Transplant International

Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

# Poor graft outcome in recipients with *de novo* donor-specific anti-HLA antibodies after living related kidney transplantation

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**Keywords**
antibody-mediated rejection, anti-HLA antibodies, living related kidney transplantation.

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Received: 9 April 2008
Revision requested: 9 May 2008
Accepted: 1 August 2008

doi:10.1111/j.1432-2277.2008.00755.x

## Summary

Antibody-mediated rejection (AMR) is now widely recognized as a major problem in organ transplantation. This study was conducted to investigate the relationship between newly developing anti-HLA antibodies post-transplantation (*de novo* Abs) and the outcome of living related kidney transplantation (LRKT). The subjects included 87 patients who had received living donor kidney allografts at our institution. Panel reactive Ab assay (Flow-PRA) and graft biopsies were performed in all the recipients before and 6 months after the LRKT. The incidence of AMR, the donor specificity and time of appearance of the *de novo* Abs were retrospectively studied. Among the 87 LRKT recipients, 47 (54%) showed negative/negative (N/N) results, 15 (17%) showed positive/positive (P/P) results, 12 (14%) showed positive/negative results (P/N), and 13 (15%) showed negative/positive (N/P) results (*de novo* Abs) in the pre-/post-transplant Flow-PRA analysis. Among the 13 cases with *de novo* Abs, 5 (38%) had donor-specific Abs (DSA) and the remaining 8 (62%) had nondonor-specific Abs, as determined by LAB single antigen analysis. Eighty percent of the recipients with DSA showed evidence of AMR in the graft biopsies. The 5-year graft survival rate of the recipients with *de novo* Abs (N/P) was 69%, as compared with 96% in the N/N, 88% in the P/N and 93% in the P/P recipient groups ($P = 0.009$). LRKT recipients developing *de novo* Abs, especially those with DSA, showed a much higher incidence of AMR and a worse prognosis. Cautious monitoring for the appearance of anti-HLA Abs should be adopted after transplantation, even in patients without anti-HLA Abs prior to the transplantation.

## Introduction

Anti-HLA antibodies (Abs) have gradually come to be recognized as a major risk factor among patients awaiting renal allografts, since the landmark study of Patel and Terasaki [1]. Preformed donor-specific anti-HLA Abs are responsible for hyperacute or accelerated acute rejection, and even nondonor-specific anti-HLA Abs are associated with an increased risk of acute rejection episodes and reduced graft survival [2–6]. Nowadays, therefore, standard HLA typing and monitoring for Abs have become routine for renal transplantation (RTx) candidates. However, what is the status after RTx?

In a preliminary multicenter study designed to prospectively evaluate the relationship between the presence of anti-HLA Abs and kidney graft loss, anti-HLA Abs were found in the serum of 20.9% of the 2278 kidney recipients evaluated at 6 months after RTx. By the 1-year follow up, 6.6% of the recipients in whom anti-HLA Abs were detected had lost their grafts, as compared with 3.3% of those in whom anti-HLA Abs were not detected ($P = 0.0007$) [7]. A correlation has also been reported

between appearance of Abs increase post-transplant and acute rejection episodes [8,9]. Furthermore, it has been suggested that Abs may play a decisive pathophysiologic role in acute rejection and may be closely involved in a considerable number of cases [10]. Although a relationship has been demonstrated between immunologic events and the appearance of anti-HLA Abs post-RTx, routine monitoring for anti-HLA Abs is still not considered at many centers. Detailed reports on the development of *de novo* anti-HLA Abs after living related kidney transplantation (LRKT) are few. Therefore, we conducted this study to investigate the relationship between *de novo* appearance of anti-HLA Abs post-RTx (*de novo* Abs) and the incidence of antibody-mediated rejection (AMR) after LRKT.

## Materials and methods

### Recipients and donors

Eighty-seven patients who underwent their first LRKT at the Tokyo Women's Medical University Hospital between January 2000 and July 2004 were included in this study. Recipients who had donor-specific anti-HLA Abs before the RTx as detected by the flow cytometry crossmatch (FCXM) test were excluded from the study. The FCXM test was negative in all the recipients at the time of the transplantation. Twenty-five ABO-incompatible recipients were also included in this study. The results of HLA typing in all the recipients were similar. Serum samples were collected sequentially from the patients before and at 6 months after the LRKT. The diagnosis of rejection was on the basis of the clinical symptoms and laboratory results and confirmed by pathologic findings. All recipients in this study were followed up strictly at our hospital. The data on proteinuria (wherever present) and the serum creatinine (SCr) concentrations of the recipients were collected prospectively. The demographic characteristics of the recipients are shown in Table 1a.

Parents (father/mother, 13/41) donated in 54 cases, siblings in 12 and other relatives in 21 cases. The mean age of the donors was 55.6 ± 10.5 years.

Sera were prepared from blood samples obtained from the recipients and donors after obtaining their informed consent. All study procedures were approved by the Ethics Committee of Tokyo Women's Medical University.

### Determination of anti-HLA antibodies and graft biopsies

In recent years, flow-cytometric methods using antigen-coated beads have been developed that enable highly specific and sensitive detection of anti-HLA Abs. Flow cytometry beads, coated with single HLA antigens pro-

**Table 1.** Recipient characteristics (a) and demographic characteristics (b) in the different groups.

| | Recipients in the study |
|---|---|
| **(a)** | |
| Number | 87 |
| Gender (female/male) | 29/58 |
| Age (years) | 38.8 ± 13.1 |
| Blood typing | |
| Compatible | 47 |
| Minor | 15 |
| Incompatible | 25 |
| HLA mismatch | |
| AB | 1.8 ± 1.0 |
| DR | 0.9 ± 0.5 |

| | N/N | P/N | P/P | N/P (*de novo*) | *P*-value |
|---|---|---|---|---|---|
| **(b)** | | | | | |
| No. patients | 47 | 12 | 15 | 13 | |
| Gender (female/male) | 11/36 | 7/5 | 8/7 | 3/10 | |
| Recipients age (years) | 39.6 ± 15.4 | 34.2 ± 12.1 | 38.7 ± 13.5 | 33.7 ± 16.9 | NS |
| Donor age (years) | 58.4 ± 10.7 | 51.9 ± 11.4 | 53.8 ± 12.2 | 52.1 ± 10.5 | NS |
| Blood typing (C/M/I) | 23/10/14 | 8/3/1 | 10/1/4 | 6/1/6 | NS |
| HLS mismatch | | | | | |
| AB | 1.8 ± 1.1 | 2.1 ± 1.2 | 1.9 ± 1.0 | 2.2 ± 0.8 | NS |
| DR | 0.9 ± 0.4 | 0.8 ± 0.3 | 0.8 ± 0.4 | 1.1 ± 0.5 | NS |

N/N, negative/negative; P/N, positive/negative; P/P, positive/positive; N/P, negative/positive; C/M/I, compatible/minor/incompatible; NS, not significant.



Journal compilation © 2008 European Society for Organ Transplantation **21** (2008) 1145–1152

duced by recombinant technologies, can also determine the specificity of the anti-HLA Abs accurately and sensitively [11–13]. In our study, blood samples were collected from the recipients prior to and at 6 months after the transplantation. The Flow-PRA Screening Test (One Lambda Inc., Canoga Park, CA, USA) was performed in all the recipients to detect the presence of anti-HLA Abs. A result of more than 10% was considered to be positive. Sequentially, samples testing positive by the Flow-PRA test were reanalyzed by LAB Screen single antigen analysis (One Lambda Inc.) to determine the donor specificity of the anti-HLA Abs.

Postoperative protocol biopsies (at 0 h, 6 months and more than 6 months), as well as episode biopsies were obtained in all recipients and the diagnoses were confirmed at the time of detection of the anti-HLA Abs. All biopsies were evaluated by light microscopy and immunofluorescence staining for C4d. The pathologic findings were classified according to the Banff 1997 working classification and Banff 2005 Update Edition [14–16] and comparatively evaluated in the recipients with and without *de novo* anti-HLA Abs.

### Immunosuppressive protocol and treatment of antibody-mediated rejection

All the recipients enrolled in this study received similar triple-therapy for immunosuppression, consisting of oral tacrolimus (FK), mycophenolate mofetil (MMF, Cellcept®; Roche, Nutley, NJ, USA) and methylprednisolone. FK and MMF were used as the alternative calcineurin inhibitor and antimetabolite immunosuppressive agent to cyclosporine (CsA) and azathioprine, respectively. For ABO-compatible and minor ABO mismatch recipients, FK (tacrolimus, Prograf®; Astellas Fujisawa, Osaka, Japan) was started 4 days before the transplantation at the dose of 0.1 mg/kg/day, and the dose was adjusted to maintain FK trough level in whole blood of between 8 and 12 ng/ml during the first month postoperatively, between 7 and 9 ng/ml during 2–3 months after the transplantation, and between 4 and 6 ng/ml thereafter. Mycophenolate mofetil was also started 4 days before the transplantation at the dose of 2000 mg/day and decreased to 1000–1500 mg/day during the first month postoperatively, depending on the count of white blood cells. Methylprednisolone (MP) was administered intravenously at the dose of 500, 250 and 125 mg/day on the day of transplantation, and on days 1 and 2 after the operation, respectively. Oral MP was started on day 3 postoperatively at the dose of 80 mg/day and then tapered to 6–8 mg/day within 1–2 months after the transplantation. Moreover, for the recipients with ABO-incompatiblities as well as with anti-HLA Abs (PRA positive), the immunosuppressive protocol was as described in detail in our previous study [17]. Briefly, three or four sessions of double filtration plasmapheresis (DFPP) in addition to conventional triple immunosuppressive regimens were performed to remove these Abs prior to transplantation.

All rejections were confirmed by graft biopsy. MP pulse therapy (500 mg QD ×2) was carried out to treat T-cell mediated rejection as well as AMR. Three sessions of plasmapheresis performed every other day and seven doses of OKT3 (5 mg/day) were used subsequently, for rejections that were resistant to MP treatment.

### Statistical analysis

The clinical outcomes, including the graft survival rate and the incidence of acute rejections, and the pathologic findings were compared between the recipients with and without *de novo* anti-HLA Abs. As for the recipients with *de novo* anti-HLA Abs, testing was conducted to determine whether the Abs were DSA or nondonor-specific Abs (NDSA). Univariate analysis was carried out using Student's *t*-test for continuous data and the chi-squared test for categorical data. Graft survival rates were estimated by Kaplan–Meier analysis and compared using Generalized-Wilcoxon's analysis. The hazard of graft survival rate in relation to the appearance of *de novo* Abs was determined by Cox Regression. Continuous data were expressed as mean ± SD, unless otherwise stated. Statistical analyses were performed using SPSS 13.0 (San Francisco, CA, USA). Values of $P < 0.01$ were considered to be statistically significant.

## Results

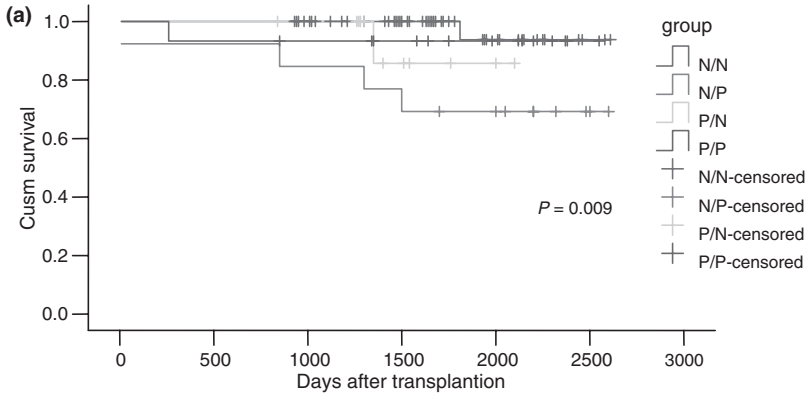
### Detection of anti-HLA antibodies

Patients were subdivided according to the presence/absence of anti-HLA Abs before and/or 6 months after the LRKT, on the basis of the results of Flow-PRA analysis. Among the 87 recipients, 47 (54%, 47/87) did not show anti-HLA Abs pre- or post-RTx (negative/negative, N/N), 12 (14%, 12/87) showed positive/negative (P/N) results, 15 (17%, 15/87) showed positive/positive (P/P) results, and 13 (15%, 13/87) showed negative/positive results (development of *de novo* anti-HLA Abs) in the testing performed pre-/post-RTx. Among the 13 recipients with *de novo* Abs, five (5/13, 38%) had DSA and the remaining eight (8/13, 62%) had NDSA, as determined by LAB single antigen analysis. Interestingly, the anti-blood type Ab titer examined post-transplant was low (below ×32) and remained stable in all of the 25 ABO-incompatible recipients, even in those with *de novo* anti-HLA Abs. The characteristics of each group are summarized in Table 1b. There were no significant differences

among the four groups of recipients in terms of the age, the donor's age, blood type distribution, or the number of HLA mismatches. Also, there were no significant differences in the positivity rates for *de novo* anti-HLA class I and class II Abs.

### Graft survival rate

All 87 recipients were alive during the period of the study, i.e. there was no significant difference in survival among the groups. In regard to the graft survival rate, as shown in Fig. 1a, the 1-, 3- and 5-year graft survival rates were 92%, 77%, and 69%, respectively, which were the lowest rates among all the groups, in the recipients with *de novo* anti-HLA Abs (group N/P). In the overall comparison of the graft survival rates, there were highly significant differences among the groups ($P = 0.009$). However, when the groups were compared in pairs, as shown in Table 2, there was a significant difference between groups N/N and N/P (*de novo* group), but not in any of the other pairwise comparisons. The cumulative hazard of graft loss in patients developing *de novo* Abs is shown in Fig. 1b, which indicates that the recipients with *de novo* Abs were at a higher risk for graft loss than those without *de novo* Abs, especially 3 years after LRKT. There was no significant difference in the graft survival rate between recipients with *de novo* anti-HLA class I and class II Abs.




| | 1 year | 3 years | 5 years | *P*-value |
|---|---|---|---|---|
| N/N | 100% | 100% | 96% | |
| P/N | 100% | 88% | 88% | |
| P/P | 93% | 93% | 93% | |
| N/P | 92% | 77% | 69% | $P = 0.009$[1] |

[1] Generalized-Wilcoxon analysis was used to compare overall proportions.

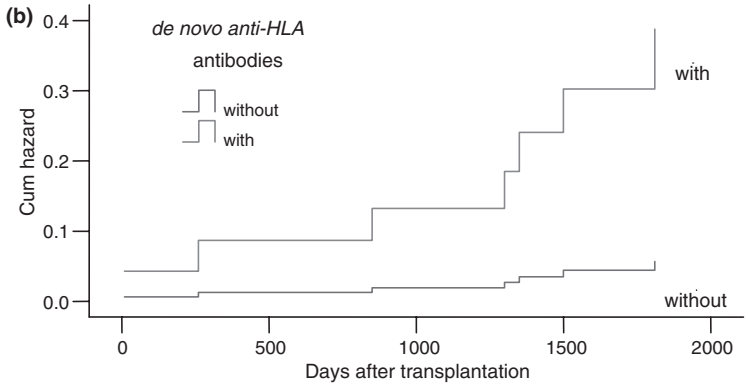



**Figure 1** (a) Kaplan–Meier graft survival; regarding the graft survival rate, as shown in (a), the 1-, 3-, and 5-year graft survival rates were 92%, 77%, and 69%, respectively, in the recipients with *de novo* Abs (group N/P), which were the lowest rates among the groups. (b) Cox-Regression Hazard Function; the cumulative hazard of developing *de novo* Abs, as shown in (b), indicated that the recipients with *de novo* Abs were at higher risk for graft loss than those without *de novo* Abs, especially 3 years after LRKT.

**Table 2.** *P*-values of Generalized-Wilcoxon pairwise comparisons.

| | N/N | P/N | P/P | N/P |
|---|---|---|---|---|
| N/N | – | 0.044 | 0.177 | 0.001* |
| P/N | 0.044 | – | 1.000 | 0.243 |
| P/P | 0.177 | 1.000 | – | 0.146 |
| N/P | 0.001[1] | 0.243 | 0.146 | – |

*There was a significant difference between groups N/N and N/P.

### Incidences of humoral rejection and graft function in the recipients with/without *de novo* Abs

The incidences of different patterns of pathologic findings in each group are shown in Table 3. Within 6 months of transplantation, there were no significant differences in the acute rejection rate among the groups. However, more than 6 months after the transplantation, the incidence of humoral rejection in the recipients with *de novo* Abs (group N/P) was much higher than that in group N/N ($P = 0.004$). Also, the frequency of no-evidence in group N/P was much lower than that in group N/N ($P = 0.003$). As for graft function, there were significant differences between the recipients with and without *de novo* Abs. The recipients with *de novo* Abs had a higher frequency of proteinuria and higher SCr level (Table 4).

### Time of appearance of *de novo* anti-HLA Abs and donor specificity

The time of appearance of *de novo* Abs varied widely. In all cases but one, they were detected more than 6 months after the LRKT; in only one case, they were detected on day 9 postoperatively. Donor-specific Abs (DSA) were detected in five of the recipients with *de novo* Abs, while the remaining eight showed nondonor-specific *de novo* Abs (NDSA *de novo* Abs). The incidence of acute AMR was 80% in the recipients with DSA within 6 months post-transplant. However, none of the recipients showing NDSA during the same period developed AMR. More than 6 months after the transplantation, one of the recipients with DSA showed persistent evidence of AMR and another three were diagnosed to have chronic active AMR. The clinical outcomes stratified by the presence of *de novo* DSA are shown in Table 5.

**Table 4.** Graft function of the recipients with/without *de novo* Abs.

| | N/N | N/P | *P*-value* |
|---|---|---|---|
| Proteinuria (>50 mg/dl) | 13% | 54% | 0.001 |
| Serum creatinine (mg/dl) | 1.52 ± 0.39 | 3.08 ± 0.71 | 0.002 |

*Graft function in group N/P remained poorer than that in group N/N.

## Discussion

With the advent of sensitive antibody-screening and cross-matching procedures and a better understanding of HLA polymorphism, the incidence of early, irreversible rejection has decreased dramatically. It is possible for us to increase the graft survival rate by using desensitization techniques and adopting comprehensive monitoring of patients pre- and post-transplantation [18]. Newly developing anti-HLA Abs post-RTx (*de novo* anti-HLA Abs) are now attracting much more attention in the field of

**Table 5.** Clinical outcomes stratified by *de novo* donor-specific antibody (DSA)*.

| | DSA ($n = 5$) | NDSA ($n = 8$) | *P*-value |
|---|---|---|---|
| A-AMR | 80% | 0% | 0.002 |
| C-AMR | 60% | 13% | 0.071 |
| 1-year GS | 80% | 100% | 0.188 |
| 5-year GS | 20% | 100% | 0.002 |

A-AMR, acute antibody-mediated rejection; C-AMR, chronic active antibody-mediated rejection; GS, graft survival.
*Specificity of antibody was determined by LAB Screen single antigen assay.

**Table 3.** Pathologic findings of the recipients within/after 6 months post-transplant.

| | N/N <6/>6 months | P/N <6/>6 months | P/P <6/>6 months | N/P <6/>6 months |
|---|---|---|---|---|
| A-AMR | 11; 23%/0 | 3; 25%/0 | 4; 27%/0 | 4; 31%/2; 17% |
| ATR | 7; 15%/0 | 2; 17%/0 | 1; 7%/0 | 1; 7%/0 |
| C-AMR | 0/5; 11% | 0/3; 25% | 1; 7%/8; 53% | 0/4; 31% |
| IF/TA | 3; 6%/8; 17% | 0/2; 17% | 0/1; 7% | 1; 8%/3; 25% |
| BC | 3; 6%/2; 4% | 1; 8%/0 | 0/0 | 0/0 |
| IgA | 0/2; 4% | 0/0 | 0/0 | 0/0 |
| NE | 23; 49%/30; 64% | 6; 50%/7; 58% | 9; 60%/5; 33% | 6; 46%/2; 17% |

A-AMR, acute antibody-mediated rejection; ATR, acute T-cell-mediated rejection; C-AMR, chronic active antibody-mediated rejection; IF/TA, interstitial fibrosis/tubular atrophy; BC, borderline changes; IgA, IgA nephropathy; NE, no evidence.

transplantation, while pre-existing anti-HLA Abs should be lessened by all possible means prior to transplantation. Pretransplant unsensitized recipients may develop *de novo* Abs, including donor-specific anti-HLA Abs, nondonor-specific anti-HLA Abs (NDSA) and donor-specific non-HLA Abs. The appearance of *de novo* anti-HLA Abs markedly increases the risk of acute and chronic rejection after RTx [19–23]. In this study of LRKT, although the patient survival rates were the same in all the four groups during the period of follow up, the graft survival rate was the lowest in the recipients with *de novo* anti-HLA Abs (group N/P) among the four groups ($P = 0.009$) and the cumulative hazard for graft loss was high in this group. The reason for the absence of significant difference when group N/P was compared with groups P/N and P/P is thought to be the relatively small number of recipients in each of the groups. Another reason is thought to be the decrease of rejection incidence rate by the Abs removal therapy, for example DFPP, prior to transplantation in P/N and P/P recipients, although N/P and N/N recipients did not receive the same treatment because of having been negative for anti-HLA Abs before transplantation. There are some possibilities that DSA in P/N and P/P recipients might disappear after a series of anti-HLA Ab removal therapy immediately before transplantation. In fact, five out of 12 recipients in P/N group and five out of 15 recipients in P/P group had DSA, however, all these recipients enrolled in this study showed negative conversion for direct crossmatch test immediately before transplantation. After short-lived negative PRA conversion before transplantation, PRA value in all 12 recipients in P/P group returned to the positive range.

A recipient undergoing ABO-incompatible LRKT can develop *de novo* anti-blood-type Abs. Thus there is the possibility of ABO-incompatible transplantation being associated with graft loss and causing AMR [17]. In this study, both anti-HLA Abs and anti-blood-type Abs were detected in some patients. However, the anti-blood-type Ab titers were below ×32 and remained stable. There was no significant difference in the percentages of ABO-incompatible recipients developing and not developing *de novo* Abs ($P$ = NS). This indicated that ABO-incompatible transplantation is safe under the currently used effective immunosuppressive protocol and is not associated with a higher incidence of humoral rejection in the recipients showing *de novo* Abs.

Recent studies have demonstrated a strong relation between the presence of anti-HLA Abs and acute humoral rejection, and patients with higher Ab titers more commonly experienced acute rejections than did patients with lower Ab titers [24,25]. Similarly, our study demonstrated that the incidence of AMR was much higher in the recipients with the appearance of *de novo* Abs more than 6 months after LRKT, while it was similar in all the groups within 6 months after LRKT. These results indicate that earlier diagnosis and treatment of humoral rejection are effective and some anti-HLA Abs developing pre- and/or post-transplant disappear after anti-rejection therapy. The mechanisms are not yet clear, but are probably related to the fact that donor-specific Abs become bound to the graft and are not found in the blood [19].

Anti-HLA Abs can be detected at any time pre- and post-transplant. However, the possibility of immediate appearance of anti-HLA Abs post-RTx decreases sharply by desensitization therapies and strict selection of transplant candidates prior to LRKT using the highly sensitive FCXM test. In our study, the *de novo* anti-HLA Abs in all, but one, cases were detected more than 1 year after the transplantation; in one recipient alone, these Abs were detected 9 days post-transplant. This demonstrated that the development of *de novo* Abs could be a chronic process. Accordingly, the risk of humoral rejection exists at any time after RTx. In a recent study, one patient developed an anti-HLA donor-specific Ab between the 22nd and 30th year after LRKT and experienced AMR [26]. Therefore, we consider that long-term monitoring for anti-HLA Abs is necessary.

It is assumed that the anti-HLA Abs can be found before the occurrence of rejection. However, the time lags among the appearance of anti-HLA Abs, RTx and the occurrence of rejection are still unclear. Some studies have shown that anti-HLA Abs do not have any obvious immediate deleterious effects on graft function [7,20]. In our study, all recipients, except one, with *de novo* Abs showed chronic gradual elevation of the SCr level and it took a long time before the grafts were finally rejected. Therefore, we consider that *de novo* anti-HLA Abs represent ideal markers of impending graft failure, because plenty of time is available before the failure to make attempts to extend the graft function by suitable intervention.

As for DSA and NDSA, both were found to be equally associated with acute and chronic rejection in one study [8], whereas a higher rate of acute rejection and graft loss was found with DSA in others [24,27]. In our study, AMR was diagnosed in 80% of the recipients with *de novo* DSAs after LRKT. The incidence was much higher than that in the recipients with *de novo* NDSAs ($P = 0.002$). Our study also demonstrated that the development of *de novo* DSA was associated with a bad outcome in terms of graft survival as compared with that of *de novo* NDSAs ($P = 0.002$). Even if the persistence of a low level of allo Ab does not appear to be necessarily associated with the development of humoral rejection [28], it plays a role as a risk factor in the long term. Thus, our study focused on the importance of detecting

DSA using sensitive techniques as well as of using immunosuppression of Abs.

Antibodies that affect the grafts may be directed against antigens other than HLA antigens. Major histocompatibility class I-related chain A (MICA) Abs are detected on endothelial cells but not on the lymphocytes in the sera of recipients with rejected kidneys [29,30]. In this study, we also examined the presence of MICA Abs in each group, and could find no relationship between the appearance of MICA Abs and the graft survival or acute rejection rate. Some Abs (for example, IgA anti-Fab auto Abs) have been shown to improve graft survival and could possibly counteract the activity of conventional cytotoxic anti-HLA Abs [31]. All these Abs mentioned above could appear as *de novo* Abs after RTx. We propose to continue to study the development and effects of *de novo* Abs in the future.

## Conclusions

Living related kidney transplantation recipients with newly developing anti-HLA Abs post-RTx (*de novo* anti-HLA Abs) show a much higher incidence of AMR and a poorer prognosis, especially those with donor-specific *de novo* Abs. Cautious monitoring for the appearance of anti-HLA Abs should be adopted after LRKT, even in recipients without anti-HLA Abs prior to the transplantation.

## Authorship

XL: collected data, analyzed data and wrote the paper. HI: designed research/study, performed research/study and collected data. YY: performed research/study. KT: contributed important reagents.

## Acknowledgements

This study was sponsored by the Japanese Ministry of Education and Tokyo Women's Medical University IREIIMS.

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