Variations in technical preference remain and modifications may be necessary to take account of anatomic variants in biliary reconstruction. Biliary complications seemed to develop more frequently in graft with multiple bile duct; however, this did not reach statistical significance in the present series. Duct-to-duct reconstruction is safely applied even in multiple bile duct reconstruction with plasty of the graft bile duct or with combined duct-to-duct and Roux-en-Y anastomosis. Contrary to an old concept, duct-to-duct reconstruction has been successfully performed even with the recipient cystic duct,[33] although the incidence of stricture in the cystic duct anastomosis was revealed to be high in our series (33.3%), and not just in a few left lobe grafts.[34]

With regard to biliary morbidity according to the reconstruction method used, we did not find any conclusive tendency to favor any mode or suture. The use of synthetic monofilament suture material was reported to be feasible for biliary reconstruction because of reduced tissue reaction by synthetic materials, as well as bacterial adherence.[35] The Paul Brousse group recommended nonabsorbable suture rather than absorbable material because the resorption of the latter might induce local inflammation and subsequent stenosis.[36] Trends remain in the Roux-en-Y group toward lower incidence of stricture in interrupted suture and lower incidence of leakage in continuous suture in the present study. Our current preference is the use of 6–0 or 7–0 nonabsorbable running suture at the posterior wall and interrupted suture at the anterior wall.

Stenting of the anastomosis is another topic for discussion in LDLT. The rationale of stent is the maintenance of biliary flow despite swelling of anastomosis and easy access for control cholangiography in case of suspected leakage or stricture.[37] The external stent tends to reduce biliary complication in the Roux-en-Y reconstruction, which was consistent with our previous series of 400 pediatric LDLTs.[27] Although our preliminary right lobe with duct-to-duct series demonstrated that the external stent significantly reduced the incidence of biliary stricture,[12,16] overall incidence of biliary stricture was considerably high (26.6%) in long-term follow-up. Scatton et al reported that employment of a T tube increased incidence of biliary complications and recommended the performance of duct-to-duct without a T tube in deceased liver transplantation.[38] The most frequent complication was leakage after T tube removal. We do not experience leakage after removal of 4-Fr biliary tube in the present series. To confirm this finding, while we formerly used a small stent tube, we ceased to use it from July 2004 and are monitoring the results.

CONCLUSION

Duct-to-duct biliary reconstruction in right lobe LDLT appears to be a feasible option with better endoscopic access for treating biliary stricture. Long-term observation may be necessary to collect sufficient data for the establishment of this treatment modality. It is hoped that increased experience and continuing refinements of the technique will lead to improved outcomes in right lobe LDLT.

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LIVER TRANSPLANTATION 12:457-464, 2006

ORIGINAL ARTICLE

Acute Humoral Rejection and C4d Immunostaining in ABO Blood Type-Incompatible Liver Transplantation

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Complement C4d deposition in graft capillaries has been reported to be associated with antibody-mediated rejection in kidney and other solid organ transplantation. The correlation of C4d deposits and humoral rejection in liver transplants, however, is not well understood. We investigated the C4d immunostaining pattern in 34 patients whose liver biopsy was taken within the first 3 postoperative weeks for suspected acute rejection after ABO blood type-incompatible liver transplantation. The staining pattern was classified as positive (portal stromal staining), indeterminate (endothelial staining only), and negative (no staining). Positive C4d immunostaining was seen in 17 (50%) patients and was significantly associated with high (×64 or more) postoperative antidonor A/B antibody (immunoglobulin M (IgM)) titers (88 vs. 35%, $P = 0.002$) and poorer overall survival rate (41 vs. 88%, $P = 0.007$). Ten of 11 (91%) cases with histological acute humoral rejection (periportal edema and necrosis (PEN) or portal hemorrhagic edema) were positive for C4d, all of which showed high postoperative antibody titers. The other histologies associated with C4d positivity was purulent cholangitis (n = 4), coagulative hepatocyte necrosis (n = 1), acute cellular rejection (n = 1), and hepatocanalicular cholestasis (n = 1). Full clinical recovery was observed in only 6 of 17 (35%) C4d-positive patients, and tended to be associated with a lower rejection activity index (RAI). In conclusion, our study indicates that C4d deposits in the portal stroma can be a hallmark of acute humoral rejection in ABO-incompatible liver transplantation, and allograft damage can be reversible in a minority of cases. *Liver Transpl 12:457-464, 2006.* © 2006 AASLD.

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In ABO blood type-incompatible liver transplantation, acute humoral rejection triggered by antibodies against donor-type isoagglutinins is the most serious form of rejection and is often associated with graft loss. With the introduction of effective antihumoral rejection therapy such as the arterial/portal infusion of prostaglandin E1 and use of anti-CD20 monoclonal antibody, as well as preoperative plasma exchange, ABO-incompatible liver transplantation is becoming a choice to overcome the paucity of liver allografts from deceased donors in Japan.[1-4] Acute humoral rejection, however, is still a major problem in patients with ABO blood type-incompatible grafts and the evaluation of humoral rejection is necessary.

We previously reported that periportal edema and necrosis (PEN) could be histological indications of the early phase of severe humoral rejection.[5] In that report, all grafts with PEN resulted in massive parenchymal or biliary necrosis. Recent papers from other institutions, however, reported that histologically proven humoral rejection could be reversible.[3,6] Biopsies also demonstrated portal edema or hemorrhage, but no significant necrosis or endothelialitis has been documented. Portal hemorrhagic edema without significant necroinflammation may represent a milder degree of humoral rejection.[6]

To obtain the full histological spectrum of humoral

Abbreviations: Ig, immunoglobulin; PEN, periportal edema and necrosis; RAI, rejection activity index.

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rejection of ABO blood type-incompatible liver transplantation, we performed clinicopathological analysis with complement C4d immunostaining. C4d is a molecule that covalently binds to tissues after activation of the complement system, and immunostaining has been widely used to demonstrate humoral immunoreactivity or antibody-mediated rejection in other solid organ transplantations.[7-10] The aim of this study was to clarify the clinicopathological features of acute humoral rejection caused by antidonor ABO blood group antigens using C4d immunostaining.

MATERIALS AND METHODS

Patient Selection

Between January 1999 and December 2004, 82 patients (35 children and 47 adults) received primary ABO blood type-incompatible living donor liver transplantation. Informed consent for ABO blood type-incompatible liver transplantation was obtained from the recipient and donor, and the Ethical Committee of the Medical School of Kyoto University approved the surgery and treatment procedures. Among them, 35 patients (13 children and 21 adults) underwent liver biopsy within the first 3 postoperative weeks. One adult patient was excluded from this study because the paraffin-embedded tissue was not available. The other 34 patients were enrolled in this study. As of April 2005, the clinical follow-up period for these patients ranged from 0.4 to 74 months (median, 16 months).

Antirejection Regimen and Clinical Monitoring

Baseline antihumoral rejection therapies included intravenous steroids and oral tacrolimus. Most adult patients underwent continuous prostaglandin E1 infusion via an intra-arterial or intravenous catheter for 2 to 3 weeks posttransplantation.[1,11]

Splenectomy was performed for adult patients whose transplantation was performed between May 1999 and January 2003. To avoid splenectomy-related portal vein thrombosis, the administration of preoperative rituximab (anti-CD 20 monoclonal antibody) without splenectomy was introduced after April 2004.[11] In addition, 2 adult patients were given postoperative rituximab as a therapy for humoral rejection.

Patients underwent preoperative plasmapheresis or blood exchange in order to reduce antidonor blood group A/B antibody (immunoglobulin M (IgM) and IgG) titers to ×8 or lower. A microhemagglutination assay was used to monitor the serum levels of antidonor ABO blood group antibodies at least 3 times per week during the first postoperative month. Patients were assigned to high-titer groups if the peak of the postoperative antidonor blood group IgM titers was ×64 or more, and to the low-titer group if it was less.[5] In this study, there were 21 patients with a high titer and 13 with a low titer. Postoperative plasmapheresis was performed when severe humoral rejection was suggested clinically or histologically. Elevation of postoperative antidonor antibody titer not associated with liver dysfunction was treated by steroid bolus therapy or was managed with watchful observation alone.

Hepatic necrosis was diagnosed by enhanced computed tomography. The findings were diffusely spreading low-density lesions in the graft. Intrahepatic biliary complications were diagnosed by cholangiography, showing irregularity and beading throughout the intrahepatic biliary tree.

Pretransplant T-cell cross-match tests were performed as previously described.[12] The test was interpreted as positive when 41% or more of donor lymphocytes were killed, weakly positive when 21 to 40% of donor lymphocytes were killed, and negative when no more than 20% of donor lymphocytes were killed. Cross-match tests were negative in 33 of 34 patients. One patient was interpreted as weakly positive.

Histological Diagnosis

Pathological diagnosis was made on a routine basis by 3 pathologists (H.H., T.S., and A.M.), with no information about immunostaining of immunoglobulins or complements, including C4d. The minimal quantitative requirement for the diagnosis of rejection was a biopsy containing at least 5 portal areas. Acute cellular rejection was diagnosed using Banff criteria.[13] Histological acute humoral rejection was suspected when PEN or portal hemorrhagic edema was associated with the elevation of antidonor A/B antibody titers.[5,6] In each case of acute cellular rejection and acute humoral rejection, portal inflammation, bile duct damage, and venular endothelialitis were evaluated separately with the rejection activity index (RAI) of Banff criteria.[13] To apply the RAI to humoral rejection, we regarded any leukocyte infiltration as portal inflammation and gave a score of 3 on periportal coagulative necrosis as part of portal inflammation.

C4d Immunostaining and Control Materials

Eighteen-gauge liver core tissue biopsies were placed in 10% buffered formalin from several hours to overnight, processed routinely, and sliced into 3-μm paraffin sections. Staining methods for routine histological evaluation included hematoxylin and eosin, Masson trichrome, and immunostaining of cytokeratin 7 (OV-TL 12/30, Dako, Glostrup, Denmark; dilution, 1:200). The polyclonal antibody against C4d complement (BI-RC4D, Biomedica, Vienna, Austria; 1:50) was used for immunostaining with an automated immunostainer (BENCHMARK XT, Ventana, Tucson, AZ). For antigen retrieval, deparaffinized and rehydrated sections were treated with protease I (Ventana; 0.5 units/mL) at 37°C for 20 minutes.

We used lymphoid tissue with follicular hyperplasia as a positive control for C4d staining.[14] The reticular staining pattern in the germinal centers was confirmed in every C4d immunostaining. Ten wedge biopsies of the liver allografts taken during graft resection (time 0

biopsy) were used as negative controls. For comparison of ABO blood type-incompatible and non-ABO blood type-incompatible cases, 10 needle biopsies with typical acute cellular rejection from ABO-identical transplants were assessed for C4d immunostaining.

Evaluation of C4d Immunostaining

Three pathologists (H.H., T.S., and A.M.) independently evaluated the C4d immunostaining slides of the initial biopsies. The identification numbers were removed from the slides and the pathologists were not given any clinical information. C4d staining was semiquantitatively evaluated in terms of the percentage of portal tracts containing distinctly stained stroma and/or endothelium. Biopsies containing 50% or more stromal-positive portal tracts were evaluated as positive. Specimens with less than 50% stromal-positive portal tracts or positive staining only in the vascular endothelium or sinusoids were classified as indeterminate. Completely negative staining was evaluated as negative. Extraportal endothelial staining was recorded but not graded. Any staining in the liver capsule or extraliver tissue was omitted to evaluate.

A couple of discordant cases were classified as indeterminate, and we did not perform additional C4d immunostaining because of the small size of the specimens. Immunostaining of the follow-up biopsies of C4d-positive cases (n = 21) and the second biopsies of C4d-negative cases (n = 17) was evaluated with clinical data and previous biopsies.

Statistical Analysis

Statistical significance of differences among the groups was assessed by Fisher's exact test. Laboratory data were analyzed using Mann-Whitney's U test. Patient survival was determined by Kaplan-Meier analysis, and differences in survival were analyzed with a log-rank test. For all analyses, P values of less than 0.05 were taken as significant.

RESULTS

C4d Immunostaining and Clinical Outcomes

Five of 10 time 0 allograft biopsies obtained at liver transplantation showed focal endothelial or stromal C4d staining. These positive stainings did not exceed 10% of the number of the portal tracts and were classified as indeterminate. The other 5 time 0 biopsies were negative for C4d. In ABO blood type-identical specimens with a diagnosis of acute rejection, 1 specimen showed strong and diffuse portal stromal staining (positive), 6 showed focal or diffuse endothelial staining (indeterminate), and 3 negative. Distinct C4d staining was seen in the perivenular areas in the C4d-positive case.

Among 34 initial biopsies from ABO blood type-incompatible transplants, 17 cases (50%) were positive for C4d in the portal stroma, 8 (24%) were indeterminate, and 9 (26%) were negative. Representative staining patterns are shown in Figure 1. Preoperative clinical features were not significantly different between the groups (Table 1). However, high-postoperative antidonor antibody titer (×64 or more) was more frequently seen in C4d-positive patients than in C4d-indeterminate and -negative ones (Table 1). When the C4d-indeterminate group was regarded as negative for C4d, C4d positivity was significantly associated with high titers (88 vs. 35%; $P = 0.002$). Hepatic necrosis demonstrated by computed tomography was seen only in the C4d-positive patients (24 vs. 0%; $P = 0.052$). Patients who underwent postoperative plasma exchange were statistically more frequently seen in C4d-positive patients than in C4d-indeterminate and -negative patients (53 vs. 6%; $P = 0.003$). The timing of postoperative plasma exchange was clinical humoral rejection, which occurred within 14 postoperative days. One to 6 (median, 3.5) courses of plasma exchange were performed, and 4 of 10 (40%) grafts recovered.

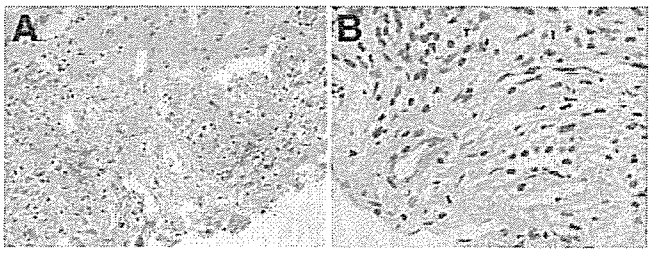

Figure 1. (A) C4d-positive staining. C4d deposited in the periportal area. Surrounding parenchyma showed coagulative necrosis (original magnification, ×200). (B) Indeterminate endothelial staining in the small artery and capillaries (×400).

Overall, patient survival was significantly worse for C4d-positive than for C4d-indeterminate and -negative patients (41 vs. 88%; $P = 0.007$; Fig. 2A). The high-titer group (n = 21) also showed poorer overall survival than the low-titer group (n = 13), which was not significant in this study (56 vs. 83%; $P = 0.072$; Fig. 2B).

C4d Immunostaining and Histology

Findings of initial biopsies are summarized in Table 2. In C4d-positive patients, the most common histology at initial biopsy was PEN/portal hemorrhagic edema. Ten of 11 cases (91%) with PEN/portal hemorrhagic edema showed C4d stromal deposition. The median postoperative day of C4d-positive PEN cases was day 7 (Table 3).

The other histology in C4d-positive cases included purulent cholangitis (n = 4; median posttransplant day 18), coagulative hepatocyte necrosis (n = 1; posttransplant day 17; clinically hepatic artery thrombosis), moderate acute cellular rejection (n = 1; posttransplant day 15), and hepatocanalicular cholestasis (n = 1; posttransplant day 13).

In C4d-indeterminate and -negative patients, the most common histology was hepatocanalicular cholestasis (n = 5; median posttransplant day 13), followed by purulent cholangitis (n = 4; median posttransplant day 18), mild lobular inflammation (n = 4; median posttransplant day 16), mild to moderate acute cellular rejection (n = 3; median posttransplant day 12), and PEN (n = 1; posttransplant day 10; RAI = 6).

TABLE 1. C4d Immunostaining Pattern and Clinical Features

C4d immunostaining	Positive (n = 17)	Indeterminate / negative (n = 17)	
		Indeterminate (n = 8)	Negative (n = 9)
Female: Male	13:4 (76%: 24%)	6:2 (75%: 25%)	6:3 (67%: 33%)
Age group			
<3 yr	3 (18%)	3 (38%)	4 (44%)
3–50 yr	10 (60%)	1 (13%)	2 (22%)
>50 yr	4 (24%)	4 (50%)	3 (33%)
Original diseases			
Chronic liver diseases	17 (100%)	6 (75%)	7 (78%)
Fulminant liver failure	0 (0%)	2 (25%)	2 (22%)
Mean HLA-A, B, DR mismatches	2.9	2.8	2.8
Preoperative treatment			
Splenectomy	4 (24%)	2 (25%)	3 (33%)
Rituximab	6 (35%)*	2 (25%)	1 (11%)
Peak of postoperative antibody titer (IgM)			
High-titer cases (x64 or more)	15 (88%)	3 (38%)	3 (30%)
Median (range)	x256 (x8–x8,192)	x12 (x1–x512)	x8 (x2–x1,024)
Mean postoperative day (range)	7 (4–13)	6 (1–10)	5 (1–18)
Radiological findings			
Hepatic necrosis	4 (24%)	0 (0%)	0 (0%)
Intrahepatic biliary complications	1 (6%)	0 (0%)	2 (22%)
Postoperative plasma exchange	9 (53%)	0 (0%)	1 (11%)
Overall patient survival	41%	100%	78%

*Used postoperatively in 2 cases.

PEN/portal hemorrhagic edema was statistically more frequently observed in C4d-positive patients than in non-C4d-positive patients (59 vs. 6%; $P = 0.0012$). Levels of serum transaminases tended to be higher in C4d-positive patients but did not reach statistical significance. Total bilirubin level was significantly higher in C4d-positive patients ($P = 0.009$).

PEN/Portal Hemorrhagic Edema Cases

Among 11 PEN/portal hemorrhagic edema cases, the histology of 5 patients (cases 3, 5, 12, 15, and 1 C4d-negative case) was PEN (Table 3). PEN included portal hemorrhage, mild to moderate portal neutrophilic infiltration, perivenular endothelialitis without significant perivenular necrosis, and focal periportal necrosis (Fig. 3A). The portal stroma adjacent to the parenchyma and peribiliary stroma were positive for C4d (Fig. 3B). The terminal hepatic venules showed occasional faint positivity, but the sinusoids in zones 2 and 3 were negative for C4d. Cholangitis with mild to moderate neutrophilic infiltration was seen in all cases, but ductopenia or duct atrophy was not observed with CK7 immunostaining. The RAI score of PEN cases was 6 or 7, and all patients with PEN resulted in graft failure or severe graft damage.

The other 6 patients showed portal hemorrhagic edema (cases 8, 11, 13, 14, 16, and 17). Cellular infiltration was mild (Fig. 3C), but all revealed a C4d staining pattern identical to that of PEN (Fig. 3D). Some cases had perivenular C4d deposition, but perivenular inflammation was none or minimal. The RAI of portal hemorrhagic edema was 4 or less (Table 3). Only 1 patient resulted in graft failure (case 14; RAI = 2).

Follow-up Biopsies

Among 17 C4d-positive patients, 5 patients (3 with PEN, 1 with periportal hemorrhagic edema, and 1 with cholangitis) resulted in rapid graft failure and follow-up biopsies were not available (cases 1, 3, 12, 14, and 15; Fig. 4).

In the other 12 C4d-positive patients, at least 1 follow-up biopsy was available at various times. Five patients (cases 2, 4, 10, 13, and 16) were negative for C4d at the last follow-up biopsy (range, 34-792 posttransplant days). The other 7 showed positive C4d immunostaining in the last follow-up biopsy (range, 52-508 posttransplant days). Six patients (case 4 with acute cellular rejection and cases 8, 11, 13, 16, and 17 with portal hemorrhagic edema) were clinically free of symptoms and achieved normalization of the levels of serum transaminases and bilirubin as of April 2005. Among them, 3 (cases 8, 11, and 17) were positive for C4d at the last biopsy (posttransplant days 77, 336, and 209, respectively).

Any second biopsy from patients whose initial biopsies were indeterminate or negative for C4d did not reveal C4d positivity in the portal stroma (median posttransplant day 31; range, 19 - 603).

Cases with Stromal C4d Immunostaining without Elevation of the Antidonor Antibody (IgM) Titers

Cases 6 and 9 showed C4d stromal positivity, but their postoperative titer of antidonor IgM antibody remained low. In case 6, the peak antidonor IgM titer was ×16, but the antidonor IgG titer was elevated up to ×256 and

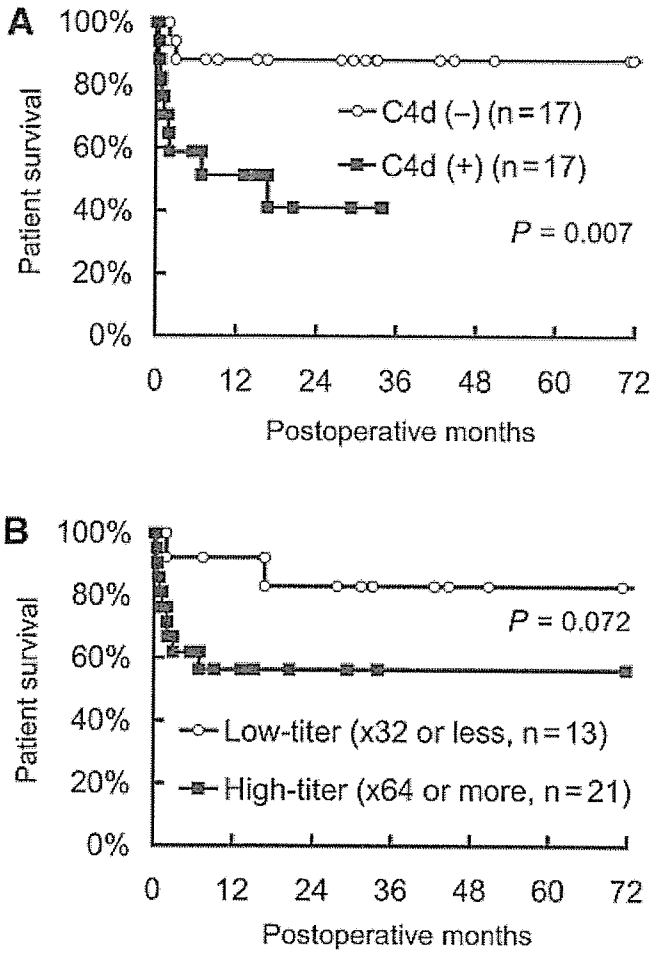

Figure 2. Survival curve in relation to (A) C4d immunostaining and (B) postoperative peak titers of antidonor A/B antibodies. Indeterminate C4d staining was regarded as negative.

lasted for 4 days. Every biopsy of case 6 showed purulent cholangitis, and subsequent computed tomography revealed hepatic necrosis. The patient died of chronic cholangitis.

Case 9 was a patient with multivisceral transplantation from 2 different donors; the liver transplantation was ABO blood type incompatible (AB to B), while the small intestine graft was ABO blood type compatible (blood type O). The postoperative peak antidonor IgM titer was low (×8) and the antidonor IgG was not detected. However, preoperative cross-match tests were interpreted as weakly positive against both donors. Liver biopsies revealed purulent cholangitis with ductular cholestasis, which was attributed to sepsis (Fig. 5A). C4d staining was positive (Fig. 5B). The ABO blood type-compatible small intestine graft showed severe acute rejection with microthrombi, and C4d deposits were observed in the endothelium (Fig. 5C and D). The patient died on posttransplant day 58. Necropsy revealed severe mucosal hemorrhage in the small intestine and monoclonal posttransplant lymphoproliferative disorder. Preoperative blood exchange and induction therapy with basiliximab were used for this patient, but no postoperative cross-match test was performed.

DISCUSSION

In ABO blood type-incompatible liver transplantation, preoperative plasma exchange was performed to lower the titers of antidonor blood group A/B antigen antibodies. The onset of acute humoral rejection is thus suspected when liver dysfunction occurs along with the elevation of titers of those antibodies. Liver biopsies soon after titer elevation usually demonstrated characteristic features such as portal edema, hemorrhage, and periportal necrosis. In some cases, however, elevation of the antidonor antibody titers was not associated with graft dysfunction, or late graft dysfunction occurred even when patients did not show significant titer elevation. In such cases, histological demonstration of deposition of immunoglobulin or complements in the allografts is important to demonstrate humoral reactivity and to decide patients' treatment.[12,15,16] Immunostaining of IgM or other complements such as C3c, however, was technically difficult in paraffin-embedded formalin-fixed liver tissue, and we were unable to make reproducible results.[4,5] Moreover, severe tissue damage other than humoral rejection can cause immunoglobulin and complement deposition.[12,17] Interpretation of the staining results can be difficult if you do not routinely perform immunofluorescent study of the allograft liver. On the other hand, C4d immunoperoxidase staining was relatively easy to perform, and the results were reliable since positive and negative controls were readily available.

Still, there have been few data about the significance of antibody-mediated rejection in clinical liver transplantation, and the criteria of histological diagnosis and therapeutic options remain unclear. Recently, a couple of studies demonstrated C4d deposition in liver allografts with acute rejection using paraffin-embedded sections, although the presence of alloreactive antibodies was not clearly stated.[18,19] Instead, they suggested that the local B-cell response might be specifically involved in C4d deposition in human liver allograft rejection. In their reports, C4d deposited in the pericapillary or periportal areas of the portal tracts, and the staining patterns were nearly identical to those of our cases. These staining patterns denoted that periportal areas may be the main targets of both humoral and acute cellular rejection, and that lobular deposition of immunoglobulin and complement may be a secondary change. In ABO blood type-incompatible transplantation, the findings may be compatible with the observation that capillaries in the portal tracts are the primary sites of ABH blood type antigen expression in the liver allografts.[20] The staining pattern may also support that periportal necrosis seen in experimental and clinical rejection can be a diagnostic histological feature of humoral rejection.[5,21]

On the other hand, the significance of C4d staining only in the endothelium was not clear in this study. Since the endothelial staining pattern was reproduc-

TABLE 2. C4d Immunostaining and Initial Biopsy Within the First 3 Postoperative Weeks

C4d immunostaining	Positive (n = 17)	Negative / indeterminate (n = 17)	*P* value
Mean postoperative day (range)	8 (5–19)	13 (5–20)	0.052
Histology			
PEN / portal hemorrhagic edema	10 (59%)	1 (6%)	0.0012
Cholangitis	4 (24%)	4 (24%)	n.s.
Cholestasis, hepatocanalicular	1 (6%)	5 (29%)	n.s.
Acute cellular rejection	1 (6%)	3 (18%)	n.s.
Coagulative hepatocyte necrosis	1 (6%)	0 (0%)	n.s.
Mild lobular inflammation	0 (0%)	4 (24%)	n.s.
Laboratory data at biopsy (median ± S.D.)			
AST (IU/L)	222 ± 305	101 ± 51	0.32
ALT (IU/L)	357 ± 388	179 ± 151	0.19
Total bilirubin (mg/dL)	11.8 ± 9.7	5.2 ± 5.6	0.009

Abbreviation: n.s., not significant.

TABLE 3. C4d-Positive Patients

Case	Age/gender	ABO (R/D)	Titer peak	Initial biopsy (POD)	Follow-up biopsy* and C4d status (POD)
1	46/F	A/AB	x8192	Cholangitis (17)	Not available
2	48/M	O/A	x128	Cholestasis (13)	Cholangitis, C4d (–) (34)
3	32/F	O/AB	x512	PEN, RAI = 6 (7)	Not available
4	1/F	B/A	x256	ACR, RAI = 6 (15)	No remarkable change, C4d (–) (792)
5	3/F	O/B	x2,048	PEN, RAI = 7 (8)	Cholangitis, C4d (+) (508)
6	55/F	O/B	x16	Cholangitis (16)	Cholangitis, C4d (+) (230)
7	11mo/M	B/AB	x128	Coagulative necrosis (17)	No remarkable change, C4d (+) (387)
8	13/M	B/A	x128	PHE, RAI = 2 (5)	No remarkable change, C4d (+) (209)
9†	1/F	B/A	x8	Cholangitis (19)	Cholangitis, C4d (+) (52)
10	56/F	B/A	x512	Cholangitis (6)	Cholangitis, C4d (–) (129)
11	41/F	A/B	x2,048	PHE, RAI = 1 (6)	Recurrent PBC or ACR, RAI = 6, C4d (+) (336)
12	50/M	O/A	x2,048	PEN, RAI = 7 (8)	Not available
13	33/F	A/AB	x256	PHE, RAI = 4 (7)	No remarkable change, C4d (–) (421)
14	29/F	O/B	x2,048	PHE, RAI = 2 (6)	Not available
15	16/F	O/A	x256	PEN, RAI = 6 (13)	Not available
16	56/F	A/AB	x1,024	PHE, RAI = 2 (6)	ACR, RAI = 5, C4d (–) (80)
17	39/F	O/AB	x256	PHE, RAI = 4 (6)	Cholangitis, C4d (+) (77)

Abbreviations: ACR, acute cellular rejection; PBC, primary biliary cirrhosis; PEN, periportal edema and necrosis; PHE, portal hemorrhagic edema; POD, postoperative day; RAI, rejection activity index (the Banff schema).
*In cases where multiple follow-up biopsies were available, the last follow-up histology is shown.
†Multivisceral transplantation case (liver and small intestine).

ible, and was common in acute cellular rejection of ABO blood type-identical cases, we speculated that it represented mild humoral reaction of the grafts. Neither patient survival nor severe liver damage, however, was associated with the endothelial staining pattern.

We were able to demonstrate in this study that C4d deposition in the portal stroma was correlated with poor patient survival and characteristic histology, as well as with postoperative titers of ABO blood type alloantibodies. Conversely, PEN/portal hemorrhagic edema showed a high percentage of C4d positivity and was probed as a useful histological feature in establishing the diagnosis of acute humoral rejection. The histology of PEN/portal hemorrhagic edema was transient, and there was a spontaneous decrease of ABO blood type alloantibody titers in the first postoperative month, but some follow-up biopsies more than a year after transplantation remained positive for C4d. This suggests that nonspecific histology such as cholangitis or coagulative necrosis can be regarded as a result of humoral rejection when C4d was detected in the allografts. For example, case 7, an 11-month-old boy, was the youngest patient with humoral rejection in this study, while hepatic artery thrombosis was a clinical cause of hepatocyte necrosis.

C4d staining was also correlated with radiological findings. It is known that there are 2 major complications associated with ABO blood type-incompatible liver transplantation.[4,15] One is massive hepatocyte necrosis occurring within the first 2 weeks, which is re-

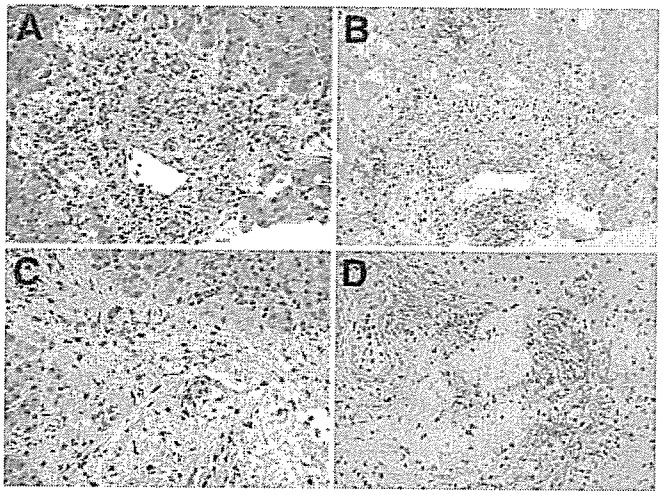

Figure 3. (A) Typical PEN (RAI = 7) and (B) C4d staining mainly in the areas of periportal edema. (C) Portal hemorrhagic edema (RAI = 2) and (D) C4d staining.

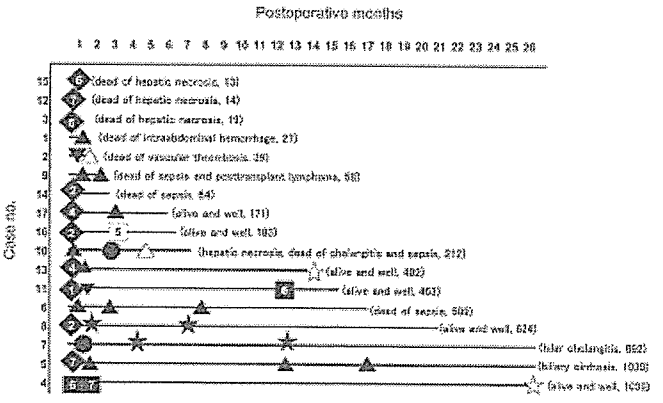

Figure 4. Histology changes in patients with C4d positivity. The histology of each case is marked on a horizontal line, the length of which denotes the follow-up period. Final patient status and postoperative day of the last follow-up are shown in parentheses. Number represents the RAI. Closed diamond (♦), PEN/portal hemorrhagic edema; closed triangle (▲/▼), cholangitis/cholestasis with C4d staining; open triangle (△), cholangitis without C4d staining; closed circle (●), coagulative parenchymal necrosis with C4d staining; closed square (■), acute cellular rejection with C4d; open square (□), acute cellular rejection without C4d; closed star (★), no remarkable finding with C4d; open star (☆), no remarkable finding without C4d.

fractory to postoperative therapy and fatal. The other is intrahepatic biliary complication that is not often associated with early liver graft dysfunction, but becomes evident several months after liver transplantation. In this study, hepatocyte necrosis found by computed tomography was associated with only C4d positivity and 3 of 4 patients revealed PEN, while no hepatocyte necrosis was found in C4d-indeterminate and -negative patients. This suggests that hepatocyte necrosis is a result of severe humoral rejection. Intrahepatic biliary complications, on the other hand, occurred in both C4d-positive and -negative patients. Since the focus of this study was concentrated on the patients showing

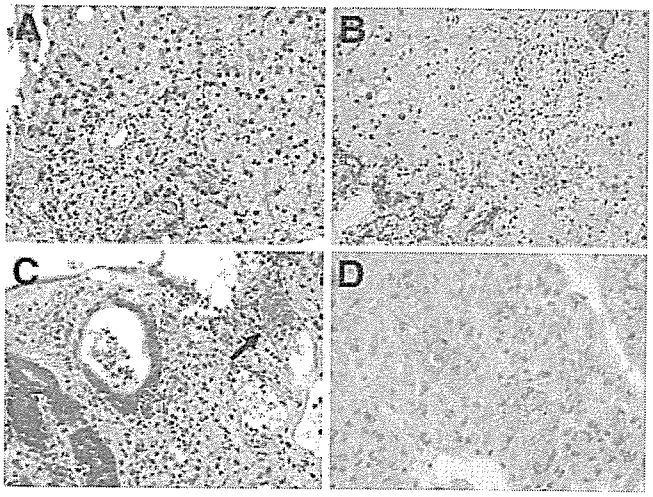

Figure 5. Multivisceral transplantation case without significant elevation of ABO alloantibodies (case 9). (A) Cholangitis with ductular cholestasis and mild portal fibrosis (×200; postoperative day 19). Clinically no biliary complications were noted and the original diagnosis was septic cholangitis. (B) C4d immunostaining revealed periportal staining (×200). (C) Small intestine allograft showing crypt apoptosis and a thrombus (arrow) (day 33; ×200). (D) The capillaries in the intestinal graft mucosa were positive for C4d (×400).

early graft dysfunction and receiving biopsy within the first 3 postoperative weeks, we think that a study based on protocol biopsy is necessary to elucidate late-onset biliary complications associated with ABO blood type-incompatible transplantation.

Once humoral rejection was defined by both morphology and C4d deposition, the RAI of the Banff schema with minor modification reliably predicted the outcome of humoral rejection. PEN corresponded to acute humoral rejection with a RAI of 6 or 7 and was associated with graft failure. On the other hand, portal hemorrhagic edema had minimal inflammatory cell infiltrate and an RAI score of 4 or less, and was related to better prognosis and complete recovery in some cases. The results suggested that the Banff schema could be useful for the evaluation of both acute cellular and acute humoral rejection. It must be kept in mind, however, that the prognosis of acute humoral rejection was much poorer than acute cellular rejection, and that even acute humoral rejection with low RAI required treatment, including repeated plasma exchange, to rescue the patients.

C4d stromal deposition in the liver allograft was demonstrated without elevation of the ABO blood type alloantibody titer in case 9. Since this patient showed a weakly positive T-cell cytotoxic cross-match and severe rejection in the ABO blood type-compatible small intestine graft, this humoral response might be mediated by lymphocytotoxic antibodies.[22,23]

We conclude from this study that the immunohistochemical detection of C4d has both diagnostic and prognostic value and could be a hallmark of antibody-mediated rejection in liver biopsy. In combination with conventional histological criteria, the demonstration of

C4d in the portal stroma may be useful to determine the indication of postoperative plasma exchange and to assess the current and future protocols for ABO blood type-incompatible liver transplantation. Further studies are needed to clarify the significance of C4d and other markers of humoral immunity in ABO blood type-matched liver transplantation.

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LIVER TRANSPLANTATION 12:912-919, 2006

ORIGINAL ARTICLE

Living Donor Liver Transplantation as a Second-Line Therapeutic Strategy for Patients With Hepatocellular Carcinoma

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Living donor liver transplantation (LDLT) has evolved to represent an important surgical strategy for patients with hepatocellular carcinoma (HCC). However, due to disadvantages, including donor risks and higher rates of perioperative complications, LDLT has been considered as a second-line treatment in Japan. The present study retrospectively evaluated clinical outcomes for 93 patients with HCC who underwent LDLT at our institute, including 44 patients who exceeded Milan criteria (MC). A total of 73 patients (78%) displayed a history of previous treatment for HCC using various nontransplant methods. Median follow-up was 24 months (range, 1-76 months). At 4 years after LDLT, overall patient survival rate was 64%, with similar rates for within-MC and over-MC groups (68% vs. 59%, respectively; $P = 0.6548$). However, cumulative recurrence rate was significantly higher in the over-MC group than in the within-MC group (35% vs. 15%, $P = 0.0190$). Regarding history of conventional treatment for HCC before LDLT, patients who had received only 1-2 previous treatments showed significantly lower recurrence rates than patients with ≥3 treatments (9% vs. 37%, $P = 0.0411$). In conclusion, LDLT may constitute an optional treatment with a chance of cure for patients with otherwise uncontrolled disease. However, repeated nontransplant treatments for recurrent HCC prior to LDLT may increase the risk of recurrence and impair the survival advantages conferred by LDLT. *Liver Transpl 12:912-919, 2006.* © 2006 AASLD.

See Editorial on Page 896

The role of orthotopic liver transplantation in the management of hepatocellular carcinoma (HCC) has evolved significantly over the past decades. Initial experiences with orthotopic liver transplantation were limited to patients with extensive unresectable tumors, and were marked by uniformly dismal outcomes due to high rates of tumor recurrence.[1,2] Those results evoked considerable interest in reexamining the staging guidelines to determine eligibility for orthotopic liver transplantation. This lead to a study by Mazzaferro et al.[3] who reported that 48 patients with a single tumor ≤5 cm in diameter or with ≤3 tumors all ≤3 cm in diameter, as identified by preoperative imaging, displayed survival rates comparable to those of non-HCC liver transplant recipients. These Milan criteria (MC) are currently widely accepted as an effective method of selecting patients with early-stage HCC for curative orthotopic liver transplantation and have been incorporated into organ allocation systems.[4]

The prevalence of HCC is high in Japan, with around 30,000 deaths annually.[5] While deceased donor liver transplantation has barely been available for patients with HCC, various treatment modalities, such as hepatic resection, percutaneous ethanol injection, radiofrequency ablation, and transarterial chemoembolization, have been developed, leading to improved outcomes.[5-8] Against this background, living donor liver transplantation (LDLT) has recently emerged as a

Abbreviations: LDLT, living donor liver transplantation; HCC, hepatocellular carcinoma; MC, Milan criteria; MELD, model for end-stage liver disease; TNM, tumor, node, metastasis; JIS, Japan Integrated Staging.

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new therapeutic option. Contrasting sharply with deceased donor liver transplantation, which utilizes a scarce public resource subject to an equitable allocation system, living donor liver grafts are dedicated to related recipients. LDLT thus allows the extension of selection criteria, since the acceptable recurrence rate is not absolute, but rather depends on donor organ availability.[9] However, LDLT involves significant disadvantages, including risks to the live donor and higher perioperative morbidity and mortality compared with other treatment modalities. Given these risks, the argument has been made that early-stage HCC should initially be managed using resection or conventional methods if comparable overall survival is available, with LDLT held in reserve as a second-line option.[10,11]

In February 1999, we started an LDLT program for patients with HCC using our own selection criteria that included tumors exceeding MC. We have previously reported initial results for 56 patients at a median follow-up of 11 months.[12] The present study reported extended results for 93 patients with HCC over a longer follow-up period. Risk factors for posttransplant mortality and recurrence were analyzed. The role of LDLT was investigated with regard to treatment strategies for cirrhotic patients with HCC.

PATIENTS AND METHODS

Patients

Patients with HCC met both of the following selection criteria for LDLT, approved by the ethics committee at Kyoto University: 1) HCC not suitable for resection or local ablation therapies due to advanced tumor spread, repeated uncontrolled recurrence or poor liver function reserve, and 2) exclusion of extrahepatic metastasis or macroscopic venous invasion on preoperative imaging. No restrictions were placed on the number or size of tumors. All patients were evaluated for the extent of tumor involvement using abdominal ultrasonography, contrast-enhanced computed tomography, brain and chest CT and bone scintigraphy.

Between February 1999 and September 2004, a total of 93 patients (64 men, 29 women) with HCC underwent LDLT at Kyoto University. During the same period, 51 patients were referred for but unable to undergo LDLT to our hospital. Reasons preventing LDLT included macroscopic vascular invasion (n = 30) or extrahepatic metastasis (n = 15) detected on imaging immediately before operation, no available donor (n = 3), and severely deteriorated patient condition (n = 3). Median age for the 93 patients who underwent LDLT was 54 years (range, 22-69 years). A total of 55 patients (59%) were hepatitis C virus antibody-positive, and 30 (32%) were hepatitis B surface antigen-positive, including 2 patients with coinfection. Child-Turcotte-Pugh classification was C for 44 patients (47%), B for 34 patients (37%), and A for 15 patients (16%). Median model for end-stage liver disease (MELD) score was 14 (range, 4-36). Of the 93 patients, 73 patients (78%) displayed a history of previous treatment for HCC using various nontransplant methods including transarterial chemoembolization (n = 63), percutaneous ethanol injection or radiofrequency ablation (n = 47), or hepatic resection (n = 11). These treatments were performed in other hospitals not as a bridge to transplant, but with intent for curative ablation before referral to our institute. The remaining 20 patients (22%) had no history of HCC treatment before LDLT, in most cases due to advanced liver dysfunction.

All 93 patients were diagnosed with HCC immediately before LDLT. Tumor staging was determined by counting only viable and enhancing nodules on pretransplant CT. For patients who had undergone previous therapies, resected tumors or nodules that were judged as nonviable after percutaneous ethanol injection, radiofrequency ablation, or transarterial chemoembolization were not counted. According to the tumor, node, metastasis (TNM) staging criteria of the International Hepato-Pancreato-Biliary Association and the Liver Cancer Study Group of Japan,[13] HCC was classified as stage I (n = 14), II (n = 35), III (n = 42) or IV-A (n = 2). A total of 49 patients (53%) met MC according to preoperative imaging, while 44 patients did not.

Of the 73 patients who had a history of previous nontransplant treatment, records of tumor stage at first diagnosis of HCC were available for 58 patients: stage I for 24 patients, stage II for 17 patients, stage III for 16 patients, and stage IV-A for 1 patient, according to TNM classification. While 42 patients met MC, 16 patients did not. Median period between first diagnosis of HCC and LDLT was 26 months (range, 2-168 months) in these 58 patients. At the time of LDLT, tumor stage was up-staged in 31 patients and 18 patients, down-staged in 5 and 6, and unchanged in 22 and 34, according to TNM classification and MC, respectively.

The Japan Integrated Staging (JIS) score combines the Child-Turcotte-Pugh classification and TNM staging by the Liver Cancer Study Group of Japan, and it has recently been proposed as a new prognostic staging system for patients with HCC.[14] JIS score is obtained by adding tumor stage score (stage I, 0; stage II, 1; stage III, 2; stage IV, 3) and Child-Turcotte-Pugh score (Child-Turcotte-Pugh A, 0; B, 1; C, 2). JIS score in the 93 patients was 0 (n = 2), 1 (n = 9), 2 (n = 26), 3 (n = 40) or 4 (n = 16).

LDLT was performed using a right lobe graft for 92 patients and a left lobe graft for 1 patient. Operative procedures for donor and recipient surgery have been described elsewhere.[15,16] Donors comprised 59 men and 44 women, with a median age of 41 years (range, 19-64 years). ABO blood-type matching was incompatible in 13 cases. Median graft-to-recipient body weight ratio was 1.03 (range, 0.73-1.69). As of the end of June 2005, median follow-up period was 24 months (range, 1-76 months).

Immunosuppression

The standard immunosuppression protocol comprised tacrolimus and low-dose steroid.[17] However, 19 patients received steroid-free tacrolimus monotherapy as

TABLE 1. Preoperative Variables and Patient Survival (Univariate Analysis)

Variables		n	Number of Deaths*	4-Year Survival Rate	P
Recipient age	≥60 years	23	1/3	80%	0.1501
	<60 years	70	9/16	59%	
Recipient gender	Male	64	7/12	65%	0.6229
	Female	29	3/7	60%	
Child-Turcotte-Pugh	A or B	49	7/9	61%	0.8806
	C	44	3/10	68%	
MELD	≥15	45	2/16	74%	0.1447
	≤14	48	8/3	53%	
Viral hepatitis	HBV	30	3/4	75%	0.4505
	HCV	55	5/12	62%	
Previous treatment	Positive	73	8/13	67%	0.2902
	Negative	20	2/6	52%	
Previous hepatectomy	Positive	11	1/2	68%	0.7421
	Negative	82	9/17	63%	
Donor age	≥50 years	32	7/4	56%	0.7590
	<50 years	61	3/15	68%	
ABO mismatch	Yes	13	2/4	23%	0.1305
	No	80	8/15	68%	
Graft-to-recipient body weight ratio.	≥1.0%	53	6/9	65%	0.6670
	<1.0%	40	4/10	62%	
Immunosuppression	With steroid	74	9/15	61%	0.5036
	Without steroid	19	1/4	72%	

*Number of dead patients: HCC-related death/HCC-nonrelated death.

an induction procedure in an attempt to reduce HCC recurrence. Patients who received ABO blood-type incompatible transplants were treated with preoperative plasma exchange or double filtration plasmapheresis to reduce anti-A or anti-B antibody titer. During the first 3 weeks postoperatively, prostaglandin E1 and additional steroids were administered via the portal vein or hepatic artery.[18] Acute rejection episodes were documented based on liver histology and treated with steroid bolus if moderate or severe.

Statistical Analysis

Differences in qualitative variables were assessed using the Fisher exact or chi-square test, while differences in quantitative variables were analyzed using the Mann-Whitney test. Cumulative probability curves of survival or HCC recurrence were calculated using Kaplan-Meier methods, and differences between these curves were compared using the log-rank test. Any variable identified as significant ($P < 0.05$) in univariate analysis using the log-rank test was considered a candidate for multivariate analysis with Cox's proportional hazard regression model. Values of $P < 0.05$ were considered statistically significant. All statistical analyses were performed using the StatView 5 statistical software package (Abacus Concepts, Berkeley, CA).

RESULTS

Patient Survival

As of the end of June 2005, a total of 64 patients remained alive. Cause of death included recurrent HCC (n = 10), sepsis (n = 7), pneumonia (n = 5), peritonitis (n = 4), chronic rejection (n = 1), and other tumor-unrelated causes (n = 2). Overall patient survival rate at 4 years was 64%. Survival rate tended to be better for hepatitis B surface antigen-positive patients (75%) than for hepatitis C virus-positive patients (62%), although no significant difference was identified ($P = 0.4505$). None of the recipient and donor variables listed in Table 1, including MELD score, history of pretransplant treatment, or ABO blood-type compatibility, were significantly associated with long-term postoperative survival rate.

Analysis of preoperative tumor characteristics and patient 4-year survival (Table 2) revealed significantly lower survival rates in patients with tumors >5 cm in diameter than in patients with smaller tumors (20% vs. 69%, respectively; $P = 0.0259$). Unexpectedly, survival rates were similar for patients who met MC and those who did not (68% vs. 59% at 4 years, respectively; $P = 0.6548$) (Fig. 1). Patients with TNM stage III also tended to display poorer survival rates than patients with less advanced tumors, although no significant difference was identified (Table 2). With respect to JIS score, patients with a JIS score of 4 showed the lowest survival rate (58%), but no significant association was observed between JIS score and survival rate.

Recurrence of HCC

As of the time of writing, postoperative recurrence of HCC had occurred in 16 patients. First sites of recurrence comprised lung (n = 5), bone (n = 3), graft liver

TABLE 2. Univariate Analysis of Patient Survival and Recurrence Rate According to Preoperative Tumor Characteristics

Variables		n	4-Year Survival Rate	P	4-Year Recurrence Rate	P
Tumor size	>5 cm	12	20%	0.0259	67%	0.0001
	≤5 cm	81	69%		19%	
Tumor number	≤3	60	66%	0.7188	21%	0.1466
	≥4	33	61%		32%	
Milan criteria	Meet	49	68%	0.6548	15%	0.0190
	Exceed	44	59%		35%	
Distribution	Unilobar	44	64%	0.7064	25%	0.5465
	Bilobar	49	64%		26%	
TNM stage*	I	14	75%	0.1967	20%	0.2024
	II	35	77%		16%	
	III	42	49%		33%	
	IVA	2	100%		50%	
JIS score	0	2	50%	0.9513	50%	NA
	1	9	78%		0%	
	2	26	69%		29%	
	3	40	62%		25%	
	4	16	58%		16%	
α-fetoprotein (ng/mL)	≥400	24	50%	0.2365	46%	0.0015
	<400	69	69%		17%	

Abbreviations: TNM, tumor, node, metastasis; NA, not applied.
*TNM stage: T factors are (1) single, (2) <2 cm, and (3) no vascular involvement. T1, fulfilling 3 factors; T2, fulfilling 2 factors; T3, fulfilling 1 factor; T4, fulfilling 0 factors. Stage I, T1N0M0; Stage II, T2N0M0; Stage III, T3N0M0; Stage IV-A, T4N0M0 or any T N1M0; Stage IV-B, any T N0-1M1.

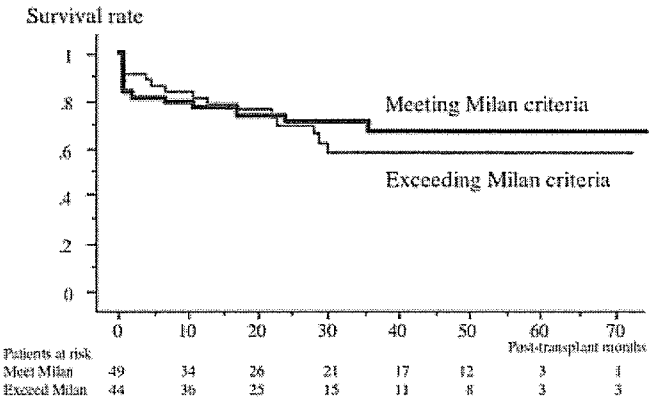

Figure 1. Patient survival according to MC. Survival rates at 4 years after LDLT were similar for patients who met MC (n = 49, 68%) and for patients who exceeded MC (n = 44, 59%; P = 0.6548).

(n = 3), abdominal lymph nodes (n = 2), adrenal gland (n = 1), brain (n = 1), and right subphrenic space (n = 1). After excluding death without recurrence, overall cumulative recurrence rate was 25% at 4 years.

Univariate analysis of risk factors for recurrence showed that recurrence rates did not differ significantly between hepatitis B surface antigen- and hepatitis C virus-positive patients (32% vs. 19%, respectively; $P = 0.3416$) or patients with and without steroid-free induction therapy (6% vs. 31%, respectively; $P = 0.1076$). Among preoperative tumor variables determined by imaging studies (Table 2), tumor diameter >5 cm, exceeding the MC and serum α-fetoprotein levels ≥400 ng/mL all represented significant predictors of higher recurrence rate. Recurrence rate was significantly higher in patients who exceeded MC than in patients who met these criteria (35% vs. 15%, respectively; $P = 0.0190$) (Fig. 2). However, multivariate analysis revealed that none of these variables represented significant independent risk factors (data not shown).

MC at First Diagnosis of HCC and Rates of Patient Survival and Recurrence after LDLT

For the 58 patients who had a history of previous nontransplant treatment for HCC and for whom records of tumor stage prior to any treatment were available, patient survival and HCC recurrence rates after LDLT were determined according to MC based on imaging at first diagnosis of HCC. Survival rates were similar for patients who had met MC (n = 42) and those who had not (n = 16) (71% vs. 69% at 4 years after LDLT, respectively; $P = 0.8259$) (Fig. 3A). Conversely, recurrence rate tended to be higher in patients who had exceeded MC than in patients who had met these criteria, although no significant difference was identified (37% vs. 13%, respectively; $P = 0.0699$) (Fig. 3B).

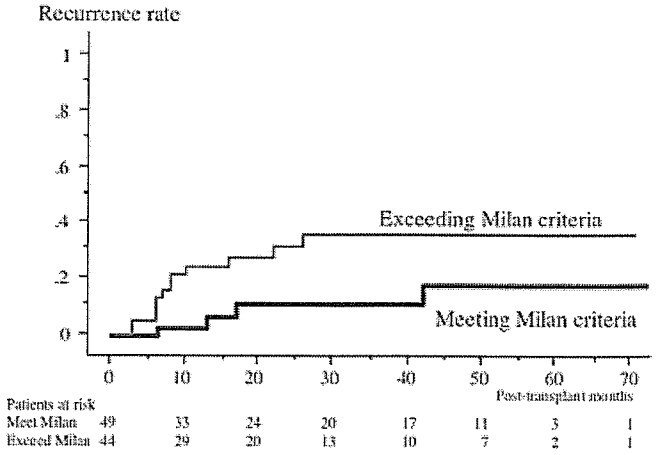

Figure 2. Cumulative recurrence rate according to MC. Cumulative HCC recurrence rate after LDLT was significantly higher for patients who exceeded MC (n = 44, 35%) than for patients who met MC (n = 49, 15%; P = 0.0190).

Pathological Findings and Recurrence

Correspondence of preoperative MC with the results of pathological analysis was evaluated in postoperative examinations of explanted livers. Completely ablated foci (100% necrosis) following pretransplant treatments were not counted as tumor. On pathological examination, 39 patients met so-called "pathological MC" and 54 did not. Accordingly, preoperative imaging underestimated the diagnosis in 13 patients (14%) and overestimated in 3 patients (3%). Of the 13 underestimated patients, HCC recurred in 4 patients. Univariate analysis of pathological findings (Table 3) revealed that tumor diameter >5 cm, tumor number ≥4, tumor grade indicating poor differentiation, and positive microvascular invasion were all significantly associated with postoperative recurrence. Using Cox's multivariate analysis (Table 4), pathological tumor number and poor differentiation were identified as independent risk factors for recurrence.

Effects of Previous Treatment Before LDLT

Patients were divided into 3 groups based on history of conventional treatment for HCC before LDLT: patients who primarily received LDLT without previous treatment (Group 1, n = 20); patients with 1-2 treatments (Group 2, n = 30); and patients with ≥3 treatments (Group 3, n = 43). Median period between first diagnosis of HCC and LDLT was 3 months in Group 1 (range, 1-15 months), 14 months in Group 2 (range, 2-70 months), and 36 months in Group 3 (range, 4-168 months). Preoperative liver function was most deteriorated in Group 1. Mean (± SD) preoperative MELD score was significantly higher in Group 1 (21 ± 8) than in Groups 2 (16 ± 7, $P = 0.0414$) or 3 (12± 6, $P = 0.002$). Conversely, the proportion of patients who exceeded MC before LDLT was significantly higher in Group 3 (63%) than in Groups 1 (35%) or 2 (33%, $P = 0.020$).

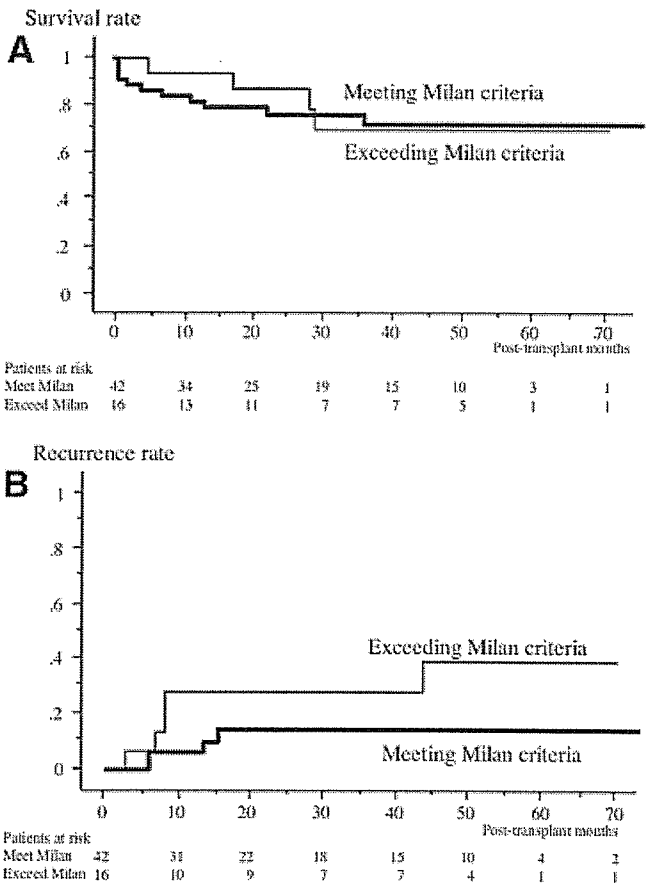

Figure 3. Survival and recurrence rates according to MC at first diagnosis of HCC.For the 58 patients who had a history of previous nontransplant treatment for HCC and for whom records of tumor stage prior to any treatment were available, patient survival and HCC recurrence rates after LDLT were determined according to MC based on imaging at first diagnosis of HCC. (A) Survival rates were similar for patients who had met MC (n = 42) and those who had not met MC (n = 16) (71% vs. 69% at 4 years after LDLT, respectively; P = 0.8259). (B) Recurrence rates tended to be higher in patients who had exceeded MC than in patients who had met these criteria, although differences were not significant (37% vs. 13%, respectively; P = 0.0699).

Based on postoperative examinations of explanted livers, microscopic venous invasion was significantly more frequent in Group 3 (51%) than in Groups 1 (21%) or 2 (33%, $P = 0.0382$). Postoperatively, HCC recurrence occurred in 3 patients in Group 1, 3 patients in Group 2, and 10 patients in Group 3. Survival rates at 4 years tended to be better for Group 2 (80%) than for Groups 1 (52%) and 3 (58%), although these differences were not significant ($P = 0.0651$ and $P = 0.1042$, respectively; Fig. 4A). Recurrence rates at 4 years were 23% for Group 1, 9% for Group 2, and 37% for Group 3. Rates were significantly lower for Group 2 than for Group 3 ($P = 0.0411$; Fig. 4B).

DISCUSSION

The present study showed that advanced stage of tumors in terms of tumor size, number, and α-fetoprotein

TABLE 3. Univariate Analysis of Pathological Tumor Characteristics and Recurrence Rate

Variables		n	4-Year Recurrence Rate	P
Tumor size	>5 cm	14	55%	0.0031
	≤5 cm	79	19%	
Tumor number	≤3	48	5%	0.0004
	≥4	45	49%	
Differentiation	Well	14	0%	
	Moderate	59	23%	0.0001*
	Poor	20	60%	
Microscopic venous invasion	Positive	37	42%	0.0026
	Negative	56	14%	

*Comparison between "Moderate" and "Poor."

TABLE 4. Multivariate Analysis of Pathological Tumor Factors and Recurrence

Variables		Risk Ratio	95% Confidence Interval	P
Tumor number	≤3	1		
	≥4	7.917	1.799-20.000	0.002
Differentiation	Well or Moderate	1		
	Poor	5.618	1.712-18.519	0.003

levels on preoperative evaluation is associated with increased postoperative recurrence. These results corroborate the findings of previous studies on deceased donor liver transplantation.[19-22] In a study by Gondolesi et al.[23] of 36 patients with HCC treated using LDLT, tumor size >5 cm showed no significant effect on recurrence. Similarly, in our preliminary report on 56 patients,[12] recurrence rate did not differ between patients within and beyond MC. However, as patient numbers increased, the present study demonstrated that patients with tumors >5 cm in diameter experienced significantly higher rates of recurrence and mortality. In addition, patients who exceeded MC displayed significantly higher risk of recurrence.

Of note was the finding that patient survival rates were similar for patients who met MC and for patients who did not (Fig. 1). This discrepancy may be due to a short follow-up period, but it can also be explained by HCC-unrelated deaths due to postoperative complications occurring within a few months after LDLT. Infectious complications such as sepsis, pneumonia, and peritonitis were the most common causes of early mortality. As a rule, patients with early HCC were referred to our hospital for LDLT due to advanced liver cirrhosis. Mortality rates within 3 months were 18% for those within MC and 9% for those beyond MC, which may be associated with poorer preoperative condition for the former group as reflected by higher MELD score (18 ± 8 vs. 13 ± 6, respectively; $P = 0.002$). Likewise, most patients who primarily received LDLT without previous treatment (Group 1, Fig. 4) were characterized by higher MELD scores and less advanced tumors. Survival rates for these patients were compromised by HCC-unrelated death due to perioperative complications.

In the present study, 78 patients had received nontransplant treatments for HCC prior to LDLT. For these patients, tumor staging was determined by counting only viable and enhancing nodules on pretransplant computed tomography. Pretransplant tumor staging thus differed from that at first diagnosis of HCC. In the 58 patients for whom records of tumor stage prior to any treatment were available, 6 patients with tumors originally beyond MC had been down-staged with treatment and were assigned to the within-MC group before LDLT. Conversely, 18 patients were up-staged before LDLT. In an attempt to analyze outcomes according to primary tumor staging, survival and recurrence curves were determined for patients meeting and patients exceeding MC based on imaging at first diagnosis of HCC for the 58 patients (Fig 3). While patient survival rates did not differ significantly, recurrence rate tended to be higher in patients who had originally exceeded MC than in patients who had met MC. However, these differences were not significant, probably due to small number of patients involved.

Another controversy remains regarding indications for patients with early HCC. On the one hand, use of LDLT has been proposed for patients with early HCC accompanied by early-stage cirrhosis (Child-Turcotte-Pugh A), as a reasonable survival rate should be expected in weighing the risk of live donation.[24] Conversely, risking the life of a live donor for a patient who has alternative options of hepatic resection or other curative treatments with comparable long-term survival is unlikely to be ethically acceptable.[25] Patient

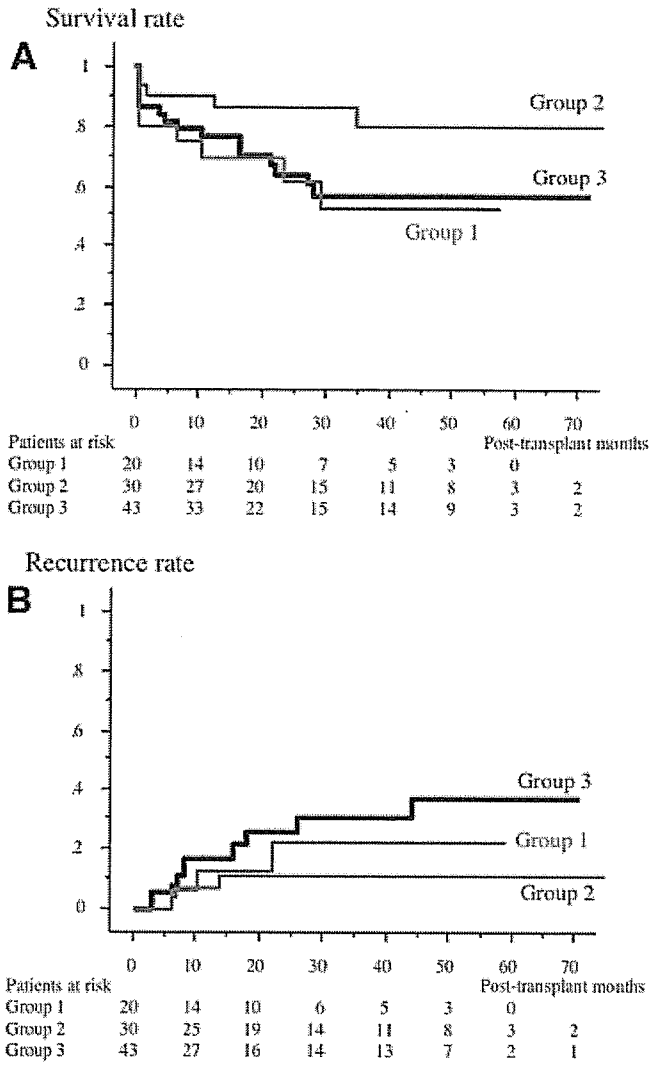

Figure 4. Survival and recurrence rates according to history of previous HCC treatment. According to history of conventional treatment for HCC before LDLT, patients were divided into 3 groups: Group 1, patients without previous treatment (n = 20); Group 2, patients with 1-2 treatments (n = 30); and Group 3, patients with ≥3 treatments (n = 43). (A) Survival rates at 4 years tended to be better for Group 2 (80%) than for Groups 1 (52%) and 3 (58%), although differences were not significant ($P = 0.0651$ and $P = 0.1042$, respectively). (B) Recurrence rate at 4 years was 23% for Group 1, 9% for Group 2, and 37% for Group 3. Recurrence rate was significantly lower for Group 2 than for Group 3 ($P = 0.0411$).

selection in our hospital is based on the latter policy: Patients with HCCs considered unsuitable for resection or local ablation therapies have been included in our program. In preoperative evaluations, a system for precise prognostic staging is essential for comparing outcomes between groups undertaking different therapeutic trials. Kudo et al.[14] recently reported that JIS score offers a better system of prognostic staging for HCC than previous systems, in terms of both stratification ability and prognostic predictive power. In an analysis of 4,525 patients with HCC who received various conventional therapies, including hepatic resection, percutaneous ablation therapies and transarterial chemoembolization, patient survival was clearly stratified according to JIS score. The 5-year survival rates for patients were 73% for JIS 0 (n = 552), 52% for JIS 1 (n = 1,399), 33% for JIS 2 (n = 1,471), 13% for JIS 3 (n = 757) and 2% for JIS 4 (n = 244).[14] This score system has not yet been validated outside Japan,[26] but it was applied to our series of LDLT recipients. As a result, both 4- and 5-year survival rates were 58%, even in JIS 4 patients. Although these figures should be carefully compared, LDLT may result in improved prognosis for patients with a JIS score ≥2.

Conversely, for patients with early HCC and preserved liver function (that is, JIS score of 0 or 1), hepatic resection or ablation therapies would represent the treatments of choice. However, high rates of recurrence occur even after curative treatment,[27] and the role of secondary or salvage transplantation has recently been discussed.[24,25,28,29] Although prior hepatic resection may complicate the operative transplant procedure and increase the risk of postoperative complications,[24] postoperative survival in the 11 patients who received hepatic resection before LDLT was comparable to survival in the other 82 patients in this series (Table 1). On the other hand, transplantability at the time of recurrence is supposedly limited due to advanced tumor extension.[24] Even for patients who are considered eligible for salvage LDLT, the present study revealed significantly higher recurrence rates for patients with a history of ≥3 treatments for HCC before LDLT (Group 3) than for patients with only 1-2 treatments (Group 2, Fig. 4B). This result implies that repeated nontransplant treatments for recurrent HCC may increase the risk of microscopic vascular invasion and impair the survival advantages conferred by LDLT. For patients who develop HCC recurrence after conventional therapies, feasibility, optimal timing, and efficacy of LDLT as a second-line treatment should be determined in further studies.

In conclusion, although efforts to decrease early mortality due to surgical complications are essential to improve outcomes for LDLT, this technique may constitute an optional treatment with a chance of cure for patients displaying otherwise uncontrolled HCC. Patients who develop recurrent HCC should be referred for LDLT before repeated nontransplant therapies, if a living donor is available.

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Identification of novel defective HCV clones in liver transplant recipients with recurrent HCV infection

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SUMMARY. Patients with recurrent hepatitis C after liver transplantation usually have a high viral load and are generally resistant to interferon (IFN)-α2b plus ribavirin (RBV) therapy. However, it remains unclear whether pretreatment viral titre determines the effectiveness of combination therapy, especially in patients with a high viral load. The aim of this study was to identify the viral factors associated with a sustained virological response (SVR) to antiviral therapy in patients with recurrent hepatitis C after living-donor liver transplantation. Twenty-three patients with recurrent hepatitis C received combination therapy of IFN-α2b plus RBV. SVR was achieved in 7 of the 23 patients (30.4%). Predictive factors for SVR included a 2 $\log_{10}$ decline in Hepatitis C virus (HCV) RNA at 2 weeks after the start of therapy and disappearance of HCV RNA at 4 or 24 weeks after the start of therapy. As the pretreatment high viral load showed no association with SVR, we asked whether other viral factor was associated with the response to the combination therapy in transplant recipients. We found the several novel defective HCV clones in 4 of 12 recipients' sera. All defective HCV clones had deletions in the envelope region. Interestingly, no patients with defective clones showed a prompt decrease in HCV RNA after the start of IFN-α2b plus RBV therapy. Thus, early decline in serum HCV RNA after treatment was closely associated with SVR. The circulating defective HCV clones are present and might be associated with the response to the combination therapy in patients with recurrent hepatitis after liver transplantation.

Keywords: hepatitis C virus genome, interferon, liver transplantation, ribavirin, sustained virological response.

INTRODUCTION

Hepatitis C virus (HCV) infection is one of the leading causes of end-stage liver disease; thus, HCV-related chronic liver disease is a common indicator for liver transplantation. However, recurrent hepatitis in the liver allograft develops in most recipients [1]. Unlike immunocompetent individuals, HCV recurrence in patients who receive a liver transplant results in severe liver damage at an accelerated rate, leading to recurrent cirrhosis within 5 years [2,3]. Moreover, several investigators have shown that HCV recurrence is more severe in living-donor liver transplantation (LDLT) than in orthotopic liver transplantation (ORLT) [4,5]. LDLT provides the only access to donor organs in countries like Japan, where cadaveric donors are not available. Therefore, establishment of highly effective treatment strategies for recurrent hepatitis C after liver transplantation is strongly required.

Hepatitis C in transplant recipients differs from that in immunocompetent individuals in several aspects [6]. Most recipients have extremely high viral loads as a result of their immunosuppressive conditions [6]. HCV baseline viral load is an independent factor associated with poor response to interferon (IFN)-α2b plus ribavirin (RBV) combination therapy [7,8]. Thus, liver transplant patients with recurrent hepatitis C are generally resistant to antiviral treatment. In fact, sustained virological response (SVR) in ORLT recipients by IFN-α2b plus RBV combination therapy has only been observed in up to 30% of patients [9,10]. Thus, resistance to

Abbreviations: IFN, interferon; RBV, ribavirin; SVR, sustained virological response; LDLT, living-donor liver transplantation; HCV, hepatitis C virus; ORLT, orthotopic liver transplantation; PMN, polymorphonuclear; HCC, hepatocellular carcinoma; HLA, human leukocyte antigen; MU, million units; RT-PCR, reverse transcriptase-polymerase chain reaction; HBV, hepatitis B virus; 5′-UTR, 5′-untranslated region; NS, nonstructural protein; E1, envelope glycoprotein-1; E2, envelope glycoprotein-2.

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IFN-α2b plus RBV combination therapy in recipients with HCV recurrence is a challenging obstacle at present. Therefore, the aim of this study was to determine viral factors other than genotype 1b and high viral load that influence responsiveness to IFN-α2b plus RBV combination therapy in patients with recurrent hepatitis C after LDLT.

MATERIALS AND METHODS

Patients

Between March 1999 and December 2004, 100 patients underwent LDLT at Kyoto University Hospital as a result of end-stage liver disease caused by HCV infection. Recurrence of chronic active hepatitis C was histologically confirmed on a biopsy sample in 49 patients. Indicators for IFN-α2b plus RBV combination therapy in these patients included high serum values of alanine aminotransferase (above upper limits of normal >27 IU/L), with haemoglobin levels >8 g/dL, total polymorphonuclear (PMN) counts >1500/mm^3, platelet counts >50 000/mm^3, normal renal function (serum creatinine <1.5 mg/dL and/or creatinine clearance >50 mg/mL) and no hepatocellular carcinoma (HCC) recurrence. Of 49 patients, 30 received treatment with IFN-α2b plus RBV combination therapy (Schering-Plough, Kenilworth, NJ, USA). Among them, 23 patients, who were sequentially monitored at the same hospital throughout the treatment period and 24 weeks after cessation of treatment, were enrolled for further analyses. At the time of entry, liver biopsy specimens were assessed by an experienced hepatopathologist using the METAVIR score; the fibrosis stage was defined as F0 (no fibrosis), F1 (mild fibrosis), F2 (moderate fibrosis), F3 (severe fibrosis) and F4 (cirrhosis) [11]. The HCV genotype was determined using a commercially available reverse transcriptase-polymerase chain reaction (RT-PCR) assay that can distinguish genotypes 1a, 1b, 2a, 2b, 3a and 3b of HCV (Monitor HCV Coregenotyping; SRL, Tokyo, Japan). Serum HCV RNA was measured before, at 2, 4, 12, 24 and 48 weeks during therapy and every 4 weeks after the end of therapy, using the Roche Amplicor HCV 2.0 assay (Roche Diagnostics, Branchburg, NJ, USA). Serum samples were diluted and retested by the same assay, when serum HCV RNA load was over 850 kIU/mL. All patients received tacrolimus-based immunosuppression [12]. The ethics committee of Kyoto University approved the studies, and informed consent for participation in the study was obtained from all patients.

Typing of human leukocyte antigen class

We performed human leukocyte antigen (HLA) typing by PCR amplification with MicroSSP HLA DNA typing trays (One Lambda, Canoga Park, CA, USA) according to the manufacturer's instructions in patients. The amplified DNA was electrophoresed and visualized on ethidium-bromide-stained 2% agarose gels. HLA analysis included 17 HLA-A, 19 HLA-B and 16 HLA-DR alleles.

Study design

Patients were treated with 6 million units (MU) or 3 MU of recombinant IFN-α2b three times a week for 48 weeks (3 MU IFN-α2b was used when platelet counts were <100 000/mm^3 or PMN counts were <2000/mm^3 before therapy) plus oral RBV for the first 24 weeks. RBV dosage was based on the weight of each patient: patients less than 60 kg were administered 600 mg, and patients greater than 60 kg received 800 mg. IFN dosage was reduced to 3 MU if platelet counts dropped to <100 000/mm^3 or if PMN counts dropped to <2000/mm^3 during therapy. When haemoglobin levels before treatment were less than 10 g/dL, RBV dosage was reduced to 400 mg in patients weighing less than 60 kg and to 600 mg in patients weighing more than 60 kg. The combination therapy was discontinued when haemoglobin levels, total PMN counts or platelet counts were observed to be less than the eligible levels mentioned above during therapy. SVR was defined as no detectable HCV RNA by qualitative assay at least 24 weeks after cessation of therapy.

Detection of defective HCV RNA

Serum samples for the detection of defective HCV RNA were taken before the start of IFN-α2b and RBV administration. Total RNA was extracted from 250 μL of serum using Sepasol® RNA II super (Nacalai Tesque, Kyoto, Japan), according to the manufacturer's protocol. The extracted RNA was resuspended in 10 μL of RNase-free water and 5 μL of each RNA sample was used in each reaction. RT-PCR was performed using the One Step RNA PCR Kit (Takara, Tokyo, Japan), as described earlier [13]. The RT-PCR primers, which were designed from the reference sequence of HCV genotype 1b (GenBank Accession No. #D90208), were shown in Table 1. All PCR products were analysed by electrophoresis in 1.2% agarose gels stained with ethidium bromide. Subcloning of purified DNA was performed using the pGEM-T easy vector (Promega, Madison, WI, USA) [14]. At least 30 colonies on each plate were picked using white/blue colony selection, and plasmid DNA was extracted and then purified by ethanol precipitation. Each purified DNA sample was sequenced at least three times using an ABI Prism Big Dye Terminator Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) [14]. To determine the defects in the HCV genome, the sequence of each sample was compared with the registered HCV genome sequence, as described above. The serum of a healthy HCV-RNA-negative volunteer was used as a negative control. The full RNA genome of HCV was synthesized *in vitro* from the pM1LE plasmid (inserted genotype 1b sequence of HCV full genome) using a MEGAscript T7 Kit (Ambion, Austin, TX, USA) and used as a positive control [15].

