Original Article

# Pathological diagnosis of antibody-mediated rejection in renal allograft without c4d staining, how much reliable?

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## Abstract

**Background:** C4d as a part of complement activation process is a marker for detecting antibody-mediated rejection (ABMR) and its positivity accompanied by positive donor specific antibody (DSA), and morphologic view of humoral rejection has been suggested to detect ABMR since 2003.

**Materials and Methods:** 41 specimens of transplanted kidney biopsies gathered from 2006 to 2008 were evaluated for morphological changes on light microscopy, and nephro-pathologist made distinct diagnosis for all of specimens then c4d staining was done for all of them. The association between primary diagnosis without c4d staining and c4d scoring on peritubular capillaries and glomerular capillaries were evaluated to determine whether morphological changes were enough for distinct diagnosis or not.

**Results:** Acute tubular necrosis (ATN) 27%, interstitial fibrosis and tubular atrophy (IF&TA) 17%, and T cell mediated rejection (TCMR) 22% were the commonest diagnosis on light microscopy, and 17% of all biopsies had diffuse positive c4d staining. There was not any report of ABMR in morphological evaluation while c4d positive staining was seen in some specimens (17%). It may result from masking of ABMR by other morphological changes such as TCMR and no specific histologic changes for ABMR on light microscopy.

**Conclusion:** We would like to emphasize that c4d staining should be done for all of renal allograft biopsies, and pathologists all over the world should consider the probability of ABMR masked by other morphological changes on light microscopic evaluation.

**Key Words:** Antibody-mediated rejection, c4d, kidney transplantation

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## INTRODUCTION

Antibody mediated rejection (ABMR) is a major cause of poor outcome in renal transplantation,[1] ABMR may be ranging from allograft injury in hyper acute rejection (within minutes after transplantation) to acute rejection (during days to weeks after transplantation) or chronic rejection (during months to years after transplantation).[2] Different therapeutic



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options for ABMR and cellular rejection[3] clarify the importance of understanding the pathologic process, which led to rejection. Allograft biopsy is still the gold standard method for diagnosis and patient management in cases of rejection by evaluation of histologic findings on the specimens.[4] Data gathered from biopsy help physicians to estimate prognosis of disease and choosing the best choice of therapy for patients,[5] but histologic features of rejection (such as margination of leukocytes in peritubular capillaries and glomerular capillaries and arterial fibrinoid necrosis) are not enough specific and sensitive for ABMR diagnosis,[6-9] and the routine procedure of renal biopsy is not a strong act to detect ABMR.[10] Therefore, by keeping together all of specimen features, in Banff consensus, the best diagnostic way to detect ABMR, suggested as presence of a triad: Morphologic evidence of acute tissue injury, serologic evidence of donor specific antibody (DSA), and positive c4d staining in peritubular capillaries (PTC).[11]

Feutch *et al.* initially demonstrated the association between positive c4d staining in PTC with inferior allograft outcome in transplanted kidney biopsy.[12,13] Studies on outcome of transplantation have reported that either focal or diffuse PTC staining had similar impacts on graft survival.[14,15] There are two different methods to detect c4d in tissue: The first is using monoclonal antibody and immunofluorescence (IF) on frozen tissue sections. The second way is using polyclonal antibody and immunohistochemistry (IHC) on formalin-fixed, paraffin tissue sections.[16] As a comparison between these methods, IF is more sensitive method to detect c4d accumulation in tissue.[17,18]

Detection of c4d, in at least 50% of PTC, is the minimal threshold for definite c4d positivity.[4,19] In renal allograft biopsy, c4d positivity is correlated with ABMR,[20] and even a focal c4d staining in the specimen is well-associated with active humoral rejection,[21] but various histological changes such as signs of acute cellular rejection may be present at microscopic evaluation in a c4d positive specimen.[19] C4d deposit may also been detectable in allograft following successful transplantation across ABO barriers.[22]

In this cross-sectional study, we would like to interpret the morphological findings in transplanted kidney biopsies without and with c4d staining results.

## MATERIALS AND METHODS

We retrospectively studied 41 cases of transplanted kidney biopsies gathered from Alzahra hospital (referral hospital of Isfahan- center of Iran) from 2006 to 2008. Paraffin-embedded specimens of biopsies were evaluated, and specimens, which had enough tissue for assessing, were included into the study. Morphologic evaluation on biopsies was done by nephro-pathologist who used Banff 09 classification[11] and clinical manifestation of patients to suggest a distinct diagnosis, which explained the pathological process of disease.

Staining for c4d was done for all of the biopsies in the pathologic laboratory of Alzahra hospital (dependent to Isfahan University of Medical Sciences) by IHC method as the following: Paraffin-embedded specimens were sectioned at 3 μ, after that, slides were de-paraffinized in xylene and rehydrated in descending alcohol. For preparing tissue antigens, slides were placed into microwave, (Tris/EDTA buffer, 20 min at 100°C) then washed by PBS buffer (PH = 7.2). Endogenous peroxidase and endogenous biotin were blocked by using Avidin/Biotin blocking kit for 30 min and consequent washing with PBS buffer. C4d antibody was added to sections [Anti-C4d Antibody (BL-RC4d), conc. 0.2 mg/dl, made in England] and after 30 min washing with PBS buffer was done. Post-antibody solution was added for 30 min, and after that using Poly solution for 30 min, 2 min of DAB solution and finally washing with PBS buffer was done. Next, hematoxilin was added to biopsies for 5 min. c4d staining results were classified due to percentage of staining positivity on the specimens: Diffuse positive (>50% of ptc with linear endothelial staining pattern), focal positive (10% to 50% of ptc with linear endothelial staining pattern), and negative (<10% of ptc with linear endothelial staining pattern).[23]

Diagnosis based on morphologic evaluation and clinical follow-up of patients were compared with c4d scoring, and data analysis was done by SPSS 16.0 software (Chicago, IL, USA).

## RESULTS

Out of the 41 specimens, 28 specimens were from male patients and 13 specimens were from female patients. Pathologic findings of all studied specimens were as the following: Acute tubular necrosis (ATN) 27%, interstitial fibrosis and tubular atrophy (IF&TA) 17%, chronic calcineurin inhibitor toxicity (CNI) 12.1%, IF&TA with T cell mediated rejection (TCMR) 4.9%, TCMR 22%, borderline changes 2.4%, and others 14.6% (including recurrence of diffuse lupus nephritis, BK virus nephropathy, primary focal segmental glomerulonephritis, recurrence of membranous glomerulonephritis, tubulointerstitial nephritis, and infarcted renal cortical tissue). ABMR was suggested for none of specimens.

Table 1 shows the score of c4d staining on specimens considering pathologic findings on light microscopic evaluation.

Another site of specimens, which evaluated for c4d positivity, was glomerular capillaries (GC). There was no statistical relationship between c4d positivity in PTC and GC. Table 2 shows the association between c4d scoring and c4d positivity in GC.

Assessment of c4d deposition in the GC and c4d positivity in the PTC due to morphologic findings of microscopic evaluation was done and in some cases; c4d deposition in the GC was seen although there was no c4d positivity on the PTC [Table 3].

## DISCUSSION

Although recent studies indicated that c4d is not enough specific as a marker in ABMR diagnosis and some cases of ABMR may be missed by c4d criteria,[24] still c4d remains as a sensitive marker of complement activation in ABMR.

A recent study has showed the important role of endothelial-associated transcripts to detect ABMR, even in c4d negative staining.[25]

Using c4d staining in renal allograft plays an important role in ABMR detection according to Banff classification[11] and ABMR diagnosis without c4d deposition may be considerably according with a misdiagnosis. In our study, there were two cases of TCMR and TCMR + IF&TA, which mimicking cellular rejection on light microscopy, but with c4d staining, they had positive c4d staining (>50%), which strongly suggest ABMR. This issue suggests that morphologic data on ABMR may be masked by those in TCMR; therefore, it is advisable that in these cases of rejection, c4d staining and DSA level should be also considered to reach a correct diagnosis.[26]

Although 17% of patients had c4d positive biopsies,

**Table 1: c4d scoring considering pathologic findings**

| Pathologic findings | C4d scoring | | | |
|---|---|---|---|---|
| | 0% | 1-10% | 10-50% | >50% |
| TCMR | 6 (66.7) | 2 (22.2) | 0 | 1 (11.1) |
| IF&TA | 5 (71.4) | 0 | 0 | 2 (28.6) |
| Chronic CNI toxicity | 2 (40) | 0 | 2 (40) | 1 (20) |
| IF&TA + TCMR | 1 (50) | 0 | 0 | 1 (50) |
| ATN | 7 (63.6) | 2 (18.2) | 1 (9.1) | 1 (9.1) |
| Borderline changes | 1 (100) | 0 | 0 | 0 |
| Recurrence of diffuse lupus nephritis (class IV) | 1 (100) | 0 | 0 | 0 |
| BK virus nephropathy | 0 | 0 | 0 | 1 (100) |
| Primary focal segmental glomerulonephritis | 1 (100) | 0 | 0 | 0 |
| Recurrence of membranous glomerulonephritis | 1 (100) | 0 | 0 | 0 |
| Tubulointerstitial nephritis | 0 | 1 (100) | 0 | 0 |
| Infarcted renal cortical tissue | 0 | 1 (100) | 0 | 0 |

TCMR = T cell mediated rejection; IF&TA = Interstitial fibrosis and tubular atrophy; CNI toxicity = Calcineurine inhibitor toxicity; ATN = Acute tubular necrosis

**Table 2: Comparison between c4d scoring and c4d positivity in GC**

| | C4d staining on GC | |
|---|---|---|
| C4d scoring (%) | Positive (%) | Negative (%) |
| 0 | 16 (64) | 9 (36) |
| 1-10 | 2 (33.3) | 4 (66.7) |
| 10-50 | 1 (33.3) | 2 (66.7) |
| >50 | 0 (0) | 7 (100) |
| All of specimens | 19 (46.3) | 22 (53.7) |

Chi-square = 9.7 *P*-value = 0.014. GC = Glomerular capillaries

**Table 3: c4d deposition in the glomerular capillaries and c4d scoring in the peritubular capillaries**

| | C4d scoring in PTC (%) | GC+ | GC- |
|---|---|---|---|
| ATN | 0 | 1 | 6 |
| | 1-10 | 2 | 0 |
| | 10-50 | 1 | 0 |
| | >50 | 1 | 0 |
| IF&TA | 0 | 2 | 3 |
| | 1-10 | 0 | 0 |
| | 10-50 | 0 | 0 |
| | >50 | 2 | 0 |
| Chronic CNI toxicity | 0 | 1 | 1 |
| | 1-10 | 0 | 0 |
| | 10-50 | 1 | 1 |
| | >50 | 1 | 0 |
| IF&TA + TCMR | 0 | 1 | 0 |
| | 1-10 | 0 | 0 |
| | 10-50 | 0 | 0 |
| | >50 | 1 | 0 |
| TCMR | 0 | 2 | 4 |
| | 1-10 | 2 | 0 |
| | 10-50 | 0 | 0 |
| | >50 | 1 | 0 |
| Infarcted renal cortical tissue | 1-10 | 0 | 1 |
| Recurrence of Diffuse Lupus nephritis | 0 | 1 | 0 |
| BK virus nephropathy | >50 | 1 | 0 |
| Tubulointerstitial nephritis | 1 - 10 | 0 | 1 |
| Recurrence of Membranous glomerulonephritis | 0 | 1 | 0 |
| Borderline changes | 0 | 0 | 1 |
| Primary Focal segmental glomerulonephritis | 0 | 0 | 1 |

GC+ = Existence of c4d deposition in glomerular capillaries; GC- = Non existence of c4d in glomerular capillaries; PTC = Peritubular capillaries; ATN = Acute tubular necrosis; IF&TA = Interstitial fibrosis and tubular atrophy; CNI toxicity = Calcineurine inhibitor toxicity; TCMR = T cell mediated rejection

but out of all biopsies, there was not any case of ABMR by light microscopic evaluation. Because of decreased survival rate in patients with c4d positive biopsies and their resistance to steroids,[27] it is really necessary to detect ABMR in all biopsies, and it will not gain without c4d staining in all renal allograft biopsies.

Although c4d deposition may be seen in tubular basement membrane of biopsies with BK virus nephropathy (because of complement activation), diffuse c4d staining on PTC suggests ABMR as an allograft destructor, even in these cases of BK virus nephropathy.[28] In our biopsies, there was one case of BK virus nephropathy, which showed positive c4d staining on PTC and positive c4d in GC with morphologic view of BK virus nephropathy. It may indicate that BK virus nephropathy may also present in ABMR.

It is said that although c4d deposition in PTC is a sensitive marker for detecting of ABMR, it is not an appropriate marker for lupus nephritis.[29] We had a case in whom recurrence of lupus nephritis occurred in her renal allograft, and her biopsy showed negative c4d staining in PTC but presence of c4d deposition in the glomerular capillaries. In the pathologic process of lupus, activation of complement results in c4d deposition in glomerular capillaries,[29] but diffuse c4d positivity in PTC strongly suggests a humoral process, which caused ABMR.

For biopsies with focal positive staining on IHC method, IF should be used to determine whether ABMR is present at biopsy. Unfortunately, there is no fresh specimen for IF method in our center. Although in Banff classification, specimens with more than 50% called to be positive, Fior *et al.* showed that focal c4d staining may also be accompanied by decreased survival rate of patients;[23] therefore, future studies may help nephro-pathologists to gain a suitable approach to these kinds of biopsies.

Lack of donor specific anti body (DSA) was another infirmity of our study to gain a distinct diagnosis of ABMR in cases of focal positivity of c4d, but based on our data, diffuse c4d positivity strongly suggests ABMR. Therefore, we considered specimens with diffuse c4d positivity as cases which highly suggest ABMR, but its definite diagnosis as ABMR certainly need DSA level.

## CONCLUSION

We strongly suggest that c4d staining for all of renal allograft biopsies should be done, and morphologic findings on light microscopic evaluation is not sensitive and specific enough for definite diagnosis of ABMR in specimens. Presence of concurrent disease on allograft should be considered, and diagnosis of a pathologic process on biopsy (such as TCMR or BK virus nephropathy) should not mislead pathologists not to consider ABMR as a differential diagnosis.

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