Immunopathologia Persa

http www.immunopathol.com

Association of glomerular C4d deposition with morphologic variables of Oxford classification in IgA nephropathy patients; a preliminary study

Hamid Nasri[1], Mahmood Rafieian-Kopaei[2], Ali Ahmadi[3], Muhammed Mubarak[4*]

[1]Nickan Research Institute, Isfahan, Iran
[2]Medical Plants Research Center, Shahrekord University of Medical Sciences, Sharhekord, Iran
[3]Department of Epidemiology and Biostatistics, School of Health, Modeling in Health Research Center, Shahrekord University of Medical Sciences, Shahrekord, Iran
[4]Department of Histopathology, Sindh Institute of Urology and Transplantation (SIUT), Karachi, Pakistan

Correspondence to
Prof. Muhammed Mubarak;
Email:
drmubaraksiut@yahoo.com

Received 19 March 2016
Accepted 29 April 2016
Published online 6 May 2016

Keywords: C4d, Oxford classification, Crescents, IgA nephropathy, End-stage renal disease

Abstract

Introduction: IgA nephropathy (IgAN) is known as the most prevalent primary chronic glomerulopathy. Hence, it is of critical importance to identify the aggravating factors impacting the disease progress, and to predict disease-specific therapy.
Objectives: The aim of our investigation was to determine whether C4d deposits in glomeruli had any correlation with various immunopathologic variables of Oxford classification in IgAN.
Patients and Methods: The characterization of IgAN requires the presence of diffuse and global IgA deposits which were graded ≥2+ with weak C1q deposition. C4d immunohistochemical (IHC) staining was conducted retrospectively on 29 renal biopsies of patients with IgAN, who were selected randomly from all biopsies. C4d IHC staining was performed on 3-µm deparaffinized and rehydrated sections of formaldehyde-fixed, paraffin-embedded (FFPE) renal tissues.
Results: In this study, a significant correlation of C4d deposits with interstitial fibrosis/tubular atrophy (IFTA; T) ($r=0.58$, $P=0.001$) was obsereved. An analysis regarding the association of C4d deposits with other deposited antibodies and complement fragments showed that only C3 had a significant negative correlation with C4d deposits ($r=-0.42$, $P=0.02$).
Conclusion: The results show that C4d deposits correlate with IFTA or T variable of Oxford classification of IgAN. There is no significant correlation of C4d deposits with other morphological variables of Oxford classification. C4d deposits showed a negative correlation with C3 deposits. These results need further studies to confirm the relationship of glomerular C4d deposits with immunopathological variables in cases of IgAN.

Citation: Nasri H, Rafieian-Kopaei M, Ahmadi A, Mubarak M. Association of glomerular C4d deposition with morphologic variables of Oxford classification in IgA nephropathy patients; a preliminary study. Immunopathol Persa. 2016;2(2):e18.

Introduction

IgA nephropathy (IgAN) is the most common glomerulopathy throughout the world (1,2). The diagnosis of IgAN is based on the predominance of IgA deposits in the mesangial areas of the glomeruli (1-3). Low intensity of IgG or IgM depositions may also be detected. C1q deposition is typically absent or insignificant and its absence is of diagnostic importance for this glomerulopathy. In some IgAN cases, the mesangial deposits may extend to the peripheral capillary walls too (2-4). Since IgAN appears to be the most prevalent primary chronic glomerulopathy and leads to end-stage renal disease (ESRD) in a significant number of cases, it is of critical importance to identify and characterize potential aggravating factors influencing the disease progress, monitor disease activity and predict disease-specific therapy (1-4). The Oxford classification of IgAN

Key point

In a preliminary study on 29 IgA nephropathy (IgAN) patients, we found C4d deposits correlated with interstitial fibrosis/tubular atrophy of Oxford classification of IgAN. There is no significant correlation of C4d deposits with other morphological variables of Oxford classification. C4d deposits showed a negative correlation with C3 deposits. These results need further studies to confirm the relationship of glomerular C4d deposits with immunopathological variables in cases of IgAN.

established mesangial hypercellularity (M), endocapillary proliferation (E), segmental glomerulosclerosis (S), and interstitial fibrosis/tubular atrophy (IFTA; T), as independent predictors of outcome (2-6). In addition to the above mentioned morphologic variables of Oxford classification, known as MEST variables, other pathologic variables and immunostaining data may also have significant implications for the pathogenesis and progression and require further investigation (1-5).

While IgAN is believed to be associated with renal immunoglobulin deposition; the exact pathogenesis still remains ill-understood (5-8). It is well known that immune complex-mediated complement activation through the classical pathway plays a major role in the pathogenesis of some glomerulonephritides (9-15). In brief, the classical pathway is activated by the binding of the complement C1 complex to the antigen-antibody complex, which is accompanied by the cleavage of the complement component C4 into C4a and C4b, and subsequently C4b is cleaved into C4c and C4d (4-9).

Recently much attention has been directed to the implication of C4d mesangial deposits and its correlation with various clinical data and morphologic variables in glomerulopathies; however, few investigations have been reported on the prevalence and significance of C4d in IgAN (4-7). C4d is generated by activation of the lectin pathway too. It has been suggested that patients with IgAN may be divided into two groups on the basis of the pattern of complement activation. Activation of the lectin pathway of complement is associated with more severe renal damage. Glomerular deposition of C4d is a sign of activation of the lectin pathway of complement (3-8). Therefore, C4d is a recognized biomarker of the complement cascade. Furthermore, C4d staining is an inexpensive and easy method for the investigation of renal biopsies (4-8).

Although C4d itself has no known biological activity, it is recognized for its function as a footprint of complement activation via the classical, alternative or lectin pathways (16,17). It has been widely used in the pathological evaluation of renal allograft biopsies, particularly in the identification of antibody-mediated rejection. However, its role in the investigation of native renal glomerulopathies has been little explored. Thus, it is conceivable to extend the investigation on C4d from kidney transplantation to glomerulonephritis, including IgAN to find the position of C4d in this disease and its clinical implication (6-9).

Objectives

The aim of our current investigation was therefore to determine whether C4d positivity in the glomerular mesangium or capillary walls had any correlation with various immunopathologic variables of Oxford classification in renal biopsies of IgAN patients.

Patients and Methods

Definition of IgAN

The pathologic identification of IgAN needs demonstration of IgA-dominant mesangial or mesangial-membranous immune deposits (≥2+) by immunofluorescence (IF) microscopy with little or no deposits of C_1q. The immune deposits are semiquantitatively assessed as0 to 3+ positive intensity of brightness on IF microscopy. None of the patients received treatment before the biopsy. Biopsies with <8 glomeruli were excluded from the study. Patients with secondary IgAN, having a history of diabetes, liver cirrhosis or collagen vascular diseases, according to the questionnaire filled at the time of biopsy admission, laboratory information in patients' records and a brief history provided by referee physicians at the time of biopsy admission, were excluded.

Immunopathologic studies

All renal biopsies were processed and prepared for light and direct IF microscopy. Tissue for light microscopy (LM) was fixed in 10% formalin for histologic sectioning. Each renal biopsy was prepared by cutting paraffin blocks into 3μm sections and staining for hematoxylin and eosin (H&E), periodic acid Schiff (PAS), Jones methenamine silver and trichrome stains. For conducting the IF study, fresh tissues were snap-frozen in liquid nitrogen. Sections (5 μm in thickness) were stained for IF study with IgG, IgM, IgA, C1q, C3, fibrin and C4. IF slides were reported on a scale of 0-3+ positive staining intensity and by a nephropathologist. IF study was performed before reviewing the slides for LM unaware of patients' data.

Definitions of morphologic variables

The renal biopsies were assessed and interpreted using standard criteria for the diagnosis and pathological reporting of IgAN. The total number of glomeruli and proportion of glomeruli with global sclerosis were recorded for each biopsy. The presence of (I) mesangial hypercellularity (M), (II) endocapillary proliferation (E), (III) segmental glomerulosclerosis (S) and (IV) the proportion of tubular atrophy and interstitial fibrosis (IF/TA [T]) was assessed as published for Oxford classification (1-3). Other important morphologic lesions were also considered, including vascular lesions and extracapillary proliferation in the glomeruli (crescents) (1-4).

C4d staining and histological study

C4d immunohistochemical (IHC) staining was carried out retrospectively on 29 renal biopsies of patients with IgAN, who were selected from all the biopsies of IgAN. C4d IHC staining was performed on 3 μm deparaffinized and rehydrated sections of formaldehyde-fixed, paraffin-embedded (FFPE) renal tissues, using rabbit polyclonal anti-human C4d (Biomedica, Vienna, Austria) as the primary antibody, diluted 1:50 in antibody diluent (Dako, Glosstrup, Denmark). In order to break cross-linking of the proteins which was caused during tissue fixation and processing and to unmask the target protein epitopes, heat-induced antigen retrieval (HIER) was performed in advance using microwave (10 minutes at high power, 10 mM citrate buffer, pH 6.0). After cooling down for 15 minutes at room temperature, endogenous peroxidase was

blocked with 3% H_2O_2 for 5 minutes, followed by washing the sections in TBS. Anti-C4d antibody was applied for 30 minutes at room temperature, followed by washing in Tris-buffered saline (TBS). The detection system used was Dako EnVision (Dako, Glosstrup, Denmark) applied for 30 min at room temperature, followed by washing in TBS. The signal was detected with DAB chromogen (Dako, Glosstrup, Denmark) for 15 minutes, resulting in a brown color. Following the washing step in tap water for a few minutes, slides were counterstained with hematoxylin (Dako, Glosstrup, Denmark) for 2 minutes. After dehydrating, the slides were cover slipped using mounting medium. C4d IHC staining was assessed by the percent of C4d positivity in the glomeruli in each biopsy, through evaluation of all glomeruli in every fragment. Involvement of ≤10% of glomeruli was considered negative. The proportion of glomerular positivity was semiquantitatively divided as 11%-25%, 26%-50%, 51%-75% and 76% to 100%.

Clinical studies and laboratory data

In this retrospective histological investigation, the medical records of patients were reviewed to gather various demographic, laboratory and clinical information at the time of biopsy and for follow-up activities. Data collected at the time of biopsy included race, gender, age, serum creatinine (Cr) and the quantity of 24-hour proteinuria.

Ethical issues

1) The research followed the tenets of the Declaration of Helsinki; 2) informed consent was obtained from all the participants of the study; and 3) the research was approved by the institutional review board of Nickan Research Institute.

Statistical analysis

Numerical variables with a normal distribution were expressed as mean ± standard deviation (SD), and numerical variables without a normal distribution were expressed as the median with minimum and maximum values. To obtain a correlation matrix for quantitative variables the Spearman correlation was used. Data were analyzed by Stata software (Stata Corp. 2011. Stata Statistical Software: Release 12. College Station, TX: Stata Corp LP). *P* values of less than 0.05 were assumed to be statistically significant.

Results

Of 29 study subjects , 68% were male. The mean± SD of patients' age were 35.03 ± 11.7 years. The frequency distribution of glomerular C4d positivity is shown in Table 1. Mean, SD, minimum, maximum, inter-quartile range and percentiles (50, 75, and 95) values for C4d were 54.2, 25.6, 12, 90, 35 and 50, 75, 90, respectively. The mean serum creatinine and 24-hour proteinuria were 1.72 ± 1.2 mg/dL and 1582 ± 1214 mg/day, respectively. The frequency distribution of Oxford variables and deposited antibody immunostaining intensity scores are indicated in Tables 2 and 3. Table 4 depicts the correlation between C4d and the Oxford MEST variables. In this study, a significant association of C4d deposits with IFTA (r = 0.58, *P* = 0.001; Figure 1) was obsereved, while there was no any correlation between C4d and the other morphological variables of Oxford classification including mesangial proliferation, endocapilary proliferation and segmental sclerosis (*P* > 0.05). Likewise, there was no any significant correlation of C4d with the proportion of crescents (*P* = 0.39). Analysis regarding the association of C4d with other deposited immunoreactants showed that only C3 had a significant but negative correlation with C4d (r = -0.42, *P* = 0.02; Figure 2; Table 5). The immunoglobulins and C1q showed no significant correlation with C4d deposits. A representative section of renal biopsy is illustrated in Figure 3.

Discussion

In this study, a significant positive correlation of C4d

Table 1. The frequency distribution of C4d glomerular positivity

C4d positivity	Number	Percent
11-25	6	20.7
26-50	10	34.4
51-75	6	20.7
76-100	7	24.2
Total	29	100

Table 2. Morphologic variables of Oxford-MEST classification

Oxford-MEST variables (N = 29)	Number	Percent	Oxford-MEST variables (N = 29)
M	M0	8	27.5
	M1	21	72.5
E	E0	16	55.2
	E1	13	44.8
S	S0	11	37.9
	S1	18	62.07
T	T0	17	58.6
	T1	7	24.2
	T3	5	17.3

Table 3. Frequency of deposited antibody intensity scores

Antibody deposition intensity				
N = 29	0	+1	+2	+3
IgA	0	0	4	25
IgG	14	11	4	0
IgM	16	11	2	0
C3	4	11	12	2

Table 4. Correlation between C4d and Oxford classification morphological variables

Variable	C4d		
	r	CI 95%	*P* value
M	0.23	-0.14–0.55	0.21
E	0.09	-0.28–0.44	0.63
S	0.27	-0.09–0.58	0.14
T(IFTA)	0.58	0.27–0.78	0.001[a]

[a] *P* value less than 0.05 was considered significant.

Table 5. Correlation of C4d with IgM, IgA, IgG and C3 immune deposits

Variable	C4d		
	r	CI 95%	*P* value
IgM	-0.17	-0.508–0.206	0.366
IgA	0.19	-0.18–0.52	0.313
IgG	-0.06	-0.42–0.305	0.72
C3	-0.42	-0.68–0.06	0.02

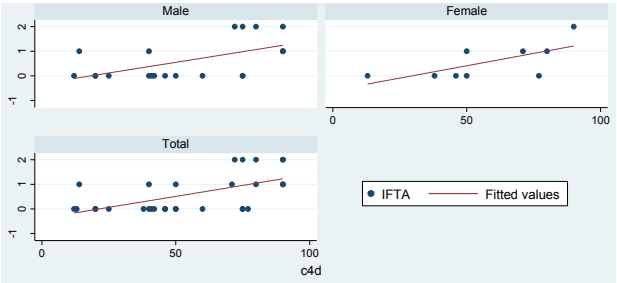

Figure 1. Association of C4d with IFTA (r=0.58, *P*=0.001).

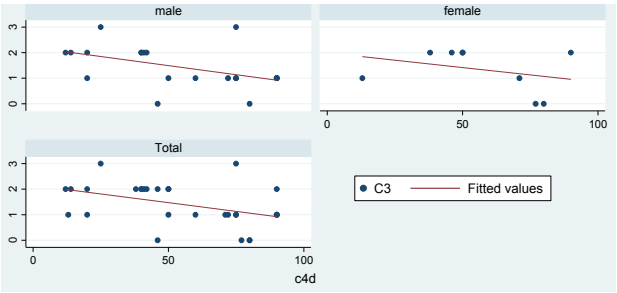

Figure 2. Association of C4d with C3 (r=-0.42, *P*=0.02).

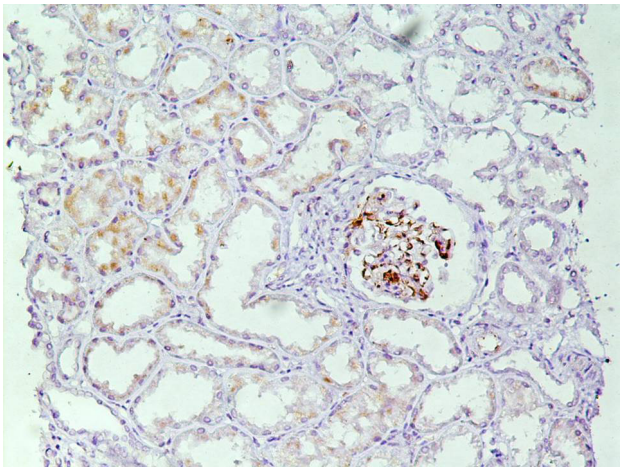

Figure 3. Immunohistochemical positivity of C4d (2+ intensity) in the mesangium of the glomerulus by peroxidase-antiperoxidase (PAP) method (PAP, anti-C4d, ×100).

deposits with IFTA or T variable of Oxford classification was obsereved, while there was no any correlation between C4d and the other three morphologic variables i.e., mesangial proliferation, endocapillary proliferation and segmental sclerosis. Likewise, there was no any significant correlation of C4d with the proportion of crescents in renal biopsies. There are very few studies on correlation of C4d deopsits with the morphological features on renal biopsies in patients with IgAN and none correlating C4d deposits with the morphological variables of the Oxford classification. The Oxford classification has been formulated to serve as the international yardstick for standardized and uniform reporting of the renal biopsy lesions in IgAN. It has been widely validated in different patient populations and different patient age groups. In the current form, it is solely dependant on morphological evaluation of renal biopsy material (1-4). A few studies have looked at the immunostaining data to fine tune the classification (7,9,11). However, the consensus at present is that further studies should be carried out before immunostaining findings can be made part of the classification. All four morphological variables of Oxford classification of IgAN have independent prognostic value. The association of C4d deposits with only IFTA variable may be relatd to the relatively small sample size of our study cohort. An analysis of the association of C4d with other deposited immune reactants showed that only C3 had a significant negative correlation with C4d deposits. The use of IHC to demonstrate the C4d complement degradation product in renal diseases has sparked substantial clinical interest recently (7,9,10). Investigations on C4d deposition have focused on transplant biopsies as an indicator of acute humoral rejection till recent past. There is little information on the role of C4d in native glomerular diseases. C4d is a piece of C4 generated during activation of lectin or classical complement pathways. This piece is highly stable, binds covalently to cell surfaces, and can be recognized using reagents that are at present easily available (7,9-11). In fact, positive C4d staining in the glomeruli may be associated with functional injury to the glomerular filtration barrier and poor renal consequence (8-12). Since IgAN is one of the most prevalent renal disorder and one that frequently leads to end-stage kidney disease, it is expedient to look for newer biomarkers to predict the clinical course of the disease. C4d has been investigated for such a role in a small number of studies (1-5,9-12). It has been hypothesized that patients with IgAN can be divided into two groups on the basis of the initiation pathway of complement activation. It has been claimed that activation of the lectin pathway of complement is associated with more severe renal damage in IgAN. Glomerular deposition of C4d is an indicator of activation of the lectin pathway of complement (7-12). Previously, Espinosa et al, suggested that C4d is a useful tool for the differential diagnosis of membranous nephropathy (13). In another retrospective analysis by Espinosa et al, on kidney biopsies of patients with IgAN, mesangial C4d staining was investigated (14). They showed that overall, 32.2% of the biopsies were C4d positive and 67.8% were C4d negative. They demonstrated that C4d positive staining was associated with progression to end-stage kidney failure on univariate analysis. Likewise, renal survival at 10 years was 43.9% in C4d positive patients vs. 90.9% in C4d negative individuals. They concluded that, negative mesangial C4d staining in

the glomeruli of IgAN patients helps to identify a subset of patients with a good long-term prognosis (14). In another retrospective study comprising of 23 IgAN patients, C4d staining and clinical and laboratory parameters including gender, age, and urine albumin were reviewed by Maeng et al (15). Thirteen patients (56.5%) were positive for C4d staining in the glomeruli. They found that glomerular C4d deposition was correlated with the quantity of albuminuria (15,16). We have also previously reported significant correlation of C4d deposits with the baseline serum creatinine and the degree of proteinuria (16). More recently, in a retrospective investigation comprising of 283 patients with IgAN by Espinosa et al, C4d was studied for an association with clinical outcomes. The results from the study showed that renal biopsies of 109 patients stained positive for C4d and 174 patients were C4d negative. The outcome analysis showed that renal survival at 20 years was 28% in C4d positive individuals vs. 85% in C4d negative patients. Their conclusion was that, C4d positive staining in the glomeruli is an independent risk factor for the development of end-stage kidney failure in IgAN patients (17).

Conclusion

The results from this study show that C4d deposits correlate with IFTA or T variable of Oxford classification of IgAN. There is no significant correlation of C4d deposits with other morphological variables of Oxford classification. C4d deposits showed a negative correlation with C3 deposits. These results need further studies to confirm the relationship of glomerular C4d deposits with immunopathological variables in cases of IgAN.

Limitations of the study

We conducted this study on 29 patients and this investigation should carry out on more patients to better find this aspect of IgAN patients.

There are some limitations in our study. It is a single center based study and cross-sectional in nature. No outcome data are available. However, the study has certain strong points too. To our best knowledge, this is the first investigation correlating C4d deposits with the morphological variables of Oxford classification and with immunostaining data in IgAN patients. The study population is racially homogeneous and the biopsy findings are not altered due to pre-biopsy treatment.

Authors' contribution

All authors wrote the paper equally.

Conflicts of interest

The authors declared no competing interests.

Ethical considerations

Ethical issues (including plagiarism, data fabrication, double publication) have been completely observed by authors.

Funding/Support

None.

References

1. Tanaka S, Ninomiya T, Katafuchi R, Masutani K, Tsuchimoto A, Noguchi H, et al. Development and validation of a prediction rule using the Oxford classification in IgA nephropathy. Clin J Am Soc Nephrol. 2013;8:2082-90.
2. Coppo R, Troyanov S, Bellur S, Cattran D, Cook HT, Feehally J, et al. Validation of the Oxford classification of IgA nephropathy in cohorts with different presentations and treatments. Kidney Int. 2014;86:828-36
3. Mubarak M. Nomenclature of the Oxford classification of IgA nephropathy: do we need to be careful? Kidney Int. 2010;77:74.
4. Roos A, Rastaldi MP, Calvaresi N, Oortwijn BD, Schlagwein N, van Gijlswijk-Janssen DJ, et al. Glomerular activation of the lectin pathway of complement in IgA nephropathy is associated with more severe renal disease. J Am Soc Nephrol. 2006;17:1724-34.
5. Couser WG. Basic and translational concepts of immune-mediated glomerular diseases. J Am Soc Nephrol. 2012;23:381-99.
6. Kim MK, Maeng YI, Lee SJ, Lee IH, Bae J, Kang YN, et al. Pathogenesis and significance of glomerular C4d deposition in lupus nephritis: activation of classical and lectin pathways. Int J Clin Exp Pathol. 2013;6:2157-67.
7. Val-Bernal JF, Garijo MF, Val D, Rodrigo E, Arias M. C4d as a diagnostic tool in membranous nephropathy. Nefrologia. 2012;32:536.
8. Mubarak M. IgA nephropathy: an update on pathogenesis and classification. J Coll Physicians Surg Pak. 2011;21;230-3.
9. Daha MR, van Kooten C. Role of complement in IgA nephropathy. J Nephrol. 2016;29:1-4.
10. Heybeli C, Unlu M, Yildiz S, Çavdar C, Sarioglu S, Camsari T. IgA nephropathy: association of C4d with clinical and histopathological findings and possible role of IgM. Ren Fail. 2015;37:1464-9.
11. Sethi S, Nasr SH, DeVriese AS, Fervenza FC.C4d as a Diagnostic Tool in Proliferative GN. J Am Soc Nephrol. 2015;26:2852-9.
12. Maillard N, Wyatt RJ, Julian BA, Kiryluk K, Gharavi A, Fremeaux-Bacchi V, et al. Current Understanding of the Role of Complement in IgA Nephropathy. J Am Soc Nephrol. 2015; 26:1503-12.
13. Espinosa-Hernández M, Ortega-Salas R, López-Andreu M, Gómez-Carrasco JM, Pérez-Sáez MJ, Pérez-Seoane C, et al. C4d as a diagnostic tool in membranous nephropathy. Nefrologia. 2012;32:295-9.
14. Espinosa M, Ortega R, Gómez-Carrasco JM, López-Rubio F, López-Andreu M, López-Oliva MO, et al. Mesangial C4d deposition: a new prognostic factor in IgA nephropathy. Nephrol Dial Transplant. 2009;24:886-91.
15. Maeng YI, Kim MK, Park JB, Cho CH, Oh HK, Sung WJ, et al. Glomerular and tubular C4d depositions in IgA nephropathy: relations with histopathology and with albuminuria. Int J Clin Exp Pathol. 2013;6:904-10.
16. Nasri H, Ahmadi A, Rafieian-Kopaei M, Bashardoust B, Nasri P, Mubarak M. Association of glomerular C4d deposition with various demographic data in IgA nephropathy patients; a preliminary study. J Nephropathol. 2015;4:19-23.
17. Espinosa M, Ortega R, Sánchez M, Segarra A, Salcedo MT, González F,etal. Association of C4d Deposition with Clinical Outcomes in IgA Nephropathy. Clin J Am Soc Nephrol. 2014;9:897-904.