Combination of Glomerular C4d and Morphologic Glomerular Lesions as a Possible Indicator in the Diagnosis of Acute or Chronic Active Antibody-Mediated Rejection

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ABSTRACT

Background. Linear C4d staining in the peritubular capillaries is considered a sensitive and useful marker of active or chronic active antibody-mediated rejection (ABMR) in transplanted kidneys. However, the diagnostic significance of glomerular C4d deposits (gC4d) is still undetermined. The aim of this study is to evaluate the association of gC4d with clinicopathologic features and to assess its diagnostic value.

Methods. From 2013 to 2018, a total of 158 cases of allograft kidney biopsy specimens were obtained from the Korea University Anam Hospital. The histologic features were evaluated according to the Banff classification. The gC4d were determined through immunohistochemical analyses and classified based on scores of 0 to 3 according to the extent of gC4d.

Results. A total of 73 cases (46.2%) showed gC4d, and 37 cases (23.4%), 23 cases (14.6%), and 13 cases (8.2%) were classified with a score of 1+, 2+, and 3+, respectively. The gC4d showed a significant correlation with antibody-associated histologic lesions, including peritubular capillaritis, glomerulitis, and transplant glomerulopathy ($P < .001$). However, gC4d showed no significant association with cell-mediated injuries such as tubulitis, interstitial inflammation, acute tubular necrosis, and thrombotic microangiopathy. Although positive gC4d alone was associated with nonspecific findings without ABMR, most cases of gC4d combined with glomerulitis or transplant glomerulopathy showed typical histologic features of ABMR, clinically with higher antibody titers and severe functional deterioration.

Conclusions. Glomerular C4d deposits may be an alternate useful marker in the diagnosis of active or chronic active ABMR when combined with histologic features of glomerular lesions.

ACUTE and chronic antibody-mediated rejection (ABMR) is an important cause of late graft failure, and, therefore, the accurate identification of antibody-associated graft damage is important for proper treatment and graft outcomes [1]. Antibody-mediated rejection is caused by the production of alloantibodies to donor-specific antigen

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(DSA), expressed on the endothelium [2,3]. Therefore, C4d, providing evidence of complement activation by DSA, has become an important biomarker for diagnosing ABMR in transplant biopsy specimens [4].

C4d is a fragment of the complement C4b and is produced during activation of the classic complement pathway [5-7]. Detection of C4d is considered to be an indirect sign of an antibody response and is strongly associated with circulating autoantibodies to donor antigens [8]. Currently, a linear C4d deposit in the peritubular capillaries (ptc-C4d), identified by immunohistochemical or immunofluorescent study, is now widely accepted as a valuable marker of ABMR in transplanted kidneys [3,6,9].

Contrarily, although nontypical C4d deposits in other compartments of the kidneys are frequently observed in graft biopsy specimens, their clinical significance is still controversial [7,9]. Because normal kidneys may show mesangial C4d staining without inflammation, it could be considered a nonspecific finding [10]. However, several studies have suggested the importance of glomerular C4d deposit (gC4d) in paraffin-embedded tissue specimens [11]. Kikic et al [3] showed that gC4d staining is correlated with glomerulitis, and Valente et al [12] observed that gC4d correlated more closely than ptc-C4d with the presence of post-transplant circulating anti-HLA antibodies. Recently, it was also demonstrated that the presence of gC4d predicts duplication of the glomerular basement membrane and glomerular remodeling 1 year later [13].

Given that antibody-related damage is a critical factor in poor graft outcome, the potential of gC4d as an ABMR diagnostic index needs to be reassessed. Therefore, we hypothesized that positive gC4d can be another indicator for acute and chronic ABMR in renal grafts and evaluated the histologic and clinical significance of gC4d.

MATERIAL AND METHODS

Study Cohorts

We retrospectively analyzed allograft kidney biopsy tissue specimens between 2013 and 2018 obtained from Korea University Anam Hospital. The biopsy specimens were obtained from post-transplant patients with clinical suspicion of acute rejection. ABO-incompatible transplant patients and polyomavirus nephropathy patients were excluded. The clinical data were collected from medical records at Korea University Anam Hospital. This study was approved by the Institutional Review Board of Korea University Anam Hospital (2018AN0307).

Histologic Analysis and Scoring of Renal Allograft Biopsy Samples

Two pathologists, blinded to the patients' clinical data and C4d results, reviewed hematoxylin and eosin–stained slides of the biopsy specimens and assessed the histologic features. These included peritubular capillaritis, glomerulitis, transplant glomerulopathy, tubulitis, interstitial inflammation, intimal arteritis, and other features, including acute tubular necrosis and thrombotic microangiopathy. These were scored according to the 2017 Banff classification, and the diagnosis of acute rejection was based on morphologic evaluation.

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The presence of gC4d was determined via immunohistochemistry with rabbit anti-C4d monoclonal antibody (Clone SP91) (Ventana Medical Systems, Tucson, Ariz, United States) used on paraffin-embedded sections, according to the manufacturer's instructions. Glomerular C4d was considered to be positive when at least 1 glomerulus showed gC4d deposits in the capillary wall and was subsequently classified based on scores of 0 to 3 according to the extent of gC4d (Fig 1).

Statistical Analysis

Data were analyzed using SPSS 20.0 software (IBM, Armonk, NY, United States). Comparison of numerical data was performed using the 1-way analysis of variance or *t* test. Categorical data were evaluated using the χ^2 test or Fisher exact test, as appropriate. A value of $P < .05$ was considered statistically significant.

RESULTS

Clinical Characteristics of Patients

A total of 158 samples from 117 patients were the subject of the study. The patients were between 15 and 73 years old, and the mean age was 47.3 (SD, 13.19) years. Of the patients, 73.4% were male. Living and deceased donor transplants consisted of 67 and 91 cases, respectively. The interval between operation and allograft biopsy ranged from 8 to 8218 days, with a mean of 1019.8 days. The total number of biopsies post-transplant was 89 (56.3%) in the first year, 18 (11.4%) between years 1 and 2, and 51 (32.3%) after 2 years. Other clinical data are listed in Table 1.

Histologic Diagnosis

In 158 cases, the morphologic evidence of peritubular capillaritis, glomerulitis, and transplant glomerulopathy was identifiable in 32.3% (n = 51), 15.2% (n = 24), and 10.1% (n = 16) of cases, respectively. Tubulitis, interstitial inflammation, intimal arteritis, thrombotic microangiopathy, and acute tubular necrosis were morphologically found in 56.3% (n = 89), 48.7% (n = 77), 5.7% (n = 9), 3.2% (n = 5), and 27.2% (n = 43) of cases, respectively. In total, 25.9% (n = 41) of the cases were diagnosed with active ABMR, 10.1% (n = 16) with chronic active ABMR, and 17.7% (n = 28) with acute T cell–mediated rejection (TCMR), while 37.3% (n = 59) were suspicions of acute TCMR; 8.2% (n = 13) were thought to have mixed rejection, and 11.4% (n=18) appeared to have mixed borderline features of TCMR and ABMR. Other histologic data are listed in Table 2.

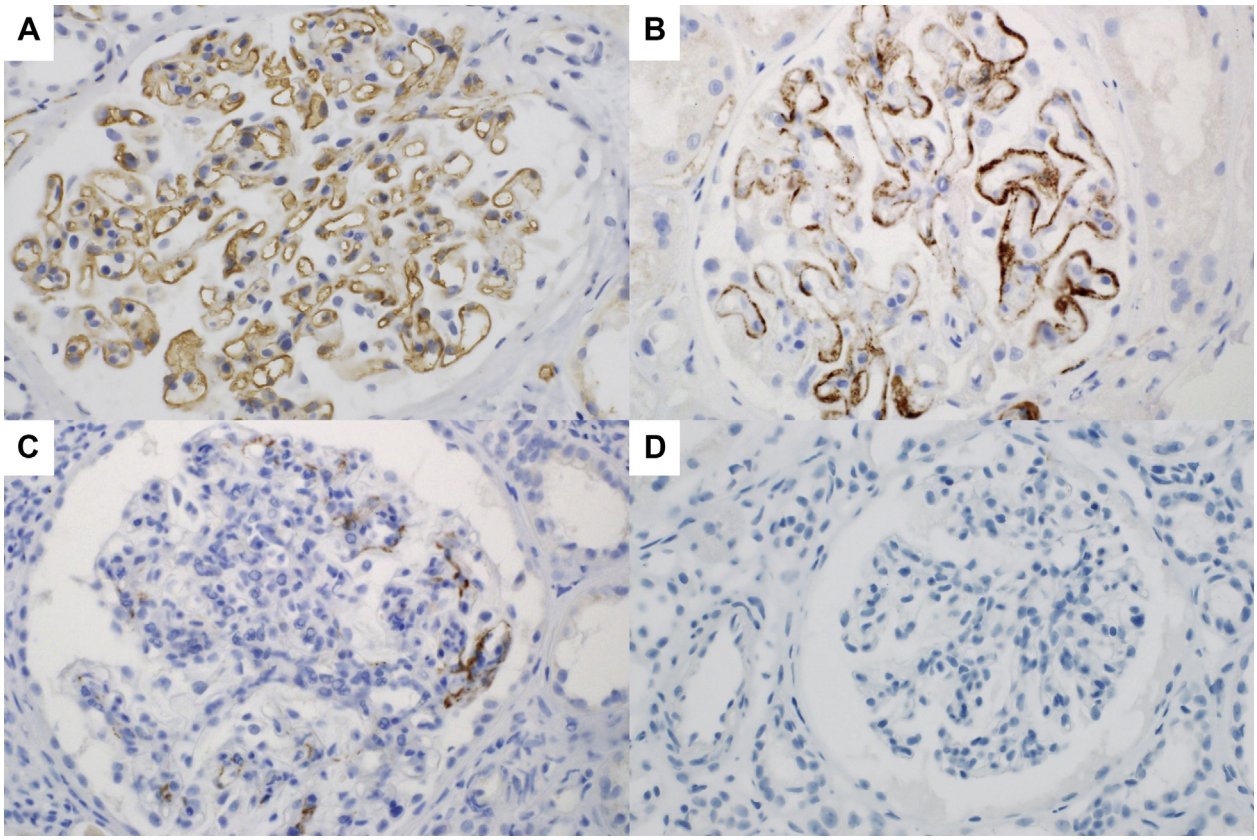

Fig 1. The representative images of glomerular C4d deposition. Glomerular C4d was considered positive when at least one glomerulus showed gC4d deposits in the capillary wall, and subsequently classified based on scores of **(A)** 3, **(B)** 2, **(C)** 1, and **(D)** 0 according to the extent of gC4d.

Immunohistochemical Staining for C4d

We found that 42 cases (26.6%) had ptc-C4d, and 73 cases (46.2%) showed gC4d; 37 cases (23.4%), 23 cases (14.6%), and 13 cases (8.2%) were classified with a score of 1+, 2+, and 3+, respectively. We found simultaneous C4d deposition on both glomeruli and peritubular capillaries in 29 cases, accounting for 39.7% of gC4d-positive cases and 69.0% of ptc-C4d–positive cases.

Table 1. Patient Characteristics

	All (n = 158)	gC4d (+) (n = 73)	gC4d (–) (n = 85)	*P* Value
Male, No. (%)	116 (73.4)	48 (65.7)	68 (72.9)	.04
Age, mean (SD), y	47.3 (13.19)	46.2 (13.21)	48.3 (13.17)	.33
Cr at the time of Bx, mean (SD), mg/dL	2.24 (0.11)	2.29 (0.15)	2.35 (0.13)	.77
Peak Cr, mean (SD), mg/dL	2.31 (0.11)	2.41 (0.17)	2.22 (0.13)	.40
Graft type, No. (%)				.19
Deceased donor	91 (57.6)	38 (52.1)	53 (62.4)	
Living donor	67 (42.4)	35 (47.9)	32 (37.6)	
Time since transplant, No. (%)				.001
< 1 y	89 (56.3)	32 (43.8)	57 (67.1)	
≤ 1, < 2 y	18 (11.4)	7 (9.6)	11 (12.9)	
> 2 y	51 (32.3)	34 (46.6)	17 (20.0)	

Abbreviations: Bx, biopsy; Cr, creatinine; gC4d, glomerular C4d deposits.

Glomerular C4d and Acute Rejection

The gC4d-positive grafts were associated with significantly more peritubular capillaritis, glomerulitis, and transplant glomerulopathy than were the gC4d-negative grafts ($P < .001$). However, proportions of tubulitis, interstitial inflammation, and intimal arteritis were not significantly different between the 2 groups (Table 2). Finally, 28 of 73 (38%) gC4d-positive tissues were diagnosed with active ABMR, which was significantly higher than 13 of 85 (15.3%) gC4d-negative tissues. In addition, of the 41 cases of active ABMR, 28 cases (68.3%) were positive for gC4d; 8 cases were 3+, 10 cases were 2+, and 10 cases were 1+.

Positive gC4d was also observed in 14 of 28 (50%) patients with morphologic evidence of acute TCMR, and 29 of 59 (42%) patients with borderline acute TCMR, respectively. There was no significant correlation between the presence of gC4d and the diagnosis of acute TCMR. Of the 13 mixed-type rejection cases, 61.5% were

Table 2. Histologic Features According to the Presence of Glomerular C4d

	gC4d(+) (n = 73)	gC4d(-) (n = 85)	*P* Value
Histologic features			
Tubulitis	38 (52.1%)	5 (5.9%)	.319
Interstitial inflammation	34 (46.6%)	43 (50.6%)	.618
Intimal arteritis	3 (4.1%)	6 (7.1%)	.428
Peritubular capillaritis	31 (42.5%)	20 (23.5%)	.011
Glomerulitis	21 (28.8%)	3 (3.5%)	<.001
Transplant glomerulopathy	16 (21.9%)	0 (0.0%)	<.001
Thrombotic microangiopathy	3 (4.1%)	2 (2.4%)	.532
Acute tubular necrosis	24 (32.9%)	19 (22.4%)	.140
Rejection			
Active ABMR	28	13	.001
Chronic active ABMR	16	0	<.001
T cell mediated rejection			.942
Borderline	25	34	
Grade I	11	9	
Grade II	2	5	
Grade III	1	0	
Mixed rejection			.022
Mixed ABMR + acute TCMR	8	5	.250
Mixed ABMR + borderline for TCMR	13	5	.019
Immunohistochemistry			
ptc-C4d	29 (39.7%)	13 (15.3%)	<.001

Abbreviations: ABMR, antibody mediated rejection; TCMR, T cell mediated rejection.

positive for gC4d, and there was also no significant correlation between the diagnosis of mixed rejection and the presence of gC4d.

Clinical Histologic Significance of gC4d with Glomerular Damage

To determine the diagnostic usefulness of gC4d, we used a combination of morphologic glomerular damage (eg, glomerulitis or transplant glomerulopathy) and gC4d as a diagnostic criterion, because histologic diagnosis of ABMR was only observed in less than 50% of gC4d-positive tissues. A total of 22 cases met the criteria. Among them, 18 cases were positive for ptc-C4d, and the combined gC4d with glomerular damage was closely related to histologic diagnosis of ABMR based on the Banff classification. Nineteen of 22 patients (86.4%) had active ABMR or chronic active ABMR, and 14 of them had 2 combined diagnoses. However, only 19.6% (10/51) of the gC4d cases alone without glomerular lesions had ABMR, including only 1 case of chronic active ABMR. The sensitivity, specificity, positive predictive, and negative predictive values using this criterion were 43.9%, 96.6%, 81.8%, and 83.1% for active ABMR, respectively, and 93.8%, 95.1%, 68.2%, and 99.3% for chronic ABMR (CABMR), respectively.

In addition to these histologic findings, at the time of histologic examination, mean fluorescence intensities (MFIs) of DSAs were significantly higher in patients with gC4d with glomerular damage than in patients with gC4d alone. These high titers of circulating antibodies were associated with severe renal functional deterioration (Fig 2C). However, regardless of glomerular damage, only presence of gC4d deposition or grades of gC4d (0–3) were not significantly associated with antibody titer or renal function (Fig 2A, B). This suggests that gC4d with glomerular damage indicates clinically significant endothelial damage due to circulating antibodies.

DISCUSSION

According to the revised Banff 2017 classification, 3 criteria are used to make a diagnosis of ABMR: 1. histologic evidence of acute tissue injury, 2. evidence of current or recent antibody interaction with vascular endothelium, and 3. serologic evidence of DSAs [9]. C4d deposition on the peritubular capillaries is associated with poor graft outcomes; thus, a C4d score greater than 0 in immunohistochemical staining can be considered evidence of interaction between circulating antibodies and the endothelium, an important marker for ABMR. Although many studies have shown prognostic values of linear ptc-C4d deposition, the diagnostic significance of C4d deposition on other structures remains controversial.

To determine the clinicopathologic implications of gC4d, we compared the presence of gC4d with clinical and histologic features. Our study showed that gC4d has a correlation with the histologic features of ABMR. On the contrary, gC4d had no significant association with other histologic features, including acute TCMR. Some previous studies have demonstrated a significant correlation between gC4d and glomerulitis [3,12,14,15], and Valente et al showed that gC4d was more strongly correlated with the presence of circulating anti-HLA antibodies than with ptc-C4d [12]. In our study, titers of circulating antibodies tended to increase with the degree of gC4d deposition. Especially in cases where glomerulitis is accompanied by gC4d deposition, the MFI level of circulating antibodies was higher than in the presence of gC4d alone. This, therefore, supports the idea that gC4d is associated with antibody-mediated activation of the complement pathway. This reaction contributes to inflammation in glomerular endothelial cells, thereby causing glomerular remodeling and transplant glomerulopathy.

However, gC4d can be reactive in other glomerular diseases, and, therefore, gC4d may be a nonspecific indicator of glomerular capillary damage [16,17]. Antibody-mediated rejection is diagnosed in only less than 50% of gC4d-positive tissues, so it is difficult to exclude the possibility of nonspecific deposition by gC4d alone, which is not useful for ABMR diagnosis.

We, therefore, tried to find possible histologic lesions that could enhance the clinical value of gC4d. In terms of the extent of gC4d, it was not proportional to the histologic degree of ABMR, and a large number of cases with a score

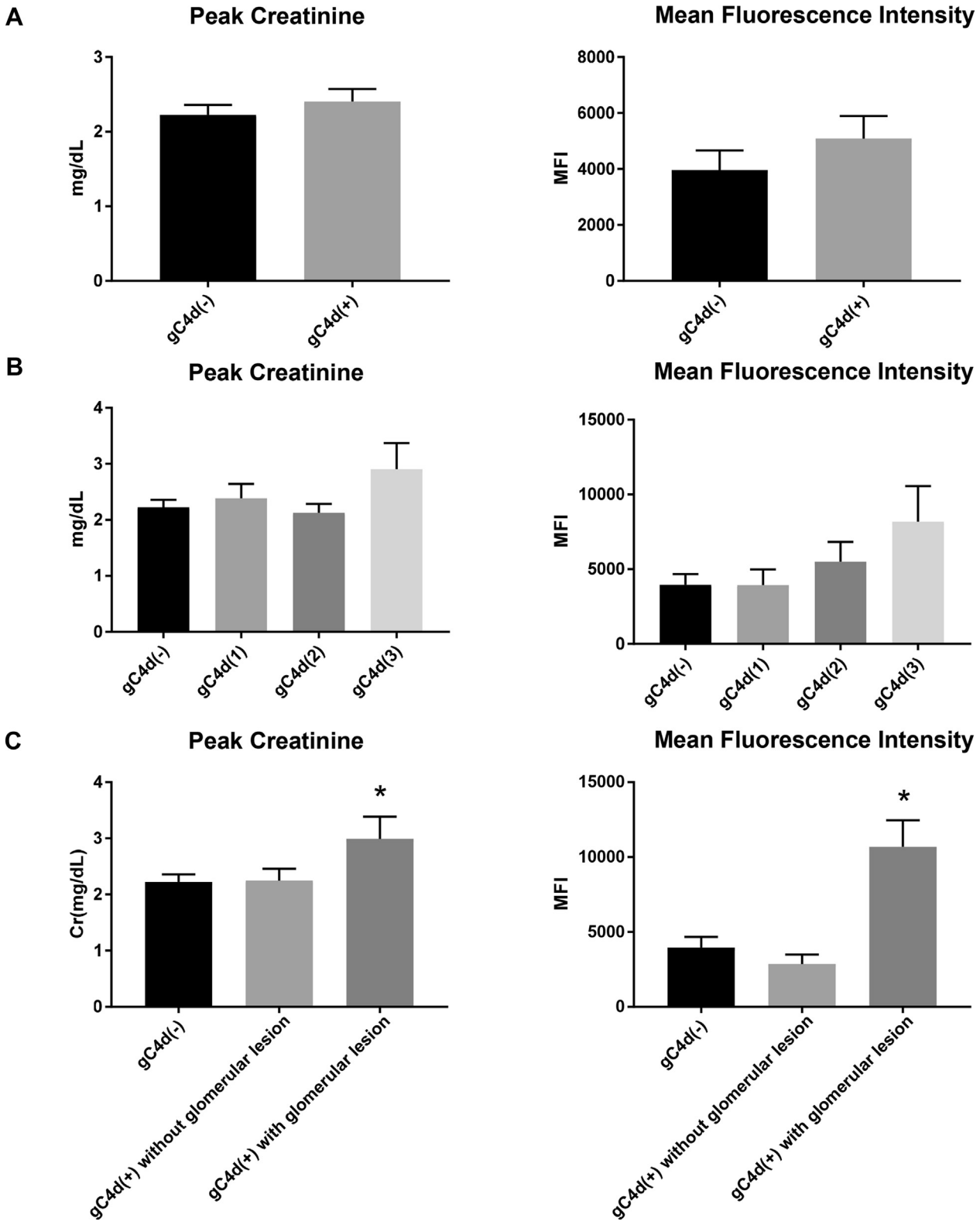

Fig 2. Levels of peak creatinine and mean fluorescence intensity (MFI) according to histologic subgroups of gC4d. **(A)** Only presence of gC4d deposition or **(B)** grade of gC4d (0–3) was not significantly associated with antibody titer or renal function. **(C)** gC4d deposition with glomerular damage was associated with significantly high titers of circulating antibodies and severe renal functional deterioration. *P-value $< .05$ compared with positive gC4d without glomerular lesion.

of 3+ gC4d were not classified as ABMR. The grade of gC4d was also not significantly associated with renal functional deterioration at the time of biopsy. This means that the presence and extent of gC4d cannot be interpreted independently as a diagnostic marker of ABMR.

Interestingly, the combination of morphologic glomerular lesions and gC4d was highly consistent with the diagnosis of ABMR, based on the Banff classification. Most cases with both glomerular damage and gC4d were diagnosed as acute or chronic active ABMR, and these lesions favored ABMR even when ptc-C4d was negative. In our data, the sensitivity of these criteria for diagnosis of ABMR is 43.9%, with a specificity of 96.6%. This, therefore, requires sufficient clinicopathologic correlation when considering a combination of glomerular damage and gC4d.

When using this criterion, we observed that many cases had chronic active ABMR combined with active ABMR. This was observed much more frequently than in cases with negative gC4d or gC4d alone, with a high sensitivity (93.8%) and specificity (95.1%) for diagnosis of chronic ABMR. Previous research has demonstrated that C4d accumulation along the peritubular capillaries can occur and vanish within several days as a dynamic process of antibody-induced graft injury but that isolated gC4d without ptc-C4d remained on repeat biopsies, thereby having different significance and supporting our results [13,18].

However, some cases showed a mismatch between glomeruli with glomerular damage and gC4d-deposited glomeruli detected histologically. Serial cutting of the slide reduced inconsistencies in only a few cases, so there are still limitations to interpreting these cases completely. Nonetheless, combined glomerular damage and gC4d deposition were associated with a clinically high titer of MFI and more functional deterioration. This suggests that morphologic glomerular lesions are indicative of clinically significant damage through interaction between antibodies and glomerular capillary endothelial cells.

Although C4d deposition provides strong evidence of endothelial damage by circulating antibodies, in 2017 Banff classification, microvascular inflammation may be substituted for ptc-C4d deposition to increase the sensitivity for ABMR diagnosis and consider C4d-negative ABMR [9]. This type of ABMR frequently develops at a late time point because of de novo DSA and is an important factor for late graft loss [2]. For cases in which ptc-C4d is negative and gC4d is positive, whether they should be classified as C4d-negative may be questionable. According to our data, ABMR with gC4d is likely to occur with CABMR, and gC4d had a significant correlation with a longer interval time between the date of biopsy and transplant. We can suggest that ABMR with gC4d would mean chronic status or late onset rejection. However, most ABMR patients with gC4d also had positive ptc-C4d, and gC4d alone without ptc-C4d was rare in our ABMR patients. Therefore, further study with a large population is needed to determine the clinical significance of gC4d in C4d-negative ABMR.

There are some important limitations to this study. First, it was a single-center study with a small number of patients. In retrospective design, treatment was not standardized, so only the clinical findings before and after biopsy were compared, and the long-term outcomes were not evaluated. It is also difficult to access the prognostic significance of single gC4d findings without a follow-up biopsy.

In conclusion, since isolated gC4d appears to have some limitations, it is difficult to use gC4d as an independent tissue marker. However, the presence of glomerular C4d deposits showed a significant correlation with ABMR. C4d can be used as an alternate useful marker in the diagnosis of active or chronic active ABMR when combined with morphologic glomerular damage, especially in cases with CABMR.

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