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Impact of the Trough Level of Calcineurin Inhibitor on the Prevalence of Donor-Specific Human Leukocyte Antigen Antibodies During Long-Term Follow-Up After Pediatric Liver Transplantation: Antibody Strength and Complement-Binding Ability

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Background. In pediatric patients, long-term immunosuppression after liver transplantation (LT) is typically minimal. However, posttransplant donor-specific HLA antibodies (DSAs) may be prevalent under these conditions. Here, we evaluated the effects of minimized calcineurin inhibitor (CNI) on DSA development to assess the validity of minimized/withdrawn immunosuppression. **Methods.** We retrospectively examined 66 patients who underwent pediatric LT at our institution between July 1991 and October 2013. Patients were divided into 2 groups based on the CNI trough level. The cutoff trough levels were 3 and 30 ng/mL for tacrolimus and cyclosporine, respectively. Luminex single-antigen bead assays were performed, and the cutoff for a positive reaction was set at a mean fluorescence intensity (MFI) of at least 1000. **Results.** The mean recipient ages at the time of LT were 29.1 and 77.2 months for the low and regular CNI groups, respectively ($P = 0.0007$). Univariate logistic regression analysis revealed that recipient age at LT younger than 3 years ($P = 0.0099$) and low CNI ($P < 0.0001$) were significantly associated with DSA development. In multivariate analysis, low CNI was an independent risk factor of DSA development ($P = 0.0011$). Of 15 high-MFI DSAs, 3 were anti-DR, and 12 were anti-DQ. Two of 3 anti-DR DSAs and 11 of 12 anti-DQ DSAs had complement-binding ability and high MFIs. **Conclusions.** CNI minimization was an independent risk factor for posttransplant DSA during long-term follow-up after pediatric LT. Adjusting CNI to appropriate levels is a safe first step to prevent the immunological effects of DSA.

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Donor-specific HLA antibodies (DSAs) have been reported to have detrimental effects on short- and long-term outcomes after organ transplantation.[1,2] Particularly for kidney transplantation, many studies have shown that de novo DSA strongly contributes to chronic antibody-mediated rejection and graft failure.[2,3] Although liver transplantation (LT) is believed to be much more immunologically tolerant than transplantation of other organs,[4,5] long-term histological examinations have revealed an association between DSA development and graft fibrosis.[6-9] Notably, after pediatric LT, long-term stable functioning of liver grafts is highly desirable. Therefore, determination of the appropriate timing and frequency of DSA screening is urgently required for long-term follow-up after pediatric LT, particularly when immunosuppression (IS) is minimized or withdrawn.

However, the significance of DSA screening during long-term follow-up after LT remains unclear, and the risk factors for DSA development have not been established. Moreover, DSA evaluation using Luminex technology is an expensive test. Therefore, the identification of high-risk groups is necessary to reduce unnecessary tests and optimize screening methods. Although we showed that younger age at the time of pediatric living donor LT (LDLT) was a risk factor for posttransplant DSA development during long-term follow-up (median follow-up period, 132 months),[10] the mechanisms mediating this effect of younger age were unclear. Furthermore, our previous study had major limitations, such as not fully assessing anti-DQ DSAs due to the unavailability of donors' HLA-DQ typing data, even though the significance of anti-DQ DSAs has been extensively reported.[1,11-13]

In our institution, many pediatric LDLT recipients with normal liver function were maintained on a low dose of calcineurin inhibitor (CNI), with trough levels under the minimum levels (3 ng/mL for tacrolimus and 30 ng/mL for cyclosporine), because of the long-term adverse effects of CNI. However, insufficient IS, such as tacrolimus withdrawal, has been reported to be associated with liver fibrosis after LDLT due to immune reactions.[14] Although several studies have shown that younger age was a risk factor for posttransplant DSA development,[1,13,15,16] researchers have only speculated as to the mechanism involved, suggesting the importance of a robust immune system or greater nonadherence.[17]

Therefore, in this study, we assessed whether the association of recipient age at the time of LDLT with DSA development was related to differences in maintained CNI trough levels. To overcome the limitations of our previous study, we extended the DSA screening to most our patients, further evaluated HLA-DQ typing data, and assessed the complement binding ability of DSAs in subgroup analyses.

MATERIALS AND METHODS

Patient Selection

We retrospectively identified 90 patients who underwent pediatric LDLT at our institution between July 1991 and October 2013. A screening for HLA antibodies was performed once for each recipient between July 2014 and March 2017. Twenty-four patients were excluded from this study for the following reasons: death before DSA screening (n = 13), follow-up at the other institutions (n = 9), discontinuation of follow-up (n = 1), and donors' HLA typing data unavailable (n = 1). Finally, a total of 66 patients were included in this study (Figure 1). Patients were divided into 2 groups based on the trough level of CNI within 3 months before or just at DSA screening: the low-CNI group included patients with trough levels of less than 3 ng/mL for tacrolimus and less than 30 ng/mL for cyclosporine, and the regular-CNI group included patients with trough levels of 3 ng/mL or more for tacrolimus and 30 ng/mL or more for cyclosporine. Patients who were free from CNI and not adherent to IS at the time of DSA screening were assigned to the low-CNI group.

For some patients, donor specificity of anti-DQ antibodies was not confirmed owing to the unavailability of donors' HLA-DQ typing data. Therefore, we performed subgroup analysis among 59 patients who were evaluated for anti-DQ DSA and assessed risk factors for anti-class II DSA development. Furthermore, for 15 of 19 patients with high-mean

fluorescence intensity (MFI) (≥ 5000) DSA, complement-binding ability was assessed using C1q-binding assays. Patients or their parents or legal guardians provided informed consent for participation in the study. The study protocol was approved by the Institutional Ethics Review Board.

Immunosuppressive Regimens

The immunosuppressive regimen was described previously.[18] Patients received induction immunosuppressive treatment consisting of steroids and a CNI with or without basiliximab. Mycophenolate mofetil or azathioprine was added based on the patient's posttransplant condition. Steroids were discontinued at 6 months after transplantation, depending on the patient's state. For 3 of 9 ABO-incompatible cases, rituximab was administered along with plasma exchange. For the treatment of acute rejection, steroid pulse therapy was conducted, and steroid recycling therapy, deoxyspergualin, and/or anti-CD3 antibody were added based on the patients' conditions.

DSA Detection

LABScreen Single Antigen class I and class II beads (One Lambda Inc., Canoga Park, CA) and a Luminex platform (One Lambda Inc.) were used to detect DSAs. The complement-binding ability of DSA was assessed using C1q binding assays (One Lambda Inc.) per the manufacturer's protocol. The cut-off for a positive reaction was set at an MFI of 1000 or more, and high MFI was defined as 5000 or more.

Statistical Analysis

Data were expressed as means (± SDs or ranges) or medians (ranges) as appropriate. Statistically significant differences were determined using Student t tests for normally distributed data, Wilcoxon signed rank tests for skewed data, and Fisher exact tests or χ^2 tests for dichotomous data. Univariate and multivariate logistic regression analyses were used to assess the risk factors for DSA development. The discriminative level of continuous variables was assessed using a receiver-operating characteristic curve based on maximizing sensitivity and specificity. Analyses were performed using JMP Pro 13 (SAS Institute, Cary, NC). Results with a P value of less than 0.05 were considered significant.

RESULTS

Patient Demographics

Of the 66 patients included in this study, 35 were in the low-CNI group, and the other 31 were in the regular-CNI group. Of the 35 patients in the low-CNI group, 5 were free from immunosuppressive drugs, and 1 patient was nonadherent to immunosuppressive drugs at the time of DSA screening. The peritransplant characteristics of the donors and recipients for the 2 groups are presented in Table 1. The mean recipient ages at the time of LDLT were 29.1 months (range, 5-170 months) for the low-CNI group and 77.2 months (range, 6-205 months) for the regular-CNI group ($P = 0.0007$). Donors were also younger in the low-CNI group than in the regular-CNI group (31.3 vs 37.3 years; $P = 0.0001$). The indications for LDLT among the 66 patients forming our study group included biliary atresia (n = 53), acute liver failure (n = 2), Alagille syndrome (n = 2), Wilson disease (n = 1), patent ductus arteriosus (n = 1), ornithine carbamoyltransferase deficiency (n = 1), nonalcoholic steatohepatitis (n = 2),
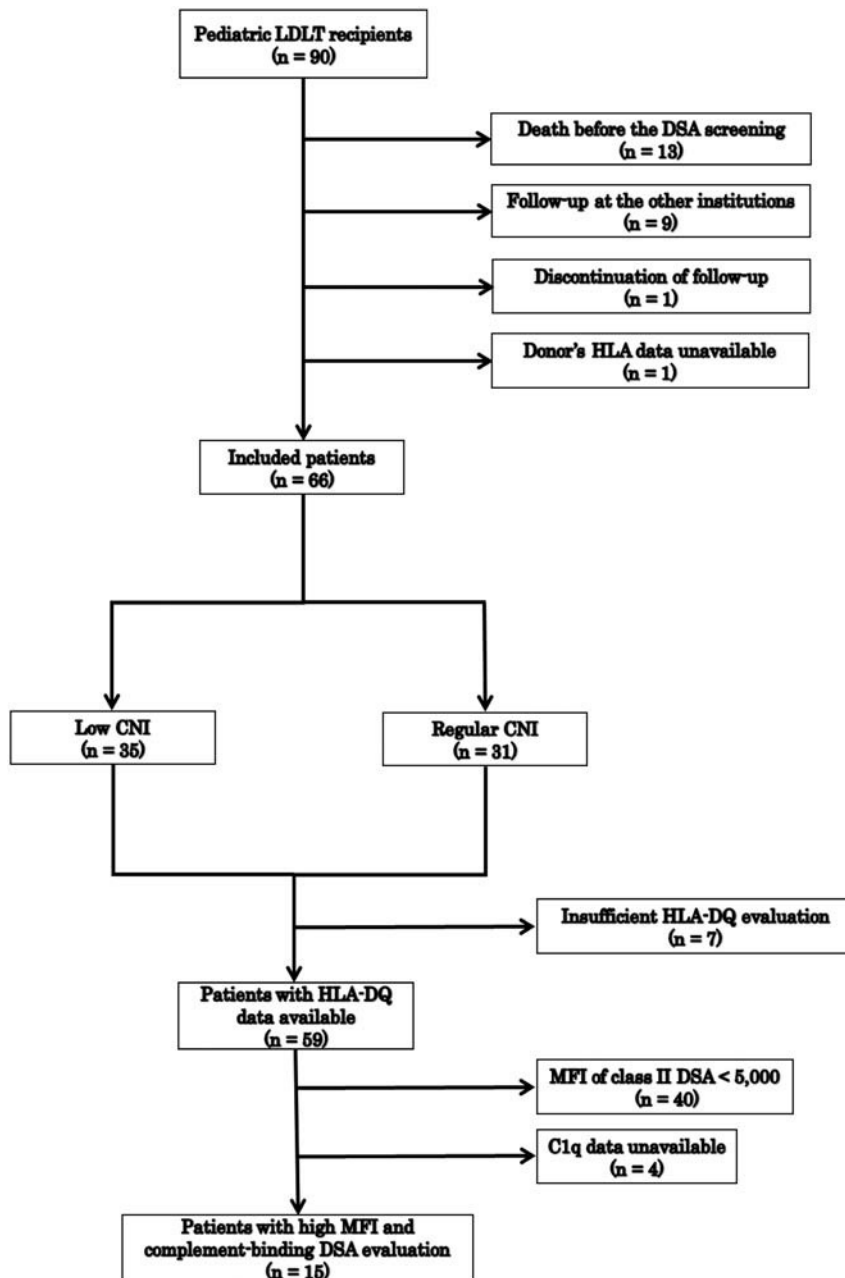
FIGURE 1. Flowchart of patient enrollment.

homozygous familial hypercholesterolemia (n = 2), and cryptogenic cirrhosis (n = 2). Groups were equivalent with regard to the distributions of recipients' and donors' sexes, primary diseases, and ABO incompatibilities.

The posttransplant characteristics of donors and recipients are presented in Table 2. The median follow-up period after LDLT was 142 months (range, 10-278 months). Seven of 66 patients had episodes of nonadherence to IS, which was diagnosed by both self-reporting by the patient and estimates by clinicians. Of 7 nonadherent patients, 1 patient was still nonadherent and classified in low-CNI group, and the other 6 were adherent to IS at the time of DSA screening. Additionally, 34 (52%) of the included 66 patients had rejection episodes; 29 patients had rejection episodes within 3 months of LDLT and 4 patients after 1 year. The mean periods from

rejection episodes to DSA screening was 142 months. For the treatment of acute rejection, steroid pulse therapy was administered for 31 patients, and the other 3 patients were treated with adjustment of maintenance immunosuppressive drugs. The mean levels of total bilirubin, aspartate aminotransferase, and alanine aminotransferase were well maintained within the normal level. There were no between-group differences in these factors. Among the 66 patients, anti-class I and anti-class II antibodies, including non-DSAs, were positive in 72.7% and 83.3% of patients, respectively. There were no between-group differences in anti-class I antibodies, whereas the positive rate of anti-class II antibodies was significantly higher in the low-CNI group. Regarding DSAs, anti-class I DSAs were positive only in 1 patient in the regular-CNI group. Significantly more patients tested positive

TABLE 1.

Peritransplant characteristics of 66 pediatric LDLT recipients

Variables	Total (n = 66)	Low CNI (n = 35)	Regular CNI (n = 31)	P
Recipient age (mo), mean	51.7 (5-205)	29.1 (5-170)	77.2 (6-205)	0.0007
Recipient sex (male/female)	25/41	11/24	14/17	0.31
Donor age (y), mean	34.1 (17-50)	31.3 (17-44)	37.3 (21-50)	0.0001
Donor sex (male/female)	25/41	13/22	12/19	1.00
Relationship (mother/father/others)	40/25/1	22/13/0	18/12/1	0.80
Primary disease				0.23
Biliary atresia	53	30	23	
Congenital metabolic disorder	2	0	2	
Acute liver failure	2	0	2	
Others	9	5	4	
ABO incompatible	9 (13.6%)	4 (11.4%)	5 (16.1%)	0.73
Positive CDC crossmatch	2 (3.1%)	2 (5.7%)	0	0.50
Use of basiliximab	6 (9.1%)	1 (2.9%)	5 (16.1%)	0.20

CDC, complement-dependent cytotoxicity.

for anti-DR DSAs in the low-CNI group than in the regular-CNI group (54.3% vs 9.7%; $P < 0.0001$). All 5 patients who were free from CNI developed anti-class II DSAs, that is, anti-DR DSAs for 2 patients, anti-DQ DSAs for 2 patients, and both anti-DR and anti-DQ DSAs for 1 patient. One patient who was nonadherent to IS developed anti-DR DSAs.

Risk Factors for DSA Development

Regarding risk factors of anti-class II antibody development including non-DSAs, logistic regression analysis identified low CNI as the only risk factor (Table 3). Recipient age, rejection episodes, and CNI type were not associated with anti-class II antibody development.

Univariate logistic regression analysis revealed that recipient age at the time of LDLT younger than 3 years ($P = 0.0099$) and low CNI ($P < 0.0001$) were significantly associated with DSA development (Table 4). In multivariate analysis, only low CNI was an independent risk factor of DSA development (odds ratio, 8.48; 95% confidence interval, 2.27-42.1; $P = 0.0011$). The association between the recipient age at LDLT younger than 3 years and DSA development did not

reach statistical significance ($P = 0.25$) after adjusting for low CNI.

Subgroup Analysis for Patients With HLA-DQ Data Available

For 7 of the 66 patients, donors' DQ typing data were not available. These 7 patients were excluded, and the remaining 59 patients for whom anti-DR and -DQ DSAs could be assessed were included in the subgroup analysis and further analyzed. Univariate logistic regression analysis for this subgroup revealed that recipient age at LDLT younger than 3 years ($P = 0.016$) and low CNI ($P = 0.0001$) were significant predictors for anti-class II DSA, consistent with the primary analysis (Table 5). Similarly, multivariate subgroup analysis showed that low CNI was an independent risk factor for the development of anti-class II DSA (odds ratio, 6.72; 95% confidence interval, 2.02-24.7; $P = 0.0017$).

Complement-Binding Assays for Patients With high-MFI Anti-Class II DSAs

From 59 patients in the subgroup, 40 patients with MFIs of anti-class II DSAs less than 5000 were excluded. Of

TABLE 2.

Posttransplant characteristics of 66 pediatric LDLT recipients

Variables	Total (n = 66)	Low CNI (n = 35)	Regular CNI (n = 31)	P
Periods from LDLT, mo	142 (10-278)	149 (12-278)	120 (10-268)	0.28
Nonadherence to IS	7 (10.6%)	3 (8.6%)	4 (12.9%)	0.70
Biliary complications	13 (20.0%)	7 (20.6%)	6 (19.4%)	1.00
Acute rejection	34 (51.5%)	18 (51.4%)	16 (51.6%)	1.00
Use of MMF at DSA evaluation	3 (4.5%)	1 (2.9%)	2 (6.5%)	0.60
Use of steroid at DSA evaluation	1 (1.5%)	0	1 (3.2%)	0.47
T bil: mean (SD), mg/dL	0.90 (0.64)	0.78 (0.46)	1.04 (0.78)	0.12
AST: mean (SD), mU/mL	28.5 (13.7)	26.7 (9.0)	30.5 (17.5)	0.28
ALT: mean (SD), mU/mL	22.1 (14.9)	21.7 (14.7)	22.6 (15.3)	0.79
Class I antibody positivity	48 (72.7%)	25 (71.4%)	23 (74.2%)	1.00
Class II antibody positivity	55 (83.3%)	33 (94.3%)	22 (71.0%)	0.018
Anti-A/B DSA positivity	1	0	1	1.00
Anti-DR DSA positivity	22 (33.3%)	18 (54.3%)	3 (9.7%)	0.0002

MMF, mycophenolate mofetil; T bil, total bilirubin at the time of DSA screening; AST, aspartate aminotransferase at the time of DSA screening; ALT, alanine aminotransferase at the time of DSA screening.

TABLE 3.

Risk factors for anti-class II antibody development

Variables	OR	95% CI	*P*
Age at LDLT < 3 y	2.10	0.56-8.15	0.27
Use of basiliximab	0.35	0.059-2.81	0.29
Acute rejection	0.86	0.23-3.20	0.83
Use of Tac at DSA evaluation	0.79	0.11-3.65	0.78
Low CNI	6.75	1.56-47.1	0.0092
Periods from LDLT ≥ 10 y	2.10	0.56-8.15	0.27

OR, odds ratio; CI, confidence interval; Tac, tacrolimus.

the remaining 19 patients with high-MFI DSAs, 15 underwent C1q-binding assay to assess the complement-binding ability of detected DSAs. Of 15 patients, 3 patients had anti-DR DSAs, and 12 had anti-DQ DSAs. Of 3 patients who had anti-DR DSAs, 2 tested positive in C1q assays, whereas 12 of the 13 anti-DQ DSAs had complement-binding ability and high MFIs (mean MFI, 24 980).

DISCUSSION

In this study, we found that CNI minimization or withdrawal was an independent risk factor for anti-DR DSA development. Additionally, subgroup analyses for patients with anti-DQ DSAs available showed that low-trough CNI was an independent risk factor for anti-class II DSA development, and that almost all high-MFI DSAs had complement-binding ability.

In our basic IS strategy for pediatric LDLT recipients who maintained normal liver function, we did not increase the CNI dose as the patients grew up, resulting in gradual tapering of the relative CNI dose. Most patients could be maintained with normal liver function using a minimized CNI dose. However, IS withdrawal during long-term follow-up has been shown to be associated with liver fibrosis,[6,14,19] potentially because of immunological effects. Based on these observations, we performed DSA screening in pediatric patients who underwent LDLT and found that younger age was associated with DSA development.[10] However, this previous study had several limitations, including small sample size and lack of HLA-DQ data for most donors. Since the significance of anti-DQ DSAs has been previously reported,[11-13,15,16] we not only extended DSA screening but also performed subgroup analysis of risk factors for anti-class II DSA development including anti-DQ DSA based on HLA-DQ typing of additional donors. By adding these data, multivariate analysis

TABLE 4.

Risk factors for anti-DR DSA development: univariate and multivariate analyses

Variables	Univariate analysis			Multivariate analysis		
	OR	95% CI	*P*	OR	95% CI	*P*
Age at LDLT < 3 y	4.50	1.31-15.5	0.0099	2.23	0.56-9.68	0.25
Use of basiliximab	0.37	0.02-2.51	0.34			
Acute rejection	1.10	0.39-3.10	0.86			
Use of Tac at DSA evaluation	1.67	0.44-8.20	0.47			
Low CNI	11.1	3.18-52.6	<0.0001	8.48	2.27-42.1	0.0011
Periods from LDLT ≥ 10 y	1.21	0.43-3.59	0.72			

TABLE 5.

Risk factors for anti-DR/DQ DSA development in 59 patients with available HLA-DQ typing data of donors: univariate and multivariate analyses

Variables	Univariate analysis			Multivariate analysis		
	OR	95% CI	*P*	OR	95% CI	*P*
Age at LDLT < 3 y	3.75	1.28-11.8	0.016	1.99	0.55-7.09	0.29
Use of basiliximab	0.38	0.050-2.14	0.28			
Acute rejection	1.39	0.50-3.92	0.53			
Use of Tac at DSA evaluation	2.92	0.71-14.9	0.14			
Low CNI	8.48	2.74-29.4	0.0001	6.72	2.02-24.7	0.0017
Periods from LDLT ≥ 10 y	1.55	0.55-4.44	0.41			

revealed that low CNI was an independent risk factor, consistent with the results of the primary analysis, which assessed risk factors of anti-DR DSA.

Few reports have evaluated the associations of CNI trough levels with DSA development, particularly during long-term follow-up after pediatric LT. For DSA development during short-term follow-up, Kaneku et al[1] analyzed DSA development within 1 year after LT and revealed that a low CNI trough concentration was a major risk factor for DSA formation. In our study, the median follow-up period was approximately 12 years, indicating that low CNI could be associated with DSA development during long-term follow-up. Moreover, Beland et al[20] revealed a strong protective effect of higher tacrolimus levels and suggested that anti-HLA antibody monitoring could facilitate optimization of maintenance IS and improvement of graft survival. Therefore, using DSAs as a marker for fibrosis progression and increasing CNI for patients with DSA development could be a promising option to prevent fibrosis and extend graft survival.

Many studies have revealed that younger age at LT is associated with DSA development,[7,13,15,16,21] and our previous studies indicated that patients who underwent LDLT when they were younger than 3 years had higher rate of DSA.[10] Univariate analysis in the present study also showed the association between younger age at LT and DSA development. However, multivariate analysis revealed that only low CNI was an independent risk factor for DSA development. These results indicated that the association between younger age at LT and DSA development was caused by the lower CNI levels among patients who underwent LT at a younger age. Several studies have demonstrated that the association between younger age and DSA development is related to nonadherence to IS.[7,13] In contrast, in our study, nonadherence was not associated with DSA development. In our study, nonadherence was defined as past episodes of discontinuing IS during long-term posttransplant follow-up. Based on these results, the most important factor affecting DSA development was the present IS status, not prior IS continuation.

The impact of complement-binding DSA has been frequently reported in kidney transplantation and LT.[15,22] However, the prevalence of complement-binding DSA during long-term follow-up after pediatric LDLT remains unclear. Furthermore, the association of MFI strength with complement-binding ability has not been fully evaluated. Therefore, we performed subgroup analysis to evaluate the complement-binding ability of high-MFI DSAs and showed

that 87% of high-MFI DSAs had complement-binding ability. This result suggested that the effects of DSA on liver graft need to be cautiously monitored among patients with high-MFI DSAs, although MFI is a semiquantitative index. In this small-sample subgroup analysis, almost all the anti-DQ DSAs had complement-binding ability, whereas 1 of 3 anti-DR DSAs did not have complement-binding ability. Further analysis of the differences in complement-binding ability between anti-DR and anti-DQ DSAs is needed.

The current study had several limitations. First, we conducted a retrospective analysis of cross-sectional data of HLA antibody screening for pediatric patients who underwent LT, resulting in a lack of longitudinal data. Considering the reported immunological impact of posttransplant DSAs on graft survival, adjusting the CNI to a normal range seems appropriate for patients who developed DSAs in our screening. However, we need to further elucidate whether increased and well-maintained CNI suppresses DSA development. Concurrently, histological examinations are essential to assess the significance of posttransplant DSA and measure the effects of adjusted CNI doses. Second, we could not determine whether the detected DSA was persistent or de novo because only a complement-dependent cytotoxicity cross-match was performed in most patients before LDLT, a test with a much lower sensitivity than single-antigen bead assays. However, for patients who were followed up for a long time after transplantation, it is intrinsically difficult to ascertain whether detected posttransplant DSAs were preformed or de novo because most detected pretransplant DSAs are decreased and become undetectable immediately after LT.[23] Third, in our study, DSA analysis was restricted to HLA-A, -B, -DR, and -DQ antigens due to the lack of HLA-Cw and -DP typing data. Although several studies have shown that the prevalence of anti-Cw and anti-DP DSAs is low,[13,24] the significance of these findings remains unclear. Although the low prevalence of anti-Cw and anti-DP antibodies in our studies (data not shown) indicated that the significance was relatively low, evaluation of anti-Cw and anti-DP DSAs is needed in future studies. Fourth, we set the cutoff level of low CNI based on the sensitivity levels at our institution. At our institution, blood levels less than 2 ng/mL for tacrolimus and 25 ng/mL for cyclosporine were undetectable. Therefore, for patients who received nonminimization IS, trough levels were controlled to more than 3 ng/mL for tacrolimus and 30 ng/mL for cyclosporine to avoid undetectable CNI trough levels. To separate patients with IS minimization from those with regular IS protocols, we chose the cutoff levels. However, the validity of the cutoff levels needs to be further examined. Finally, in the present study, complement-binding assays were performed for subgroup patients with high-MFI DSAs, demonstrating that 87% of high-MFI DSAs had complement-binding ability. This result indicated that high-MFI DSA could have immunological effects on liver grafts through complement activation. However, histological examination is required to reveal the evidence of complement activation. Furthermore, the complement-binding ability of moderate MFI, such as 1000 < MFI < 5000, was not examined in the subgroup analysis. Therefore, we were unable to compare differences in complement-binding ability between high- and moderate-MFI DSAs.

In conclusion, CNI minimization was an independent risk factor for posttransplant DSA development during long-term follow-up after pediatric LDLT. Because long-term stable functioning of transplanted livers is required for pediatric LDLT recipients, CNI needs to be rigidly maintained at appropriate levels, particularly in patients for whom DSAs have been detected.

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