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Diagostic Accuracy of C4d-IHC in Diagnosis of Membranous Glomerulonephritis

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Introduction. Membranous glomerulonephritis (MGN) is the most common cause of nephrotic syndrome in adults. The gold standard techniques for diagnosis of MGN are based on a constellation of findings given by light microscope, electron microscope (EM), and immunofluorescence (IF). Occasionally, only formalin-fixed tissues are available for the analysis by light microscopy, which have limitations in differentiating minimal change diseases from MGN. Recently, the usage of C4d immunohistochemistry (IHC) has been proposed for the diagnosis of MGN. The aim of this study was to evaluate the accuracy of C4d-IHC in diagnosis of MGN.
Methods. The present investigation conducted on patients with nephrotic syndrome who underwent renal biopsy in Labbafinejad hospital, from 2016 to 2017. The entire specimens were examined by light microscope, immunofluorescence, and electron microscope as a gold standard method for diagnosis of MGN. The samples were then stained for C4d immunohistochemical analysis. Eventually, the sensitivity, specificity, positive, and negative predictive value for C4d-IHC was determined.
Results. The sensitivity and specificity of the C4d-IHC in order to differentiate MGN from other glomerulopathies were 95% and 87.5%, respectively. In addition, the negative and positive predictive values were 97.2% and 79.16%, respectively.
Conclusion. It was ultimately attained that C4d-IHC has more accuracy in identification and diagnosis of MGN, in contrary to EM and IF, this method is more usable and cost effective, which requires a lower level of skill and advanced equipment. Indeed, this technique does not require fresh specimen.

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INTRODUCTION

Membranous nephropathy is one of the most recognized etiologies of nephrotic syndrome which accounts for 20-30% of nephrotic syndrome in adults and 1-9% in pediatric population. The peak incidence is from 30 to 50 years and is two times more common in males than females.[1,2]

Primary type of MGN is more common, but in 25% is secondary to other pathologies like infection, neoplasms and systemic lupus erythematosus (SLE), and is more common in children and elderly patients.[3]

The most indispensable site of pathologic changes is glomerular basement membrane (GBM), including diffuse thickening with spike formation in the basement membrane.[4] Pathologic changes are dynamic; furthermore, in early stages the glomerulus appear normal and is difficult to distinguish from minimal change diseases; therefore, in this case, glomerulus should be examined by

immunofluorescence or electron microscope to determine from minimal change diseases.[4] Diagnosis of membranous nephropathy would not be certain by IF or electron microscope solely if there would not be access to fresh tissue or there would not be any glomeruli in the received specimen. On the other hand, electron microscope is not accessible in the entire centers.

According to the aforementioned limitations and acknowledging that C4d deposition in sub epithelial areas of basement membrane because of complement activation; it could be attained that C4d-IHC may be a useful modality in diagnosis of MGN. To the best of our knowledge this is the first report regarding diagnostic accuracy of C4d-IHC in diagnosis of MGN.

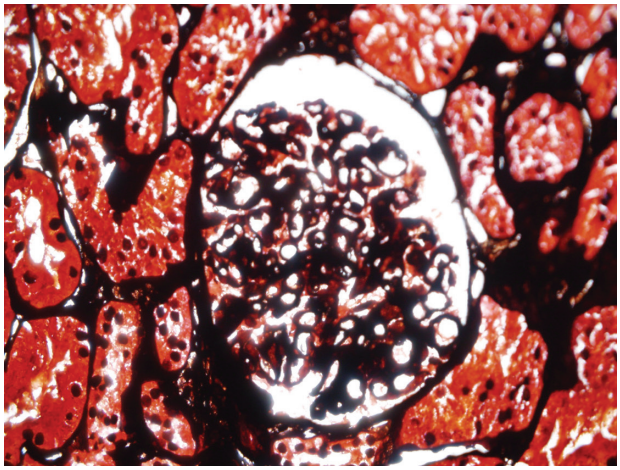

Figure 1. This figure shows Jones' staining of a paitient with membranous nephropathy (×40).

MATERIALS AND METHODS

The present study included patients with nephrotic syndrome and proteinuria who underwent renal biopsy in Labbafinejad medical center, Shahid Beheshti University of medical sciences, from 2016 to 2017. Following data were documented in the case reports: age, gender, lab findings (included creatinine and protein excretion) in all cases if they were available. All specimens were examined with light microscope, electron microscope, and immunofluorescence as the elements of a gold standard method for diagnosis of MGN.

For light microscopy assessment, 3-5 micrometer slices prepared from formaldehyde-fixed paraffin-embedded tissue, and stained with hematoxylin and eosin, periodic acid-Schiff, Masson trichrome and methenamine silver (Figure 1). For IF study, 1-3 micrometer slices prepared from snap freezed tissue in -20 centigrade temperature and then prepared by anti-IgM, IgG, IgA, C3,C4, C1q, albumin, and fibrinogen antibody (Figure 2). The EM was performed on ultrathin slices (50 nm) which were prepared from glutaraldehyde 2.5%-fixed renal tissue according to the standard laboratory protocol (Figure 3). Finally all cases were immunostained with C4d anti-human (clone C4D204, isotype IgG1, Diagnostic biosystem) polyclonal antibodies obtained from rabbit (Figure 4). Sensitivity, specificity, negative, and positive predictive value for C4d-IHC determined. The data were analyzed by using SPSS software version 24 and the significant *P* value was .05.

RESULTS

In this study, 60 patients (28 male and 32 female) with the mean age of 40 years (14 to 76 years old), were randomly selected. The mean serum creatinine level was 1.9 mg /dL (ranging from 0.66 to 9 mg/dL). The mean protein excretion was 3573.5 mg/d (480 to 11000 mg/d). There were no differences between males and females in degree of proteinuria according to the Mann Whitney test.

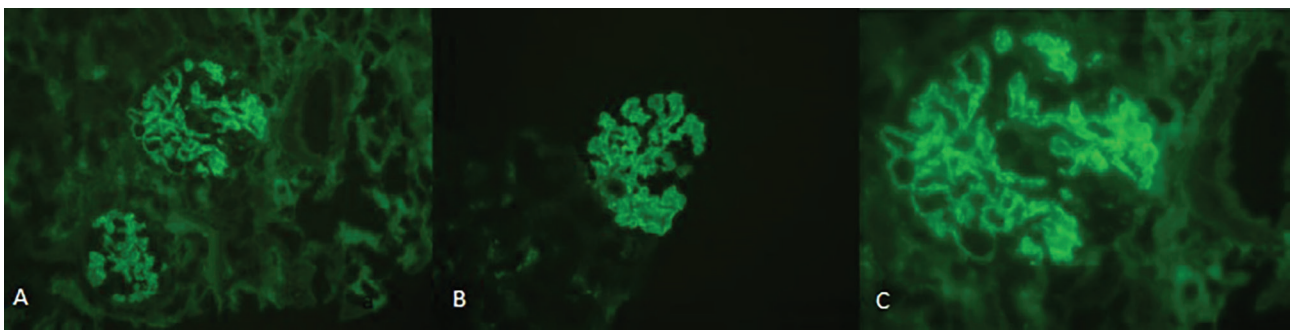

Figure 2. It mentions immunofluorescence findings in MGN. A) staining for C3 reveal granular positivity involving the basement membrane, B) a similar distribution but more intense staining for IgG is present along the GBM, C) total Ig.

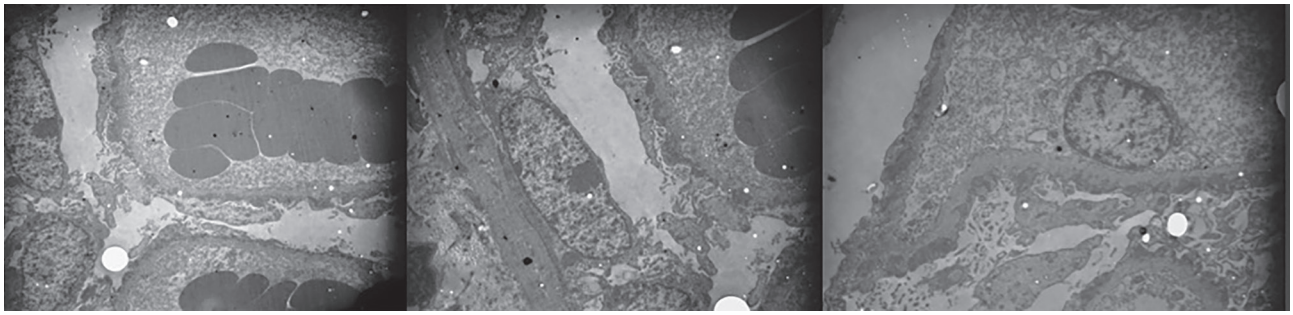

Figure 3. It determines electron microscope study in a patient with membranous nephropathy.

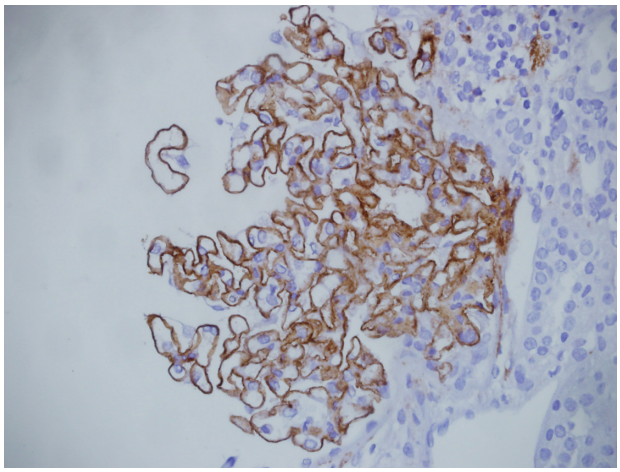

Figure 4. Immunohistochemistry with anti-C4d (polyclonal) in MGN reveals positive immunoreactivity along BM with granular pattern.

These samples including 20 cases of MGN (33.3%), 14 cases of FSGS (23.3%), 7 cases of MCD (11.7%), 5 cases of diabetic nephropathy (8.3%), 6 cases of lupus nephritis (10%), 6 cases of IgA nephropathy (10%), and 2 cases of MPGN (3%).

Considering the mean age of patients, we used independent samples t-test to compare MGN with other diseases. Apparently, no difference was found statistically ($P > .05$). Lack of normal data distribution in serum creatinine level and proteinuria, we had to use Mann-Whitney test to compare these parameters in MGN and other diseases. Serum creatinine level in MGN was significantly lower than other diseases group ($P > .05$); nonetheless, there was no significant difference in proteinuria ($P > .05$).

19 out of 20 cases of MGN had positive immunoreaction for C4d antibody. All cases of MCD, FSGS, IgA nephropathy, and MPGN were negative for C4d. Apparently, 1 out of 5 cases of diabetic nephropathies and 4 out of 6 cases of lupus nephritis were positive in C4d-IHC (Table 1).

There was a significant correlation between positive and negative C4d IHC results ($P < .001$). We divided the participants into 2 groups. The primary group was MGN patients and the secondary groups were MCD, FSGS, DM, lupus nephritis, MPGN, and IgA nephropathy. After that, according to the positive and negative IHC results, sensitivity, specificity, negative, and positive predictive value were calculated which are present in Table 2.

Sensitivity of C4d-IHC in diagnosis of MGN was 95% and the specificity was 87.5%. The negative and positive predictive values were 97.2 %and 79.16%, respectively.

DISCUSSION

The GBM is the main site of involvement in MGN, and detection of it's thickening in light microscopy is helpful in pathologic diagnosis but in early stages of the disease, light microscope findings may be normal; therefore, other modalities such as direct immunofluorescence or electron microscope may be necessary to rule out of other differential diagnosis like minimal change disease. In recent studies, C4d-IHC was suggested as a desirable marker in MGN diagnosis that could be a rapid method with low cost and good availability in most laboratory centers.[5,6] Estimation of diagnostic accuracy of this method was our goal in this study to have better understanding in its proper use in diagnosis of MGN.

60 patients were included in our study with mean age of 40 (17 to 76 years), 51.7% were female and 48.3% were male. Mean serum creatinine was 1.9 mg/dL (0.6 to 9) and most of the patients did not have a high serum creatinine level; in addition, they had approximately normal renal function.

The rate of proteinuria is one of the indicators of disease progression, and daily urine protein level lower than 4g is considered as low risk;

Table 1. Frequency and Percentage of Positive and Negative C4d-IHC Results Among Different Nephropathies

Disease	IHC Result		All	*P*
	Positive	Negative		
MGN				
number	19	1	20	
Disease Ratio, %	95.0%	5.0%	100.0%	
Result Ratio, % IHC	79.2%	2.8%	33.3%	
FSGS				
number	0	14	14	
Disease Ratio, %	0.0%	100.0%	100.0%	
Result Ratio, % IHC	0.0%	38.9%	23.3%	
MCD				
number	0	7	7	
Disease Ratio, %	0.0%	100.0%	100.0%	
Result Ratio, % IHC	0.0%	19.4%	11.7%	
DM				
number	1	4	5	
Disease Ratio, %	20.0%	80.0%	100.0%	
Result Ratio, % IHC	4.2%	11.1%	8.3%	
Lupus Nephritis				< .001
number	4	2	6	
Disease Ratio, %	66.7%	33.3%	100.0%	
Result Ratio, % IHC	16.7%	5.6%	10.0%	
IgA Nephropathy				
number	0	6	6	
Disease Ratio, %	0.0%	100.0%	100.0%	
Result Ratio, % IHC	0.0%	16.7%	10%	
MPGN				
number	0	2	2	
Disease Ratio, %	0.0%	100.0%	100.0%	
Result Ratio, % IHC	0.0%	5.6%	3.3%	
All				
number	24	36	60	
Disease Ratio, %	40.0%	60.0%	100.0%	
Result Ratio, % IHC	100.0%	100.0%	100.0%	

Notes:
• Diseases Ratio = number of positive cases in each group ÷ total number of cases in the group
• IHC Result Ratio = number of positive C4d cases in each group ÷ total number of positive C4d in all groups

Table 2. Frequency of Positive and Negative C4d-IHC Results Between MGN and Other Nephropathies

Disease	Result IHC		All
	Positive	Negative	
MGN			
number	19	1	20
Disease Ratio, %	95.0%	5.0%	100.0%
Result Ratio, % IHC	79.2%	2.8%	33.3%
Other Nephropathies			
number	5	35	40
Disease Ratio, %	12.5%	87.5%	100.0%
Result Ratio, % IHC	20.8%	97.2%	66.7%
All			
number	24	36	60
Disease Ratio, %	40.0%	60.0%	100.0%
Result Ratio, % IHC	100.0%	100.0%	100.0%
All, %	40.0%	60.0%	100.0%

furthermore, the level more than 8 g/24h is considered as high risk for disease progression. The mean daily urine protein level in the patients was 4.1 mg/24h; therefore, most of the patients in the present study were in low to moderate risk of disease progression.

There was negative C4d immunoreactivity in all of the cases of FSGS, MCD, IgA nephropathy and MPGN. Most of the positive C4d-IHC results were regarding primary MGN and lupus nephritis.

The results in the present investigation are similar to a recent study conducted by Hui *et al.* in which C4d-IHC was positive in the primary MGN and also secondary MGN due to lupus and hepatitis C.[6] In contrast, there are some reports of

positive C4d-IHC in IgA nephropathy in which it had been shown that it could be an indicator of the progression of the disease.[7,8] Similarly Shahin OZ *et al.* showed the possibility of prediction of the state of the disease activity in lupus nephritis by the means of C4d-IHC.[9] We also had similar result, as 66.7% of the patients with lupus nephritis in class V, as a secondary membranous nephropathy, showed positive immunoreactivity along BM with granular pattern .

Cases with diabetic nephropathy had 20% positive immuonoreactivity for C4d-IHC with nonspecific pattern including mesangial deposits due to mesangial cell damage and protein in sudation in sub-epithelial and mesangial spaces. In a recent research, Bus *et al.* showed that C4d deposits in glomeruli of 46% of autopsied diabetic nephropathies, which was significantly higher than control autopsies without diabetic nephropathy.[10]

C4d-IHC was positive for all cases of MGN in some studies; however, in our study, 95.2% of MGN showed positive immunoreactivity, although considered as a great number of positive results; nonetheless, it implies the possibility of negative IHC staining MGN. The pattern of IHC staining was similar to the recent studies and in the entire cases of MGN, there were granular pattern along GBM.[5,6]

In this study, sensitivity and specificity of C4d-IHC in diagnosis of MGN were 95% and 87%, respectively. Although, specificity is not as high as sensitivity; however, it is considered remarkable. Considering positive likelihood ratio of 7.3, the probability of positive C4d-IHC result in MGN is much more than other glumerolopathies with nephrotic syndrome

High negative predictive value (97.2%) emphasizes the importance of negative results, and the test efficiency is also high (90%). Wang and collogues showed that 95.6% of the MGN cases were positive for IgM and 88.9% were positive for C3,which to some extent are close to C4d-IHC result in our study (95% positive for C4d-IHC) that emphasizes the similarity of the results of these two methods in diagnosis of MGN.[11]

In our study, 5 out of 40 cases of nephrotic syndrome had positive C4d-IHC result in the absence of primary MGN. Distinctly, one of them was diabetic nephropathy in which there were scattered deposits in mesangium and along GBM with-sub endothelial pattern that could easily be differentiated from MGN. Four out of the five aforementioned cases of non-MGN with positive C4d-IHC result were in lupus nephritis in class V, which was predictable because as primary MGN, there is complement activation in lupus nephritis. If we want to differentiate the primary MGN from membranous lupus nephritis, electron microscopy and immunofluorescence findings beside LM study can be helpful. Typical light microscopy findings in primary membranous nephropathy are GBM thickening and spike formation but the presence of such atypical findings such as mesangial hyper cellularity, endocapillary proliferation, and GBM duplication are in favor of membranous lupus nephritis. According to the recent studies, there is greater prevalence of positive staining for IgM, IgA, C3, and specially C1q with more than 2+ intensity, in membranous lupus nephritis rather than primary MGN.[12] On the other hand, it is mentioned that combined IgA, IgM, and C3 staining, have 29% sensitivity and 87% specificity and immunoreactivity for c1q with more than 2+ intensity, have 67 % sensitivity and 88% specificity in differentiating lupus nephritis from primary MGN. Distinctly, immunofluorescence could not always differentiate the two mentioned diagnoses.[12]

Another study proposed IgG subtypes, as a modality to differentiate primary MGN and secondary forms of MGN in which IgG4 is the predominant subclass in primary MGN while IgG1, IgG2, and IgG3 stain more intensely than igG4 in secondary MGN.[13-5] Electron microscopy plays an important role in diagnosis of MGN; however, it is quite expensive and is not accessible in all centers.

CONCLUSION

It was magnificently attained that C4d-IHC has high accuracy for the identification and diagnosis of MGN. In comparison to the electron microscope and immunofluorescence, this method is more available and less expensive,which require a lower level of skill and advanced equipment; therefore, this technique could be used instead of other expensive and complex modalities such as IF or electron microscope.

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