



Title	Impact of Donor-Specific Antibodies on Graft Fibrosis after Pediatric Living Donor Liver Transplantation for Biliary Atresia
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Citation	Transplantation Proceedings. 2016, 48(4), p. 1095-1099
Version Type	AM
URL	<a href="https://hdl.handle.net/11094/98564">https://hdl.handle.net/11094/98564</a>
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# **Impact of Donor-Specific Antibodies on Graft Fibrosis after Pediatric Living Donor Liver Transplantation for Biliary Atresia**

## **Introduction**

Liver transplantation has become the definitive treatment for children with end-stage liver disease. It is now important to focus on improving long-term outcomes for pediatric transplant recipients. The major indication for liver transplantation among pediatric patients is biliary atresia. This condition is characterized by the obliteration or discontinuity of the extrahepatic biliary system, sometimes resulting in hepatic fibrosis. Although biliary atresia is essentially a non-recurrent disease, pathological changes are sometimes detected even when blood tests are within the normal range. Therefore, a liver biopsy is an essential follow-up procedure to identify pathological changes in patients after living donor liver transplant (LDLT). We have shown that most patients with biliary atresia develop graft fibrosis after pediatric LDLT. Patients with biliary atresia after LDLT can develop biopsy-proven fibrosis, even in the context of normal liver function as demonstrated by blood tests [1].

Donor-specific antibodies (DSA) have recently been shown to play a potential role in graft damage after liver transplantation. Patel et al. revealed the importance of cross-matching in renal transplants in 1969 [2], and DSA testing is now required before

kidney transplantation. There is increasing evidence that the presence of DSA may impact long-term graft survival [3]. The presence of DSA is associated with poorer graft outcomes in kidney and cardiac transplantation [4]. A recent study assessed the effects of positive cross-matching and the presence of DSA on allograft function in LT, including influence on graft survival [5]. However, the role of DSA in the pediatric population is poorly understood. The relationship of progressive graft fibrosis and DSA in pediatric liver grafts has been reported in various original disease[6]. In particular, maternal chimerism may play a role in the pathogenesis of biliary atresia [7]. Auto immune mechanism may be partly responsible. The effect of DSA in biliary atresia is interesting. Biliary atresia is essentially a non-recurrent disease, different from major adult disease like hepatitis. Therefore we studied pathological findings to figure out pathogenesis of graft fibrosis for biliary atresia patients.

At our institution, we perform protocol biopsies even if patients have normal liver function tests. The aim of this study was to evaluate the pathological findings and DSA associated with fibrosis after LDLT for biliary atresia and to demonstrate the role and importance of DSA after pediatric LDLT.

## **Methods**

### *Patients*

Patients under 18 years old who received LDLT at our institution from 1998 to 2009 were identified. The target population was patients who underwent liver transplantation due to biliary atresia and who were followed for at least 5 years after LDLT. Protocol liver biopsies were performed every 2 or 3 years in all patients, even those with normal liver function tests. Patients who complied poorly with medications or scheduled clinic visits were excluded from this study. In total, 23 patients were enrolled. Patients were divided in two groups, DSA positive and DSA negative. Graft fibrosis after LDLT were assessed according to DSA groups.

### *Immunosuppression*

All patients received steroids and standard tacrolimus-based immunosuppression. The standard protocol for tacrolimus tapering was as follows: the tacrolimus trough level was 10–15 ng/ml for the first month, 5–10 ng/ml for one year, and then 3–5 ng/ml thereafter. Methylprednisolone was given as a bolus dose (20 mg/kg) at the time of transplantation, and then fully tapered by 4 months after transplantation. Prednisolone was continued in patients who had a biopsy-proven acute cellular rejection episode or post-transplant hepatitis. No

patients were withdrawn from immunosuppressive therapy.

#### *Donor-specific antigen assessment*

Post-transplant DSA were measured by single antigen beads with a Luminex assay at least 5 years after LDLT. The DSA assay was performed by HLA Laboratory (Kyoto, Japan). Results were expressed as the mean fluorescence intensity (MFI). A normalized MFI greater than 1000 after LDLT was defined as the threshold for a positive value, based on antibody reactivity against HLA molecules bound to Luminex beads. Pre-transplant DSA were evaluated by cross-matching.

#### *Histological assessment*

The last available biopsies were assessed with hematoxylin–eosin (HE) and Masson’s trichrome (MT) stains. Liver biopsies were obtained percutaneously with a 16-gauge biopsy needle. The specimens were fixed in 4% phosphate-buffered formaldehyde and embedded in paraffin. After hematoxylin–eosin staining and Masson’s trichrome staining, the liver specimens were examined microscopically. The degree of liver fibrosis was assessed based on the Metavir scoring system[8]. Chronic allograft rejection was defined according to the Banff criteria [3].

#### *Statistical analysis*

Data were analyzed using the JMP Ver.11 software package (SAS, USA). Pearson's chi-squared test or Fisher's exact test was used for categorical variables. A *P* value less than 0.05 was considered statistically significant.

## **Results**

### *Patient profiles*

All 23 patients (18 females, 5 males) received related LDLT. Their mean age at the time of transplant was 2.6 years. The follow-up period ranged from 5.4 to 16.9 years (mean 9.7 years). The mean time from transplantation to the latest biopsy was 8.2 years. Donors were fathers (*n* = 15) and mothers (*n* = 8).

### *Pathological findings*

Typical hematoxylin–eosin-stained biopsy specimens of graft fibrosis (F1 and F2) are shown in **Fig. 1**. The pathological results of the last available biopsies are shown in **Fig. 2**. Among the last available biopsies from 23 patients, 8 (35%), 8 (35%), and 1 (4%) showed portal fibrosis of grades F1, F2, and F3, respectively. There was no cirrhosis (F4). There were no findings to suspect acute or chronic rejection in any of the examined patients.

### *Correlation between post-transplant DSA and fibrosis progression*

**Fig. 3** shows the profiles of the latest DSA and its class. DSA were observed in 12

patients (52%). Most of DSA were class2. Patient demographic data of ~~two~~ the DSA positive and negative groups are shown in **Table 1**. **Although there were no differences in age at transplant, age of donor, or sex of donor between the two groups, male patient was more common in DAS negative group.** Fig 4 shows the relation of DSA to the presence and grades of fibrosis. Moderate graft fibrosis (F2 and F3) was found in 7 (58%) of DSA-positive patients, whereas only 2 (18%) DSA-negative patients developed moderate fibrosis, indicating a stastically significant difference ( $P=0.049$ ).

#### *Correlation between pre-transplant DSA and fibrosis progression*

Results of pre-transplant cross-matching and presence of post-transplant DSA are shown in **Fig. 5**. Pre-transplant cross-matching was performed in 19 patients, 4 of whom demonstrated positive results. These 4 patients were DSA positive after LDLT. Six cross-match-negative patients developed *de novo* DSA after LDLT. Two (50%) patients with preformed DSA had severe fibrosis (50%), compared to 3 with *de novo* DSA. There was no difference between preformed and *de novo* DSA in terms of graft fibrosis.

#### *Correlation between donor and graft fibrosis*

Maternal graft developed moderate graft fibrosis in 5 (62.5%) out of 8 patient. Paternal graft developed moderate graft fibrosis 4 (26.6%) out of 15 patients. Maternal grafts

tended to develop fibrosis, with an odds ratio of 4.58. However, the chi-squared test yielded a *P* value of 0.09, indicating no statistical significance.

## **Discussion**

Follow-up biopsies for adult liver graft patients were previously associated with a high frequency of histological abnormalities [9]. In adults, these abnormalities were mainly related to recurrent primary disease. However, biliary atresia, which is the major indication for LDLT in Japan, is not considered a recurrent disease.

There are only limited data on the long-term histological status of grafts after pediatric liver transplantation for biliary atresia. Some studies reported portal fibrosis in pediatric liver transplant biopsies [10-12]. Scheenstra et al. [13] reported that the prevalence of fibrosis increased from 31% to 65% (*n* = 66) from 1 to 5 years after liver transplantation, but was stable thereafter (69% at 10 years, *n* = 55). However, the percentage of patients with severe fibrosis increased from 10% at 5 years after liver transplantation to 29% at 10 years. Among the 69% of children who did not have fibrosis at 1 year post-transplant, 64% (*n* = 39) developed some degree of fibrosis at 10 years post-transplant. We previously reported that 71% of patients showed some degree of fibrosis at the 5-year follow-up exam, but there was



no severe fibrosis [1]. In this study, we showed that graft fibrosis was highly prevalent in pediatric LDLT patients.

We evaluated the relationship between histological results and DSA during the follow-up period. Liver transplants are considered to be less affected by humoral rejection than other organs [14]. However, DSA may have a detrimental effect on outcomes after liver transplants [15]. DSA affect acute and chronic rejection rates [5]. Grabhorn et al. reported that 68% of patients with histological signs of chronic rejection and the need for re-transplantation exhibited high levels of DSA [16].

At present, minimal data are available regarding the role of DSA in pediatric liver transplant graft fibrosis. Pediatric studies have demonstrated an association between DSA and the development of graft fibrosis. Miyagawa-Hayashino et al. evaluated 79 pediatric liver transplant recipients with protocol liver biopsies, and detected DSA in 32 individuals (48%). These patients had a higher frequency of bridging fibrosis or cirrhosis (88%) than DSA-negative patients (17%) [6]. Yamada et al. performed an immunohistochemical study of 28 pediatric patients who had undergone LDLT. Their results suggest that DSA also influence the development of pericentral fibrosis [17]. Girnita et al. determined DSA levels in liver transplant recipients who had been weaned off immunosuppression. Clinically tolerant

children and adults lacked DSA, whereas the prevalence of DSA was significantly higher in nontolerant patients [18]. Our current study identified DSA-positive patients at a higher rate than in other studies. Compared to previous reports, we used a rather low threshold MFI of 1000 after LDLT. Perhaps due to a shorter observation period, we observed lower rates of fibrosis progression. Furthermore, we found more *de novo* DSA than preformed DSA, but did not evaluate preformed DSA with cross-matching. Results using Luminex with single beads should be accumulated for preformed DSA.

The presence of maternal cells in offspring may promote tolerance to maternal antigens. Children with biliary atresia have increased proportions of maternal cells in their livers, which may impact tolerance. Nijagal et al. reviewed all pediatric liver transplants recorded in the Scientific Registry of Transplant Recipients database from 1996 to 2010, and compared patients with and without biliary atresia who received maternal livers versus paternal livers in terms of the incidences of graft failure and re-transplantation. Biliary atresia patients receiving maternal grafts had lower rates of graft failure compared to those receiving paternal grafts (3.7% vs. 10.5%,  $P = 0.02$ ), and consequently, had fewer episodes of re-transplantation (2.7% vs. 7.5%,  $P = 0.04$ ). In biliary atresia patients only, paternal liver transplantation was associated with an increased incidence of refractory rejection compared

to maternal liver transplantation [7]. Our data showed that maternal grafts tended to develop fibrosis though the difference compared to paternal grafts was not statistically significant. More cases are needed to determine the effects of maternal grafts.

In our study, all patients remained on immunosuppressive therapy with a calcineurin inhibitor. Many patients nonetheless developed fibrosis, even those with normal graft function. Therefore, we believe that therapy with low doses of immunosuppressive drugs is necessary for all patients in order to prevent graft fibrosis progression, although some patients may discontinue the immunosuppressive therapy. In a study of heart, lung, kidney, and liver transplant recipients, Perbos et al. analyzed the incidence of rejection and the evolution of HLA antibodies and *de novo* DSA after patients switched to everolimus [19]. They found no statistical differences in rejection or in the evolution of preformed anti-HLA antibodies or *de novo* DSA after conversion to everolimus. The ability of a superior new immunosuppression regimen other than or adding to tacrolimus is being evaluated in terms of preventing DSA formation and fibrosis. In our program, if patients show graft fibrosis, tacrolimus levels are raised to the 3–5 ng/ml range, and low-dose maintenance steroids at an approximate dose of 1 mg/day is restarted. The results of these interventions will be reported in the future.

The normal life expectancy of a child receiving a liver transplant is still unknown.

We hope that children will survive for at least 50 to 60 years with the same graft. However, the progression of fibrosis to cirrhosis may endanger long-term graft function and graft survival. This complication is critical since it is difficult for LDLT patients to undergo re-transplantation due to limited donor availability. Progressive fibrosis with a gradual decline in liver function threatens the potential for long-term graft survival and must be carefully studied.

## **Conclusions**

Graft fibrosis was observed after LDLT for biliary atresia in long-term follow-up. DSA were found in patients after LDLT, and these individuals tended to develop graft fibrosis. DSA may play a role in fibrosis formation. Further follow-up is required to determine the relationship between fibrosis progression and DSA.

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