deposition disease (Table 2). Results of serological studies were available in 40 patients of MN and hence equal numbers of controls were tested after random selection. The results are depicted in Table 1 and 2. The distribution of PLA2R positivity and serological status is represented in Table 3.

PLA2R as Diagnostic Test: Performance of PLA2R as diagnostic test was evaluated by determining the sensitivity, specificity, positive and negative predictive values. positive- and negative likelihood ratios. Combination of histopathology, DIF, electron microscopy and clinical evaluation was taken as gold standard. For

analysis, the variables taken were primary MN versus secondary MN (n=60) and primary MN versus secondary MN + Control group. These groups were evaluted for immunohistochemistry and serology. The results are depicted in Table 4. The ROC-AUC for primary MN diagnosis by immunohistochemistry was 0.885 (0.783-0.987, 95% CI) and for serology was 0.776 (0.610-0.921, 95% CI).

Table 1: Distribution of membranous nephropathy cases with respect to Phospholipase A2 receptor expression (PLA2R) (n=60).

S. No.	Diagnosis	No of Cases	PLA2R Positivity (Immuno histochemistry)	PLA2R Positivity (Serology)
1	Primary Membranous Nephropathy	39	37/39 (94.8%)	20/28 (71.42%)
2	Secondary Membranous Nephropathy	21	5/21 (23.8%)	1/12 (8.3%)
	Lupus nephritis Class V	04	0/4	0/1
	Lupus nephritis Class IV+V	02	0/2	0/2
	Hepatitis C virus associated	04	2/4	0/1
	Hepatitis B virus associated	02	0/2	0/2
	Nonsteroidal anti inflammatory drugs	02	0/2	0/1
	Juvenile rheumatoid arthritis	01	1/1	1/1
	Filariasis	01	0/1	0
	Thyroiditis	02	0/1	0/1
	Mixed connective tissue disorder	01	0/1	0/1
	Graft Vs Host disease	01	1/1	0
	Myasthenia gravis	01	1/1	0/1

Table 2: Distribution of Phospholipase A2 receptor (PLA2R) positivity in control group.

S. No.	Diagnosis	PLA2R Positivity (Immuno Histochemistry) n=60	PLA2R Positivity (Serology) n=40
1	Minimal change disease	0/25	15
2	Focal segmental glomerulosclerosis	0/6	10
3	IgA Nephropathy	2/3	2/2
4	Diffuse proliferative glomerulonephritis	0/1	0/1
5	Mesangioproliferative glomerulonephritis	1/3	0
6	Membranoproliferative glomerulonephritis	0/2	0/1
7	Tubulointerstitial nephritis	0/2	0
8	Lupus nephritis	0/9	0/1
9	Renal amyloidosis	0/2	0
10	Light chain deposition disease	1/1	0
11	End stage renal disease	0/2	0
12	Autopsy tissue of normal renal parenchyma	0/4	0
	TOTAL	**4/60 (6.6%)**	**2/40 (5%)**

Table 3: Distribution of results of serology with immunohistochemistry cases, n=40.

Distribution of cases		Immunohistochemistry		Total
		Negative	Positive	
SEROLOGY	Negative	10	9	19
	Positive	0	21	21
Total		**10**	**30**	**40**

Table 4: Performance of Phospholipase A2 receptor antibody as a diagnostic test in Membranous Nephropathy and Control Group:

Categories	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	PLR	NLR	Diagnostic Odds Ratio
Primary Vs Secondary MN (IHC)	94.87	76.19	88.10	88.89	3.98	0.07	14.85
95% CI	82.68-99.37	52.83-91.78	77.43-94.10	67.01-96.92	1.85-8.59	0.02-0.27	
Primary Vs Secondary MN + Controls (IHC)	94.87	92.59	82.22	97.33	12.81	0.06	168.81
95% CI	82.64-99.37	84.57-97.23	74-93	90.68-99.60	5.91-27.76	0.01-0.22	
Primary Vs Secondary MN (Serology)	71.43	91.67	95.25	57.89	8.57	0.31	27.5
95% CI	51.33-86.78	61.52-99.79	75.11-99.25	42.76-71.68	1.29-56.80	0.17-0.57	
Primary Vs Secondary MN + Controls (Serology)	71.40	94.43	86.96	85.96	12.38	0.30	
95% CI	51.33-86.78	61.52-99.79	75.11-99.25	42.76-71.68	1.29-56.80	0.17-0.57	40.83

Legends to Table
MN – Membranous Nephropathy
PPV- Positive Predictive value *NPV- Negative Predictive Value*
PLR-Positive Likelihood ratio *NLR- Negative Likelihood ratio*

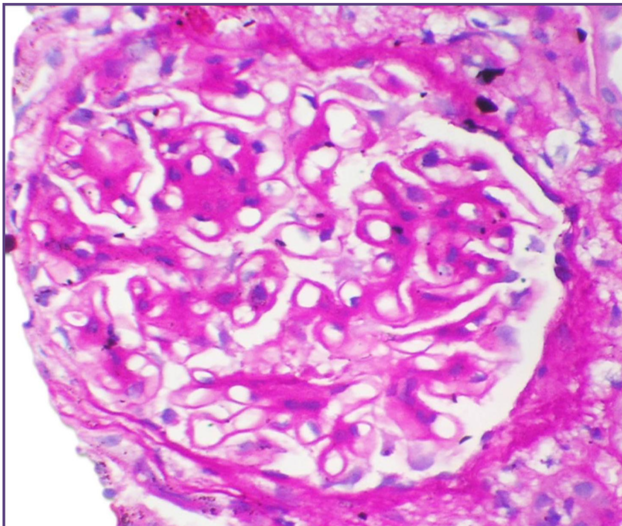

Fig. 1: Glomerulus showing diffuse thickening of glomerular basement membrane. (PAS stain, magnification 200x).

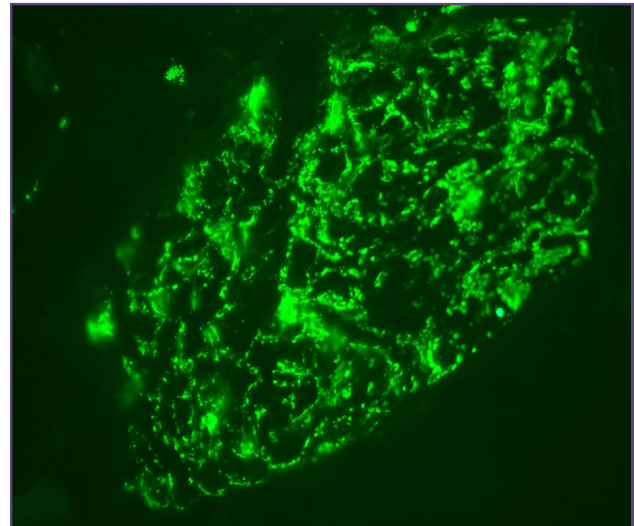

Fig. 2: Diffuse granular positivity 3+ for IgG along the glomerular basement membrane, direct immunofluorescence for IgG antibody (Flourescein isothiocyanate, magnification 200x).

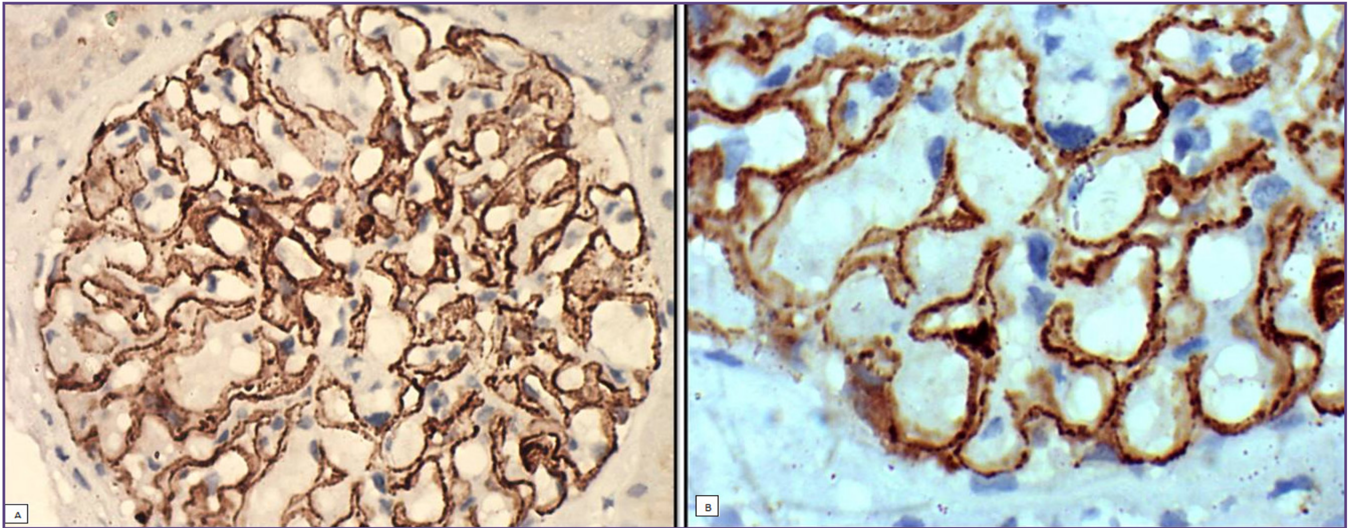

Fig. 3: (A) Phopholipase A2 receptor antibody staining (intensity-3) exhibiting staining along the glomerular basement membrane in a granular pattern. (Immunoperoxidase stain with DAB, magnification 400x) (B) Prominent granularity noted in oil immersion (magnification 1000x).

Discussion

Primary MN needs clear delineation from secondary MN due to its autoimmune etiology evidenced by identification of the anti-PLA2R antibody, an IgG4 subclass antibody deposition in glomeruli.[17] There are important management and prognostic issues between the two categories as management is targeted towards the underlying disease in cases of secondary MN while primary MN requires upfront management by immunosuppressant and other supportive measures. Differentiation between the two categories is largely carried out by excluding the secondary etiologies using a detailed medical history, laboratory studies and clinicoradiological examination. Anti-PLA2R antibody is fast becoming a standard of care for objectively categorizing the cases of MN. However the performance of PLA2R as diagnostic test is variable with conflicting results from different center.[18] The immunohistochemical staining of renal biopsies and its correlation with serological studies has also not been clearly defined with very few centers using immunohistochemical staining of renal biopsies. [19] This study was aimed at analyzing performance of PLA2R antibody in our cohort of patients with equal number of controls by immunohistochemical and serological studies.

Anti-PLA2R Antibody in Primary vs Secondary MN vs Control Group: Ninety four percent (37/39) cases of primary MN exhibited diffuse granular GBM staining of varying intensities. One negative case on careful questioning had received immunosuppression in the past while the second patient, a 24 year old male, probably had a secondary cause of MN which has not manifested clinically. Limited data is available for interpretation of PLA2R staining on paraffin embedded sections. We followed the sharp granular GBM staining pattern with scoring of intensity as described by Hoxha *et al* and Larsen *et al.*[13,14] Another agreement was presence of faint linear GBM staining which we considered as negative.

All cases which stained positive on biopsy were positive on serology (n=21) while 9 cases negative on serology stained positive on renal biopsy. Rapid antibody clearance from the blood with persistent deposition in the glomeruli may explain the seronegative cases. Secondary MN group and non-membranous group also exhibited positive glomerular staining in few cases. Within the subgroups, positivity was noted in cases where it might have resulted consequent to polyclonal hypergammaglobulinaemia as an aberrant expression of PLA2R. Subgroup analysis by Huanzi Dai *et al* showed significant discrepancy between different forms of secondary MN with highest diagnostic accuracy when secondary MN was lupus MN.[20] We did not carry out subgroup analysis but all our cases of Lupus Nephritis Class V were negative for PLA2R.It suffices to say that clinical significance of anti-PLA2R1 antibodies in secondary MN with positive PLA2R staining requires wider sampling and screening is necessary for specific subgroups especially in older patients even if serological anti-PLA2R and/or histological PLA2R antigen is positive.

Performance of PLA2R as Diagnostic Tool: Our assessment of PLA2R was largely based on immunohistochemical staining with subset tested for circulating PLA2R in variance to most of the studies where serological analysis was the dominant test. The index study used secondary MN cases, non-MN cases including

autopsy tissue as controls and analyzed variables against them. Overall tissue staining for PLA2R exhibited high sensitivity (ROC-AUC :0.885 of IHC vs ROC-AUC: 0.776 of serology), modest specificity with moderate negative and positive predictive values for differentiating primary from secondary MN. The performance was optimal with good sensitivity and specificity when non-MN patients were used as controls. However the confounding factor of having received immunosuppressive therapy prior to biopsy remains. Tissue staining was found to be more sensitive than serology for both the categories while serology was found to be more specific. It needs to be emphasized that standardization of the staining, careful interpretation of the granular pattern is of utmost importance to reduce the false positives.

Debiec and Ronco evaluated anti-PLA2R antibody in glomerular deposits and serum with sensitivity of 57% and 74%, respectively, with 21/42 cases exhibiting concordance between serum and glomerular deposits.[1] An updated meta-analysis of 19 studies and 1160 patients was conducted to clinically evaluate the value of serological anti-PLA2R test and histological PLA2R staining. This study found an overall sensitivity, specificity, diagnostic odds ratio and ROC-AUC of serum anti-PLA2R to be 0.68 (95% CI, 0.61–074), 0.97 (95% CI, 0.85–1.00), 73.75 (95% CI, 12.56–432.96) and 0.82 (95% CI, 0.78–0.85), respectively, with substantial heterogeneity (I^2 = 86.42%) between the studies.[20] Another similar study for PLA2R displayed 99% specificity and 78% sensitivity for the diagnosis of primary MN with tissue staining and serology distinguishing most cases of primary MN from secondary.[21] Larsen *et al*. in his series of 85 primary MN and 80 secondary MN found sensitivity of 75% (95% CI: 65−84%) and specificity of 83% (95% CI: 72−90%).[14]

Hence absence of circulating anti-PLA2R autoantibody at the time of kidney biopsy does not rule out a diagnosis of PLA2R-related MN. Therefore, assessment of both circulating anti-PLA2R antibody and PLA2R in biopsy samples will facilitate better categorization of patients with prognostic and therapeutic implications. Immunostaining by other newly discovered autoantibodies like thrombospondin type 1 domain-containing 7A (THSD7A) may help further characterize the heterogeneous nature of membranous nephropathy[22-24]

Limitations of The Study

The study was limited by heterogeneous population within the groups of secondary MN and non-membranous etiologies. Besides, evenly matched biopsy and serological numbers may have provided optimal results. We tried sincerely to exclude cases who had received immunosuppression but likelihood of prior treatment remains.

Conclusion

PLA2R testing has generated tremendous interest in utilization of this marker for disease diagnosis, monitoring and for risk stratification. Our study concludes that tissue staining for PLA2R is a valuable diagnostic tool as the glomerular deposits of PLA2R persist longer and even after clearance of circulating antibody. It has an additional advantage over IIF as it can be carried out on retrospective cases. However the evaluation of biopsies requires standardization and expertise for correct interpretation and may result in false positives and lower specificity. Assessment of both circulating PLA2R antibodies and PLA2R antigen in biopsy specimens might be a better discriminator between primary and secondary MN than only assessing the levels of anti-PLA2R antibodies.

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