Pre-Transplant Angiotensin II Type 1 Receptor Antibodies and Anti-Endothelial Cell Antibodies Predict Graft Function and Allograft Rejection in a Low-Risk Kidney Transplantation Setting

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Background: Non-HLA antibodies, anti-angiotensin II type 1 receptor antibodies (anti-AT1R) and anti-endothelial cell antibodies (AECA), are known to play a role in allograft rejection. We evaluated the role of both antibodies in predicting post-transplant outcomes in low-risk living donor kidney transplantation (LDKT) recipients.

Methods: In 94 consecutive LDKT recipients who were ABO compatible and negative for pre-transplant HLA donor-specific antibodies, we determined the levels of anti-AT1Rs using an enzyme-linked immunosorbent assay and the presence of AECA using a flow cytometric endothelial cell crossmatch (ECXM) assay with pre-transplant sera. Hazard ratio (HR) was calculated to predict post-transplant outcomes.

Results: Pre-transplant anti-AT1Rs (≥11.5 U/mL) and AECA were observed in 36 (38.3%) and 22 recipients (23.4%), respectively; 11 recipients had both. Pre-transplant anti-AT1Rs were a significant risk factor for the development of acute rejection (AR) (HR 2.09; P = 0.018), while a positive AECA status was associated with AR or microvascular inflammation only (HR 2.47; P = 0.004) throughout the follow-up period. In particular, AECA (+) recipients with ≥11.5 U/mL anti-AT1Rs exhibited a significant effect on creatinine and estimated glomerular filtration rate (P < 0.001; P = 0.028), although the risk of AR was not significant.

Conclusions: Pre-transplant anti-AT1Rs and AECA have independent negative effects on post-transplant outcomes in low-risk LDKT recipients. Assessment of both antibodies would be helpful in stratifying the pre-transplant immunological risk, even in low-risk LDKT recipients.

Key Words: Non-HLA antibodies, Anti-angiotensin II type 1 receptor antibodies, Anti-endothelial cell antibodies, Endothelial cell crossmatch, Kidney transplantation, Outcome, Low-risk
INTRODUCTION

Human leukocyte antigen (HLA) system antigens are major barriers for the acceptance of kidney transplants. HLA donor-specific antibodies (DSA), present before or induced after kidney transplantation (KT), have been associated with hyper-acute and acute humoral rejection episodes, graft vasculopathy, graft loss, and poor long-term graft survival [1]. Appropriate donors can be chosen based on several histocompatibility assessments. However, both allograft rejections and graft failures can occur after transplantation even in HLA-identical sibling transplantations [2].

Previous studies have provided evidence for the association between non-HLA antibodies, such as angiotensin II type 1 receptor antibodies (anti-AT1Rs) and anti-endothelial cell antibodies (AECAs), and acute rejection (AR) and/or graft failure [3–13]. Indeed, the allograft endothelium is the first barrier between self and non-self in vascularized solid organ transplantations and an active target of the host immune response [14]. Anti-AT1Rs can also cause excessive activation of signal transduction pathways in the vessel endothelium, which is associated with vascular inflammatory damage [15]. AECAs activate the vascular endothelium, amplifying allo-immune responses, such as the increased expression of adhesion molecules and the production of inflammatory cytokines, which increase the degree of microvascular injury [11].

Several studies examining the significance of anti-AT1Rs or AECAs and their contribution to transplant outcomes have inevitably analyzed the antibodies in the presence of HLA antibodies because of the appearance of de novo DSA in recipients with pre-transplant non-HLA antibodies [12, 13, 16]. Considering the mechanism of action [11, 15], anti-AT1Rs or AECAs may frequently work together with DSA to exacerbate allo-immune responses in KT [7, 17]. The significance of pre-transplant non-HLA antibodies in low-risk recipients is poorly understood [16]. We aimed to investigate the significance of pre-transplant anti-AT1Rs and AECAs that affect KT outcome in ABO compatible low-risk living donor kidney transplantation (LDKT) recipients without preformed DSA. In addition, we attempted to identify a correlation between serum anti-AT1R levels and AECAs.

MATERIALS AND METHODS

Recipient population

This prospective study included total 94 recipients among 201 consecutive recipients who underwent an ABO-compatible LDKT at Samsung Medical Center (SMC), Seoul, Korea, between January 2012 and September 2014. A negative, complement-dependent microcytotoxicity crossmatch (CDC-XM) for T and B donor cells, without historic and pre-transplant HLA-DSA, was required for inclusion in this study. Furthermore, recipients who underwent desensitization owing to high panel reactive antibodies without DSA and multi-organ or combined kidney and bone marrow transplantation cases were excluded. This study was approved by the Institutional Review Board of SMC (approval number: SMC 2011-05-084), consistent with the Declaration of Helsinki, and written informed consent was obtained from all participants prior to their inclusion in the study.

Measurement of anti-AT1Rs using enzyme-linked immunosorbent assay (ELISA)

Anti-AT1R levels were measured in 123 sera using an ELISA (One Lambda Inc., Luckenwalde, Germany); 94 pre-transplant sera were acquired at the time of CDC-XM, and 29 post-transplant sera were acquired at the time of the kidney biopsy from recipients who had experienced biopsy proven rejection. Anti-AT1R level in each sample was derived from a standard curve and was defined as positive when >17 U/mL, at-risk when 10–17 U/mL, and negative when <10 U/mL, according to the manufacturer’s recommendations. Each run was validated with one positive and one negative control included in the kit.

Detection of AECAs using flow cytometric endothelial cell crossmatch (ECXM) assay

The presence of pre-transplant IgG and IgM antibodies against human endothelial progenitor cells (EPCs) was assessed using the XM-ONE assay (Olerup SSP AB [previously Absorber AB], Stockholm, Sweden) according to the manufacturer’s instructions. A total of 94 pairs of peripheral blood samples, which were obtained from donors and recipients at the time of the final crossmatch (XM) before KT, were used for the XM-ONE assay. Donor peripheral blood mononuclear cells separated from 32 mL of whole blood were collected in CPT tubes (Becton Dickinson, Heidelberg, Germany) and incubated at 4°C for 30 minutes with paramagnetic nanobeads coated with antibodies against the Tie-2 receptor, an angiotropin receptor, in order to isolate the EPCs. The isolated donor EPCs were incubated with recipient serum, as well as positive and negative control sera, for 30 minutes. After washing, the EPCs were incubated with fluorescein isothiocyanate-conjugated secondary antibodies against IgG and IgM at 4°C for 20 minutes. The cells were analyzed using a FACSCanto ll flow cytometer and FACSdiva software v1.6 (Bec
Ron Dickinson, San Jose, CA, USA). The cut-offs for positive EPC XM were ≥50 and ≥80 fluorescence channel-shift above the negative control for IgG and IgM, respectively.

**HLA testing**

All transplant donors and recipients were typed for HLA-A, -B, and -DRB1 using LIFECODES HLA-SSO Typing Kits (Immucor Transplant Diagnostics, Inc., Stamford, CT, USA). HLA-DQB1 was typed retrospectively only in recipients who had HLA-DQB1 antibodies pre-transplant or developed them post-transplant. Recipient HLA antibodies were evaluated as HLA class I or II using the LIFECODES LifeScreen Deluxe assay and/or the LIFE CODES LSA class I and II ID Single Antigen kit (Immucor Transplant Diagnostics, Inc.). The definition of de novo HLA antibodies included DSAs against HLA-A, -B, or -DRB1 identified by single antigen identification assay and HLA-DQB1 antibodies that developed in post-transplant sera.

**Kidney histology**

AR included biopsy-proven AR (BPAR), according to the revised Banff 2017 classification [18], and clinically suspected AR (CSAR; ≥25% increase in blood creatinine from baseline, or proteinuria > 0.5 g/day), which improved following empirical steroid pulse therapy. Microvascular inflammation (MVI) was defined as a sum score of glomerulitis (g) and peritubular capillaritis (ptc) ≥2, which is one of the three criteria for antibody-mediated rejection (ABMR), according to the revised Banff 2017 classification [18]. Protocol biopsies were performed in 65 recipients at 1, 3, 6, and 12 months post-transplant and indication biopsies for renal dysfunction were performed in 15 recipients. Fourteen recipients who had not been subjected to kidney biopsy during the follow-up period were considered as having no signs of clinical rejection.

**Data collection and statistical analysis**

All clinical data were obtained from medical records. Creatinine levels and estimated glomerular filtration rate (eGFR), calculated using the modification of diet in renal disease equation were analyzed up to three years post-KT.

Statistical analysis was performed using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). Fisher’s exact tests were used for categorical variables. Wilcoxon signed-rank tests and Kruskal-Wallis tests were used for non-parametric continuous variables. The optimal cut-off value for anti-AT1Rs was determined using a Log-rank test and Youden’s index method. The repeated measurements of serum creatinine levels and eGFR at 1, 3, 6, 12, 24, and 36 months post-transplant were analyzed using the generalized estimating equation; P<0.05 was considered significant. The potential risk factors of AR or MVI only within 1, 3, 6, and 12 months post-transplant and during the follow-up period were analyzed using Logistic regression analysis and Cox pro-

### Table 1. LDKT recipient characteristics

| Characteristics | Recipients (N = 94)* |
|-----------------|----------------------|
| Age (yr)        | 49.5 (39-56)         |
| Gender, male    | 57 (60.6%)           |
| BMI             | 23.1 ± 3.8           |
| Diagnosis       |                      |
| Diabetic nephropathy | 25 (29.8%)     |
| IgA nephropathy | 16 (17.0%)           |
| Hypertensive nephrosclerosis | 15 (16.0%) |
| Glomerulonephritis | 10 (10.6%)      |
| Other causes    | 7 (7.4%)             |
| Unknown         | 18 (19.2%)           |
| Re-transplantation | 1 (1.1%)       |
| Induction therapy regimen |          |
| Anti-thymocyte globulin | 10 (10.6%)  |
| Basiliximab     | 84 (89.4%)           |
| Maintenance regimen immunosuppressants | |
| CsA+MMF+PD      | 13 (13.8%)           |
| FK+MMF+PD       | 79 (84.0%)           |
| Sirolimus or everolimus combination | 2 (2.1%)  |
| HLA mismatches  |                      |
| Class 1 (HLA-A, -B) | 2 (1-3)          |
| Class 2 (HLA-DR) | 1 (0-1)             |
| cPRA            |                      |
| 0%              | 72 (76.6%)           |
| < 50%           | 18 (19.1%)           |
| ≥ 50%           | 4 (4.3%)             |
| Pre-transplant AECA (+) | 22 (23.4%)     |
| Pre-transplant anti-AT1R levels, U/mL | 10.2 ± 4.8 |
| AR, during F/U period | 43 (45.7%) |
| MVI only (g+ptc ≥ 2), during F/U period | 6 (6.4%) |

*Continuous variables are reported as mean±SD or median (interquartile range) and categorical variables are listed as total number (%). Kolmogorov-Smirnov test was employed for testing normality assumption. Abbreviations: LDKT, living donor kidney transplantation; BMI, body mass index; CsA, cyclosporine A; MMF, mycophenolate mofetil; PD, prednisolone; FK, tacrolimus; cPRA, calculated panel reactive antibodies; AECA, anti-endothelial cell antibodies; Anti-AT1R, anti-angiotensin II type 1 receptor antibodies; MVI, microvascular inflammation; g, glomerulitis; ptc, peritubular capillaritis; AR, acute rejection; F/U, follow-up.
portional-hazard regression analysis, respectively, and the proportional hazard assumption was determined using a Supremum test. Factors with \( P < 0.2 \) in the univariate analysis were included in the multivariate analysis, and multicollinearity was determined using a variance inflation factor. \( P \) and 95% confidence intervals were corrected using Bonferroni's method in cases of multiple analyses.

### Table 2. Recipient characteristics and post-transplant outcomes according to anti-AT1R levels and AECAs using ECXM assay

| Anti-AT1R-negative (\(<10\) U/mL) (N = 45)* | Anti-AT1R at-risk (10–17 U/mL) (N = 41)* | Anti-AT1R-positive (\(>17\) U/mL) (N = 8)* | \( P \) | AECA (-) (N = 72)* | AECA (+) (N = 22)* | \( P \) |
|-------------------------------------------|------------------------------------------|---------------------------------|---------|-----------------|-----------------|---------|
| Age (yr)                                  | 50 (40-55)                               | 50 (39-56)                      | 45.5 (39.5-59.5) | 0.975 | 50 (40-56) | 46 (37-53) | 0.437 |
| Gender, male                              | 32 (71.1%)                               | 22 (53.7%)                      | 3 (37.5%) | 0.097 | 43 (59.7%) | 14 (63.6%) | 0.807 |
| BMI                                       | 23.6 ± 3.5                               | 22.9 ± 4.2                      | 20.7 ± 2.4 | 0.0069 | 23.5 ± 4.0 | 21.6 ± 2.9 | 0.05 |
| HLA mismatches                            |                                          |                                 |          |      |            |             |        |
| Class 1 (HLA-A, -B)                       | 2 (1-3)                                  | 2 (2-3)                         | 2 (1-2.5) | 0.683 | 2 (1-3)  | 2 (2-3)   | 0.712 |
| Class 2 (HLA-DR)                          | 1 (1-1)                                  | 1 (0-2)                         | 1 (0.5-1) | 0.749 | 1 (1-1)  | 1 (0-2)   | 0.683 |
| cPRA                                      |                                          |                                 |          |      |            |             |        |
| 0%                                        | 39 (86.7%)                               | 29 (70.7%)                      | 4 (50%) | 0.54 | 54 (75.0%) | 18 (81.8%) |        |
| <50%                                      | 6 (13.3%)                                | 8 (19.5%)                       | 4 (50%) | 0.14 | 14 (19.4%) | 4 (18.2%)  |        |
| ≥50%                                      | 0 (0%)                                   | 4 (9.8%)                        | 0 (0%)  | 0.002 | 4 (5.6%)  | 0 (0%)    |        |
| Induction therapy regimen                 |                                          |                                 |          |      |            |             | 1.0     |
| Anti-thymocyte globulin                   | 5 (11.1%)                                | 4 (9.8%)                        | 1 (12.5%) | 0.84 | 8 (11.1%) | 2 (9.1%)  |        |
| Basiliximab                               | 40 (88.9%)                               | 37 (90.2%)                      | 7 (87.5%) | 0.118 | 64 (88.9%) | 20 (90.9%) |        |
| Maintenance regimen immunosuppressants    |                                          |                                 |          |      |            |             | 0.118   |
| CsA+MMF+PD                                | 10 (22.2%)                               | 2 (4.9%)                        | 1 (12.5%) | 0.62 | 11 (15.3%) | 2 (9.1%)  |        |
| FK+MMF+PD                                 | 34 (75.6%)                               | 38 (92.7%)                      | 7 (87.5%) | 0.62 | 59 (81.9%) | 20 (90.9%) |        |
| Sirolimus or everolimus combination       | 1 (2.2%)                                 | 1 (2.4%)                        | 0 (0%)  | 0.002 | 2 (2.8%)  | 0 (0%)    |        |
| AR                                        |                                          |                                 |          |      |            |             | 1.0     |
| Within 1 month post-transplant            | 8 (17.8%)                                | 10 (24.4%)                      | 0 (0%)  | 0.46 | 13 (18.1%) | 5 (22.7%) | 0.627 |
| Within 3 months post-transplant           | 8 (17.8%)                                | 13 (31.7%)                      | 1 (12.5%) | 0.24 | 16 (22.2%) | 6 (27.3%) | 0.625 |
| Within 6 months post-transplant           | 9 (20.0%)                                | 16 (39.0%)                      | 2 (25.0%) | 0.15 | 19 (26.4%) | 8 (36.4%) | 0.368 |
| Within 12 months post-transplant          | 10 (22.2%)                               | 17 (41.5%)                      | 2 (25.0%) | 0.15 | 21 (29.2%) | 8 (36.4%) | 0.523 |
| During F/U period                         | 17 (37.8%)                               | 23 (56.1%)                      | 3 (37.5%) | 0.13 | 29 (40.3%) | 14 (63.6%) | 0.062 |
| AR or MVI only                            |                                          |                                 |          |      |            |             | 1.0     |
| Within 1 month post-transplant            | 9 (20.0%)                                | 13 (31.7%)                      | 0 (0%)  | 0.24 | 16 (22.2%) | 6 (27.3%) | 0.625 |
| Within 3 months post-transplant           | 11 (24.4%)                               | 16 (39.0%)                      | 1 (12.5%) | 0.19 | 19 (26.4%) | 9 (40.9%) | 0.196 |
| Within 6 months post-transplant           | 12 (26.7%)                               | 19 (46.3%)                      | 2 (25.0%) | 0.13 | 22 (30.6%) | 11 (50.0%) | 0.099 |
| Within 12 months post-transplant          | 13 (28.9%)                               | 20 (48.8%)                      | 2 (25.0%) | 0.12 | 24 (33.3%) | 11 (50.0%) | 0.161 |
| During F/U period                         | 20 (44.4%)                               | 25 (63.4%)                      | 3 (37.5%) | 0.10 | 32 (44.4%) | 17 (77.3%) | 0.008 |
| Pre-transplant anti-AT1R levels, U/mL     | 6.26 ± 2.2                               | 12.50 ± 1.6                     | 20.72 ± 3.1 | <0.001 | 10.0 ± 4.8 | 11.0 ± 5.1 | 0.412 |
| Pre-transplant AECA (+)                   | 10 (22.2%)                               | 9 (22.0%)                       | 3 (37.5%) | 0.616 |            |             |        |

*Continuous variables are reported as mean±SD or median (interquartile range) and categorical variables are listed as total number (%). Kolmogorov-Smirnov test was employed for test of normality assumption. Abbreviations: ECXM, endothelial cell crossmatch; Anti-AT1R, anti-angiotensin II type 1 receptor antibodies; AECA, anti-endothelial cell antibodies; BMI, body mass index; cPRA, calculated panel reactive antibodies; CsA, cyclosporine A; MMF, mycophenolate mofetil; PD, prednisolone; FK, tacrolimus; AR, acute rejection; MVI, microvascular inflammation.
RESULTS

Characteristics of the study population
Of the 94 KT recipients, 93 (98.9%) had received their first kidney transplant and one (1.1%) had received a second kidney transplant following primary graft failure due to renal vein thrombosis. The main demographic characteristics of the recipients are presented in Table 1. No graft failure occurred during the follow-up period (996±292 days); however, one recipient (1.1%) died owing to septic shock irrespective of an immunologic event. Nine of the 94 recipients (9.6%) developed de novo HLA antibodies. Of the five recipients who experienced rejection episodes, three had pre-transplant anti-AT1R levels of 10–17 U/mL; however, none had positive pre-transplant AECA. There was no significant association between de novo HLA antibodies and rejection (AR, P=0.555; AR or MVI only, P=0.392).

Correlation between clinical outcomes and anti-AT1R levels and AECA results
The demographic characteristics of the recipients based on anti-AT1R levels and AECA using ECXM assay are presented in Table 2. No significant differences in anti-AT1R levels were found between AECA (-) and AECA (+) recipients. Additionally, there were no significant differences in the AECA (+) rate among the three anti-AT1R groups as well as between the groups using the new optimal anti-AT1R level cut-off value, 11.5 U/mL (19.0% in

Table 3. Risk factors associated with AR based on Cox proportional-hazards regression analysis

|                     | Univariate analysis | Multivariate analysis* | Multivariate analysis† |
|---------------------|---------------------|------------------------|------------------------|
|                     | HR 95% CI           | P                       | HR 95% CI           | P                       | HR 95% CI       | P                       |
| AR within 6 months post-transplant |                     |                         |                       |                         |                       |
| HLA mismatches      |                     |                         |                       |                         |                       |
| Class 1 (HLA-A, -B) | 1.47 0.98-2.20 0.060‡ | 1.44 0.84-2.46 0.181 | 1.36 0.83-2.25 0.225 |
| Class 2 (HLA-DR)    | 2.50 1.23-5.02 0.010‡ | 2.02 0.86-4.75 0.106 | 1.89 0.84-4.24 0.124 |
| Pre-transplant anti-AT1R ≥ 11.5 U/mL | 2.74 1.09-6.86 0.032‡ | 4.11 1.44-11.79 0.009 | - - -                  |
| AECA (+)            | 1.59 0.28-4.40 0.368 | -                       | -                       | 2.09 0.66-6.59 0.208 |
| AR within 12 months post-transplant |                     |                         |                       |                         |                       |
| HLA mismatches      |                     |                         |                       |                         |                       |
| Class 1 (HLA-A, -B) | 1.50 1.01-2.23 0.046‡ | 1.52 0.92-2.52 0.103 | 1.44 0.89-2.33 0.134 |
| Class 2 (HLA-DR)    | 2.14 1.11-4.16 0.024‡ | 1.59 0.72-3.51 0.251 | 1.54 0.72-3.32 0.267 |
| Pre-transplant anti-AT1R ≥ 11.5 U/mL | 2.25 0.92-5.49 0.077‡ | 3.11 1.15-8.43 0.026 | - - -                  |
| AECA (+)            | 1.39 0.51-3.50 0.523 | -                       | -                       | 1.73 0.57-5.24 0.335 |
| AR during F/U period |                     |                         |                       |                         |                       |
| HLA mismatches      |                     |                         |                       |                         |                       |
| Class 1 (HLA-A, -B) | 1.26 0.98-1.61 0.069‡ | 1.20 0.89-1.62 0.228 | 1.18 0.88-1.58 0.260 |
| Class 2 (HLA-DR)    | 1.55 1.01-2.39 0.045‡ | 1.41 0.86-2.29 0.173 | 1.36 0.85-2.17 0.207 |
| Pre-transplant anti-AT1R ≥ 11.5 U/mL | 1.85 1.02-3.37 0.044‡ | 2.09 1.14-3.85 0.018 | - - -                  |
| AECA (+)            | 1.84 0.97-3.48 0.062‡ | -                       | -                       | 1.92 1.01-3.66 0.046 |
| AR or MVI only during F/U period |                     |                         |                       |                         |                       |
| HLA mismatches      |                     |                         |                       |                         |                       |
| Class 1 (HLA-A, -B) | 1.32 1.05-1.66 0.018‡ | 1.21 0.92-1.60 0.175 | 1.27 0.96-1.67 0.095 |
| Class 2 (HLA-DR)    | 1.74 1.15-2.62 0.008‡ | 1.50 0.94-2.39 0.090 | 1.41 0.91-2.20 0.127 |
| Pre-transplant anti-AT1R ≥ 11.5 U/mL | 1.32 1.75-3.23 0.342 | 1.47 0.83-2.62 0.185 | - - -                  |
| AECA (+)            | 2.23 1.23-4.02 0.008‡ | -                       | -                       | 2.47 1.35-4.53 0.004 |

*The pre-transplant anti-AT1R ≥ 11.5 U/mL results were included as an independent variable; †The AECA result by ECXM assay was included as an independent variable; ‡P<0.2 was subjected to a backward stepwise Cox regression model (multivariate analysis).

Abbreviations: AR, acute rejection; HR, hazard ratio; CI, confidence interval; Anti-AT1R, anti-angiotensin II type 1 receptor antibodies; AECA, anti-endothelial cell antibodies; MVI, microvascular inflammation; ECXM, endothelial cell crossmatch.
the anti-AT1R <11.5 U/mL group vs. 44.0% in the anti-AT1R >11.5 U/mL group, \( P=0.218 \). The prevalence of AR within 12 months post-transplant did not differ significantly based on anti-AT1R levels and AECA results. However, when a cut-off value of 11.5 U/mL was applied, it independently predicted a higher risk for AR. AECA (+) recipients had a higher risk for AR or MVI only during the follow-up period (Table 3). Recipient characteristics based on combined immunologic status of pre-transplant anti-AT1R levels and AECA using ECXM assay are shown in Table 4.

Kaplan–Meier analysis demonstrated that recipients with anti-AT1R ≥11.5 U/mL had a higher risk of AR than those with anti-AT1R <11.5 U/mL, AECA (+) recipients had a higher risk for AR or MVI only than AECA (-) recipients, and AECA (+) recipients

### Table 4. Recipient characteristics and post-transplant outcomes according to combined immunologic status of pre-transplant anti-AT1R levels and AECA using ECXM assay

|                        | Anti-AT1R <11.5 U/mL and AECA (-) \( N = 47 \)* | Anti-AT1R ≥11.5 U/mL and AECA (-) \( N = 25 \)* | Anti-AT1R <11.5 U/mL and AECA (+) \( N = 11 \)* | Anti-AT1R ≥11.5 U/mL and AECA (+) \( N = 11 \)* | \( P \) |
|------------------------|-------------------------------------------------|-------------------------------------------------|-----------------------------------------------|-----------------------------------------------|--------|
| **Age (yr)**           | 47.9±10.8                                       | 47.4±12.3                                       | 50.2±10.3                                     | 40.8±13.0                                     | 0.331  |
| **Gender, male**       | 30 (63.8%)                                      | 13 (52.0%)                                      | 9 (81.8%)                                     | 5 (45.5%)                                     | 0.242  |
| **BMI**                | 23.6±3.6                                        | 23.3±4.7                                        | 22.6±2.9                                      | 20.6±2.8                                      | 0.089  |
| **HLA mismatches**     |                                                |                                                |                                               |                                               |        |
| Class 1 (HLA-A,-B)     | 2 (1-3)                                         | 2 (1-3)                                         | 3 (2-3)                                       | 2 (1-2)                                       | 0.666  |
| Class 2 (HLA-DR)       | 1 (1-1)                                         | 1 (0-1)                                         | 1 (0-2)                                       | 1 (0-2)                                       | 0.822  |
| **cPRA**               |                                                |                                                |                                               |                                               | 0.526  |
| 0%                     | 37 (78.7%)                                      | 17 (68.0%)                                      | 10 (90.9%)                                    | 8 (72.7%)                                     |        |
| <50%                   | 9 (19.2%)                                       | 5 (20.0%)                                       | 1 (9.1%)                                      | 3 (27.3%)                                     |        |
| ≥50%                   | 1 (2.1%)                                        | 3 (12.0%)                                       | 0 (0%)                                        | 0 (0%)                                        |        |
| **Induction therapy regimen** |                                      |                                                |                                               |                                               | 0.61   |
| Anti-thymocyte globulin| 5 (10.6%)                                       | 3 (12.0%)                                       | 2 (18.2%)                                     | 0 (0%)                                        |        |
| Basiliximab            | 42 (89.4%)                                      | 22 (88.0%)                                      | 9 (81.8%)                                     | 11 (100%)                                     |        |
| **Maintenance regimen immunosuppressants** |                                      |                                                |                                               |                                               | 0.704  |
| CsA+MMF+PD             | 8 (17.0%)                                       | 3 (12.0%)                                       | 2 (18.2%)                                     | 0 (0%)                                        |        |
| FK+MMF+PD              | 37 (78.7%)                                      | 22 (88.0%)                                      | 9 (81.8%)                                     | 11 (100%)                                     |        |
| Sirolimus or everolimus combination | 2 (4.3%)                                      | 0 (0%)                                          | 0 (0%)                                        | 0 (0%)                                        |        |
| **AR**                 |                                                |                                                |                                               |                                               |        |
| Within 1 month post-transplant | 8 (17.0%)                                      | 5 (20.0%)                                       | 2 (18.2%)                                     | 3 (27.3%)                                     | 0.893  |
| Within 3 months post-transplant | 8 (17.0%)                                      | 8 (32.0%)                                       | 3 (27.3%)                                     | 3 (27.3%)                                     | 0.526  |
| Within 6 months post-transplant | 8 (17.0%)                                      | 11 (44.0%)                                      | 4 (36.4%)                                     | 4 (36.4%)                                     | 0.099  |
| Within 12 months post-transplant | 10 (21.3%)                                     | 11 (44.0%)                                      | 4 (36.4%)                                     | 4 (36.4%)                                     | 0.238  |
| During F/U period      | 15 (31.9%)                                      | 14 (56.0%)                                      | 7 (63.6%)                                     | 7 (63.6%)                                     | 0.071  |
| **AR or MVI only**     |                                                |                                                |                                               |                                               |        |
| Within 1 month post-transplant | 11 (23.4%)                                     | 5 (20.0%)                                       | 3 (27.3%)                                     | 3 (27.3%)                                     | 0.952  |
| Within 3 months post-transplant | 11 (23.4%)                                     | 8 (32.0%)                                       | 6 (54.5%)                                     | 3 (27.3%)                                     | 0.258  |
| Within 6 months post-transplant | 11 (23.4%)                                     | 11 (44.0%)                                      | 7 (63.6%)                                     | 4 (36.4%)                                     | 0.069  |
| Within 12 months post-transplant | 13 (27.7%)                                     | 11 (44.0%)                                      | 7 (63.6%)                                     | 4 (36.4%)                                     | 0.152  |
| During F/U period      | 18 (38.3%)                                      | 14 (56.0%)                                      | 10 (90.9%)                                    | 7 (63.6%)                                     | 0.012  |

*Continuous variables are reported as mean±SD or median (interquartile range), and categorical variables are listed as number (%). Kolmogorov-Smirnov test was employed for testing normality assumption.

Abbreviations: Anti-AT1R, anti-angiotensin II type 1 receptor antibodies; AECA, anti-endothelial cell antibodies; ECXM, endothelial cell crossmatch; BMI, body mass index; cPRA, calculated panel reactive antibodies; CsA, cyclosporine A; MMF, mycophenolate mofetil; PD, prednisolone; FK, tacrolimus; AR, acute rejection; MVI, microvascular inflammation.
Fig. 1. Clinical outcomes according to the anti-AT1R levels and AECA status using ECXM assay. (A) No significant differences in AR or MVI only free survival rates are seen between recipients with anti-AT1R ≥ 11.5 U/mL (N=36) and those with anti-AT1R < 11.5 U/mL (N=58). (B) Recipients with anti-AT1R ≥ 11.5 U/mL have a higher risk of AR than those with anti-AT1R < 11.5 U/mL (P=0.039). (C) AECA (+) recipients (N=22) have a higher risk of AR or MVI only than AECA (-) recipients (N=72) (P=0.006). (D) There is no significant difference in the AR free survival rates. (E) AECA (+) recipients with anti-AT1R < 11.5 U/mL (N=11) have a higher risk of AR or MVI only than other recipients (P=0.006); (F) There are no significant differences in AR free survival rates among the four groups.

Abbreviations: Anti-AT1R, anti-angiotensin II type 1 receptor antibodies; AECA, anti-endothelial cell antibodies; ECXM, endothelial cell crossmatch; AR, acute rejection; MVI, microvascular inflammation.
Fig. 2. Effect of anti-AT1R levels and AECA status using ECXM assay on renal function during the post-KT period. Recipients with pre-transplant anti-AT1R ≥11.5 U/mL show significantly lower eGFR (B) but not creatinine levels (A) at 6 and 12 months post KT (\(P=0.012; P=0.012\), respectively), compared with those at one month post-KT. AECA (+) recipients have significantly higher creatinine levels (C) and lower eGFRs (D) at six (\(P=0.003; P=0.028\), respectively) and 12 months (\(P<0.001; P=0.011\), respectively), compared with those at one month post-KT. The change in the pattern of creatinine levels in AECA (+) recipients from one to 12 months post-KT is significantly different compared with that in AECA (-) recipients (\(P=0.038\)) (C). AECA (+) recipients with anti-AT1R ≥11.5 U/mL show significantly different changes in the pattern of creatinine levels (E) from one to 12 months post-KT (\(P=0.045\)) compared with other recipients, and significantly higher creatinine levels and lower eGFRs (F) at 12 months (\(P<0.001; P=0.028\)) compared with those at one month post-KT.

Abbreviations: Anti-AT1R, anti-angiotensin II type 1 receptor antibodies; AECA, anti-endothelial cell antibodies; ECXM, endothelial cell crossmatch; KT, kidney transplantation; eGFR, estimated glomerular filtration rate; MVI, microvascular inflammation.
with anti-AT1R <11.5 U/mL had a higher risk of AR or MVI only than other recipients (Fig. 1). Based on multivariate analysis, pre-transplant AECA (-) recipients with anti-AT1R ≥11.5 U/mL had a higher risk of AR within six months post-transplant (HR 5.68; \(P=0.018\)), and pre-transplant AECA (+) recipients with anti-AT1R <11.5 U/mL had a higher risk of AR or MVI only within 6 months post-transplant (HR 7.37; \(P=0.037\)) and during the follow-up period (HR 4.14; \(P=0.002\)).

We measured anti-AT1R levels in the sera of 29 BPAR recipients at the time of kidney biopsy. Only two recipients had an anti-AT1R ≥11.5 U/mL at the time of AR and were among the 12 recipients who had an anti-AT1R ≥11.5 U/mL pre-KT. Seventeen recipients had an anti-AT1R <11.5 U/mL both pre-KT and at the time of AR. The median pre-transplant anti-AT1R level of these 29 recipients was 11.2 U/mL (range: 3.8–25.4 U/mL), which was higher than that at the time of AR (7.9 U/mL; range, 4.4–12.7 U/mL; \(P=0.029\)).

There was no correlation between pre-transplant anti-AT1R levels and post-transplant creatinine levels; however, recipients with pre-transplant anti-AT1R ≥11.5 U/mL had a significantly lower eGFR at both six and 12 months post-transplant (\(P=0.012; \ P=0.028\) compared with recipients with pre-transplant anti-AT1R <11.5 U/mL post-transplant (Fig. 2A and B). Additionally, recipients with anti-AT1R ≥11.5 U/mL showed significantly lower eGFR for six months post-transplant compared with recipients with pre-transplant anti-AT1R <11.5 U/mL (\(P=0.010\)), which continued until 24 months post-transplant. Renal function, estimated using post-transplant creatinine and eGFR, differed depending on AECA results. AECA (+) recipients showed a rapid increase in creatinine and decrease in eGFR at approximately three months; after this point, they showed persistently higher creatinine and lower eGFR until 20 months (Fig. 2C and D). Interestingly, pre-transplant AECA (+) recipients with anti-AT1R ≥11.5 U/mL were not associated with AR or MVI only at any time point during the entire follow-up period; however, they had significantly different changes in the creatinine level pattern from one to 12 months post-transplant (\(P=0.045\)), as well as significantly higher creatinine and a lower eGFR at 12 months (\(P<0.001; \ P=0.028\)) compared with levels at one month post-transplant (Fig. 2E and F).

DISCUSSION

Our aim was to evaluate the impact of pre-transplant anti-AT1R and AECA on post-transplant outcomes in low-risk LDKT recipients. The target antigen for AECA detected in the ECXM assay is unknown; thus, all antigens expressed on endothelial cells are possible candidates [4]. Previous studies have reported that ECXM assay can detect anti-AT1R as well as antibodies against targets other than AT1R [11–13]. Philogene, et al. [12, 13] reported that recipients who were positive for AECA have higher anti-AT1R levels; however, we found that there was no correlation between pre-transplant anti-AT1R levels and AECA status, and that KT outcomes are affected differently by pre-transplant anti-AT1R levels and AECA status. The presence of pre-transplant anti-AT1R was a significant risk factor for the development of AR, whereas AECA status was associated with post-transplant renal function, estimated using creatinine levels or eGFR and AR or MVI only. MVI, which was included as a sign of ABMR in the revised Banff 2017 classification [18], can be observed not only in ABMR but also in acute tubular necrosis, glomerulonephritis, and acute T-cell-mediated rejection [19, 20]. However, an MVI ≥2 is significantly associated with a histological diagnosis of acute and chronic ABMR [21]. Therefore, the progression to ABMR should be carefully monitored in recipients with MVI only who are not yet BPAR-compatible.

Recent studies have drawn very diverse conclusions regarding the effect of pre-transplant anti-AT1R on KT outcomes [16, 24, 25]. We identified an optimal cut-off value for anti-AT1R of 11.5 U/mL, which independently predicted a higher risk of AR in low risk LDKT recipients within six and 12 months post-transplant. We hypothesize that these AR episodes contributed to the decreased eGFR in the early stages of KT (Fig. 2B). The difference in eGFR between anti-AT1R ≥11.5 and <11.5 U/mL is offset, most likely by proper management for increased creatinine and AR episodes. However, recipients with anti-AT1R ≥11.5 U/mL appear to have persistently lower eGFR compared with those with <11.5 U/mL until 36 months. AECA (+) recipients demonstrated decreased eGFR at approximately three months and maintained lower eGFR than AECA (-) recipients until 24 months, regardless of AR episodes. Although it remains unclear why eGFR decreases and serum creatinine increases regardless of AR in AECA (+) recipients, we have observed that unlike AT1R, an AECA (+) result was significantly associated not only with AR but also with MVI only during the follow-up period. A previous study has reported that endothelin-1 type A receptor antibody, one of the AECA candidates, reduces renal function and increases intimal arteritis post-transplant [24]. Long term follow-up is needed to clarify the effect of anti-AT1R and AECA status on graft outcome.

Several studies have explored an association between HLA-DSA and anti-AT1R [7, 12, 27]. A few studies have shown that recipients with both pre-transplant HLA-DSA and anti-AT1R had
lower graft survival rates compared with recipients with either one, suggesting that there is a synergistic effect between pre-transplant HLA-DSA and anti-AT1R [7, 12]. Determining the mechanism by which anti-AT1R affect KT outcome is difficult, as pre-transplant HLA-DSA have a greater effect on KT outcome [14, 15]. Thus, we enrolled low-risk LDKT recipients, without pre-transplant HLA-DSA, in order to exclude the possibility that HLA-DSA affects KT outcomes. Several previous studies have reported that the de novo development of HLA-DSA is significantly higher in recipients with positive anti-AT1R [7, 24, 27]. We identified six recipients (6.4%) who developed de novo HLA-DSA; however, none were positive for AECA or anti-AT1R (>17 U/mL). This may suggest that the development of de novo HLA-DSA does not require pre-existing AECA or anti-AT1R. We did not observe a correlation between de novo HLA-DSA and AR, probably because of the small number of recipients who developed de novo HLA-DSA. This suggests that the presence of pre-transplant anti-AT1R and AECA have a greater effect on rejection risk and graft function than de novo HLA-DSA in low-risk LDKT recipients. In our study, the renal function of the recipients tended to improve after 12 months post-transplant (Fig. 2); this might be due to the prospective management employed according to the biopsy findings in the 73 recipients who underwent the one-yr protocol biopsy. Recipients under subclinical rejection were treated with steroid pulse therapy. In the cases of tacrolimus toxicity or BK virus-associated nephritis, the immunosuppressant was replaced with a lower intensity immunosuppressant such as sirolimus.

The limitation of this study is that we evaluated pre-transplant anti-AT1R levels and AECA status in all recipients, except for the post-transplant anti-AT1R levels in 29 recipients who experienced a biopsy proven rejection. Thus, we cannot exclude the impact of post-transplant anti-AT1R and AECA on transplant outcomes. However, except for two recipients with BPAR, the anti-AT1R levels at the time of rejection were <11.5 U/mL. This is consistent with previous study demonstrating that the anti-AT1R levels at the time of rejection were lower than the pre-transplant levels in most recipients with rejection episodes [8], probably owing to absorption of anti-AT1R to the graft.

In conclusion, we found that the presence of pre-transplant anti-AT1R and AECA, has a significant impact on the post-transplant outcomes in low-risk LDKT recipients. Pre-transplant anti-AT1R level was a significant risk factor for the development of AR, while AECA status was associated with impaired renal function regardless of AR. Therefore, evaluation of anti-AT1R levels and AECA before KT would be necessary to stratify the risk of graft dysfunction and predict the risk of AR due to non-HLA antibodies, particularly in a low-risk LDKT setting.

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AUTHOR CONTRIBUTIONS

SY collected and analyzed the data and wrote the paper; HJH designed the study; KWL, JBP, SJK, WH, HRJ, GYK, and HHM performed the study; and ESK designed and performed the study and wrote the paper.

CONFLICT OF INTEREST

No potential conflicts of interest relevant to this paper were reported.

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