Donor-specific antibodies, glomerulitis, and human leukocyte antigen B eplet mismatch are risk factors for peritubular capillary C4d deposition in renal allografts

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Abstract

Background: The complement system plays an important role in the immune response to transplantation, and the diagnostic significance of peritubular capillary (PTC) C4d deposition (C4d+) in grafts is controversial. The study aimed to fully investigate the risk factors for PTC C4d+ and analyze its significance in biopsy pathology of kidney transplantation.

Methods: This retrospective study included 124 cases of kidney transplant with graft biopsy and donor-specific antibody (DSA) testing from January 2017 to December 2019 in a single center. The effects of recipient pathological indicators, eplet mismatch (MM), and DSAs on PTC C4d+ were examined using univariate and multivariate logistic regression analyses.

Results: In total, 35/124 (28%) were PTC C4d+, including 21 with antibody-mediated rejection (AMR), eight with renal tubular injury, three with T cell-mediated rejection, one with glomerular disease, and two others. Univariate analysis revealed that DSAs (P < 0.001), glomerulitis (P < 0.001), peritubular capillaritis (P < 0.001), and human leukocyte antigen (HLA) B eplet MM (P = 0.010) were the influencing factors of PTC C4d+. According to multivariate analysis, DSAs (odds ratio [OR]: 9.608, 95% confidence interval [CI]: 2.742–33.668, P = 0.001), glomerulitis (OR: 3.581, 95% CI: 1.246–10.289, P = 0.018), and HLA B eplet MM (OR: 1.166, 95% CI: 1.005–1.353, P = 0.042) were the independent risk factors for PTC C4d+. In receiver operating characteristic curve analysis, the area under the curve was increased to 0.831 for predicting PTC C4d+ when considering glomerulitis, DSAs, and HLA B eplet MM. The proportions of HLA I DSAs and PTC C4d+ in active antibody-mediated rejection were 12/17 and 15/17, respectively. Furthermore, the higher the PTC C4d+ score was, the more serious the urinary occult blood and proteinuria of recipients at the time of biopsy.

Conclusions: PTC C4d+ was mainly observed in AMR cases. DSAs, glomerulitis, and HLA B eplet MM are the independent risk factors for PTC C4d+.

Keywords: Kidney transplantation; C4d deposition; donor-specific antibody; Glomerulitis; human leukocyte antigen eplet

Introduction

The role of the complement system in the immune response to transplantation is very complex and involves many aspects. In complement activation, C4d is a common marker of the classical antibody-mediated pathway and non-antibody-mediated lectin pathway. Peritubular capillary C4d deposition (PTC C4d+) is used as a specific marker of alloantibody-dependent graft injury in kidney allografts and is a marker for antibody-mediated rejection (AMR) in Banff 2003 diagnosis criteria. The lectin pathway is activated in transplant biopsies with delayed graft function (DGF), AMR, and T cell-mediated rejection (TCMR). Thus, C4d is not only an indicator of the interaction between antibodies and tissues but also a marker of tissue damage, whether caused by ischemia-reperfusion injury or by other immune factors.

Human leukocyte antigen (HLA) eplet mismatch (MM) load has been suggested as improving HLA antigen MM determination for organ selection. Indeed, several recent publications show that HLA eplet matching associated...
with allograft outcomes prevents donor-specific antibody (DSA) development and graft rejection.[3,4] Nevertheless, it remains unknown whether there is a relationship between HLA MM and PTC C4d+.

In this study, 124 cases with pathological biopsy and HLA eplet matching data as well as DSA detection were collected to analyze the impact of PTC C4d+ in transplanted kidneys. The main objective of the study was to reveal the risk factors for PTC C4d+ in transplanted kidneys.

Methods

Ethics approval and consent to participate
This retrospective study was approved by the Institutional Review Board/Ethics of The First Affiliated Hospital of Xi’an Jiaotong University, Xi’an, China (No. XJTU1AF2015LSL-058). This study was performed in accordance with the ethical standards of the Declaration of Helsinki. All patients signed an informed consent form, were informed about the study, and agreed to have their clinical information used in the reported research.

Study cohort
This study reviewed the records of 124 recipients of a single kidney transplant in a single center from January 2017 to December 2019 for which biopsy pathology and DSA testing were available. Deceased donation (DD) organs were obtained by the Organ Procurement Organization of the First Affiliated Hospital of Xi’an Jiaotong University and were allocated by the China Organ Transplant Response System (COTRS, version 2.0, Healthcare Commission, Xizhimen Wai Nan Lu, Xicheng District, Beijing, China).

HLA typing and HLA eplet MM assessment
High-resolution HLA typing was performed using sequence-specific primer technology (LABType HD SSO, Micro SSP; One Lambda, Canoga Park, CA, USA). HLAMatchmaker software (version 3.1, http://www.epitopes.net/downloads.html, University of Pittsburgh Medical Center: 200 Lothrop St, Pittsburgh, PA 15213, USA) was used to define eplet MM between donor and recipient HLA alleles. First, donor and recipient HLA eplets were analyzed with HLAMatchmaker software and compared, and then the number of different HLA eplets was calculated. The HLA eplet MM numbers of donors and recipients were obtained.

Anti HLA antibody monitoring
Posttransplant monitoring of DSAs was implemented for all kidney transplant patients. As routine clinical practice in our program since 2017, serum samples were collected at 0, 1, 3, 6, 9, and 12 months and then yearly or at the time of biopsy for graft dysfunction. DSA screening was performed using flow cytomtery panel reactive antibody (PRA) beads representing HLA-A, -B, -Cw, -DR, -DQ, and -DP antigens (One Lambda). HLA antibody specificities were validated by LABScreen single-antigen beads (one lambda) using a threshold mean fluorescence intensity (MFI) ≥1000.

Immunosuppression and postoperative management
The patients were treated with rabbit anti-human thymocyte globulin (rabbit anti-thymocyte globulin [rATG], Sanofi: Bridgewater, NJ, USA) (1.25–1.50 mg·kg⁻¹·d⁻¹, intravenously) for induction therapy on the day of surgery and were then tapered until discontinuation on postoperative day 5. Methylprednisolone (Pfizer, Burtt Rd, Andover, MA 01810, USA) was administered on the day of surgery, tapered along (500, 250, 120, and 80 mg after the operation, respectively) with the rATG and then replaced by prednisone (10 mg/day). The basic immunosuppressive regimen was Tacrolimus (TAC, Astellas Pharma Inc. Tokyo: 2-5-1, Nihonbash-Honcho, Chuo-Ku, Tokyo, Japan) (0.06–0.08 mg·kg⁻¹·d⁻¹) or cyclosporine A (CsA, Novartis, 181 Massachusetts Ave, Cambridge, MA 02139, USA) (4.0–4.5 mg·kg⁻¹·d⁻¹), mycophenolate mofetil (MMF, Roche, Switzerland) (1500–2000 mg/d), and prednisone (10 mg/d).

Biopsy pathology
Clinically indicated allograft biopsies were performed on patients whose proteinuria was >0.5 g/d or whose serum creatinine (sCr) rose >25% from baseline without a known cause. Ultrasound-guided percutaneous biopsy was performed with an 18G puncture needle to puncture two tissues. To be qualified for adequate puncture tissue, >10 glomeruli and >2 arterioles with smooth muscle layers are required. All tissues were routinely fixed, embedded, sliced, and treated with immunofluorescence antibodies, hematoxylin and Eosin, periodic acid–Schiff, Masson, periodic Schiff-Methenamine, and immunohistochemical staining. C4d+ was determined using immunohistochemistry with rabbit anti-human C4d monoclonal antibody (ab136921, Abcam, 152 Grove Street Waltham, MA 02453, USA). Histology was evaluated based on Banff criteria 2017[7] by two experienced renal transplant pathologists (Hui-Lin Gong and Yan-Xia Sui).

Graft rejection diagnosis and treatment
Graft rejection was identified on biopsy and classified according to the Banff 2017 criteria.[7] Recipients with DSAs and/or graft rejection were treated via optimization of TAC trough levels and mycophenolate doses. A steroid bolus with a taper was given when clinical or subclinical TCMR and/or AMR was present on biopsy. Occasionally, rATG was administered to patients with severe TCMR. Recipients with active antibody-mediated rejection (aAMR) received high-dose (2 g/kg) intravenous immunoglobulin (IVIG) and plasmapheresis combined with rituximab (375 mg/m² BSA) or bortezomib (1.3 mg/m²). For chronic active AMR (caAMR), IVIG and plasmapheresis were given.

Quantitative criteria for C4d scores
According to the Banff criteria, PTC C4d+ is linear C4d staining in peritubular capillaries (C4d2 or C4d3 by
immunofluorescence in frozen sections or C4d >0 by immunohistochemistry in paraffin sections). Quantitative criteria for the C4d score used in the study were according to Banff 2015 criteria as follows: C4d0, no staining of PTCs; C4d1, minimal C4d staining (>0 but <10% of PTCs); C4d2, focal C4d staining (10%–50% of PTCs); and C4d3, diffuse C4d staining (>50% of PTCs).

**Statistical analysis**

The results are expressed as numerical values and percentages for categorical variables and as the mean ± standard error for continuous variables. Differences in the clinical characteristics of recipients and donors were examined using Student t test if data match normal distribution and homogenous variance. If the normal distribution is not followed, the Mann-Whitney U test is used. Univariate and multivariate logistic regression analyses were applied to analyze the influencing factors for PTC C4d+ in grafts. Receiver operating characteristic (ROC) curves were produced to compare the predictive value of variables for PTC C4d+.

**Results**

**Cohort characteristics**

During the study period, 954 patients received a kidney allograft at our center. We excluded 830 cases without pathological biopsy and DSA testing, cases of ABO blood group (ABO) incompatible (ABOi) transplants, and cases with comorbidities (infection, hepatitis, diabetes, autoimmune disease, and tumor). The final cohort consisted of 124 patients, including 108 cases of DD kidney transplant and 16 cases of living relative kidney transplant. The study cohort included 33 TCMRs, 31 renal tubular injuries (TIs), 28 AMRs (including 17 aAMRs and 11 chronic active antibody-mediated rejection [caAMRs]), 12 glomerular diseases (GDs), 12 BK virus nephritis, and eight others [Figure 1A]. There was a total of 35 cases of PTC C4d+, including 21 AMRs, eight TIs, three TCMRs, one GD, and two other cases (diabetic kidney injury and thrombotic microangiopathy) [Figure 1B]. The PTC C4d+ scores of the AMR cases were significantly higher than those of the other diagnoses (P < 0.001) [Figure 1C].

**Clinical information for PTC C4d+ and PTC C4d0 cases**

The characteristics of the cohort grouped by C4d+ and C4d0 are summarized in Table 1. There were no significant differences between the PTC C4d+ and PTC C4d− groups regarding recipient age, sex, body mass index (BMI), primary disease, dialysis type and duration, induction therapy, maintenance immunosuppression, stability of drug concentration, or time from kidney transplantation to biopsy. Additionally, no significant differences were found in donor age, BMI, cause of death, type of donation, terminal creatinine, hypertension, or diabetes history. However, significant differences between the PTC C4d+ and PTC C4d0 groups were found with regard to Banff diagnosis criteria, including glomerulitis (g) (P < 0.001), tubulitis (t) (P = 0.001), interstitial inflammation (i) (P = 0.003), and perivascular capillaritis (ptc) (P < 0.001). Regardless, no difference in intimal arteritis (v) (P = 0.578) was found between the two groups. The positive percentage rate of PRA and DSAs in the PTC C4d+ group was significantly higher than that of PTC C4d0 group (P < 0.001). For HLA eplet MM, only the HLA B eplet MM in the PTC C4d+ group was markedly higher than that of PTC C4d0 group (P = 0.015).

**Risk factors for PTC C4d+**

According to univariate analysis of the influencing factors for PTC C4d+, recipient ptc, g, PRA, DSAs, and HLA B eplet MM were associated with a higher risk of PTC C4d+, especially ptc (odds ratio [OR]: 6.594, 95% confidence interval [CI]: 2.319–18.746, P < 0.001), g (OR: 7.915, 95% CI: 3.023–20.725, P < 0.001), and DSAs (OR: 4.038, 95% CI: 2.120–7.693, P < 0.001) [Table 2]. Variables with P values <0.1 and with the highest OR values for similar variables were selected for multivariate analysis, and as shown in Table 3, independent risk factors for PTC C4d+ included DSAs (OR: 9.608, 95% CI: 2.742–33.668, P < 0.001), g (OR: 3.581, 95% CI: 1.246–10.289, P = 0.018), and HLA B eplet MM (OR: 1.166, 95% CI: 1.005–1.353, P = 0.042).

**Predictive value of composite parameters for PTC C4d+**

The coevaluation of PTC C4d+ was based on g, DSAs, and HLA B eplet MM using ROC curves, with calculated area under the curves (AUCs) of 0.686, 0.738, and 0.640. However, the AUC increased to 0.831 when all variables
Table 1: Clinical information for PTC C4d+ and PTC C4d0 recipients.

| Characteristics                        | C4d+ (n = 35) | C4d0 (n = 89) | Statistics | P value |
|----------------------------------------|---------------|---------------|------------|---------|
| **Recipient-related information**      |               |               |            |         |
| Age (years)                            | 35.8 ± 10.4   | 35.6 ± 9.1    | -0.132^t   | 0.895   |
| Male/female                            | 21/14         | 68/21         | 2.576^t    | 0.108   |
| BMI (kg/m²)                            | 20.19 ± 3.33  | 21.24 ± 3.30  | 1.573^t    | 0.118   |
| Primary disease (%)                    |               |               | 0.426^t    | 0.980   |
| Glomerulonephritis                     | 65.7          | 64.0          |            |         |
| Hypertensive nephropathy               | 11.4          | 13.5          |            |         |
| Membranous nephropathy                 | 11.4          | 11.2          |            |         |
| IgA nephropathy                        | 8.6           | 9.0           |            |         |
| Diabetes                               | 0             | 0             |            |         |
| Others                                 | 2.9           | 2.3           |            |         |
| Dialysis type (%)                      |               |               | 0.358^t    | 0.549   |
| Hemodialysis                           | 94.9          | 88.8          |            |         |
| Peritoneal dialysis                     | 5.1           | 11.2          |            |         |
| Dialysis duration (months)             | 25.45 ± 24.79 | 19.17 ± 19.43 | -1.230^t   | 0.165   |
| Induction therapy (%)                  |               |               | 0.027^t    | 0.871   |
| Anti-thymocyte globulin                | 85.7          | 80.9          |            |         |
| Basiliximab                            | 14.3          | 19.1          |            |         |
| Maintenance immuno-suppression (%)     |               |               | 0.039^t    | 0.844   |
| FK506/MPA/Pred                         | 91.4          | 94.4          |            |         |
| CsA/MPA/Pred                           | 8.6           | 5.6           |            |         |
| Stability of drug concentration (%)    |               |               | 0.543^t    | 0.461   |
| Time from KTx to biopsy (days)         | 163.37 ± 62.24 | 298.63 ± 37.81 | -4.015^t   | <0.001 |
| **Donor-related information**          |               |               |            |         |
| Age (years)                            | 51.65 ± 13.76 | 49.92 ± 15.19 | -0.577^t   | 0.565   |
| BMI (kg/m²)                            | 22.65 ± 2.93  | 21.73 ± 2.83  | -1.731^t   | 0.112   |
| Cause of death (%)                     |               |               | 1.932^t    | 0.748   |
| Trauma                                 | 27.1          | 40.5          |            |         |
| Hematencephalon                        | 34.3          | 40.4          |            |         |
| Hypoxic encephalopathy                 | 5.7           | 2.3           |            |         |
| Tumor                                  | 5.7           | 5.6           |            |         |
| Others                                 | 17.1          | 11.2          |            |         |
| Type of donation (%)                   |               |               | 0.343^t    | 0.558   |
| Deceased donation                      | 82.9          | 88.8          |            |         |
| Living relative donation               | 17.1          | 11.3          |            |         |
| Terminal creatinine (µmol/L)           | 87.21 ± 8.44  | 99.76 ± 7.00  | -0.848^t   | 0.396   |
| Hypertension (%)                       | 34.3          | 38.2          | 0.040^t    | 0.842   |
| Diabetes (%)                           | 5.7           | 3.4           | 0.205^t    | 0.650   |
| Banff criteria                         |               |               |            |         |
| g                                      | 0.51 ± 0.10   | 0.09 ± 0.03   | -4.697^t   | <0.001  |
| t                                      | 0.32 ± 0.11   | 0.73 ± 0.07   | 3.279^*    | 0.001   |
| i                                      | 0.54 ± 0.10   | 0.95 ± 0.07   | 3.036^*    | 0.003   |
| v                                      | 0.14 ± 0.08   | 0.09 ± 0.05   | -0.559^t   | 0.578   |
| ptc                                    | 0.40 ± 0.09   | 0.07 ± 0.03   | -4.426^t   | <0.001  |
| Anti-HLA antibody (%)                  |               |               |            |         |
| PRA                                    | 60.0          | 10.1          | 31.423^t   | <0.001  |
| DSA                                    | 54.3          | 6.7           | 32.386^t   | <0.001  |
| HLA mismatches (eplet)                 |               |               |            |         |
| A                                      | 5.77 ± 0.85   | 6.89 ± 0.39   | -0.956^t   | 0.339   |
| B                                      | 6.57 ± 0.64   | 4.82 ± 0.34   | -2.425^t   | 0.015   |
| C                                      | 2.71 ± 0.33   | 2.56 ± 0.20   | -0.411^t   | 0.681   |
| DRB1                                   | 5.45 ± 0.62   | 5.01 ± 0.37   | -0.465^t   | 0.642   |
| DQB1                                   | 2.77 ± 0.53   | 2.08 ± 0.24   | -0.761^t   | 0.447   |
| HLA I                                  | 15.06 ± 1.29  | 14.28 ± 0.80  | -0.406^t   | 0.685   |
| HLA II                                 | 8.23 ± 0.93   | 7.10 ± 0.45   | -0.732^t   | 0.464   |
| HLA I +II                              | 23.29 ± 1.85  | 21.38 ± 0.99  | -0.659^t   | 0.510   |

Values were shown as mean ± standard error, n, or percentage. ^ Student t test. † Chi-square test. ¶ Mann-Whitney U test. BMI: Body mass index; C4d+: C4d deposition; CsA: Cyclosporine A; DD: Deceased donation; DSA: Donor-specific antibody; g: Glomerulitis; HLA: Human lymphocyte antigen; i: Interstitial inflammation; MPA: mycophenolic acid; MMs: Mismatches; MN: Membranous nephropathy; PRA: Panel reactive antibody; PTC C4d+: Peritubular capillary C4d deposition; ptc: Peritubular capillaritis; PTC: Peritubular capillary; Pred: prednisone; t: Tubulitis; v: Intimal arteritis; KTx: Kidney transplantation.
Furthermore, the specificity and sensitivity of predictive PTC C4d+ were 0.816 and 0.818, respectively.

PTC C4d+ and HLA antibody type in aAMR and caAMR

PTC C4d+ and the type of serum DSA in aAMR and caAMR were also analyzed. In total, 15/17 of the aAMR cases were PTC C4d+, but only 7/12 of caAMR cases were PTC C4d+. Moreover, the PTC C4d+ grade in aAMR was significantly higher than that of caAMR (2.16 ± 0.25 vs. 0.88 ± 0.35, Z = −2.522, P = 0.013). HLA class I DSAs were found in 12/17 of aAMR cases, whereas HLA class II DSAs were mainly detected in 8/12 of caAMR cases. Moreover, the average MFI of DSAs in aAMR cases was lower than that of caAMR cases (t = −1.152, P = 0.265), but there was no significant difference.

Effect of PTC C4d+ on allograft function at the time of biopsy

It has been reported that PTC C4d+ is associated with poorer graft function. Therefore, this study also analyzed renal function at the time of biopsy, including sCr, the estimated glomerular filtration rate (eGFR), urinary occult blood, and urinary protein. The urinary occult blood (t = −4.315, P < 0.001) and urinary protein (t = −2.900, P = 0.004) levels of PTC C4d+ cases were significantly higher than that of PTC C4d0 cases, but no

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**Table 2: Univariate analysis of risk factors affecting PTC C4d+ (N = 124)**.

| Variables                      | Odds ratios | Lower bound | Upper bound | P value |
|--------------------------------|-------------|-------------|-------------|---------|
| **Recipient’s variables**      |             |             |             |         |
| Age                            | 1.003       | 0.962       | 1.045       | 0.894   |
| Sex                            | 2.159       | 0.937       | 4.794       | 0.071   |
| BMI                            | 0.905       | 0.798       | 1.026       | 0.120   |
| Primary disease                | 3.013       | 0.685       | 1.498       | 0.955   |
| Dialysis type                  | 2.082       | 0.404       | 10.726      | 0.384   |
| Dialysis duration              | 1.013       | 0.994       | 1.033       | 0.171   |
| Induction therapy              | 0.823       | 0.481       | 1.141       | 0.478   |
| Maintenance immunosuppression  | 1.494       | 0.388       | 5.409       | 0.581   |
| Stability of drug concentration| 0.742       | 0.291       | 1.892       | 0.537   |
| Time from KTx to biopsy        | 0.999       | 0.997       | 1.000       | 0.073   |
| **Donors’ variables**          |             |             |             |         |
| Age                            | 1.008       | 0.981       | 1.037       | 0.562   |
| BMI                            | 1.120       | 0.973       | 1.290       | 0.114   |
| Cause of death                 | 0.867       | 0.575       | 1.305       | 0.493   |
| Type of donation               | 1.468       | 0.779       | 2.766       | 0.235   |
| Terminal creatinine            | 0.996       | 0.989       | 1.004       | 0.316   |
| Hypertension                   | 0.805       | 0.355       | 1.823       | 0.603   |
| Diabetes                       | 1.717       | 0.274       | 10.745      | 0.563   |
| **Banff variables**            |             |             |             |         |
| ptc                            | 6.594       | 2.319       | 18.746      | <0.001  |
| g                              | 7.915       | 3.023       | 20.725      | <0.001  |
| t                              | 0.327       | 0.160       | 0.669       | 0.002   |
| i                              | 0.397       | 0.211       | 0.747       | 0.004   |
| v                              | 1.243       | 0.578       | 2.673       | 0.578   |
| **HLA antibody variables**     |             |             |             |         |
| PRA                            | 3.180       | 1.976       | 5.118       | <0.001  |
| DSA                            | 4.038       | 2.120       | 7.695       | <0.001  |
| **HLA MM variables**           |             |             |             |         |
| HLA A eplet MM                 | 0.961       | 0.892       | 1.036       | 0.300   |
| HLA B eplet MM                 | 1.163       | 1.033       | 1.308       | 0.010   |
| HLA C eplet MM                 | 1.042       | 0.851       | 1.277       | 0.689   |
| HLA DRB1 eplet MM              | 1.036       | 0.928       | 1.157       | 0.526   |
| HLA DQB1 eplet MM              | 1.104       | 0.953       | 1.279       | 0.186   |
| HLA I eplet MM                 | 1.014       | 0.963       | 1.068       | 0.605   |
| HLA II eplet MM                | 1.053       | 0.968       | 1.144       | 0.227   |
| HLA I+II eplet MM              | 1.020       | 0.980       | 1.062       | 0.332   |

*Logistic regressions were performed; odds ratios and 95% confidence intervals were reported. BMI: Body mass index; CI: Confidence interval; DSA: Donor-specific antibody; g: Glomerulitis; HLA: Human lymphocyte antigen; t: Interstitial inflammation; MM: Mismatch; PRA: Panel reactive antibody; PTC C4d+: Peritubular capillary C4d deposition; ptc: Peritubular capillaritis; PTC: Peritubular capillary; t: Tubulitis; v: Intimal arteritis; KTx: Kidney transplantation.

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were combined (Table 4). Furthermore, the specificity and sensitivity of predictive PTC C4d+ were 0.816 and 0.818, respectively.
Recipient gender 0.780 0.237 2.569 0.684
Time from KTx to biopsy 0.999 0.998 1.001 0.244
HLA B eplet MM 1.166 1.005 1.353 0.042

Variables Odds ratios 95% CI for mean

| Variables       | Odds ratios | Lower bound | Upper bound | P value |
|-----------------|-------------|-------------|-------------|---------|
| DSA             | 9.608       | 2.742       | 33.668      | <0.001  |
| g               | 3.381       | 1.246       | 10.289      | 0.018   |
| HLA B eplet MM  | 1.166       | 1.005       | 1.353       | 0.042   |
| Time from KTx to biopsy | 0.999 | 0.998 | 1.001 | 0.244 |
| Recipient gender| 0.780       | 0.237       | 2.569       | 0.684   |

Variables Odds ratios Lower bound Upper bound
DSA 0.738 0.628 0.847
HLA B eplet MM 0.640 0.527 0.753

**Table 4: ROC curves for g, DSA, and HLA B eplet MM as predictors of PTC C4d+**

| Variables       | Area | Lower bound | Upper bound |
|-----------------|------|-------------|-------------|
| g               | 0.686| 0.572       | 0.800       |
| DSA             | 0.738| 0.628       | 0.847       |
| HLA B eplet MM  | 0.640| 0.527       | 0.753       |

**Discussion**

Our study included 124 cases of kidney transplant with graft biopsy and DSA testing. We identified that DSAs, glomerulitis, and HLA B eplet MM are the risk factors for PTC C4d+. The proportion of PTC C4d+ was higher in aAMR than that of caAMR. In addition, HLA class I DSAs were mostly associated with aAMR, whereas HLA class II DSAs were associated with caAMR. PTC C4d+ obviously affected graft sCr, urinary occult blood, and urinary protein; and the PTC C4d+ score was higher.

Complement deposition strongly correlates with histopathological changes observed in renal transplants, and all three complement recognition pathways are involved. Regarding organ ischemia-reperfusion injury that activates the lectin pathway, antibodies, such as IgM, immunoglobulin G (IgG1, IgG2, and IgG3), activate the classical pathway associated with most AMRs; and C4d is a common marker of both pathways.

Based on the Banff diagnostic criteria, the only glomerulitis was the independent risk factor for PTC C4d+ in this study. Tubulitis and interstitial inflammation are the important diagnostic indicators of TCMR according to the Banff criteria and mainly induce tubular epithelial cell damage. Arteritis, mainly occurring in small arteries, is also an important index of TCMR according to the Banff diagnostic criteria, and glomerulonephritis and peritubular capillaritis (ptc) are common in AMR. These phenomena were also observed in our study. As shown in Supplementary Table 1, http://links.lww.com/CM9/A726, the glomerulonephritis and peritoneal capillaritis scores of

marker for activation of the alternative complement pathway. All three markers have been detected in biopsies of transplant cases with DGF, AMR, and TCMR. In the present study, AMR and TI accounted for 60% and 22.9% of PTC C4d+ cases, respectively. We also found three TCMR cases to be PTC C4d+. In general, AMR was most common among the PTC C4d+ cases, and the PTC C4d scores of AMR cases were significantly higher than that of TI and TCMR. Hence, the main factors causing graft injury after transplantation are immune factors.

HLA eplet MM load has been suggested as an improvement of HLA antigen MM determination for organ selection. More recently, the term “eplet MM load” was introduced in publications. Donor–recipient matching based on predicted indirectly recognizable HLA eplets independently predicts the incidence of de novo donor specific antibody (dnDSA) following renal transplantation. In this study, among all the HLA eplet MMs of the HLA locus, we found that only HLA B eplet MM affects PTC C4d+. Furthermore, when we combined DSAs, glomerulitis, and HLA B eplet MM, the AUC of predicted PTC C4d+ increased to 0.831. This indicates that HLA B eplet MM also plays a certain role in PTC C4d+, even though DSAs and glomerulitis were found to be the main risk factors for PTC C4d+. We speculated the reasons that are as follows: (1) this may be related to the limited sample size and the short observation follow-up time of this study; (2) as the HLA B locus has more antigens than other loci, the probability of donor and recipient matching is relatively small; and (3) the effect of HLA B eplet MM on PTC C4d+ is still mediated by the immune response. Because the risks of DSAs and AMR are higher in recipients with a higher donor HLA eplet MM, the probability of PTC C4d+ also increases correspondingly.
AMR cases were significantly higher than that of all the other diagnoses. It has been reported that the total number of infiltrating cells in glomeruli and PTC are associated with PTC C4d+ and that infiltrating cells in glomeruli and PTC are predominantly macrophages and T cells with completely cytotoxic phenotype. Both macrophages and cytotoxic T cells can damage endothelial cells and induce lectin-mediated complement pathway activation, which findings support our results.

In our study, the proportion of PTC C4d+ in aAMR was higher than that of caAMR. In general, HLA class I DSAs were mostly associated with aAMR, whereas HLA class II DSAs were associated with caAMR. It has been reported that antibodies that activate the classical pathway of complement include IgM, IgG1, IgG2, and IgG3, which are mainly related to aAMR. IgA, IgE, IgG4, and LPS activate the alternative pathway, which is associated with aAMR. HLA class I DSAs mostly include IgG1 and IgG3, which are primarily involved in the classical pathway of complement activation and PTC C4d+, which is a reason why most cases of aAMR have PTC C4d+. However, IgG sub-types among HLA class II DSAs are mostly IgG2 and IgG4, and these IgGs mainly participate in complement alternative pathways, which explains why PTC C4d0 can occur in some cases of caAMR. In this study, the proportions of HLA class I DSAs and PTC C4d+ in aAMR were 12/17 and 15/17, respectively; the proportions of HLA class II DSAs and PTC C4d0 in caAMR were 8/12 and 7/12, respectively. Although these data are rather consistent with the reported findings, further studies are needed to clarify the IgG sub-types among DSAs.

It has been reported that PTC C4d+ is associated with poor graft function and inferior graft survival. In our study, PTC C4d+ was associated with more severe urinary occult blood and urinary protein, indicating a poor graft function. On the other hand, some studies have shown that C4d+ may be a manifestation of graft accommodation. For example, anti-HLA-class I ligation may induce cytotoxic genes, but anti-A/B ligation enhances complement regulatory molecules, such as CD55 and CD59, which may inhibit the formation of membrane attack complexes. Additionally, anti-A/B ligation may reduce the activation of mitogen-activated protein kinase (MAPK) and mechanistic target of rapamycin (mTOR) pathways, induce programmed death factor 1 (PD-1) production, and inhibit the allogenic immune response of CD4+ T cells. Hence, a small degree of anti-HLA-class I antibody and anti-A/B antibody ligation has the potential to induce accommodation. There were no ABO-incompatible renal transplants among the patients in the study, which is also a limitation. Nonetheless, some cases of caAMR were included with insignificant clinical symptoms, continuous DSA positivity, and pathological manifestations such as graft glomerulopathy and PTC C4d+. The presence of graft accommodation remains to be further studied and discussed.

C4d+ in glomeruli is a common manifestation of renal disease in a disguised form. Except for systemic lupus erythematosus, PTC C4d+ is very rare in primary renal diseases. In fact, only one case of graft GD was PTC C4d+ in this study. It has been reported that in addition to C4d+ in glomeruli and tubules, C4d+ in PTC also occurs in primary Sjögren’s syndrome-related membranous nephropathy and IgA nephrosis. Therefore, differential diagnosis of recurrent or new nephropathy with PTC C4d+ should be considered when graft pathology is used for the diagnosis of AMR.

All patients in this study underwent pathological biopsy, HLA molecular genotyping, and high-resolution monitoring of HLA antibodies. The collection of these data provided comprehensive supporting materials for the comprehensive analysis of the risk factors for PTC C4d+ in this study. However, as a clinical study, the cases in this study were from a single center, and the number of included cases was only 124, which was a limitation of this study. Our next plan is to conduct a national multicenter study to increase the sample size and extend the follow-up time to reveal the influencing factors of PTC C4d+ and the relationship between PTC C4d+ and renal graft function, as well as long-term survival.

In summary, our study identified that DSAs, glomerulitis, and HLA B eplet MM are risk factors for PTC C4d+ and that DSAs are the main risk factors for PTC C4d+. The AUC of predicted PTC C4d+ increased when combining glomerulitis, DSAs, and HLA B eplet MM. PTC C4d+ was associated with more severe urinary occult blood and urinary protein.

Availability of data and material

The data and material used and/or analyzed during the current study are available from the corresponding author.

The contents of this article were communicated in the form of oral speech at the organ transplantation annual meeting of the Chinese Medical Association in 2020.

Acknowledgements

The authors very much appreciate the help of Mr. Jian Yang who helped to analyze data and revise the manuscript.

Funding

This article was supported by the Clinical Research Award of the First Affiliated Hospital of Xi’an Jiaotong University, China (No. XJU1AF-CRF-2018-026) and the Natural Science Foundation of China (No. 82070768).

Conflicts of interest

None.

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How to cite this article: Zheng J, Guo H, Gong HL, Lan P, Ding CG, Li Y, Ding XM, Xue WJ. Donor-specific antibodies, glomerulitis, and human leukocyte antigen B eplet mismatch are risk factors for peritubular capillary C4d deposition in renal allografts. Chin Med J 2021;134:2874–2881. doi: 10.1097/CME9.0000000000001685