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＜短　報＞

# オルセイン染色の実態調査結果と標準化への試み

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緒言：原発性胆汁性肝硬変（PBC）の診断に際し，慢性胆汁うっ滞の指標である銅沈着は病初期から出現する重要な組織所見であり，またPBCの組織学的病期分類の指標として用いられつつある[1)~3)]．オルセイン染色は銅関連蛋白を敏感に染め出す染色法[4)]であり，PBC病理診断時の特殊染色として一般化しつつあるが，染色結果は施設間でのばらつきが大きい．我々はオルセイン染色の一般化と標準化を目指し，各施設での本染色法の現状を把握すべく実態調査を行った．

対象と方法：銅の沈着を伴うPBC 2症例の肝未染色標本を北陸10施設に送付し，各施設および我々のプロトコールに従い染色を依頼．各施設のプロトコールおよび染色標本を回収し，銅関連蛋白および弾性線維の染色性について検討した．なお，銅関連蛋白の染色性の評価は，陽性顆粒が明らかでないものを陰性，弱拡大（40倍視野）で容易に認識できるものを強陽性，40倍では認識困難であるが100倍視野で認識できるものを弱陽性とした．

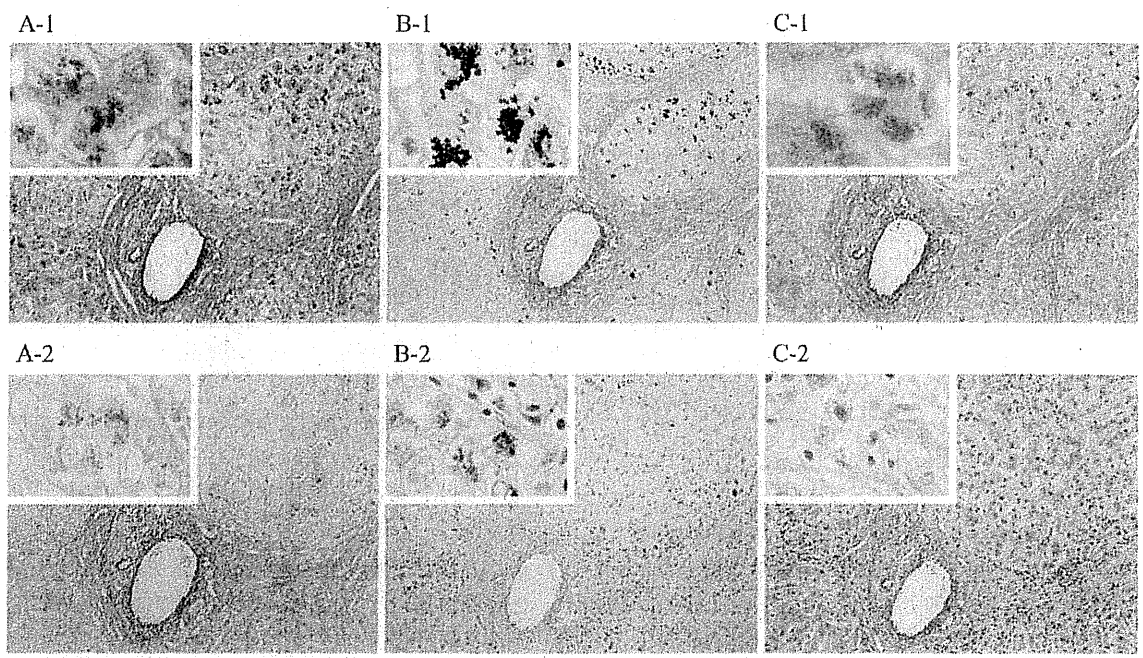


**Fig. 1** Difference of orcein staining results between institutions. (A) Laboratories using Merck's orcein. (A-1) Reducer: Oxalic acid, Laboratory A (A-2) Reducer: Sodium bisulfite, Laboratory F (B) Laboratories using Tokyo Chemical Industry's orcein. (B-1) Reducer: Oxalic acid (3%), Laboratory G (B-2) Reducer: Oxalic acid (5%), with hematoxylin stain, Laboratory D (C) Laboratories using Muto Pure Chemical's orcein. (C-1) Reducer: Oxalic acid, Laboratory J (C-2) Reducer: Sodium bisulfite, with hematoxylin stain, Laboratory H.

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Table 1

| Laboratory | Orcein | | | Oxidizer | | | Reducer | | | Results | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Manufacturer | % | Staining time | Reagent | % | Time | Reagent | % | Time | copper-binding protein | elastic fiber |
| A | Merck | 0.5% | 2-3 hours | potassium permanganate & sulfuric acid | 0.25% | 1 min | oxalic acid | 2% | 1 min | good | good |
| B | Merck | 0.5% | 2 hours | potassium permanganate & sulfuric acid | 0.20% | 1 min | oxalic acid | 1.5% | 1 min | good | good |
| C | Merck | 0.5% | 1 hour 30 min | potassium permanganate & sulfuric acid | 0.20% | 2 min | oxalic acid | 1.5% | 1 min | good | good |
| D | Tokyo Chemical Industry | 1% | 1 hour | potassium permanganate & sulfuric acid | 0.28% | 2 min | oxalic acid | 5% | 2 min | weak | weak |
| E | Muto Pure Chemicals | 1% | 1 hour | potassium permanganate & sulfuric acid | 0.15% | 2 min | sodium bisulfite | 3% | 1 min | weak | good |
| F | Merck | 1% | 30 min | potassium permanganate & sulfuric acid | 0.15% | 3 min | sodium bisulfite | 3% | 1 min | weak | good |
| G | Tokyo Chemical Industry | 1% | 2 hours 30 min | potassium permanganate & sulfuric acid | 0.15% | 3 min | oxalic acid | 3% | 1 min | good | good |
| H | Muto Pure Chemicals | 1% | 2-3 hours | potassium permanganate & sulfuric acid | 0.15% | 2 min | sodium bisulfite | 3% | 1 min | poor | good |
| I | Merck | 0.5% | 2-3 hours | potassium permanganate & sulfuric acid | 0.25% | 1 min | oxalic acid | 2% | 1 min | good | good |
| J | Muto Pure Chemicals | 1% | 2 hours | potassium permanganate & sulfuric acid | 0.15% | 2 min | oxalic acid | 5% | 30 sec | weak | good |
| K | Muto Pure Chemicals | 1% | 2 hours 30 min | potassium permanganate & sulfuric acid | 0.15% | 2 min | oxalic acid | 5% | 30 sec | poor | good |

結果：オルセイン染色の工程は，いずれの施設でもほぼ同じで，脱パラフィン後，酸化液，還元液に反応させオルセイン試薬にて染色していた．しかし，使用するオルセイン試薬，酸化液，還元液は施設によって異なっていた．オルセイン試薬は，メルク，東京化成工業，武藤化学の製品が使用され，メルクが当教室を含む5施設，東京化成工業が2施設，武藤化学が4施設で，濃度は0.5又は1%であった．染色時間は30分～3時間と幅がみられた．酸化液はすべての施設で過マンガン酸カリウム硫酸混合液が使用され，濃度は0.15～0.28%，時間は1～3分であった．還元液はシュウ酸が8施設，重亜硫酸ナトリウムが3施設で使用され，濃度はシュウ酸が1.5～5%，重亜硫酸ナトリウムが3%で時間は30秒～2分であった．Fig. 1およびTable 1に，当教室を含めた施設間の染色性の相違をオルセイン試薬と還元液別に示す．メルク使用施設では，還元液にシュウ酸を使用した施設は銅関連蛋白が強陽性であったが，重亜硫酸ナトリウム使用施設は，弱陽性であった．また，弾性線維は染色良好だった．東京化成工業使用施設は，還元液はともにシュウ酸を使用していたが，1施設は銅関連蛋白が強陽性，弾性線維が良好に染色され，他の1施設は各々弱陽性，不良であった．武藤化学使用施設は，還元液にシュウ酸，重亜硫酸ナトリウムを使用している施設共に，弾性線維はすべて良好であったが，銅関連蛋白は弱陽性と陰性であった．また，ヘマトキシリン染色を併せて行う施設が4施設（施設

D，E，H，K）あったが，Fig. 1B-2およびC-2に示した如く，核染色を行うと陽性顆粒が見にくい印象であった．当教室を含む11施設による染色結果は，銅関連蛋白に関しては5施設で強陽性，4施設で弱陽性，2施設で陰性であった．また，弾性線維では10施設で良好，1施設で不良であった．他施設での我々のプロトコールによる銅関連蛋白の染色結果は11施設中7施設が強陽性，3施設が弱陽性となった．そのうち3施設では自施設の方法で弱陽性であったものが強陽性に，2施設では自施設の方法で陰性であったものが弱陽性と，染色結果に改善が見られた．さらに，我々のプロトコールを用いて弱陽性であった3施設中1施設にプロトコールの徹底のもと再施行を依頼したところ染色結果が強陽性となり，試薬の反応時間や水洗時間も含めた標準化が必要と考えられた．

考察：銅関連蛋白の検出を目的としたオルセイン染色に関しては，メルクまたは東京化成工業のオルセイン試薬で，還元液に1.5%～3%のシュウ酸を使用することが良好な染色方法と考えられた．また，ヘマトキシリンによる核染色は，陽性顆粒が見にくくなるので不要と考えられた．推奨される染色方法は次の如くである．

1. 脱パラフィン
2. 流水水洗5分
3. 0.25%過マンガン酸カリウム＋0.2%硫酸混合液（酸化液）1分
4. 流水水洗5分
5. 2%シュウ酸（還元液）1分
6. 流水水洗5分
7. 蒸留水1分
8. オルセイン液［オルセイン（メルクまたは東京化成）0.5 g＋70%エタノール100 m*l*＋濃塩酸0.8 m*l*］2～3時間
9. 70%エタノール1分
10. 脱水，透徹，封入

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索引用語：原発性胆汁性肝硬変，オルセイン染色，銅関連蛋白

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本論文内容に関連する著者の利益相反：なし

## 英文要旨

## Standardized protocol of orcein staining based on survey of technical procedures in different institutions

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Copper deposition of periportal hepatocytes indicates chronic cholestasis and it is regarded as a very important histological finding to diagnose primary biliary cirrhosis (PBC) and define its stage. Orcein stain makes copper-binding protein visible clearly and sensitively. However, there are variations in orcein staining results every institutions. In order to generalize and standardize staining method, we surveyed the methods of orcein stain at 11 institutions including our laboratory. Consequently, there were differences in staining result according to the kinds of orcein reagents and reducers. As for staining of copper-binding protein, combination of orcein reagent manufactured by Merck or Tokyo Chemical Industry and 1.5 to 3% oxalic acid as reducer is recommended.

**Key words**: primary biliary cirrhosis, orcein stain, copper-binding protein

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Research Article

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# Efficacy of pegylated interferon alpha-2b and ribavirin treatment on the risk of hepatocellular carcinoma in patients with chronic hepatitis C: A prospective, multicenter study☆

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**Background & Aims**: The effects of pegylated interferon (PegIFN) α and ribavirin (RBV) treatment of chronic hepatitis C on the incidence of hepatocellular carcinoma (HCC) have not been well established. This study investigated the impact of treatment outcome on the development of HCC by chronic hepatitis C patients treated with PegIFNα2b and RBV.

**Methods**: This large-scale, prospective, multicenter study consisted of 1013 Japanese chronic hepatitis C patients with no history of HCC (non-cirrhosis, n = 863 and cirrhosis, n = 150). All patients were treated with PegIFNα2b and RBV and the follow-up period started at the end of the antiviral treatment (median observation period of 3.6 years). The cumulative incidence rate of HCC was estimated using the Kaplan–Meier method, according to treatment outcome.

**Results**: Forty-seven patients (4.6%) developed HCC during the observation period. In the non-cirrhosis group, the 5-year cumulative incidence rates of HCC for the sustained virological response (SVR) (1.7%) and transient virological response (3.2%) (TVR: defined as relapse or breakthrough) groups were significantly lower than those of the non-virological response (NVR) group (7.6%) ($p = 0.003$ and $p = 0.03$, respectively). A significantly low rate of incidence of HCC by TVR patients in comparison with NVR patients was found for patients aged 60 years and over, but not for those under 60 years of age. In the cirrhosis group, the 5-year cumulative incidence rates of HCC for the SVR (18.9%) and TVR groups (20.8%) were also significantly lower than those of the NVR group (39.4%) ($p = 0.03$ and $p = 0.04$, respectively).

**Conclusions**: SVR and complete viral suppression during treatment with relapse (TVR) were associated with a lower risk of HCC development when compared with NVR.



Keywords: Hepatitis C; Pegylated interferon; Ribavirin; Hepatocellular carcinoma.

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*Abbreviations:* HCV, hepatitis C virus; HCC, hepatocellular carcinoma; SVR, sustained virological response; IFN, interferon; PegIFN, pegylated interferon; RBV, ribavirin; NVR, non-virological response; TVR, transient virological response; KULDS, Kyushu University Liver Disease Study; AFP, α-fetoprotein; HIV, human immunodeficiency virus; EASL, European Association for the Study of the Liver; ALT, alanine aminotransferase; HbA1c, hemoglobin A1c; EPV, events per predictor variable; HR, hazard ratio; CI, confidence interval; DAAs, direct acting antivirals.

## Introduction

Hepatitis C virus (HCV) is a major human pathogen responsible for chronic hepatitis, which often progresses to cirrhosis and hepatocellular carcinoma (HCC) [1–3]. While recent advances in HCV have led to a markedly improved treatment, HCC is at present the sixth most common cancer and the third cause of cancer death worldwide [4]; moreover, its incidence is increasing due to HCV infection [5].

Previous studies have reported that patients who achieved a sustained virological response (SVR) after interferon (IFN) monotherapy demonstrated improvement in liver fibrosis and a

reduction in the incidence of decompensated liver disease and HCC compared with non-SVR patients [6–9]. In the past 10 years, a combination of pegylated IFN (PegIFN) α and ribavirin (RBV) has become the standard treatment and has resulted in an increased SVR rate [10–12]. Therefore, whether or not PegIFNα and RBV treatment is effective in preventing HCC is important, but its effect on the incidence of HCC has not been adequately studied, particularly in a large prospective study.

A recent prospective study from the United States reported that the cumulative incidence rate of HCC in an SVR group was significantly lower than in a non-virological response (NVR) group. It was also lower in a transient virological response (TVR) group than in an NVR group, although the difference did not reach statistical significance [13]. The number of aging chronic hepatitis C patients has been increasing in Japan, earlier than in other countries [14], thus investigation into the development of HCC by Japanese chronic hepatitis C patients treated with PegIFNα and RBV is highly important. Furthermore, the risk factors for the development of HCC by patients who achieve an SVR after treatment with PegIFNα and RBV have not been adequately clarified in a prospective study, although a recent report suggested that SVR reduced the risk of all-cause mortality in patients treated with PegIFNα and RBV [15]. Clarification of the demographic and clinical factors associated with HCC development, such as advanced age, lower albumin, lower platelet count and higher α-fetoprotein (AFP) level, is important.

The aim of this large-scale, multicenter, prospective study was to evaluate the relationships among pretreatment clinical factors, virological response, and development of HCC by chronic hepatitis C patients with no history of HCC, who were treated with PegIFNα2b and RBV.

Cancer

## Patients and methods

### *Patients*

The Kyushu University Liver Disease Study (KULDS) Group consists of the Kyushu University Hospital and affiliated hospitals in the Northern Kyushu area of Japan. We conducted a prospective study to investigate the efficacy and safety of PegIFNα2b and RBV for chronic hepatitis C patients. The design of the KULDS project has been described previously [12,16,17]. This prospective study consisted of 1013 Japanese patients with chronic HCV infection aged 18 years or older, treated with PegIFNα2b and RBV between December 2004 and November 2009.

The exclusion criteria were: (1) history of HCC; (2) HCC development during antiviral treatment; (3) previous PegIFNα and RBV treatment; (4) positivity for antibody to human immunodeficiency virus (HIV) or positivity for hepatitis B surface antigen; (5) clinical or biochemical evidence of hepatic decompensation at entry; (6) excessive active alcohol consumption (a daily intake of more than 40 g of ethanol) or drug abuse; (7) other forms of liver disease (e.g., autoimmune hepatitis, alcoholic liver disease, hemochromatosis); or (8) treatment with antiviral or immunosuppressive agents prior to enrollment.

The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and was approved by the Ethics Committee of each participating hospital. Informed consent was obtained from all patients before enrollment.

### *Antiviral treatment and patient follow-up*

All HCV genotype 1 patients received a combination treatment of PegIFNα2b (PEG-Intron; MSD, Tokyo, Japan) and RBV (Rebetol; MSD) for 48 weeks: the same regimen was prescribed for 24 weeks for genotype 2 patients. In order to investigate the incidence of HCC after treatment, the length of the follow-up period was calculated from the end of antiviral treatment to the diagnosis of HCC or last follow-up visit. Serum AFP and abdominal imaging (ultrasonographic examination, or computed tomography) were performed every 3–6 months, for each patient. The HCC diagnosis was based on histology or non-invasive criteria according to the guidelines of the European Association for the Study of the Liver (EASL) [18].

### *Clinical and laboratory assessment*

Clinical parameters included serum albumin, alanine aminotransferase (ALT), serum AFP, hemoglobin, platelet count, hemoglobin A1c (HbA1c), HCV genotype, and HCV RNA. All were measured by standard laboratory techniques in a commercial laboratory (SRL Laboratory, Tokyo, Japan). The HbA1c levels that we report are expressed as National Glycohemoglobin Standardization Program units (%). Body mass index was calculated as weight in kilograms/height in square meters.

### *Assessment of liver fibrosis*

Liver biopsy for 613 (60.5%) of the 1013 patients was performed by experienced hepatologists. The antiviral treatment was initiated within 1 month after liver biopsy. The minimum length of liver biopsy was 15 mm and at least 10 complete portal tracts were necessary for inclusion. For each specimen, the stage of fibrosis was established according to the METAVIR score [19]. Liver cirrhosis in patients with no liver biopsy was diagnosed by ultrasonographic findings (nodules in the hepatic parenchyma, portal vein >16 mm) (mandatory inspection) at the time of antiviral treatment initiation. Moreover, the diagnosis of liver cirrhosis was made based on at least one of the following: (1) endoscopic findings (varices, portal gastropathy); (2) serological markers (aspartate aminotransferase to platelet ratio index >2.0; the cut-off value that indicates a negative predictive value for cirrhosis is 93%) [20]; or (3) transient elastography (FibroScan value ⩾14.9 kiloPascal; the cut-off value that indicates that the negative predictive value for cirrhosis is 100%) [21]. The EASL HCV guidelines of 2011 describe the accuracy of these non-invasive tests of liver fibrosis as sufficient for identifying patients with cirrhosis [22].

### *Efficacy of treatment*

Successful treatment was an SVR, defined as undetectable HCV RNA at 24 weeks after the end of treatment. A TVR was defined as relapse of serum HCV RNA after treatment of patients whose HCV RNA level was undetectable at the end of treatment and the reappearance of HCV RNA at any time during treatment after virological response (breakthrough). An NVR was defined as a decrease in the HCV RNA level of less than 2 $\log_{10}$ IU/ml at week 12 (null response) and a more than 2 $\log_{10}$ IU/ml decrease in the HCV RNA level from baseline at week 12, but detectable HCV RNA at weeks 12 and 24 (partial response).

### *HCV RNA level and HCV genotype*

Clinical follow-up of HCV viremia was done by real-time reverse transcriptase PCR assay (COBAS TaqMan HCV assay) (Roche Diagnostics, Tokyo, Japan), with a lower limit of quantitation of 15 IU/ml and an outer limit of quantitation of $6.9 \times 10^7$ IU/ml (1.2 to 7.8 log IU/ml referred to $\log_{10}$ IU/ml). HCV genotype determination was by sequence determination in the 5′ non-structural region of the HCV genome, followed by phylogenetic analysis [23].

### *Statistical analysis*

Statistical analyses were conducted using SPSS Statistics 19.0 (IBM SPSS Inc., Chicago, IL, USA). Baseline continuous data are expressed as median (first-third quartiles) and categorical variables are reported as frequencies and percentages. Univariate analyses were performed using the Chi-square, Fisher's Exact, Mann–Whitney U tests or analysis of variance (ANOVA) as appropriate. Variables with $p < 0.05$ in univariate analysis were evaluated using multivariate logistic regression to identify those significantly associated with the incidence of HCC. As a rule of thumb, 10 events per predictor variable (EPV) are needed when performing a logistic regression analysis. However, 5 to 9 EPV with a large sample size (over 1000) showed robust results of as much as 10 to 16 EPV [24]. Thus, our sample size and 5 to 9 EPV might be sufficient to insure the robustness of our model. Results are expressed as hazard ratios (HR) and their 95% confidence interval (CI).

The main outcome of this study was HCC incidence. Cumulative incidence curves of HCC according to response to antiviral treatment were plotted using the Kaplan–Meier method. Differences between groups were assessed using

log-rank tests. The time frame for HCC incidence was defined as the time from the end of antiviral treatment to the diagnosis of HCC. A $p$ value less than 0.05 was regarded as statistically significant in all analyses.

## Results

### *Patient characteristics*

The baseline characteristics of the 1013 studied patients at the start of antiviral treatment, as classified by the existence of cirrhosis and treatment outcome, are shown in Table 1. HCV genotype 1 was detected in 710 patients and genotype 2 in 303. Of all patients, 151 (14.9%) discontinued antiviral treatment because of adverse effects or other reasons (e.g., poor virological response, economic reasons, or dropout). The discontinuation rate of patients with HCV genotype 1 (129 of 710, 18.2%) was significantly higher than that of those with HCV genotype 2 (22 of 303, 7.3%) ($p$ <0.001). Of the studied patients, 557 achieved SVR (55.0%), 304, including 20 with breakthrough, were TVR (30.0%), and 152 (15.0%) were NVR. The SVR rate of patients infected with HCV genotype 1 was 43.9% (312 of 710), significantly lower than the 80.9% (245 of 303) found for patients with genotype 2 ($p$ <0.001).

In the non-cirrhosis group (n = 863), the three treatment outcome groups differed significantly for age, sex, HCV genotype, and laboratory values associated with liver and metabolic disease (e.g., ALT, platelet count, AFP and HbA1c). The SVR group was more likely to be infected with HCV genotype 2 and to have mild liver fibrosis, but less likely to have laboratory values associated with advanced liver and metabolic disease (e.g., low platelet count, or high AFP and HbA1c level) than the TVR and NVR groups. Independent comparisons of SVR and TVR patients extracted age ($p$ <0.001), sex distribution ($p$ = 0.01), ALT level ($p$ = 0.01), platelet count ($p$ <0.001) and HCV genotype ($p$ <0.001). Likewise, independent comparisons of TVR and NVR patients extracted only AFP level ($p$ = 0.01).

Liver cirrhosis was diagnosed according to clinical (n = 77) and histological (n = 73) findings. In the cirrhosis group (n = 150), however, no significant differences, except for ALT and HCV genotype, were found among the clinical and biochemical parameters of the three treatment outcome groups.

SVR and TVR patients had fewer deaths from any cause (four [0.7%] and four [1.3%], respectively) in comparison to NVR patients (six [3.9%]). Similarly, the frequency of SVR and TVR patients who developed ascites and encephalopathy, symptoms of hepatic decompensation, was lower than that of NVR patients (ascites: two [0.4%], six [2.0%] and eight [5.3%], and encephalopathy: two [0.4%], two [0.7%] and five [3.3%] patients with SVR, TVR and NVR, respectively). None of the patients underwent liver transplantation during the observation period.

### *Risk of HCC classified by treatment outcome*

Of 1013 patients who were followed for a median of 3.6 (range 0.3–7.0) years, 47 (4.6%) developed HCC during the observation period. The baseline characteristics of these patients classified by the development of HCC are shown in Table 2. By univariate analysis, the development of HCC was associated with older age, male sex, higher ALT level, lower serum albumin, lower platelet count, higher AFP level, cirrhosis, and NVR. No significant difference in the duration of HCV RNA negativity was found between the HCC (median [first-third quartiles]: 30.0 [24.0–48.5] weeks) and non-HCC group (41.0 [27.0–48.0] weeks) ($p$ = 0.36) in patients with TVR.

Multivariable logistic regression analysis of possible predictors of HCC development is shown in Table 3. We examined eight factors (age [<60 *vs.* ⩾60 years], sex [men *vs.* women], ALT [<40 *vs.* ⩾40 IU/L], platelet count [<150 *vs.* ⩾150 × $10^9$/L], AFP [<10 *vs.* ⩾10 ng/ml], serum albumin [<40 *vs.* ⩾40 g/L], liver pathophysiology [non-cirrhosis *vs.* cirrhosis] and treatment outcome [SVR *vs.* TVR *vs.* NVR]). Significant independent pretreatment predictors of HCC were age 60 years and over (HR 2.81; 95%CI 1.39–5.69; $p$ = 0.004), male sex (HR 2.98; 95%CI 1.46–6.05; $p$ = 0.003), low platelet count (<150 × $10^9$/L) (HR 4.04; 95%CI 1.57–10.44; $p$ = 0.004), higher AFP level (⩾10 ng/ml) (HR 2.50; 95%CI 1.09–5.78; $p$ = 0.03), cirrhosis (HR 3.22; 95%CI 1.28–8.13; $p$ = 0.01), and NVR (HR 3.72; 95%CI 1.69–8.18; $p$ = 0.001). Baseline ALT level, serum albumin level, and TVR were not associated with the development of HCC.

**Table 1. Pretreatment characteristics of 1013 patients with chronic hepatitis C classified by the existence of cirrhosis and treatment outcome.**

| Characteristic | Non-cirrhosis n = 863 | | | | Cirrhosis n = 150 | | | |
|---|---|---|---|---|---|---|---|---|
| | SVR n = 504 | TVR n = 255 | NVR n = 104 | $p$ value* | SVR n = 53 | TVR n = 49 | NVR n = 48 | $p$ value* |
| Age (yr) | 54 (46-63) | 61 (55-67) | 61 (53-67) | <0.001 | 61 (57-67) | 63 (53-68) | 60 (54-68) | 0.94 |
| Male, n (%) | 263 (52.2) | 109 (42.7) | 52 (50.0) | 0.05 | 30 (56.6) | 19 (38.8) | 25 (52.1) | 0.18 |
| Body mass index (kg/m²) | 22.9 (20.8-25.2) | 23.3 (21.3-25.7) | 23.1 (21.2-25.1) | 0.12 | 23.0 (20.4-25.6) | 23.7 (21.9-26.7) | 24.6 (22.8-26.9) | 0.07 |
| ALT (IU/L) | 52 (34-91) | 47 (33-78) | 51 (31-80) | 0.02 | 88 (69-127) | 65 (53-107) | 66 (48-102) | 0.01 |
| Albumin (g/L) | 42 (40-44) | 42 (39-44) | 42 (39-44) | 0.26 | 37 (35-39) | 37 (35-40) | 37 (33-39) | 0.87 |
| Platelet count (x$10^9$/L) | 177 (144-212) | 158 (129-194) | 159 (130-197) | <0.001 | 103 (89-116) | 97 (84-111) | 99 (84-118) | 0.26 |
| Hemoglobin (g/L) | 137 (129-148) | 136 (128-147) | 138 (127-149) | 0.49 | 130 (122-140) | 133 (123-142) | 137 (126-147) | 0.37 |
| Ferritin (ng/ml) | 156 (75-280) | 174 (92-316) | 213 (116-361) | 0.16 | 200 (127-317) | 202 (134-327) | 250 (170-452) | 0.05 |
| α-fetoprotein (ng/ml) | 4.1 (2.9-6.0) | 4.8 (2.9-7.8) | 5.9 (3.4-8.9) | <0.001 | 14.0 (9.2-36.0) | 14.1 (9.3-31.3) | 30.2 (15.4-42.9) | 0.24 |
| Hemoglobin A1c (%) | 5.8 (5.7-6.3) | 5.9 (5.7-6.4) | 6.0 (5.7-6.7) | 0.005 | 5.8 (5.4-6.4) | 5.6 (5.3-6.4) | 6.0 (5.4-6.6) | 0.73 |
| HCV genotype (1/2), n (%) | 288/216 (57.1/42.9) | 220/35 (86.3/13.7) | 92/12 (88.5/11.5) | <0.001 | 24/29 (45.3/54.7) | 43/6 (87.8/12.2) | 43/5 (89.6/10.4) | <0.001 |

Data are expressed as number (%) or median (first-third quartiles).
SVR, sustained virological response; TVR, transient virological response; NVR, non-virological response; HCV, hepatitis C virus; ALT, alanine aminotransferase.
*Comparison among the three groups.

**Table 2. Risk factors for the development of HCC by chronic hepatitis C patients treated with PegIFNα2b and RBV.**

| Characteristic | All patients n = 1013 | HCC n = 47 | non-HCC n = 966 | *p* value* |
|---|---|---|---|---|
| Age (yr) | 58 (50-65) | 67 (58-71) | 58 (49-65) | <0.001 |
| Male, n (%) | 498 (49.2) | 32 (68.1) | 466 (48.2) | 0.007 |
| Body mass index (kg/m²) | 23.0 (21.1-25.2) | 23.6 (21.6-25.7) | 23.0 (21.1-25.2) | 0.15 |
| ALT (IU/L) | 54 (35-89) | 74 (46-100) | 54 (34-89) | 0.008 |
| Albumin (g/L) | 41 (39-44) | 40 (37-42) | 44 (41-46) | 0.002 |
| Platelet count (x10⁹/L) | 159 (120-199) | 110 (88-132) | 161 (123-201) | <0.001 |
| Hemoglobin (g/L) | 136 (127-147) | 136 (128-149) | 136 (127-147) | 0.89 |
| Ferritin (ng/ml) | 165 (84-376) | 187 (80-462) | 167 (80-306) | 0.68 |
| α-fetoprotein (ng/ml) | 4.9 (3.0-9.3) | 11.7 (6.8-32.7) | 4.8 (3.0-8.7) | <0.001 |
| Hemoglobin A1c (%) | 5.5 (5.3-5.9) | 5.8 (5.4-6.3) | 5.5 (5.3-5.9) | 0.96 |
| HCV genotype (1/2), n (%) | 710/303 (70.1/29.9) | 38/9 (80.9/19.1) | 672/294 (69.6/30.4) | 0.09 |
| Non-cirrhosis/cirrhosis, n | 863/150 (85.2/14.8) | 19/28 (40.4/59.6) | 844/122 (87.4/12.6) | <0.001 |
| Treatment duration (wk) | 47 (24-48) | 43 (23-48) | 47 (24-48) | 0.58 |
| Virological response (SVR/TVR/NVR), n (%) | 557/304/152 (55.0/30.0/15.0) | 13/13/21 (27.7/27.7/44.7) | 544/291/131 (56.3/30.1/13.6) | <0.001 |

Data are expressed as number (%) or median (first-third quartiles).
All demographic and clinical data are those at the start of antiviral treatment.
HCV, hepatitis C virus; HCC, hepatocellular carcinoma; SVR, sustained virological response; TVR, transient virological response; NVR, non-virological response; ALT, alanine aminotransferase.
*Comparison between HCC and non-HCC.

*Overall cumulative incidence of HCC classified by treatment outcome*

The 5-year cumulative incidence rates of HCC of the SVR (3.1%) and TVR groups (5.8%) were significantly lower than those of the NVR group (18.8%) (both $p < 0.001$), and the rate of the SVR group was lower, but not significantly, than that of the TVR group ($p = 0.21$).

*Cumulative incidence of HCC classified by treatment outcome in the non-cirrhosis group*

The Kaplan–Meier curves for the incidence of HCC classified by treatment outcome in the non-cirrhosis group are shown in Fig. 1A ($p = 0.009$ by log-rank test). The 5-year cumulative incidence rates of HCC in the SVR (1.7%) and TVR groups (3.2%) were significantly lower than those of the NVR group (7.6%) ($p = 0.003$ and $p = 0.03$, respectively), and the rate of the SVR group was lower, but not significantly, than that of the TVR group ($p = 0.47$).

*Cumulative incidence of HCC classified by treatment outcome in the cirrhosis group*

The Kaplan–Meier curves for the incidence of HCC classified by treatment outcome in the cirrhosis group are shown in Fig. 1B ($p = 0.03$ by log-rank test). The 5-year cumulative incidence rates of HCC in the SVR (18.9%) and TVR groups (20.8%) were significantly lower than those of the NVR group (39.4%) ($p = 0.03$ and $p = 0.04$, respectively), and the rate of the SVR group was lower, but not significantly, than that of the TVR group ($p = 0.94$).

*Adjusted rates of HCC incidence classified by treatment outcome of non-cirrhotic patients under 60 years of age*

The Kaplan–Meyer curves of the estimation of the incidence of HCC by non-cirrhosis patients under 60 years of age, classified by treatment outcome, are shown in Fig. 2A ($p = 0.51$ by log-rank test). The 5-year cumulative incidence rates of HCC in the SVR (0.9%) and TVR groups (1.7%) were lower, but not significantly, than those of the NVR group (2.6%) ($p = 0.25$ and $p = 0.45$, respectively).

**Table 3. Multivariate logistic regression analysis of possible predictors of HCC development.**

| Parameter | Hazard ratio | 95% CI | *p* value |
|---|---|---|---|
| Age | | | |
| <60 yr | 1 | | |
| ≥60 yr | 2.81 | 1.39-5.69 | 0.004 |
| Sex | | | |
| Female | 1 | | |
| Male | 2.98 | 1.46-6.05 | 0.003 |
| Platelet count | | | |
| ≥150 x 10⁹/L | 1 | | |
| <150 x 10⁹/L | 4.04 | 1.57-10.44 | 0.004 |
| α-fetoprotein | | | |
| <10 ng/ml | 1 | | |
| ≥10 ng/ml | 2.50 | 1.09-5.78 | 0.03 |
| Liver pathophysiology | | | |
| Non-cirrhosis | 1 | | |
| Cirrhosis | 3.22 | 1.28-8.13 | 0.01 |
| Treatment outcome | | | |
| SVR | 1 | | |
| TVR | 1.50 | 0.65-3.44 | 0.34 |
| NVR | 3.72 | 1.69-8.18 | 0.001 |

HCC, hepatocellular carcinoma; SVR, sustained virological response; TVR, transient virological response; NVR, non-virological response.

*Adjusted rates of HCC incidence classified by treatment outcome of non-cirrhotic patients aged 60 years and over*

The Kaplan–Meyer curves of the estimation of the incidence of HCC in non-cirrhosis patients, aged 60 years and over classified by treatment outcome, are shown in Fig. 2B ($p = 0.05$ by log-rank test). The 5-year cumulative incidence rates of HCC in the SVR (3.5%) and TVR groups (4.2%) were significantly lower than those

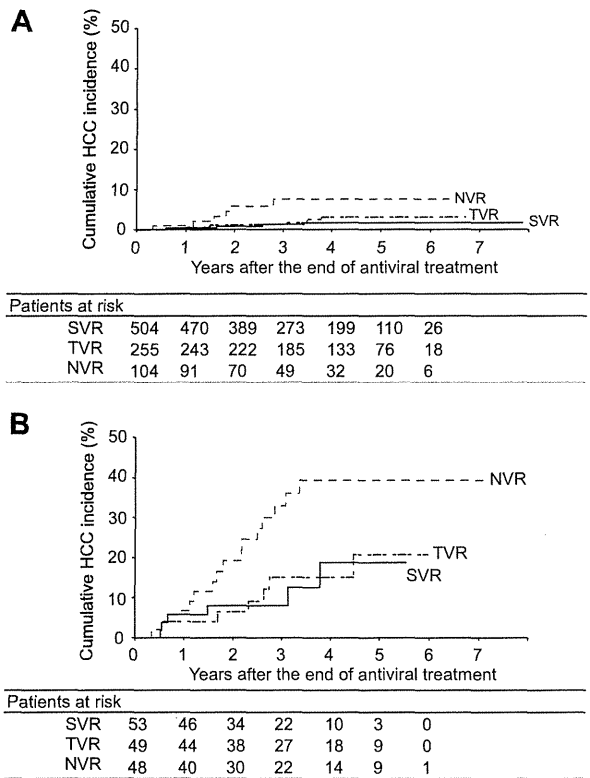


**Fig. 1. Cumulative incidence of HCC after PegIFNα2b and RBV treatment stratified by treatment outcome (SVR: continuous line, TVR: long dashed-dotted line, NVR: dashed line).** (A) Non-cirrhosis group (overall: $p = 0.009$; SVR *vs.* TVR: $p = 0.47$; SVR *vs.* NVR: $p = 0.003$; and TVR *vs.* NVR: $p = 0.03$ by log-rank test). (B) Cirrhosis group (overall: $p = 0.03$; SVR *vs.* TVR: $p = 0.94$; SVR *vs.* NVR: $p = 0.03$; and TVR *vs.* NVR: $p = 0.04$ by log-rank test).

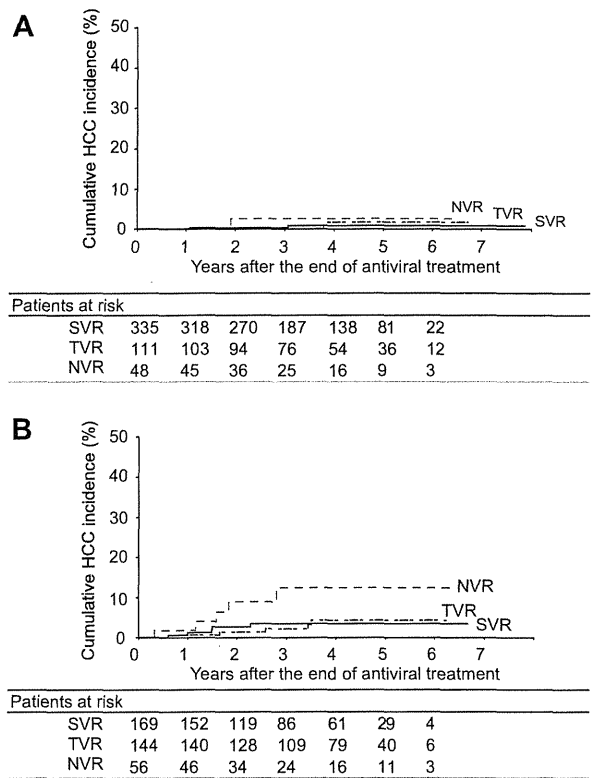


**Fig. 2. Cumulative incidence of HCC after PegIFNα2b and RBV treatment stratified by treatment outcome of the non-cirrhosis group (SVR: continuous line, TVR: long dashed-dotted line, NVR: dashed line).** (A) Under 60 years of age (overall: $p = 0.51$; SVR *vs.* TVR: $p = 0.94$; SVR *vs.* NVR: $p = 0.25$; and TVR *vs.* NVR: $p = 0.45$ by log-rank test). (B) Aged 60 years and over (overall: $p = 0.05$; SVR *vs.* TVR: $p = 0.96$; SVR *vs.* NVR: $p = 0.04$; and TVR *vs.* NVR: $p = 0.03$ by log-rank test).

of the NVR group (12.4%) ($p = 0.04$ and $p = 0.03$, respectively), and the rate of the SVR group was slightly lower, but not significantly, than that of the TVR group ($p = 0.96$).

*The development of HCC by SVR patients*

Thirteen patients who achieved SVR (2.3%) (6 non-cirrhotic and 7 cirrhotic patients) developed HCC during the follow-up period. Their individual pretreatment characteristics are shown in Table 4. Of these patients, 3 (patients 1–3) under 55 years of age had liver cirrhosis and the period from the end of antiviral treatment to the diagnosis of HCC was over 3 years. Of the remaining 10 patients (patients 4–13) aged 55 years and over, 6 did not have cirrhosis and the period from the end of antiviral treatment to the diagnosis of HCC was under 2.5 years.

## Discussion

We here report the results of a prospective, long-term follow-up study done to evaluate the effect of treatment outcome on the development of HCC in a large cohort of Japanese patients with chronic hepatitis C, who were treated with PegIFNα2b and RBV. We found that those patients who achieved SVR or TVR had a lower risk of developing HCC within 5 years after the end of PegIFNα2b and RBV treatment when compared with NVR, in both cirrhosis and non-cirrhosis groups. Although SVR patients have been reported to have little risk of HCC incidence, a small number of our patients who achieved SVR did develop HCC, showing the necessity of a continued screening of patients with SVR.

Previously, the likelihood of HCC development by PegIFNα- and RBV-treated patients was difficult to determine because of the paucity of adequate long-term prospective studies. Based on the results of this prospective study, sex, age, platelet count, AFP level, and treatment outcome are significant, independent factors for the development of HCC. In addition to our present data, the incidence rate of HCC has been shown to be significantly lower for patients with TT genotype at rs8099917 and CC genotype at rs12979860 near the *IL28B* gene, which are associated with good response to antiviral treatment (data not shown). Of particular interest, the adjusted cumulative incidence of HCC was not significantly different between SVR and TVR for the 5 years after the end of treatment. Two randomized studies of maintenance therapy with low-dose PegIFNα to prevent hepatic decompensation and HCC have been recently reported [25,26]. However, maintenance therapy did not prevent HCC in presence of HCV viremia for at least 5 years, regardless of the degree of viral suppression. Our results showed that complete HCV sup-

**Table 4. Individual characteristics of SVR patients who developed HCC.**

| Patient number | Age (yr) | Sex | Liver pathophysiology | Time to HCC* (yr) | HCV genotype | ALT (IU/L) | Albumin (g/L) | Platelet count (x10$^9$/L) | AFP (ng/ml) | HbA1c (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 47 | F | Cirrhosis | 3.1 | 1 | 44 | 40 | 134 | 3.3 | 7.1 |
| 2 | 53 | M | Cirrhosis | 3.1 | 2 | 105 | 42 | 68 | 31.0 | 6.1 |
| 3 | 54 | M | Cirrhosis | 3.8 | 1 | 86 | 36 | 88 | 13.9 | 5.9 |
| 4 | 59 | M | Non-cirrhosis | 1.1 | 2 | 227 | 44 | 131 | 4.4 | 6.6 |
| 5 | 63 | F | Cirrhosis | 1.5 | 2 | 81 | 33 | 130 | 16.3 | 5.3 |
| 6 | 64 | F | Non-cirrhosis | 1.5 | 2 | 72 | 38 | 120 | 6.6 | 6.8 |
| 7 | 64 | M | Non-cirrhosis | 1.5 | 1 | 29 | 46 | 124 | 20.7 | 5.1 |
| 8 | 66 | F | Cirrhosis | 0.7 | 2 | 169 | 42 | 105 | 106.0 | 6.4 |
| 9 | 66 | M | Non-cirrhosis | 0.6 | 1 | 36 | 35 | 147 | 6.2 | 5.5 |
| 10 | 71 | M | Cirrhosis | 0.6 | 2 | 80 | 32 | 106 | 10.6 | 5.5 |
| 11 | 71 | M | Non-cirrhosis | 1.0 | 1 | 47 | 42 | 108 | 4.3 | 5.7 |
| 12 | 74 | M | Non-cirrhosis | 2.3 | 1 | 47 | 43 | 143 | 12.9 | 6.9 |
| 13 | 77 | M | Cirrhosis | 0.5 | 1 | 73 | 30 | 124 | 11.6 | 5.4 |

All data are those at the start of antiviral treatment.
SVR, sustained virological response; HCC, hepatocellular carcinoma; F, female; M, male; HCV, hepatitis C virus; ALT, alanine aminotransferase; AFP, α-fetoprotein; HbA1c, hemoglobin A1c.
*The time frame for HCC incidence starts from the end of antiviral treatment.

pression during antiviral treatment played an important role in preventing the development of HCC.

A recent prospective study that included Caucasian, Hispanic, and Black patients treated with PegIFNα2a and RBV reported that the adjusted mortality from any cause or liver transplantation, or of any liver-related outcome, was significantly lower in TVR patients than in NVR patients [13]. Similarly, the risk of decompensated liver disease, HCC and liver-related death was also lower in TVR patients than in NVR patients, although these differences did not reach statistical significance [13]. Therefore, the significantly low incidence rate of HCC, for the patients of this study with TVR in comparison with NVR, is an original finding, but the trend was true for cirrhotic patients of all ages and for non-cirrhotic patients aged 60 years and over. One possible explanation for this difference may be related to the rising incidence of HCC for NVR patients aged 60 years and over. Our results indicate that the duration of clinical benefit may outlast the period of actual viral suppression in the 5 years after treatment, however, it remains unclear how older age would explain why TVR resulted in a lower incidence of HCC that matched the incidence in SVR. Therefore, it will be necessary to investigate the development of HCC in SVR and TVR patients beyond five years.

Recently, a number of direct-acting antivirals (DAAs) have been designed and developed. Among them, telaprevir and boceprevir, non-structural 3/4A protease inhibitors, have shown promising results in various clinical trials and have led to an increased SVR rate when given in combination with PegIFNα and RBV, as compared with PegIFNα and RBV alone [27,28]. Furthermore, several IFN-free clinical trials, using regimens that combine several potent DAAs, are ongoing. As a result of advances in antiviral treatment, almost all patients can experience complete HCV suppression during treatment. We showed that TVR patients had a lower incidence rate of HCC than did NVR patients. It will be necessary to study the impact of virological response on the development of HCC by patients who undergo DAAs with and without IFN antiviral treatment.

Findings on the effect of SVR on liver-related preferable clinical outcomes have been reported in many previous reports [13,29–31], however, the analysis of the effect of SVR on the development of HCC is statistically difficult, because the number of events is too small to draw meaningful conclusions. In fact, there were only 13 patients with SVR who developed HCC during the observation period, reducing the validity of the analysis. Additional prospective studies that include a larger number of patients with SVR will be necessary to evaluate the relationship between SVR and the development of HCC.

Risk factors for HCV-related HCC have been reported previously, such as older age, male sex, obesity, diabetes mellitus, alcohol consumption, HCV genotype 1b, insulin resistance, complicated hepatic steatosis, and co-infection with hepatitis B virus or HIV [32,33]. Unfortunately, this study lacks data on insulin resistance and hepatic steatosis. Homeostasis Model Assessment of Insulin Resistance value is also related to a profound effect on PegIFNα2b and RBV treatment outcome [34], thus, there may be a significant difference in HbA1c level between the SVR, TVR and NVR non-cirrhotic groups, indicating differences in glucose metabolism. Moreover, it is known that hepatic steatosis occurs in about 40% of the chronic hepatitis C patients, when all common factors of fatty liver, such as alcohol abuse, obesity, and diabetes, have been excluded [35]. Therefore, it remains unclear whether or not there is a significant bias due to different rates of patients with insulin resistance or hepatic steatosis. Another limitation is the generalizability of the extremely high cumulative incidence rate of HCC, especially for cirrhotic NVR patients. The reasons for this exceedingly high rate are not well understood, although it may be explained by the increasing number of aging chronic hepatitis C patients in Japan, earlier than other countries [14]. Our results, therefore, may not be generalized to other ethnic groups that do not have such high rates of HCC.

In summary, this prospective study demonstrated that SVR and TVR patients had a significantly lower rate than NVR patients of HCC incidence within five years after the end of treatment, both for patients with and without cirrhosis. Because the risk of developing HCC remains present even after HCV eradication, long-term screening of patients with SVR is important.

### Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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***Original Article***

# Early extensive viremia, but not rs8099917 genotype, is the only predictor for cholestatic hepatitis C after living-donor liver transplantation

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*Aim:* Cholestatic hepatitis C is one of the most serious but still unaddressed disorders after liver transplantation.

*Methods:* In this study, we analyzed 49 patients who underwent living-donor liver transplantation (LDLT) to treat hepatitis C virus (HCV) infection.

*Results:* Five patients developed cholestatic hepatitis C, with total bilirubin of 15.2 ± 3.1 mg/dL at diagnosis 6.2 ± 1.0 weeks after LDLT. Univariate analysis showed that larger graft to standard liver volume ratio, higher HCV RNA titer at 2 weeks, earlier peak HCV RNA titer and cytomegalovirus infection were the significant risk factors. The development of cholestatic hepatitis C was not significantly associated with interleukin-28B genotype (rs8099917); four out of five affected patients had the T/T genotype. Multivariate analysis showed that higher HCV RNA titer at 2 weeks was the only significant factor ($P = 0.026$) for the development of cholestatic hepatitis C. Receiver–operator curve analysis showed that that HCV RNA titer of more than 7.2 $\log_{10}$IU/mL was the optimal cut-off for characterizing cholestatic hepatitis C. All of the patients were serum HCV RNA negative after treatment with pegylated interferon and ribavirin and all the patients are alive.

*Conclusion:* Early extensive viremia, but not the rs8099917 genotype, was the only predictor for cholestatic hepatitis C after LDLT.

**Key words:** cholestatic hepatitis, hepatitis C, interleukin 28B, liver transplantation, living donor, splenectomy

## INTRODUCTION

ALTHOUGH END-STAGE LIVER disease secondary to hepatitis C virus (HCV) is the leading indication for liver transplantation (LT), re-infection of HCV is a widespread, unaddressed and serious event.[1] It has been reported that approximately one-quarter of patients develops cirrhosis within 10 years after LT for HCV; therefore, graft outcomes after LT for HCV are inferior to those for other indications.[2]

Nevertheless, recurrent hepatitis C after LT is represented by a spectrum of disorders, including mild to severe inflammation with various degrees of fibrosis progression over several years.[1,2] Of note, HCV re-infection can result in very aggressive hepatitis in a small number of patients, and is usually characterized by rapid progression of cholestasis with fibrosis resulting in graft failure and death.[3,4] This outcome has been termed post-transplant cholestatic hepatitis C and its risk factors include higher donor age, HCV genotype 1, extremely high viral titers and bolus steroid administration for acute rejection.[3,4] More recently, two reports

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have shown that single nuclear polymorphism (SNP) in the interleukin (IL)-28B gene was a significant risk factor for the disease process.[5,6] To date, however, the pathogenesis of recurrent cholestatic hepatitis C after LT has not been elucidated.

Therefore, in the current study, we examined the clinical characteristics of patients who developed this rare type of recurrent cholestatic hepatitis C after living-donor liver transplantation (LDLT). We investigated whether its pathogenesis could be attributed to viral factors, host factors, including IL-28B genotypes or graft-related factors.

## METHODS

### Patients

LIVING-DONOR LIVER TRANSPLANTATION was performed in 54 patients positive for the HCV antibody at Kyushu University Hospital between February 2007 and July 2012. All procedures were approved by the Ethics and Indications Committee of Kyushu University. Forty-nine patients who were HCV RNA positive before LDLT were included in the current study. The mean follow-up time was 2.8 ± 1.1 years.

### Transplantation and postoperative care

The surgical procedures for both the donors and the recipients are described in more detail elsewhere.[7,8] The graft type, either left or right lobe, was determined based on the need for a graft volume (GV) of more than 35% of the recipient's standard liver volume (SLV).[7] Splenectomy was performed for 47 (95.9%) recipients to prevent pancytopenia caused by interferon (IFN) therapy.[9] A biliary stent over the biliary anastomosis was placed during the surgery and was kept in place for 3–4 months after LDLT to prevent early stricture.[10]

The immunosuppression regimen consisted of tacrolimus or cyclosporin with mycophenolate mofetil and steroids as previously reported.[8] The immunosuppression level was maintained at a standard level to prevent acute rejection; unfortunately, this hinders the diagnosis and treatment of hepatitis C after LDLT. The tacrolimus level was maintained at 10–14 ng/mL for 1 month after LDLT and was then decreased to 7–10 ng/mL over the next few months. The cyclosporin level was maintained at 150–250 ng/mL for 1 month after LDLT and then decreased to 100–150 ng/mL over the next few months. Mycophenolate mofetil at the dose of 2 g/day, was then tapered down to 1 g daily over 1–3 months and tapered off at 6 months. All the patients received steroids during the study period. Methylprednisolone (1 g) was given after reperfusion, and titrated from 200 mg/day to 20 mg/day in a week, then switched to oral prednisolone, and tapered off by 6 months. The immunosuppression protocol for blood type-incompatible LDLT consisted of pretransplant rituximab and plasma exchanges with tacrolimus or cyclosporin and mycophenolate mofetil and steroids, as previously described.[11]

### Antiviral treatment

Interferon was indicated for recurrent hepatitis C associated with serum HCV RNA positivity, abnormal liver function tests and histological evidence of recurrent hepatitis C. Preemptive antiviral treatment was not performed.

Antiviral treatment consisted of pegylated (PEG) IFN-α-2b with ribavirin (Pegintron with Rebetol; Merck, Whitehouse Station, NJ, USA) or PEG IFN-α-2a with ribavirin (Pegasys with Copegus; Chugai Pharmaceutical, Tokyo, Japan) was used for antiviral treatment. Although PEG IFN-α-2b was primarily used for post-transplant induction of antiviral treatment, PEG IFN-α-2a could also be used for refractory or severe cases. The type of PEG IFN drug, regarding conversion between the products, was determined for individual cases. PEG IFN-α-2b and ribavirin were started at doses of 0.5–1.0 mcg/kg per week and 200–400 mg/day, respectively. The doses were escalated in a stepwise manner, in accordance with the individual's tolerability, to 1.5 mcg/kg per week and 800 mg/day, respectively. PEG IFN-α-2a and ribavirin were started at doses of 90–120 mcg/week and 200–400 mg/day, respectively, to 180 mcg/week and 800 mg/day respectively. The recommended duration of treatment was 48 weeks after achieving viral response (VR), defined as undetectable serum HCV RNA.

### Measurement of the serum HCV RNA titer

The serum HCV RNA titer was determined by a real-time HCV assay (AccuGene HCV; Abbott Molecular, Des Plaines, IL, USA). The lower and higher limits of quantification for this assay are 1.08 log IU/mL and 8.0 log IU/mL, respectively. The serum HCV RNA titer was measured before LDLT, 2 weeks after LDLT and monthly thereafter.

### IL-28B genotyping assay

DNA from the donors and the recipients was extracted from a biopsy or explanted liver tissue obtained during LDLT, and genotyping was performed using TaqMan

GTX press Master Mix (Life Technologies, Tokyo, Japan), in accordance with the manufacturer's instructions. The Custom TaqMan SNP Genotyping Assay (Life Technologies) was used to identify IL-28B genetic polymorphisms. We used rs8099917 as the representative SNP for IL-28B because of its higher sensitivity and specificity for IFN sensitivity in Asian individuals.[12] The T/T genotype of rs8099917 was defined as the major allele, while the T/G and G/G genotypes were regarded as the minor alleles.

### Diagnosis of cholestatic hepatitis

Cholestatic hepatitis C was defined according to the factors as proposed by Wiesner *et al.*[13] with minor modifications: (i) total bilirubin of more than 6 mg/dl; (ii) elevated biliary enzymes with alkaline phosphatase (ALP) and/or γ-glutamyltransferase (GGT) of more than 5 times the upper limit of normal; (iii) very high serum HCV RNA titer of more than 6 log IU/mL; (iv) histological findings that include predominant ballooning of hepatocytes in the perivenular zone and limited inflammation; (v) occurring between 1 and 6 months after LT; and (vi) absence of surgical complications at the time of diagnosing cholestatic hepatitis C.

Percutaneous liver biopsy was obtained and evaluated for patients with abnormal liver function tests suggestive of recurrent hepatitis C or acute rejection. Biopsies were also obtained every year in accordance with the established protocol.

### Statistical analysis

Values are expressed as the mean ± standard deviation. Variables were analyzed using the $\chi^2$-test for categorical values or the Mann–Whitney *U*-test for continuous variables. Multivariate analyses were performed using the logistic regression model and odds ratios were calculated. $P < 0.05$ was considered statistically significant.

## RESULTS

### Characteristics of patients with cholestatic hepatitis C

FIVE PATIENTS DEVELOPED cholestatic hepatitis C after LDLT (Table 1). The mean ages of the donors and the recipients were 58.2 ± 7.7 years and 29.2 ± 10.0 years, respectively. The mean GV/SLV ratio was 45.0 ± 7.3%. Donor age was less than 40 years old in all of the cases except for case 5. GV/SLV was more than 35% in all of the cases, except in case 3. Splenectomy was performed in all five cases.

Hepatitis C virus genotype was type 1b, except in case 4 (2a) and the mean HCV RNA titer before LDLT was 5.2 ± 0.7 $\log_{10}$IU/mL. The HCV RNA titer was more than 5 $\log_{10}$IU/mL in all the cases except case 5. The IL-28B (rs8099917) genotype was T/T in both the donors and recipients except in case 2, where the donor and recipient both had the T/G genotype.

The mean values of liver function parameters were 15.2 ± 3.1 mg/dL for total bilirubin, 357 ± 79 IU/L for aspartate aminotransferase (AST) and 859 ± 497 IU/L for GGT. The peak HCV RNA titer was 7.9 ± 0.1 $\log_{10}$IU/mL and more than 7.7 $\log_{10}$IU/mL in all five patients at diagnosis of cholestatic hepatitis C, 6.2 ± 1.0 weeks after LDLT. Although cases 1 and 5 had biliary anastomotic stenosis after LDLT, this complication occurred after treatment for cholestatic hepatitis C.

All of the five patients were treated with PEG IFN with ribavirin after histological confirmation of cholestatic hepatitis C. PEG IFN-α-2b was used in two patients and PEG IFN-α-2b was used in three patients. VR was observed in all of the patients. Among the patients who received IFN ($n = 41$) after LDLT, the total dosage of IFN was larger in patients with ($n = 5$) cholestatic hepatitis C (10.5 ± 3.0 vs 6.0 ± 4.6 mg, $P = 0.040$), compared with those without ($n = 36$). However, the total dosage of ribavirin (24.6 ± 26.1 vs 24.4 ± 20.7 g, $P = 0.981$) and the treatment period (90.0 ± 44.7 vs 62.2 ± 38.8 g, $P = 0.147$) was not different between the groups. Discontinued antiviral treatment was observed in no case in the patients with cholestatic hepatitis ($n = 5$) and 10 cases (27.8%) in the patients without ($n = 36$) due to intolerance and adverse reactions. Dose modification of IFN during the treatment course was observed in three patients (60%) and 18 patients (50.0%), respectively.

### Risk factors for cholestatic hepatitis C

We next determined possible risk factors for cholestatic hepatitis C after LDLT. In univariate analyses, larger GV/SLV (45.0 ± 7.3% vs 39.2 ± 5.9%, $P = 0.049$), higher HCV RNA titer at 2 weeks after LDLT (7.7 ± 0.4 vs 5.8 ± 1.3 $\log_{10}$IU/mL, $P = 0.002$), earlier period for having peak HCV RNA titer (3.7 ± 2.3 vs 9.4 ± 5.6 weeks, $P = 0.031$) and cytomegalovirus infection (80.0% vs 27.2%, $P = 0.017$) were significantly associated with cholestatic hepatitis C after LDLT. By contrast, donor and recipient age, cold and warm ischemic time, HCV genotype, and donor and recipient IL-28B genotype were not associated with the occurrence of cholestatic hepatitis C (Table 2).

**Table 1** Clinical characteristics of the five cases of cholestatic hepatitis C

| Case | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Recipient age, sex | 54, F | 62, F | 52, M | 53, F | 70, F |
| MELD score | 16 | 18 | 8 | 18 | 12 |
| Hepatocellular carcinoma | Yes | Yes | Yes | No | Yes |
| Splenectomy | Yes | Yes | Yes | Yes | Yes |
| Donor age, sex | 21, F | 36, M | 20, M | 23, M | 43, F |
| Immunosuppression regimen | FK-based | CyA-based | CyA-based | CyA-based | CyA-based |
| ABO incompatible | No | Yes | Yes | No | No |
| Graft type | Left | Left | Left | Right | Right |
| GV (g) | 460 | 440 | 510 | 598 | 502 |
| GV/SLV (%) | 39.9 | 44.0 | 37.0 | 55.4 | 48.9 |
| HCV genotype | 1b | 1b | 1b | 2a | 1b |
| HCV RNA titer ($log_{10}$IU/mL) | 5.7 | 5.7 | 5.3 | 5.5 | 3.9 |
| Recipient IL-28B genotype | T/T | T/G | T/T | T/T | T/T |
| Donor IL-28B genotype | T/T | T/G | T/T | T/T | T/T |
| Peak liver function tests | | | | | |
| Total bilirubin (mg) | 17.4 | 13.6 | 19.1 | 16.7 | 9.0 |
| AST (IU/L) | 354 | 382 | 486 | 163 | 399 |
| GGT (IU/L) | 519 | 1939 | 415 | 1023 | 401 |
| HCV RNA ($log_{10}$IU/mL) | 7.7 | 7.7 | 8.0 | 8.0 | 7.7 |
| Weeks after LDLT | 4 | 8 | 6 | 6 | 7 |
| Histological findings | | | | | |
| Hepatocyte ballooning | ++ | ++ | ++ | +++ | ++ |
| Cholestasis | + | – | – | – | – |
| Perivenulitis | +++ | + | ++ | + | – |
| Portal infiltration | + | + | – | – | + |
| Ductular reaction | + | + | + | – | + |
| Interferon treatment | | | | | |
| Type and dose (μg/week) | α-2b (50) | α-2a (180) | α-2b (90) | α-2a (180) | α-2a (180) |
| Ribavirin dose (mg/day) | 400 | 0 | 400 | 200 | 200 |
| Response (weeks) | VR (130) | VR (17) | VR (15) | VR (49) | VR (23) |
| On treatment (weeks) | Yes (170) | Yes (74) | Yes (70) | Yes (69) | Yes (68) |
| Graft outcomes (years) | Alive (3.4) | Alive (1.6) | Alive (1.5) | Alive (1.5) | Alive (1.5) |

AST, aspartate aminotransferase; CyA, cyclosporin; FK, tacrolimus; GGT, γ-glutamyltransferase; GV, graft volume; HCV, hepatitis C virus; IL, interleukin; LDLT, living-donor liver transplantation; MELD, Model for End-Stage Liver Disease; SLV, standard liver volume; VR, viral response.

In multivariate logistic regression analysis, higher HCV RNA titer at 2 weeks after LDLT ($P = 0.026$) was the only significant factor associated with having cholestatic hepatitis C. The other factors identified in univariate analyses, including earlier peak of HCV RNA titer ($P = 0.317$), larger GV/SLV ($P = 0.382$) and cytomegalovirus infection ($P = 0.936$) were not significantly associated with cholestatic hepatitis C after LDLT. Receiver–operator curve (ROC) analysis showed that HCV RNA titer of more than 7.2 $log_{10}$IU/mL at 2 weeks after LDLT was the optimal cut-off for discriminating cholestatic hepatitis C after LDLT. The area under the ROC for this value was 0.989 (Fig. 1).

## Histological characteristics of cholestatic hepatitis C after LDLT

The histological characteristics of the five cases of cholestatic hepatitis C are summarized in Table 1. Although hepatocyte ballooning was prominent in all of the five patients (Fig. 2), portal infiltration and cholestasis were relatively minor or absent, despite the high serum bilirubin level. Perivenulitis was observed in four cases and was significantly more common in patients with recurrent cholestatic hepatitis C than in patients with recurrent non-cholestatic hepatitis C (80.0% vs 20.5%, $P = 0.004$, Table 2). Ductular reaction was observed in four cases.

Table 2 Factors associated with cholestatic hepatitis C

| Factors | Cholestatic hepatitis | | *P*-value |
|---|---|---|---|
| | No ($n = 44$) | Yes ($n = 5$) | |
| Recipient age (years) | 57.4 ± 8.0 | 58.2 ± 7.7 | 0.839 |
| Recipient sex, male | 22 (50.0) | 1 (20.0) | 0.203 |
| Hepatocellular carcinoma, yes | 31 (70.5) | 3 (60.0) | 0.631 |
| MELD score | 14.8 ± 7.0 | 14.4 ± 4.3 | 0.908 |
| History of IFN treatment, yes | 34 (80.9) | 3 (60.0) | 0.602 |
| Donor age (years) | 34.5 ± 10.9 | 29.2 ± 10.0 | 0.302 |
| Donor sex, male | 31 (70.5) | 3 (60.0) | 0.631 |
| ABO incompatible, yes | 5 (11.4) | 2 (40.0) | 0.083 |
| Graft type, left lobe | 17 (38.6) | 2 (40.0) | 0.952 |
| GV (g) | 461 ± 91 | 502 ± 61 | 0.341 |
| GV/SLV (%) | 39.2 ± 5.9 | 45.0 ± 7.3 | 0.049 |
| Splenectomy, yes | 42 (95.5) | 5 (100.0) | 0.626 |
| Cold ischemic time (min) | 100 ± 62 | 83 ± 43 | 0.551 |
| Warm ischemic time (min) | 39 ± 10 | 37 ± 9 | 0.631 |
| Operative time (min) | 793 ± 136 | 740 ± 107 | 0.404 |
| Blood loss (L) | 4.5 ± 6.5 | 4.9 ± 3.2 | 0.894 |
| Recipient IL-28B genotype, T/T | 23 (60.5) | 4 (80.0) | 0.393 |
| Donor IL-28B genotype, T/T | 27 (64.3) | 4 (80.0) | 0.483 |
| HCV genotype 1, yes | 34 (80.9) | 3 (60.0) | 0.279 |
| HCV RNA titer ($log_{10}$IU/mL) | | | |
| Before LDLT | 5.4 ± 1.2 | 5.2 ± 0.7 | 0.813 |
| At 2 weeks after LDLT | 5.8 ± 1.3 | 7.7 ± 0.4 | 0.002 |
| Peak titer | 6.8 ± 1.3 | 7.9 ± 0.1 | 0.089 |
| Time to peak HCV RNA titer (weeks) | 9.4 ± 5.6 | 3.7 ± 2.3 | 0.031 |
| Viral response (%) | 22 (64.7) | 5 (100.0) | 0.110 |
| Tacrolimus use, yes | 22 (50.0) | 1 (20.0) | 0.202 |
| Acute rejection, yes | 1 (2.3) | 0 (0.0) | 0.733 |
| Bile duct stenosis, yes | 8 (18.2) | 2 (40.0) | 0.251 |
| Cytomegalovirus infection, yes | 12 (27.2) | 4 (80.0) | 0.017 |
| Central perivenulitis on biopsy, yes | 9 (20.5) | 4 (80.0) | 0.004 |

GV, graft volume; HCV, hepatitis C virus; IL, interleukin; LDLT, living-donor liver transplantations; MELD, Model for End-Stage Liver Disease; SLV, standard liver volume; SNP, single nuclear polymorphism; VR viral response.

## DISCUSSION

IN THE CURRENT study, HCV RNA titer of more than 7.2 $log_{10}$IU/mL at 2 weeks after transplantation was the only predictive factor for recurrent cholestatic hepatitis C after LDLT. None of the other donor or recipient factors, including IL-28B (rs8099917) genotypes were associated with this severe disease in multiple regression analysis. Cholestatic hepatitis C was diagnosed in all five patients based on early extensive viremia and histological findings (e.g. pan-lobular hepatocyte ballooning). VR was achieved in all of the cases following immediate treatment with PEG IFN with ribavirin.

Although cholestatic hepatitis C is an uncommon (2–5%) form of HCV recurrence, it is usually associated with rapid progression of cholestasis with fibrosis, and often results in graft failure within 1 year after transplantation.[3–6] Early and accurate diagnosis of cholestatic hepatitis C and immediate treatment is essential to save the transplanted grafts, although diagnosis is often difficult.[14–16] The difficulties in diagnosis are mainly due to the differential diagnoses, including acute rejection, biliary stenosis or primary graft dysfunction, for which the treatments are opposite or are very different from those used for cholestatic hepatitis C.[3] We think that the combination of HCV RNA titer of more than 7.2 $log_{10}$IU/mL at 2 weeks after LDLT and pan-lobular ballooning of the hepatocytes are key factors for identifying cholestatic hepatitis C.

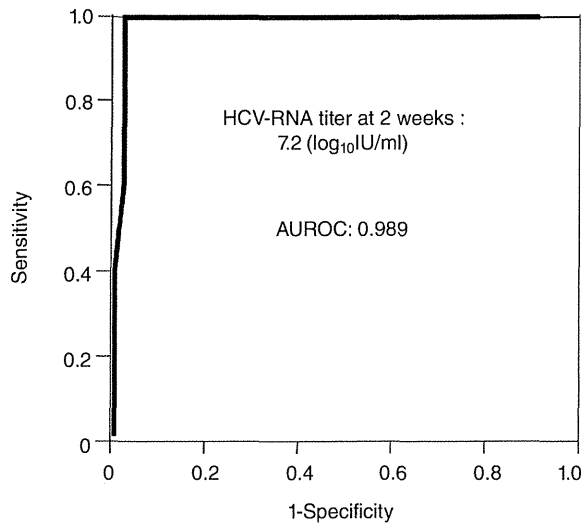


**Figure 1** Receiver–operator curve analysis showed that HCV RNA titer of more than 7.2 $\log_{10}$IU/mL at 2 weeks after LDLT was the optimal cut-off for discriminating cholestatic hepatitis C. AUROC, area under the receiver–operator curve; HCV, hepatitis C virus; LDLT, living-donor liver transplantations.

Extensive HCV infection in hepatocytes and the direct cytopathological effects of HCV, together with a relative absence of inflammation, are thought to be the major mechanisms involved in the development of cholestatic hepatitis C.[17] Therefore, a very high HCV RNA titer was proposed as one of the diagnostic criteria for cholestatic hepatitis after LT in a consensus statement published in 2003.[13] However, the cut-off level for a very high HCV RNA titer was not reported in that consensus statement. More recently, Shackel *et al.*[18] reported that a peak HCV RNA titer of more than 7.0 $\log_{10}$IU/mL within 1 year of LT was a predictor of HCV-associated graft failure. Moreover, Granziadei *et al.*[5] showed that HCV RNA titer of more than 6.0 $\log_{10}$IU/mL 2 weeks after transplantation is the most significant risk factor for the development of cholestatic hepatitis. However, they did not report how they selected this value. We used ROC analysis and found that a HCV RNA titer of more than 7.2 $\log_{10}$IU/mL at 2 weeks after LDLT was the optimal cut-off for predicting cholestatic hepatitis C after transplantation.

Histological features are also important for the diagnosis of cholestatic hepatitis C.[3,14] Hepatocyte ballooning with limited inflammation is considered to be a typical finding, and it was observed in all of our cases with pan-lobular distribution. However, the international consensus criteria stated that ballooning predominantly occurred in the perivenular zone.[14] In LDLT, perivenular hepatocyte ballooning with cholestasis is often observed in dysfunctional grafts associated with small graft size, older donor or systemic inflammation.[19] Hepatocyte cholestasis was apparent in just one case (20%) in our series, and it might be attributed to the early biopsy before becoming fully established and irreversible.

Perivenulitis with centrilobular hepatocyte dropouts is a distinct histopathological process that could occur after LT, and is associated with post-transplant processes, including cytotoxic drugs, acute or chronic rejection, recurrent or de novo autoimmune hepatitis, and viral hepatitis.[20] Recent research focused on its immunological significance with significant graft injuries.[21] In hepatitis C after LT, Khettry *et al.*[22] reported that perivenulitis was significantly recognized in cases with severe recurrent hepatitis C associated with other pathological features with autoimmune hepatitis. Antonini *et al.*[23] reported that this phenomenon was more common in cholestatic patients than in non-cholestatic patients (36% vs 4%). Taking into account that cholestatic type recurrent hepatitis C causes significant hepatocyte injuries with vigorous cytokine production with unspecified immune reactions,[20–23] perivenulitis could be a significant pathological marker in cholestatic hepatitis C.

Interleukin-28B genotyping is an important predictor for the viral response to IFN. We previously reported that the T/T genotype of rs8099917 in donors and recipients is a positive predictor of the response to IFN after LDLT for hepatitis C.[12] In the current series, however, the T/T genotype was not associated with the recurrence of cholestatic hepatitis C. By contrast, Graziadei *et al.*[5] reported that rs12979860 genotypes, other than the favorable C/C genotype, in the recipients were significantly associated with cholestatic hepatitis C after LT, although the relevance of rs12979860 in donors has not been exclusively investigated. Hanouneh *et al.*[6] reported that the favorable T/T genotype of rs8099917 in the donor was associated with cholestatic recurrence. Based on these results, no consensus can be reached regarding the impact of IL-28B genotype on recurrence of cholestatic recurrent hepatitis C. Additionally, because there is a discrepancy between the IL-28B genotype, IL-28B transcription and the expression of IFN-stimulated genes,[24] further studies are needed to clarify the role of IL-28B in anti-HCV therapy.

It is still unclear why HCV can infect and replicate so vigorously, and cause cholestatic recurrence in a small number of patients after LT. We consider that

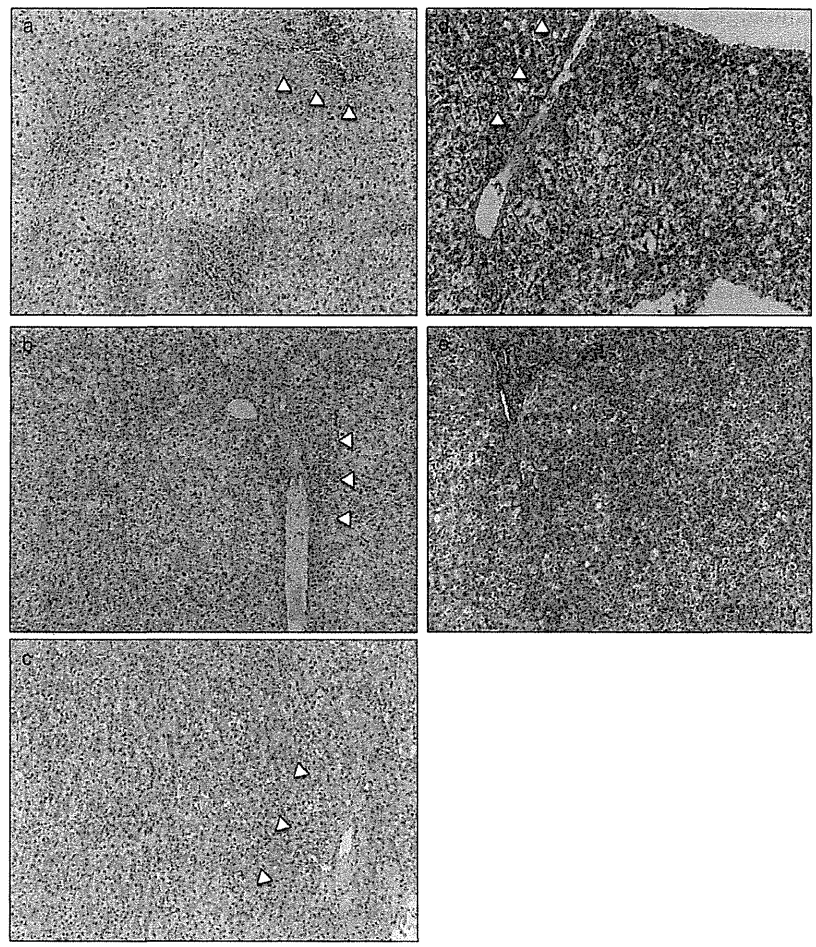

**Figure 2** Histological findings of cases 1–5 (a–e, respectively) with recurrent cholestatic hepatitis C. Pan-lobular hepatocyte ballooning was prominent in all of the five patients. Perivenulitis was observed in cases 1–4 (a–d, white arrowheads) (hematoxylin–eosin, original magnification ×100).

quasispecies of HCV may play some role in this process. Previous studies showed that the number of quasispecies increased following transplantation and onset of mild recurrence, but the species distribution was more homogenous in patients with severe recurrence.[25,26] It was also reported that HCV infection becomes more severe in patients infected with HIV type 1 with decreased or homogenous quasispecies.[23,27] Because an increased number of quasispecies is thought to represent the response of HCV to a strong immune pressure, induction of the local non-specific histocompatibility independent immune system may also mediate the disease process. Although viral mutations with increased capability of antiviral drug resistance as observed in cholestatic hepatitis B may have roles,[28] we regard it as doing little in cholestatic recurrent hepatitis C after LT because it becomes evident very early after transplantation before antiviral treatment is initiated. Therefore, we regard mechanisms in higher replication property against natural immune pressure including quasispecies as playing an important role.[23–27]

In terms of treatment, we think that PEG IFN with ribavirin should be the first choice of regimen for cholestatic hepatitis C, considering its clinically relevant outcomes. Nevertheless, the important point is that antiviral treatment should only be initiated once clinical cholestasis is evident, and histological cholestasis and fibrosis are established.[4–6,14] If started too late, the tolerability of IFN may become a major problem for decompensated liver grafts. Satapathy *et al.*[4] reported that seven out of eight patients (88%) with cholestatic hepatitis discontinued IFN because of decompensation or complications. The important key step to initiate early antiviral treatment for cholestatic hepatitis C is the accurate

pathological diagnosis differentiating acute rejection, although it is not an easy task. Bolus steroids for severe hepatitis C could terminate a transplanted graft.[29] Therefore, we maintain an appropriate immunosuppression level for the first 3 months after LT for HCV-associated liver diseases and never perform rapid tapering, making pathological interpretation easier. If treatment is started early, routine splenectomy of HCV patients during LDLT is reported to increase their tolerability of intense antiviral therapies.[9]

In conclusion, HCV viremia of more than 7.2 $\log_{10}$IU/mL at 2 weeks after transplantation was the predictor of recurrent cholestatic hepatitis C after LDLT in this study. IL-28B (rs8099917) genotype and other donor and recipient factors were not associated with its recurrence. Early diagnosis followed by antiviral treatment using PEG IFN with ribavirin is important to achieve VR and graft survival.

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