Chronic Rejection Associated with Antiviral Therapy for Recurrent Hepatitis C after Living-Donor Liver Transplantation

Yoshihide Ueda,[1,4] Toshimi Kaido,[2] Takashi Ito,[2] Kohei Ogawa,[2] Atsushi Yoshizawa,[2] Yasuhiro Fujimoto,[2] Akira Mori,[2] Aya Miyagawa-Hayashino,[3] Hironori Haga,[3] Hiroyuki Marusawa,[1] Tsutomu Chiba,[1] and Shinji Uemoto[2]

Background. Chronic rejection (CR) has been reported to be associated with antiviral therapy for recurrent hepatitis C in liver transplant (LT) recipients. The aims of this study were to clarify the details of antiviral therapy-associated CR after living-donor liver transplantation (LDLT) and to identify the factors associated with CR.

Methods. A retrospective chart review was performed on 125 recipients who had received antiviral therapy for recurrent hepatitis C after LDLT between January 2001 and September 2012. The characteristics of patients who developed CR during or within 6 months after antiviral therapy were compared with those of 76 patients who did not develop CR despite receiving antiviral therapy for more than 1 year.

Results. Seven of 125 (6%) patients developed CR during or within 6 months after the end of antiviral therapy. CR was diagnosed after a median (range) of 9 (1–16) months of antiviral therapy. In five patients, rejection progressed rapidly and resulted in death within 3 months after diagnosis. Analysis revealed two significant factors associated with CR: reduction of the immunosuppressant dose during antiviral therapy and a low fibrosis score as the indication for antiviral therapy.

Conclusions. CR developed in association with antiviral therapy for recurrent hepatitis C after LDLT. This complication may be prevented by ensuring that the immunosuppressant dose is not reduced during antiviral therapy.

Keywords: Chronic rejection, Hepatitis C, Liver transplantation, Living donor, Antiviral therapy.

(*Transplantation* 2014;97: 344–350)

Hepatitis C virus (HCV) infection, which leads to liver cirrhosis and hepatocellular carcinoma, is the most common indication for liver transplantation (LT) in Japan, the United States, and western Europe. Most patients who undergo LT for HCV-related liver disease develop recurrent viral infection, and 70% to 90% suffer from histologically proven recurrent hepatitis (*1–6*). The progression of recurrent hepatitis C is often rapid. Without appropriate antiviral therapy, 10% to 25% of patients develop cirrhosis within 5 years after transplantation, and this explains the relatively poor prognosis for HCV-positive recipients compared with HCV-negative recipients (*7*). Interferon (IFN)–based combination therapy is commonly administered to prevent the progression of hepatitis C after LT (*8, 9*), but its efficacy in LT recipients is limited. The mean (range) sustained virologic response (SVR) rate in patients with recurrent hepatitis C after LT is only 30% (8%–50%) (*10*). One reason for the low SVR rate is the high rate of treatment withdrawal, particularly because of the unique adverse effects of IFN therapy for transplant recipients, including chronic rejection (CR) (*11, 12*).

CR is characterized by progressive ductopenia, with atrophy and loss of the bile ducts in the portal tracts and by arteriopathy with foamy cell infiltration (*13–15*). A cholestatic liver enzyme pattern suggests the diagnosis of CR. If bile duct enlargement and/or hepatic artery changes are excluded by imaging studies as potential causes of abnormal liver function tests, then CR is confirmed or excluded by liver biopsy examination. The incidence of CR after LT is

This work was supported by the Japan Society for the Promotion of Science (JSPS) Grants-in-Aid for Scientific Research 21229009 and 23590972, Health and Labor Sciences Research Grants for Research on Intractable Diseases, and Research on Hepatitis from the Ministry of Health, Labor and Welfare, Japan.
The authors declare no conflicts of interest.

[1] Department of Gastroenterology and Hepatology, Graduate School of Medicine, Kyoto University, Kyoto, Japan.
[2] Department of Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan.
[3] Department of Diagnostic Pathology, Graduate School of Medicine, Kyoto University, Kyoto, Japan.
[4] Address correspondence to: Yoshihide Ueda, M.D., Ph.D., Department of Gastroenterology and Hepatology, Graduate School of Medicine, Kyoto University, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan.
E-mail: yueda@kuhp.kyoto-u.ac.jp

Y.U. participated in the research design, researched relevant data, performed the data analysis, and wrote the article. T.K., T.I., K.O., A.Y., Y.F., A.M., A.M.-H., and H.M. researched relevant data. H.H., T.C., and S.U. participated in the research design and article review.
Received 5 August 2013. Revision requested 13 August 2013.
Accepted 28 August 2013.
Copyright © 2013 by Lippincott Williams & Wilkins
ISSN: 0041-1337/14/9703-344
DOI: 10.1097/01.TP.0000435702.61642.0a

Copyright © 2014 Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

approximately 3% to 5%. This event does not simply represent end-stage acute cellular rejection (ACR), although the two may be temporally related. The pathogenesis of CR is not completely understood, although its association with donor-specific human leukocyte antigen antibodies was recently reported (*16*). Additional immunosuppressive therapy is unlikely to be beneficial for CR patients, particularly those with late disease in which bile duct loss affects more than 50% of the portal tracts, and retransplantation is required (*15*).

Several studies have suggested an association of CR with IFN-based antiviral therapy (*17–20*). Two recent reports found that CR was associated with antiviral therapy for recurrent hepatitis C after LT (*11, 12*). Stanca et al. (*12*) reported that 12 of 70 LT recipients with HCV infection treated with pegylated IFN (peg-IFN) and ribavirin developed CR. Their study indicated that ACR and CR are not strongly associated and that CR progresses rapidly, terminating in graft failure. Fernandez et al. (*11*) reported that 7 of 79 (9%) patients developed CR during antiviral therapy. They found that the use of cyclosporine in immunosuppression therapy, achievement of an SVR, and ribavirin discontinuation were factors associated with CR development.

Although the details of patients with antiviral therapy-associated CR after deceased-donor liver transplantation (DDLT) have been reported (*11, 12*), no study of antiviral therapy-associated CR in patients receiving living-donor liver transplantation (LDLT) has been published thus far. The features specific to LDLT, including blood-relative donors, posttransplantation liver regeneration, and ABO-incompatible LT, might result in characteristic differences between LDLT and DDLT patients.

We aimed to clarify the details of antiviral therapy-associated CR after LDLT and to identify the factors associated with CR.

RESULTS

Patient Characteristics and Treatment Outcomes

The study included 125 HCV-infected LT patients treated with standard IFN and/or peg-IFN in combination with ribavirin for recurrent hepatitis C after LDLT. Of these, 69 (55%) were men (median [range] age at the beginning of therapy, 57 [32–70] years). Most patients were infected with HCV genotype 1b (n=101 [81%]). The HCV genotype for the remaining patients was 2a (n=14), 2b (n=6), 3a+3b (n=1), and indeterminate (n=2). Genotype was not examined in one patient. The median (range) serum HCV RNA load at the beginning of antiviral therapy after LDLT was 3980 (31 to <69,000) kIU/mL. The median (range) donor age was 42 (19–65) years. Seventy-three (58%) donors were men, and 84 (67%) were blood relatives of the recipients. The graft type was the right lobe for 108 (86%) patients and the left lobe for 17 (14%) patients. The blood type combination was incompatible for 27 (22%) patients. Thirty-six (29%) patients had histologically diagnosed ACR before antiviral therapy, 16 of whom had moderate or severe ACR. No patient had shown ACR findings in the liver biopsy examination immediately before antiviral therapy. The median (range) time to treatment initiation after LDLT was 8.9 (1.1–72.4) months. Before treatment, necroinflammatory activity of levels A1, A2, and A3 based on the METAVIR score was found in 82 (66%), 40 (32%), and 3 (2%) patients, respectively. Fibrosis scores of F0, F1, F2, and F3 were found in 19 (15%), 82 (66%), 19 (15%), and 5 (4%) patients, respectively. Tacrolimus-based immunosuppression was administered to 117 (94%) patients and cyclosporine was administered to 7 (6%) patients. Mycophenolate mofetil (MMF) without calcineurin inhibitor (CNI) was administered to one patient because of renal failure at the beginning of antiviral therapy. In the patients who received tacrolimus, the mean (range) serum trough level at therapy initiation was 6.2 (2.0–12.7) ng/mL. In addition to CNIs, MMF and prednisolone were administered at the start of the antiviral treatment to 39 (31%) and 21 (17%) patients, respectively.

Of the 123 patients in whom the final treatment outcomes could be evaluated, 54 (44%) patients achieved SVR, 12 (10%) relapsed, 30 (24%) were nonresponders, and 27 (22%) withdrew from treatment. The remaining two patients were still undergoing treatment during the analysis.

Characteristics of Patients with Antiviral Therapy-Associated CR

Seven of 125 (6%) patients developed CR during or within 6 months after the end of antiviral therapy. The characteristics and clinical courses of these seven patients are shown in Table 1. Although four patients had a history of ACR before antiviral therapy was initiated (three of whom had moderate or severe ACR), three had no previous ACR episodes. The METAVIR score-based fibrosis level before antiviral therapy was F0 in three of the seven patients, F1 in three patients, and F2 in one patient, indicating that the antiviral therapy had been initiated at an early stage of fibrosis. The median (range) time from transplantation to initiation of antiviral therapy in these seven recipients was 9 (2–72) months. Tacrolimus was administered to five patients and cyclosporine was administered to one patient when the antiviral therapy was initiated. One patient did not receive a CNI because of renal failure (patient 7). Four patients received MMF, and one patient received prednisolone in combination with tacrolimus and MMF. The trough levels of tacrolimus and cyclosporine were within the therapeutic range. Standard amounts of immunosuppressant were therefore used for all patients, except for patient 7 who received MMF only. Immunosuppressant doses were reduced during therapy in five of seven patients. The tacrolimus dose was reduced for two patients (patients 2 and 3), as a result of which the blood trough level of tacrolimus decreased by approximately 2 ng/mL. In patient 3, MMF (500 mg/day) was also stopped during treatment. In patient 4, the MMF dose was reduced from 1000 to 250 mg per day, and prednisolone treatment (2.5 mg/day) was also terminated during treatment. In patient 5, MMF (1000 mg/day) was stopped immediately after initiation of antiviral therapy. Patient 6 received no CNI, and MMF dose was reduced from 500 to 250 mg per day during treatment. Three patients received standard IFN, and four received peg-IFN. Ribavirin was not administered to three patients immediately before the diagnosis of CR because of anemia.

CR was diagnosed after a median (range) of 9 (1–16) months of antiviral therapy. Two patients were diagnosed

Copyright © 2014 Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

TABLE 1. Characteristics of patients with CR associated with antiviral therapy

Patient	1	2	3	4	5	6	7
Age (years)	62	41	45	67	50	59	49
Gender	Female	Male	Female	Female	Female	Male	Male
ABO mismatch with donor	Match	Match	Match	Mismatch	Match	Mismatch	Match
Relation to donor	Related	Related	Nonrelated	Related	Nonrelated	Nonrelated	Nonrelated
Graft type (lobe)	Right	Right	Right	Right	Left	Right	Right
Splenectomy	No	No	No	No	Yes	Yes	No
Previous ACR	Yes	Yes	Yes	No	Yes	No	No
Previous moderate/severe ACR	Yes	No	Yes	No	Yes	No	No
Previous steroid pulse	Yes	No	No	No	Yes	No	No
HCV genotype	1b	1b	1b	2a	1b	1b	1b
HCV RNA (kIU/mL) before IFN	>850	3620	1790	>5000	<5000	<5000	16,000
METAVIR score before IFN	A2 F2	A2 F0	A1 F0	A1 F1	A2 F1	A1 F0	A1 F1
Months from LT to IFN	13	2	5	13	7	9	72
Months from initiation of IFN to diagnosis of CR	9	1	16	10	15	8	7
Immunosuppressant at initiation of IFN	Tacrolimus	Tacrolimus,	Tacrolimus, MMF	Tacrolimus, MMF, PSL	Cyclosporine, MMF	Tacrolimus, MMF	MMF
Trough level of CNI	7.8	7.9	7.9	6.8	152	5.9	—
Reduction of immunosuppressant during IFN (reduced drugs)	No	Yes (tacrolimus)	Yes (tacrolimus, MMF)	Yes (MMF, PSL)	Yes (MMF)	No	Yes (MMF)
Type of IFN	Standard	Standard	Standard	Pegylated	Pegylated	Pegylated	Pegylated
Ribavirin discontinuation	No	No	Yes	Yes	No	No	Yes
IFN at diagnosis of CR	On treatment	On treatment	1 month after end of IFN	5 months after end of IFN	On treatment	On treatment	On treatment
At diagnosis of CR							
Liver biopsy	Foam cell arteriopathy, bile duct atrophy	Bile duct atrophy	Bile duct atrophy	Bile duct atrophy, bile duct loss	Bile duct atrophy, bile duct loss	Bile duct atrophy, bile duct loss	Foam cell arteriopathy, bile duct atrophy
AST (IU/L)	121	90	53	73	331	124	36
ALT (IU/L)	67	37	43	63	288	52	32
ALP (IU/L)	2034	906	494	1751	2143	528	1164
γ-GTP (IU/L)	561	768	155	209	515	27	1489
Bilirubin (mg/dL)	18.6	18.8	31.5	38.1	11.8	16.4	22.6
HCV RNA (kIU/mL)	Undetectable	460	Undetectable	Undetectable	16,000	Undetectable	0.40
Treatment for CR	Tacrolimus, MMF	Tacrolimus	Tacrolimus, steroid pulse, MMF	Tacrolimus, MMF, PSL	Tacrolimus, MMF, rapamycin, steroid pulse	Tacrolimus, steroid pulse, MMF	Tacrolimus, MMF, rapamycin, steroid pulse
Outcome	Died	Alive	Died	Died	Died	Died	Died
Months from diagnosis of CR to death	64	—	1	1	1	3	1

ALT, alanine aminotransferase; AST, aspartate aminotransferase; PSL, prednisolone.

Copyright © 2014 Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

with CR after antiviral therapy was terminated. Antiviral therapy was discontinued in the remaining five patients. Of note, six patients were treated with IFN for more than 7 months, suggesting that long-term administration of IFN is associated with CR. Liver biopsy was performed for diagnosis of CR because of abnormal liver function tests in all cases. All patients with documented CR had high levels of alkaline phosphatase (ALP). Total bilirubin levels were extremely high (11.8–38.1 mg/dL) at diagnosis, suggesting a delayed diagnosis of CR. All liver biopsies showed atrophy affecting most bile ducts as well as hepatocanalicular cholestasis. Two patients (patients 1 and 7) showed foam cell obliterative arteriopathy. Bile duct loss was shown in 100%, 67%, and 20% of the portal tracts in patients 4, 5, and 6, respectively. In none of the seven patients was evidence of ACR found in the biopsy specimens. Hepatic artery or biliary tract obstruction or structuring was excluded by imaging in all patients.

Serum HCV RNA was undetectable in four patients at CR diagnosis and remained undetectable in all four patients during the follow-up period. Two of the four patients were considered to have SVR. Final outcomes could not be determined in the remaining two patients who died within 24 weeks after termination of treatment.

Various intensive treatment protocols were used for these seven patients after CR diagnosis, including increase of tacrolimus dose, addition or increase in MMF and/or prednisolone dose, administration of steroid pulse therapy, and inclusion of rapamycin in the therapy. CR progressed rapidly to liver failure in five patients (patients 3–7). These five patients died within 3 months after diagnosis of CR due to liver failure and infection. The liver damage in patient 1 gradually progressed to liver failure, and the patient died at 64 months after CR was diagnosed. Only one patient (patient 2) recovered from CR and survived, although a follow-up liver biopsy showed chronic hepatitis C.

Risk Factors of CR Associated with Antiviral Therapy

Factors associated with the development of CR during and after antiviral therapy were analyzed by comparing the features of 7 CR patients with those of 76 patients who did not develop CR despite receiving antiviral therapy for more than 1 year (Table 2). A reduction of the immunosuppressant dose during antiviral therapy (P=0.034) and a low fibrosis stage before antiviral therapy (P=0.045) were significantly associated with antiviral therapy-related CR. No significant associations were found with other variables, including donor factors, ribavirin discontinuation, and undetectable HCV RNA. The rate of previous ACR (P=0.065), rate of previous moderate or severe ACR (P=0.059), ALP level (P=0.121), and γ-glutamyl transpeptidase (γ-GTP) level (P=0.051) before antiviral therapy was higher in the patients who developed CR, but the differences from patients without CR were not significant.

DISCUSSION

Of the 125 patients, 7 (6%) who received antiviral therapy for hepatitis C after LDLT developed CR. CR progressed rapidly, resulting in death within 3 months after diagnosis, in 5 of these 7 patients.

The risk of rejection have been suggested to increase with IFN administration because of the drug's theoretical immunomodulatory actions, such as up-regulation of human leukocyte antigen class II antigens and induction of proinflammatory cytokines (*21*). Previous studies have reported that the frequency of CR in patients who received IFN was substantially higher compared with patients who did not receive antiviral therapy (*11, 12, 17*). In the present study, the rate of antiviral therapy-associated CR was 6%. This rate is high, because no CR occurred in the entire study period other than during or within 6 months after termination of antiviral therapy in the 230 HCV-positive recipients analyzed. Some cases showed sudden onset of CR after a long transplantation period in the absence of preexisting ACR, supporting the association of antiviral therapy with CR.

In our analysis, the two significant risk factors for CR were reduction of the immunosuppressant dose during antiviral therapy and low fibrosis score at antiviral therapy initiation. Additional characteristics associated with CR were elevated cholestatic enzyme levels at the time of diagnosis, onset of CR more than 7 months after treatment initiation (excluding one patient) and poor prognosis after the diagnosis. The MMF dose was reduced or stopped during antiviral therapy in four of five patients who had received MMF at the start of the treatment. We had initially tried to reduce the MMF dose during antiviral therapy, because MMF is known to suppress the bone marrow and could therefore augment the cytopenic effects of IFN and ribavirin. We had reduced immunosuppressant according to our reduction protocol even during antiviral therapy. Based on the data, we subsequently changed our strategy to maintaining the MMF dose and increasing the trough level of CNIs during antiviral therapy. The reason for the association between the low fibrosis score and CR is currently unclear. Although some institutions recommend early introduction of antiviral therapy (*8, 9*), our data suggest that antiviral therapy should not be administered to patients with no or mild fibrosis. On the contrary, it is reported that tolerance to therapy decreases significantly in patients with a fibrosis stage ≥3 on baseline liver biopsy (*22*). Therefore, the antiviral therapy should be initiated in patients with a fibrosis stage 2, as the recent review articles recommended (*23, 24*).

All our patients underwent LDLT, but no characteristics specific to LDLT, including blood-relative donors, graft size, and ABO incompatibility, were identified as risk factors for CR in our study. This appears to indicate that LDLT and DDLT patients do not differ with respect to antiviral therapy-associated CR.

Early diagnosis of CR, as well as prevention, is important for ensuring improved outcomes in LT recipients. CR was diagnosed in our patients after liver damage had already progressed. Histologic diagnosis of CR was difficult in all these cases, despite repeated liver biopsy examination. However, all the patients had elevated ALP and γ-GTP levels before jaundice was observed. CR should therefore be suspected when a cholestatic liver enzyme pattern develops during antiviral therapy for hepatitis C. When imaging has excluded large bile duct and/or hepatic artery changes as the

Copyright © 2014 Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

TABLE 2. Risk factors for CR

	CR (n=7)	No CR (n=76)	*P*
Age at LT (years)	50 (41−67)	56 (36−69)	0.506[a]
Gender, male/female	3/4	44/32	0.352[b]
HCV genotype, 1/non-1	6/1	71/5	0.421[b]
Donor age at LT (years)	46 (28−60)	42 (21−65)	0.857[a]
Donor gender, male/female	4/3	40/36	0.568[b]
Sex mismatch, match/mismatch	0/7	26/50	0.064[b]
ABO mismatch, match/mismatch	5/2	59/17	0.507[b]
Relation to donor, related/nonrelated	3/4	48/28	0.254[b]
HLA-A matched number, 0/1/2/unknown	0/5/2/0	13/44/16/3	0.332[a]
HLA-B matched number, 0/1/2/unknown	2/4/1/0	21/47/5/3	0.778[a]
HLA-DR matched number, 0/1/2/unknown	3/3/1/0	18/47/8/3	0.487[a]
Graft type, left lobe/right lobe	1/6	9/67	0.608[b]
Splenectomy, yes/no	2/5	38/38	0.247[b]
Previous ACR, yes/no	4/3	17/59	0.065[b]
Previous moderate/severe ACR, yes/no	3/4	9/67	0.059[b]
Previous steroid pulse therapy, yes/no	2/5	8/68	0.198[b]
Months from LT to therapy	9.0 (1.8−72.4)	9.1 (2.2−68.8)	0.694[a]
Valuables at initiation of IFN			
Age (years)	55 (41−68)	57 (37−70)	0.599[a]
CNI tacrolimus/cyclosporine	5/1	71/5	0.376[b]
Trough level for tacrolimus (ng/mL)	7.3 (0−7.9)	6.2 (2.6−10.9)	0.641[a]
AST (IU/L)	68 (24−464)	76 (21−331)	0.908[a]
ALT (IU/L)	88 (25−354)	79 (20−392)	0.842[a]
ALP (IU/L)	878 (283−2977)	462 (168−2818)	0.121[a]
γ-GTP (IU/L)	317 (48−1623)	112 (15−1704)	0.051[a]
Bilirubin (mg/dL)	0.8 (0.3−10.4)	0.9 (0.3−4.6)	0.861[a]
Activity grade, A1/A2/A3	4/3/0	50/24/2	0.693[a]
Fibrosis stage, F0/F1/F2/F3	3/3/1/0	4/56/13/3	0.045[a]
Reduction of immunosuppressant during IFN, yes/no	5/2	22/54	0.034[b]
Ribavirin discontinuation during IFN, yes/no	3/4	26/50	0.468[b]
Undetectable HCV RNA during IFN, yes/no	4/3	51/25	0.439[b]

[a] Wilcoxon rank-sum test.
[b] Chi-square test.
Comparison was made between 7 patients with CR and 76 patients without CR despite receiving antiviral therapy for more than 1 year (No CR). Qualitative variables expressed as number. Quantitative variables expressed as median (range).
ALT, alanine aminotransferase; AST, aspartate aminotransferase; HLA, human leukocyte antigen.

potential etiology of abnormal liver function, we believe that cessation of antiviral therapy and initiation of intensive immunosuppressive therapy should be considered, even without histologic confirmation of CR.

Some limitations of this study are its retrospective nature and relatively small sample size. Because the frequency of CR was low, the sample size was not adequate for multivariate analysis.

In conclusion, CR developed in association with antiviral therapy for recurrent hepatitis C after LDLT. Reduction of the immunosuppressant dose during antiviral therapy should be avoided and antiviral therapy should not be administered to patients with no or mild fibrosis to prevent antiviral therapy-associated CR. Early CR diagnosis should be suspected when a cholestatic liver enzyme pattern develops during antiviral therapy. In these cases, discontinuation of antiviral therapy and increase in the immunosuppressant dose are recommended when other causes of liver dysfunction are excluded.

MATERIALS AND METHODS

Patients

A total of 232 patients with HCV-related end-stage liver disease underwent LDLT at Kyoto University Hospital between March 1999 and September 2012. Two patients who received a liver graft from an identical twin were excluded from this study, because they did not require immunosuppression because of genetic identity. Of the remaining 230 patients, 157 patients were followed up for more than 6 months after LDLT in our hospital. Antiviral therapy was administered to 125 of the 157 patients with recurrent hepatitis C between January 2001 and September 2012. They were diagnosed with recurrent hepatitis C after LDLT via serum HCV RNA analysis and histologic evidence. The remaining 32 patients did not receive antiviral therapy for various reasons: serum HCV RNA negative after LDLT (n=4), no histologic hepatitis C recurrence in the follow-up period (n=13),

Copyright © 2014 Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

no fibrosis seen by liver histology (n=8), and ongoing treatment for the other complications (n=7). CR was defined histologically according to the updated International Banff Schema for Liver Allograft Rejection with the following criteria: (a) the presence of bile duct atrophy/pyknosis affecting most of the bile ducts with or without bile duct loss, (b) convincing foam cell obliterative arteriopathy, or (c) bile duct loss affecting more than 50% of the portal tracts (*13*). Patients who were diagnosed with CR based on these diagnostic criteria during or within 6 months after terminating antiviral therapy were examined for antiviral therapy-associated CR. The clinical features of these 7 patients with CR were compared with those of 76 patients who did not have CR despite receiving antiviral therapy for more than 1 year to determine the risk factors for CR.

The study protocol was approved by the ethics committee at Kyoto University and performed in compliance with the Helsinki Declaration.

Treatment Protocol and Definition of Responses to Treatment

Between January 2001 and April 2004, 40 patients with recurrent hepatitis C after LDLT received treatment with IFN-α-2b plus ribavirin (*25*). From May 2004 to June 2011, patients received combination therapy with peg-IFN-α-2b plus ribavirin (*26*). Patients who acquired a negative serum HCV RNA status within 12 months after treatment initiation continued to receive the treatment for an additional 12 months. Patients who tested negative for serum HCV RNA for more than 6 months after completing IFN therapy were defined as having achieved SVR. For those who tested positive for serum HCV RNA after 12 months of treatment, therapy was discontinued or switched to maintenance therapy with low-dose peg-IFN (*27*), and patients were classified as having shown no response.

Histologic Assessment

Liver biopsy examination was performed when patients showed abnormal liver function tests, or at yearly intervals, with informed consent. Biopsy specimens were evaluated by two pathologists (H.H. and A.M.-H.) with extensive experience in the pathology of LT. Necroinflammatory activity (A0–A3) and fibrosis stage (F0–F4) were assessed using METAVIR scores (*28*).

Immunosuppression

Tacrolimus with low-dose steroid or MMF was administered to most patients for immunosuppression (*25*). The target whole blood lower level for tacrolimus was 10 to 15 ng/mL during the first 2 weeks, 10 ng/mL thereafter, and 5 to 8 ng/mL starting from the second month. Steroid therapy was initiated at a dose of 10 mg/kg methylprednisolone before graft reperfusion then tapered down from 1 mg/kg per day on days 1 to 3, to 0.5 mg/kg per day on days 4 to 6, and to 0.3 mg/kg per day on day 7. Subsequently, oral prednisolone was continued at 0.3 mg/kg per day until the end of the first month, and this was followed by 0.1 mg/kg per day until the end of the third month. After that, steroid administration was terminated. MMF was initiated at a starting dose of 10 to 15 mg/kg on day 1, which was gradually increased to a target dose of 30 mg/kg, and this was continued for 6 months. Thereafter, MMF administration was terminated. Four patients received cyclosporine microemulsions instead of tacrolimus. MMF and/or prednisolone was administered again to patients who experienced refractory rejection or required reduction of the tacrolimus or cyclosporine dose because of adverse events and then tapered down gradually. Twenty-seven patients who received ABO-incompatible transplants were treated with rituximab, plasma exchange, and hepatic artery or portal vein infusion with prostaglandin E1 and methylprednisolone (*29*).

Virologic Assays

HCV genotype was determined using a genotyping system based on polymerase chain reaction (PCR) to amplify the core region using genotype-specific primers (*30*). The serum HCV RNA load was evaluated before LDLT, before IFN treatment, once a month during treatment, and 24 weeks after treatment using PCR and an Amplicor HCV assay (Cobas Amplicor HCV Monitor; Roche Molecular Systems, Pleasanton, CA) until April 2008. A real-time PCR-based quantitation method for HCV (COBAS AmpliPrep/COBAS TaqMan HCV Test; Roche Molecular Systems) was used alternatively from May 2008.

Statistical Analysis

To evaluate the association between patient characteristics and CR, the characteristics were defined and compared between patients with and without CR. Medians and ranges were determined for continuous variables, and data were analyzed using the Wilcoxon rank-sum test. Categorical variables were expressed as counts, and data were analyzed using the chi-square test. A significance level of $P<0.05$ was considered significant. Statistical analyses were performed using PASW Statistics version 18.0.0 (SPSS, an IBM company).

REFERENCES

1. Berenguer M, Prieto M, San Juan F, et al. Contribution of donor age to the recent decrease in patient survival among HCV-infected liver transplant recipients. *Hepatology* 2002; 36: 202.
2. Feray C, Caccamo L, Alexander GJ, et al. European collaborative study on factors influencing outcome after liver transplantation for hepatitis C. European Concerted Action on Viral Hepatitis (EUROHEP) Group. *Gastroenterology* 1999; 117: 619.
3. Forman LM, Lewis JD, Berlin JA, et al. The association between hepatitis C infection and survival after orthotopic liver transplantation. *Gastroenterology* 2002; 122: 889.
4. Gane E. The natural history and outcome of liver transplantation in hepatitis C virus-infected recipients. *Liver Transpl* 2003; 9: S28.
5. Prieto M, Berenguer M, Rayon JM, et al. High incidence of allograft cirrhosis in hepatitis C virus genotype 1b infection following transplantation: relationship with rejection episodes. *Hepatology* 1999; 29: 250.
6. Sanchez-Fueyo A, Restrepo JC, Quinto L, et al. Impact of the recurrence of hepatitis C virus infection after liver transplantation on the long-term viability of the graft. *Transplantation* 2002; 73: 56.
7. Velidedeoglu E, Mange KC, Frank A, et al. Factors differentially correlated with the outcome of liver transplantation in HCV+ and HCV-recipients. *Transplantation* 2004; 77: 1834.
8. Gordon FD, Kwo P, Vargas HE. Treatment of hepatitis C in liver transplant recipients. *Liver Transpl* 2009; 15: 126.
9. Terrault NA. Hepatitis C therapy before and after liver transplantation. *Liver Transpl* 2008; 14: S58.
10. Berenguer M. Systematic review of the treatment of established recurrent hepatitis C with pegylated interferon in combination with ribavirin. *J Hepatol* 2008; 49: 274.
11. Fernandez I, Ulloa E, Colina F, et al. Incidence, risk factors, and outcome of chronic rejection during antiviral therapy for post-transplant recurrent hepatitis C. *Liver Transpl* 2009; 15: 948.
12. Stanca CM, Fiel MI, Kontorinis N, et al. Chronic ductopenic rejection in patients with recurrent hepatitis C virus treated with pegylated interferon alfa-2a and ribavirin. *Transplantation* 2007; 84: 180.
13. Demetris A, Adams D, Bellamy C, et al. Update of the International Banff Schema for Liver Allograft Rejection: working recommendations for the histopathologic staging and reporting of chronic rejection. An International Panel. *Hepatology* 2000; 31: 792.
14. Demetris AJ. Distinguishing between recurrent primary sclerosing cholangitis and chronic rejection. *Liver Transpl* 2006; 12: S68.
15. O'Grady J. The immunoreactive patient: rejection and autoimmune disease. *Liver Transpl* 2011; 17: S29.
16. O'Leary JG, Kaneku H, Susskind BM, et al. High mean fluorescence intensity donor-specific anti-HLA antibodies associated with chronic rejection postliver transplant. *Am J Transplant* 2011; 11: 1868.
17. Berenguer M, Palau A, Fernandez A, et al. Efficacy, predictors of response, and potential risks associated with antiviral therapy in liver transplant recipients with recurrent hepatitis C. *Liver Transpl* 2006; 12: 1067.
18. Dousset B, Conti F, Houssin D, et al. Acute vanishing bile duct syndrome after interferon therapy for recurrent HCV infection in liver-transplant recipients. *N Engl J Med* 1994; 330: 1160.
19. Feray C, Samuel D, Gigou M, et al. An open trial of interferon alfa recombinant for hepatitis C after liver transplantation: antiviral effects and risk of rejection. *Hepatology* 1995; 22: 1084.

Copyright © 2014 Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

20. Stravitz RT, Shiffman ML, Sanyal AJ, et al. Effects of interferon treatment on liver histology and allograft rejection in patients with recurrent hepatitis C following liver transplantation. *Liver Transpl* 2004; 10: 850.
21. Selzner N, Guindi M, Renner EL, et al. Immune-mediated complications of the graft in interferon-treated hepatitis C positive liver transplant recipients. *J Hepatol* 2011; 55: 207.
22. Roche B, Sebagh M, Canfora ML, et al. Hepatitis C virus therapy in liver transplant recipients: response predictors, effect on fibrosis progression, and importance of the initial stage of fibrosis. *Liver Transpl* 2008; 14: 1766.
23. Berenguer M, Schuppan D. Progression of liver fibrosis in post-transplant hepatitis C: mechanisms, assessment and treatment. *J Hepatol* 2013; 58: 1028.
24. Coilly A, Roche B, Samuel D. Current management and perspectives for HCV recurrence after liver transplantation. *Liver Int* 2013; 33: 56.
25. Ueda Y, Takada Y, Haga H, et al. Limited benefit of biochemical response to combination therapy for patients with recurrent hepatitis C after living-donor liver transplantation. *Transplantation* 2008; 85: 855.
26. Ueda Y, Takada Y, Marusawa H, et al. Individualized extension of pegylated interferon plus ribavirin therapy for recurrent hepatitis C genotype 1b after living-donor liver transplantation. *Transplantation* 2010; 90: 661.
27. Ueda Y, Marusawa H, Kaido T, et al. Effect of maintenance therapy with low-dose peginterferon for recurrent hepatitis C after living donor liver transplantation. *J Viral Hepat* 2012; 19: 32.
28. Bedossa P, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. *Hepatology* 1996; 24: 289.
29. Raut V, Mori A, Kaido T, et al. Splenectomy does not offer immunological benefits in ABO-incompatible liver transplantation with a preoperative rituximab. *Transplantation* 2012; 93: 99.
30. Ohno O, Mizokami M, Wu RR, et al. New hepatitis C virus (HCV) genotyping system that allows for identification of HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a. *J Clin Microbiol* 1997; 35: 201.

Copyright © 2014 Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

OPEN ACCESS Freely available online PLOS | ONE

Pretransplant Serum Hepatitis C Virus RNA Levels Predict Response to Antiviral Treatment after Living Donor Liver Transplantation

Yoshihide Ueda[1]*, Toshimi Kaido[2], Yasuhiro Ogura[2], Kohei Ogawa[2], Atsushi Yoshizawa[2], Koichiro Hata[2], Yasuhiro Fujimoto[2], Aya Miyagawa-Hayashino[3], Hironori Haga[3], Hiroyuki Marusawa[1], Satoshi Teramukai[4], Shinji Uemoto[2], Tsutomu Chiba[1]

1 Department of Gastroenterology and Hepatology, Graduate School of Medicine, Kyoto University, Kyoto, Japan, 2 Department of Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan, 3 Department of Diagnostic Pathology, Kyoto University Hospital, Kyoto, Japan, 4 Division of Clinical Trial Design and Management, Translational Research Center, Kyoto University Hospital, Kyoto, Japan

Abstract

Background: Given the limited efficacy and high adverse event rate associated with treatment of recurrent hepatitis C after liver transplantation, an individualized treatment strategy should be considered. The aim of this study was to identify predictors of response to antiviral therapy for hepatitis C after living donor liver transplantation (LDLT) and to study the associated adverse events.

Methods: A retrospective chart review was performed on 125 hepatitis C virus (HCV)-positive LDLT recipients who received interferon plus ribavirin and/or peginterferon plus ribavirin therapy at Kyoto University between January 2001 and June 2011.

Results: Serum HCV RNA reached undetectable levels within 48 weeks in 77 (62%) of 125 patients, and these patients were defined as showing virological response (VR). Of 117 patients, 50 (43%) achieved sustained VR (SVR). Predictive factors associated with both VR and SVR by univariate analysis included low pretransplant serum HCV RNA levels, a non-1 HCV genotype, and low pretreatment serum HCV RNA levels. In addition, LDLT from ABO-mismatched donors was significantly associated with VR, and white cell and neutrophil counts before interferon therapy were associated with SVR. Multivariate analysis showed that 2 variables–pretransplant serum HCV RNA level less than 500 kIU/mL and a non-1 HCV genotype–remained in models of both VR and SVR and that an ABO mismatch was associated with VR. No variables with a significant effect on treatment withdrawal were found.

Conclusions: Virological response to antiviral therapy in patients with hepatitis C recurring after LDLT can be predicted prior to transplant, based on pretransplant serum HCV-RNA levels and HCV genotype. LDLT from ABO-mismatched donors may contribute to more efficacious interferon therapy.

Trial Registration: UMIN-CTR UMIN000003286.

Citation: Ueda Y, Kaido T, Ogura Y, Ogawa K, Yoshizawa A, et al. (2013) Pretransplant Serum Hepatitis C Virus RNA Levels Predict Response to Antiviral Treatment after Living Donor Liver Transplantation. PLoS ONE 8(3): e58380. doi:10.1371/journal.pone.0058380

Editor: Hak Hotta, Kobe University, Japan

Received August 30, 2012; **Accepted** February 4, 2013; **Published** March 7, 2013

Copyright: © 2013 Ueda et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by Japan Society for the Promotion of Science (JSPS) Grants-in-aid for Scientific Research 21229009 and 23590972, and Health and Labour Sciences Research Grants for Research on Intractable Diseases, and Research on Hepatitis from the Ministry of Health, Labour and Welfare, Japan. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: yueda@kuhp.kyoto-u.ac.jp

Introduction

Hepatitis C virus (HCV) infection, leading to liver cirrhosis and hepatocellular carcinoma, is the leading indications for liver transplantation in Japan, the United States, and Western Europe. However, almost all patients who undergo liver transplantation for HCV-related liver disease develop recurrent viral infection, and 70–90% of patients suffer from histologically proven recurrent hepatitis [1,2,3,4,5,6]. The progression of recurrent hepatitis C is often accelerated and, without appropriate antiviral therapy, 10–25% of patients develop cirrhosis within 5 years after transplantation, resulting in poorer prognoses for HCV-positive recipients than HCV-negative recipients [7]. To prevent the progression of hepatitis C after liver transplantation, interferon-based combination therapy is commonly administered [8,9]. However, its efficacy in liver transplant recipients is limited, with the mean sustained virological response (SVR) rate among patients with recurrent hepatitis C after liver transplantation being only 30% (range, 8–50%) [10]. One of the reasons for the low SVR rate is the high rate of treatment withdrawal. Several severe adverse events have been reported in transplant recipients after interferon therapy,

including chronic rejection and *de novo* autoimmune hepatitis [11,12,13].

To improve the efficacy of anti-HCV treatment in patients with hepatitis C after liver transplantation, an individualized treatment strategy based on efficacy prediction and adverse events should be attempted. In several studies, an analysis of predictors associated with SVR was conducted in patients with recurrent hepatitis C after deceased donor liver transplantation (DDLT) [10,14,15,16,17,18,19,20]. In these studies, variables most frequently associated with SVR were early virological response (EVR) at 3 months of therapy, HCV genotype 2, adherence to therapy, and baseline viremia [14,15,16,17,18,19,20]. Of these factors, EVR and adherence to therapy can only be recognized after the initiation of treatment. However, to enable decisions on treatment indications and strategy, predictors of response that are available before initiation of therapy are more valuable. Thus, an individualized treatment strategy could be based on the identification of baseline predictive factors before interferon therapy. Moreover, no study of factors predictive of response to the interferon therapy in patients with recurrent hepatitis C after living donor liver transplantation (LDLT) has been reported so far. Characteristics specific to LDLT, including blood-relative donors, post-transplant liver regeneration, and ABO-incompatible liver transplantation, might cause the antiviral effects of interferon therapy in these patients to differ from those who received DDLT.

The direct-acting antiviral agents telaprevir and boceprevir recently became available for clinical use. The results of clinical trials of these agents in combination with peginterferon plus ribavirin in nontransplant patients with HCV were promising [21,22,23,24]. SVR rates to telaprevir-based combination therapy were significantly higher than those to the peginterferon-ribavirin combination. The efficacy in the patients who had suffered a relapse after a previous treatment by peginterferon plus ribavirin was especially striking [21,24]. The SVR rate to telaprevir based-therapy in patients who had a previous relapse was more than 80%, while that in patients who had no response to previous treatment was around 30% [24]. These results suggest that patients who show a virological response (VR) to peginterferon plus ribavirin are expected to achieve SVR after telaprevir-based therapy. Therefore, identification of factors predictive of virological response to peginterferon plus ribavirin should also prove useful when making the clinical decision about telaprevir usage. In liver transplant recipients, the use of telaprevir and boceprevir poses risks because of their inhibitory action on the enzyme cytochrome P450 3A, responsible for the metabolism of both tacrolimus and cyclosporine. In fact, the phase I study of telaprevir in healthy individuals revealed that it significantly increased the blood concentrations of both tacrolimus and cyclosporine [25]. Therefore, the selection of the patients for whom telaprevir is prescribed is especially important in liver transplant recipients.

Recently, a polymorphism in the interleukin-28B (IL28B) gene region, encoding interferon-lambda 3, was identified as a strong predictive factor for response to antiviral treatment in nontransplant patients with hepatitis C [26,27,28]. In post-transplant patients, the IL28B polymorphism in both recipients and donors was shown to be associated with response to antiviral treatment [29,30]. In addition, HCV-RNA mutations, including those affecting amino acid (aa) residues 70 and 91 in the core region of HCV and those in the interferon sensitivity determining region (ISDR) in nonstructural protein 5A (NS5A), were also demonstrated to be predictors of response to interferon therapy in transplant recipients, as well as in nontransplant settings [31,32,33]. These factors could be used to predict response to antiviral therapy, but these are presently not part of a routine clinical examination and require special techniques not covered by health insurance. Moreover, probing individual genetic information poses potential ethical issues.

The aims of this study were, therefore, to identify noninvasively obtained regular baseline factors associated with VR, SVR, and treatment withdrawal, in order to elucidate the factors associated purely with response to interferon therapy, to identify the valuables related to final outcomes, and to clarify the factors associated with adverse events.

Methods

A retrospective chart review was performed for all HCV-positive liver transplant patients who received antiviral therapy with standard interferon and/or pegylated interferon in combination with ribavirin after liver transplantation at Kyoto University between January 2001 and June 2011.

Patients

Between March 1999 and June 2011, 214 HCV-positive recipients underwent LDLT at Kyoto University. Of these, 157 patients were followed up for more than 6 months after LDLT in our hospital. Anti-viral therapy was administered to 125 of the 157 patients with recurrent hepatitis C between January 2001 and June 2011. The remaining 32 patients did not receive anti-viral therapy for various reasons: serum HCV-RNA negative after LDLT (n = 4), no histological hepatitis C recurrence in the follow-up period (n = 13), no fibrosis seen by liver histology (n = 8), and ongoing treatment for the other complications (n = 7). HCV RNA concentrations and histological evidence were used to diagnose patients with recurrent hepatitis C after LDLT. These patients were given combination therapies with interferon plus ribavirin and/or peginterferon plus ribavirin at Kyoto University between January 2001 and June 2011. The study protocol was approved by the Ethics Committee at Kyoto University and performed in compliance with the Helsinki Declaration. Written informed consent for participation in this study was not obtained, because this study is an observational study without use of human specimen. Our institutional review board waived the need for written informed consent from the participants of the initial study.

Treatment Protocol and Definition of Responses to Treatment

Between January 2001 and April 2004, patients with recurrent hepatitis C after LDLT received treatment with interferon-α-2b (3 or 6 mega units, 3 times/week) plus ribavirin (400–800 mg/day orally), for the first 6 months. This was followed by interferon monotherapy for 6 months [34]. Forty patients received this treatment. Of the 40 patients, 14 patients achieved SVR and 9 withdrew from the treatment protocol. The remaining 17 patients, including 2 who relapsed and 15 nonresponders were retreated by the following protocol with peginterferon and rebavirin. Between May 2004 and June 2011, patients received combination therapy with peginterferon-α-2b (1.5 μg/kg) plus ribavirin (400–800 mg/day orally) [35]. Patients who acquired a negative serum HCV RNA status within 12 months after treatment initiation continued to receive the treatment for an additional 12 months before treatment termination. Total 102 patients, including 17 patients who had previously treated with standard interferon plus ribavirin and did not achieve SVR, were treated with this treatment protocol. Patients who were negative for serum HCV RNA for more than 6 months after completion of interferon therapy were defined as having achieved SVR. If serum HCV RNA was positive after 12 months of treatment, therapy was discontinued or

switched to maintenance therapy with low-dose peginterferon [36], and the patient was classified as having shown no response. Treatment was discontinued in patients with severe adverse events. Additionally, peginterferon treatment was discontinued when neutrophil and platelet counts fell below 500/µL and 30000/µL, respectively, and ribavirin was discontinued when hemoglobin levels fell below 8 g/dL.

We studied the final outcomes of the treatment with peginterferon plus ribavirin (n = 102) and with standard interferon plus ribavirin (n = 23).

Histological Assessment

Liver biopsies were performed when patients' alanine aminotransferase (ALT) levels were more than twice the normal upper limit, or at yearly intervals, with informed consent. Biopsy specimens were evaluated by 2 pathologists (H.H. and A.M-H.) with extensive experience in the pathology of liver transplantation. Necroinflammatory activity (A0–A3) and fibrosis stage (F0–F4) were assessed using METAVIR scores [37,38]. Activity was graded as A0 (no activity), A1 (mild activity), A2 (moderate activity), or A3 (severe activity); Fibrosis was staged as F0 (no fibrosis), F1 (mild fibrosis), F2 (moderate fibrosis), F3 (severe fibrosis), or F4 (cirrhosis).

Immunosuppression

Tacrolimus and low-dose steroid therapy were administered to induce immunosuppression in most patients [34]. Four patients received cyclosporine microemulsions instead of tacrolimus. Mycophenolate mofetil was administered to patients who experienced refractory rejection or required reduction of tacrolimus or cyclosporine doses due to adverse events. Patients who received ABO blood-type incompatible transplants were treated with rituximab, plasma exchange, and hepatic artery or portal vein infusion with prostaglandin E1 and methylprednisolone [39].

Virological Assays

HCV genotype was determined using a genotyping system based on polymerase chain reaction (PCR) to amplify the core region using genotype-specific PCR primers [40]. Serum HCV RNA load was evaluated before LDLT, before interferon treatment, once a month during treatment, and 24 weeks after treatment, using PCR and an Amplicor HCV assay (Cobas Amplicor HCV Monitor, Roche Molecular Systems, Pleasanton, CA, USA) until April 2008, or a real-time PCR-based quantitation method for HCV (COBAS AmpliPrep/COBAS TaqMan HCV Test, Roche Molecular Systems, Pleasanton, CA, USA) from May 2008. Detection of amino acid substitutions in the HCV core region was performed using the method reported previously [31].

Statistical Analysis

To evaluate the association between the patient characteristics and the outcomes (VR, SVR, or withdrawal), the Wald test was performed based on a logistic regression model. Multivariate logistic regression analysis with backward variable selection was used to identify independent and significant predictors for the outcomes, and to estimate the odds ratio (OR) ant its 95% confidence interval (CI). A p-value of 0.05 was used for variable selection and was regarded as significant. Statistical analyses were performed using SAS version 9.2 (SAS Institute Inc., Cary NC).

Results

Patient Characteristics

This study included 125 HCV-infected liver transplant patients treated with standard interferon and/or pegylated interferon in combination with ribavirin for recurrent hepatitis C after LDLT. Of the 125 patients, 69 (55%) were male, and the median age was 57 years (range: 15–70) at the beginning of the therapy. Most patients were infected with HCV genotype 1b (n = 103, 82%). HCV genotypes of the remaining patients were 2a (n = 13), 2b (n = 5), 3a plus 3b (n = 1), not determined (n = 2), and not examined (n = 1). Median serum HCV RNA load was 410 kIU/mL (range: <0.5–5000<kIU/mL) before LDLT, and 3260 kIU/mL (range: 31–69000<kIU/mL) at the beginning of the interferon therapy after LDLT. The median donor age was 41 (range: 19–65) years. Seventy-two donors (58%) were male, and 86 (69%) were related to the recipients. The graft type was the right lobe in 109 patients (87%), and the left lobe in 16 patients (13%). The blood type combination was incompatible in 26 patients (21%). The median time to treatment initiation after LDLT was 9.0 months (1.1–85.3 months). Before treatment, the necroinflammatory activity was A1 or greater in all patients, and 104 patients (83%) had a fibrosis score of F1 or greater (METAVIR score). Tacrolimus-based immunosuppression was used in 116 patients (93%). Among patients receiving tacrolimus for immunosuppression, the mean serum trough level was 6.0 ng/mL (range: 2.0–12.7) at the initiation of interferon therapy. In addition to calcineurin inhibitors, mycophenolate mofetil and prednisolone were used at the initiation of the interferon treatment in 36 (29%) and 19 (15%) patients, respectively.

Efficacy of Interferon Therapy

Of the 125 patients who received interferon therapy, serum HCV RNA reached undetectable levels (less than 0.05 kIU/mL) within 48 weeks in 77 patients (62%) (Figure 1). These patients were defined as showing virological response (VR). Of the remaining 48 patients, 2 patients received treatment for less than 48 weeks, and 15 patients withdrew from the treatment protocol within 48 weeks because of worsening of liver function (n = 5), recurrent hepatocellular carcinoma (n = 2), ascites (n = 2), anemia (n = 1), leucopenia (n = 1), brain hemorrhage (n = 1), biliary complication (n = 1), sepsis (n = 1), or myocardial infarction (n = 1). The remaining 31 patients with detectable HCV RNA in the serum 48 weeks after the initiation of the treatment were placed in the non-VR group. All patients in the non-VR group received peginterferon plus ribavirin therapy, including 9 patients who had previously treated with standard interferon plus ribavirin and did not achieve SVR. Of the patients with VR, 11 discontinued the treatment protocol within 24 weeks after serum HCV-RNA became negative, and 6 patients are still under treatment. The reasons for discontinuation were biliary complications (n = 2), worsening of liver function (n = 2), general fatigue (n = 2), recurrent hepatocellular carcinoma (n = 1), leucopenia (n = 1), hemoptysis (n = 1), brain tumor (n = 1), and depression (n = 1). Of 60 patients who achieved VR and completed the treatment protocol, 50 achieved SVR and 10 relapsed. None of the non-VR patients achieved VR even after more than 48 weeks of treatment, and were classified as nonresponder (NR).

In summary, among the 117 patients in whom the final outcomes of the treatment could be evaluated, 50 patients (43%) achieved SVR, and the remaining 67 patients, including 10 who relapsed (9%), 31 NR (26%), and 26 withdrawals (22%), were classified as non-SVR.

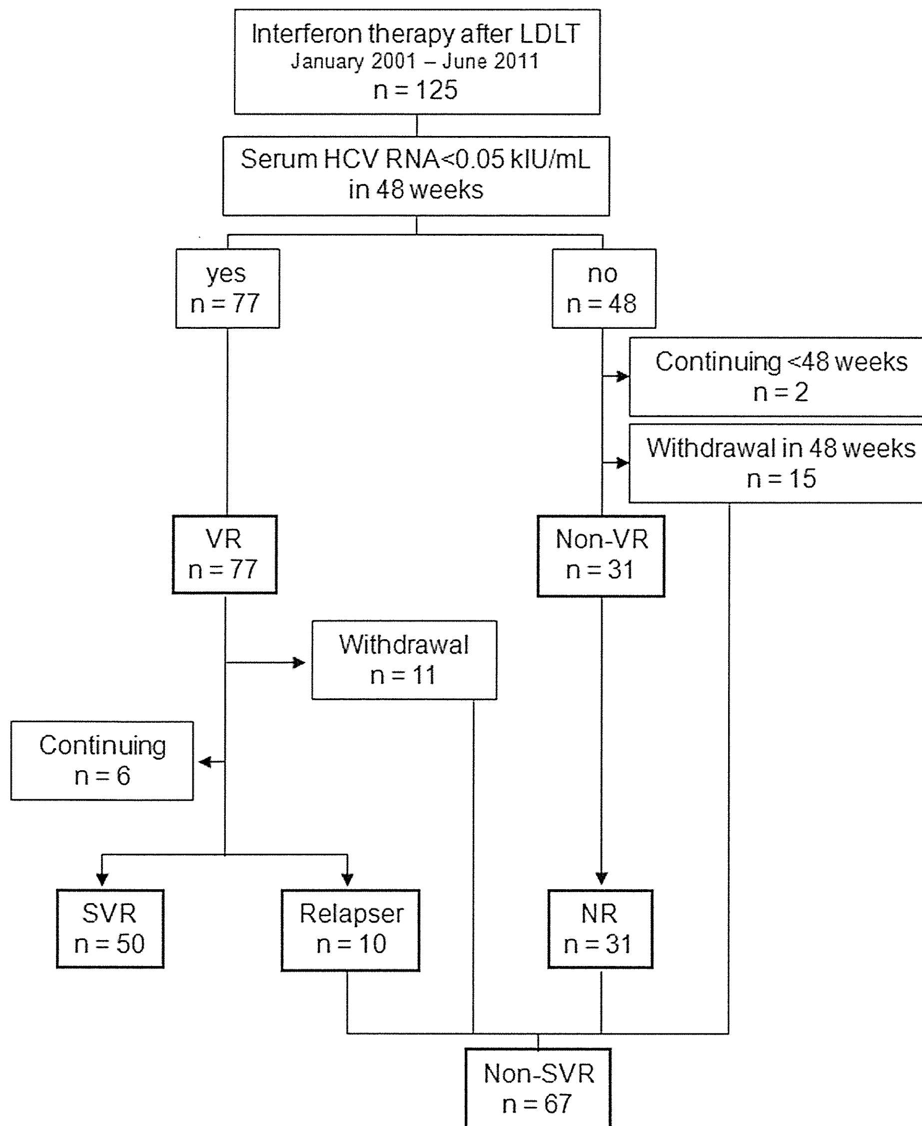

Figure 1. Flow diagram showing the outcome of interferon therapy in patients with recurrent hepatitis C after living donor liver transplantation (LDLT) and indicating the classification of patients in this study. N, number of patients; VR, virological response; SVR, sustained virological response; NR, nonresponder.
doi:10.1371/journal.pone.0058380.g001

Factors Predictive of Virological Response

Factors that could predict virological response were analyzed by comparing patients in the VR (n = 77) and non-VR (n = 31) groups (Table 1). Univariate analysis demonstrated that a low pretransplant serum HCV RNA level (less than 500 kIU/mL, $P<0.001$; and less than 1000 kIU/mL, $P<0.001$), an ABO-mismatched donor ($P = 0.036$), HCV genotype (non-1, $P = 0.001$), and a low pretreatment serum HCV RNA level (less than 5000 kIU/mL, $P = 0.020$) were significantly associated with VR. There were no significant associations with any other variables, including donor factors. Multivariate analysis revealed that the 3 variables that retained a significant association in the model were a pretransplant serum HCV RNA level less than 500 kIU/mL [odds ratio (OR): 0.178, 95% confidence interval (CI): 0.054–0.535, $P = 0.001$], a non-1 HCV genotype (OR: 0.087, 95% CI: 0.000–0.589, $P = 0.008$), and an ABO-mismatched donor (OR: 5.492, 95% CI: 1.004–58.06, $P = 0.049$) (Table 2). All 20 patients with a non-1 HCV genotype achieved VR, while VR rate in patients with the HCV genotype 1 was 65% (57 out of 88 patients). In the patients with HCV genotype 1, VR rate was 80% (36 of 45 patients) when pretransplant serum HCV-RNA level was less than 500 kIU/mL and 42% (15 of 36 patients) when it was 500 kIU/mL or more. Among 22 recipients from ABO-mismatched donors, 20 patients (91%) showed VR, while 57 (66%) out of 86 patients who underwent LDLT from an ABO-matched (identical and compatible) donor achieved VR.

Factors Predictive of SVR

The same variables were analyzed to clarify factors that predicted SVR by comparing patients in the SVR (n = 50) and non-SVR (n = 67) groups (Table 1). By univariate analysis, the same variables that had a significant association with VR were identified as significant predictive factors for SVR–low pretransplant serum HCV RNA levels (less than 100 kIU/mL, $P = 0.028$;

Table 1. Baseline predictive factors before liver transplantation (pre-LT), at liver transplantation (at LT), and before interferon therapy (pre-IFN) associated with virorogical response (VR) and sustained VR (SVR): Univariate analysis.

		VR	non-VR	*P*	SVR	non-SVR	*P*
		n = 77	n = 31		n = 50	n = 67	
Age at LT (years)		55 (8−67)	56 (37−69)	0.462	54.5 (8−67)	56 (30−69)	0.212
Gender	Male	45 (74%)	16 (26%)	0.518	30 (46%)	35 (54%)	0.404
	Female	32 (68%)	15 (32%)		20 (38%)	32 (62%)	
HCC pre-LT	No	29 (71%)	12 (29%)	0.919	18 (43%)	24 (57%)	0.984
	Yes	48 (72%)	19 (28%)		32 (43%)	43 (57%)	
MELD pre-LT		15.5 (3−51)	15 (6−25)	0.403	16 (3−51)	15 (0−43)	0.616
Child-Pugh pre-LT	A/B	35 (74%)	12 (26%)	0.488	25 (49%)	26 (51%)	0.192
	C	41 (68%)	19 (32%)		24 (37%)	41 (63%)	
	unknown	1	0		1	0	
Serum HCV RNA pre-LT	<100 kIU/mL	16 (89%)	2 (11%)	0.063	11 (65%)	6 (35%)	0.028
	100 kIU/mL≤	52 (65%)	28 (35%)		31 (35%)	57 (65%)	
	unknown	9	1		8	4	
Serum HCV RNA pre-LT	<500 kIU/mL	50 (85%)	9 (15%)	<0.001	30 (55%)	25 (45%)	0.002
	500 kIU/mL≤	18 (46%)	21 (54%)		12 (24%)	38 (76%)	
	unknown	9	1		8	4	
Serum HCV RNA pre-LT	<1000 kIU/mL	56 (81%)	13 (19%)	<0.001	34 (49%)	36 (51%)	0.013
	1000 kIU/mL≤	12 (41%)	17 (59%)		8 (23%)	27 (77%)	
	unknown	9	1		8	4	
HCV genotype	Non-1	20 (100%)	0 (0%)	0.001	15 (79%)	4 (21%)	0.002
	1	57 (65%)	31 (35%)		35 (36%)	62 (64%)	
	unknown				0	1	
Donor age at LT (years)		42 (20−63)	38 (21−61)	0.504	43 (20−60)	38 (19−63)	0.748
Donor gender at LT	Male	41 (67%)	20 (33%)	0.287	27 (40%)	40 (60%)	0.538
	Female	36 (77%)	11 (23%)		23 (46%)	27 (54%)	
Sex mismatch	Match	28 (72%)	11 (28%)	0.932	18 (43%)	24 (57%)	0.984
	Mismatch	49 (71%)	20 (29%)		32 (43%)	43 (57%)	
ABO mismatch	Match	57 (66%)	29 (34%)	0.036	38 (40%)	56 (60%)	0.310
	Mismatch	20 (91%)	2 (9%)		12 (52%)	11 (48%)	
Relation of donor	Nonrelated	24 (73%)	9 (27%)	0.827	16 (44%)	20 (56%)	0.803
	Related	53 (71%)	22 (29%)		34 (42%)	47 (58%)	
Graft type	Left lobe	13 (81%)	3 (19%)	0.347	8 (62%)	5 (38%)	0.155
	Right lobe	64 (70%)	28 (30%)		42 (40%)	62 (60%)	
Splenectomy	No	38 (68%)	18 (32%)	0.413	25 (39%)	39 (61%)	0.378
	Yes	39 (75%)	13 (25%)		25 (47%)	28 (53%)	
Age pre-IFN (years)		57 (15−68)	57 (41−70)	0.494	56 (15−68)	57 (32−70)	0.200
Months from LT to therapy		9.2 (1.1−85.3)	8.9 (1.8−59.0)	0.846	9.0 (1.3−85.3)	9.0 (1.3−72.4)	0.879
Trough level for tacrolimus (ng/mL) pre-IFN		5.9 (2.0−10.9)	6.4 (3.3−10.6)	0.323	6.2 (2.2−9.5)	5.9 (2.0−12.7)	0.933
MMF pre-IFN	No	55 (71%)	23 (29%)	0.772	36 (43%)	48 (57%)	0.966
	Yes	22 (73%)	8 (27%)		14 (42%)	19 (58%)	
Prednisolone pre-IFN	No	64 (70%)	28 (30%)	0.347	41 (41%)	60 (59%)	0.245
	Yes	13 (81%)	3 (19%)		9 (56%)	7 (44%)	
Serum HCV RNA pre-IFN	<1000 kIU/mL	17 (89%)	2 (11%)	0.064	8 (38%)	13 (62%)	0.583
	1000 kIU/mL≤	58 (67%)	29 (33%)		42 (45%)	52 (55%)	
	unknown	2	0		0	2	
Serum HCV RNA pre-IFN	<5000 kIU/mL	52 (78%)	15 (22%)	0.020	36 (50%)	36 (50%)	0.030
	5000 kIU/mL≤	18 (55%)	15 (45%)		10 (28%)	26 (72%)	
	unknown	7	1		4	5	

Table 1. Cont.

		VR	non-VR	*P*	SVR	non-SVR	*P*
		n = 77	**n = 31**		**n = 50**	**n = 67**	
White cell count (102/mL)		51 (13−114)	49 (17−98)	0.135	49 (18−114)	48.5 (13−99)	0.049
Neutrophil count (102/mL)		26 (8−89)	22 (11−58)	0.127	26 (11−89)	23 (8−61)	0.044
Hemoglobin (g/dL)		12.0 (9.2−17.2)	12.0 (8.9−17.9)	0.638	12.0 (9.4−17.2)	11.8 (8.9−17.9)	0.157
Platelet count (104/mL)		21.7 (4.7−58.1)	15.1 (4.3−40.0)	0.153	20.3 (5.0−58.1)	15.8 (4.3−45.8)	0.165
AST (IU/L)		78 (19−352)	72 (25−464)	0.677	85 (21−352)	75 (24−547)	0.887
ALT (IU/L)		93 (18−395)	82 (21−392)	0.544	106 (22−395)	82 (18−597)	0.251
ALP (IU/L)		461 (199−1985)	433 (168−2977)	0.345	470 (204−1985)	470 (168−2977)	0.610
g-GTP (IU/L)		118.5 (15−1623)	114 (20−1827)	0.856	141 (15−1623)	115 (20−1827)	0.356
Bilirubin (mg/dL)		0.9 (0.3−11.0)	0.9 (0.3−10.4)	0.827	0.9 (0.4−11.0)	1.0 (0.3−13.7)	0.611
Activity grade pre-IFN	A1	54 (75%)	18 (25%)	0.448	35 (47%)	40 (53%)	0.517
	A2	22 (65%)	12 (35%)		14 (36%)	25 (64%)	
	A3	1 (50%)	1 (50%)		1 (33%)	2 (67%)	
Fibrosis stage pre-IFN	F0	9 (60%)	6 (40%)	0.446	6 (32%)	13 (68%)	0.530
	F1	54 (75%)	18 (25%)		34 (46%)	40 (54%)	
	F2/3	14 (67%)	7 (33%)		10 (42%)	14 (58%)	
Steatosis (5%<) pre-IFN	No	40 (69%)	18 (31%)	0.609	27 (42%)	38 (58%)	0.633
	Yes	36 (73%)	13 (27%)		23 (46%)	27 (54%)	
	unknown	1	0		0	2	
Cholestasis pre-IFN	No	58 (71%)	24 (29%)	0.903	38 (42%)	53 (58%)	0.577
	Yes	18 (72%)	7 (28%)		12 (48%)	13 (52%)	
	unknown	1	0		0	1	

NOTE. Qualitative variables are shown in number; and quantitative variables expressed as median (range). P-values are calculated by Wald test for logistic regression analysis.
LT, liver transplantation; HCC, hepatocellular carcinoma; MELD, model for end-stage liver disease; HCV, hepatitis C virus; MMF, mycophenolate mofetil; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; g-GTP, gamma-glutamyl transpeptidase.
doi:10.1371/journal.pone.0058380.t001

less than 500 kIU/mL, $P = 0.002$; and less than 1000 kIU/mL, $P = 0.013$), HCV genotype (non-1, $P = 0.002$), and low pretreatment serum HCV RNA levels (less than 5000 kIU/mL, $P = 0.030$). In addition, white cell count ($P = 0.049$) and neutrophil count ($P = 0.044$) before interferon therapy were significantly associated with SVR. Multivariate analysis showed that 2 variables were independently associated with SVR–a non-1 HCV genotype (OR: 0.182, 95% CI: 0.054–0.614, $P = 0.006$), and pretransplant serum HCV RNA levels lower than 500 kIU/mL (OR: 0.310, 95% CI: 0.130–0.742, $P = 0.009$) (Table 3). SVR rate among patients with a non-1 HCV genotype was 79% (15 of 19 patients) on average, 83% (10 of 12 patients) when pretransplant serum HCV-RNA level was less than 500 kIU/mL, and 50% (2 of 4 patients) when it was 500 kIU/mL or more. In patients with HCV genotype 1, SVR rate was 36% (35 of 97 patients) on average, 47% (20 of 43 patients) when pretransplant serum HCV-RNA level was less than 500 kIU/mL, and 22% (10 of 45 patients) when it was 500 kIU/mL or more.

Amino Acid Substitutions in Core Region of HCV

To determine the viral factors that predicted VR and SVR in patients infected with HCV genotype 1b, association of aa substitutions at aa 70 of arginine or glutamine/histidine and aa

Table 2. Predictive factors associated with virological response (VR): Multivariate analysis.

		Odds Ratio	95% confidence intervals	P-value
Serum HCV RNA pre-LT	<500 kIU/mL	1	-	-
	500 kIU/mL≤	0.178	0.054–0.535	0.001
HCV genotype	Non-1	1	-	-
	1	0.087	0.000–0.589	0.008
ABO mismatch	Match	1	-	-
	Mismatch	5.492	1.004-58.06	0.049

HCV, hepatitis C virus; LT, liver transplantation.
doi:10.1371/journal.pone.0058380.t002

Table 3. Predictive factors associated with sustained virological response (SVR): Multivariate analysis.

		Odds Ratio	95% confidence intervals	P-value
HCV genotype	Non-1	1	-	-
	1	0.182	0.054–0.614	0.006
Serum HCV RNA pre-LT	<500 kIU/mL	1	-	-
	500 kIU/mL≤	0.310	0.130–0.742	0.009

HCV, hepatitis C virus; LT, liver transplantation.
doi:10.1371/journal.pone.0058380.t003

91 of leucine or methionine with VR and SVR were analyzed in 40 patients, whose pre-treatment sera were stored (Table 4). As a result, substitutions of both aa 70 and aa 91 were not significantly associated with VR and SVR.

Predictors of Withdrawal from Therapy

Predictive factors for withdrawal from the treatment protocol were evaluated by comparing 26 patients who withdrew from the treatment protocol and the patients who completed the treatment including patients with SVR, patients who relapsed, and NR. None of the variables analyzed had a significant effect on withdrawal (Data not shown).

Discussion

In this study, we identified 2 independent predictors of SVR in patients with recurrent hepatitis C after LDLT by multivariate analysis: A non-1 HCV genotype and pretransplant serum HCV-RNA levels lower than 500 kIU/mL. The same factors were identified as predictors for VR, which purely indicates response to interferon therapy, by excluding the influences of the premature termination of the therapy and virological relapse after termination of the treatment. In addition, an ABO-incompatible LDLT was identified as an independent variable predicting VR.

In non-transplant settings, pretreatment predictors of response to interferon therapy have been analyzed in many studies, and the viral genotype and pretreatment viral load have been almost invariably shown to be 2 major predictors of SVR [41,42,43,44]. SVR rates were higher in patients infected with a non-1 HCV genotype and in those with a low pretreatment viral load. These 2 factors have been also identified in several reports [16,17,18,19] as factors predicting SVR in patients with recurrent hepatitis C after DDLT. In the present study, a non-1 HCV genotype was again identified as an independent predictive factor for both VR and SVR in patients with recurrent hepatitis C after LDLT by multivariate analysis. A pretreatment viral load <5000 kIU/mL was also a significant predictive factor by univariate analysis, but it was not an independently associated variable by multivariate analysis. On the other hand, pretransplant viral load was identified as an independent variable predictive of both VR and SVR by multivariate analysis.

While reports of factors that can control viral load exist, the mechanism by which serum HCV-RNA levels are regulated has not yet been completely clarified. A correlation between mutations in the ISDR sequence in the NS5A region of the HCV genome and serum HCV RNA levels has been reported. We did not analyze this viral factor in the current study; however, it is possible that the HCV genome sequence determines both pretransplant viremia and response to interferon therapy. The host polymorphism in IL28B, which was identified as a strong predictor of virological response to interferon therapy in patients with hepatitis C, was recently reported to be associated with baseline viral load [26,45]. The allele associated with a better treatment response is associated with a higher baseline viral load. This finding does not correspond with our results showing that a low HCV load predicts a better response to treatment. We speculate that the balance between host immunity and HCV replication regulates the serum HCV load, and that this balance also determines VR. As pretreatment viral load in post-transplant patients is influenced by immunosuppressive agents, the original host-virus balance

Table 4. Association of amino acid substitutions in the core region with virorogical response (VR) and sustained VR (SVR) in 40 patients infected with HCV genotype 1b: Univariate analysis.

		VR	non-VR	*P*	SVR	non-SVR	*P*
		n = 22	n = 13		n = 14	n = 24	
Core aa 70	Arg	9 (75%)	3 (25%)	0.289	7 (50%)	7 (50%)	0.204
	Gln/His	13 (57%)	10 (43%)		7 (29%)	17 (71%)	
Core aa 91	Leu	14 (64%)	8 (36%)	0.902	9 (38%)	15 (63%)	0.912
	Met	8 (62%)	5 (38%)		5 (36%)	9 (64%)	
Core aa 70 and 91	70 Arg and 91 Leu	6 (67%)	3 (33%)	0.784	5 (50%)	5 (50%)	0.320
	Others	16 (62%)	10 (38%)		9 (32%)	19 (68%)	
Core aa 70 and 91	70 Gln/His and 91 Met	5 (50%)	5 (50%)	0.324	3 (30%)	7 (70%)	0.603
	Others	17 (68%)	8 (32%)		11 (39%)	17 (61%)	

NOTE. Data are shown in number. P-values are calculated by Wald test for logistic regression analysis.
Arg, Arginine; Gln, glutamine; His, histidine; Leu, leucine; Met, methionine.
doi:10.1371/journal.pone.0058380.t004

would be reflected better by serum HCV levels before transplantation than by those after transplantation. It is unclear whether this result is specific to LDLT or holds true for both DDLT and LDLT. The significance of pretransplant viral load in DDLT as a predictor for virological response to post-transplant interferon therapy has not been analyzed in most previous studies [10]. Further analysis in patients who receive DDLT could help clarify the underlying mechanism.

Liver transplantation across the ABO blood-type barrier (ABO-incompatible) is generally contraindicated because of the possibility of graft loss caused by antibody-mediated rejection and is performed under exceptional circumstances as a rescue option in an emergent situation. However, ABO-incompatible LDLT has been performed in Japan to overcome organ shortage problems. Recently, rituximab prophylaxis and local infusion of prostaglandin E1 and steroids were established as therapeutic measures for recipients who underwent ABO-incompatible LDLT, and these treatments improved outcomes [46]. Interestingly, in this study, we found that an ABO-mismatched donor is associated with VR to interferon therapy. The reason for this interesting finding is unclear, but it is possible that either subclinical antibody-mediated rejection or drugs such as rituximab and prostaglandin E1 used in ABO-incompatible recipients may contribute to the higher VR to interferon therapy. There is hope that future studies to clarify the basic mechanism underlying this result will lead to a novel strategy to improve the efficacy of interferon therapy in patients with hepatitis C.

Amino acid substitutions of core region of HCV were not associated with treatment response in our analysis. We do not know the reason for the difference of impact of substitution of core aa 70 and aa 91 on virological response to interferon therapy from a previous report, in which SVR rate were significantly higher in transplant recipients with aa 70 of arginine and aa 91 of leucine of core region of HCV [33]. As sample size of both the previous study and our present study are small, and our present study did not assess the other HCV RNA mutations, including ISDR [32] and interferon/ribavirin resistance-determining region [47] in NS5A, and IL28B polymorphism in recipients and donors, further analysis should be required in larger cohorts.

Another aim of this study was to identify predictive variables for adverse events during interferon therapy, but none of the studied factors proved to be statistically significant predictors of withdrawal from the treatment protocol. As patients withdrew from the treatment for diverse reasons, it would be difficult to predict each adverse event before the initiation of interferon therapy. Therefore, careful follow-up during the treatment procedure is important for early detection of adverse events and to prevent progression to severe complications.

In this study, the final outcomes of the treatment including standard interferon plus ribavirin and peginterferon plus ribavirin were analyzed. Difference of the efficacy between standard interferon and peginterferon might affect the results of our present study. We predicted that patients who had virological response to standard interferon would also show the same response to peginterferon, because it is reported that the efficacy of peginterferon plus ribavirin is higher than that of standard interferon plus ribavirin [44,48]. Accordingly, the patients who achieved SVR by standard interferon were included in the present study. On the other hand, all nonresponders and all patients who relapsed by standard interferon plus ribavirin were retreated with peginterferon plus ribavirin, and we analyzed the final outcomes of the peginterferon plus ribavirin therapy. Therefore, we conclude that the difference of treatment regimen has little influence on our results.

In conclusion, SVR to antiviral therapy in patients with recurrent hepatitis C after LDLT is predictable before transplant by serum HCV-RNA level and HCV genotype. In addition, patients who undergo ABO-incompatible LDLT appear to have a better VR to interferon therapy after liver transplantation. Mechanisms underlying these interesting results are unknown at present, but these findings are likely to be useful for improved clinical assessment of patients with hepatitis C after liver transplantation, and could lead to development of new strategies for better outcomes in LDLT recipients with the HCV genotype 1 and/or a higher pretransplant viral load.

Author Contributions

Conceived and designed the experiments: YU HM. Performed the experiments: YU TK YO KO AY KH YF AMH HH HM. Analyzed the data: YU ST. Contributed reagents/materials/analysis tools: YU TK YO KO AY KH YF AMH HH HM. Wrote the paper: YU HM SU TC.

References

1. Berenguer M, Prieto M, San Juan F, Rayon JM, Martinez F, et al. (2002) Contribution of donor age to the recent decrease in patient survival among HCV-infected liver transplant recipients. Hepatology 36: 202–210.
2. Feray C, Caccamo L, Alexander GJ, Ducot B, Gugenheim J, et al. (1999) European collaborative study on factors influencing outcome after liver transplantation for hepatitis C. European Concerted Action on Viral Hepatitis (EUROHEP) Group. Gastroenterology 117: 619–625.
3. Forman LM, Lewis JD, Berlin JA, Feldman HI, Lucey MR (2002) The association between hepatitis C infection and survival after orthotopic liver transplantation. Gastroenterology 122: 889–896.
4. Gane E (2003) The natural history and outcome of liver transplantation in hepatitis C virus-infected recipients. Liver Transpl 9: S28–34.
5. Prieto M, Berenguer M, Rayon JM, Cordoba J, Arguello L, et al. (1999) High incidence of allograft cirrhosis in hepatitis C virus genotype 1b infection following transplantation: relationship with rejection episodes. Hepatology 29: 250–256.
6. Sanchez-Fueyo A, Restrepo JC, Quinto L, Bruguera M, Grande L, et al. (2002) Impact of the recurrence of hepatitis C virus infection after liver transplantation on the long-term viability of the graft. Transplantation 73: 56–63.
7. Velidedeoglu E, Mange KC, Frank A, Abt P, Desai NM, et al. (2004) Factors differentially correlated with the outcome of liver transplantation in hcv+ and HCV- recipients. Transplantation 77: 1834–1842.
8. Gordon FD, Kwo P, Vargas HE (2009) Treatment of hepatitis C in liver transplant recipients. Liver Transpl 15: 126–135.
9. Terrault NA (2008) Hepatitis C therapy before and after liver transplantation. Liver Transpl 14 Suppl 2: S58–66.
10. Berenguer M (2008) Systematic review of the treatment of established recurrent hepatitis C with pegylated interferon in combination with ribavirin. J Hepatol 49: 274–287.
11. Berardi S, Lodato F, Gramenzi A, D'Errico A, Lenzi M, et al. (2007) High incidence of allograft dysfunction in liver transplanted patients treated with pegylated-interferon alpha-2b and ribavirin for hepatitis C recurrence: possible de novo autoimmune hepatitis? Gut 56: 237–242.
12. Fernandez I, Ulloa E, Colina F, Abradelo M, Jimenez C, et al. (2009) Incidence, risk factors, and outcome of chronic rejection during antiviral therapy for posttransplant recurrent hepatitis C. Liver Transpl 15: 948–955.
13. Stanca CM, Fiel MI, Kontorinis N, Agarwal K, Emre S, et al. (2007) Chronic ductopenic rejection in patients with recurrent hepatitis C virus treated with pegylated interferon alfa-2a and ribavirin. Transplantation 84: 180–186.
14. Berenguer M, Palau A, Fernandez A, Benlloch S, Aguilera V, et al. (2006) Efficacy, predictors of response, and potential risks associated with antiviral therapy in liver transplant recipients with recurrent hepatitis C. Liver Transpl 12: 1067–1076.
15. Carrion JA, Navasa M, Garcia-Retortillo M, Garcia-Pagan JC, Crespo G, et al. (2007) Efficacy of antiviral therapy on hepatitis C recurrence after liver transplantation: a randomized controlled study. Gastroenterology 132: 1746–1756.
16. Neumann U, Puhl G, Bahra M, Berg T, Langrehr JM, et al. (2006) Treatment of patients with recurrent hepatitis C after liver transplantation with peginterferon alfa-2B plus ribavirin. Transplantation 82: 43–47.
17. Oton E, Barcena R, Moreno-Planas JM, Cuervas-Mons V, Moreno-Zamora A, et al. (2006) Hepatitis C recurrence after liver transplantation: Viral and

histologic response to full-dose PEG-interferon and ribavirin. Am J Transplant 6: 2348–2355.
18. Picciotto FP, Tritto G, Lanza AG, Addario L, De Luca M, et al. (2007) Sustained virological response to antiviral therapy reduces mortality in HCV reinfection after liver transplantation. J Hepatol 46: 459–465.
19. Rodriguez-Luna H, Khatib A, Sharma P, De Petris G, Williams JW, et al. (2004) Treatment of recurrent hepatitis C infection after liver transplantation with combination of pegylated interferon alpha2b and ribavirin: an open-label series. Transplantation 77: 190–194.
20. Sharma P, Marrero JA, Fontana RJ, Greenson JK, Conjeevaram H, et al. (2007) Sustained virologic response to therapy of recurrent hepatitis C after liver transplantation is related to early virologic response and dose adherence. Liver Transpl 13: 1100–1108.
21. Bacon BR, Gordon SC, Lawitz E, Marcellin P, Vierling JM, et al. (2011) Boceprevir for previously treated chronic HCV genotype 1 infection. N Engl J Med 364: 1207–1217.
22. Jacobson IM, McHutchison JG, Dusheiko G, Di Bisceglie AM, Reddy KR, et al. (2011) Telaprevir for previously untreated chronic hepatitis C virus infection. N Engl J Med 364: 2405–2416.
23. Poordad F, McCone J, Jr., Bacon BR, Bruno S, Manns MP, et al. (2011) Boceprevir for untreated chronic HCV genotype 1 infection. N Engl J Med 364: 1195–1206.
24. Zeuzem S, Andreone P, Pol S, Lawitz E, Diago M, et al. (2011) Telaprevir for retreatment of HCV infection. N Engl J Med 364: 2417–2428.
25. Garg V, van Heeswijk R, Lee JE, Alves K, Nadkarni P, et al. (2011) Effect of telaprevir on the pharmacokinetics of cyclosporine and tacrolimus. Hepatology 54: 20–27.
26. Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, et al. (2009) Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 461: 399–401.
27. Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, et al. (2009) IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet 41: 1100–1104.
28. Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, et al. (2009) Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet 41: 1105–1109.
29. Charlton MR, Thompson A, Veldt BJ, Watt K, Tillmann H, et al. (2011) Interleukin-28B polymorphisms are associated with histological recurrence and treatment response following liver transplantation in patients with hepatitis C virus infection. Hepatology 53: 317–324.
30. Fukuhara T, Taketomi A, Motomura T, Okano S, Ninomiya A, et al. (2010) Variants in IL28B in liver recipients and donors correlate with response to peg-interferon and ribavirin therapy for recurrent hepatitis C. Gastroenterology 139: 1577–1585, 1585 e1571–1573.
31. Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, et al. (2005) Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and non-virological response to interferon-ribavirin combination therapy. Intervirology 48: 372–380.
32. Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, et al. (1996) Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. N Engl J Med 334: 77–81.
33. Fukuhara T, Taketomi A, Okano S, Ikegami T, Soejima Y, et al. (2010) Mutations in hepatitis C virus genotype 1b and the sensitivity of interferon-ribavirin therapy after liver transplantation. J Hepatol 52: 672–680.
34. Ueda Y, Takada Y, Haga H, Nabeshima M, Marusawa H, et al. (2008) Limited benefit of biochemical response to combination therapy for patients with recurrent hepatitis C after living-donor liver transplantation. Transplantation 85: 855–862.
35. Ueda Y, Takada Y, Marusawa H, Egawa H, Uemoto S, et al. (2010) Individualized extension of pegylated interferon plus ribavirin therapy for recurrent hepatitis C genotype 1b after living-donor liver transplantation. Transplantation 90: 661–665.
36. Ueda Y, Marusawa H, Kaido T, Ogura Y, Oike F, et al. (2010) Effect of maintenance therapy with low-dose peginterferon for recurrent hepatitis C after living donor liver transplantation. J Viral Hepat.
37. Bedossa P, Poynard T (1996) An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. Hepatology 24: 289–293.
38. Poynard T, Bedossa P, Opolon P (1997) Natural history of liver fibrosis progression in patients with chronic hepatitis C. The OBSVIRC, METAVIR, CLINIVIR, and DOSVIRC groups. Lancet 349: 825–832.
39. Raut V, Mori A, Kaido T, Ogura Y, Taku I, et al. (2012) Splenectomy does not offer immunological benefits in ABO-incompatible liver transplantation with a preoperative rituximab. Transplantation 93: 99–105.
40. Ohno O, Mizokami M, Wu RR, Saleh MG, Ohba K, et al. (1997) New hepatitis C virus (HCV) genotyping system that allows for identification of HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a. J Clin Microbiol 35: 201–207.
41. Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, et al. (2002) Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 347: 975–982.
42. Ghany MG, Strader DB, Thomas DL, Seeff LB (2009) Diagnosis, management, and treatment of hepatitis C: an update. Hepatology 49: 1335–1374.
43. Hadziyannis SJ, Sette H, Jr., Morgan TR, Balan V, Diago M, et al. (2004) Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. Ann Intern Med 140: 346–355.
44. Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, et al. (2001) Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet 358: 958–965.
45. Thompson AJ, Muir AJ, Sulkowski MS, Ge D, Fellay J, et al. (2010) Interleukin-28B polymorphism improves viral kinetics and is the strongest pretreatment predictor of sustained virologic response in genotype 1 hepatitis C virus. Gastroenterology 139: 120–129 e118.
46. Egawa H, Teramukai S, Haga H, Tanabe M, Fukushima M, et al. (2008) Present status of ABO-incompatible living donor liver transplantation in Japan. Hepatology 47: 143–152.
47. El-Shamy A, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, et al. (2008) Sequence variation in hepatitis C virus nonstructural protein 5A predicts clinical outcome of pegylated interferon/ribavirin combination therapy. Hepatology 48: 38–47.
48. Triantos C, Samonakis D, Stigliano R, Thalheimer U, Patch D, et al. (2005) Liver transplantation and hepatitis C virus: systematic review of antiviral therapy. Transplantation 79: 261–268.

uicc global cancer control

IJC International Journal of Cancer

A model of liver carcinogenesis originating from hepatic progenitor cells with accumulation of genetic alterations

Soo Ki Kim[1], Akihiro Nasu[1], Junji Komori[2], Takahiro Shimizu[1], Yuko Matsumoto[1], Yasuko Minaki[1], Kenji Kohno[3], Kazuharu Shimizu[4], Shinji Uemoto[2], Tsutomu Chiba[1] and Hiroyuki Marusawa[1]

[1] Department of Gastroenterology and Hepatology, Graduate School of Medicine, Kyoto University, Kyoto, Japan
[2] Department of Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan
[3] Graduate School of Biological Sciences, Nara Institute of Science and Technology, Nara, Japan
[4] Department of Nanobio Drug Discovery, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan

Activation-induced cytidine deaminase (AID) contributes to inflammation-associated carcinogenesis through its mutagenic activity. In our study, by taking advantage of the ability of AID to induce genetic aberrations, we investigated whether liver cancer originates from hepatic stem/progenitor cells that accumulate stepwise genetic alterations. For this purpose, hepatic progenitor cells enriched from the fetal liver of AID transgenic (Tg) mice were transplanted into recipient "toxin-receptor mediated conditional cell knockout" (TRECK) mice, which have enhanced liver regeneration activity under the condition of diphtheria toxin treatment. Whole exome sequencing was used to determine the landscape of the accumulated genetic alterations in the transplanted progenitor cells during tumorigenesis. Liver tumors developed in 7 of 11 (63.6%) recipient TRECK mice receiving enriched hepatic progenitor cells from AID Tg mice, while no tumorigenesis was observed in TRECK mice receiving hepatic progenitor cells of wild-type mice. Histologic examination revealed that the tumors showed characteristics of hepatocellular carcinoma and partial features of cholangiocarcinoma with expression of the AID transgene. Whole exome sequencing revealed that several dozen genes acquired single nucleotide variants in tumor tissues originating from the transplanted hepatic progenitor cells of AID Tg mice. Microarray analyses revealed that the majority of the mutations (>80%) were present in actively transcribed genes in the liver-lineage cells. These findings provided the evidence suggesting that accumulation of genetic alterations in fetal hepatic progenitor cells progressed to liver cancers, and the selection of mutagenesis depends on active transcription in the liver-lineage cells.

Carcinogenesis

Tumorigenesis comprises multiple processes with a stepwise accumulation of genetic alterations that drive the progressive transformation of normal cells into highly malignant derivatives.[1] Recent studies of a large number of genomes in human cancer tissues clarified that cancer cells generally possess hundreds of somatic mutations and dysregulated gene expression profiles.[2–4] Although the origin of cancer cells remains mostly unsolved at present, it might be difficult for fully differentiated cells to acquire these large numbers of nucleotide alterations during their limited life-span to achieve malignant transformation. In contrast, stem/progenitor cells have a long lifetime to supply the differentiated progenies in each organ. Thus, it appears reasonable to assume that long-lived tissue stem/progenitor cells can accumulate genetic alterations and hence could be the origin of tumor cells. Consistent with this hypothesis, a number of studies have provided evidence that the mutations would most likely result in expansion of the altered stem cells, perpetuating and

Key words: liver cancer, hepatic progenitor cells, activation-induced cytidine deaminase (AID), mutation, liver carcinogenesis
Abbreviations: AFP: alpha-fetoprotein: AID: activation-induced cytidine deaminase; hHB-EGF: human heparin binding epidermal growth factor-like growth factor: CK: cytokeratin; DT: diphtheria toxin: GFP: green fluorescent protein; HCC: hepatocellular carcinoma: ICC: intrahepatic cholangiocarcinoma; MAPK: mitogen-activated protein kinase: PPAR: peroxisome proliferator-activated receptor; PCR: polymerase chain reaction: SNV: single nucleotide variant; Tg: transgenic: TRECK: toxin-receptor mediated conditional cell knockout
Additional Supporting Information may be found in the online version of this article.
S.K.K. and A.N. contributed equally to this work.
Grant sponsor: JSPS KAKENHI, Research program of the Project for Development of Innovative Research on Cancer Therapeutics (P-Direct) from Ministry of Education, Culture, Sports, Science and Technology of Japan, Health and Labour Sciences Research Grants for Research from the Ministry of Health, Labour and Welfare, and Takeda Science Foundation
DOI: 10.1002/ijc.28445
History: Received 7 Mar 2013; Accepted 7 Aug 2013; Online 19 Aug 2013
Correspondence to: Hiroyuki Marusawa, Department of Gastroenterology and Hepatology, Graduate School of Medicine, Kyoto University, Kyoto, Japan, Tel.: +81-75-751-4319, Fax: +81-75-751-4303, E-mail: maru@kuhp.kyoto-u.ac.jp

What's new?
The accumulation of stepwise genetic aberrations is a defining feature of cancer. To better understand this process in liver cancer, the present study leveraged the mutagenic ability of activation-induced cytidine deaminase (AID) by using fetal hepatic progenitor cells from AID transgenic mice. The progenitor cells were transplanted into "toxin-receptor mediated conditional cell knockout" mice, where they accumulated genetic alterations sufficient to induce liver tumor formation, for both HCC and cholangiocarcinoma. The landscape of accumulated alterations was revealed by whole exome sequencing. The findings lend support to the idea that cancer arises from tissue stem/progenitor cells.

increasing the chances of additional mutations, leading to malignant transformation.[5–8]

Several studies have provided evidence that hepatocellular carcinoma (HCC) might originate from hepatic stem/progenitor cells.[9–12] A histologic study of clinical specimens also revealed that a substantial number of human HCC tissues have bipotential characteristics with coexpression of biliary and hepatocytic markers such as cytokeratin 7 (CK7), CK19, alpha-fetoprotein (AFP) and albumin.[13,14] Conversely, all cholangiocarcinoma tissues examined showed hepatocellular differentiation in part of the tumor and expression of hepatic progenitor cell markers.[15] Findings from a recent study also suggested that human HCC could arise as a consequence of the dysregulated proliferation of hepatic progenitor cells when the TGF-β and IL-6 signaling pathway was disrupted.[16]

Activation-induced cytidine deaminase (AID) can induce genetic alterations in human genome DNA sequences.[17,18] Under physiological condition, AID is expressed almost exclusively in B lymphocytes, and plays a critical role not only in class switch recombination but also in somatic hypermutation of immunoglobulin genes. We recently demonstrated that inflammatory stimulation triggers aberrant AID expression in epithelial cells and initiates and/or promotes oncogenic pathways by inducing genetic alterations in various tumor-related genes.[19,20] Indeed, AID expression is induced by proinflammatory cytokine stimulation and/or hepatitis C virus infection through NF-kB activation in hepatocytes,[21] and the resultant AID upregulation leads to the accumulation of somatic mutations in *TP53* and *c-MYC* genes, both of which are frequently mutated in human cancer tissues.[21,22] These findings suggest that aberrant AID production induced by chronic inflammation in the liver contributes to hepatocarcinogenesis via the accumulation of genetic aberrations in tumor-related genes.[23]

The fact that it usually takes over a year for AID transgenic (Tg) mice to accumulate the genetic aberrations required for carcinogenesis[24,25] prompted us to speculate that constitutive expression of AID in the cells with long life-span might possess the higher risk for malignant transformation compared to that in the cells with the limited life-span. Therefore, in our study, we took advantage of the AID-mediated stepwise genotoxicity that recapitulates human hepatitis-associated carcinogenesis to investigate whether liver cancer originates from fetal hepatic progenitor cells with constitutive AID expression. Accordingly, we separated hepatic progenitor cells enriched from the fetal liver of AID Tg mice followed by transplantation into recipient mice and examined whether recipient mice receiving AID-expressing hepatic progenitor cells develop liver tumors. Furthermore, to unveil the overall landscape of genetic alterations that accumulate in hepatic progenitor cells during the process of malignant transformation, we applied whole exome sequencing and determined the whole picture of genetic aberrations that accumulated in liver cancer cells originating from hepatic stem/progenitor cells.

Material and Methods

Animals

The "toxin-receptor mediated conditional cell knockout" mice, which are homozygous for the albumin enhancer/promoter driven-human heparin binding epidermal growth factor-like growth factor (hHB-EGF) alleles, achieve the specific and conditional ablation of hepatocytes under the treatment of diphtheria toxin (DT).[26] AID Tg mice were previously described.[24] All animals were maintained in a specific pathogen-free facility at the Kyoto University Faculty of Medicine. All animal experiments were approved by the ethics committee for animal experiments and performed under the Guidelines for Animal Experiments of Kyoto University.

Isolation of enriched hepatic progenitor cells, cell transplantation and administration of diphtheria toxin

Hepatic progenitor cells were obtained from the fetal liver of pregnant wild-type, AID Tg and green fluorescent protein (GFP) Tg mice on gestational day 13.5 and were enriched through sphere formation as previously described.[27] Briefly, after the digestion of fetal liver tissues using a 0.5% collagenase solution (Invitrogen, Carlsbad, CA), fetal liver cells were subjected to floating culture to form spheres in RPMI 1640 (Invitrogen) supplemented with 10% fetal calf serum. After 16 h incubation, the formed spheres were selected by gravity sedimentation and inoculated on Type-I collagen-coated culture plates (Asahi Glass, Chiba, Japan). After 24 h of incubation, floating hematopoietic cells were removed by washing and adhered cells were collected using trypsin-ethylenediaminetetraacetic acid (EDTA) solution (Sigma-Aldrich, St. Louis, MO) for 3 min. The dissociated cells were counted and suspended in a Ca^{2+}-free Hank's balanced salt solution (Invitrogen) with fetal calf serum at a density of 5.0

$\times\ 10^6$ cells/mL as the enriched hepatic progenitor cells. To characterize the enriched hepatic progenitor cells, expression levels of fetal liver stem/progenitor markers, including albumin, AFP, DLK1, CK19 and CD133 were examined using both immunohistochemistry and RT-PCR. In addition, the lack of expression of the hematopoietic cell marker CD45 in sphere-derived hepatic progenitor cells was also confirmed by both immunostaining and RT-PCR.

To achieve efficient engraftment of transplanted hepatic progenitor cells to livers of the recipient mice, we used TRECK mice as a liver-specific regeneration model.[26] TRECK mice express DT receptor under control of the albumin promoter, and treatment with DT selectively and efficiently ablates the hepatocytes, resulting in enhanced liver regeneration and efficient colonization of transplanted hepatic progenitor cells.[27] The enriched hepatic progenitor cells were transplanted into 7- to 9-week old TRECK mice using an intrasplenic approach.[27,28] We injected 0.2 mL of a cell suspension containing 1.0×10^6 hepatic progenitor cells. The DT was purified as described previously[26] and a total of 75 ng/kg DT was administered by intraperitoneal injection into recipient mice twice a week for 25 weeks from the day of cell transplantation.

Whole exome capture and massively-parallel sequencing

Massively-parallel sequencing was performed using the Illumina Genome Analyzer IIx (Illumina, San Diego, CA) as described.[29] End-repair of DNA fragments, addition of adenine to the 3' ends of DNA fragments, adaptor ligation and PCR amplification were performed according to the instructions. Exome capture was performed according to the NimbleGen Arrays Users Guide (Roche, Basal, Switzerland). The DNA library was hybridized to the custom designed NimbleGen Seq Cap arrays targeting a total of 17,089 genes, including 157,728 exons. These libraries were enriched independently using a minimal PCR amplification step of 18 cycles with Phusion High-Fidelity DNA polymerase. The concentration of enriched DNAs was measured by Quant-iT PicoGreen Reagent and Kits (Invitrogen) to make a working concentration of 10 nM. Cluster generation and sequencing was performed for 76 cycles on the Illumina Genome Analyzer IIx as described using the pair-end protocol and collecting 76 bases from each read.[29] The obtained images were analyzed and base-called using GA pipeline software version 1.4 with the default settings provided by Illumina. All sequence reads were deposited in the DNA Data Bank of Japan Sequence Read Archive; accession number DRA000601.

RNA preparation and hybridization to the microarray

Total RNA was extracted from adult mice (12-week old) liver tissues, bone marrow and the fetal liver at Day 13.5 of gestation using RNeasy Mini Kit (Qiagen, Valencia, CA). The details of the procedures for hybridization to the microarray were described previously.[30] RNA amplification and labeling were performed according to the manufacturer's instructions (Agilent Technologies, Palo Alto, CA). Array image acquisition and feature extraction were performed using an Agilent G2505C scanner with feature extraction software (Agilent Technologies). Microarray data were deposited in the GEO database; accession number GSE39213.

Genome Analyzer sequence data analysis and variant filtering.

Semiquantitative reverse transcription PCR and quantitative real-time genomic and reverse transcription-PCR

Histology and immunohistochemistry

Southern blot analysis

Statistical analysis

These procedures are described in Supporting Information Materials and Methods.[31–33]

Results

Enrichment of hepatic progenitor cells derived from fetal mouse liver

Enriched hepatic progenitor cells were obtained from the fetal liver of wild-type, AID Tg and GFP Tg mice through the formation of cell spheres, and the dissociated cells were cultured, counted and then transplanted into recipient mice (Fig. 1*a*). To characterize the sphere-derived hepatic cells used for the transplantation procedure, we first examined the expression of various marker genes in the fetal liver of wild-type mice. Immunohistochemistry revealed that expression of both the liver cell marker albumin and the hematopoietic cell marker CD45 were detectable in the fetal liver tissues (Fig. 1*b*). Cells expressing DLK1, a cell surface marker for hepatic stem/progenitor cells, comprised ~10% of the total cells of the fetal liver parenchyma (Supporting Information Fig. 2*a*). The enriched cell population specifically contained cells expressing the hepatocyte-lineage cell markers such as albumin and AFP, but no expression of CD45 was detectable in these sphere-forming cells (Fig. 1*c*). In addition, we confirmed that almost the entire enriched sphere-derived cell population expressed E-cadherin and DLK1, and a subset of those enriched cells expressed CK19 and CD133 (Supporting Information Fig. 2*b*). On the other hand, the floating cells that did not form spheres strongly expressed CD45 (Fig. 1*d*). RT-PCR also revealed that the sphere-forming cells prepared for the transplantation procedure expressed albumin, AFP, DLK1, CK19 and CD133 transcripts, but not CD45 (Fig. 1*e*). Similar results were obtained in the fetal liver of AID Tg mice (data not shown). These expression profiles of the collected sphere-derived cells were consistent with those found in previous studies[34] and indicated that the enriched cells derived from the fetal liver fully contained hepatic lineage progenitor cells.

Efficient engraftment of transplanted hepatic progenitor cells in the recipient liver

To enhance engraftment of the transplanted cells in the liver, we used TRECK mice as a liver-specific regeneration model.

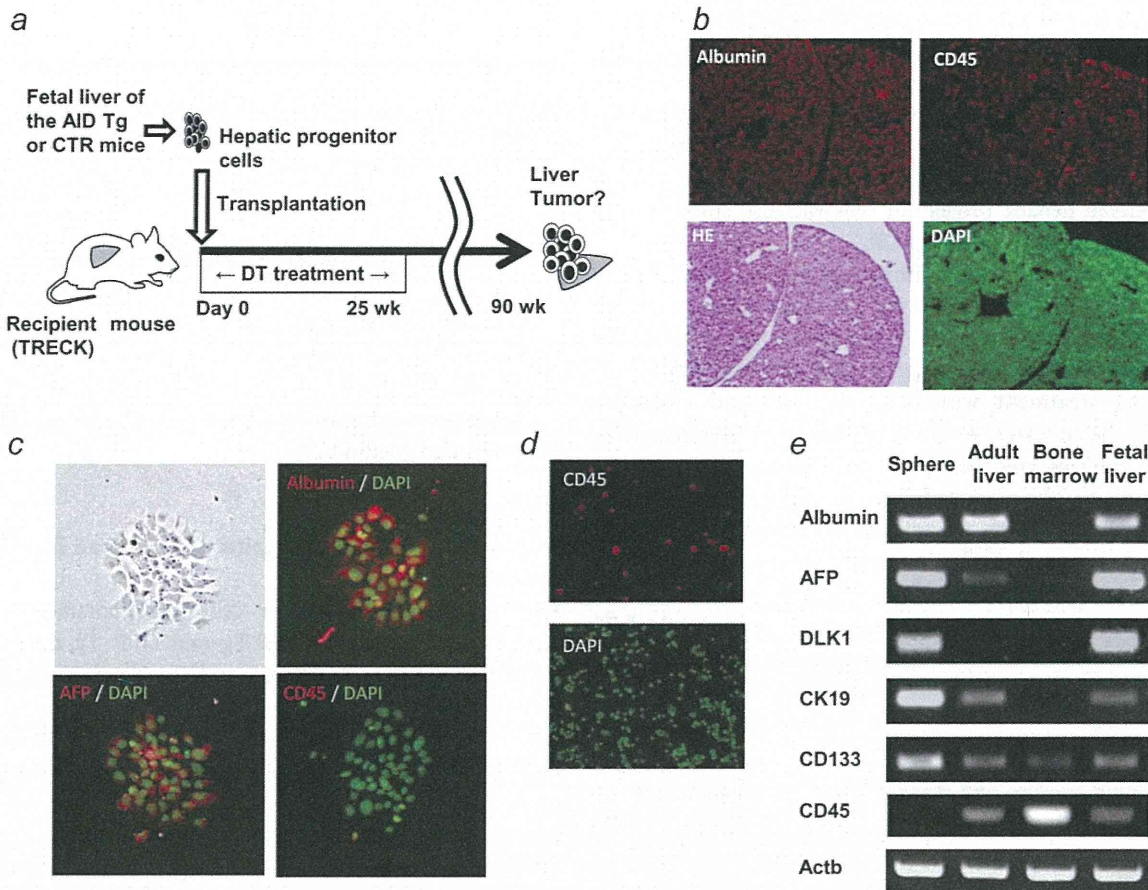

Figure 1. Enrichment of hepatic stem/progenitor cells from the fetal liver. (*a*) Schematic diagram showing the transplantation of the enriched hepatic stem/progenitor cells of AID Tg mice or control (CTR) mice into the recipient TRECK mice. DT was administered intraperitoneally twice a week to recipient (TRECK) mice for 25 weeks from the day of cell transplantation. The phenotypes were examined 90 weeks after transplantation. (*b*) Microscopic image (H&E staining) of the fetal liver tissues. Immunohistochemical staining for both the liver cell marker albumin and the hematopoietic cell marker CD45 are shown. (*c*) Immunohistochemical staining of the enriched cell population from the fetal liver via sphere formation for albumin, AFP and CD45. (*d*) Immunohistochemical staining of floating cells that did not form spheres for CD45. (*e*) Representative RT-PCR for the various phenotypic expression: albumin, AFP, DLK1, CK19, CD133, CD45 and control Actb (β-actin). Total RNA was extracted from the spheres of the enriched cell population from the fetal liver, adult liver tissue, bone marrow and fetal liver tissue.

These mice express hHB-EGF precursor, which functions as a DT receptor, under the control of an albumin promoter, and thus the hepatocytes of these mice are selectively ablated by the administration of DT.[26] We confirmed that the transcripts of hHB-EGF were specifically detectable in the liver of the TRECK mice (Fig. 2*a*), and immunohistochemistry also revealed that hHB-EGF protein expression was present in the TRECK mouse liver tissues (Fig. 2*b*). Serum alanine aminotransferase levels of a TRECK mouse were increased at 24 h after 75 ng/kg of DT administration, peaked at 48–72 h and subsequently returned to basal levels after 120 h (Fig. 2*c*). After repeated trials, we found that twice-weekly DT administration maintained the sublethal liver injury, resulting in the constitutive hepatic regeneration process. Under these experimental conditions, DT-mediated ablation of hepatocytes in TRECK mice resulted in the expansion of cells expressing E-cadherin, EpCAM and HNF4α accompanied by an increased number of the Ki67-positive cells, suggesting enhanced proliferation activity of hepatocyte-lineage cells including hepatic progenitor cells and mature hepatocytes in the TRECK liver tissues (Fig. 2*d*, and data not shown).

To examine the repopulation of the transplanted cells in the recipient liver, the hepatic progenitor cells of the GFP Tg fetal livers obtained in a similar way were introduced into the TRECK mice, followed by the repeated DT administration. At Day 7, the GFP-positive cells were observed as clusters, and at Day 30 the cluster of the GFP-positive cells was large enough to view macroscopically (Fig. 2*e*). Moreover, the cluster of hepatocytes derived from the transplanted GFP-positive enriched hepatic progenitor cells was detectable in the recipient liver even 90 days after the transplantation while no such cells were observed in the liver of mice