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Significance of C4d expression in peritubular capillaries concurrent with microvascular inflammation in for-cause biopsies of ABO-incompatible renal allografts

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Background: Pathologic diagnosis of antibody-mediated rejection (ABMR) in ABO-incompatible (ABOi) transplantation patients is often challenging because patients without ABMR are frequently immunopositive for C4d. The aim of this study was to determine whether C4d positivity with microvascular inflammation (MVI), in the absence of any detectable donor-specific antibodies (DSAs) in ABOi patients, could be considered as ABMR.

Methods: A retrospective study of 214 for-cause biopsies from 126 ABOi kidney transplantation patients was performed. Patients with MVI score of ≥2 and glomerulitis score of ≥1 (n = 62) were divided into three groups: the absolute ABMR group (DSA-positive, C4d-positive or C4d-negative; n = 36), the C4d-positive group (DSA-negative, C4d-positive; n = 22), and the C4d-negative group (DSA-negative, C4d-negative; n = 4). The Banff scores, estimated glomerular filtration rates (eGFRs), and graft failure rates were compared among groups.

Results: C4d-positive biopsies showed higher glomerulitis, peritubular capillaritis, and MVI scores compared with C4d-negative specimens. The C4d-positive group did not show significant differences in eGFRs and graft survival compared with the absolute ABMR group.

Conclusion: The results indicate that C4d positivity, MVI score of ≥2, and glomerulitis score of ≥1 in ABOi allograft biopsies may be categorized and treated as ABMR cases.

Keywords: ABO-incompatible, C4d, Kidney transplantation, Transplant rejection

Introduction

Severe shortages of organs available for transplantation have resulted in increased number of ABO-incompatible (ABOi) kidney transplantations, which have shown comparable outcomes with ABO-compatible kidney transplan-

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tations [1–9]. With the increase in ABOi kidney transplants, the diagnosis of rejection in ABOi allograft biopsies has become crucial for ensuring better clinical outcomes, including graft survival. Pathologic evaluation of biopsied tissue is essential for diagnosing graft rejection along with the presence of donor-specific antibodies (DSAs) [10]. Linear C4d expression in peritubular capillaries is indicative of antibody-vascular endothelial cell interactions and a surrogate for DSAs in antibody-mediated rejection (ABMR) [10–13]. Therefore, C4d expression in peritubular capillaries is the currently adopted feature of ABMR according to the Banff classification system, the most widely used kidney allograft pathology scoring system [10,14].

However, the significance of C4d staining in ABOi renal allograft remains unclear because it can be observed in these allografts even without histologic evidence of ABMR [15–18], which hinders the diagnosis of ABMR in ABOi allografts. Although potentially due to accommodation, C4d expression in ABOi allografts is not necessarily indicative that complement activation is absent. The Banff Kidney Meeting Report recommends the use of molecular diagnostics when ABOi allografts show microvascular inflammation (MVI) scores of ≥2 without detectable DSAs; however, molecular diagnostics are not widely available in daily clinical practice. Furthermore, the usefulness of these tools in the diagnosis of ABMR in ABOi patients, especially with negative DSAs, has yet to be validated [19].

In the present study, the C4d staining pattern in for-cause biopsies was evaluated and whether C4d positivity with MVI and no detectable DSAs in ABOi patients should be considered ABMR determined.

Methods

Patients included in the study and their clinical parameters

From February 2009 to January 2016, a total of 501 patients underwent ABOi renal transplant at Asan Medical Center (Seoul, Republic of Korea). During follow-up, a total of 214 for-cause biopsies were performed on 126 patients that were included in this study. Exclusion criteria were the following: occurrence of polyomavirus nephropathy, recurrence of previous nephropathy, and only undergoing protocol biopsies and/or zero-hour biopsies.

Several parameters were collected and assessed from electronic medical records such as age at transplantation, sex, posttransplantation time (time elapsed since kidney transplantation until for-cause biopsy), the initial cause of renal failure, donor age, human leukocyte antigen (HLA) mismatch status between donor and recipient, baseline isoagglutinin titer, ABO group, body mass index (BMI), pathologic diagnosis, graft survival, and follow-up periods. The Institutional Review Board of Asan Medical Center approved this retrospective study (No. 2021-0702). The need for written consent was formally waived due to the retrospective and anonymous nature of the study.

Histological evaluation of pathologic parameters and patient grouping

Slides of biopsied material were stained with hematoxylin and eosin, periodic acid-Schiff, methenamine silver, Masson's trichrome, and C4d and SV40 immunohistochemical (IHC) staining and independently evaluated and graded using the Banff 2017 criteria [10] by two nephropathologists. IHC staining was performed on 4-μm-thick sections from 10% formalin-fixed, paraffin-embedded blocks. For the IHC protocol, the rabbit polyclonal anti-C4d antibody (1:32 dilution; Cell Marque) and anti-SV40 antibody (1:32 dilution; Cell Marque) in the Ventana BenchMark XT autostainer (Ventana Medical Systems) were used following the manufacturer's protocols. A representative image of the C4d staining is shown in Fig. 1.

For each biopsy, the MVI score was calculated as the sum of the glomerulitis (g) score and the peritubular capillaritis (ptc) score. C4d positivity was defined as a C4d score of >0. Patients with MVI score of ≥2 and g score of ≥1 were divided into three groups: absolute ABMR group (MVI score of ≥2, g score of ≥1, C4d-positive or C4d-negative, DSA-positive), C4d-positive group (MVI score of ≥2, g score of ≥1, C4d-positive, DSA-negative), and C4d-negative group (MVI score of ≥2, g score of ≥1, C4d-negative, DSA-negative). In patients with multiple biopsies, the highest MVI score was used for group distribution.

Desensitization and immunosuppressive protocols

The desensitization protocol for ABOi kidney transplantation consisted of rituximab administration combined with plasmapheresis [20]. A single dose of 200 mg of rituximab

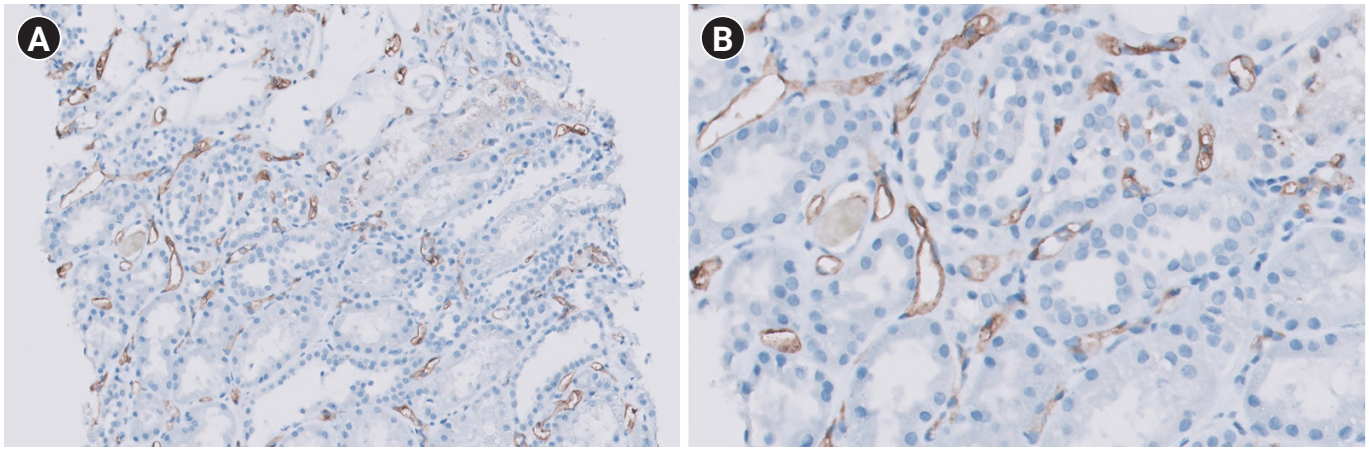


Figure 1. Representative image of C4d staining. Linear and circumferential C4d staining in peritubular capillaries and/or medullary vasa recta is interpreted as C4d positivity. (A) ×200, (B) ×400 magnification.

was administered 7 days before the first plasmapheresis, which was performed 3 to 14 days prior to surgery, until the isoagglutinin titer decreased to ≤1:4. Postoperative plasmapheresis was performed when the isoagglutinin titer was ≥1:16. Tacrolimus, mycophenolate mofetil, and methylprednisolone were administered 7 to 10 days before surgery. As an induction therapy, basiliximab (anti-CD25 monoclonal antibody) was administered on the day of the surgery and 4 days after the procedure.

Treatment regimens for antibody-mediated rejection

To treat ABMR, intravenous methylprednisolone was administered for 3 days, 500 mg per day, followed by plasmapheresis daily or every other day for a maximum of nine sessions based on changes in the DSA titer. Intravenous immunoglobulin (IVIG) was administered at a dose of 100 to 300 mg/kg after each plasmapheresis session. Finally, a single dose of rituximab, 200 mg or 375 mg/m^2, was administered after plasmapheresis and IVIG. All patients in the three aforementioned groups were subjected to this treatment regimen.

Donor-specific antibodies

Blood samples were collected 1 week prior to the biopsy for DSA screening in 97 patients (158 biopsies). DSAs were screened using the Luminex single antigen bead assay, with LABScreen Single Antigen HLA Class I and Class II (One Lambda, Inc.). The cutoff for DSA presence was a mean fluorescence intensity of >1,000. In 10 biopsies from seven patients, the donor HLA class II was not available for analysis. DSAs were not tested in 57 biopsies from 49 patients.

Statistical analysis

All data analyses were conducted using the IBM SPSS version 24.0 (IBM Corp.). The chi-square test and Fisher exact test were used for comparison of categorical variables. The Mann-Whitney U test and Student t test were used for comparison of continuous variables. Graft survival was calculated using the Kaplan-Meier method and compared between groups using the log-rank test. For graft function, estimated glomerular filtration rate (eGFR) was measured and the mean sequential changes of eGFR were compared between groups and plotted.

Multivariable regression analysis was performed using the Cox proportional hazards model. Probability values of <0.05 were considered statistically significant.

Results

Clinical characteristics of the patients

The clinical characteristics of the 126 study patients who underwent for-cause biopsy are summarized in Table 1. The mean age at the time of transplant was 51.6 ± 11.2

Table 1. Clinical characteristics of ABOi recipients who underwent for-cause biopsy

Characteristic	Total	Absolute ABMR group	C4d-positive group	p-value
No. of patients	126	36	22	
Age at transplantation (yr)	51.6 ± 11.2	50.2 ± 12.7	54.1 ± 7.8	0.16
Body mass index (kg/m^2)	22.85 ± 3.05	22.81 ± 3.25	22.92 ± 2.78	0.89
Sex				0.39
Male	87 (69.0)	24 (66.7)	17 (77.3)	
Female	39 (31.0)	12 (33.3)	5 (22.7)	
Cause of renal failure				0.15
Diabetic nephropathy	31 (24.5)	14 (38.9)	5 (22.7)	
IgA nephropathy	17 (13.5)	6 (16.7)	2 (9.1)	
Hypertensive nephropathy	11 (8.7)	2 (5.6)	3 (13.6)	
Polycystic kidney disease	6 (4.8)	0 (0)	3 (13.6)	
FSGS	2 (1.6)	0 (0)	0 (0)	
Unknown/other	59 (46.8)	11 (30.6)	5 (22.7)	
Donor age (yr)	47.9 ± 8.6	48.0 ± 11.1	46.3 ± 11.1	0.57
HLA mismatch	3.84 ± 1.43	3.94 ± 1.41	3.86 ± 1.32	0.84
Baseline isoagglutinin titer, ≥1:128	34 (27.0)	7 (19.4)	7 (31.8)	0.29
ABO group				0.92
A to B	28 (22.2)	8 (22.2)	7 (31.8)	
A to O	23 (18.3)	8 (22.2)	5 (22.7)	
B to A	24 (19.0)	10 (27.8)	4 (18.2)	
B to O	16 (12.7)	3 (8.3)	2 (9.1)	
AB to A	16 (12.7)	4 (11.1)	3 (13.6)	
AB to B	16 (12.7)	1 (2.8)	1 (4.5)	
AB to O	2 (1.6)	2 (5.6)	0 (0)	
No. of biopsies	1.71 ± 1.30	2.17 ± 1.45	1.64 ± 0.90	0.01
Follow-up (yr)	5.9 ± 2.8	4.9 ± 2.4	5.4 ± 2.3	0.48
Posttransplantation time until first biopsy (mo)	18.3 ± 23.0	29.0 ± 27.8	24.7 ± 28.2	0.55
Graft failure				
Dialysis restart/retransplantation	22 (17.5)	7 (19.4)	6 (27.3)	0.49
Death from any cause	11 (8.7)	4 (11.1)	4 (18.2)	0.45

Data are expressed as mean ± standard deviation or number (%).
ABMR, antibody-mediated rejection; ABOi, ABO-incompatible; FSGS, focal segmental glomerulosclerosis; HLA, human leukocyte antigen; IgA, immunoglobulin A.

years, with a mean BMI of 22.85 ± 3.05 kg/m^2. Among the patients, 87 (69.0% were male. The most common cause of end-stage renal disease (ESRD) was diabetic nephropathy (24.5%) followed by immunoglobulin A nephropathy (13.5%). All donors were living donors (related or unrelated) with a mean age of 47.9 ± 8.6 years. The mean number of HLA mismatches was 3.84 ± 1.43. Thirty-four patients (27.0%) showed baseline isoagglutinin titers of ≥1:128. The mean number of biopsies was 1.7 ± 1.3 and the mean follow-up duration was 5.9 ± 2.8 years. The posttransplantation time until the first biopsy was 18.34 ± 22.99 months. Twenty-two patients (17.5%) experienced graft failure (e.g., restarting dialysis, retransplantation) and 11 patients (8.7%) died during the follow-up period.

Association between C4d expression and Banff scores

Each biopsy specimen was characterized either as C4d-positive or C4d-negative and compared based on the Banff scores, presence of DSAs, and posttransplantation time until biopsy (Table 2). Among the 214 biopsies, 162 (75.7%) showed C4d positivity. The g, ptc, and total MVI (g + ptc) scores were significantly higher in the C4d-positive biopsies than in C4d-negative biopsies. The proportion

Table 2. Differences in DSA status and histological features according to C4d positivity

Variable	C4d-positive (n = 162)	C4d score, 1 or 2 (n = 118)	C4d score, 3 (n = 44)	C4d-negative (n = 52)	p-value	
					C4d-positive vs. C4d-negative	C4d score 1 or 2 vs. 3
DSA					0.61	0.098
DSA-negative	54 (33.3)	42 (35.6)	12 (27.3)	16 (30.8)		
DSA-positive	66 (40.7)	51 (43.2)	15 (34.1)	16 (30.8)		
Class I	26 (16.0)	14 (11.9)	12 (27.3)	5 (9.6)		
Class II	52 (32.1)	41 (34.7)	11 (25)	11 (21.2)		
NA or ND	42 (25.9)	25 (21.2)	17 (38.6)	20 (38.5)		
MVI score	2.44 ± 2.12	2.18 ± 2.00	2.80 ± 2.17	1.46 ± 2.02	<0.001	<0.001
Glomerulitis	1.10 ± 1.14	1.02 ± 1.14	1.32 ± 1.12	0.58 ± 1.02	0.004	<0.001
Peritubular capillaritis	1.35 ± 1.23	1.30 ± 1.21	1.48 ± 1.28	0.89 ± 1.18	0.006	<0.001
MVI ≥ 2 and g ≥ 1	81 (50.0)	55 (46.6)	26 (59.1)	14 (26.9)	0.004	0.22
Other Banff scores						
t	1.53 ± 1.06	1.55 ± 1.03	1.45 ± 1.15	1.23 ± 1.04	0.08	0.06
i	1.43 ± 1.01	1.47 ± 0.98	1.34 ± 1.10	1.23 ± 1.10	0.22	0.27
v	0.19 ± 0.54	0.16 ± 0.47	0.25 ± 0.69	0.14 ± 0.53	0.09	0.12
ci	1.17 ± 0.91	1.27 ± 0.92	0.89 ± 0.81	1.04 ± 0.77	0.36	<0.001
ct	1.10 ± 0.94	1.20 ± 0.96	0.80 ± 0.85	0.94 ± 0.83	0.31	<0.001
Posttransplantation time until biopsy (mo)	25.40 ± 25.10	28.02 ± 24.71	18.43 ± 25.20	19.70 ± 22.3	0.15	<0.001

Data are expressed as number (%) or mean ± standard deviation.
DSA, donor-specific antibody; MVI, microvascular inflammation; NA, not available; ND, not done.

of cases with MVI score of ≥2 and g score of ≥1 was also significantly higher in the C4d-positive biopsies (50.0% vs. 26.9%, p = 0.004). Other Banff scores, including t, i, v, ct, ci, and posttransplantation time were not significantly different between C4d-positive and C4d-negative biopsies.

C4d-positive biopsies were further divided in diffuse C4d-positive specimens (C4d score of 3, n = 44) and focal C4d-positive specimens (C4d score of 1 or 2, n = 118). Diffuse C4d positivity was accompanied with significantly higher MVI (2.8 vs. 2.2, p < 0.001), g (1.32 vs. 1.02, p < 0.001), and ptc (1.48 vs. 1.30, p < 0.001) scores and a higher proportion of cases with lower ci (1.17 vs. 1.04, p < 0.001) and ct (1.10 vs. 0.94, p < 0.001) scores compared with the C4d-negative specimens. The posttransplantation time until biopsy was significantly shorter in diffuse C4d-positive specimens than in the C4d-negative or focal C4d-positive specimens.

Associations between donor-specific antibodies and Banff scores and clinical outcomes

Each patient was defined as DSA-positive or DSA-negative and Banff scores and clinical outcomes were compared (Table 3). Among 126 patients, 49 (38.9%) were positive for DSAs and 77 (61.1%) were negative. DSA-positive patients had a significantly higher total MVI (3.56 vs. 1.36, p < 0.001), g (1.60 vs. 0.58, p < 0.001), ptc (1.96 vs. 0.78, p < 0.001), and i (1.68 vs. 1.20, p = 0.001) scores than DSA-negative patients. Furthermore, C4d scores and other Banff scores including t, v, ct, and ci were not significantly different between the two groups.

Among DSA-positive patients, 12 (24.5%) lost their graft function during the follow-up period and 6 (12.2%) died. Among DSA-negative patients, 10 (13.0%) lost their graft function and five (6.5%) died.

Clinicopathological characteristics based on microvascular inflammation, C4d positivity, and donor-specific antibody status

C4d positivity in the diagnosis of antibody-mediated rejection in ABO-incompatible patients

Among the 126 study patients, 62 (49.2%) had MVI score of ≥2 with at least mild g (≥1). Among the 62 patients, 36

Table 3. Banff scores in DSA-positive and DSA-negative patients

Variable	DSA-positive (n = 49)	DSA-negative (n = 77)	p-value
MVI score	3.56 ± 2.02	1.36 ± 1.74	<0.001
Glomerulitis	1.60 ± 1.14	0.58 ± 0.33	<0.001
Peritubular capillaritis	1.96 ± 1.16	0.78 ± 1.04	<0.001
C4d score			0.15
0	12 (24.5)	21 (27.3)	
1	12 (24.5)	21 (27.3)	
2	15 (30.6)	11 (14.3)	
3	10 (20.4)	24 (31.2)	
Other Banff scores			
t	1.56 ± 1.02	1.39 ± 1.09	0.25
i	1.68 ± 0.95	1.20 ± 1.04	0.001
v	0.26 ± 0.70	0.17 ± 0.38	0.22
ci	1.16 ± 0.94	0.99 ± 0.90	0.34
ct	1.22 ± 0.87	1.08 ± 0.87	0.22
Graft failure			
Restart dialysis/retransplantation	12 (24.5)	10 (13.0)	0.097
Death from any cause	6 (12.2)	5 (6.5)	0.34

Data are expressed as mean ± standard deviation or number (%).
DSA, donor-specific antibody; MVI, microvascular inflammation.

patients (58.1%) were DSA-positive and diagnosed with active ABMR regardless of the C4d positivity status. Among the 26 (41.9%) DSA-negative patients, 22 (84.6%) met the diagnostic criteria for active ABMR based on C4d positivity and were categorized into the C4d-positive MVI score of ≥2 group.

Clinicopathological characteristics

Baseline data of the absolute ABMR group and the C4d-positive MVI score of ≥2 group are summarized in Table 1. Significant differences were not observed in the clinical characteristics between the two groups including age, BMI, sex, cause of ESRD, donor age, number of HLA mismatches, proportion of baseline isoagglutinin titer of ≥1:128, ABO group of the donor and recipient, mean follow-up period, and posttransplantation time until first biopsy. In contrast, the number of for-cause biopsies was significantly higher in the absolute ABMR group than in the C4d-positive MVI score of ≥2 group (2.17 ± 1.45 vs. 1.64 ± 0.90, p = 0.01). In the absolute ABMR group, seven patients (19.4%) lost their graft function during the follow-up period and four (11.1%) died. In the C4d-positive MVI score of ≥2 group, six patients (27.3%) lost their graft function and four (18.2%) died.

Banff scores of the absolute ABMR and C4d-positive MVI score of ≥2 groups are summarized in Table 4; MVI (4.80 vs. 3.91, p = 0.003) and ptc (2.53 vs. 2.00, p = 0.008) scores were significantly higher in the absolute ABMR group than in the C4d-positive MVI score of ≥2 group. Other Banff scores including g, t, i, v, ct, and ci did not significantly differ between the two groups. Among the C4d-positive MVI score of ≥2 group, nine cases (40.9%) showed diffuse C4d positivity (C4d score, 3) and 13 (59.1%) showed focal C4d positivity (C4d score, 1 or 2).

In the absolute ABMR group, recurrence of ABMR was observed in 14 patients (38.9%), and in the C4d-positive group, recurrence of ABMR was observed in seven patients (31.8%); the difference was non-significant (p = 0.59). Furthermore, in the absolute ABMR group, concurrent acute T-cell-mediated rejection was observed in 10 patients (27.8%), and in the C4d-positive group, was only observed in four patients (18.1%) but without statistical significance (p = 0.69).

Graft function and survival in the absolute antibody-mediated rejection and C4d-positive groups

Graft function was measured at 3 and 6 months and then yearly after transplant by analyzing eGFR according to

Table 4. Banff scores in the absolute ABMR and the C4d-positive MVI score of ≥2 groups

Score	Absolute ABMR (n = 36)	C4d-positive group (n = 22)	p-value
MVI scores	4.80 ± 1.15	3.91 ± 0.97	0.003
Glomerulitis	2.24 ± 0.83	1.91 ± 0.75	0.13
Peritubular capillaritis	2.56 ± 0.73	2.00 ± 0.82	0.008
Other Banff scores			
t	1.68 ± 0.94	1.73 ± 1.03	0.85
i	2.00 ± 0.91	1.73 ± 0.83	0.26
v	0.28 ± 0.66	0.14 ± 0.48	0.30
ci	1.11 ± 0.77	1.18 ± 0.85	0.74
ct	1.00 ± 0.85	1.14 ± 0.88	0.56
C4d score			0.06
0	8 (22.2)	0 (0)	
1	13 (36.1)	9 (40.9)	
2	8 (22.2)	4 (18.2)	
3	7 (19.4)	9 (40.9)	

Data are expressed as mean ± standard deviation or number (%).
ABMR, antibody-mediated rejection; MVI, microvascular inflammation.

the Modification of Diet in Renal Disease equation. The eGFR sequential changes in the two groups are illustrated in Fig. 2. The mean eGFR of the absolute ABMR group at 3 months and 7 years after transplant was 60.55 ± 14.4 mL/min/1.73 m^2 and 39.80 ± 20.34 mL/min/1.73 m^2, respectively. In the C4d-positive group, the mean eGFR at 3 months and 7 years was 60.11 ± 17.3 mL/min/1.73 m^2 and 38.17 ± 15.92 mL/min/1.73 m^2, respectively. Throughout the follow-up period, eGFR was not significantly different between the two groups.

Graft survival in both groups is plotted as Kaplan-Meier curves in Fig. 3. The 5-year graft survival rate for the absolute ABMR group was 79.1% and for the C4d-positive group was 84.0%. The log-rank test indicated no significant difference in graft survival between the groups (p = 0.40).

Patient age (p = 0.03) and MVI score of ≥4 (p = 0.04) were associated with graft loss and patient death based on multivariable Cox proportional hazards regression analysis (Table 5).

Graft function and survival in the C4d-negative group (MVI score of ≥2, g score of ≥1, C4d-negative, DSA-negative)

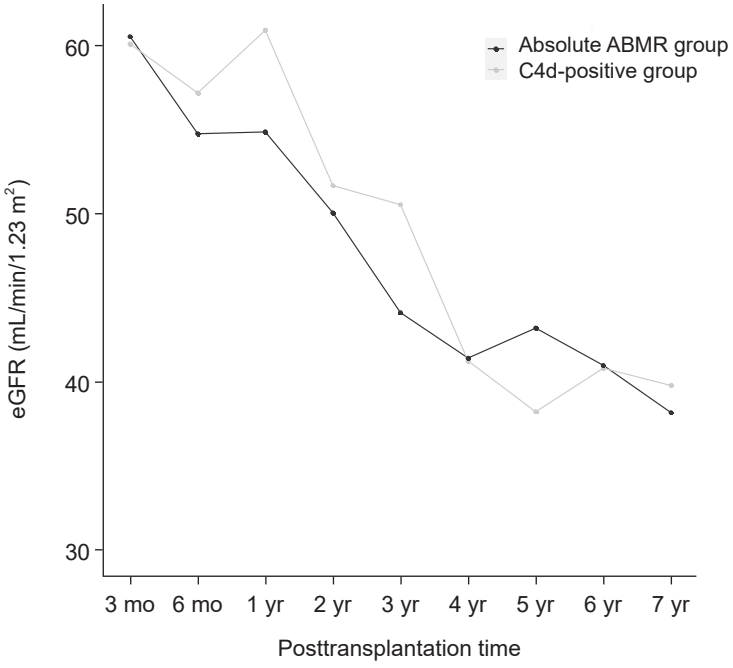
The number of cases in the C4d-negative group was too small (n = 4) to statistically compare their prognostic differences with other groups. The mean eGFR of the four cases in the C4d-negative group was similar to the mean eGFR in the absolute ABMR group and the C4d-positive group (Supplementary Table 1, available online). None of the four patients restarted dialysis or underwent retransplantation.

Mild g only with C4d positivity

Among the 214 biopsies, 10 (4.7%) specimens from nine patients showed mild g (g = 1), no ptc, and C4d positivity. Among these nine patients, four underwent another biopsy due to poor graft function and three of the four patients had an MVI score of ≥2. Additional biopsy was not performed in the other five patients because signs of graft function deterioration were not observed.

Discussion

ABOi kidney transplantation is currently considered a viable option for ESRD patients and produces similar outcomes to ABO-compatible kidney transplantation [1–8]. ABMR is one of the main causes of graft loss in ABOi kidney transplantation [15,18,21,22], and as the number of ABOi kidney transplantations increases, accurate diagnosis of ABMR in ABOi allograft has become particularly important. In ABMR, DSAs interact with the donor endothelium activating the classical complement pathway, which leads to graft injury. C4d is a split product of the C4 component of the classical complement pathway and does not have a known biological function; however, C4d staining in peritubular capillaries was shown correlated with the pres-



Absolute ABMR	36	36	33	33	32	27	18	11	10
C4d-positive	22	22	22	22	21	15	11	8	6
p-value	0.927	0.642	0.313	0.755	0.312	0.994	0.512	0.224	0.405

Figure 2. Mean serum eGFR in the absolute ABMR group and the C4d-positive group.
ABMR, antibody-mediated rejection; eGFR, estimated glomerular filtration rate.

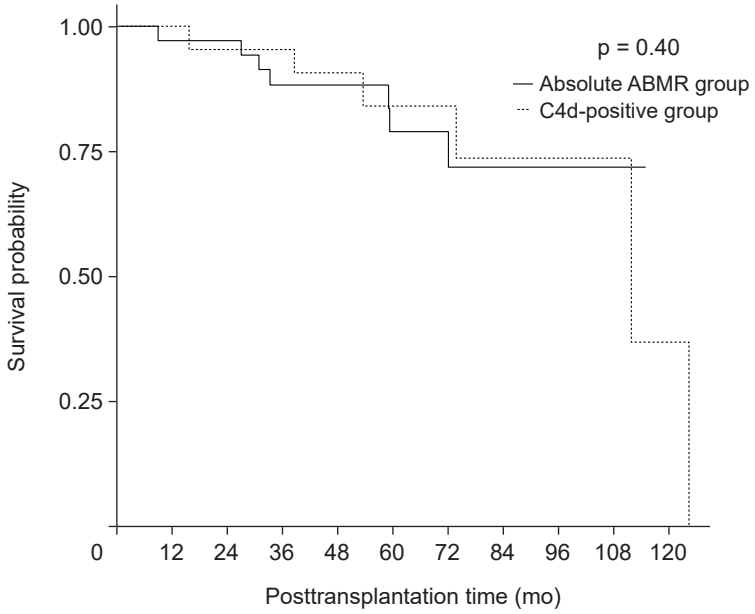


Figure 3. Graft survival in the absolute ABMR group and the C4d-positive group during the months after transplantation. Graft survival was calculated using the Kaplan-Meier method and compared with the long-rank test.
ABMR, antibody-mediated rejection.

Table 5. Multivariable Cox proportional hazard analysis for patient survival

Parameter	Hazard ratio (95% CI)	p-value
Age	1.04 (1.01–1.08)	0.03
Donor age	1.02 (0.98–1.06)	0.64
Sex	1.56 (0.55–4.38)	0.56
Body mass index	0.99 (0.85–1.18)	0.51
HLA mismatch ≥ 3	0.963 (0.73–1.28)	0.20
Baseline isoagglutinin titer, ≥1:128	1.14 (0.45–2.88)	0.49
Presence of donor-specific antibody	1.54 (0.63–3.77)	0.83
MVI score, ≥4	2.55 (1.05–6.17)	0.04
C4d-positivity	0.69 (0.24–1.96)	0.18
Acute T-cell–mediated rejection	2.12 (0.64–7.01)	0.39

CI, confidence interval; HLA, human leukocyte antigen; MVI, microvascular inflammation.

ence of DSAs and is considered evidence for antibody-tissue interactions [11,13,23]. Accordingly, C4d staining was incorporated in the 2003 Banff classification as an ABMR diagnostic marker and recognized as a DSA equivalent in the 2017 Banff classification [10,24].

However, in ABOi allografts, C4d positivity has been considered irrelevant to ABMR or MVI [11,15–18,25]. Haas et al. [17] considered C4d deposition without rejection a sign of accommodation in ABOi allografts [17,26]. The unclear significance of C4d staining in ABOi allografts results in diagnostic difficulties; in the present study, allograft biopsies of 62 patients with MVI score of ≥2 and g score of ≥1, 36 (58.1%) were DSA-positive and the remaining 26 (41.9%) were DSA-negative and required DSA-equivalent evidence to be diagnosed with ABMR. In cases of biopsies from ABOi allografts with a positive MVI score (g + ptc > 0), C4d positivity, and no DSAs, the 2017 Banff classification recommends molecular testing (i.e., the ABMR classifier) [10]. However, the cost and technical complexity of these tests render their application in daily clinical practice difficult. Furthermore, the validation of molecular testing in the diagnosis of ABMR in ABOi has not yet been clarified.

In the present study, 75.7% of the 214 biopsy specimens showed C4d positivity, a similar number to previous reports [15–18,25]. However, unlike previous studies, C4d positivity was associated with higher g and ptc scores and higher rate of MVI score of ≥2 + g score of ≥1, possibly because only for-cause biopsies were collected for this study, while in previous studies, both for-cause and protocol biopsies were analyzed. The insignificance of C4d positivity in ABOi allografts has been reported in numerous studies [15–18]. However, in two studies, a possible role of C4d positivity in predicting graft survival was suggested. Couzi et al. [27] reported that C4d positivity with tubulointerstitial inflammation in ABOi allografts was associated with chronic graft dysfunction. Ishihara et al. [25] reported that a high C4d score was an independent predictor of MVI score of ≥2 in ABOi allografts. Based on the results of the present study and these previous studies, we suggest that among for-cause biopsies and biopsies with rejection, C4d positivity may be a predictor of poor graft outcome even in ABOi allografts.

In the present study, C4d-positive cases were further divided into diffuse C4d-positive cases (C4d, 3) and focal C4d-positive cases (C4d, 1 or 2). Diffuse C4d-positive cases showed significantly higher MVI scores and significantly lower ct and ci scores. This may be because diffuse C4d-positive cases had a shorter posttransplantation duration (i.e., fewer days between transplantation and biopsy day).

Among the absolute ABMR and C4d-positive groups, multivariable Cox proportional hazards regression analysis was performed and patient age and MVI score of ≥4 were associated with poor outcome. This result is consistent with previous studies in which higher MVI score was associated with poor graft outcome [12,25,28].

In addition, the C4d-positive group did not show significant differences from the absolute ABMR group in terms of eGFR and graft survival. We suggest that ABOi allografts with MVI and C4d positivity without identifiable DSAs may be classified as ABMR and should be treated as such.

Several limitations should be mentioned. Only for-cause biopsies were included in the study and zero-hour biopsies and protocol biopsies were excluded; however, we believe this may be more appropriate for interpreting ABOi allograft biopsy in cases of graft deterioration. Furthermore, this study included biopsies and patients from a single center which limited the sample size. In addition, only four C4d-negative, MVI score of ≥2, g score of ≥1, C4d-negative, DSA-negative cases were included in this study, which did not allow statistical comparison with the other groups. Furthermore, molecular diagnostics in the C4d-positive MVI score of ≥2 cases was not performed. A multicenter study is required to further confirm the validity of our results.

In summary, the results indicate that cases of ABOi allograft biopsies that are C4d-positive, with MVI score of ≥2 and g score of ≥1 may be categorized and treated as ABMR cases. Larger studies and molecular research are required to determine the prognostic effect of C4d positivity/negativity in MVI score of ≥2 and g score of ≥1 cases in ABOi allograft.

Conflicts of interest

All authors have no conflicts of interest to declare.

Data sharing statement

The data presented in this study are available on request from the corresponding author.

Authors' contributions

Conceptualization: HC, SKP, HG
Data curation: HC, CHB, SKP, HG
Formal analysis: HC, HG
Methodology: CHB, SKP
Supervision: HK, HG
Validation: HC, HK
Writing–original draft: HC
Writing–review & editing: HK, HG
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