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# Steroid-free living donor liver transplantation for HCV – a multicenter prospective cohort study in Japan

Marubashi S, Umeshita K, Asahara T, Fujiwara K, Haga H, Hashimoto T, Hatakeyama K, Ichida T, Kanematsu T, Kitajima M, Kiyosawa K, Makuuchi M, Miyagawa S, Satomi S, Soejima Y, Takada Y, Tanaka N, Teraoka S, Monden M. Steroid-free living donor liver transplantation for HCV – a multicenter prospective cohort study in Japan

Abstract: This prospective, non-randomized, multicenter cohort study analyzed the safety and efficacy of a steroid-free immunosuppressive (IS) protocol for hepatitis C virus (HCV)-positive living donor liver transplant (LDLT) recipients in Japan. Of 68 patients enrolled from 13 transplant centers, 56 fulfilled the inclusion/exclusion criteria; 27 were assigned the steroid-free IS protocol (Fr group) and 29 the traditional steroid-containing IS protocol (St group). Serum HCV RNA levels increased over time and were higher in the St group until postoperative day 90 (POD 14, $p = 0.013$). Preemptive anti-HCV therapy was started in a higher percentage of recipients (59.3%) in the Fr group than in the St group (31.0%, $p = 0.031$), mainly due to early HCV recurrence. The incidence of HCV recurrence at one yr was lower in the Fr group (22.2%) than in the St group (41.4%; $p = 0.066$). The incidence of acute cellular rejection was similar between groups. New onset diabetes after transplant, cytomegalovirus infection, and renal dysfunction were significantly less frequent in the Fr group than in the St group ($p = 0.022$, $p < 0.0001$, $p = 0.012$, respectively). The steroid-free IS protocol safely reduced postoperative morbidity and effectively suppressed both the HCV viral load in the early post-transplant period and HCV recurrence in HCV-positive LDLT recipients.

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## Introduction

The safety of a steroid-free immunosuppression (IS) protocol in liver transplantation has been reported in several studies (1–8). The impact of a steroid-free IS protocol on hepatitis C virus (HCV) recurrence in HCV-positive liver transplant recipients has also been investigated in cohort studies (9–15) and randomized control studies (16–19), but the results are controversial, and it remains unclear whether HCV recurrence or the progression of fibrosis is decreased by the steroid-free IS protocol.

A previous study of living donor liver transplantation (LDLT) (15) reported that a combination of steroid-free IS and preemptive anti-HCV therapy was necessary to suppress HCV activity because steroid-free IS suppressed HCV activity only temporarily in the early post-transplant period, making it necessary to introduce preemptive low-dose interferon and ribavirin combination therapy to prolong the suppression of HCV activity for more than one month after transplantation.

Two issues regarding the post-transplantation IS strategy for HCV-positive liver transplant recipients were raised in a previous report. First, it is unclear whether steroid treatment is really avoidable for liver transplant recipients, because many liver transplant physicians still feel that it is challenging to avoid steroids completely during and after liver transplantation because of the lack of experience. Rapid tapering of steroids might accelerate HCV progression (20–22), making physicians reluctant to administer steroid-free IS for liver transplantation. Second, preemptive anti-HCV therapy is controversial because of the high drop-out rate after liver transplantation (23–25), although there are some reports describing its positive effect of suppressing the progression of hepatitis and fibrosis (15, 26). These concerns influence the decision-making processes regarding the appropriate protocol to use, and many centers hesitate to apply these new protocols. Under this medical and social background, this study was designed among the most active liver transplant centers in Japan.

The goal of this prospective, non-randomized, multicenter cohort study was to analyze the safety and efficacy of a steroid-free IS protocol for HCV-positive liver transplant recipients and to compare the efficacy of a steroid-free IS protocol with that of a traditional steroid-containing IS protocol either with or without preemptive anti-HCV therapy in the most active liver transplant centers in Japan.

## Patients and methods

### Study design

This study was conducted at the 13 most active multiple transplant centers in Japan. The trial was an open-label, prospective, cohort study and conducted by intention-to-treat analysis. Each center determined the IS protocol, either a steroid-free IS (Fr group) or steroid-containing IS (St group) protocol. All protocols were approved by the ethics committee at each center and followed the tenets of the Declaration of Helsinki concerning medical research.

### Endpoint of the study

The primary endpoint of the study was a comparison of HCV recurrence and serum HCV

RNA levels between patients on the two different IS protocols. The secondary endpoint was the incidence of acute cellular rejection (ACR) and other postoperative morbidities, and graft and patient survival.

### Patients

Patients were enrolled into this study from October 2004 to March 2007. Eligible criteria for this study were patients undergoing liver transplant for end-stage liver disease, age over 18 yr old, anti-HCV antibody-positive, serum HCV RNA-positive preoperatively, and informed consent for the study. Exclusion criteria were ABO-incompatible liver transplantation, re-transplantation, anti-HIV antibody-positive, anti-HBsAg- or anti-HBcAb-positive donor, and other patients who were not appropriate for this study as determined by the investigators.

### IS protocol

The IS protocol was either a steroid-containing protocol (St group) and/or a steroid-free protocol (Fr group). The selection of the IS protocol used for each patient was decided by the primary physician at each center.

In the Fr group, a calcineurin inhibitor, either tacrolimus or emulsified cyclosporin A, was administered titrated according to the protocol at each center with a recommended trough level of 8–12 ng/mL (tacrolimus) or 200–300 ng/mL (cyclosporin A) within three months after transplant, and 5–10 ng/mL (tacrolimus) or 100–200 ng/mL (cyclosporin A) thereafter, combined with mycophenolate mofetil (MMF), for which the recommended dose was 500 mg twice daily, and basiliximab, an anti-CD25 monoclonal antibody, at a dose of 20 mg during and four d after transplant. Corticosteroids were completely avoided perioperatively and were not administered unnecessarily because of ACR. MMF was tapered and discontinued within three months unless it was deemed necessary as a renal sparing protocol or other reasons.

In the St group, corticosteroids were administered, tapered, and continued or discontinued at the dose according to the protocol of each center. Steroids could be used for ACR or other conditions. The IS protocol in the St group comprised a calcineurin inhibitor, either tacrolimus or cyclosporin A, with or without MMF or basiliximab. Anti-lymphocyte antibodies, such as OKT3, Thymoglobulin, and daclizumab were not used.

### Evaluation of graft dysfunction

Liver biopsy was recommended whenever the patient developed liver dysfunction and ACR, or recurrent HCV was suspected. A protocol liver biopsy at one yr after liver transplantation was also recommended unless there were any reasons not to do liver biopsy. Liver biopsy specimens were evaluated with hematoxylin and eosin (HE) staining and Masson trichrome staining by special liver pathologists at each center. Recurrent HCV was histologically diagnosed by portal infiltrates, hepatocyte necrosis, lymphoid aggregates, and fibrous expansion of the portal field. ACR was diagnosed by mixed lymphoid cell infiltrates, endothelialitis, and eosinophil infiltrates in portal tracts and bile ducts. In the absence of a liver biopsy, recurrence of HCV was determined by the primary physician with the criteria of elevated aspartate aminotransferase (AST) and alanine aminotransferase (ALT), high serum HCV RNA level, and exclusion of ACR or other causes of liver dysfunction.

### Serum HCV RNA level

Peripheral blood was obtained and sent to the laboratory (SRL, Inc, Tokyo, Japan) for determination of HCV RNA levels preoperatively, in the anhepatic period during surgery, and at POD 7, 14, 28, 90, 180, and one yr. HCV RNA levels were quantified using the COBAS AMPLICOR$^{TM}$ HCV Test version 2.0 (Roche Diagnostics K.K., Tokyo, Japan). Serum HCV RNA levels were evaluated until initiation of anti-HCV treatment either as preemptive therapy or treatment for HCV recurrence.

### Anti-HCV preemptive therapy and anti-HCV treatment after HCV recurrence

Preemptive anti-HCV therapy consisting of pegylated interferon and ribavirin was permitted in centers where it is common. The doses of the drugs were subject to the protocol of each center. Upon diagnosing HCV recurrence in each center, anti-HCV treatment was started at the standard or adjusted treatment dose and titrated based on the patients' conditions according to the protocol of each center. In the analysis of HCV viral loads, the time points during anti-HCV treatment as either preemptive or therapy for HCV recurrence were excluded.

### Histological evaluation

Liver biopsy at one yr after LDLT in each recipient was collected and evaluated by an expert

Table 3. Postsurgical Data

| | Fr group (n = 27) | St group (n = 29) | p value |
|---|---|---|---|
| Immunosuppressive drugs (initial drugs) | | | |
| Calineurin inhibitor (Tac/CsA) | 18/9 | 20/9 | 0.854 |
| Mycophenolate mofetil (yes/no) | 23/4 | 7/20 | <0.0001 |
| Basiliximab (yes/no) | 24/2 | 6/20 | <0.0001 |
| Anti-cytomegalovirus (CMV) prophylaxis (Y/N) | 13/14 | 15/14 | 0.789 |
| Postoperative morbidities | | | |
| Hepatitis C virus (HCV) recurrence (within 12 months after LT) | 12 (44.4%) | 16 (55.2%) | 0.422 |
| Acute cellular rejection (ACR) | 6 (22.2%) | 10 (34.5%) | 0.310 |
| Steroid pulse for ACR | 4 (14.8%) | 6 (20.7%) | 0.566 |
| Anti-HCV therapy | | | |
| Preemptive therapy | 16 (59.3%) | 9 (31.0%) | 0.034 |
| Treatment after HCV recurrence | 7 (25.9%) | 11 (37.9%) | 0.337 |
| De novo hypertension | 4/23 (17.3%) | 8/26 (30.8%) | |
| NODAT | 0/19 (0%) | 6/19 (31.6%) | |
| Infection | | | |
| Bacterial infection | 3 (11.1%) | 9 (31.0%) | 0.069 |
| CMV infection | 0 (0%) | 14 (51.9%) | <0.0001 |
| Fungal infection | 1 (3.7%) | 1 (3.4%) | 0.959 |
| Surgical complications | | | |
| Intra-abdominal bleeding | 2 (7.4%) | 2 (6.9%) | 0.941 |
| Hepatic artery | 0 (0%) | 1 (3.4%) | 0.33 |
| Portal vein | 1 (3.7%) | 0 (0%) | 0.296 |
| Bile duct | 3 (11.1%) | 6 (20.7%) | 0.329 |
| Other complication | | | |
| Renal dysfunction | 0 (0%) | 6 (20.7%) | 0.012 |
| Heart failure | 1 (3.7%) | 1 (3.4%) | 0.959 |
| Respiratory failure (ARDS) | 1 (3.7%) | 0 (0%) | 0.296 |
| CNS complication | 1 (3.7%) | 1 (3.4%) | 0.959 |
| GI bleeding | 0 (0%) | 0 (0%) | – |
| Hepatocellular carcinoma recurrence | 2 (7.4%) | 0 (0%) | 0.136 |

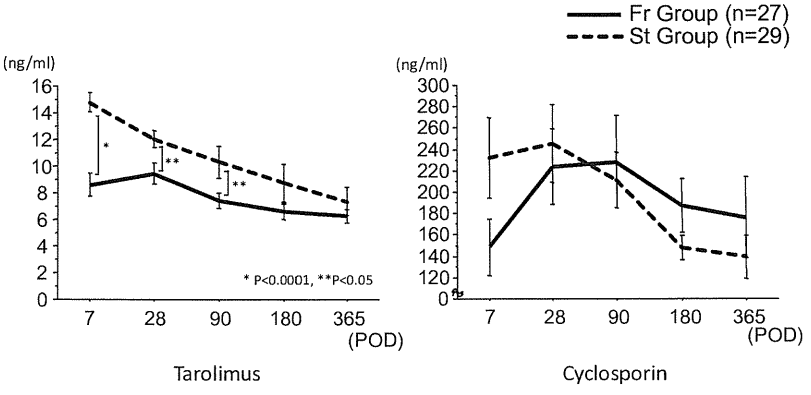


*Fig. 1.* Serum trough level of calcineurin inhibitor. The time course of the trough level (left, tacrolimus; right, cyclosporin A) after liver transplantation. The tacrolimus trough level was significantly lower in the Fr group than in the St group at postoperative day (POD) 7, POD 28, and POD 90 ($p < 0.0001$, $p < 0.05$, $p < 0.05$, respectively), while the cyclosporine trough level was similar between groups.

(Table 3). The calcineurin inhibitor trough level in the peripheral blood is shown in Fig. 1. Tacrolimus trough levels were significantly lower in the Fr group than in the St group at POD7, POD28, and POD90, while the cyclosporine trough level was similar between groups, although it was slightly lower in the Fr group at POD7.

Total dose of steroid administration is shown in Fig. 2. Steroid treatment was continued in most patients in the St group throughout the study

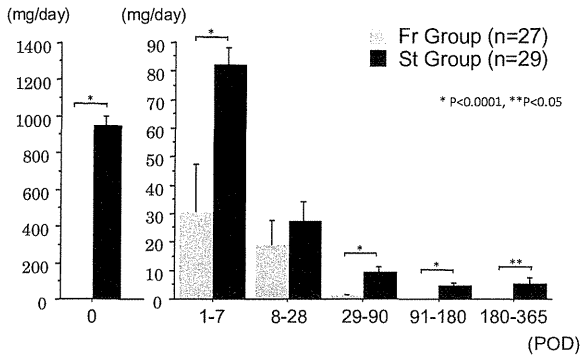


*Fig. 2.* Dose of corticosteroid (methyl prednisolone/predonisolone). Comparison of administered amount of corticosteroid between the Fr and St groups based on measurements obtained at different time points. The doses of steroids differed significantly between groups at all time periods except postoperative days 8–28.

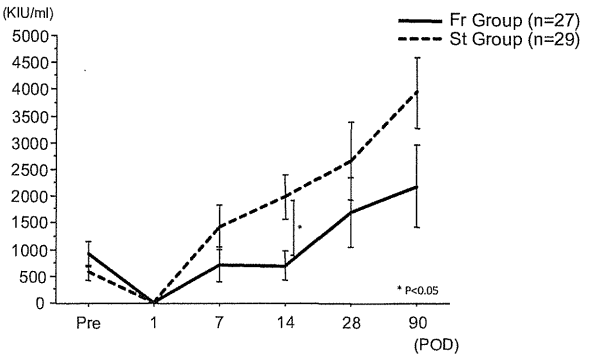


*Fig. 4.* Serum HCV RNA viral load. The time course of the serum HCV RNA viral load after liver transplantation. The increase in HCV RNA remained as low as around 600 kIU/mL between postoperative days (POD) 7 and 14 and then gradually increased to around 2000 kIU/mL on POD90 in Fr group. It was more rapid in St group. There was significant difference between groups on POD 14 ($p = 0.013$).

period and tapered to 5.2 ± 10.0 mg/d on POD 181-365. Steroids were not administered during surgery or in the early post-transplant period in the Fr group but were temporarily administered in eight recipients (29.6%) because of ACR (n = 4), drug allergy (n = 1), and physicians' decision without ACR (n = 3). The total steroid dose was significantly lower in the Fr group, and there were significant differences between groups at all time periods except POD 8-28.

MMF was administered to 85.1% of the patients in the Fr group and only 25.9% of the recipients in the St group ($p < 0.0001$). The MMF dose was started at 796.3 ± 574.3 mg/d in the first week and tapered to 174.2 ± 258.9 mg/d in POD 181–365 in the Fr group. On the other hand, the MMF dose was gradually increased toward the end of the one yr follow-up to 267.7 ± 557.4 mg/d in the St group. The MMF dose was significantly higher in the Fr group than in the St group until POD 90 and was then similar between groups (Fig. 3).

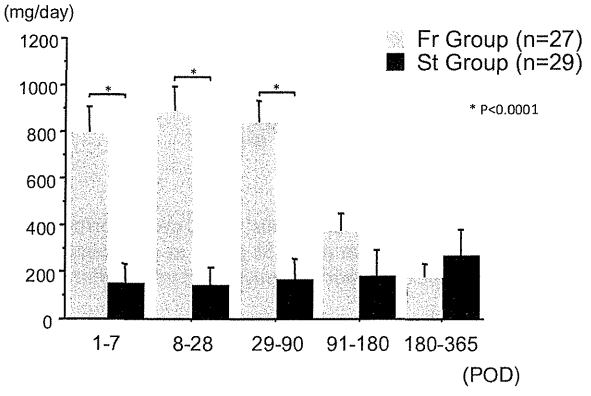


*Fig. 3.* Dose of mycophenolate mofetil. Comparison of administered amount of corticosteroid between Fr and St groups based on measurements obtained at different time points. The doses of steroids differed significantly between groups until postoperative day 90 and then became similar between groups.

Sixteen (59.3%) of the patients in the Fr group received preemptive anti-HCV therapy consisting of pegylated interferon with ribavirin at the dose of each institutional protocol, while only 9 (31.0%) in the St group received preemptive therapy ($p = 0.034$). The reasons for not receiving preemptive therapy in the St group were HCV recurrence prior to therapy (n = 11, 37.9%) and institutional policy (n = 9, 31.0%). The ratio of the recipients that received anti-HCV therapy after HCV recurrence was similar between groups [Fr group, n = 7 (25.9%); St group, n = 11 (37.9%)].

### Serum HCV RNA levels

Serum HCV RNA levels decreased to undetectable levels on POD 1 in both groups and increased gradually after that (Fig. 4). HCV RNA in the Fr group remained around 600 kIU/mL between POD 7 and 14 and then gradually increased to around 2000 kIU/mL on POD 90. The increase in the HCV RNA in the St group was more rapid and reached almost 1500 kIU/mL on POD 7 and 2000 kIU/mL on POD 28. Despite the lower HCV viral loads preoperatively, serum HCV RNA levels were higher throughout the postoperative period until POD 90 in the St group. There was significant difference between groups on POD 14 ($p = 0.013$).

### HCV recurrence

The recurrence rate of HCV diagnosed by each institute is shown in Fig. 5. There were 28 HCV

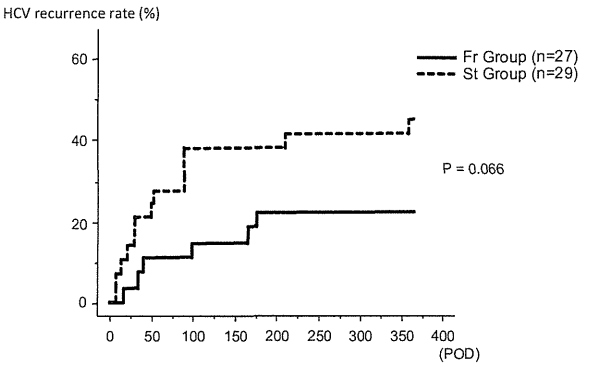


*Fig. 5.* Cumulative HCV recurrence rate. The incidence of HCV recurrence at one yr was 22.2% in the Fr group and 41.4% in St group (p = 0.066).

recurrences diagnosed (12 in the Fr group, 16 in the St group). Twelve (42.9%) patients with HCV recurrence were diagnosed by liver biopsy, and 16 (57.1%) were diagnosed based on clinical data without liver biopsy. Most HCV recurrence was diagnosed during the first six months after liver transplantation. The incidence of HCV recurrence was 22.2% in the Fr group and 41.4% in the St group at one yr. The difference between them was remarkable, but it did not reach statistical significance (p = 0.066). A comparison between tacrolimus (n = 38) and cyclosporin A (n = 18) as the initial calcineurin inhibitor indicated no difference between them in the HCV recurrence rate (Fig. 5).

Protocol liver biopsy at one yr after liver transplantation was performed in 22 cases (12 cases (44.4%) in the Fr group, and 10 cases (34.5%) in the St group. HCV recurrence, which was confirmed during the period by one yr after liver transplantation, was diagnosed in five cases (41.7%) in the Fr group and eight cases (80%) in the St group. Eight cases (66.7%) in the Fr group

Table 4. Modified HAI score and fibrosis score of one yr protocol liver biopsies (n = 22, n = 12 in the Fr group and n = 10 in the St group)

| | Hepatitis C virus (HCV) recurrence (−) | HCV recurrence (+) |
|---|---|---|
| (a) HAI score | | |
| Fr group | n = 7 | n = 5 |
| | 0, 0, 0, 1, 1, 1, 3 | 1, 2, 3, 4, 6 |
| St group | n = 2 | n = 8 |
| | 3, 4 | 0, 1, 1, 1, 2, 3, 3, 6 |
| (b) Ishak fibrosis stage | | |
| Fr group | n = 7 | n = 5 |
| | 0, 0, 0, 0, 0, 2, 3 | 0, 1, 2, 2, 3 |
| St group | n = 2 | n = 8 |
| | 2, 2 | 0, 0, 1, 1, 1, 1, 1, 2 |

Table 5. Postoperative mortality

| Reason for mortality | Fr group (n = 27) | St group (n = 29) | p value |
|---|---|---|---|
| Graft failure | 0 | 2 | |
| Infection | 2 | 3 | |
| Portal vein thrombosis | 1 | 0 | |
| Hepatocellular carcinoma recurrence | 1 | 0 | |
| Total | 4 (14.8%) | 5 (17.2%) | 0.805 |

received preemptive anti-HCV therapy, while only four cases (40%) received in the St group. Both HAI score and Ishak fibrosis stage were the least in the subgroup of HCV recurrence-free cases in the Fr group (n = 7), compared with HCV recurrence-free cases in the St group (n = 2) or HCV recurrence cases in the both groups (n = 13) (Table 4a, b).

### ACR incidence

The incidence of ACR was similar between groups (22.2% in the Fr group and 34.5% in the St group). Bolus steroid administration as a treatment for ACR was implemented in four cases (14.8%) in the Fr group and six cases (20.7%) in the St group, which was similar between the two groups.

### Post-transplant morbidities and mortalities

The incidence of de novo hypertension was similar between the two groups (Tables 3 and 5). New onset diabetes mellitus after transplantation (NODAT) occurred in 31.6% of the St group, and in 0 patients in the Fr group (p = 0.022). Cytomegalovirus (CMV) infection was observed in 51.9% in the St group, whereas none of the patients in the Fr group developed CMV infection (p < 0.0001), although anti-CMV prophylaxis was administered to a similar percentage of the population between the two groups. Surgical complications, in terms of hepatic artery, portal vein, bile duct complications, and intra-abdominal bleeding, were similarly observed between the two groups. Renal dysfunction was observed in the St group (20.7%), while no recipients in the Fr group developed renal dysfunction (p = 0.012). Heart failure, respiratory failure, central nervous system complications, gastrointestinal bleeding, and HCC recurrence were similarly observed between the two groups.

Four recipients (14.8%) in the Fr group died within one yr after transplant because of infection (n = 2), portal vein thrombosis (n = 1), and HCC recurrence (n = 1), while six recipients (17.2%) in

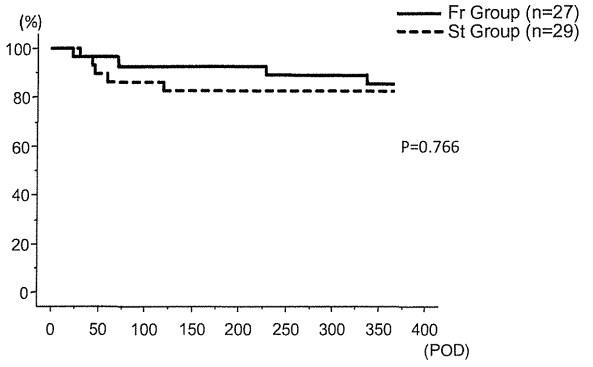


*Fig. 6.* Cumulative patient survival. The patient survival was similar between groups (p = 0.766).

the St group died because of graft failure (n = 2) and infection (n = 3). Patient survival was similar between groups: three and 12 month patient survival was 92.6% and 85.2% in the Fr group, and 86.2% and 82.8% in the St group (Fig. 6).

## Discussion

HCV recurrence after liver transplantation is one of the main obstacles in liver transplantation for HCV-positive recipients. Many factors, such as donor age (28, 29), steroid use (30–32), OKT3 (33, 34), genotype (30, 35, 36), preoperative HCV RNA level (30, 32, 37, 38), and rejection episode (30, 33, 37, 39), are reported risk factors associated with HCV activity and recurrence after liver transplantation. We focused on the IS protocol, particularly on corticosteroid usage. Corticosteroids are considered strong activators of HCV in the setting of post-liver transplant (30–32).

The safety and efficacy of the complete avoidance of corticosteroids for adult liver transplantation have been confirmed in both deceased and LDLT in several studies (9–14), including a single center study in one of our study groups (7). The impact of a steroid-free protocol on HCV-positive recipients has rarely been investigated. A prospective randomized controlled study (RCT) study (HCV-3 study) by Klintmalm et al. showed the safety and efficacy of a steroid-free regimen for HCV-positive liver transplant recipients but failed to show a positive impact on HCV recurrence (17, 19). Another prospective RCT study by Llado et al. showed the safety and efficacy of a cyclosporine-based steroid-free IS protocol in terms of ACR, and patient and graft survival but failed to demonstrate a difference in HCV recurrence or histological fibrosis progression at one or two yr after liver transplantation (18). In an RCT study, Kato et al. failed to show the efficacy of a steroid-free protocol in terms of HCV recurrence or fibrosis progression at one or two yr after liver transplantation (16). One of the key differences between our study and these studies is the implementation of preemptive anti-HCV therapy.

Contrary to these previous studies, we allowed anti-HCV treatment according to the institutional preference in this study. There were 16 (59.3%) patients in the Fr group who received preemptive pegylated interferon with ribavirin therapy, while only nine (31.0%) patients in the St group received preemptive therapy (p = 0.034). The main reason for not receiving preemptive therapy in the St group was HCV recurrence prior to therapy (n = 11, 37.9%). These findings suggested that recipients in the St group had rapid HCV recurrence before anti-HCV preemptive therapy could be initiated, while rapid HCV recurrence was relatively rare in patients in the Fr group.

Steroid-free IS should not be confused with steroid withdrawal or tapering for liver transplant recipients. Several studies have evaluated steroid tapering for liver transplant recipients in a stable condition (20–22). These studies concluded that slow tapering of corticosteroids is favorable with less aggressive progression of HCV in a population of stable post-transplant recipients. Withdrawal of corticosteroids in stable post-transplant recipients is different from the use of a steroid-free protocol used as primary immunosuppression for liver transplantation. Therefore, these data are not comparable.

This study was the first national multicenter prospective study in the field of liver transplantation in Japan, participated in by most of the major liver transplant centers in Japan. Although the study protocol strongly recommended several standard protocols, including a steroid-free IS protocol using the combination of a calcineurin inhibitor, MMF, and basiliximab, protocol liver biopsy at one yr post-transplant, diagnostic liver biopsy upon HCV recurrence, or other graft dysfunction suspected, we did not make any protocol mandatory so that we could recruit as many study patients as possible from each transplant center. Therefore, four of 12 transplant centers implemented a steroid-free protocol, while eight (67%) centers did not implement a steroid-free protocol because of the lack of experience or other reasons.

Background characteristics of recipients in both groups were similar in both recipient and donor demography, except the ratio of HCC exceeding the Milan criteria in the Fr group that was higher than that in the St group. Although surgical duration and blood loss were significantly shorter and lower, respectively, in the Fr group, the differences

between the two groups did not influence the final results of HCV recurrence, suggesting that this is not important factor in HCV recurrence.

This study is novel in that all of the recipients underwent LDLT. There has been debate about the impact of deceased and living donors on HCV recurrence after liver transplantation (40–43). The results may differ depending on the donor type, and further studies are needed.

Although the target trough level of tacrolimus was the same in the two groups, the actual trough level was significantly higher in the St group than in the Fr group until 90 d post-transplant (Fig. 1). On the other hand, the trough level of cyclosporine A was similar between groups (Fig. 1). The difference in the tacrolimus level might have to do with the dose adjustments at different centers and might have resulted in the better outcome of preserved renal function in the Fr group.

In the Fr group, no steroids were administered as during the transplant as defined, but a fair amount of steroids were administered between POD1 and POD28 (Fig. 2). On the other hand, MMF was almost routinely administered in the Fr group and tapered after 90 d to the level in the St group (Fig. 3).

Preoperative serum HCV RNA level, which was reported to be a risk factor for HCV recurrence after liver transplantation (30, 32, 37, 38), was higher in the Fr group than in the St group. Despite the initial higher viral load of HCV, the time course of serum HCV RNA showed that it was suppressed for the first 14 d then gradually increased until 90 d after the transplant in the Fr group, while it was rapidly replicated in the St group (Fig. 4). This result was almost to the same as that in a previous report from a single institute (15). Llado et al. reported in their RCT study that the viral load six months after liver transplantation was not different between patients receiving IS with or without steroids, but they did not investigate the viral load within 90 d after liver transplant. We therefore consider that suppression of HCV viral load after liver transplantation by a steroid-free IS was effective only in the early period until 90 d after liver transplantation.

HCV recurrence was diagnosed in each institute by either (1) liver biopsy and the clinical course after HCV treatment or (2) clinical manifestation of HCV recurrence with elevated liver function and serum HCV RNA level, as well as ruling out ACR and cause of graft dysfunction, and the clinical course after HCV treatment. HCV recurrence was lower in the Fr group than in the St group, although the difference was not significant (Fig. 5). The HCV recurrence rate at one yr was 22.2% in the Fr group, much lower than that in the St group (41.4%). The incidence of HCV recurrence in the St group was comparable to that in the previous reports, about 50% at one yr (44), and that in the Fr group was suppressed. Although the difference in the incidence of HCV recurrence between the Fr and St groups by logrank analysis did not reach statistical significance, it was a substantial difference and would likely be statistically significant with a greater number of study patients. The comparison between tacrolimus and cyclosporin A showed no difference in HCV recurrence between them, consistent with the previous report (44).

Histological findings of 22 protocol liver biopsies (12 in Fr group and 10 in St group) showed that HCV recurrence-free cases in Fr group ($n = 7$) had the least HAI score and Ishak fibrosis stage compared with cases in St groups ($n = 10$) and HCV recurrent cases in Fr group ($n = 5$). It was noted that all the seven HCV recurrence-free cases in Fr group were on the preemptive anti-HCV therapy at the time of one yr post-transplant protocol biopsy, whereas six cases (40%) of 15 cases in the rest of the cases were on preemptive anti-HCV treatment. These results suggested that combination of steroid-free IS protocol with preemptive anti-HCV treatment might be the key point of preventing HCV recurrence and histological fibrosis and hepatitis activity.

The incidence of ACR was similar between the groups. New onset diabetes mellitus after transplantation and CMV infection were not observed in the Fr group, while they occurred in a substantial number of the recipients in the St group ($p = 0.022$, $p < 0.0001$, respectively). This result was compatible with those of other reports comparing steroid avoidance (16), suggesting that steroid has negative impact on diabetes and CMV infection after liver transplantation. Bacterial infection tended to be more frequent and sometimes life threatening in the St group compared with the Fr group, where none of the recipients died from bacterial infection. Renal dysfunction was more frequent in the St group than in the Fr group and might be one reason why the trough level of tacrolimus was lower in the Fr group than in the St group. Other complications such as heart failure, central nervous system complication, and HCC recurrence were not different between the groups. These results confirmed that steroid-free IS consisting of calcineurin inhibitor of a standard dose, MMF, and basiliximab was safe and resulted in a lower incidence of bacterial infection, CMV infection, and renal toxicity.

As a result of compromising the regulation of the protocol biopsy to recruit as many study

patients as possible, the rate of liver biopsy at one yr after liver transplant was only 46.8% (52.1% of one yr survivors in the Fr group and 41.7% of one yr survivors in the St group). Although this may somewhat weaken the interpretation of the results of this study, we believe that the findings are relevant because the diagnosis of allograft dysfunction, including HCV and ACR, was made based on the institutional criteria by professionally skilled pathologists and physicians at each transplant center. Our study has other weaknesses as well. First, this was not a randomized control study. It was too early to perform randomization of a steroid-free IS for liver transplantation because of the fear of using steroid-free IS in some centers without previous experience using the protocol. There could be an institutional bias as well as a patient bias. Second, the small sample size is also a weakness. To confirm the finding of this study that a steroid-free IS protocol reduces the incidence of HCV recurrence after LDLT, a randomized control study with more patients is necessary. This study provides statistical evidence for planning such an RCT study, which should be undertaken in the near future.

In conclusion, this multicenter prospective cohort study in Japan confirmed that a steroid-free IS regimen consisting of a calcineurin inhibitor of a standard dose, MMF, and basiliximab safely reduced new onset diabetes mellitus after transplantation and bacterial or CMV infection, spared renal dysfunction. It is also observed that steroid-free IS regimen was effective toward suppressing the HCV viral load in early post-transplant period as well as HCV recurrence in HCV-positive LDLT recipients.

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今月のテーマ　PBC診療の最前線

# 原発性胆汁性肝硬変に対する肝移植

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**要旨**：原発性胆汁性肝硬変（primary biliary cirrhosis；PBC）は肝移植の主要な適応疾患のひとつである．近年は，内科的治療の進歩のためか，欧米でのPBC移植数は減少傾向にある．しかし，末期肝硬変に至ったPBCでは，現在でも肝移植は唯一の救命法であり，適切な移植時期は予後予測モデルなどから計られる．移植成績は他の疾患と比較して良好で，5年生存率はいずれの報告でも70%を越える．また，その成績に生体ドナーと脳死ドナーの差は認められない．PBCは移植後グラフト肝に再発するが，頻度，危険因子，長期予後など不明な部分もいまだに多い．

**索引用語**：原発性胆汁性肝硬変，肝移植，予後予測，成績，再発

## はじめに

原発性胆汁性肝硬変（primary biliary cirrhosis；PBC）は，小葉間胆管障害による進行性の肝内胆汁うっ滞による肝障害をきたし，緩除に進行して高度の黄疸，腹水，静脈瘤出血を呈する非代償性肝硬変に至る原因不明の疾患である．臨床症状を認めない無症候性PBCの場合，10年生存率は50%から70%と高いが，臨床症状を有する症候性PBCの場合，生存期間の中央値は5年から8年と報告されている[1)2)]．主な死因は，肝不全か門脈圧亢進症による食道静脈瘤破裂とされ[3)]．組織学的に進行した例では肝細胞癌の合併もあり得る[4)]．いまだに根治的治療は確立されていないが，ウルソデオキシコール酸（ursodeoxycholic acid；UDCA）はPBCの進行抑制効果が複数のランダム化比較試験で確認されており，臨床現場では第一選択薬として用いられている．UDCAの継続的な内服はPBC患者の生化学検査値を改善させるばかりでなく，組織学的な進行を遅らせ生存率の改善をもたらすことが明らかになっているが[5)〜7)]，その予後改善効果はすべてのPBC患者にもたらされるわけではない．組織学的に進行してから投与を開始した例や，UDCA投与によっても生化学検査結果の改善効果が乏しい例の生存率は，無治療例の生存率と差がないことが判明している[7)8)]．すなわち，PBC患者が末期肝硬変まで進行した段階では予後を改善する有効な内科的治療法はなく，肝移植のみが唯一の救命法となる．本稿ではPBCに対する肝移植の現況を概説した．

## I　PBCに対する肝移植の動向

PBCによる末期肝硬変は，肝移植が臨床応用された初期から主要な適応疾患のひとつであった．1980年代に報告されたIwatsukiら[9)]の1000例の肝移植の中でPBCは適応疾患の16.5%を占め，二番目に頻度の高い適応疾患であった．ELTR（European Liver Transplant Registry）による1988年から2001年までの欧州全体の統計では，

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Liver transplantation for primary biliary cirrhosis

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PBCは全移植症例39196例中2969例（8%）を占め，アルコール性肝硬変，C型肝硬変，急性肝不全に次ぐ頻度となっている[10]．米国のOPTN（Organ Procurement and Transplant Network）の最も新しい統計報告によると，2009年12月末時点でPBCを含む胆汁うっ滞性疾患は，脳死肝移植待機している成人患者の7.9%を占めている[11]．これらの報告からわかるように，初期の頃と比較して最近では移植適応疾患に占めるPBCの割合は減少傾向にある．移植適応疾患が変化したことも一因かもしれないが，欧州においても米国においても肝移植数全体は増加傾向にあるにもかかわらず，PBCの肝移植自体は，全体に対する割合のみならず絶対数も減少傾向にある[12][13]．興味深いことに，同じ肝内胆汁うっ滞性疾患である原発性硬化性胆管炎（primary sclerosing cholangitis；PSC）の割合はほぼ不変であり，このようなPBCに対する肝移植数の減少傾向はUDCAの治療効果によりもたらされたものではないかと考えられている．

わが国において行われた世界初の成人生体肝移植は，PBC患者に対して行われた[14]．このことからもわかるように，PBCは日本においても肝移植が臨床現場に広まるきっかけとなった，重要な適応疾患である．わが国では脳死肝移植普及の遅れから，この成功を契機にして生体肝移植が爆発的に広まった．2010年末までの累計移植数では脳死肝移植95例に対し生体肝移植は6097例であり，わが国の肝移植は98.5%が生体ドナーからの提供で行われた計算になる．このうち2010年末までに初回成人生体肝移植は3796例に行われたが，その中でPBC症例は535例14%を占めており，PBCが本邦でも肝移植の主要な対象疾患であることがわかる[15]．PBCの移植全体に対する頻度は欧米と比較してやや高い印象であるが，各統計の詳細を比較すると欧米において頻度の高いアルコール性肝硬変がわが国ではほとんどなく，このためにPBCの相対的な割合が日本で高くなっているのではないかと考えられる．

## II PBCの移植適応と移植時期

PBCによる肝硬変の移植適応は難治性腹水，繰り返す静脈瘤からの出血や肝性脳症，（ミラノ基準内の）肝細胞癌の合併など，その他の疾患による肝硬変と変わるところはない．それ以外に，胆汁うっ滞性肝障害に比較的特徴的な皮膚掻痒感や慢性的な倦怠感も，はなはだしい場合はPBC患者の生活の質（quality of life；QOL）を著しく障害するため，移植適応となる場合がある[16][17]．

**Table 1.** Child-Turcotte-Pugh（CTP）スコア

| points | 1 | 2 | 3 |
|---|---|---|---|
| Encephalopathy | None | 1 and 2 | 3 and 4 |
| Ascites | Absent | Slight | Moderate |
| Bilirubin（mg/dL） | 1～2 | 2～3 | >3 |
| Albumin（g/dL） | >3.5 | 2.8～3.5 | <2.8 |
| INR | <1.7 | 1.7～2.3 | >2.3 |
| For PBC：bilirubin | 1～4 | 4～10 | >10 |

一般に肝移植は，移植を受けない場合に予想される生存率を移植後に期待し得る生存率が上回るタイミングで行われる．食道静脈瘤に対する食道離断術のリスク評価のために作成されたChild-Turcotte-Pugh（CTP）スコア（Table 1）[18][19]や，経静脈的肝内門脈大循環短絡術後の予後予測のために作成されたmodel for end-stage liver disease（MELD）スコアは（Table 2）[20]，慢性肝疾患の一般的予後予測スコアとして有用性と汎用性が認められている．これらのスコアを用いると，CTPスコアが7もしくはMELDスコアが15を越えた場合に，移植を受けた場合の生存率が移植を受けない場合の生存率を上回ると判断され，肝移植の施行が推奨される[21]．

PBCは症例間で自然経過の差が少ないため，さまざまな予後予測モデルの作成が試みられてきた．Mayo Clinicにおいて中央値5.5年の経過観察を受けた無治療PBC 312例のデータを用いて同定された因子から作成された予後予測式がMayo natural history modelで，年齢，血清ビリルビン値，アルブミン値，プロトロンビン時間，浮腫の有無，利尿薬の有無から計算される（Table 2）[22]．Mayo modelから得られたリスクスコア（R）で7年後までの予測生存率が計算可能であり，このモデルの有用性は，Mayo Clinicにおける別の

**Table 2.** PBCに用いられる予後予測式

1. Model for end-stage liver disease (MELD) score
   $9.57\times\log_e$ (creatinine mg/dL) $+3.78\times\log_e$ (bilirubin mg/dL) $+11.2\times\log_e$ (international normalized ratio [INR]) $+6.43$.
   Laboratory values less than 1.0 were set to 1.0 and the maximum serum creatinine was set to 4.0 mg/dL.

2. The Mayo natural history model for PBC
   $R=0.039$ (age) $+0.871\ \log_e$ (bilirubin) $-2.53\ \log_e$ (albumin) $+2.38\ \log_e$ (prothrombin time) $+0.859$ (edema*)
   *0 = no edema without diuretic therapy ; 0.5 = edema without diuretic therapy or edema resolved with diuretic therapy ; 1 = edema with diuretic therapy.
   (http://www.mayoclinic.org/gi-rst/mayomodel1.html)

3. The updated Mayo natural history model for PBC
   $R=0.051$ (age) $+1.209\ \log_e$ (bilirubin) $-3.304\ \log_e$ (albumin) $+2.754\ \log_e$ (prothrombin time) $+0.675$ (edema*)
   (http://www.mayoclinic.org/gi-rst/mayomodel2.html)

4. 日本肝移植適応研究会の予後予測式
   $\lambda=-4.33+1.2739\ \log_e$ (total bilirubin) $+4.4880\ \log_e$ (AST/ALT)
   6カ月後の死亡確率 (%) $=1/(1+e-\lambda)\times100$

106例のPBC患者と，他の施設の176例で確認された[23]．それ以前に発表された予後予測式と異なり[24][25]，このMayo modelは変数に組織学的評価を含まないという臨床的に有利な点もあり，一般に広く用いられるようになった．また，自然経過による予後を予測するばかりでなく，移植前のリスクスコアが7.8を越えると移植後の死亡リスクが上昇し，集中治療室入室日数，在院日数，輸血量など医療資源の必要量も有意に増加することが示されており，このスコアを越える前の移植施行が適切とされている[26][27]．

Mayo modelにより得られるリスクスコアを時系列で観察すると，患者死亡の2年以上前は年率0.23の割合で増加するが，死亡の2年以内になるとその割合が年率1.4に急増することが判明した．この結果は，短期間で死亡するリスクの高い患者に対してはMayo modelが生存率を過大に評価している可能性を示している．そこでMurtaughらは患者の時系列での観察から得られたデータを用いてupdated Mayo modelを作成した(Table 2)[28]．このupdated modelは2年以内の生存予測に関しては元のmodelより優れていることが示されているため，短期的な予後予測にはupdated modelを用いることが勧められる．

一方，わが国では日本肝移植適応研究会において症候性PBC 141例を解析し，血清ビリルビン値とAST/ALTの2つを変数とする独自の予後予測式が作成されている (Table 2)[29]．現行のわが国の脳死肝移植レシピエント登録基準では，この日本肝移植適応研究会モデルでの死亡確率を用いて医学的緊急性に関する点数配分が行われる．

## III PBCの移植成績

1980年代に，英国King's College病院のグループと米国Pittsburgh大学のグループが，予後予測モデルにより得られた予測生存と比較して実際の移植後生存が明らかに上回っていることを示し，末期肝硬変に至ったPBC患者に対する有効な治療としての肝移植が確立した[30][31]．1980年代前半のPBCに対する移植成績は1年生存率76%，2年生存率75%であったが，1990年代には周術期管理の進歩もあり，それぞれ93%，90%と更に改善している[26]．ELTRからの2001年までの統計では，欧州全体でPBCの生存率は移植後1年，5年，10年でそれぞれ83%，77%，69%

**Table 3.** 移植後再発PBCに関する報告

| | 報告年 | N | 観察期間（中央値，月） | 肝生検 | 再発率（%） | 再発までの期間 |
|---|---|---|---|---|---|---|
| Liermann Garcia[35] | 2001 | 400 | 56 | protocol | 17 | 36 |
| Sanchez[39] | 2003 | 156 | 72 | protocol | 11 | 50 |
| Sylvestre[40] | 2003 | 100 | 44 | protocol | 17 | 56 |
| Neuberger[41] | 2004 | 485 | 79 | protocol | 23 | |
| Jacob[42] | 2006 | 100 | 118 | protocol | 14 | 61 |
| Charatcharoenwitthaya[13] | 2007 | 154 | | protocol | 34 | |
| Morioka[44] | 2007 | 50 | 29 | ad hoc | 18 | 36 |
| Montano-Loza[45] | 2010 | 108 | 83 | ad hoc | 26 | 70 |
| Manousou[46] | 2010 | 103 | 108 | protocol | 26 | 44 |
| Kaneko[47] | 2012 | 81 | 74 | ad hoc | 1 | 61 |

と報告されている[10]．この結果は，ウイルス性肝硬変の移植後5年生存率72%，急性肝不全の59%，肝細胞癌の58%と比較して明らかに良い成績で，代謝性疾患などと並んで移植成績の最も良い疾患のひとつであることがわかる．OPTNの統計でも1997年から2004年に移植を受けたPBCを含む胆汁うっ滞性疾患の成績は1年生存率89.8%，5年生存率79.7%と極めて良好である[32]．UNOS(United Network for Organ Sharing)データベースを使った疾患別の移植成績の検討でもPBCの移植成績が最も良好である[33]．PBC患者の各年齢での死亡数を解析した結果では，女性PBC患者の死亡数には1980年代は50歳代後半と70歳代の二峰性のピークが認められたが，1990年代になると若年のピークがなくなり，健常人と同じように加齢にともなう死亡数の上昇のみが認められるようになる[34]．このようなPBC患者の若年での死亡数の減少の一因は，肝移植の普及と成績向上にともなうものと考えられている．なお，PBC患者の移植後死亡原因としては，6カ月以内の早期死亡は主に敗血症，多臓器不全などが原因となり，6カ月以降の晩期死亡は主に敗血症，悪性腫瘍の発生，腎不全，慢性拒絶などが報告されている[35]．

一方，わが国での移植成績は，全体で5年，10年生存率はそれぞれ76.5%，55.6%と報告され[15]，単施設では3年，5年生存率がそれぞれ88%，80%と報告されている[36]．欧米では脳死ドナーを用いた肝移植が主流であるのに対し，わが国では肝移植の98%以上が生体ドナーを用いて行われるという背景の違いはあるが，これらの結果をみるとPBCの肝移植に関しては脳死ドナーと生体ドナーで移植成績に差はないと考えられる．

PBCをはじめとする胆汁うっ滞性肝疾患は，皮膚掻痒感や倦怠感といった症状が他の疾患より高頻度で認められ，これらの症状によるQOLの低下が移植適応とされる場合もある．このようなQOLに対する肝移植の治療成績に関し，Grossらによる肝移植を受けたPBCもしくはPSC患者157人を対象とした検討が報告されている[37]．この報告では術前51%の症例で耐えがたい疲労感，不眠あるいは皮膚掻痒感が認められていたが，移植1年後にはその割合は25%まで減少している．ただし，皮膚掻痒感は最も改善効果が高いが，慢性的な疲労感は移植後も比較的残る場合が多いとされている．また，「ほぼ正常な日常生活を送ることができる」と感じている患者の割合は，移植前の29%から移植後は61%に増加している．このように肝移植は生命予後ばかりでなく，皮膚掻痒感や倦怠感といった症状，あるいは患者のQOLをも改善させるが，術前要因でこのようなQOLの改善効果を予測する因子は見出されていない．

## IV PBCの移植後再発

肝移植後におこるPBCのグラフト肝への再発

は不明な部分が多い．なぜなら，報告により再発の診断基準が一定していないからである．移植前の状態であれば，①アルカリフォスファターゼ（ALP）値の上昇に代表される胆汁うっ滞を示す生化学検査所見，②抗ミトコンドリア抗体（AMA）陽性．③慢性非化膿性破壊性胆管炎（chronic non-suppurative destructive cholangitis；CNSDC）に代表される組織学的所見，という3項目の診断基準が一般的に用いられる[17]．しかし，血清ALP値の上昇は慢性拒絶やサイトメガロウイルス感染，薬剤性胆汁うっ滞などの移植後のさまざまな病態で認められる．AMAに関しても，特にM2抗体は，組織学的な再発がなくても移植後に陽性で経過することが報告されている[38]．このような理由から，組織学的な診断が移植後にPBCの再発を診断する唯一の方法となっている．

これまでの移植後再発PBCに関する主な報告をTable 3[35)39)~47)]に示す．PBC再発頻度はおおむね10%から30%，再発までの期間は3年から6年と一定の傾向はない．しかし，組織学的なPBCの再発は必ずしも臨床症状や検査値異常をともなわないため[35]，肝生検が症状出現時のみに行われたのか（ad hoc），プロトコール肝生検を行ったかで結果は異なると考えられる．再発関連因子としてレシピエントの年齢[35)42)]，性別[43]，免疫抑制剤の種類[41)~43)]，ドナーとレシピエントのHLA適合[44]などが報告されているが，いまだに確立されたものはない．

グラフト肝への再発は，現時点では患者の予後に与える影響は少ないと考えられている．Liermann Garciaらは，68例の移植後再発PBC患者のうち，肝硬変に至るか再移植を要した例は2例のみであったと報告している[35]．しかし今後，移植後観察期間が長期になった場合の影響はいまだに明らかではないと思われる．また，移植後再発PBCに対する治療としてはUDCAを用いた報告が多いが[35)39)43)]，生化学検査の改善以外の組織学的進展や予後への影響はやはり明らかではない．

## おわりに

UDCAの登場により末期肝硬変に至るPBC患者が減少していることは実臨床上でも実感される．しかし，一部の症例は内科的治療に抵抗し，このような患者が肝硬変に至ると現時点では移植以外に治療法がないことも事実である．肝移植の黎明期から適応疾患のひとつとされてきたPBCに対する移植成績はほぼ確立したものになりつつあるが，移植後再発PBCの病態，治療，予後など新しい課題が生まれつつある．

本論文内容に関連する著者の利益相反
：なし

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Research Article

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# Bak deficiency inhibits liver carcinogenesis: A causal link between apoptosis and carcinogenesis

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**Background & Aims**: Hepatocyte apoptosis is a key feature of chronic liver disease including viral hepatitis and steatohepatitis. A previous study demonstrated that absence of the Bcl-2 family protein Mcl-1 led to increased hepatocyte apoptosis and development of liver tumors in mice. Since Mcl-1 not only inhibits the mitochondrial pathway of apoptosis but can also inhibit cell cycle progression and promote DNA repair, it remains to be proven whether the tumor suppressive effects of Mcl-1 are mediated by prevention of apoptosis.

**Methods**: We examined liver tumor development, fibrogenesis, and oxidative stress in livers of hepatocyte-specific knockout (KO) of *Mcl-1* or *Bcl-xL*, another key antagonist of apoptosis in hepatocytes. We also examined the impact of additional KO of *Bak*, a downstream molecule of Mcl-1 towards apoptosis but not the cell cycle or DNA damage pathway, on tumor development, hepatocyte apoptosis, and inflammation.

**Results**: *Bcl-xL* KO led to a high incidence of liver tumors in 1.5-year-old mice, similar to *Mcl-1* KO. Bcl-xL- or Mcl-1-deficient livers showed higher levels of TNF-α production and oxidative stress than wild-type livers at as early as 6 weeks of age and oxidative DNA damage at 1.5 years. Deletion of Bak significantly inhibited hepatocyte apoptosis in *Mcl-1* KO mice and reduced the incidence of liver cancer, coinciding with reduction of TNF-α production, oxidative stress, and oxidative DNA damage in non-cancerous livers.

**Conclusions**: Our findings strongly suggest that chronically increased apoptosis in hepatocytes is carcinogenic and offer genetic evidence that inhibition of apoptosis may suppress liver carcinogenesis in chronic liver disease.

Keywords: Bcl-xL; Mcl-1; 8-OHdG.
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*Abbreviations:* HCC, hepatocellular carcinoma; ALT, alanine aminotransferase; RT-PCR, reverse-transcription PCR; HO-1, heme oxygenase-1; NQO1, NAD(P)H:quinone oxidoreductase 1; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling.



## Introduction

Apoptosis of epithelial cells, as well as infiltration of inflammatory cells or deposits of fibers, is frequently observed in the chronic diseased liver, which is a high-risk condition for hepatocellular carcinoma (HCC) [1]. For example, Fas-mediated hepatocyte apoptosis is a mechanism of cell death in chronic hepatitis C virus infection and hepatitis B virus infection [2,3]. Hepatocyte apoptosis shows correlation with inflammation and fibrosis in non-alcoholic steatohepatitis [4]. Cytokeratin 18 neoepitope, a well-established marker of caspase activity in serum, is elevated and associated with liver injury in chronic viral hepatitis and non-alcoholic steatohepatitis [5–7]. Although viral factors and overt organ inflammation linked to liver cancer development have been extensively studied [8,9], less information is available on the involvement of hepatocyte apoptosis in liver cancer development.

Bcl-xL and Mcl-1 are among the anti-apoptotic members of the Bcl-2 family, which antagonizes the pro-apoptotic function of Bak and/or Bax at the mitochondrial outer membrane. We previously reported that hepatocyte-specific *Bcl-xL* or *Mcl-1* knockout (KO) mice showed persistent apoptosis of hepatocytes in the adult liver and mild fibrotic responses [10,11]. A very recent study by Weber *et al.* [12] demonstrated that hepatocyte-specific *Mcl-1* KO mice developed liver tumors in old age. This observation raised the important possibility that apoptosis in hepatocytes could lead to the development of liver cancer. However, as Mcl-1 has been reported to possess functions other than anti-apoptosis, such as cell cycle inhibition [13,14] and DNA damage repair [15,16], it is difficult to conclude that the phenotypes observed in *Mcl-1* KO are simply ascribable to apoptosis. Indeed, *Mcl-1* KO mice showed not only increased apoptosis but also increased regeneration in the liver [12]. In the present study, we demonstrated that hepatocyte-specific *Bcl-xL* KO mice also develop liver cancer in old age and that deficiency of Bak, a downstream effector molecule of Mcl-1 towards the

**Table 1. Incidence of liver tumors in KO mice.**

| Age (yr) | Genotype | Tumor incidence |
|---|---|---|
| 1.5 | | |
| | *Bcl-xL*$^{+/+}$ | 0% (0/10) |
| | *Bcl-xL*$^{-/-}$ | 88% (7/8)* |
| 1 | | |
| | *Bcl-xL*$^{+/+}$ | 0% (0/4) |
| | *Bcl-xL*$^{-/-}$ | 27% (3/11) |
| 1.5 | | |
| | *Mcl-1*$^{+/+}$ | 0% (0/22) |
| | *Mcl-1*$^{-/-}$ | 100% (16/16)* |
| 1 | | |
| | *Mcl-1*$^{-/-}$ *Bak*$^{+/+}$ | 64% (14/22) |
| | *Mcl-1*$^{-/-}$ *Bak*$^{-/-}$ | 0% (0/7)* |

*$p$ <0.05 *vs.* control.

mitochondrial pathway of apoptosis, clearly suppresses hepatocyte apoptosis and liver carcinogenesis in *Mcl-1* KO mice. We also considered possible mechanisms involving oxidative stress that underlie elevated malignant transformation in the apoptosis-prone liver. The present study offers strong support for the hypothesis that chronically increased apoptosis in hepatocytes is carcinogenic. It also provides genetic evidence that inhibition of apoptosis may suppress liver carcinogenesis in chronic liver disease.

## Materials and methods

*Mice*

Conditional *Bcl-xL* KO mice (*bcl-x*$^{flox/flox}$ *Alb-Cre*) and *Mcl-1* KO mice (*mcl-1*$^{flox/flox}$ *Alb-Cre*) were previously described [11]. We purchased *Bak* KO mice (*bak* $^{-/-}$) from the Jackson Laboratory (Bar Harbor, ME). We generated hepatocyte-specific Bak/Mcl-1 double KO mice (*bak* $^{-/-}$ *mcl-1*$^{flox/flox}$ *Alb-Cre*) by mating the strains. They were maintained in a specific pathogen-free facility and treated with humane care with approval from the Animal Care and Use Committee of Osaka University Medical School. Measurement of serum alanine aminotransferase (ALT) level, caspase-3/7 activity and histological analyses have been previously described [11].

*Western blot analysis*

For immunodetection, the following antibodies were used: anti-Bcl-xL antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Mcl-1 antibody (Rockland, Gilbertsville, PA), anti-Bak antibody (Millipore, Billerica, MA), anti-Bax antibody, anti-ERK antibody, anti-phospho-ERK antibody, anti-p38 antibody, anti-phospho-p38 antibody, anti-JNK antibody, anti-phospho-JNK antibody, anti-PCNA antibody (Cell Signaling Technology, Danvers, MA), and anti-beta-actin antibody (Sigma-Aldrich, Saint Louis, MO).

*Real-time reverse-transcription PCR (RT-PCR)*

The following TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) were used: mouse-AFP (Mm00431715_m1), mouse-glypican-3 (Mm00516722_m1), mouse-IL-6 (Mm00446190_m1), mouse-TNF-α (Mm00443258_m1), mouse-MCP-1 (Mm00441242_m1), mouse-CD68 (Mm03047343_m1), mouse-CD4 (Mm00442754_m1), mouse-CD8 (Mm01182108_m1), mouse-heme oxygenase-1 (HO-1) (Mm00516005_m1), mouse-NAD(P)H:quinone oxidoreductase 1 (NQO1) (Mm00500821_m1), and mouse-Beta actin (Mm00607939_s1). All expression levels were corrected with the quantified expression level of beta actin.

*Immunohistochemistry*

8-Hydroxy-2′-deoxyguanosine (8-OHdG), cleaved caspase-9, PCNA, and ki-67 were labeled in paraffin-embedded liver sections using anti-8-OHdG antibody (Nikken Seil, Tokyo, Japan), anti-cleaved caspase-9 antibody, anti-PCNA antibody (Cell Signaling Technology), and anti-ki-67 antibody (Dako, Tokyo, Japan), respectively. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) was performed according to a previously reported procedure [17].

*Statistical analysis*

Data are presented as mean ± SD. Differences between two groups were determined using the Student's *t*-test for unpaired observations. Carcinogenesis rates were analyzed using the Chi-square test. Multiple comparisons of *Bak/Mcl-1* double KO mice were performed by ANOVA followed by Scheffe *post hoc* correction. Fisher *post hoc* correction was used for the other multiple comparisons. A $p$ <0.05 was considered statistically significant.

## Results

*Bcl-xL KO mice develop liver tumors in old age*

We previously reported that hepatocyte-specific *Bcl-xL* KO mice developed spontaneous hepatocyte apoptosis by the mitochondrial pathway (Supplementary Fig. 1A) at as early as 1 month of age with a gradual increase in the liver fibrotic response from 3 to 7 months [10]. To examine the phenotypes at later time points, we sacrificed *Bcl-xL* KO mice and their control littermates at 1 and 1.5 years of age. Macroscopic tumors had developed in the liver of 27% and 88% of the KO mice, respectively, but not in the control littermates (Fig. 1A and Table 1). Most of the *Bcl-xL* KO mice had multiple tumors and the liver body-weight ratio for *Bcl-xL* KO mice was significantly higher than that of the control mice (Fig. 1B and C). Tumors were histologically defined as well-differentiated HCCs (Fig. 1D). To find out whether the *bcl-x* gene is really targeted in the tumors, we performed Western blot analysis for the expression of the Bcl-2 family proteins (Fig. 1E and Supplementary Fig. 2A). The tumors were confirmed to be deficient for Bcl-xL, excluding the possibility that transformed cells arising from hepatocytes in which the *bcl-x* gene was not deleted had expanded to form tumors. Interestingly, most of these tumors showed apparently higher levels of Mcl-1 expression than the wild-type liver or the non-cancerous surrounding tissues. Reciprocal overexpression of Mcl-1 may explain the possible survival advantage of these tumors. Tumors in *Bcl-xL* KO mice expressed higher levels of α-fetoprotein (Fig. 1F) and frequently showed activation of ERK and JNK (Fig. 1G), which are observed in human HCC [18,19].

*Liver tumors in Mcl-1 KO mice show similar characteristics to human HCC*

We have previously reported phenotypes of hepatocyte-specific *Mcl-1* KO mice, which display spontaneous hepatocyte apoptosis by the mitochondrial pathway (Supplementary Fig. 1B) and liver fibrotic responses at an early age [11]. Since our *Mcl-1* floxed mice differed from those of Weber *et al.* [12] in origin, we next examined the development of liver tumors in our hepatocyte-specific *Mcl-1* KO mice. All the *Mcl-1* KO mice, but none of the control littermates, developed liver tumors at 1.5 years of age, with a significant increase of liver body-weight ratio (Fig. 2A–C

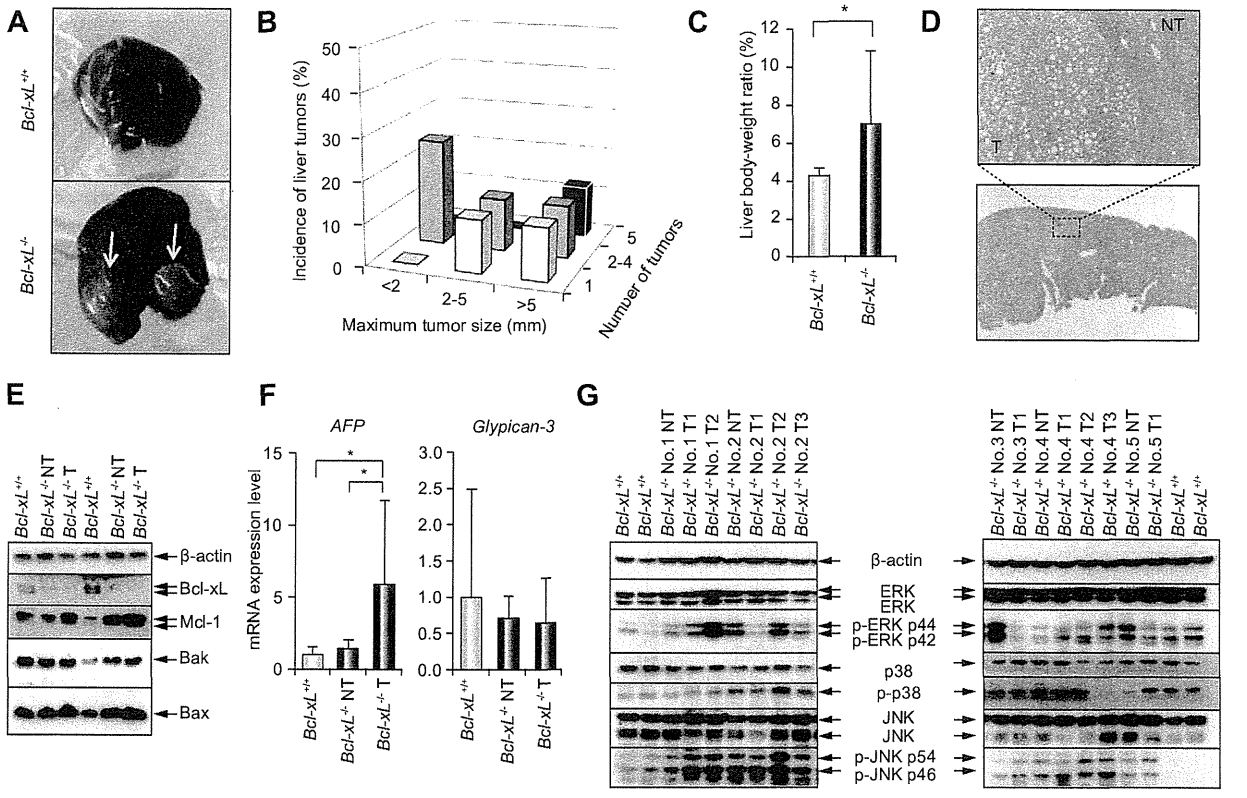


**Fig. 1. Liver tumors in *Bcl-xL* KO mice.** (A–E) Hepatocyte-specific *Bcl-xL*-deficient mice (*Bcl-xL* $^{-/-}$) (N = 8) and their control littermates (*Bcl-xL*$^{+/+}$) (N = 10) were sacrificed at 1.5 years of age. (A) Representative macroscopic view of the livers with arrows indicating tumors. (B) Incidence of liver tumors separated by maximum tumor size and number of tumors. (C) Liver body-weight ratio. (D) Representative histology of liver tumors in *Bcl-xL* KO mice. (E) Western blot of the Bcl-2 family proteins in tumors (T) and surrounding non-cancerous livers (NT) of *Bcl-xL* KO mice and livers of control mice. (F and G) Characteristics of liver tumors in *Bcl-xL* KO mice. (F) Real-time RT-PCR analysis of the expression levels of α-fetoprotein (*AFP*) and *glypican-3* mRNA (N = 9 or 10 per group). (G) Expression and activation of mitogen-activated protein kinases. *$p$ <0.05.

and Table 1). As in the case of tumors of *Bcl-xL* KO mice, liver tumors that developed in *Mcl-1* KO mice were deficient for Mcl-1 expression and, in most cases, reciprocally overexpressed Bcl-xL (Fig. 2E and Supplementary Fig. 2B). These tumors expressed higher levels of α-fetoprotein and glypican-3 (Fig. 2F) and frequently showed activation of ERK and JNK (Fig. 2G).

*Inflammatory response and oxidative stress occur in Bcl-xL- or Mcl-1-KO livers*

To examine the molecular mechanism of tumor development, we examined gene expression in the livers of 6-week-old *Bcl-xL* or *Mcl-1* KO mice. Real-time RT-PCR analysis revealed increases of inflammatory cytokine TNF-α, but not IL-6, and chemokine MCP-1 in *Bcl-xL* and *Mcl-1* KO livers (Fig. 3A and B), despite overt histological inflammation (data not shown). Together with an increase of MCP-1, CD68 expression was significantly higher in KO livers than in control livers (Fig. 3C and D). In contrast, there was no difference in the expression of CD4 and CD8 between the groups. These findings suggest that activation or infiltration of myeloid-derived cells and production of TNF-α are characteristic of the *Bcl-xL* or *Mcl-1* KO liver. Together with the previous study reporting that TNF-α promotes cellular transformation [20], these results suggest that the increase in TNF-α may be one of the mechanisms of tumor development.

Since oxidative stress is also reported to cause carcinogenesis [21], we examined the expression of HO-1 and NQO1, inducible anti-oxidant enzymes, and 8-OHdG in the liver tissues. Real-time RT-PCR analysis revealed that HO-1 and NQO-1 expressions were significantly increased in *Mcl-1* KO livers at 6 weeks (Fig. 3E). 8-OHdG staining revealed that there were few 8-OHdG positive nuclei in both *Mcl-1* KO and the control liver at 6 weeks of age. However, scattered positive nuclei were observed in KO livers at 1.5 years of age, but not in the tumors, and the number of positive nuclei was significantly higher in KO livers than in control livers (Fig. 3F and Supplementary Fig. 3). Similarly, the number of 8-OHdG positive nuclei was significantly higher in *Bcl-xL* KO livers at 1.5 years of age than in control livers (Fig. 3G). These results suggest that oxidative stress may occur at as early as 6 weeks of age in KO livers and that oxidative injury arises at a later time point.

*Bak deficiency significantly ameliorates hepatocyte apoptosis and reduces tumor development in Mcl-1 KO mice*

Bak is a proapoptotic Bcl-2 family protein, which is able to oligmerize to form pores at the outer membrane of mitochondria. To understand whether inhibition of apoptosis could reduce the carcinogenic potential, we crossed *Mcl-1* KO mice and *Bak* KO mice and generated *Bak Mcl-1* double KO mice. As expected, *Bak* KO significantly suppressed hepatocyte apoptosis in *Mcl-1*