

Immunohistochemical Scoring of C4d in Egyptian Patients with Chronic Renal Allograft Dysfunction

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Abstract: C4d is a degradation product of complement component C4. Since the beginning of the current century, C4d has been used as a marker of acute antibody mediated rejection in renal allograft and considered as an ominous portent for poor graft survival. The ninth Banff meeting emphasized the value of C4d in renal allograft biopsy assessment and adopted a grading system for the staining degree of C4d (0 through 3). The twelfth Banff meeting revised the proposed grading system. The literature data about the role of C4d in chronic renal allograft damage is variable. We conducted a retrospective study for cases with biopsy proven chronic renal allograft damage diagnosed at our nephropathology units, between Jan. 2007 and Sep. 2013 in order to detect and score C4d positive cases comparing the variability between C4d scores in correlation with the available clinical data and histopathological parameters of chronicity. Twenty eight cases (54.9%) were C4d positive. The frequency of cases with scored positive staining was as follows: C4d1 (12 cases, 23.5%), C4d2 (2 cases, 3.9%) and C4d3 (14 cases, 27.5%). No significant clinical or chronic histopathological findings between score (1) and score (3).

Key words: C4d • Banff schema • Chronic renal allograft rejection

INTRODUCTION

The chronic allograft damage stands as the chief culprit of late loss of a renal allograft [1]. The presence of C4d is considered the best in situ evidence for an active humoral immunologic process in transplanted kidney and is associated with poor prognosis unless treated aggressively [2]. C4d is a degradation product of the complement component C4, which binds to the vascular endothelium when the classical complement pathway is triggered [3]. The association of C4d with antibody mediated rejection (AMR) was initially described by Feucht *et al.* when they showed a significant association of C4d with preformed anti-HLA antibodies in patients with acute rejection [4]. Collins *et al.* [5] reported that C4d in peritubular capillaries (PTC) is a useful diagnostic marker of acute humoral rejection. Crespo *et al.* [6] evaluated the donor specific antigens (DSA) and C4d in steroid-resistant rejections and found positive DSA in 37% of the cases. Among these, 95% had positive PTC C4d-staining. C4d antibodies, currently in the market,

include a monoclonal antibody suitable for immunofluorescence (IF) performed on frozen tissue and a polyclonal antibody works on formalin fixed paraffin embedded tissue and with immunoperoxidase detection (IP) [7]. The ninth Banff meeting recommended C4d staining for every renal allograft biopsy. The meeting adopted C4d grading (0 through 3) for IF and IP techniques (Table 1) [8].

In 2013, the twelfth Banff meeting revised this grading system considering the presence of any PTC positive staining using IP technique is an immunohistologic evidence for antibody mediated reaction [9]. This study was designed to detect and score C4d staining using IP technique in cases of biopsy proven chronic renal allograft damage presented to our nephropathology units, according to the ninth Banff classification schema and to determine whether the formerly proposed grading system significantly correlated with presented clinical data as well as other associated histopathologic parameters indicative of chronicity.

Table 1: Banff '07 C4d scores (% of biopsy with PTC+)

C4d score	% of cortex or medulla	IF	IHC
0 Negative	None	-	-
1 Minimal	<10	-	Unknown
2 Focal	10-50	Unknown	Positive
3 Diffuse	>50	Positive	Positive

MATERIALS AND METHODS

Fifty one paraffin blocks of renal allograft biopsies diagnosed as interstitial fibrosis/tubular atrophy (IF/TA) at our nephropathology units-between January 2007 and September 2013 were collected; those with recurrent/de novo glomerulonephritis-diagnosed on clinical and histopathological bases- were excluded. Local ethics committee approved the study.

Patients' Data: Clinical data were obtained from patients' medical reports included age, sex, duration of the allograft and the serum creatinine level.

Material Preparation

For Histopathological Evaluation: The available archived slides stained with (Hematoxylin and eosin, Masson's trichrome and Periodic acid Schiff) were collected.

For Immunohistochemical Evaluation: Additional sections (3 micron thick) were prepared from paraffin blocks on charged glass slides, treated for antigen retrieval and then treated with antibodies using avidin-biotin peroxidase technique. DAB was used as a substrate and chromogen. Hematoxylin was used as a counterstain. Sections were treated with [C4d] "polyclonal antibody, ready to use" (Spring bioscience, Pleasanton, CA, US): an immunological marker for the humoral alloresponse [10]. Sections from a lymph node with florid reactive follicular hyperplasia were used as a control material.

Histopathological Examination: All cases were examined using light microscope by two observers, the percent of glomerular sclerosis was estimated and the following items were scored (0-3) as defined in the Banff schema [11]: transplant glomerulopathy (cg), tubular atrophy (ct), interstitial fibrosis (ci), chronic vascular changes (cv).

Immunohistochemical Examination: C4d staining was scored (0-3) [9].

Statistical Analysis: Data were handled using the Graphpad Instat computer software version 3.10 and summarized using the mean and standard deviation for

quantitative variables while the frequency and percentages were used for the qualitative ones. Differences between groups were tested using One-way Analysis of Variance (ANOVA).

RESULTS

This study involved fifty one renal allograft biopsies histologically documented to have 'IF/TA'. These cases included 43 males (84.3%) and 8 females (15.6%), with M:F ratio (5.3:1) and wide age range (10-62) years; their biopsies obtained between (3-180) post-transplant months. Twenty eight cases (54.9%) were C4d positive, while twenty three cases (45.1%) were negative. The frequency of cases with scored positive staining was as follows: C4d1 (12 cases, 23.5%), C4d2 (2 cases, 3.9%) and C4d3 (14 cases, 27.5%). The main demographic and clinical data are presented in Table 2 and Fig. 1-2.

Transplant glomerulopathy (TG) was encountered in 25% of C4d positive cases. Marked IF/TA was only encountered in association with strong C4d staining. Similarly the chronic vascular changes; where half of cases with such changes were also seen in association with strong C4d positivity (Table 3).

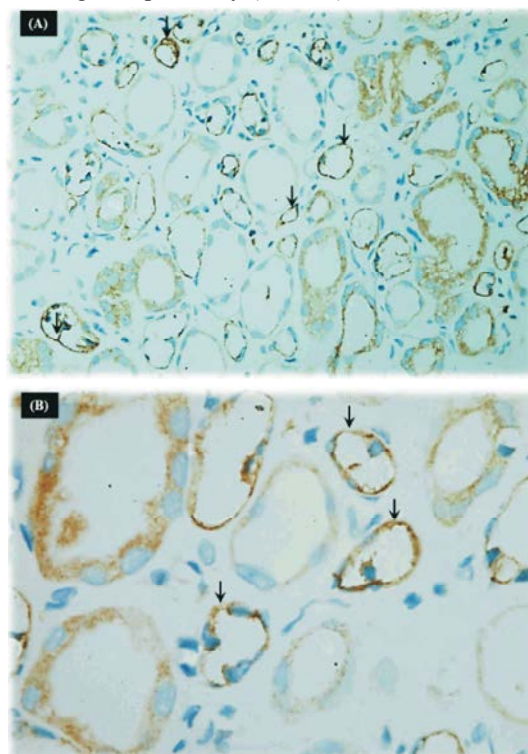


Fig. 1: A case of chronic antibody mediated rejection: Renal tissue shows dilated PTCs with positive diffuse circumferential staining for C4d "arrows" (A- IP-x400, B- IP-x1000).

Table 2: Main demographic and clinical data

Item	C4d0	C4d1	C4d2	C4d3
Patients (n)	23	12	2	14
Recipient age range (years)*	10-6233±13	10-5031±12	24-5037±18	10-5632±14
Recipient gender (M/F)	18/5	9/3	2/0	14/0
Time post-transplantation (months)**	4-18048±49	3-15655±54	36-9666±42	12-15668±50
Serum creatinine level (mg/dl)***	4.0±4.2	3.3±2.1	9.0±4.2	5.2±4.1

*The P value was 0.94, denoting non-significant age differences among the scores.

**No significant differences were detected in time post-transplant among the scores. The P values were (0.58, 0.78 & 0.71) for (C4d1 vs. C4d2, C4d1 vs. C4d3 & C4d2 vs. C4d3 respectively).

***The P value comparing all categories was 0.25 (non-significant).

Table 3: Frequency of encountered chronic histopathological findings in positively stained cases.

Item	C4d1	C4d2	C4d3
Global sclerosis (%)*	15.1±12.5	28.8±21.9	21.1± 28.4
TG	2 (28.6%)	1 (14.3%)	4 (57.1%)
IF/TA (g1/g2/g3) (%) Chronic vascular changes	66.7/33.3/0.02 (25.0%)	0/100.0/02 (25.0%)	64.3/21.4/14.34 (50.0%)

*No significant difference in global sclerosis percent between C4d1 and C4d3 was detected (P value = 0.51)

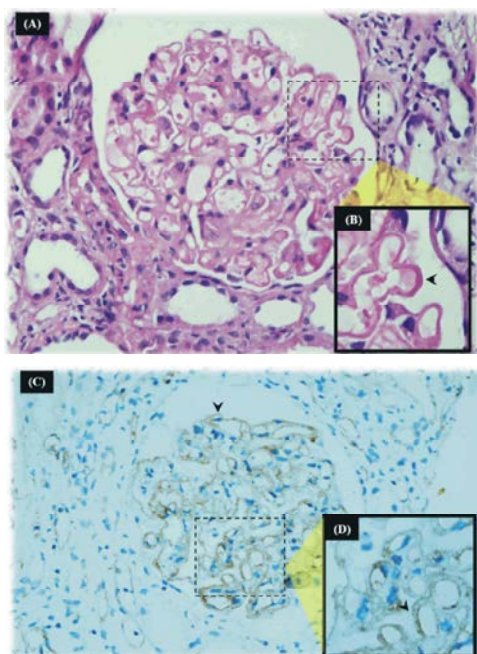


Fig. 2: A case of chronic antibody mediated rejection: A glomerulus shows mesangial expansion and irregular thickening of the GBM (transplant glomerulopathy) with formation of double contours "arrow heads" (A- H&E-x400, B- H&E-x1000, C- IP-x400, D- IP-x1000).

DISCUSSION

In the present study, cases of renal allograft biopsy diagnosed as IF/TA at our units were revised and subjected for further C4d immunohistochemical evaluation to be scored according to the ninth Banff schema [8].

The data for C4d-staining in chronic antibody mediated renal allograft rejection is variable, with few studies showing strong correlation, while others showing poor correlation. Mauiyyedi *et al.* [12] supported the use of C4d to separate the antibody mediated group of chronic rejection from the nonspecific chronic allograft nephropathy. Regele *et al.* [13] suggested that complement activation in renal microvasculature, indicating humoral alloreactivity, contributes to chronic rejection. On the other hand, Al Aly *et al.* [14] stated that C4d staining of the peritubular capillaries appears to be rare in patients with pure chronic renal allograft damage. In this study more than half of cases showed C4d positivity highlighting a strong role of antibody mediated rejection in our cases with chronic allograft damage. This frequency is very close to that reported by Rotman *et al.* [15] where approximately 50% of their IF/TA cases were C4d-positive. In 2007, the ninth Banff meeting [8] adopted C4d grading based on studies provided by Mihatsch [10] and Nadasdy [16]. At the same time, the minimal C4d by IP technique had unknown clinical significance. On the same line, in 2010 Crary *et al.* [17] defined C4d positivity as more than or equal to 10% by immunoperoxidase technique. The concept of "C4d-negative AMR" has been raised in 2009 together with identification of a number of endothelial-associated transcripts in kidney transplants which were found to be higher in all types of rejection and more so in AMR. This, along with reported low sensitivity of C4d for chronic AMR, the concept of "C4d-negative AMR" has appeared to be as common, if not more than the C4d-positive AMR, with similar poor prognosis in terms of graft survival [18]. The twelfth Banff meeting [9]

revised C4d grading results based on multiple studies strongly supported the existence of antibody mediated rejection with negative or minimal C4d deposition within the peritubular capillaries. In the current study, C4d3 was the most frequent score detected in positively stained cases (50%) followed by C4d1 (42.9%). Only two cases showed moderate C4d staining (7.1%). The patients' age, post-transplant durations and the serum creatinine levels at time of graft biopsy were not significantly different among the C4d scores. In our work, there was no significant difference in global sclerosis percent between C4d1 and C4d3. The transplant glomerulopathy was encountered in (25%) of C4d positive cases. Multiple studies reported C4d positive TG [19-22] and C4d negative TG [23,24] and few theories were proposed to explain such variation [19, 23, 25]. Although more than half of cases with TG and half of those with chronic vascular changes were seen in association with C4d3 staining in this work, these changes were also encountered with other scores. Marked IF/TA was only encountered with strong C4d staining predicting poorer graft prognosis. Intuitively, the frequency and extent of such chronic changes is directly correlated with the burden of antibody mediated allograft damage. These findings are concordant with the results of previous studies reported by Regele *et al.*[13], Kawamura *et al.* [26], Kieran *et al.* [27] and David-Neto *et al.* [28].

CONCLUSION

From this study, we concluded that:

- Humeral immune mechanisms are implicated in the development of chronic renal allograft damage.
- No significant clinical or chronic histopathological findings between C4d stainig score (1) and score (3) using the Immunoperoxidase technique in our cases.

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