# Clinical relevance of donor-specific human leukocyte antigen antibodies after pediatric liver transplantation

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**Abstract.** Donor-specific human leukocyte antigen (HLA) antibodies (DSAs) have a significant role in graft survival after pediatric liver transplantation. To understand the significance of DSAs, a retrospective cohort study of 48 pediatric liver transplant recipients with posttransplant serum samples that were analyzed for DSAs was performed. According to their test results, the recipients were divided into a DSA-positive group and a DSA-negative group. Postoperative liver transplantation biopsies were performed in patients with abnormal liver function. The liver condition and prognosis of the recipients were recorded, and their association was analyzed. A total of 48 recipients were followed up for 2.7±0.8 years. DSA positivity was detected in 10 cases (20.8%). One case was positive for HLA class I and HLA class II antibodies, whereas 9 cases were positive for HLA class II antibodies, and the gene loci were HLA-DR and/or DQ. Antibody-mediated rejection (AMR) occurred in four of 10 patients in the DSA-positive group. Liver function was abnormal in 3 of 38 cases in the DSA-negative group. Multivariate analysis revealed that DSA positivity was an independent risk factor for liver insufficiency and long-term survival of recipients. In addition, Kaplan-Meier survival analysis demonstrated that there were significant differences in the survival of graft recipients between the DSA-positive group and the DSA-negative group (P<0.05). The positivity of DSAs after pediatric liver transplantation was closely related to the occurrence of AMR. These results suggested that DSAs should be routinely monitored post-operatively, and that DSA-positive recipients should be screened as soon as possible and given appropriate treatment.

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## Introduction

Increasing evidence has indicated that the existence of donor-specific human leukocyte antigen (HLA) antibodies (DSAs) may adversely affect the long-term survival of grafts (1). DSAs in kidney and heart transplant recipients have been associated with acute T-cell-mediated rejection, antibody-mediated rejection (AMR), progression to chronic rejection, late graft dysfunction, vasculopathy and allograft loss (2-6).

In adult liver transplantation, the effect of DSAs on long-term survival is controversial, but DSAs may be a risk factor for poor survival (7-9). Patients undergoing liver transplantation with preformed DSAs have been indicated to be at increased risk of hyperacute rejection (10) and AMR within the first weeks after transplantation (11-13). In addition, DSAs have been associated with chronic rejection (14,15), accelerated fibrosis (16,17) and anastomotic biliary strictures (18). Compared with adult recipients, pediatric liver transplant recipients exhibit a higher incidence of DSAs after liver transplantation. It has been reported that the positive rate of DSAs in pediatric liver transplant recipients can be as high as 54% (19). However, the relationship between the presence of DSAs and AMR after pediatric liver transplantation and how it affects the survival of allogeneic liver transplant recipients is not clear.

The purpose of the present study was to analyze the effect of DSAs on the liver function and survival of 48 pediatric patients undergoing liver transplantation at the Tianjin First Central Hospital (Tianjin, China).

## Materials and methods

*Patients and samples.* A retrospective analysis of 48 pediatric patients undergoing liver transplantation (age, 0-11 years) at the Tianjin First Central Hospital, enrolled between January 2015 and December 2018, was conducted. The following criteria were used to select patients for the present study: i) All children with complete follow-up data after liver transplantation; and ii) all pediatric liver transplant recipients who received immunosuppressive therapy consisting of tacrolimus combined with corticosteroids.

*Immunosuppression.* All recipients were treated with tacrolimus and corticosteroids. Methylprednisolone and

basiliximab were also used to treat patients following pediatric liver transplantations; basiliximab was given on the fourth day post-operatively. Sequential treatment with methylprednisolone was administered post-operatively and stopped at 6 months. Tacrolimus was administered on the second day after liver transplantation, and the trough concentration (7-10 ng/ml) was maintained up to 3 months after pediatric liver transplantation. Following detection of DSAs, the recipients were treated with mycophenolate mofetil (MMF; 600 mg/m$^2$ per dose twice daily).

*Diagnostic criteria for AMR following pediatric liver transplantation.* The diagnostic criteria for AMR after pediatric liver transplantation included the following: i) early liver transplantation insufficiency; ii) diffuse vascular endothelial injury and small perivascular inflammation; iii) high DSA positivity; iv) strong diffuse C4d linear staining of liver tissue; and v) improvement in liver function (measured using standard biochemical tests), decreased DSA level, improvement in liver histology (such as fibrosis and inflammation) and disappearance of C4d deposition after anti-AMR treatment.

*HLA typing and HLA antibody determination.* All patients and donors were typed for HLA-A, -B, -DRB1 and -DQB1. HLA class I and II typing was performed by molecular methods (PCR-sequence-specific oligonucleotide techniques; One Lambda Inc.) (20). The detection of HLA antibodies was performed using Lifecodes single-antigen beads class I and II (Immucor Inc.), according to the manufacturer's protocol (21). Antibody results of liver transplantation recipients were compared with the HLA of the corresponding donor to determine whether they were donor-specific. The interpretation criteria for positive samples were as follows: Mean fluorescence intensity (MFI) >10,000 was strongly positive, 4,000< MFI <10,000 was moderately positive, 750< MFI <4,000 was weakly positive and <750 was negative.

*Liver histology.* Liver tissue specimens obtained by ultrasound-guided biopsy were fixed for 12 h in 4% formalin solution at room temperature, and 3-$\mu$m paraffin-embedded sections were cut according to the conventional procedure. For H&E staining, hematoxylin staining was performed for 5 min, followed by immersion in 1% hydrochloric acid alcohol for 30 sec, light ammonia water for 30 sec, eosin staining for 1 min and gradient alcohol dehydration at room temperature (all from Wexis Group Limited). For Masson's trichrome, the slides were stained with hematoxylin for 3-5 min, followed by Ponceau acid red for 5-8 min at room temperature. After washing with water, the slides were sequentially immersed in phosphomolybdic acid for 3 min, acetic acid for 20 sec, water-soluble aniline blue for 30 sec, 95% ethanol and xylene for dehydration at room temperature (all from Beijing Yili Fine Chemicals co., Ltd.). For C4d staining, after antigen retrieval (high pressure for 3 min at 120˚C), the anti-C4d antibody (cat no. ab187931; Abcam) was incubated at 4˚C overnight, followed by HRP-labeled anti-mouse IgG antibody (provided in the ultraView Universal DAB Detection Kit; cat. no. 760-500;

Table I. Characteristics of pediatric liver transplant recipients (n=48).

| Characteristics | Data at transplantation |
|---|---|
| Age, mean ± SD (range) | 3.7±2.6 (1-11) |
| Sex (male/female) | 27/21 |
| Clinical diagnosis (n) | |
| Biliary atresia | 38 |
| Autoimmune liver cirrhosis | 1 |
| Congenital bile duct dilatation | 1 |
| Bile cirrhosis | 1 |
| Alagille syndrome | 1 |
| Cholestasis | 6 |
| Liver transplant type (n) | |
| Parental transplantation | 35 |
| Liver transplantation after donor death | 13 |
| Blood type combination (n) | |
| Identical | 31 |
| Compatible | 17 |

Roche Diagnostics) at 37˚C for 30 min, incubation with 3,3-diaminobenzidine at room temperature for 5 min for color development and hematoxylin counterstain for 30 sec (both from Roche Applied Science).

*Statistical analysis.* Statistical analysis was performed using SPSS (version 20.0; IBM Corp.) and GraphPad Prism (version 5.0; GraphPad Software, Inc.) software. Continuous variables are presented as the mean ± standard deviation for normally distributed data and as median (range) when the values were not normally distributed. The differences between the groups were tested by independent samples t-test or $\chi^2$ test, as appropriate. The Mann-Whitney U test was used when parameters exhibited a non-normal distribution. Kaplan-Meier survival analysis was performed to analyze clinical events following liver transplantation, and statistical significance was determined by log rank testing. Multivariate analyses were performed using the Cox proportional hazards regression model. P<0.05 was considered to indicate a statistically significant difference.

## Results

*Baseline characteristics.* All patients were children who underwent a primary liver transplant. Patient characteristics are outlined in Table I. The mean recipient age was 3.7±2.6 years. The primary disease of 38 (79.2%) patients was biliary atresia. A total of 35 livers were from parental donors and 13 livers were from deceased organ donors.

*Prevalence of DSAs in pediatric liver transplantation.* It was observed that 10 of 48 (20.8%) pediatric patients developed DSAs post-liver transplantation (Table II). One of the 10 patients (10%) developed DSAs against HLA class I and II antigens, and nine (90%) developed DSAs against HLA class II antigens. Six of the 10 patients (60%) developed one DSA,

Table II. Demographic and clinical data and specifications of patients with DSA (n=10).

| Patient characteristics | | Patients | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| Sex (M/F) | | F | M | M | F | F | M | M | M | F | F |
| Age at LT (years) | | 2 | 3 | 11 | 1 | 1 | 1 | 1 | 2 | 2 | 2 |
| Donor age (years) | | 1 | 34 | 34 | 32 | 29 | 34 | 32 | 31 | 1 | 28 |
| Donor type (DCD/P) | | DCD | DCD | P | P | P | P | P | P | DCD | P |
| Disease type | | BA | AS | ALC | BA | BA | BA | BA | BA | BA | BA |
| Amount of blood transfusions during surgery | | | | | | | | | | | |
| Erythrocytes (U) | | 2 | 4 | 4 | 4 | 2 | 2 | 4 | 4 | 4 | 2 |
| Fresh-frozen plasma (ml) | | 400 | 400 | 600 | 400 | 200 | 200 | 400 | 400 | 400 | 200 |
| Time post-LT, months | | 7 | 4 | 1 | 1 | 1 | 2 | 4 | 24 | 12 | 8 |
| AMR (Y/N) | | N | N | N | Y | Y | Y | Y | N | N | N |
| Type of DSA (MFI) | | DQ6 (18,045) | DQ5 (807) DQ6 (2,573) DR15 (761) | DQ7 (3,496) | DQ7 (15,527) | DR7 (6,351) DQ8 (13,707) | DQ4 (18,640) | A2 (2,463) DR7 (18,231) DQ8 (19,356) | DR7 (1,397) DR9 (1,365) | DQ7 (1,894) | DQ6 (1,297) |
| Immunosuppression | | Tac | Tac | Tac | Tac | Tac | Tac | Tac | Tac | Tac | Tac |
| Tacrolimus level in the presence of DSAs (ng/ml) | | 3.5 | 6.9 | 12.6 | 4.3 | 6.5 | 8.1 | 7.6 | 5.0 | 3.4 | 7.6 |
| Biochemical liver test in the presence of DSAs | | | | | | | | | | | |
| AST (U/l) | | 288.6 | 418 | 280.2 | 331.6 | 273.9 | 208.9 | 277.1 | 40.4 | 21.7 | 40.8 |
| ALT (U/l) | | 381 | 317.4 | 232.4 | 307.8 | 237.5 | 160.2 | 301.1 | 50.9 | 35.9 | 48.8 |
| ALP (U/l) | | 279 | 382 | 181 | 463.7 | 231 | 360.3 | 287.5 | 240 | 331 | 294 |
| Bilirubin (mg/dl) | | 5.4 | 223.7 | 13.38 | 207.2 | 234.8 | 174.9 | 156.4 | 14.9 | 3.5 | 3.58 |

ALC, autoimmune liver cirrhosis; ALT, alanine aminotransferase; ALP, alkaline phosphatase; AMR, antibody-mediated rejection; AS, Alagille syndrome; AST, aspartate aminotransferase; BA, biliary atresia; DCD, donation after cardiac death; DSA, donor-specific human leukocyte antigen antibodies; F, female; LT, liver transplantation; M, male; MFI, mean fluorescence intensity; N, no; Tac, tacrolimus; Y, yes.

Table III. Comparison of demographic and clinical parameters between pediatric transplant patient groups.

| Characteristics | DSA group | Non-DSA group | P-value |
|---|---|---|---|
| Number of patients | 10 | 38 | |
| Sex (male/female) | 5/5 | 22/16 | 0.6543 |
| Age at LT, mean ± SD (years) | 2.6±0.9 | 2.6±0.4 | 0.9961 |
| Donor age, mean ± SD (years) | 25±4.2 | 26±1.9 | 0.9321 |
| Donor type (DCD/Parental) | 3/7 | 10/28 | 0.8156 |
| Amount of blood transfusions during surgery | | | |
|   Erythrocytes (U) | 3.2 (2-4) | 2.9 (2-4) | 0.4745 |
|   Fresh-frozen plasma (ml) | 360 (200-400) | 340 (200-600) | 0.8329 |
| Number of transfusions, mean ± SD (n) | 0.9±0.32 | 0.74±0.45 | 0.0110 |
| Time post-LT, mean ± SD (months) | 7.4±4.5 | 17.5±7.8 | 0.0091 |
| Acute rejection (Y/N) | 6/4 | 3/35 | 0.0010 |
| Antibody-mediated rejection (Y/N) | 4/6 | 0/38 | 0.0010 |
| Tacrolimus level in the presence of DSA (ng/ml) | 6.6±0.9 | 3.9±0.3 | 0.0005 |
| Biochemical liver test at biopsy | | | |
|   ALT (U/l) | 227.4±46.0 | 43.0±7.5 | <0.0001 |
|   AST (U/l) | 198.1±36.4 | 43.6±3.8 | <0.0001 |
|   ALP (U/l) | 305.0±26.0 | 261.8±17.9 | 0.2684 |
|   Bilirubin (mg/dl) | 103.8±32.7 | 7.6±1.0 | <0.0001 |

ALC, autoimmune liver cirrhosis; AS, Alagille syndrome; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate amino-transferase; BA, biliary atresia; DCD, donation after cardiac death; DSA, donor-specific human leukocyte antigen antibodies; LT, liver transplantation; MFI, mean fluorescence intensity; N, no; Y, yes.

Table IV. Cox regression analysis of patient survival predictors.

| Variables | Multivariate analysis | |
|---|---|---|
| | 95% CI | P-value |
| DSAs | 0.182-0.952 | 0.038 |
| Patient age <2-years-old | 0.678-0.996 | 0.045 |
| Donor age <2-years-old | 0.967-1.042 | 0.837 |
| Donor type | 0.124-1.071 | 0.066 |

CI, confidence interval.

2 patients (20%) developed two DSAs and 2 patients (20%) developed three DSAs. The locus-specificity of DSAs was as follows: One (10%) against the A locus, 4 (40%) against the DR locus and 9 (90%) against the DQ locus. AMR occurred after pediatric liver transplant in in 4 of the 10 patients (40%). In patients with AMR, the mean MFI of HLA-DQ was 16,807 (range, 13,707-19,356) and the mean MFI of HLA-DR was 12,291 (range, 6,351-18,231). In patients with no AMR, the mean MFI of HLA-DQ was 5,461 (range, 1,297-18,045) and the mean MFI of HLA-DR was 1,079 (range, 761-1,397).

*Differences in various indexes in different pediatric liver transplantation groups.* According to the HLA classification of donors and recipients, and the DSA detection results, the recipients were divided into two groups: A DSA-positive group and a DSA-negative group. There was no significant difference in age, sex, donor age, donor type and intraoperative blood transfusion between the two groups (P>0.05). There were significant differences in the tacrolimus level in the presence of DSA, postoperative alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin and follow-up time (time post-LT) between the two groups (P<0.05; Table III).

*Impact of DSAs on patient and allograft survival.* The role of DSAs in predicting patient survival was then analyzed using Cox proportional hazard analysis (Table IV). The multivariate model identified DSAs and recipient age <2-years-old as independent predictors of patient death (P<0.05).

Liver biopsies were performed in 10 patients with abnormal liver function in the DSA-positive group (7 cases) and DSA-negative group (3 cases). Among 7 cases in the DSA-positive group, the pathological results of 4 cases indicated an antibody-mediated rejection, and 3 of the 4 cases were positive for C4d (Fig. 1). Two cases demonstrated T-cell-mediated rejection. The 3 cases in the DSA-negative group indicated a T-cell-mediated rejection. Kaplan-Meier survival analysis revealed that there was a significant difference in the survival of graft recipients between the DSA-positive and the DSA-negative group (Fig. 2; P<0.05).

*Treatment and outcome of AMR in DSA-positive recipients.* Of the 10 DSA-positive recipients, four had AMR. Liver function returned to normal after hormone therapy. All four patients with AMR were treated with MMF and 3 of the patients were treated with plasma exchange 1-3 times. During
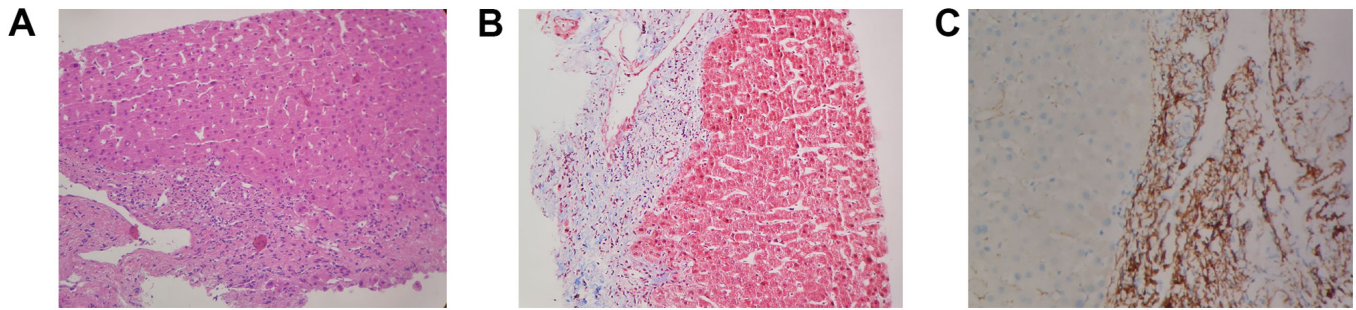
Figure 1. Pathological manifestations of antibody-mediated rejection in liver allografts. (A) Hematoxylin and eosin staining. Proliferation and expansion of small veins in the portal area, and lymphocyte and neutrophil infiltration were detected. Lymphocyte and neutrophil infiltration were also seen in the liver sinusoid. (B) Masson staining. Fibrous tissue was observed. (C) C4d staining. C4d positive expression was detected in the venule and hepatic sinusoid in the portal area.
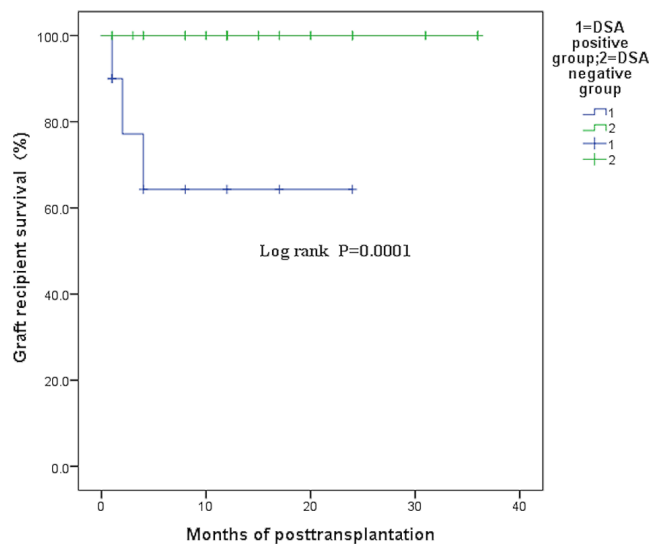


Figure 2. Graft recipient survival of the DSA-positive and the DSA-negative group. DSA, donor-specific human leukocyte antigen antibodies.

and after plasma exchange therapy, human immunoglobulin (intravenous immunoglobulin, 2.5 g/day) was administered intravenously. One patient was treated with bortezomib five times, and the other three patients were treated with rituximab once each. During treatment, except for the routine daily use of MMF, other treatment methods were carried out alternately. Among the patients with AMR, only one case was cured after treatment, and the other three cases finally underwent re-transplantation.

## Discussion

The liver is recognized as an immune-privileged organ (22); therefore, AMR is less common after liver transplantation (23). However, the humoral immunological mechanism of acute rejection after liver transplantation is consistent with that of other organ transplantations (24). A re-evaluation of the importance of DSAs in liver transplants was required, and the present study demonstrated a higher risk of rejection and decreased allograft survival in DSA-positive patients.

In the present study, it was observed that DSAs against HLA class II antigens were more common than those against class I antigens and represented 90% of all DSAs. The HLA-II antigen is dominated by the HLA-DQ site. It was observed that in patients with AMR, the mean value of HLA-DQ MFI was >15,000 and the mean value of HLA-DR MFI was >10,000. Therefore, a high MFI value was closely related to the occurrence of AMR. Previous research has demonstrated that the occurrence of AMR, bile duct complications and hepatic fibrosis in DSA-positive liver transplantation recipients is closely related to the characteristics, intensity and IgG classification of antibodies (25). In a retrospective cohort study, such as that by Kaneku et al (26), DSA analysis of serum samples before and after transplantation was performed in 749 adult liver transplant recipients. It was found that 8.1% of patients developed DSAs 1 year after transplant. Almost all DSAs were targeted at HLA II antigens, most of which were targeted at HLA-DQ antigens. O'Leary and Klintmalm (27) retrospectively analyzed 79 children with good graft function >5 years after transplantation. The donor specificity of antibodies was identified in 67 patients; specifically, DSAs were detected in 32 cases (48%). The DSAs were usually targeted at HLA class II (30 cases) and rarely against class I (2 cases). Although the liver has the ability to absorb pre-stored HLA class I antibodies, the persistence of HLA class II antibodies increases the incidence of acute and chronic rejection (27).

Another finding of the present study was that DSA-positive recipients exhibited abnormal liver function before AMR and exhibited abnormal elevation of serum transaminase, followed by hypercholanemia. O'Leary et al (28) retrospectively evaluated 1,270 cases of liver transplantation and demonstrated that immunosuppressant concentration and hormone dosage were closely related to rejection. Another study reported that the detection rate of DSAs in the non-tolerant group was 54%, mainly targeting HLA class II antigens (DR, 41%; DQ, 53%). The average levels of AST, ALT, total bilirubin and γ-glutamyltransferase were also higher in DSA-positive patients (19).

The formation of DSAs in the early stages after organ transplantation suggests a poor prognosis, but the pathogenesis between DSAs and rejection has not been determined. In other organ transplants, such as renal, complement C4d has been recognized as a sensitive index to predict rejection; however, the role of C4d in liver transplantation has been controversial (29).

Although it has been reported that C4d serves an important role in the prediction of rejection in DSA-positive cases, it is highly specific but not highly sensitive. Some research has shown that acute rejection after liver transplantation is closely related to the accumulation of vascular endothelial cells in the portal area. However, Ali *et al* (30) revealed that positive staining of C4d can be found in different liver lesions, i.e., acute cell rejection (52%), chronic catheterization rejection (50%), recurrent liver disease (48%), preservation injury (18%) and liver necrosis (54%). Furthermore, C4d positivity was rarely found in ABO blood group-compatible liver transplantation, but it was closely related to liver fibrosis after transplant. Musat *et al* (15) performed liver biopsies in 43 patients with acute or chronic rejection after liver transplantation. There was a significant positive association between C4d deposition and DSA intensity (MFI). The incidence of chronic rejection among DQ-DSA-positive patients was higher than that among DQ-DSA-negative patients. The higher the MFI value of DSAs, the higher the risk of chronic rejection. Del Bello *et al* (31) performed a retrospective analysis of 152 patients with liver transplantation and demonstrated that 21 patients (14%) developed DSAs, including 5 patients with C1q-DSA (24%) and 9 patients (43%) with AMR. The positive rate of C4d staining was higher in liver biopsies from patients with AMR (P<0.0001). In the present study, liver biopsies were performed in 10 patients with abnormal liver function in the DSA-positive group. The pathological results of four cases revealed that the cellular rejection was grade 0, and three cases were positive for C4d, suggesting that systemic fluid rejection should be further assessed. C1q-DSA should also be further assessed. In recent years, additional knowledge on complement C1q has been obtained (32). It has been revealed that the damage caused by DSA was related to its own ability to bind C1q. C1q-DSA is considered to exhibit potential cytotoxicity and has been associated with acute rejection and graft loss (33).

In the present study, all the patients with AMR were treated with MMF, and 3 of them were treated with plasma exchange. During and after plasma exchange therapy, human immunoglobulin was administered intravenously. One patient was treated with bortezomib, and the other 3 patients were treated with rituximab. Among the patients with AMR, only one case was cured after treatment, and the other three cases finally underwent re-transplantation. DSAs are closely related to rejection after liver transplantation. Common treatments for abnormal liver function caused by DSAs include plasma exchange, immunoglobulin, rituximab and bortezomib. Bortezomib is used to treat AMR; however, considering the increased risk of viral hepatitis and other infections, the pros and cons should be assessed prior to treatment (34-36). In a study by Paterno *et al* (13), bortezomib was used to treat three ABO-compatible liver transplant recipients with AMR. These patients developed a severe, acute rejection of steroids and antithymocyte globulin resistance; they also had histological evidence of plasma cell infiltration. After treatment with bortezomib, the liver function of all patients with C4d positivity and high DSA levels improved or was normal, the deposition of C4d disappeared and the level of DSAs decreased significantly.

The present study had some limitations. Firstly, HLA antibody analysis in pediatric liver transplants is not a routine test for all patients before transplant. Thus, the authors were unable to determine whether the detected antibodies in patients were preformed or *de novo*. Secondly, the sample size in the present study was small; more samples need to be collected to further analyze the relationship between DSAs and graft loss in patients. Thirdly, the follow-up time of pediatric liver transplants needs to be extended to study the 5-year graft survival rate of children. Finally, more clinical data and practical experience should be accumulated in the diagnosis and treatment of AMR after liver transplantation in children. When the diagnosis of AMR is unknown or highly suspected, liver biopsies and DSA tests should be performed in a timely manner, and C4d, IgG subclass, C1q and C3d should be assessed at the same time (37). In conclusion, regular monitoring of DSA levels may have an important role in predicting graft survival and DSA treatment choice.

## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

DHL conceived the study. WL performed the research, analyzed the data and wrote the first draft of the manuscript. KW collected the clinical data and revised the draft. YLX and CL performed the measurement of DSA and HLA typing. WG screened the clinical data. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

Written informed consent was obtained from the patients' parents/guardians. The present study was approved by the Medical Ethics Committee of Tianjin First Central Hospital (approval no. 2015017S).

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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