Title	Application of complement component 4d immunohistochemistry to ABO-compatible and ABO-incompatible liver transplantation.
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Citation	Liver transplantation (2014), 20(2): 200-209
Issue Date	2014-01-27
URL	http://hdl.handle.net/2433/196634
Right	This is the accepted version of the following article: Salah, A., Fujimoto, M., Yoshizawa, A., Yurugi, K., Miyagawa-Hayashino, A., Sumiyoshi, S., Minamiguchi, S., Uemoto, S., Maekawa, T. and Haga, H. (2014), Application of complement component 4d immunohistochemistry to ABO-compatible and ABO-incompatible liver transplantation. Liver Transpl, 20: 200–209. doi: 10.1002/lt.23789, which has been published in final form at http://onlinelibrary.wiley.com/doi/10.1002/lt.23789/epdf
Type	Journal Article
Textversion	author

Application of C4d Immunohistochemistry in ABO-Compatible and ABO-Incompatible Liver Transplantations

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Short title: C4d immunohistochemistry in liver allografts

Key words:

accommodation

antibody-mediated rejection

donor-specific antibody

human leukocyte antigen

graft fibrosis

Abbreviations:

AMR; antibody-mediated rejection

ABO-C; ABO-compatible or ABO-identical

ABO-I; ABO-incompatible

ACR; acute cellular rejection

C4d; complement component 4d

DSA; donor-specific anti-HLA antibody

HCV; hepatitis C virus

HLA; human leukocyte antigen

LT; liver transplantation

POD; postoperative day

Grant support: This project was supported by a Grant-in-Aid from the Japan Society for the Promotion of Science (to Aya Miyagawa-Hayashino and Atsushi Yoshizawa)

Disclosures:

The authors of this manuscript have no conflicts of interest to disclose.

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Abstract (271 words)

Antibody-mediated rejection (AMR) is difficult to diagnose after ABO-compatible or ABO-identical (ABO-C) liver transplantation. To confirm whether C4d immunostaining is useful for diagnosing AMR, we compared the results of C4d immunohistochemistry in allograft biopsies with assays for anti-donor antibodies performed at the time of biopsies. A total of 114 patients with ABO-C grafts and 29 patients with ABO-incompatible (ABO-I) grafts were included. Linear C4d endothelial staining identifiable by x4 objective lens or staining seen in >50% of portal tracts was considered positive.

Five of 114 (4%) patients with ABO-C and 15 of 29 (52%) patients with ABO-I showed C4d positivity. In ABO-C cases, C4d positivity was associated with ≥stage 2 fibrosis (METAVIR score) and presence of donor-specific anti-HLA DR antibodies (HLA-DR DSA) with more than 5000 mean fluorescence intensity (MFI) by the Luminex single antigen bead assay in late (≥30 days posttransplantation) biopsies (p=0.01 and 0.04, respectively). Conversely, presence of HLA-DR DSA was associated with presence of ≥stage 2 fibrosis, acute cellular rejection, and C4d positivity. During two-year follow-up, neither C4d positivity nor HLA-DR DSA was related to graft loss. In ABO-I, C4d positivity was not associated with allograft dysfunction or fibrosis. Only three of 15 (20%) C4d-positive patients showed periportal hemorrhagic edema, which could be a histological sign of AMR in ABO-I, and were the only cases associated with elevations in anti-donor A/B antibody titers. In conclusion, C4d endothelial positivity in ABO-C was an uncommon event that could be associated with chronic graft damage with or without clinical AMR. C4d positivity is common in ABO-I grafts, and may not be associated with allograft dysfunction if alloantibody titers are not elevated.

Antibody-mediated rejection (AMR) in liver allografts is recognized as a possible cause of early and late allograft injury, and poor prognosis (1-8). However, unlike acute cellular or chronic rejection, the diagnosis of AMR in liver allografts is often difficult to establish. One of the main reasons for this is due to the difficulty in interpreting C4d deposition, which is the most widely used marker of clinical AMR in renal, cardiac, and pancreatic transplantations (8-11).

The specificity of C4d staining in liver allografts is controversial. Ali S et al. (3) and Lunz J et al. (4) correlated diffuse portal tract vascular endothelial C4d deposition with AMR. However, C4d positivity was also reported in other medical conditions, such as acute cellular rejection (ACR) (1,3-5,12,13), chronic rejection (CR) (3,5,12,13), ischemic injury (1,3,12), hepatitis (1,3,4,14) and cholangitis (1,3,4). Unfortunately, most of these previous studies performed C4d staining on non-consecutive biopsies from unstable liver grafts (15). A more comprehensive study is required to understand the significance of C4d and its utility in AMR, in combination with tests for alloantibodies.

In addition, sites of C4d deposition differ between and within these reports, including portal vessels (1-6,12,14), portal stroma (1,2,5), and sinusoids (3,4,5,12). The lack of agreement in staining patterns may also be related to the low specificity of C4d for AMR and may prevent clinicians and pathologists from using C4d in the routine histological diagnosis of liver allografts.

Kozlowski et al. (7) recently suggested that strong linear staining in the sinusoid, rather than the portal tract, was a better marker for AMR and recommended the use of immunofluorescence on frozen sections. As they pointed out, immunoperoxidase staining is insensitive and frozen sections may be a better tool to demonstrate C4d deposition. However, frozen sections are not suitable for conventional histological evaluations, and formalin-fixed paraffin embedded tissue is additionally required. Considering the rarity

of clinical AMR in liver transplantation (LT), we suggest that establishing a method to evaluate C4d by immunoperoxidase alone may be practical.

Here, we designed a non-selective prospective study in which we performed C4d staining on all liver allograft biopsies obtained over four consecutive months, and every clinically indicated biopsy was included in this study. The presence of anti-blood group (Anti-A/B) antibodies or anti-human leukocyte antigen (anti-HLA) antibodies was evaluated during the same period. All patients were followed up for 2 years to clarify the significance of C4d in liver allografts. We adopted endothelial staining for this study although we previously reported the stromal deposition of C4d as an ominous sign of ABO-I LT (2). The main reason to exclude stromal staining in this study was that only endothelial staining has been used as the standard in other solid organ transplantations (16). The second reason is that stromal staining alone is often difficult to differentiate from the non-specific staining seen in elastic fibers or necrotic tissue (1, 17). When we picked up every portal stromal or endothelial staining, C4d staining was seen in various types of liver allograft injuries and did not show clinical significance (1). Since extensive C4d staining covers the endothelia of portal, sinusoidal, and perivenular areas (1, 2), we now assume that endothelial staining alone is adequate for evaluating C4d.

Materials and methods

Study population and biopsies

In a prospective and non-selective manner, regardless of indication, we studied all liver allograft biopsies obtained between July and October 2011 at Kyoto University Hospital. Patients who underwent LT outside Kyoto University hospital were not included. Liver allograft biopsies were performed to determine allograft dysfunction or evaluate graft fibrosis when immunosuppression weaning was intended. If a patient underwent multiple biopsies during this period, the first biopsy that showed C4d positivity was

selected for analysis. When all biopsies were negative for C4d, the first biopsy was selected. In each case, the biopsy specimen for analysis was classified as early (taken within 30 days after transplantation) or late (taken 30 days or more after transplantation). All patients were followed up until July 2013. Clinical and serological data were obtained from electronic patient charts. The Institutional Review Board of Kyoto University approved this study.

Immunosuppression

The baseline immunosuppression protocol consisted of tacrolimus and oral prednisolone in both ABO-C and ABO-I patients. The lower limit of the target for whole blood tacrolimus levels was 10 to 15 ng/mL during the first 2 weeks, 10 ng/mL thereafter, and 5 to 8 ng/mL from the second month on. Acute rejection was treated by a 3-day course of intravenous methylprednisolone bolus therapy (10 mg/kg). Mycophenolate mophetil was administered to patients who underwent refractory rejection or plasma cell hepatitis simulating autoimmune hepatitis. Immunosuppression was weaned in selected pediatric patients, according to the previously described protocol (18). All ABO-I patients underwent preoperative plasmapheresis or blood exchange in order to reduce antidonor A/B antibodies to 1:8 or lower. In addition, patients who underwent ABO-I transplantation after 2006 received rituximab (anti-CD 20 monoclonal antibody) approximately two weeks before transplantation (19). Adult patients were given prostaglandin E1 and methylprednisolone via a portal vein or hepatic artery. Clinical AMR, consisted of an elevation in postoperative anti-donor A/B antibody titers and graft dysfunction, was treated for about 5 days by plasmapheresis or intravenous immunoglobulin, with steroid bolus therapy.

Histopathology

Liver allograft biopsies were processed for routine light microscopy. Biopsy specimens were fixed in 10% buffered formalin, sliced 3 μm thick, and stained with hematoxylin and eosin (H&E), Masson Trichrome, and Cytokeratin 7 (clone OV-TL 12/30, Dako, Glostrup, Denmark; dilution 1:200).

ACR and chronic rejection were diagnosed according to Banff criteria (20, 21). AMR was diagnosed according to the criteria used in other solid organ transplantations; i) clinical evidence of graft dysfunction, ii) histologic evidence of graft injury, iii) immunopathologic evidence of antibody action (C4d deposition), and iv) serologic evidence of anti-HLA or anti-donor antibodies at time of the biopsy (22). A combination of periportal edema, hemorrhage, and neutrophilic infiltration was regarded as an indicator of AMR in ABO-I patients (8,23). Allograft fibrosis was staged according to the METAVIR scoring system (24).

C4d immunohistochemical staining

A rabbit polyclonal anti-human C4d antibody (BIOMEDIA, Bl-RC4D, 1:50 dilution) was used to detect C4d. Staining was performed on an autostainer machine (Ventana Benchmark ULTRA). Sections were treated with protease (Ventana, 0.5 U/mL) at 37 °C for 20 minutes for antigen retrieval. C4d immunostaining using formalin-fixed, paraffin embedded tissue was available in our laboratory since August 2003, but it was applied only to selected cases and was not used routinely before this study.

C4d interpretation

Staining was recorded as diffuse when linear C4d deposition in the portal tract vascular endothelium was seen in 50% or more of portal tracts. Staining of fewer than 50% of portal tracts was considered focal. We also evaluated the intensity of staining, which was

recorded as strong when linear C4d deposition was seen on low power magnification (x4 objective lens), and weak when staining was confirmed only on higher magnification. Completely negative (Score 0) or focally weak (Score 1) staining was considered negative and equivocal, respectively. Diffuse or strong (Score 2) as well as diffuse and strong (Score 3) staining was considered positive for statistical analysis. Staining in hepatocytes, portal stroma, and elastic fibers was recorded but not included for statistical analysis. All stained slides were interpreted by M. F. and H. H. without clinical data.

Assays for alloantibodies

The lymphocyte cross-match test was only conducted before transplantation (25). After LT, the anti-HLA antibody titer was analyzed using Luminex multiplex technology at the time of the biopsy. The specificity of positive tests was determined using the LABScreen Single Antigen test (LABScreen Mixed and LABScreen Single Antigen, One Lambda, Canoga Park, CA) and the results were displayed as mean fluorescence intensity (MFI). MFI of more than 5000 was regarded as positive (13). The anti-HLA antibody was then compared with the patient's HLA type to decide whether it was a donor-specific antigen (DSA) or non-DSA.

In ABO-I cases, serum levels of anti-A/B antibodies were evaluated before and after LT using the microhemagglutination assay. This test was conducted at least 3 times per week during the first postoperative month. A postoperative anti-donor blood group immunoglobulin M titer of 1:32 or more was defined as an elevated titer.

Statistical analysis

Associations between categorical variables were assessed using Fisher's exact test. Descriptive statistical methods (mean, median, standard deviation, range) as well as the

Mann-Whitney U test were used to assess the distribution of variables. For all analyses, a P value of less than 0.05 was regarded as significant.

Results

Patient Characteristics

A total of 219 biopsies obtained from 163 patients (range: 1 to 9 per patient) during this study period. After excluding 20 ABO-C patients whose Luminex assays for anti-HLA antibodies were not available at the time of index biopsy, 143 patients with a total of 194 biopsies were enrolled in this study. Seven ABO-I patients who underwent isoagglutinin tests but not Luminex assays were not eliminated.

The demographic of patients is summarized in Table 1. Most patients (98%) underwent living donor LT. The most common indications for transplantation in pediatric and adult groups were biliary atresia and chronic hepatitis C, respectively. In the ABO-C group, there were 114 patients and had a higher percentage of children (being less than 18 years old, 74% vs. 38%) and most (91%) of their index biopsy were taken more than 30 days after transplantation. In the ABO-I group, there were 29 patients, and acute cellular rejection, C4d positivity, and graft loss were more commonly seen than the ABO-C group. All patients were lymphocyte cross-match negative before transplantation. No significant difference was observed in the percentage of positivity for anti-HLA-DSA antibodies between the ABO-C group and the ABO-I group. We also checked the data using cut-off point of 1000 MFI and there was no difference between the two groups (data not shown). The distribution of HLA-DSA by class among patients was as follows: 1 class I, 36 class II, and 3 class I and II. Among 39 patients with anti-class II antibodies, antibodies against DR loci were most commonly observed (n = 27, 69%). Among 96 HLA-DSA-negative patients, 22 showed non-donor-specific HLA antibody (>1000 MFI), 7 weak class II

(>1000 and ≤5000 MFI against donor DR locus), 2 weak class I, and 65 patients were completely negative for anti-HLA antibody.

Three ABO-C patient and 6 ABO-I patients died during the follow-up period, and none of them showed positivity for the anti-HLA antibody or high anti-A/B antibody titers. For 2 ABO-I patients, data of Luminex assays were not performed before death. All the ABO-C patients were negative for C4d, while five of the six (83%) ABO-I showed C4d positivity. Four patients died of severe bacterial or fungal infection within six months after LT. The other five died of severe acute cellular rejection (7 months after LT), graft-versus-host disease (14 months after LT), fibrosing cholestatic hepatitis C (15 months after LT), ischemic cholangiopathy after rupture of the hepatic artery (6 years after LT), and cirrhosis due to de novo autoimmune hepatitis (14 years after LT), respectively.

Characteristics of C4d-positive cases in ABO-compatible or identical transplantation

Table 2 lists the clinical and histological characteristics of 20 patients exhibiting C4d positivity at index biopsy. In early biopsies of the ABO-C group, only one of 10 patients was positive for C4d (Case C1) and statistical analysis was not suitable for this subgroup (Table 3). The previous biopsy of C1 (POD 7) showing moderate degree of acute cellular rejection was also C4d-positive but was out of this study period.

In late biopsies of the ABO-C group, C4d immunoreactivity was significantly correlated with graft bridging fibrosis (P = 0.01) but not with histology of acute cellular rejection, levels of serum transaminases or total bilirubin (Table 3). Although positivity for anti-DSA antibody itself was not statistically associated with C4d positivity, presence of DSA against DR loci was correlated with C4d status (P=0.04). Inclusion of anti-HLA-DQ antibody status made the difference statistically insignificant (data not shown). When late biopsies were divided in terms of donor-specific anti-HLA-DR antibody, Presence of donor-specific anti-HLA-DR antibody was significantly associated with fibrosis, acute

cellular rejection and C4d score but not with levels of serum transaminase or total bilirubin (Table 4).

C4d-positive cases in late biopsy included heterogeneous histology with various possible causes of fibrosis (C2, C3, C4, C5 in Table 2); C2 and C3 were pediatric protocol biopsies with minimal or no inflammatory cell infiltration, and C4d positivity was thought be related to suboptimal immunosuppression.

Case C4 was obtained from a patient whose recurrent hepatitis C was treated with interferon since 7 months after LT at stage 1 fibrosis. Although sustained virus response was achieved, the biopsy taken five years after LT revealed progression of fibrosis and focal ductopenia (Figure 1A and 1B). This patient was found to have low titer of anti-nuclear antibody, but histology was different from autoimmune hepatitis and compatible with chronic cholangiopathy (Figure 1C). There was a history of biliary anastomotic stricture 2 year after LT and the patient underwent a successful removal of biliary casts. Diffuse C4d staining was noted in the fibrous portal tracts (Figure 1D), and C4d positivity persisted in the biopsy taken a year after this study period. Another patient with a history of recurrent hepatitis C, Case C5, was DSA-negative at the time of index biopsy with interferon therapy. When follow-up biopsy was done after cessation of unsuccessful interferon therapy, C4d became negative (Table 2).

Although no patient was diagnosed with clinical AMR in the patients with ABO-C LT in this study period, one patient was revealed to have persistent graft dysfunction along with persistent DSA, and a history of sporadic C4d staining. Before transplantation, the lymphocyte cross-match test was negative and the Luminex test was not available. Three allograft biopsies within three months posttransplantation showed acute cellular rejection and C4d staining was negative each time. In spite of the long-term use of triple immunosuppressants (tacrolimus, prednisolone, and mycophenolate mofetil), graft dysfunction persisted and histological diagnosis after 6 months was mild acute cellular

rejection with perivenular hemorrhage (Figure 2A). Diffuse endothelial C4d staining with some stromal staining was seen in biopsies taken at postoperative day (POD) 185, 192, and 227 (Figure 2B). The Luminex test revealed DSA at POD 229 (B59, 3932; DR4, 15840; DR53; 8061; DQ4, 4747). During this study period (POD 524), portal inflammation was mild (Figure 2C) and C4d staining was faint and considered negative (Figure 2D). DSA remained positive (B59, 3434; DR4, 12318; DR53, 2444) and portal and perivenular fibrosis progressed (Figure 2E). Serum bilirubin levels remained at 2 to 3 mg/dL. On the last follow-up biopsy taken at POD 986, DSA remained positive (B59, 4509; DR4, 6458; DR53, 23557; DQ4, 23738) with persistent fibrosis and ductular reaction. Bile duct loss was not observed. C4d endothelial staining returned (Figure 2F).

Characteristics of C4d-positive cases in ABO-incompatible transplantation

In both early and late biopsies, C4d status in ABO-I LT was not statistically associated with any clinical parameters possibly related to rejection (Table 5). The majority of C4d-positive patients did not show postoperative elevations in anti-donor A/B antibody titers in spite of C4d endothelial staining (I1-15, Table 2). Only 3 patients (I1, I8, and I12) showed anti-A/B antibody titer elevations, and they were the only patients that fulfilled the criteria for AMR: 1) detectable anti-donor antibody (1:32 or more anti A/B antibody with or without the presence of an anti-HLA antibody), 2) C4d in the graft endothelium, 3) graft pathology, and 4) graft dysfunction. These three patients showed typical ABO-I-associated injuries, characterized by portal edema and hemorrhage, with foci of necrosis (Figure 3A). Sinusoidal C4d staining was also observed in Case I8 (Figure 3B). All ABO-I AMR cases responded well to steroid pulse therapy with or without plasmapheresis and immunoglobulin bolus administration. The level of isoagglutinin decreased to 1:4 or lower after therapy for AMR. Follow-up biopsies showed diffuse C4d

positivity in Case I8, equivocal (score 1) staining in Case I1, and complete negativity in Case I12 (Table 5) 59, 390, and 169 days after index biopsies, respectively.

All C4d staining in ABO-I LT tended to fade in the follow-up biopsies. Only in 3 of 11 last follow-up biopsies, C4d scores remained the same as those of index biopsy (I1, I6, and I15, Table 5).

Discussion

This study showed that C4d positivity without an elevation in anti-donor A/B antibodies was not uncommon among patients with ABO-I LT. Before the use of rituximab, we observed that postoperative isoagglutinin titer elevations were often associated with fatal AMR, which was characterized by periportal edema, necrosis, and hemorrhage (2, 23). C4d deposition was commonly seen in portal stroma as well as the endothelium. In contrast, all ABO-I transplant recipients in this study underwent planned preoperative intravenous rituximab administration as well as plasmapheresis or blood exchange. As a result, most of the C4d-positive ABO-I cases had low Anti-A/B antibody titers at the time of biopsy and did not show histological evidence of critical graft injury. This is partly similar to the findings in ABO-I kidney allografts by Haas M et al. (26). The reason for this result may also be explained by considering the liver's ability to absorb, eliminate, and neutralize antibodies. Mild alloantibody reactions may cause C4d deposition, but not significant allograft injury (8, 27). Another possibility is the presence of the accommodation phenomenon. In ABO-I renal allografts, graft resistance to the acute pathological effects of graft-specific antibodies even after the rebound of antibody concentrations has been referred to as accommodation (9). However, in our series, cases with postoperative elevations in anti-A/B antibody titers were associated with periportal changes that were compatible with acute antibody-mediated allograft injury accompanied by the focal or diffuse deposition of C4d. This suggests that postoperative titer monitoring

may be practical to predict acute AMR in patients with ABO-I transplantation and that the routine application of C4d immunostaining in ABO-I LT may not be necessary to detect acute AMR.

Diffuse or strong C4d staining was uncommon in ABO-C cases, and none of the C4d-positive cases during the study period were associated with typical severe allograft rejection. We previously reported that lymphocyte cross-match positive transplantation without preventive conditioning against AMR could result in clinical AMR (1, 25). In that report, lymphocyte cross-match positive cases often showed diffuse C4d positivity and common histology were ACR, neutrophilic cholangitis/cholangiolitis, and hepatocanalicular cholestasis (1). After encountering some fatal clinical AMR cases, we tried to avoid lymphocyte cross-match positive transplantation. Therefore, patients in this study were all negative for lymphocytic cross-match tests before LT; C4d positivity was not associated with severe inflammation or cholestasis, which could suggest acute AMR after ABO-C LT. We suggest that avoiding cross-match-positive LT reduced critical AMR, but C4d positive cases may still be observed without severe graft damage.

As in renal allografts, association of DSA and chronic rejection has been recognized in some studies in LT (5, 13). We have reported that anti-class II DSA was related to late graft fibrosis and C4d positivity (6). This study also proved that DSA against HLA-DR was associated with late-onset acute rejection, graft fibrosis and C4d deposition. While previous study focused on pediatric cases and excluded fibrosis with apparent causes such as steatohepatitis, this study included all biopsies of adult and pediatric patients whose fibrosis could be attributable to non-rejection episodes. It is of note that two adult patients who were treated with interferon for recurrent Hepatitis C were included among C4d-positive cases. Since chronic hepatitis C itself is associated with graft fibrosis, it seems difficult to determine if C4d has a role in graft fibrosis or not. Interferon therapy alone may be related to C4d positivity (14). In one of the two patients, however,

progression of fibrosis was observed even after sustained viral response and successful treatment of biliary stricture. Diffuse C4d positivity and persistent anti-class II (DR locus) DSA might be related to progressive fibrosis and bile duct loss. In addition, a pediatric case in which C4d positivity was found before this study was also associated with progressive fibrosis, which was a clue to prove HLA-DSA. These findings suggest that C4d can be a tool to detect possible DSA-related fibrosis; the causes of fibrosis can be multifactorial, especially among adults who may have recurrent original disease and positive DSA status at the same time. Since C4d positivity was rare and was not associated with graft loss or severe graft dysfunction, C4d immunohistochemistry seems to be useful only in limited situations for the evaluation of late allograft biopsies, such as immunosuppression weaning or unusual allograft fibrosis. However, C4d staining is inexpensive and can be easily evaluated using conventional biopsy, and would be more practical than applying HLA assays in all the cases after LT. The exact prognostic significance and contribution to optimization of immunosuppressants needs to be determined in further studies.

Our study has several limitations for analysis of DSA. Pre-operative data of HLA assay other than lymphocyte crossmatch test were not available in most cases. Postoperative HLA assays were not performed in a fixed period of time after LT. Although negativity of preoperative lymphocyte crossmatch test suggests that most DSA found at late biopsies was associated with de novo DSA, definitive data is lacking in this study. Since presence of DSA did not correlated with the level of serum transaminases or total bilirubin, further study for alloantibody and autoantibody is also required in order to clarify the presence of chronic-antibody mediated rejection of the liver; assays for immunoglobulin subclass or complement fixation might be more important rather than simple quantification of those antibodies (28).

In conclusion, our study is the first to compare the prevalence of C4d positivity in liver allografts between ABO-C and ABO-I by applying C4d immunohistochemistry to routine anatomic pathology practice. In ABO-C LT, diffuse or strong endothelial C4d positivity is uncommon and may be associated with graft fibrosis and the presence of DSA against HLA DR. In ABO-I LT, C4d positivity is common with or without elevations in postoperative anti-A/B antibody titers, and is of little value to detect acute AMR.

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Figure Legends

Figure 1. A case of C4d-positive liver allograft biopsy after liver transplantation for hepatitis C cirrhosis. 1A, Biopsy taken 5 years after transplantation showing bridging portal fibrosis (Trichrome stain, x10 objective lens). HCV-RNA was negative in the serum; 1B, cytokeratin 7 immunostaining demonstrating focal bile duct loss and cytokeratin 7-positive hepatocytes (x 10); 1C, Mild lymphocytic portal infiltration without definite interface activity (H & E stain, x20); 1D, Diffuse C4d staining in the capillaries of the portal tract (x20).

Figure 2. A case of chronic allograft injury associated with persistent donor-specific HLA antibodies. 2A, Biopsy taken 185 days after transplantation revealed portal lymphocytic inflammation and perivenular hemorrhage, suggesting acute cellular rejection (H&E stain, ×4 objective lens); 2B, C4d was positive along the endothelium and stroma (postoperative day 185, x4, with inset highlighting the C4d-positive endothelium, ×20); 2C, Follow-up biopsy showing portal fibrosis with focal lymphocytic portal infiltration (postoperative day 524, H&E stain, ×10); 2D, Faint C4d staining (postoperative day 524, ×10); 2E, Last biopsy showing bridging perivenular and periportal fibrosis (postoperative day 968, Masson-Trichrome stain, ×4); 2F, C4d positivity returned (postoperative day 968, ×40).

Figure 3. A case of acute antibody-mediated rejection after ABO-incompatible transplantation (postoperative day 9). 1A, Periportal edema and hemorrhage with mild neutrophilic infiltration (H&E stain, ×20 objective lens); 1B, C4d staining was seen along the endothelia of portal vessels (×20). Focal periportal sinusoidal staining was also observed.

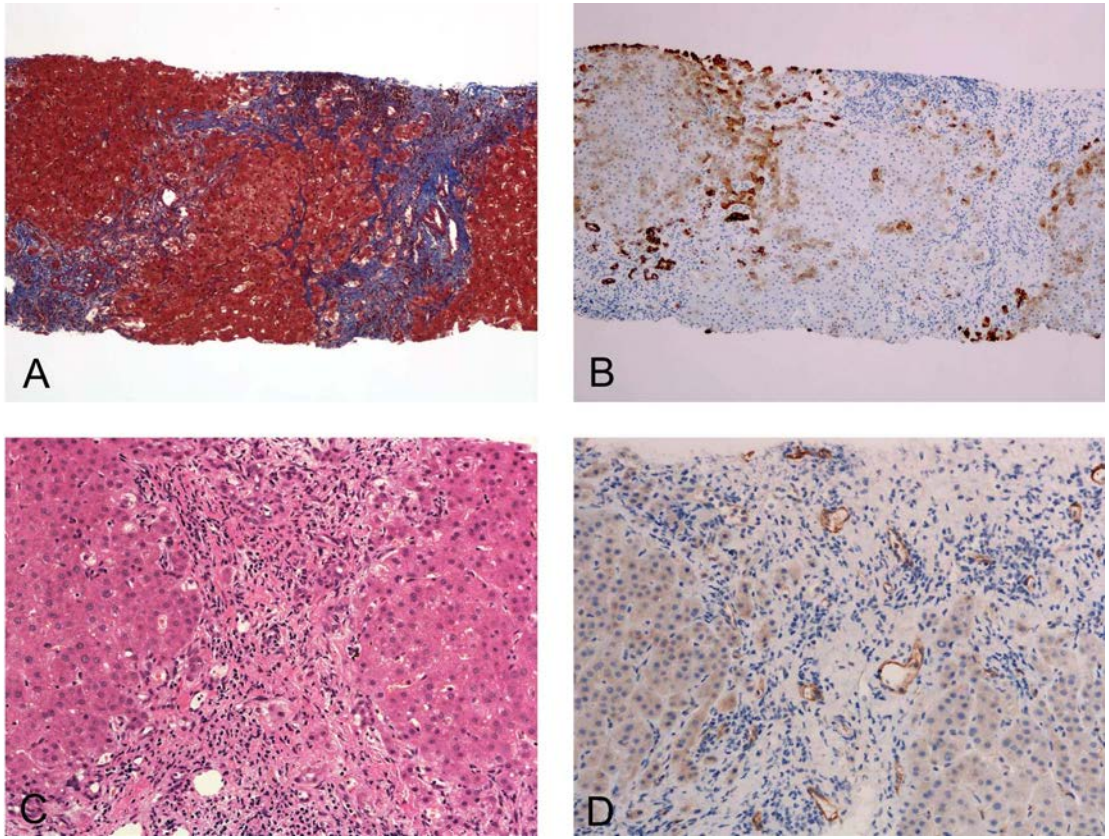

Figure 1

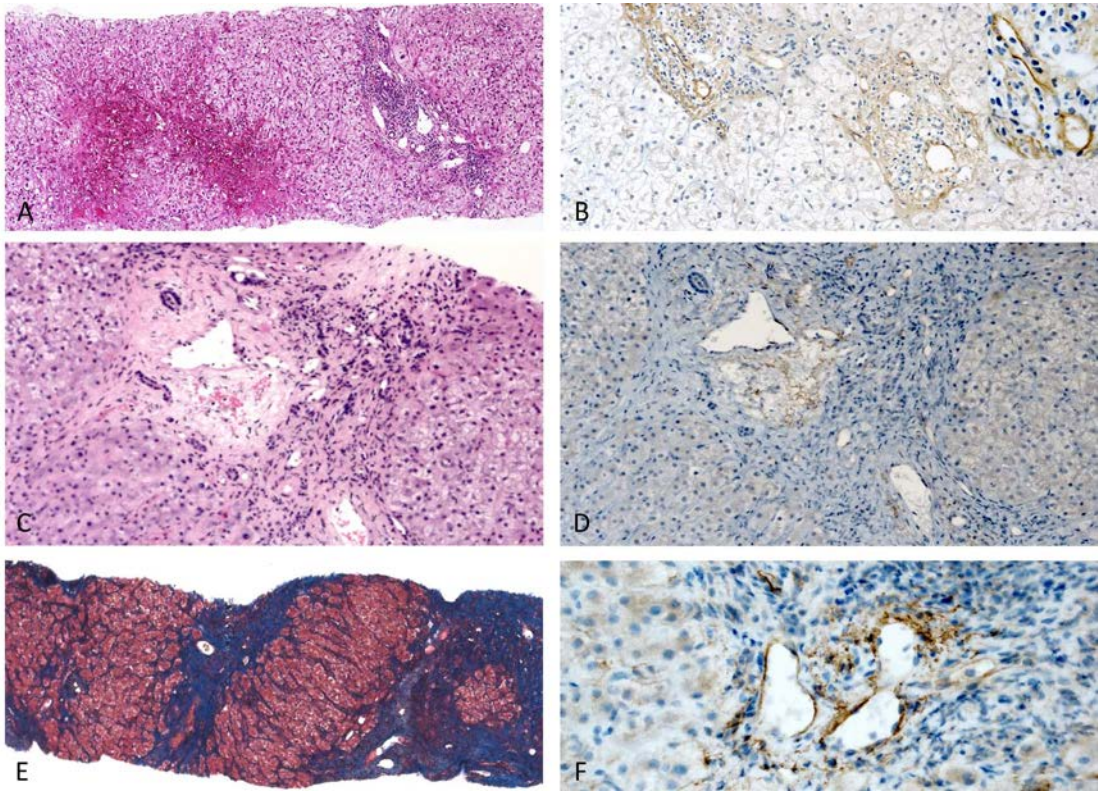

Figure 2

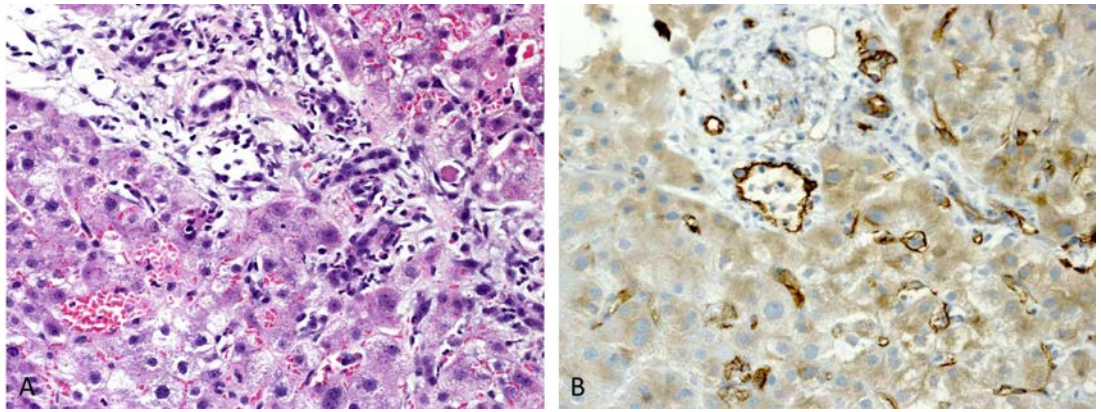

Figure 3

Table 1. The comparison of the ABO-compatible/identical patients and ABO-incompatible patients.

	ABO-C (n = 114)	ABO-I (n = 29)	P value
Age at LT (median, range)	4.7, 0.1–67.5	26.3, 0.1–66.7	-
<18 years old	74%	38%	0.0007
Female	49%	38%	0.2
Indication for LT	BA (70%), HCV (12%)	BA (31%), HCV (10%)	0.03, 1.0
Biopsy >30 POD	91%	76%	0.05
ACR	18%	42%	0.07
C4d score 1-3*	35%	72%	0.0006
C4d score 2-3*	4%	52%	<0.0001
> 5000 MFI of DSA	32%	14%	0.1
> 5000 MFI of DSA at DR locus	22%	9%	0.2
Graft loss	3%	20%	0.002

Abbreviations: ABO-C, ABO compatible/identical; ABO-I, ABO-incompatible; ACR, acute cellular rejection; DSA, donor-specific anti-HLA antibodies; HLA, human leukocyte antigen; LT, liver transplantation; POD, posttransplantation days

*C4d score in the endothelium of portal areas: score 0, completely negative; score 1, focal and weak staining; score 2, diffuse or strong staining; score 3, diffuse and strong staining.

Table 2. Characteristics of patients showing C4d positivity in the endothelium

Case *	Sex	Age at LT	Original disease	POD	DSA Locus, MFI	A/B titer	ANA	Histology of index biopsy (F stage)	C4d pattern (score)	Follow-up histology; DSA status and/or anti-A/B titer; C4d score (POD)
C1	M	0.8	BA	14	DR8, 1329	-	N/A	Hepatocyte ballooning (1)	Focal (2)	Portal inflammation; NA; C4d socre 0 (447)
C2	M	1.6	BA	4964	DR15, 8961	-	Negative <1:40	Perivenular fibrosis (3)	Focal (2)	N/A
C3	M	4.8	FHF	3245	DR8, 22701	-	N/A	ACR0 (2)	Diffuse (3)	Late ACR; DR DSA (+); C4d score 1 (3634)
C4	M	57.7	HCV LC	2113	DR51, 18195	-	Positive 1:40	Biliary stenosis (3)	Diffuse (2)	Biliary stenosis; DR DSA (+); C4d score 3 (2505)
C5	F	58.8	HCV LC	1812	Negative	-	Negative	HepC (2)	Focal (2)	HepC; N/A; Score 0 (2162)
I1	M	0.6	BA	8	Negative	1:32	N/A	AMR (1)	Focal (2)	Mild ACR; DSA (-), anti-B, 1:2; C4d score 2 (398)
I2	F	0.6	BA	2289	NDSA (DR52, 1495)	<1:1	N/A	ACR1 (1)	Focal (2)	Mild perivenular fibrosis; DSA (-); C4d score 0 (3000)
I3	M	1.2	FHF	5	Negative	1:8	N/A	ACR1 (1)	Diffuse (2)	Steatosis; DSA N/A, anti-B, 1:2; C4d score 0 (115)
I4	M	6.9	PSC	1077	DR15, 5513; DR51, 21178; DQ6; 24806	1:2	N/A	ACR2 (1)	Diffuse (2)	N/A
I5	F	17.8	BA	680	Negative	<1:1	Positive 33.6	ACR3 (2)	Diffuse (3)	ACR0; DSA (-), anti-A, 1:4; C4d score 2 (1160)
I6	F	19.3	BA	174	Negative	1:2	N/A	Cholangitis (2)	Diffuse (3)	Cholangitis; DSA (-), anti-A, 1:2; C4d score 3 (545)

I7	M	26.1	IPH	68	N/A	1:4	N/A	Congestion, hepatocyte inclusions (2)	Focal (2)	Liver abscess; DSA N/A, anti-A, <1:1; C4d score 0 (180)
I8	F	33.3	EHE	9	Negative	1:256	N/A	AMR (1)	Diffuse (3)	ACR0; N/A; C4d score 2 (68)
I9	F	43.2	HBV LC	864	N/A	<1:1	Negative	ACR0 (1)	Focal (2)	N/A
I10	F	45.7	BCS	34	Negative	1:2	N/A	Cholangitis (2)	Diffuse (3)	Cholangitis; DSA N/A, anti-A <1:1; C4d score 2 (101)
I11	M	46.0	PSC	2373	N/A	<1:1	Positive 87.6	Cholangitis (1)	Focal (2)	N/A, (died of sepsis on POD 2404)
I12	F	47.6	Alcoholic LC	12	A31, 19571; DR9, 18175	1:256	N/A	AMR (1)	Focal (2)	ACR0; DSA (-), anti-A, 1:4; C4d score 0 (675)
I13	F	48.0	PBC	4903	Negative	<1:1	Positive 1:80	Bile duct atrophy (1)	Focal (2)	N/A
I14	F	51.6	HCV LC	6	Negative	1:2	N/A	Cholangitis (1)	Diffuse (2)	Cholestatic hepatitis C; DSA N/A, anti-A, 1:2; C4d score 1 (452)
I15	F	54.3	HCV LC	719	DR9; 1830; DR53; 2452; DQ9; 6156	<1:1	N/A	HepC (1)	Focal (2)	Chronic hepatitis C; DSA (-), anti-A, 1:4; C4d score 2 (1262)

Abbreviations: ACR, Acute cellular rejection; (ACR0; indeterminate; ACR1, mild; ACR2, moderate; ACR3, severe); AMR, antibody-mediated rejection; ANA, anti-nuclear antibody; BA, biliary atresia; BCS, Budd-Chiari syndrome; DSA, donor-specific anti-hunan leukocyte antigen antibody; EHE, epithelioid hemangioendothelioma; F, fibrosis; FHF, fulminant hepatic failure; HCC, hepatocellular carcinoma; HCV, Hepatitis C virus; HBV, Hepatitis B virus; HepC, chronic hepatitis C; LC, liver cirrhosis; LT, Liver transplantation; MFI, mean fluorescence intensity; NDSA, non-donor-specific antibody; N/A, Not Available; P, positive; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; POD, postoperative day.

*C1 to C5 are ABO-compatible/identical cases; I1 to I15 are ABO-incompatible transplantation cases.

Table 3. Correlation of C4d positivity and clinicopathological parameters in ABO-compatible or ABO-identical liver transplantation

	Early biopsy (<30 days posttransplantation)			Late biopsy (≥30 days posttransplantation)		
C4d status	C4d+ (n=1)	C4d- (n=9)	P value	C4d+ (n=4)	C4d- (n=100)	P value
Age at LT, years*	0.8	34 ± 26	-	31 ± 32	14 ± 20	0.11
Age at biopsy, years*	0.8	34 ± 26	-	38 ± 28	24 ± 20	0.18
POD, days*	14	13 ± 7	-	3034 ± 1427	2830± 1883	0.83
AST (IU/L)*	164	83 ± 58	-	41 ± 24	39 ± 29	0.89
ALT (IU/L)*	300	129 ± 132	-	39 ± 26	39 ± 50	1.00
Total bilirubin (mg/dL)*	0.4	6 ± 4	-	0.6 ± 0.3	1.2± 1.9	0.53
≥Stage 2 Fibrosis	0%	17%	1.00	100%	30%	0.01
Acute cellular rejection	0%	58%	1.00	0%	14%	1.00
>5000 MFI of DSA	0%	0%	1.00	75%	34%	0.12
>5000 MFI of DSA at DR locus	0%	0%	1.00	75%	22%	0.04
Graft loss	0%	11%	1.00	0%	2%	1.00

Abbreviations: ALT, alanine Aminotransferase; AST, aspartate aminotransferase; DSA, donor-specific anti-human leukocyte antigen antibodies; LT, liver transplantation; MFI, mean fluorescence intensity.

*Mean ± standard deviation.

Table 4. Correlations of donor specific anti-HLA DR antibodies and clinicopathological parameters in late biopsies of ABO-compatible or identical patients

	>5000 MFI (n = 25)	MFI ≤5000 (n = 79)	P value
Age at LT, years*	7.9 ± 14.5	16.5 ± 22.0	0.07
postoperative days*	3012 ± 1899	2782 ± 1859	0.74
AST (IU/L)*	45 ± 37	45 ± 36	0.99
ALT (IU/L)*	49 ± 63	52 ± 72	0.88
TB (mg/dL)*	0.9 ± 0.5	1.7 ± 2.9	0.16
≥Stage 2 Fibrosis	52%	27%	0.03
ACR%	32%	8%	0.004
C4d Score 2-3	12%	1%	0.04
C4d Score 1-3	56%	27%	0.01

Abbreviations: ACR, acute cellular rejection; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HLA, human leukocyte antigen; LT, liver transplantation; TB, Total Bilirubin.

*Mean ± standard deviation.

Table 5. Correlation of C4d positivity and clinicopathological parameters in ABO-incompatible liver transplantation

	Early biopsy (<30 days posttransplantation)			Late biopsy (≥30 days posttransplantation)		
C4d status	C4d+ (n=5)	C4d- (n=2)	P value	C4d+ (n=10)	C4d- (n=12)	P value
Age at LT, years*	27± 25	27 ± 33	0.9	31 ± 19	27 ± 27	0.7
Age at biopsy, years*	26 ± 25	26 ± 33	0.9	34 ± 20	32 ± 32	0.8
POD, days*	8 ± 3	18 ± 11	0.08	1318 ± 1508	1906 ± 1903	0.4
AST (IU/L)*	82 ± 43	92 ± 23	0.8	68 ± 59	102 ± 170	0.8
ALT (IU/L)*	129 ± 75	242 ± 193	0.3	74 ± 66	84 ± 136	0.7
Total bilirubin (mg/dL)*	9 ± 8	10 ± 7	0.8	5 ± 8	2 ± 2	0.2
≥ Stage 2 Fibrosis	0%	50%	0.3	60%	50%	0.8
Acute cellular rejection	20%	100%	0.1	30%	25%	1.0
Antibody-mediated rejection	60%	0%	0.4	0%	0%	-
>5000 MFI of DSA	20% (1/5)	0% (0/2)	1.0	14% (1/7)	13% (1/8)	1.0
>5000 MFI of DSA at DR locus	20% (1/5)	0% (0/2)	1.0	14% (1/7)	0% (0/8)	0.5
>1:16 isoagglutinin titer	60%	0%	0.4	0%	0%	-
Graft loss	2	0	1.0	3	1	0.3

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; DSA, donor-specific anti-human leukocyte antigen antibodies; LT, liver transplantation; MFI, mean fluorescence intensity.

*Mean ± standard deviation.