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ORIGINAL ARTICLE

Refinement of the criteria for ultrastructural peritubular capillary basement membrane multilayering in the diagnosis of chronic active/acute antibody-mediated rejection

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SUMMARY

Chronic active/acute antibody-mediated rejection (cABMR) is the main cause of late renal allograft loss. Severe peritubular capillary basement membrane multilayering (PTCML) assessed on electron microscopy is one diagnostic feature of cABMR according to the Banff 2013 classification. We aimed to refine the PTCML criteria for an earlier diagnosis of cABMR. We retrospectively investigated ultrastructural features of 159 consecutive renal allografts and 44 nonallografts. The presence of serum donor-specific antibodies at the time of biopsy of allografts was also examined. Forty-three patients (27.0%) fulfilled the criteria of cABMR, regardless of PTCML, and comprised the cABMR group. Forty-one patients (25.8%) did not exhibit cABMR features and comprised the non-cABMR allograft control group. In addition, 15 zero-day wedge resections and 29 native kidney biopsies comprised the nonallograft control group. When the diagnostic accuracies of various PTCML features were assessed using the cABMR and non-cABMR allograft control groups, ≥4 PTCML, either circumferential or partial, in ≥2 peritubular capillaries of the three most affected capillaries exhibited the highest AUC value (0.885), greater than the Banff 2013 classification (0.640). None of the nonallograft control groups exhibited PTCML features. We suggest that ≥4 PTCML in ≥2 peritubular capillaries of the three most affected cortical capillaries represents the proper cutoff for cABMR.

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Key words
chronic antibody-mediated rejection, electron microscopy, kidney allograft, peritubular capillary

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Introduction

Chronic antibody-mediated rejection (cABMR) is the major contributor of late renal allograft dysfunction and accounts for 64% of late allograft failures [1,2]. The identification of antibody-mediated injury in the active or early chronic stages, which may be reversible and benefit from therapy, is important to improve late graft survival [3].

Severe peritubular capillary basement membrane multilayering (PTCML) is one diagnostic feature of cABMR. According to the Banff 2013 classification, severe PTCML is defined as ≥7 layers in one cortical peritubular capillary (PTC) and ≥5 layers in two additional

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capillaries assessed via electron microscopy (EM) [4]. These criteria have rarely been identified in native kidney disease and are well suited for the specific detection of chronic antibody-mediated injury [5]. Moreover, these criteria represent a very severe form of PTCML, which primarily occurs in the late stage of antibody-mediated chronic tissue injury [5].

The diagnostic use of EM is not widespread for renal allograft biopsies. However, ultrastructural alterations in microcirculation have recently gained increased attention because they may be detected early during antibody-mediated chronic tissue injury and may guide early treatment [3,6–8]. For example, transplant glomerulopathy (TG), another diagnostic feature of cABMR, may be detected early as duplication of the glomerular basement membrane via EM. It is worth noting that the treatment of ABMR in cases that exhibited the early ultrastructural changes of TG significantly reduced the development of overt TG [3]. Considering that overt TG is associated with a poor prognosis with as much as 40% graft loss in 5 years, the recognition of early chronic features of cABMR, not only TG but also likely PTCML, may be important to improve graft survival [9,10].

During pathologic examination of renal allograft biopsies at our institution, severe PTCML was uncommon, even in cases with advanced cABMR, which prompted us to reconsider the cutoff for PTCML. We retrospectively analyzed various ultrastructural features of the glomerular capillaries and PTCs in the renal allografts of cABMR cases and compared these cases with non-cABMR allograft cases, zero-day donors, and native kidney disease cases. This study suggests that ≥4 PTCML in ≥2 PTCs of the three most affected cortical capillaries is the cutoff for PTCML with the highest diagnostic accuracy for cABMR.

Patients and methods

Patients

This retrospective study was approved by the Asan Medical Center Institutional Review Board (2015-1367). Allograft cases, which were investigated using light microscopy (LM), EM, C4d immunohistochemistry (IHC), and serum donor-specific antibodies (DSAs) performed at the time of biopsy between January 2014 and September 2015, were searched from the archives of the Department of Pathology at Asan Medical Center. One hundred and fifty-nine allograft cases with needle biopsies for clinical indications from 123 recipients were retrieved. Ninety, 30, and three patients were biopsied one, two, and three times, respectively. Presensitized patients were not included in this study.

In addition, 44 cases were included as nonallograft controls, which were composed of 15 consecutively selected cases of wedge resections from zero-day donors and 29 cases of needle biopsies for the diagnosis of native kidney disease during the same period. Therefore, 203 cases were ultimately included in this study. The clinical information was obtained from patient medical records.

Pathologic examination

All biopsies contained at least seven glomeruli and one artery, which was adequate to diagnose and categorize rejection. All 203 cases were evaluated via immunofluorescence (IF) and EM, as well as LM using standard procedures. Sections for IF were prepared using snap-frozen acetone-fixed tissues and stained for immunoglobulin (Ig) G, IgM, IgA, C3, C4, C1q, and fibrinogen. C4d IHC (1:100, rabbit polyclonal; Cell Marque, Rocklin, CA, USA) was performed on paraffin-embedded, formalin-fixed tissues using the Ventana Benchmark XT automated staining system (Ventana Medical Systems, Tucson, AZ, USA) according to the manufacturer's protocol [11]. All allograft cases and zero-day donor cases were investigated regarding histologic features and C4d accumulation according to the Banff 2013 classification and Banff 07 criteria, respectively [4,12].

Detection of anti-HLA donor-specific antibodies (DSAs)

The 159 allograft cases were tested for antibody specificity at the time of allograft dysfunction using LABScreen® single antigen beads (LS1A04 and LS2A01; One Lambda, Canoga Park, CA, USA). This laboratory protocol is used to type for HLA class I and II antigens -A, -B, -Cw, -DR, -DQ, and DP.

Ultrastructural examination of glomerular capillaries

Ultrastructural TG was defined as glomerular endothelial cell swelling (GESW), glomerular endothelial cell serration (GESR), and glomerular basement membrane multilayering (GBMML) via EM according to the previous study [3]; no or small amounts of immune-complex deposition via IF; and the absence of hepatitis C infection and the clinical features of thrombotic microangiopathy [13]. The cases with GESW and GESR without

GBMML comprised the early ultrastructural TG group; the cases with GBMML comprised the overt ultrastructural TG group.

Ultrastructural examination of peritubular capillaries

Initially, the cortices were scanned to identify the three most affected PTCs; the number and circumference (≥50% vs. <50%) of their basement membrane layers were subsequently recorded (Fig. 1a–c). In the cases in which the number of layers varied along the circumference, the number of maximum layers that avoided corners and crosscut sections was recorded. Similar to the early ultrastructural TG, endothelial cell swelling or serration of the PTC was designated as endothelial activation [3]. The presence of inflammatory cells including mononuclear cells and polymorphonuclear leukocytes within the PTC was also recorded.

Statistical analyses

The statistical analyses were performed using R 3.2.2 (R Development Core Team, Vienna, Austria). The selection of the PTCML cutoff for cABMR relative to the controls was based on a receiver operating characteristic (ROC) curve and misclassification cost term (MCT) using the OptimalCutpoints library. The MCT is defined as

$$\mathrm{MCT}(c) = \frac{\text{Cost of false negative}}{\text{Cost of false positive}}(\text{prevalence})(1 - \text{Sensitivity}) + (1 - \text{prevalence})(1 - \text{Specificity}).$$

The relationships between the groups were compared using Pearson's Chi-squared test, Fisher's exact test, and Student's *t*-test as needed. All statistical tests were two-sided, and differences with $P < 0.05$ were regarded as statistically significant.

Results

Patient characteristics

The clinical features of the patients are summarized in Table 1. In the 159 renal allograft biopsy cases from 123 patients, the mean duration between transplantation and allograft biopsy was 76.8 months (range, 0.5–310.9 months), with 101 cases (82.4%) more than 1 year post-transplantation. Forty-nine recipients (49/123, 39.8%) received kidneys from living-related donors; 35 recipients (35/123, 28.5%) received kidneys from living-unrelated donors; and 39 recipients (39/123, 31.7%) received kidneys from deceased donors. Ten patients (10/123, 8.1%) were recipients of ABO-incompatible (ABOi) living donors. None of the patients had malignancy or viral infections, such as hepatitis B virus, hepatitis C virus, cytomegalovirus, and human immunodeficiency virus. In patient with *de novo* DSA, 46.7% of patients had HLA class II such as DR, DQ, or DP, 21.7% of patients had HLA class I such as A, B, or C, and the remaining patients had both HLA class I and class II.

Of the 15 cases of zero-day donor wedge resection, the majority of cases were male (12 cases, 80.0%) and deceased (11 cases, 73.3%). The mean ischemic time of the kidney from the living donors was 88.3 min (range, 72–105 min) compared with 292 min (range, 211–479 min) from the deceased donors. Of the 29 cases of various native kidney disease biopsies, minimal change disease (six cases, 20.7%) and ANCA-associated glomerulonephritis (five cases, 17.2%) were the two most frequent diseases.

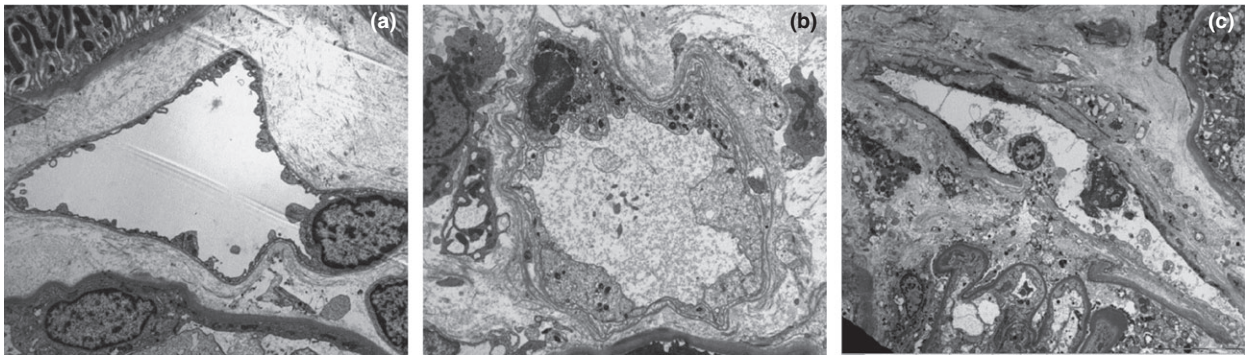

Figure 1 Ultrastructural features of peritubular capillary basement membrane multilayering (PTCML). (a) A peritubular capillary from the cortex without features of PTCML; (b) four circumferential layers of PTCML with an activated endothelial cell; (c) more than 10 circumferential layers of PTCML with an activated endothelial cell and inflammatory cell infiltration. (Original magnification: a, ×4000; b, ×5000; and c, ×2000).

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Table 1. Clinicopathologic features of all 203 cases from 167 patients.

Characteristics	*N* (%)
Allograft needle biopsy	*N* = 159 cases, 123 patients*
Recipient sex	
Female	56 (45.5)
Male	67 (54.5)
Donor age (year), mean (range)	40.0 (2–71)
Type of transplantation	
Living-related	49 (39.8)
Living-unrelated	35 (28.5)
Deceased	39 (31.7)
ABO compatibility	
Compatible	107 (87.0)
Incompatible	10 (8.1)
Not available	6 (4.9)
Previous kidney transplantation	
None	115 (93.5)
Once	7 (5.7)
Twice	1 (0.8)
Recipient age at biopsy (year), mean (range)	43.9 (14–72)
Duration after transplantation at biopsy	
<1 year	22 (17.6)
≥1 year	101 (82.4)
Serum Cr level at biopsy (mg/dL), mean (range)	2.95 (0.56–19)
de novo DSA†	
HLA class I	20 (12.7)
HLA class II	43 (27.4)
HLA class I + class II	29 (18.5)
Absent	65 (41.4)
Zero-day donor wedge resection	*N* = 15 cases/patients
Donor sex	
Female	3 (20.0)
Male	12 (80.0)
Donor age (year), mean (range)	48.9 (27–65)
Donor type	
Living-related	3 (20.0)
Living-unrelated	1 (6.7)
Deceased	11 (73.3)
Ischemic time (min), mean (range)	234 (72–479)
Native kidney disease biopsy	*N* = 29 cases/patients
Sex	
Female	16 (55.2)
Male	13 (44.8)
Age (year), mean (range)	43.9 (3–77)
Pathologic diagnosis	
Minimal change disease	6 (20.7)
ANCA-glomerulonephritis	5 (17.2)
Focal segmental glomerulosclerosis	3 (10.3)
IgA nephropathy	3 (10.3)
Lupus nephritis	2 (6.9)
Acute interstitial nephritis	2 (6.9)
Postinfectious glomerulonephritis	1 (3.4)
Membranous glomerulonephritis	1 (3.4)
MPGN	1 (3.4)
C3 glomerulonephritis	1 (3.4)
Myeloma cast nephropathy	1 (3.4)
Hypertensive nephrosclerosis	1 (3.4)
Thrombotic microangiopathy	1 (3.4)
Chronic glomerulonephropathy	1 (3.4)

*Because the renal allografts of 33 patients were biopsied more than once, 159 cases from 123 patients were analyzed in this study.

†DSA status was not checked in two cases.

Cr, creatinine; DSA, donor-specific antibody; ANCA-glomerulonephritis, anti-neutrophil cytoplasmic antibody-associated glomerulonephritis; IgA, immunoglobulin A; MPGN, membranoproliferative glomerulonephritis.

Grouping of allograft cases for PTCML analysis

To define the PTCML cutoff for cABMR, the 159 renal allograft cases were first analyzed according to the Banff 2013 classification to identify the cases that belong to the cABMR group, regardless of PTCML. The following three features should be present for inclusion in the cABMR group: (i) evidence of current/recent antibody interaction with the endothelium, such as linear C4d staining via PTC or at least moderate microvascular inflammation {[glomerulitis (g) + peritubular capillaritis (ptc)] ≥ 2}, (ii) serologic evidence of DSA, and (iii) morphologic evidence of chronic tissue injury, such as allograft glomerulopathy [(cg) ≥ 1] via LM or ultrastructural overt TG via EM. Renal allograft cases with none of these three features were defined as the non-cABMR allograft control group. Of the 159 renal allograft cases, 43 cases (27.0%) comprised the cABMR group, whereas 41 cases (25.8%) comprised the non-cABMR allograft control group. Patients' configuration and the definition of patients' group are summarized in Fig. S1. Clinicopathologic features of renal allograft cases except cABMR and non-cABMR groups are summarized in Table S1.

Comparison between cABMR and non-cABMR allograft control groups

The age, sex, transplantation type, ABOi frequency, previous transplantation history, mean serum creatinine (Cr) level at biopsy, concurrent Bk viral nephropathy

(BKVN), and chronic calcineurin inhibitor (cCNI) toxicity were not different between the two groups (Table 2). There was a tendency toward concurrent acute T-cell-mediated rejection (TCMR) and cABMR; however, it did not reach statistical significance ($P = 0.064$).

The mean time between transplantation and biopsy was significantly longer in the cABMR group (mean ± standard deviation, 9.8 ± 5.9 years) than in the non-cABMR allograft control group (3.0 ± 3.2 years) ($P < 0.001$). As expected, the scores of g ($P < 0.001$), ptc ($P < 0.001$), cg ($P < 0.001$), and ultrastructural TG ($P < 0.001$) were significantly increased in the cABMR group compared with the control group. Linear C4d expression via IHC ($P < 0.001$) and endothelial activation of PTC via EM ($P = 0.008$) were frequently noted in the cABMR group (Table 2). Interstitial fibrosis (ci) and tubular atrophy (ct) were also significantly advanced in the cABMR group compared with the control group ($P = 0.010$ and $P = 0.001$, respectively). Even in late allograft cases of ≥1 year post-transplantation, which consisted of 41 cases in the cABMR group and 25 cases in the non-cABMR allograft control group, the clinicopathologic differences remained (Table S2).

Evaluation of PTCML criteria for chronic antibody-mediated tissue injury

To determine the proper criteria for PTCML, we investigated more than 20 diagnostic criteria of PTCML by comparing the cABMR and non-cABMR allograft control groups using ROC analysis (Table S3). The area under the curves (AUC) of several representative criteria is illustrated in Fig. 2a. The highest PTCML number, either circumferential or partial, of the second most severely affected PTC of the three most affected PTCs exhibited the highest AUC (0.885). It was followed by the mean PTCML number and the mean percentage of PTCML circumference of the three most affected PTCs (AUC = 0.859 and 0.846, respectively). In contrast, the severe PTCML of the Banff 2013 classification demonstrated a relatively lower AUC (0.640). Even in late allograft cases of ≥1 year post-transplantation, the PTCML number of the second most severely affected PTC also exhibited the highest AUC (0.880), whereas the severe PTCML of the Banff 2013 classification exhibited a lower AUC (0.646).

The mean PTCML numbers of the second most severely affected PTCs in the cABMR and non-cABMR allograft control groups were 4.95 ± 2.87 and 1.95 ± 0.83, respectively ($P < 0.001$; Fig. 2b). The proper cutoff value of the PTCML number to reduce false positivity was 4, which was supported by a low MCT value (0.131) compared with the Banff 2013 criteria (0.185). Therefore, ≥4 PTCML in ≥2 PTCs of the three most affected PTCs was designated as PTCML(+), whereas <4 PTCML was designated as PTCML(−). The positive predictive value (PPV), negative predictive value (NPV), and accuracy of this new PTCML criterion for cABMR were 95.6%, 68.4%, and 76.2%, respectively (Fig. 3a). In comparison, the PPV, NPV, and accuracy of the severe PTCML of the Banff 2013 classification were 100%, 56.9%, and 63.1%, respectively (Fig. 3b).

When the new PTCML(+) criterion was applied as morphologic evidence of chronic tissue injury to the 159 renal allograft cases, 51 cases were classified as PTCML(+). Among them, 25 cases belonged to previously defined "cABMR group" and 2 cases to "non-cABMR allograft control" group. Fourteen cases could be newly classified as cABMR. The remaining 10 PTCML(+) cases did not meet the criteria to cABMR. In contrast, only three cases could be newly classified as cABMR with the severe PTCML criteria of the Banff 2013 classification.

There were two PTCML(+) cases in the non-cABMR allograft control group (false positivity, 4.88%). One case was BKVN without evidence of allograft rejection. The other case exhibited an isolated v-lesion with cCNI toxicity and severe interstitial fibrosis and tubular atrophy (IF/TA).

PTCML status in renal allograft, zero-day donor, and native kidney disease cases

The mean PTCML number of second severe PTC in the 159 renal allograft cases (3.23 ± 2.13) was similar to the selected 84 allograft cases for the previously described analysis (3.49 ± 2.60) ($P = 0.430$) (Fig. 4a,b). However, the means of the nonallograft control groups were significantly lower compared with the allograft cases ($P < 0.001$, both): 1.62 ± 0.68 for native kidney disease cases and 1.07 ± 0.26 for zero-day donor cases (Fig. 4c,d). Of note, native kidney disease and zero-day donor cases did not exhibit PTCML(+).

Comparison of PTCML(+) cases and PTCML(−) cases in all allograft cases

When the new criteria "≥ 4 PTCML in ≥2 PTCs of the three most affected PTCs" were applied, the 159 allograft

Table 2. Comparison of the clinicopathologic features at biopsy between the chronic active/acute ABMR (cABMR) and non-cABMR allograft control groups.

	Total (84 cases, 69 patients)	Control (41 cases, 35 patients)	cABMR (43 cases, 34 patients)	*P*
Age (year), mean (range)	43.6 (14–67)	45.6 (26–67)	41.6 (14–67)	0.187
Sex				
Female	38 (55.1)	20 (57.1)	18 (52.9)	0.913
Male	31 (44.9)	15 (42.9)	16 (47.1)	
Type of transplantation				
Living-related	23 (33.3)	11 (31.4)	12 (35.3)	0.204
Living-unrelated	21 (30.4)	8 (22.9)	13 (38.2)	
Deceased	25 (36.2)	16 (45.7)	9 (26.5)	
ABO compatibility*				
Compatible	62 (91.2)	30 (90.9)	32 (94.1)	0.673
Incompatible	5 (8.8)	3 (9.1)	2 (5.9)	
Previous kidney transplantation				
None	65 (94.2)	34 (97.1)	31 (91.2)	0.356
Once	4 (5.8)	1 (2.9)	3 (8.8)	
Serum Cr level (mg/dL), Mean (range)	2.85 (1.18–19)	2.71 (1.2–15.6)	2.98 (1.18–19)	0.633
Concurrent TCMR				
Absent†	52 (61.9)	30 (73.2)	22 (51.2)	0.064
Present	32 (38.1)	11 (26.8)	21 (48.8)	
Concurrent Bk nephropathy				
Absent	79 (94.0)	37 (90.2)	42 (97.7)	0.197
Present	5 (6.0)	4 (9.8)	1 (2.3)	
Concurrent cCNI toxicity				
Absent	72 (85.7)	36 (87.8)	36 (83.7)	0.824
Present	12 (14.3)	5 (12.2)	7 (16.3)	
Duration after transplantation				
<1 year	18 (21.4)	16 (39.0)	2 (4.6)	<0.001
≥1 year	66 (78.6)	25 (61.0)	41 (95.4)	
g Score				
0	18 (21.4)	18 (43.9)	0 (0)	<0.001
1–3	66 (78.6)	23 (56.1)	43 (100)	
ptc score				
0	23 (27.4)	23 (56.1)	0 (0)	<0.001
1–3	61 (72.6)	18 (43.9)	43 (100)	
ct score				
0	9 (10.7)	9 (21.9)	0 (0)	0.001
1–3	75 (82.3)	32 (78.1)	43 (100)	
ci score				
0	15 (17.9)	12 (29.3)	3 (7.0)	0.010
1–3	69 (82.1)	29 (70.7)	40 (93.0)	
mm score				
0	53 (63.1)	33 (80.5)	20 (46.5)	0.003
1–3	31 (36.9)	8 (19.5)	23 (53.5)	
cv score				
0	25 (29.8)	17 (41.5)	8 (18.6)	0.040
1–3	59 (70.2)	24 (58.5)	35 (81.4)	
ah score				
0	35 (41.7)	23 (56.1)	12 (27.9)	0.016
1–3	49 (58.3)	18 (43.9)	31 (72.1)	
cg score				
0	43 (51.2)	41 (100)	2 (4.6)	<0.001
1–3	41 (48.8)	0 (0)	41 (95.4)	

Table 2. Continued.

	Total (84 cases, 69 patients)	Control (41 cases, 35 patients)	cABMR (43 cases, 34 patients)	*P*
Ultrastructural TG‡				
Absent	45 (53.6)	41 (100)	4 (9.3)	<0.001
Overt§	32 (38.1)	0 (0)	32 (74.4)	
C4d-(+) PTC (%)[e], Mean (range)	9.92 (0–90)	1.46 (0–35)	18.0 (0–90)	<0.001
C4d score¶				
0	52 (61.9)	36 (87.8)	16 (37.2)	<0.001
1–3	32 (38.1)	5 (12.2)	27 (62.8)	
Endothelial activation of PTC				
Not identified	30 (35.7)	21 (51.2)	9 (20.9)	0.008
Identified	54 (64.3)	20 (48.8)	34 (79.1)	
Inflammatory cells within PTC				
Not identified	32 (38.1)	20 (48.8)	12 (27.9)	0.081
Identified	52 (61.9)	21 (51.2)	31 (72.1)	

Values are presented as *n* (%), unless otherwise indicated.

Electron microscopy was used to evaluate transplant glomerulopathy (TG), endothelial cell activation, and inflammatory cells within the peritubular capillary (PTC).

*ABO compatibility information was not available in two cases because these patients received renal transplantation in other countries, including China and the United States of America.

†These cases did not exhibit evidence of T-cell-mediated rejection or borderline changes.

‡Seven cases displaying early ultrastructural transplant glomerulopathy (TG) features were not included in this analysis. Early TG group defined as exhibited glomerular endothelial cell swelling (GESW) and glomerular endothelial cell serration (GESR) without glomerular basement membrane multilayering (GBMML) [3].

§Overt ultrastructural TG group included cases with GBMML.

¶Linear C4d staining was evaluated via immunohistochemistry.

Cr, creatinine; TCMR, T-cell-mediated rejection; cCNI toxicity, chronic calcineurin inhibitor toxicity.

cases were divided into 51 cases (32.1%) of PTCML(+) and 108 cases (67.9%) of PTCML(−). The PTCML(+) cases significantly exhibited a frequent cg ≥ 1 ($P = 0.002$), overt ultrastructural TG ($P < 0.001$), and the presence of serum DSA ($P < 0.001$) compared with the PTCML(−) cases. Furthermore, $g \geq 1$ ($P = 0.042$), ptc ≥ 1 ($P < 0.001$), and C4d-positivity ($P = 0.002$) were also significantly common in the PTCML(+) cases. However, the cv score, ah score, and concurrent TCMR were not different between the PTCML(+) and PTCML(−) cases (Table 3). The severe PTCML of the Banff 2013 classification also exhibited significant differences in the cg score ($P = 0.003$), overt ultrastructural TG ($P < 0.001$), ptc score ($P = 0.043$), and serum DSA ($P = 0.004$) but not in the *g* score ($P = 0.470$) or C4d expression ($P = 0.108$) (Table S4).

Of the 51 PTCML(+) cases, 39 cases (76.5%) were diagnosed as cABMR with the new criterion of PTCML (+), whereas all cases (100%) among the 15 cases with severe PTCML according to the Banff 2013 classification were diagnosed as cABMR. Furthermore, 18 (11.3%) of the 108 cases of PTCML(−) were diagnosed as cABMR compared with 33 (22.9%) of the 144 cases of nonsevere PTCML according to the Banff 2013 classification.

Discussion

This study indicates that the highest PTCML number, either circumferential or partial, of the second most severely affected PTC of the three most affected PTCs is helpful to detect antibody-mediated chronic tissue injury. In addition, ≥4 vs. <4 is the proper cutoff for PTCML of the second most severely affected PTCML.

Because endothelial cell injury and multilayering of BM in PTCs in cABMR is a continuous process and progressive in nature in accordance with chronic tissue injury, the determination of its proper cutoff is a challenging issue [7,14]. A high cutoff level is necessary to specifically identify a fully established cABMR and to avoid overtreatment. However, a relatively lower cutoff level is necessary to detect chronic tissue injury at an early reversible stage and to enable recipients at risk to

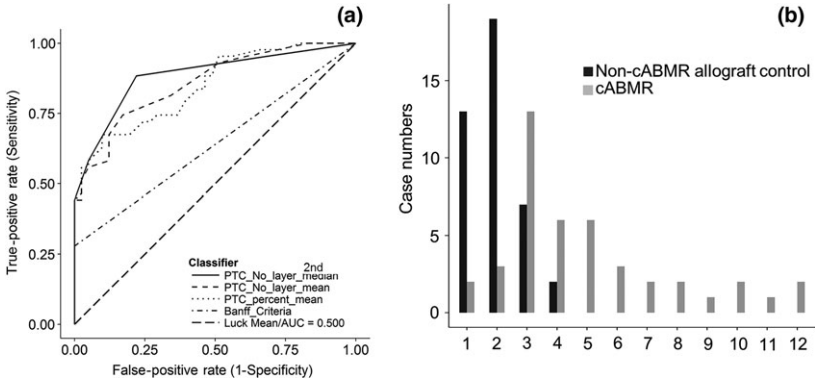

Figure 2 Determination of the cutoff of peritubular capillary basement membrane multilayering (PTCML) for chronic active/acute antibody-mediated rejection (cABMR). (a) ROC analysis of representative PTCML criteria: PTC_No_layer_second, PTCML number of the second most severely affected PTC; PTC_No_layer_mean, mean PTCML number of the three most affected PTCs; PTC_percent_mean, mean percentage of the PTCML circumference of the three most affected PTCs; (b), distribution of the PTCML numbers of second severe PTC in the cABMR and non-cABMR allograft control groups.

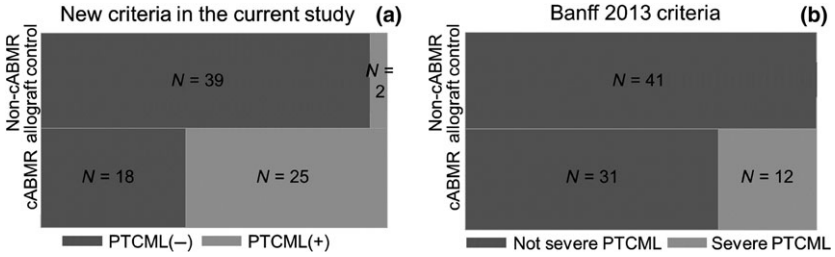

Figure 3 PTCML status in the cABMR and non-cABMR allograft control groups. Comparison of the PTCML status according to the criteria suggested in the current study (a) and the Banff 2013 classification (b) in the cABMR and non-cABMR allograft control groups.

obtain appropriate clinical, histologic, and serological surveillance. Consequently, immunosuppressive therapeutic options may be tailored to a recipient's specific immune status, which may subsequently minimize irreversible graft damage or loss.

Previous studies have suggested various cutoffs for PTCML in the diagnosis of cABMR over the last 20 years [3,5,6,15–27]. The major issues for the cutoffs included the number of counted PTCs, definition of circumference, number of BM layers, and development of a scoring system using these variables [13]. The number of counted PTCs has varied from 2 to 32. The definition of the PTC circumference has ranged from 50% to >75%. Recently, PTCML was defined in the Banff 2013 classification. It recommends that the evaluation of PTCML should be performed on the three most affected PTCs. It provides 50% as the cutoff degree of circumferential multilayering [4,5]. To minimize the influence of confounding factors on the development of PTCML criteria, we followed the recommendation and tested the severe PTCML criteria of Banff 2013 as one of the various candidate criteria.

Several previous studies have included allograftectomy specimens, as well as protocol biopsies in their cohorts [5,7,14]. We only incorporated allograft biopsies for the clinical indication to determine the PTCML criteria and did not include allograftectomy specimens or protocol biopsies. This approach was implemented because allograftectomy specimens were inappropriate for the early detection of cABMR because they typically accompany severe chronic rejection with irreversible chronic graft injury. The protocol biopsy cases were not included because this study aimed to define PTCML cutoff in practical clinical settings to manage dysfunctional renal allografts. However, wedge resections of zero-day donors and native kidney disease needle biopsies were included as the control groups to demonstrate the number of PTCML and the endothelial cell changes in the normal kidney and native kidney disease status, respectively; nevertheless, only a small number of these cases were included.

Of the more than 20 criteria analyzed, we conclude that ≥4 PTCML in ≥2 peritubular capillaries of the three most affected cortical capillaries is a useful marker for cABMR based on the best AUC and low MCT, regardless of the post-transplantation period. Although the Banff 2013 criteria did not result in false-positive cases, its NPV and AUC were lower compared with the new

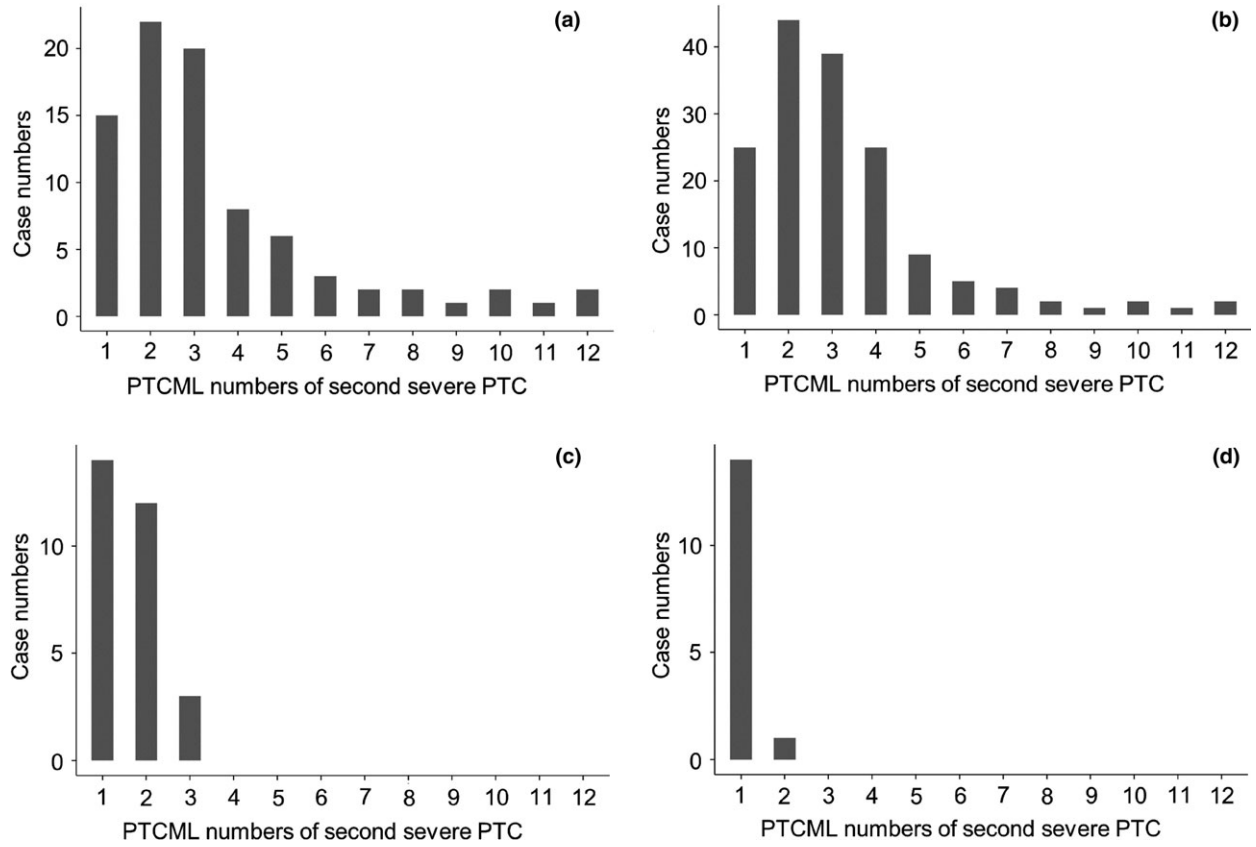

Figure 4 Distribution of PTCML numbers of the second most severely affected PTC in various renal conditions. (a), Eighty-four cases in the cABMR and non-cABMR allograft control groups; (b) all 159 allograft cases; (c) 29 cases of native kidney diseases; (d) 15 cases of zero-day donors.

criteria in this present study. Of the cases categorized as acute/active ABMR according to the Banff 2013 classification, irrespective of PTCML status, three and 14 additional cases were newly included in the diagnosis of cABMR when the severe PTCML of the Banff 2013 classification and the new PTCML(+) of the present study were applied, respectively. Consequently, the new PTCML(+) criteria identified additional cases of cABMR compared with the Banff 2013 criteria. Thus, the new PTCML(+) criterion is useful to detect chronic antibody-mediated tissue injury, particularly at an earlier time, compared with the Banff 2013 classification.

As noted by a previous study, obstructive uropathy, chronic tubulointerstitial nephritis, thrombotic microangiopathy, radiation nephritis, analgesic nephropathy, and Balkan nephropathy may cause multilayering of BM of PTCs [6]. In addition, Liapis *et al.* [5] reported that C4d-negative acute cellular rejection, C4d-negative chronic active/inactive TCMR, and cCNI toxicity may also cause severe PTCML (subgroup C3). In the present study, there were seven PTCML(+) cases that did not exhibit chronic glomerulopathy (cg score ≥ 1 or overt TG), C4d expression, or DSA. Some of these cases exhibited other conditions: There was one case each of TCMR, BKVN, cCNI toxicity, and IgA nephropathy. However, no pathologic alteration was identified in the remaining three cases. Therefore, these cases are in concordance with the previous reports and suggest various mechanisms other than cABMR may induce PTCML.

The limitations of the present study are mainly associated with its retrospective nature. Although all recipients were investigated for anti-HLA antibody using bead assays, the DQ profile of the donors was not available in some patients. This study included ABOi cases; however, the case number was relatively small (8% of the recipients). In addition, we could not analyze the graft survival or recipient survival because our cohort consisted of recent indication biopsies. Of the criteria for chronic tissue injury, arterial intimal fibrosis with a new onset could not be tested because a zero-day biopsy and protocol biopsy were not performed in most cases. Circular proof and lack of the external validation are other weaknesses of this study. We defined a new PTCML criterion with patients of cABMR who fulfilled

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Table 3. Comparison of the PTCML(+) and PTCML(−) groups using the new PTCML criteria.

	Total N = 159	PTCML(−) *N* = 108 (67.9)	PTCML(+) *N* = 51 (32.1)	*P*
cg score				
0	104 (65.4)	80 (74.1)	24 (47.1)	0.002
1–3	55 (34.6)	28 (25.9)	27 (52.9)	
g score				
0	27 (17.0)	23 (21.3)	4 (7.8)	0.042
1–3	132 (83.0)	85 (78.7)	47 (91.2)	
ptc score				
0	35 (22.0)	32 (29.6)	3 (5.9)	<0.001
1–3	124 (78.0)	76 (70.4)	48 (94.1)	
cv score				
0	43 (27.0)	30 (27.8)	13 (25.5)	0.911
1–3	116 (73.0)	78 (72.2)	38 (74.5)	
ah score				
0	74 (46.5)	53 (49.1)	21 (41.2)	0.446
1–3	85 (53.5)	55 (50.9)	30 (58.8)	
Ultrastructural TG*				
Absent	98 (62.0)	81 (75.7)	17 (33.3)	<0.001
Early	25 (15.8)	11 (10.3)	14 (27.5)	
Overt	35 (22.2)	15 (14.0)	20 (39.2)	
C4d†				
0	108 (68.4)	82 (76.6)	26 (51.0)	0.002
1–3	50 (31.6)	25 (23.4)	25 (49.0)	
DSA‡				
Absent	65 (41.4)	56 (51.9)	9 (13.4)	<0.001
Present	92 (58.6)	52 (48.1)	40 (81.6)	
TCMR				
Absent	81 (50.9)	60 (55.6)	21 (41.2)	0.128
Present	78 (49.1)	48 (44.4)	30 (58.8)	

Values are presented as n (%), unless otherwise indicated.

*Ultrastructural TG was not available in one case because of a suboptimal glomerulus for the ultrastructural study.

†C4d immunostaining results were not available in one patient.

‡DSA status was not available in two cases.

PTCML, peritubular capillary basement membrane multilayering; TG, transplant glomerulopathy; EM, electron microscopy; DSA, donor-specific antibody; TCMR, T-cell-mediated rejection.

the criteria of cABMR of Banff 2013 classification regardless of PTCML and non-cABMR groups who showed none of cABMR features. However, because of the lack of patients involved, we included the patients of cABMR and non-cABMR groups in the process of validating the new PTCML criteria and this could cause errors in the external validation. For clinical application, the newly proposed PTCML criteria for chronicity in ABMR should be confirmed in an independent cohort. It could be a subject of a new working group in Korean society of renal pathology or in Banff Meeting 2017.

In conclusion, ≥4 PTCML in ≥2 peritubular capillaries of the three most affected cortical capillaries represents the proper cutoff for cABMR. We suggest that this new PTCML criterion may be used as an earlier marker for cABMR in conjunction with other ABMR features.

Authorship

HG and YMC: contributed to the design of the study. HG and YMC: participated in the writing of the manuscript. HG, SS, YHK, DJH, and YMC: participated in the performance of the research. HG, SS, and YMC: participated in data analysis.

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Conflicts of Interest

The authors have declared no conflicts of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Patients' configuration and the definition of the patients' group.

Table S1. Clinicopathologic features of renal allograft cases except the chronic active/acute ABMR (cABMR) and the non-cABMR groups.

Table S2. Comparison of the clinicopathologic features between the cABMR group and the non-cABMR allograft control group of late renal allograft cases.

Table S3. Area under the curve values of various PTCML criteria in the comparison of the cABMR and non-cABMR allograft control groups.

Table S4. Comparison of the severe and nonsevere PTCML groups according to the Banff 2013 classification.

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