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Original Article

Diagnostic utility of C4d immunohistochemistry in membranous nephropathy

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Abstract: Objectives: Membranous nephropathy (MN), also called membranous glomerulopathy, is one of the leading causes of nephrotic syndrome in adults which is defined by the presence of subepithelial immune complex deposits with a spectrum of changes in the glomerular basement membrane (GBM). It is known that C4d is a byproduct of the classic and lectin pathway. There is deposition of C4d noted in the cases of immune complex-mediated glomerulonephritis involving the classical/lectin pathway including MN. The main objective of this study is to assess the utility C4d as an immunohistochemical (IHC) stain in MN. Materials: A total of 43 cases of MN (primary & secondary) were taken, and 39 cases of minimal change disease (MCD)/focal segmental glomerulosclerosis (FSGS) were used as the control group. All the relevant data were retrieved from the hospital database. C4d immunohistochemistry was performed in the cases as well as the control group. Results: A diffuse continuous staining pattern in the glomeruli was observed in cases of primary MN whereas a discontinuous staining in the glomerull favors a secondary MN. 26/29 cases of MCD showed positivity in the podocytes. Among the cases of FSGS, 7/10 cases showed positivity in the podocytes with 3 cases showing an associated mesangial blush pattern of staining. Conclusion: Very few studies are available demonstrating the importance of C4d IHC in MN. C4d IHC can be a useful adjunct for immunofluorescence, especially in cases of early MN.

Keywords: C4d, immunohistochemistry, membranous nephropathy

Introduction

Membranous nephropathy (MN), also called membranous glomerulopathy, is of the leading causes of nephrotic syndrome in adults. MN is defined by the presence of subepithelial immune complex deposits with a spectrum of changes in the glomerular basement membrane (GBM).

IgG4, the predominant subtype of antibody in the sub-epithelial deposits of MN, has a poor ability to fix complement, which underscores an alternate pathway of complement activation in the activation of the membrane attack complex (MAC). As C4d is a byproduct of the classic and lectin pathway, the deposition of C4d is noted in the cases of immune complex-mediated glomerulonephritis involving the classical/lectin pathway [1].

The association of glomerular disease with complement abnormalities is emerging as a vital tool in deriving new perspectives pertaining to the underlying pathogenesis. It is a known fact that C4d immunostaining is used as an adjunct in the diagnoses of antibody-mediated rejection in graft biopsies. C4d, a byproduct of C4 activation, has the propensity to bind to the cell surface because of its thioester bond serving as a marker of complement activation. Detection of C4d deposition by either immunofluorescence (IF) or immunohistochemistry (IHC) technique can be employed in demonstrating evident/masked antigen-antibody complex deposits in native kidney diseases [2].

C4d deposits in immune complex glomerulonephritis have become a topic of discussion in recent years and found to correlate with the presence/absence of antigen-antibody complex deposition in glomerular capillaries to a variable extent [3-6]. In addition, C4d IHC can be used as an indirect marker for the demonstration of immune complex deposition in these conditions

when fresh tissue for IF is unavailable, or in a few cases to reveal masked antigens.

The pattern of variable C4d staining in glomerular diseases like IgAN, focal segmental glomerulosclerosis (FSGS), and minimal change disease (MCD) has been elucidated in recent studies [6-8]. However, very occasional studies have elucidated the role of C4d in MGN. Demonstration of C4d deposits in glomerular lesions in podocytopathies aids in understanding immune-mediated damage by complement activation in the absence of evident antigen-antibody complex. Extraglomerular staining in peritubular capillaries and arterioles has been recorded especially in lupus nephritis and Hemolytic uremic syndrome (HUS) [9, 10]. In combination with C1q, C4d stain can help in delineating various complement pathway activations and may assist in the prognostication of various renal diseases that can influence treatment outcomes [11]. Here we discuss the various uses of C4d in glomerular disease. With the available literature, we focus on the utility of C4d in MGN in our study.

The purpose is to highlight the utility of C4d as an IHC marker in cases of early MGN when there is a diagnostic dilemma with MCD and access to immunofluorescence is not possible.

Materials and methods

Renal biopsies received and diagnosed from 2017 to 2020 that were diagnosed as MGN (primary & secondary) and cases of MCD and FSGS were taken as the control group were reviewed from the Department of Pathology, JIPMER, Puducherry. The cases with a complete immunofluorescence profile of IgG, IgA, IgM, C3, C1q, kappa, lambda, and electron microscopy findings were included in this study. The glomerular histology was classified as MGN (primary & secondary) or a non-proliferative pattern of glomerular injury (MCD/FSGS) in correlation with immunofluorescence findings.

C4d immunohistochemistry

Formalin-fixed paraffin-embedded tissue sections were stained using the mouse anti-human C4d monoclonal antibody (clone: C4D204) by the immunoperoxidase method using Ventana BenchMark system. 3-µm-thick sections were deparaffinized followed by heat-induced antigen retrieval in citrate buffer (pH 6) for 20 minutes. Primary antibody C4d was applied for 1 hour at a dilution of 1:100 followed by DAB-based detection. Slides were counterstained with haematoxylin and mounted with DPX.

Glomerular C4d staining intensity was scored semi-quantitatively on a scale of 0 to 3+ as 0 (negative), 1+, 2+, and 3+. A variable staining pattern among glomeruli was interpreted as segmental/global and focal/diffuse similar to glomerular lesions. The location of C4d staining was mapped out based on location in the capillary wall, mesangium, podocytes, tubular basement membrane, and peritubular capillaries. The character of the capillary wall C4d stain was labeled as either a continuous or irregular granular pattern. Evaluation was performed by 2 renal pathologists and a consensus was reached.

Ethical approval was obtained before beginning the study from the institutional ethics committee.

Results

Glomerular C4d staining was assessed in 43 cases of MGN (primary and secondary) and 39 cases of MCD/FSGS which included 29 cases of MCD and 10 cases of FSGS as the control group.

Among the 43 cases of MGN, there were 31 cases of primary MGN & 12 cases of secondary MGN. The 31 cases of primary MGN showed a diffuse continuous positivity in the glomerular tuft whereas the secondary MGN cases showed a discontinuous granular positivity in the glomerular tuft (**Table 1**; **Figures 1**, **3**).

Among the control cases, 26/29 (89.6%) cases of MCD showed weak positivity in the podocytes with 3 completely negative cases (10.4%). 25/26 cases of MCD were associated with nephrotic range proteinuria. All three cases negative for C4d did not have nephrotic range proteinuria (**Tables 2**, **3**; **Figure 2**).

7/10 cases of FSGS showed similar positivity in the podocytes with 3 cases showing additional focal mesangial blush positivity. All the cases of FSGS were associated with nephrotic range proteinuria.

These findings were correlated with the light microscopic and immunofluorescence findings to reach a consensus.

Table 1. Pattern of GBM staining of C4d IHC among MN cases

Membranous Nephropathy (MN) (n=43)	C4d immunohistochemical staining	
	Diffuse, continuous	Discontinuous, granular
Primary MN (n=31)	31 (100%)	0
Secondary MN (n=12)	0	12 (100%)

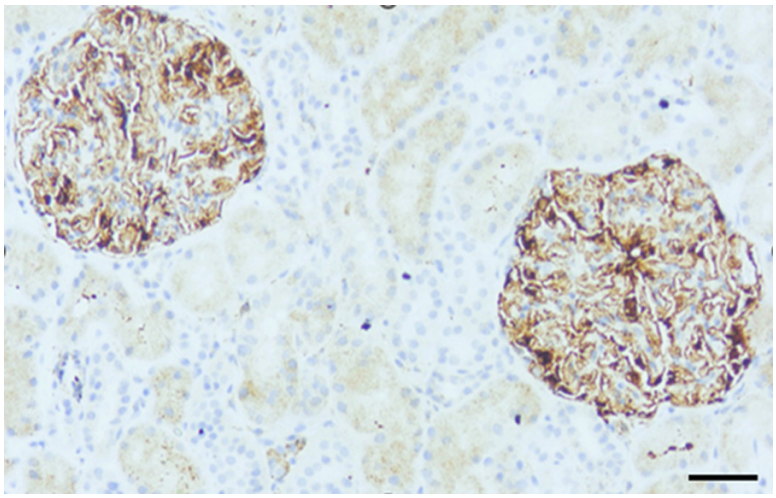

Figure 1. C4d staining in primary membranous nephropathy (MN): C4d immunohistochemistry shows continuous granular positivity in the glomerular basement membrane (H&E-200×) (Scale bar-100 μm).

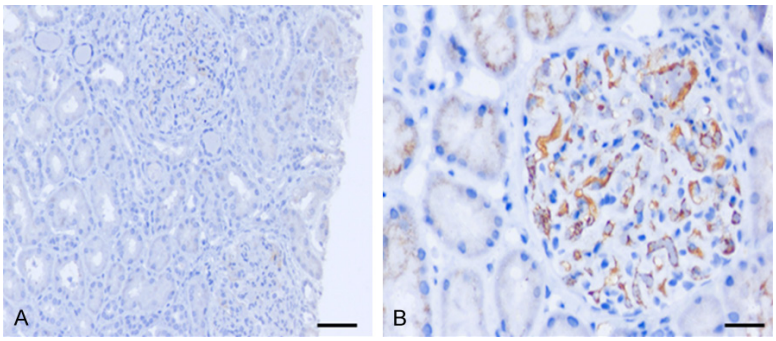

Figure 2. A: C4d staining in minimal change disease (MCD): C4d immunohistochemistry is completely negative in the glomerular basement membrane (H&E-40×) (Scale bar-100 μm). B: C4d immunohistochemistry shows positivity in the podocytes in MCD (H&E-400×) (Scale bar-400 μm).

Discussion

Membranous nephropathy (MN), also called Membranous glomerulopathy, is one of the leading causes of nephrotic syndrome. It is defined by the presence of subepithelial immune complex deposits with a spectrum of changes in the glomerular basement membrane (GBM).

IgG4, the predominant subtype of antibody in the subepithelial deposits of MN, has a poor ability to fix complement, which underscores its role in the activation of the membrane attack complex (MAC) [12]. Activation of the classical and lectin pathway leads to activation of C4 complement protein [3].

Antigen-antibody complex activates the complement pathway followed by the cleavage of C4 domains into C4a and C4b. This is followed by leaving into C4c and C4d. Thus, C4d staining is an adjunct marker for activation of the classical/lectin complement pathway.

Peritubular capillary staining of C4d has been used as a diagnostic marker of humoral rejection [13]. Given the ease of performing IHC, C4d is being used as an adjunct technique in demonstrating evident and masked immune complex deposits in native glomerular diseases [14]. In a resource-poor setting, this can be used as a standalone marker to establish the etiology of glomerular disease. As described earlier, the location, pattern, intensity, and character of C4d deposition in MN and minimal change disease/focal segmental glomerulosclerosis are important for a definite diagnosis [4, 5].

A limited number of studies have documented the deposition of C4d in MN which is due to complement activation and C5b-9 deposition.

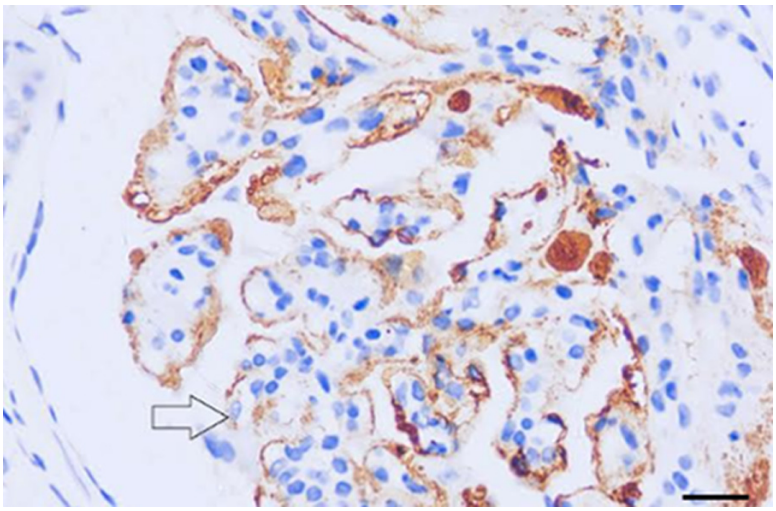

Figure 3. C4d staining in secondary membranous nephropathy (MN): C4d immunohistochemistry shows discontinuous granular positivity in the glomerular basement membrane (H&E-400×) (Scale bar-400 µm).

Table 2. C4d IHC staining pattern among MCD/FSGS cases

Total cases of MCD & FSGS (n=39)	C4d immunohistochemical staining		
	Positive		Negative
	Podocyte	Podocyte + mesangial	
MCD (n=29)	26 (89.6%)	0	3 (10.4%)
FSGS (n=10)	07 (70%)	03 (30%)	0

Table 3. Correlation of C4d staining with nephrotic range proteinuria among MCD & FSGS cases

Total cases of MCD & FSGS (n=39)	Nephrotic range proteinuria	
	C4d positive cases	C4d negative cases
MCD (n=29)	25/26 (96.15%)	0/3
FSGS (n=10)	10/10 (100%)	nil

C4d deposits were localized in GBM with a continuous, granular positivity in the GBM. The above pattern was observed in all cases of primary MN [15]. The cases of secondary MN showed discontinuous granular staining of the GBM. The irregular staining in cases of secondary membranous nephropathy may be attributed to the minimal endocapillary or mesangial hypercellularity. However, there were no mesangial deposits of C4d although there was immune complex deposition by immunofluorescence [5-7].

In addition, we performed C4d staining in cases of MCD and FSGS which acted as a control group. C4d staining in cases of MCD and FSGS was variable. Hence, cautious interpretation of C4d staining in the mesangium is recommended in non-proliferative glomerular diseases. Very limited literature is available to substantiate C4d positivity in MCD & FSGS [16]. In addition, C4d deposition can precede the development of FSGS [11]. In our study, all the cases of MCD and FSGS with C4d positivity showed nephrotic range proteinuria. The three negative cases did not present with nephrotic range proteinuria. This result was similar to the study by Son et al. [16]. In a study by Heybeli et al., it was found that the mesangial pattern of staining of C4d is associated with poorer survival in cases of primary FSGS [17]. The same was questioned in a study by Aksali et al. [18]. In addition, earlier studies showed that C4d deposition in glomerular diseases is associated with a poorer prognosis and evolves into end-stage renal disease, especially in FSGS and IgA nephropathy [19]. The drawback of our study is that we did not do a thorough follow-up which would have helped in our understanding of the disease. Further studies need to be done to help provide conclusive evidence about the diagnostic and prognostic significance of C4d staining in glomerular disease.

Conclusion

Immunohistochemical staining for C4d has revolutionized the field of renal pathology. Though it is a simple diagnostic test, its utility can be of utmost importance, especially in a resource-poor setting where access to immunofluorescence may not be possible. The staining pattern and location will help us to confirm the diagnosis of early MGN as well as explain the pathogenesis of MCD and FSGS.

Disclosure of conflict of interest

None.

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