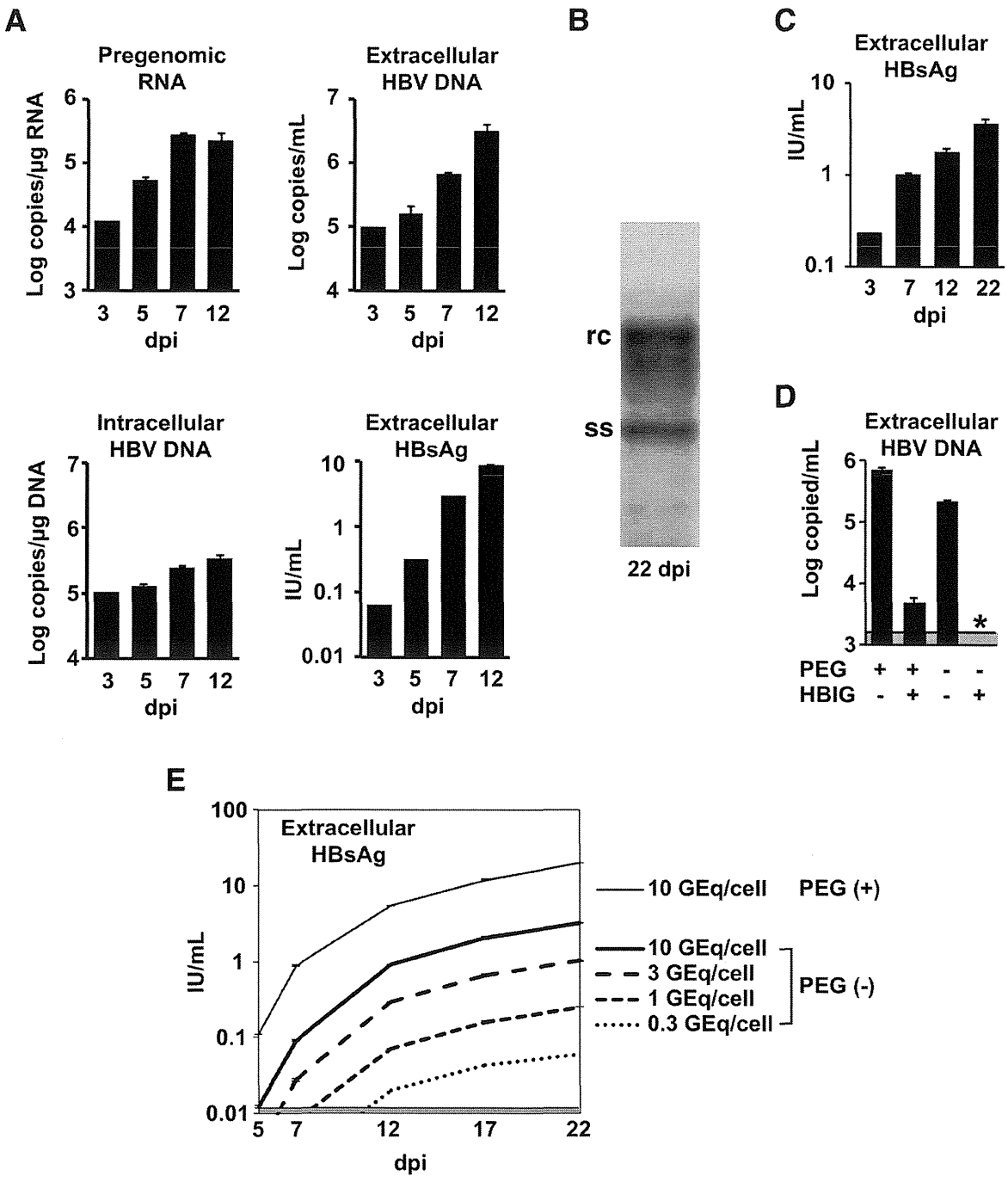

Fig 3. In vitro HBV infection model using PHHs isolated from chimeric mice with human hepatocytes. (A) PHHs were inoculated with HBV gt-C at 5 genomes per cell in the presence of PEG and intracellular pregenomic RNA, intracellular HBV DNA, extracellular HBV DNA and extracellular HBsAg were monitored by real-time quantitative PCR, or by automated ELISA. dpi, days post infection. **(B)** 20 μg of total DNA was extracted from PHHs 22 days after infection with HBV and analyzed by Southern blotting. Single-stranded HBV DNA (ss), a replication intermediate, and relaxed circular HBV DNA (rc) were detected. **(C)** Freshly prepared PHHs were inoculated with the day 52 supernatant from other HBV-infected PHHs. HBsAg secretion was monitored. **(D)** The use of PEG on HBV infection could mask the specificity of neutralization of HBV infection. Residual HBV infection was observed when PHHs were inoculated with a mixture of HBV and HBIG in the presence of PEG. An asterisk indicates a value below detection limit. **(E)** The efficacy of HBV infection without PEG was proportional to the size of the inoculum.

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HB0478 efficiently blocks HBV infection by both gt-C and gt-A

To evaluate the neutralizing activity of HB0478 against HBV infection, various amounts of HB0478 were preincubated with HBV gt-C or gt-A at 10 HBV genomes per cell (6.7×10^5 genomes/well) for 2 hours and exposed to PHHs for 48 hours without PEG (Fig. 4A). Fig. 4B shows the levels of HBV DNA in the supernatants harvested at 22 dpi. HB0478 in the amounts of 550 and 55 mIU completely blocked the infection by both gt-C and gt-A (HBV DNA was never detected in the supernatant). 5.5 mIU of HB0478 also completely inhibited gt-C infection, while it strongly reduced but did not completely inhibit gt-A infection. These results indicate that mAb HB0478 has powerful neutralizing activity against HBV infection and that HB0478 generated by the gt-C type vaccine could protect against HBV infection by both gt-C and gt-A, although less effectively against gt-A.

Discussion

Although the HBV vaccine strain used predominantly worldwide is genotype A2, genotype C strains are prevalent in Japan, where a selective vaccination program for high risk individuals with a gt-C-based vaccine is ongoing. A potential problem is that genotype A2 has been increasing recently as a cause of acute hepatitis B in Japan [10] and little is known about the efficacy of the gt-C-based vaccine against non-C HBV infection. In this report, we demonstrated that two mAbs, HB0478 and HB0116, derived from individuals immunized with the gt-C vaccine (Biimugen) that has been approved in Japan, neutralized HBV infections by both gt-C and gt-A in vitro and in vivo, suggesting that immunization with the gt-C vaccine could prevent infection by non-C HBV strains.

Epidemiological studies have shown that, in countries operating universal childhood vaccination programs using the gt-A2 vaccine, vertical transfer and/or incident infection of non-A2 were prevented efficiently [7]. Some studies have produced data supporting cross-genotype protection by immunization. An analysis of 221 mAbs isolated from volunteer HB vaccinees showed that 97% of them recognized common epitopes shared by all HBV genotypes [5]. The C(K/R)TC motif (amino acids 121–124), located in the N-terminal portion of the first loop of the "a" determinant of HBsAg, is conserved among all HBV genotypes (except for residue 122, K or R determining the serological subtype d or y, respectively) and highly immunogenic [26]. Moreover, a single mouse monoclonal Ab protected chimpanzees from infection by both adr (gt-C) and ayw (genotype D) strains [27].

Along with these findings, our results showed that the mAbs HB0478 and HB0116, generated following immunization with the gt-C type vaccine, neutralized the infectivity of both gt-C and gt-A HBV. In vitro experiments investigating dose dependency using freshly isolated PHHs also demonstrated that HB0478, at doses above 55 mIU, completely protected against both gt-C and gt-A infection, whereas HB0478, at a lower dose, 5.5 mIU, protected against gt-C infection only. It has been reported that analysis of nine HBV DNA positive blood donors in the United States revealed that 5 individuals who had been immunized with an A2-type vaccine were not protected against infections by non-A2 HBV [28]; however, the serum anti-HBs levels of these individuals (3–96 mIU/mL) were relatively low. Interestingly, the infections remained at a subclinical level in these vaccines, who subsequently resolved the HBV infection, suggesting that gt-A2 vaccination could not prevent non-A2 infection but can inhibit the development of clinical manifestations [28]. Therefore, it is possible that HBV specific antibodies, induced by gt-C vaccines, might be able to protect against clinical hepatitis caused by infection with non-C genotypes, even with lower anti-HBs concentrations. Further investigations are needed to determine clinical effectiveness of gt-C vaccine to induce cross-genotype immune responses.

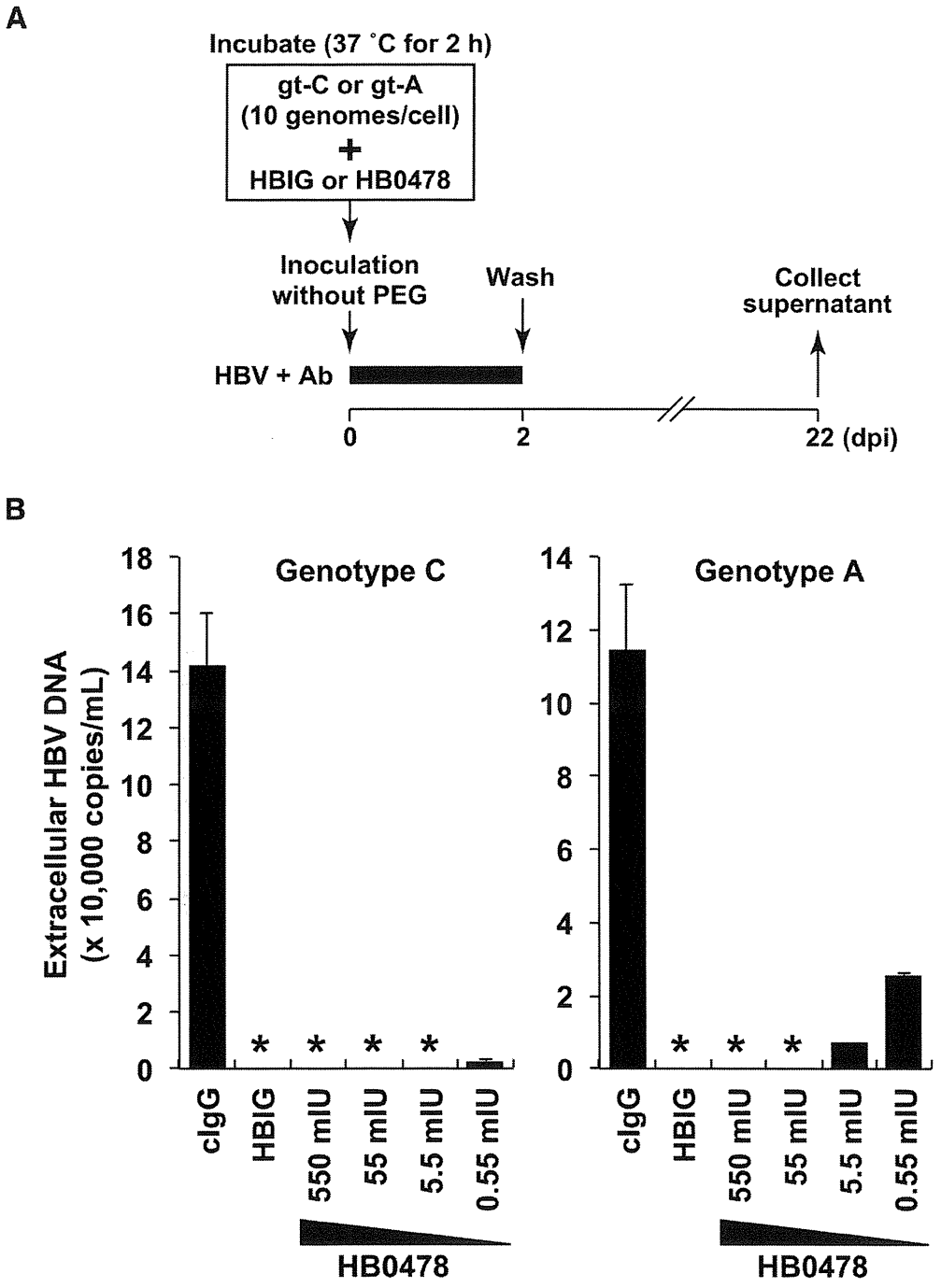

Fig 4. Titration of neutralization of gt-C and gt-A infection by mAb HB0478. HBV gt-C and gt-A were preincubated for 2 hours with 670 ng of control human IgG (cIgG), 100 mIU of HBIG, or 670, 67, 6.7 or 0.67 ng HB0478 (corresponding to 550, 55, 5.5, and 0.55 mIU) and PHHs were inoculated with the products at 10 genomes per cell. The Y-axis depicts the levels of extracellular HBV DNA in the supernatant harvested on 12 days post infection. Asterisks indicate values under the detection limit.

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Meanwhile, virus strains with amino acid substitutions in HBsAg often escape from HB vaccine-induced antibody and HBIG treatment during vertical transmission of HBV [19,20,29]. The substitution reported most frequently is residue 145, glycine to arginine (G145R), located in the second loop of the "a" determinant of HBsAg. This study demonstrated that HB0478 also recognized HBsAg with the G145R substitution and protected against G145R infection in vivo, whereas HB0116 did not bind to the G145R substituted protein or neutralize the mutant. Although how G145R in the second loop affects mAb-binding to the first loop is largely unknown, it is possible that the C(K/R)TC-dependent HB0478 epitope might be more distant from the second loop than that of HB0116, suggesting that HB0478 might not be affected by the conformational change of HBsAg induced by substitution of glycine at residue 145. It is noted that epitopes other than "a" determinant such as those within pre-S2 region [30] could also contributed to the neutralization of escape mutants.

In conclusion, this study raises the possibility that active immunization with a gt-C-based vaccine confers prophylaxis against gt-A, which is spreading in Japan, and against escape mutants such as G145R, when the anti-HBs responses are sufficient. Note that PHHs isolated from chimeric mice with human hepatocytes enabled us to investigate precisely the inhibitory effects of the mAbs, or any antiviral compounds, against HBV infection in vitro.

Author Contributions

Conceived and designed the experiments: SHT EI SM KT TJ YT. Performed the experiments: SHT EI TW SM KM KT TO. Analyzed the data: SHT EI TW SM MI SI TI KM. Contributed reagents/materials/analysis tools: HK AM. Wrote the paper: SHT EI TW YT.

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Hepatology Research

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Original Article

Factors associated with the effect of interferon-α sequential therapy in order to discontinue nucleoside/nucleotide analog treatment in patients with chronic hepatitis B

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Aim: The factors associated with the outcome of sequential therapy with interferon-α (IFN-α) in order to halt nucleoside/nucleotide analog (NUC) maintenance treatment for chronic hepatitis B were analyzed.

Methods: A total of 50 patients with chronic hepatitis B who underwent IFN-α sequential therapy for cessation of NUC were enrolled retrospectively. The subjects received NUC plus IFN-α for 4 weeks followed by IFN-α alone for 20 weeks. Natural IFN-α of 6-MU doses was administrated three times a week. A successful response to NUC/IFN-α sequential therapy was defined as serum hepatitis B virus (HBV) DNA below 4.0 log copies/mL, serum alanine aminotransferase (ALT) below 30 IU/L, and hepatitis B e-antigen negativity at 24 months after completing the treatment.

Results: Multivariate analysis revealed that hepatitis B surface antigen (HBsAg) of 3.0 log U/mL or more ($P < 0.002$) and hepatitis B core-related antigen (hepatitis B core-related antigen [HBcrAg]) of 4.5 log U/mL or more ($P < 0.003$) at the start of IFN-α administration were significant factors associated with a 24-month non-response. Maximal levels of ALT and HBV DNA during the follow-up period after completing IFN-α therapy were significantly related ($P < 0.001$), and receiver operating characteristic analysis showed that both maximal ALT ($P < 0.001$) and HBV DNA ($P < 0.001$) were significantly related to the final 24-month response.

Conclusion: The combinational use of HBsAg and HBcrAg levels may be useful to predict the 24-month outcome of NUC/IFN-α sequential therapy. Maximal levels of ALT and HBV DNA during post-treatment follow-up may also help monitor responses to IFN-α sequential therapy.

Key words: hepatitis B core-related antigen, hepatitis B surface antigen, interferon-α, nucleoside/nucleotide analogs, sequential therapy

INTRODUCTION

HEPATITIS B VIRUS (HBV) infection is a widespread health problem with an estimated 350–400 million carriers worldwide. Prolonged infection with HBV can cause chronic hepatitis, which may eventually develop into liver cirrhosis and hepatocellular carcinoma (HCC).[1-3] Currently available antiviral treatments for hepatitis B include nucleoside/nucleotide analogs (NUC) and interferon-α (IFN-α).[4] NUC are p.o. administrated and are associated with low rates of adverse effects. Although treatment with NUC, such as lamivudine (LVD), adefovir dipivoxil and entecavir (ETV), induces virological and biochemical responses in most patients, NUC therapy also carries the risk of drug resistance. Furthermore, patients with hepatitis B are required to undergo extended

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treatment with NUC because early discontinuance often leads to relapse.[5,6] In contrast, the remission of chronic hepatitis B by IFN-α is prolonged, but is achieved only in a small percentage of patients.

Serfaty *et al.*[7] conducted a pilot study on sequential therapy using LVD and IFN-α and concluded that this treatment could induce a sustained virological response in patients with chronic hepatitis B who did not respond to IFN-α alone. However, ensuing reports[8-12] were unable to confirm such a cooperative effect. Because the clinical backgrounds of the enrolled patients also differed among the above reports, it has become necessary to clarify the factors associated with the outcome of IFN-α sequential therapy in order to estimate its clinical significance.

We previously analyzed patients with chronic hepatitis B who ceased NUC therapy and showed that lower hepatitis B surface antigen (HBsAg) and hepatitis B core-related antigen (HBcrAg) levels were associated with a favorable clinical outcome in subjects negative for hepatitis B e-antigen (HBeAg) and HBV DNA at NUC discontinuation.[13,14] Although we identified patients in whom NUC could be safely halted with high reliance, such patients accounted for a relatively minor percentage. Therefore, we conducted the present study to analyze the effect of IFN-α sequential therapy on successfully stopping NUC.

This report retrospectively analyzes the factors associated with outcome of IFN-α sequential therapy following NUC treatment. As the subjects were followed long term, treatment responses at 24 months after stopping IFN-α were evaluated and compared with those at 6 and 12 months.

METHODS

Patients

A TOTAL OF 50 patients with chronic hepatitis B who underwent IFN-α sequential therapy in order to halt NUC therapy between May 2002 and September 2010 were enrolled. Subjects received NUC plus IFN-α for 4 weeks followed by IFN-α alone for 20 weeks (Fig. 1). Natural IFN-α (Sumiferon; Sumitomo Dainippon Pharma, Tokyo, USA) at a dose of 6 MU was administrated three times a week. Doses were reduced to 3 MU during exceptional circumstances, such as side-effects. All patients completed 24 weeks of IFN-α administration and received over 80% of the scheduled dose. Patients were recruited retrospectively from eight hospitals across Japan (Shinshu University Hospital, National Hospital Organization Nagasaki Medical Center, Toranomon Hospital, Hiroshima University Hospital, Chiba University Hospital, The Hospital of Hyogo College of Medicine, Kumamoto Shinto General Hospital,and Teine Keijinkai Hospital). The demographic data of the subjects are presented in Table 1. The median age at NUC cessation was 35 years. Approximately three-fourths of the patients were men. Genotype C HBV was predominant as has earlier been reported for Japan.[15] Eighty-six percent of patients began NUC therapy with LVD and 14% did so with ETV. The duration of NUC administration ranged from 4 to 121 months. The follow-up period was defined as the point of stopping IFN-α administration up until the last visit or to when NUC were re-administrated due to reactivation of hepatitis B. NUC were recommenced in 25 (50%) of the 50 patients enrolled. Among them, 17 were treated before judgment of the 24-month response to sequential therapy. All patients requiring re-administration

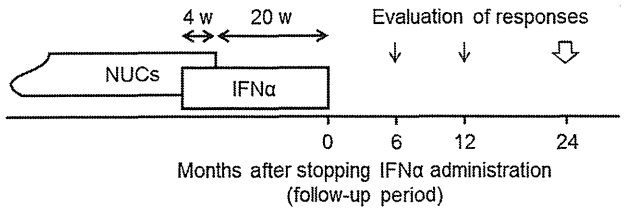

Figure 1 Experimental design of the present study. IFN, interferon; NUC, nucleoside/nucleotide analog; w, weeks.

Table 1 Demographic data of 50 enrolled patients

Characteristic	Value
Age at start of NUC administration (years)†	34 (21–57)
Age at end of NUC administration (years)†	35 (22–62)
Sex (male : female)	38:12
Genotype (B : C : undetermined)	3:36:11
NUC at start (LVD : ETV)	43:7
NUC at end (LVD : ETV : LAM + ADV : ETV + ADV)	40:8:1:1
Duration of NUC administration (months)†	6 (4–121)
HBeAg positivity at start of NUC‡	70% (35/50)
HBeAg positivity at end of NUC‡	42% (21/50)
Follow-up period after stopping IFN-α administration (months)†	28 (2–102)
Patients requiring re-administration of NUC‡	50% (25/50)
Patients developing HCC‡	0% (0/50)

†Data are expressed as the median (range).
‡Data are expressed as a positive percentage (positive number/total number).
ADV, adefovir dipivoxil; ETV, entecavir; HBeAg, hepatitis B e-antigen; HCC, hepatocellular carcinoma; IFN, interferon; LAM, lamivudine; LVD, lamivudine; NUC, nucleoside/nucleotide analog.

of NUC possessed alanine aminotransferase (ALT) levels of over 80 IU/L and HBV DNA levels of over 5.8 log copies/mL at or just before the point of NUC re-continuation, which fulfilled the established requirements for restarting NUC.[13,14,16]

Hepatitis B surface antigen was confirmed to be positive on at least two occasions at least 6 months apart in all patients before NUC treatment. Tests for hepatitis C virus and HIV antibodies were all negative. Patients complicated with HCC or signs of hepatic failure at the cessation of NUC administration were excluded from the study. No such complications were observed during follow up.

With few exceptions, patients were seen at least once a month during the first year of follow up, at least once every 3 months during the second year and at least once every 6 months afterwards. No patient developed HCC or hepatic failure during the follow-up period. Stored serum samples were kept frozen at −20°C or below until assayed. This study was approved by the ethics committees of all participating institutions (approval reference 1117 for Shinshu University Hospital, 24085 for National Hospital Organization Nagasaki Medical Center, 758 for Toranomon Hospital, 321 for Hiroshima University Hospital, 934 and 977 for Chiba University Hospital, 779 for The Hospital of Hyogo College of Medicine, 411 for Kumamoto Shinto General Hospital, and "Analysis of efficacy of IFN- to stop NUC in patients with chronic hepatitis B" for Teine Keijinkai Hospital).

Hepatitis B viral markers

Serological markers for HBV, including HBsAg, HBeAg and antibody to HBeAg, were tested using commercially available enzyme immunoassay kits (Abbott Japan, Tokyo, Japan; Fujirebio, Tokyo, Japan; and/or Sysmex, Kobe, Japan) at each hospital. Quantitative measurement of HBsAg[17] was performed using a chemiluminescence enzyme immunoassay (CLEIA)-based HISCL HBsAg assay manufactured by Sysmex (Kobe, Japan). The assay had a quantitative range of -1.5 to 3.3 log IU/mL. End titer was determined by diluting samples with normal human serum when initial results exceeded the upper limit of the assay range.

Serum HBV DNA was determined using a COBAS TaqMan HBV kit (Roche, Tokyo, Japan)[18] with a quantitative range of 2.1–9.0 log copies/mL. According to the manufacturer's instructions, detection of a positive signal below the quantitative range was described as a positive signal, and no signal detection was regarded as a negative signal. Six HBV genotypes (A–F) were evaluated according to the restriction patterns of DNA fragments from the method reported by Mizokami *et al.*[19]

Serum HBcrAg levels were measured using a CLEIA HBcrAg assay kit with a fully automated Lumipulse System analyzer (Fujirebio) as described previously.[20,21] The HBcrAg assay measures all antigens transcribed and translated from the precore and core genes of the HBV genome, which include hepatitis B e, core and p22cr antigens.[14,20] HBcrAg concentration was calculated based on a standard curve generated using recombinant pro-HBeAg. The immunoreactivity of pro-HBeAg at 10 fg/mL was defined as 1 U/mL. We expressed HBcrAg in terms of log U/mL, with a quantitative range set at 3.0–6.8 log U/mL.

Evaluation of response to NUC/IFN-α sequential therapy

The clinical conditions of a successful response to NUC/IFN-α sequential therapy were set at serum HBV DNA below 4.0 log copies/mL, serum ALT below 30 IU/L and negative HBeAg, according to established Japanese guidelines in which patients who meet these conditions are not recommended to start antiviral therapy.[22] We assessed the final response at approximately 24 months after completing IFN-α sequential therapy and compared results to those at 6 and 12 months after the treatment.

Statistical analyses

Fisher's exact and Pearson's χ^2-tests were adopted to test for differences between subgroups of patients. The Mann–Whitney *U*-test was employed to compare continuous data. Each cut-off value was decided using receiver operating characteristic (ROC) analysis, and results were evaluated by measuring the area under the ROC (AUC). Multivariate analysis was performed using a logistic model for the 24-month response to NUC/IFN-α sequential therapy. Correlations between maximal values of ALT and HBV DNA were calculated using Spearman's rank correlation coefficient test. The non-relapse rate was analyzed by the Kaplan–Meier method.

All tests were performed using the IBM SPSS Statistics Desktop for Japan version 19.0 (IBM Japan, Tokyo, Japan). $P < 0.05$ was considered to be statistically significant.

RESULTS

Factors associated with the 24-month response to NUC/IFN-α sequential therapy

OF THE 50 patients enrolled, 18 were judged as responders at 24 months after completing IFN-α sequential therapy (i.e. 24-month responders), while the

remaining 32 were classified as 24-month non-responders. The clinical backgrounds of both groups are compared in Table 2. The median age at NUC commencement and sex distribution did not differ remarkably between the groups. Genotype C was similarly predominant. The types of NUC administrated at the start and end of treatment were comparable between the groups, but the duration of NUC administration was significantly longer in responders. Re-administration of NUC due to aggravation of hepatitis B before judgment of the 24-month response was observed in approximately half of the 32 non-responders. After the final evaluation at 24 months, re-continuation of NUC was seen in only one of the 18 responders versus roughly half of the 15 non-responders who had previously not required it. The follow-up period was significantly longer in responders because observation was discontinued when NUC were re-administrated.

Biochemical and virological markers were compared between 24-month responders and non-responders at the start of NUC, at the start of IFN-α and at the end of IFN-α (Table 3). Positivity for the HBeAg was significantly lower in responders at all time points. HBsAg and HBcrAg levels did not differ between the groups at the start of NUC, but became significantly lower in responders at the start and end-points of IFN-α administration. A significant difference in HBV DNA level was seen between the groups at the end of IFN-α administration only. ALT levels did not differ between the groups at any point.

Multivariate analysis revealed that HBsAg and HBcrAg levels of 3.0 or more and 4.5 log U/mL or more, respectively, at the start of IFN-α administration were significant factors associated with a 24-month non-response to NUC/IFN-α sequential therapy (Table 4). The factors adopted for this logistic model were as follows: age at end of NUC of 37 years or more, duration of NUC administration of 18 months or more, sex, type of NUC at start, HBV genotype, HBeAg positivity at the start of IFN-α, HBsAg level at the start of IFN-α of 3.0 log IU/mL or more, and HBcrAg level at the start of IFN-α of 4.5 log U/mL or more. The corresponding cut-off values for each factor were determined by ROC analysis.

Of the 50 patients enrolled, 23 (46%) had HBsAg of 3.0 log IU/mL or more and HBcrAg of 4.5 log U/mL or more, 27 (54%) had HBsAg of less than 3.0 log IU/mL or HBcrAg of less than 4.5 log U/mL, and none had HBsAg of less than 3.0 log IU/mL and HBcrAg of less than 4.5 log U/mL at the start of IFN-α administration. Whereas none of the 23 patients with the highest HBsAg and HBcrAg levels were responders, 18 (67%) of the remaining 27 patients responded to NUC/IFN-α sequential therapy ($P = 0.005$).

Comparison of responses to NUC/IFN-α sequential therapy at different time points

We assessed the responses to NUC/IFN-α sequential therapy at 6 and 12 months after completing IFN-α administration using the same criteria as those for determining the 24-month outcome. Responses were in 78% agreement ($P < 0.001$) between 6 and 24 months and 80% agreement ($P < 0.001$) between 12 and 24 months.

Prediction of response to NUC/IFN-α sequential therapy using maximal levels of ALT and HBV DNA

The maximal levels of ALT and HBV DNA during follow up were found to be significantly related ($r = 0.777$, $P < 0.001$). ROC analysis showed that both maximal ALT

Table 2 Comparison of clinical backgrounds between 24-month responders and non-responders

Clinical background	24-month responders ($n = 18$)	24-month non-responders ($n = 32$)	*P*
Age at start of NUC (years)†	36 (21–56)	34 (21–57)	0.486
Sex (male : female)	15:3	23:9	0.497
Genotype (B:C:undetermined)	1:16:1	2:20:10	0.101
NUC at start (LVD : ETV)	16:2	27:5	1.000
NUC at end (LVD : ETV : LAM + ADV : ETV + ADV)	16:2:0:0	24:6:1:1	0.610
Duration of NUC administration (months)†	51 (5–121)	5 (4–72)	0.001
Follow-up period after stopping IFN-α administration (months)†	30 (23–102)	22 (2–81)	0.014
Re-administration of NUC before judging 24-month response‡	0% (0/18)	53% (17/32)	<0.001
Re-administration of NUC after judging 24-month response‡	6% (1/18)	47% (7/15)	0.012

†Data are expressed as the median (range).
‡Data are expressed as a positive percentage (positive number/total number).
ADV, adefovir dipivoxil; ETV, entecavir; HBeAg, hepatitis B e-antigen; HCC, hepatocellular carcinoma; IFN, interferon; LAM, lamivudine; LVD, lamivudine; NUC, nucleoside/nucleotide analog.

Table 3 Comparison of ALT level and viral markers between 24-month responders and non-responders at the time points of starting NUC administration, starting IFN-α administration and stopping IFN-α administration

ALT/viral marker	24-month responders ($n = 18$)	24-month non-responders ($n = 32$)	*P*
At start of NUC administration			
ALT (IU/L)†	242 (32–2274)	281 (22–1044)	0.872
HBeAg‡	44% (8/18)	84% (27/32)	0.008
HBV DNA (log copies/mL)†	8.0 (<2.1–>9.0)	7.8 (<2.1–>9.0)	0.866
HBsAg (log IU/mL)†	3.5 (1.8–4.9)	3.5 (2.5–4.4)	1.000
HBcrAg (log U/mL)†	>6.8 (3.7–>6.8)	>6.8 (<3.0–>6.8)	0.121
At start of IFN-α administration			
ALT (IU/L)†	29 (12–103)	29 (12–111)	0.779
HBeAg‡	11% (2/18)	59% (19/32)	0.001
HBV DNA (log copies/mL)†	<2.1 (neg.–3.9)	<2.1 (neg.–4.8)	0.142
HBsAg (log IU/mL)†	2.9 (1.5–4.1)	3.7 (2.5–4.3)	0.028
HBcrAg (log U/mL)†	3.6 (<3.0–5.9)	5.6 (<3.0–>6.8)	0.002
At end of IFN-α administration			
ALT (IU/L)†	25 (10–48)	28 (12–134)	0.384
HBeAg‡	6% (1/18)	59% (19/32)	<0.001
HBV DNA (log copies/mL)†	<2.1 (neg.–4.1)	4.6 (<2.1–>9.0)	<0.001
HBsAg (log IU/mL)†	2.8 (1.9–4.0)	3.6 (2.6–4.7)	0.007
HBcrAg (log U/mL)†	3.4 (<3.0–5.5)	5.5 (<3.0–>6.8)	0.017

†Data are expressed as the median (range).
‡Data are expressed as a positive percentage (positive number/total number).
ALT, alanine aminotransferase; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e-antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; IFN, interferon; neg., negative; NUC, nucleoside/nucleotide analog.

Table 4 Multivariate analysis of factors associated with 24-month non-responders to NUC/IFN-α sequential therapy

Selected factor	Odds ratio	95% CI	*P*
HBsAg ≥3.0 log IU/mL at start of IFN-α	17.7	2.9–108.2	0.002
HBcrAg ≥4.5 log U/mL at start of IFN-α	15.0	2.5–88.6	0.003

CI, confidence interval; HBcrAg, hepatitis B core-related antigen; HBsAg, hepatitis B surface antigen; IFN, interferon; neg., negative; NUC, nucleoside/nucleotide analog.

and HBV DNA levels were significantly associated with the treatment response (Fig. 2), with an AUC for each parameter of over 0.8. The cut-off values providing the highest significance in ROC analysis were 128 IU/L for ALT and 4.5 log copies/mL for HBV DNA. The existence of a second cut-off value was also identified for HBV DNA (6.0 log copies/mL) to discriminate between 24-month responders and non-responders. These results indicated that patients reaching a maximal ALT level of over 128 IU/L or maximal HBV DNA level of over 6.0 log copies/mL during post-treatment follow up were likely to be non-responders.

Lastly, we analyzed the changes in cumulative non-relapse rate of hepatitis B during and after IFN-α administration by tentatively defining relapse as ALT level exceeding 128 IU/L during follow up. We selected maximal ALT instead of maximal HBV DNA because: (i) the inflection point to distinguish a response was clear for maximal ALT but ambiguous for maximal HBV DNA; (ii) the value for "sensitivity + specificity − 1" as calculated by ROC analysis was larger for maximal ALT (7.5 vs 6.5); and (iii) the maximal levels of ALT and HBV DNA were closely associated, and thus ALT values were considered to represent those of HBV DNA. The cumulative non-relapse rate decreased rapidly after completely halting NUC until just prior to 6 months after stopping IFN-α and then was seen to plateau until the study end-point (Fig. 3). This suggests that the recurrence of hepatitis associated with a 24-month non-response can be expected to occur primarily during the first 6 months after stopping IFN-α administration.

DISCUSSION

THE COOPERATIVE EFFECT of NUC/IFN-α sequential therapy has been controversial.[7-12] Enomoto *et al.*[10] first analyzed the results of ETV/IFN-α sequential therapy in patients with HBeAg positive chronic hepatitis B and detected several differences. Although their results were negative, they witnessed that patients who had achieved HBeAg

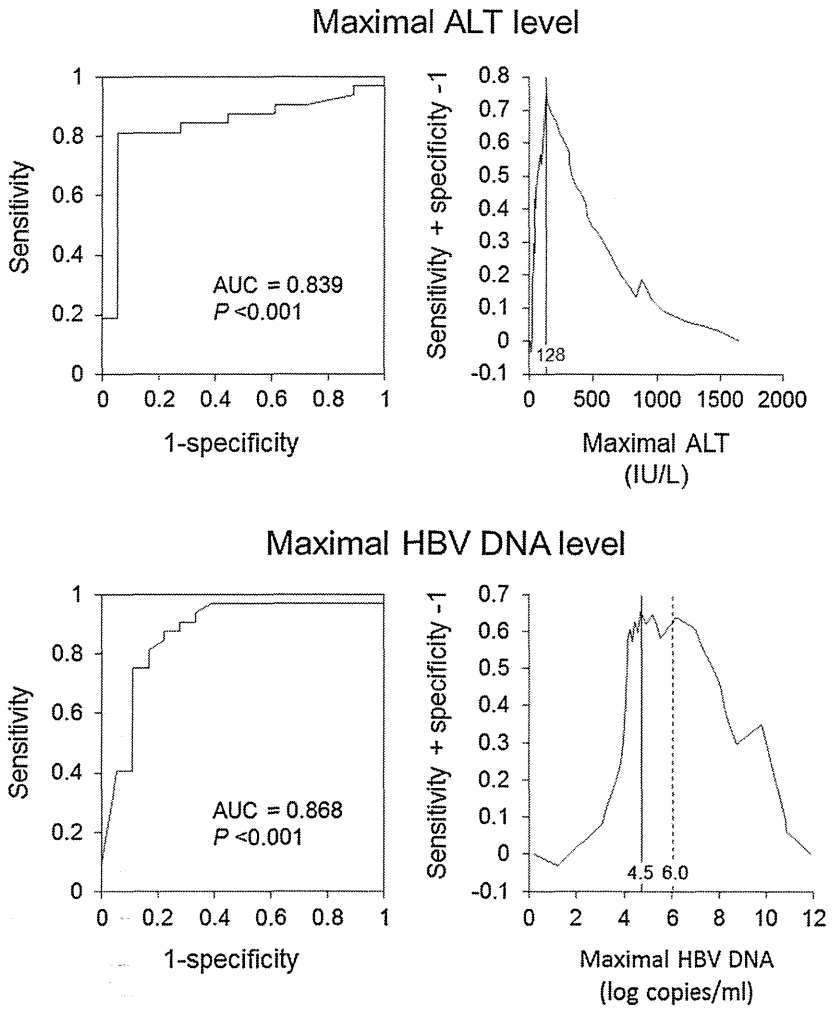

Figure 2 Receiver operating characteristic analysis of maximal alanine aminotransferase (ALT) and hepatitis B virus (HBV) DNA levels to discriminate between 24-month responders and non-responders. Vertical solid lines indicate the actual values of markers corresponding to main inflection points and the vertical broken line indicates the actual value of the marker corresponding to a second inflection point; AUC, area under the receiver operating characteristic curve.

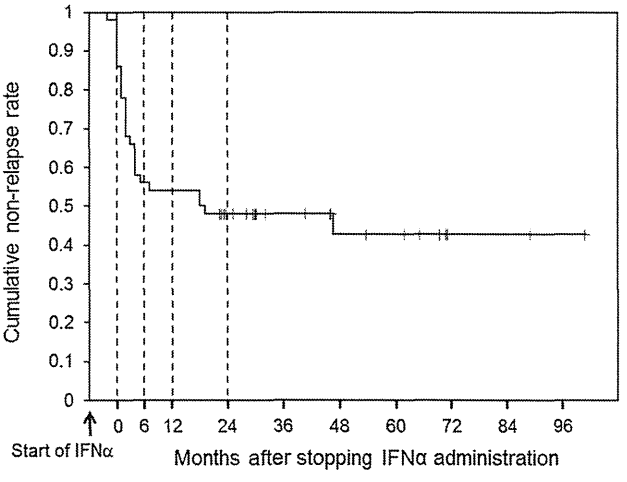

Figure 3 Kaplan–Meier analysis of the non-relapse rate after stopping interferon (IFN)-α administration by defining relapse of hepatitis B as alanine aminotransferase (ALT) level exceeding 128 IU/L.

seroconversion by the time of IFN-α commencement experienced a significantly higher sustained virological response rate than those in whom the HBeAg persisted. Thus, it appeared beneficial to further clarify the factors associated with the response to NUC/IFN-α sequential therapy.

The present study analyzed the factors associated with a long-term response to IFN-α sequential therapy in order to safely discontinue NUC therapy. All patients were treated with natural IFN-α for 6 months and followed for at least 24 months after completing the sequential therapy, with the exception of those who required re-administration of NUC due to aggravation of hepatitis B. The type and duration of NUC administration were not fixed in this study because IFN-α sequential therapy was implemented to discontinue NUC in patients who were undergoing maintenance treatment. Although a prospective study would have been ideal to elucidate the factors associated with

IFN-α sequential therapy outcome, we undertook this retrospective trial because no variables have been sufficiently analyzed to date. Furthermore, we were able to address the long-term response to IFN-α sequential therapy in relation to the results of earlier retrospective studies. It has been reported that pegylated IFN-α (PEG IFN-α) provides a higher HBV response rate than does conventional IFN-α.[23] Therefore, additional prospective studies of sequential therapy using PEG IFN-α are needed as well.

Both HBsAg and HBcrAg levels at the time of NUC cessation were factors significantly associated with the response to NUC/IFN-α sequential therapy. HBsAg has been closely linked with PEG IFN-α therapy outcome.[24-27] Moucari *et al.*[26] analyzed HBeAg negative hepatitis B patients who had been treated with PEG IFN-α for 48 weeks and concluded that an early serum HBsAg drop was strongly predictive of a sustained virological response. Sonneveld *et al.*[24] assessed HBeAg positive hepatitis B patients who had received PEG IFN-α with or without LVD for 52 weeks and observed that patients who experienced no decline in HBsAg level from baseline at week 12 had little chance of achieving a sustained response and no possibility of HBsAg loss. HBcrAg includes antigens that are transcribed and translated from precore and core genes of the HBV genome, and HBeAg is a primary component of these antigens. Thus, our results were consistent with those described by Enomoto *et al.*[10] that the proportion of patients losing HBeAg positivity during ETV treatment was significantly higher in responders to ETV/IFN-α sequential therapy than in non-responders.

Hepatitis B surface antigen and HBcrAg levels have both been associated with intrahepatic HBV cccDNA, which is a key molecule in HBV replication whose value is closely related to HBV replication activity.[21,27,28] Several reports[27,29,30] have shown that HBV cccDNA level is associated with the response to antiviral therapy, such as with PEG IFN-α and NUC. Sung *et al.*[29] analyzed HBeAg positive hepatitis B patients who had been treated with either LVD monotherapy or a combination of PEG IFN-α and LVD and concluded that intrahepatic HBV cccDNA level at the end of therapy was superior to serum HBV DNA in predicting a sustained virological response. Serum HBV DNA is associated with intrahepatic HBV cccDNA and is widely used as a marker for HBV replication activity. However, such associations may be incompatible with antiviral therapies, and especially NUC treatment, because NUC directly hamper production of the HBV virion by inhibiting reverse transcription of pre-genomic RNA without affecting HBV cccDNA directly. As serum levels of HBsAg and HBcrAg are easier to measure than intrahepatic HBV cccDNA, these two antigen assays may be more suitable as surrogate markers for HBV replication activity in patients undergoing antiviral therapy. We previously reported that the combinational use of HBsAg and HBcrAg was beneficial to forecast the risk of hepatitis relapse after discontinuation of NUC.[13,14] The present study confirms this notion; it is possible that HBsAg and HBcrAg have complimentary roles in monitoring antiviral effects because the production of these two antigens is regulated by alternative enhancer–promoter systems in the HBV genome.

It is noteworthy that ROC analysis revealed maximal levels of ALT and HBV DNA to be closely associated with the 24-month response to NUC/IFN-α sequential therapy. We observed that patients with ALT higher than 128 IU/mL or HBV DNA higher than 6.0 log copies/mL during follow up were likely to be non-responders. When a relapse of hepatitis B was tentatively defined as ALT exceeding 128 IU/L during observation, relapses occurred frequently during the first 6 months after ceasing IFN-α and then became more sporadic afterwards. The timing of judgment of a virological response to NUC/IFN-α sequential therapy is critical when evaluating treatment efficacy. As this period is usually set at 6 months after completing therapy, our results confirm that 6 months is indeed appropriate. Our findings also suggest that maximal levels of ALT and HBV DNA are useful for monitoring the results of NUC/IFN-α sequential therapy. Accordingly, patients who are likely to be non-responders can now be identified as early as 24 weeks in advance and alternative strategies for treatment may be considered in a more timely fashion.

In conclusion, the combinational use of HBsAg and HBcrAg levels may be useful to predict the response to NUC/IFN-α sequential therapy. Maximal levels of ALT and HBV DNA during follow up may also be employed for monitoring the results of IFN-α sequential therapy.

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Original Article

Risk factors for hepatitis B virus recurrence after living donor liver transplantation: A 17-year experience at a single center

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Aim: The incidence of hepatitis B virus (HBV) recurrence after liver transplantation (LT) has been reduced by prophylaxis with hepatitis B immunoglobulin (HBIG) and nucleoside analogs, but the factors associated with HBV recurrence are unclear. The aim of this study was to determine the risk factors associated with HBV recurrence after living donor LT (LDLT).

Methods: A retrospective review was performed for 45 patients (28 male and 17 female; median age, 54 years) who underwent LDLT for HBV-related liver disease and were followed up for at least 6 months between October 1996 and June 2013. The virological data, tumor burden, antiviral therapy and immunosuppressive therapy were evaluated and compared between the HBV recurrence ad non-recurrence groups.

Results: Seven of the 45 patients (15.6%) developed post-LT HBV recurrence. The median interval between LDLT and HBV recurrence was 23.7 months (range, 0.8–35.9). Three of the seven patients (42.9%) developed recurrence after cessation of HBIG, and three (42.9%) were cases with hepatocellular carcinoma (HCC) recurrence after LDLT. The remaining case underwent transplantation from a donor with positive hepatitis B surface antigen. Based on the univariate and multivariate analyses, HBIG cessation (hazard ratio [HR], 20.17; 95% confidence interval [95% CI], 2.091–194.593; $P = 0.009$) and HCC recurrence (HR, 30.835; 95% CI, 3.132–303.593; $P = 0.003$) were independent risk factors for HBV recurrence after LDLT.

Conclusion: In LDLT patients, cessation of HBIG and HCC recurrence were risk factors associated with HBV recurrence, so careful monitoring for serological HBV markers is needed in patients with these factors.

Key words: hepatitis B immunoglobulin, hepatitis B virus recurrence, hepatocellular carcinoma, living donor liver transplantation

INTRODUCTION

HEPATITIS B IS a leading cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) worldwide. Before the advent of an effective means for preventing the virtually universal re-infection of the graft, the outcome of liver transplantation (LT) for hepatitis B virus (HBV)-related liver diseases was dismal, and this often led to HBV recurrence rates greater than 80% and mortality rates of 50% at 2 years.[1] Prophylaxis with hepatitis B immunoglobulin (HBIG) and nucleoside analogs, such as lamivudine, has markedly decreased the recurrence rate of HBV through their synergistic effects.[2] However, approximately 10% of transplanted patients still develop HBV recurrence.[3,4] In previous studies, the factors associated with HBV recurrence were reported to be a high pre-LT HBV DNA level,[5,6] hepatitis B e-antigen (HBeAg) positivity,[7] non-fulminant hepatitis B,[8] immunosuppression from steroids and systemic chemotherapy,[9] and pre-LT HCC and post-LT HCC recurrence.[10–12]

In this study, we noted that a group of patients still developed HBV recurrence after living donor LT (LDLT). We analyzed a retrospective series of 45 patients who underwent LDLT for HBV-related liver disease and were followed for at least 6 months, and we evaluated their virological and biochemical data, tumor burden, antiviral therapy and immunosuppressive therapy, as well as the eventual development of HCC recurrence. The aim of this

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study was to determine the risk factors associated with HBV recurrence after LDLT.

METHODS

Immunoprophylaxis

ALL PATIENTS WERE treated with a combination HBIG (Hebsbulin-IH; Japan Blood Products Organization, Tokyo, Japan) and at least one nucleoside agent (lamivudine, adefovir, entecavir or a combination thereof) for HBV prophylaxis after transplantation.

Nucleoside analog therapy was initiated when the patients were referred to the hospital and indicated for LDLT, if they had not yet been treated with this agent. For fulminant hepatitis B patients, lamivudine or entecavir was initiated when the etiology was verified to be HBV. HBIG at 10 000 U was administrated i.v. during the anhepatic phase during the operation, followed by 5000 U/day for 1 week after the LDLT. Thereafter, 3000–5000 U of HBIG was administrated every 2–3 months. The targeted level of hepatitis B surface antibody (HBsAb) was more than 500 IU/L for the cases before March 2004, and was more than 200 IU/L for the first year and more than 100 IU/L thereafter for the cases after April 2004.[13]

Immunosuppression

A calcineurin inhibitor, such as cyclosporin or tacrolimus, with or without mycophenolate mofetil, was used as the immunosuppressive therapy after LDLT. The immunosuppressive dosing was adjusted according to the therapeutic drug levels and renal function. A gram of methylprednisolone was given after reperfusion, and the dose was tapered from 200 mg to 20 mg daily in a week, then switched to oral prednisolone, and finally tapered off in 6 months.

Serological monitoring

The recurrence of the HBV was defined as the appearance of the hepatitis B surface antigen (HBsAg) in the serum after LDLT.[8,10] Standard biochemical tests of liver function were performed at each follow-up visit. The serum HBsAg, HBsAb and HBV DNA were tested monthly. From 1996 until March 2004, the HBV DNA levels were quantified with a transcription-mediated amplification assay (Mitsubishi Chemical Medience, Tokyo, Japan), which has a detection range of 3.7–8.7 log genome equivalents (LGE)/mL. Thereafter, all HBV DNA levels were tested with a polymerase chain reaction (PCR) assay (SRL, Tokyo, Japan), which has a detection range of 2.6–7.6 log copies/mL. The YMDD mutant was detected using a PCR enzyme-linked minisequence assay (SRL).

Surveillance for HCC recurrence

After LDLT, patients with known HCC were followed regularly in our outpatient hepatology clinic. Surveillance with computed tomography was performed every 3–4 months. If there were concerns about HCC recurrence, whole-body computed tomography or magnetic resonance imaging was ordered at the discretion of the patient's physician.

Statistical analysis

Continuous variables were compared by the Mann–Whitney *U*-test. Categorical variables were compared by the χ^2-test and Fisher's exact tests. A Cox regression analysis was used to determine the predictors of the time to HBV recurrence. The variables reaching statistical significance by the univariate analysis were then included in the multivariate analysis. The cumulative incidence of patient survival and HBV recurrence after LDLT were calculated using the Kaplan–Meier method, and the difference was evaluated by the log-rank test. A value of $P < 0.05$ was considered significant. Statistical analyses were performed using the SPSS version 17.0 software package (SPSS, Chicago, IL, USA).

RESULTS

Demographics

A RETROSPECTIVE REVIEW of the medical record database was performed for 45 patients (28 male and 17 female; median age, 54 years) who underwent LDLT for HBV-related liver disease and were followed up at least 6 months between October 1996 and June 2013 at Kyushu University. Table 1 summarizes the patients' data at the time of LDLT. The median follow-up time after LDLT was 66 months (range, 9–174). Hepatitis C virus co-infection was present in three patients (6.7%). HCC was present in 28 patients (62.2%). Twenty-five patients with HCC (89.3%) were diagnosed by preoperative computed tomography or magnetic resonance imaging, whereas three patients (10.7%) were diagnosed incidentally by a pathological examination of the explant. Six patients (21.4%) had evidence of vascular invasion. HCC beyond the Milan criteria was present in eight patients (28.8%) by preoperative imaging, and in 15 patients (53.8%) by explant pathology, respectively.

Pre-LT HCC therapy was administrated to 17 of the 28 patients with HCC (60.7%). Among them, two patients (7.1%) received pre-LT systemic chemotherapy, which consisted of a combination of an antimetabolite (5-fluorouracil), platinum-based agent (cisplatin) and anthracycline (epirubicin). Fourteen patients (50%) received a combination of local ablative therapy in the

Table 1 Characteristics of the 45 patients with hepatitis B virus-related liver disease

	HBV-related transplantation ($n=45$)
Age (years)	54 (31–67)
Sex	
Male	28 (62.2%)
Female	17 (37.8%)
HCV co-infection	3 (6.7%)
Primary disease	
Acute liver failure	12 (26.7%)
Liver cirrhosis	33 (73.3%)
HBsAg positivity	41 (91.1%)
HBeAg positivity	10 (22.2%)
HBV DNA (log copies/mL)	
Unknown	5 (11.1%)
<2.6	20 (44.4%)
2.6–5	8 (17.8%)
>5	12 (26.7%)
HCC	28 (62.2%)
By preoperative imaging	25 (89.3%)
Incidental on explant	3 (10.7%)
Vascular invasion	6 (21.4%)
Beyond Milan criteria by:	
preoperative imaging	8 (28.8%)
explant pathology	15 (53.8%)
Pre-LT systemic chemotherapy	2 (7.1%)
Post-LT systemic chemotherapy	3 (10.7%)
Pre-LT antiviral therapy	
None	4 (8.9%)
LAM	22 (48.9%)
LAM + ADV	6 (13.3%)
ETV	13 (28.9%)
Corticosteroid therapy >6 months	12 (26.7%)
Median follow-up period (months)	66 (9–174)

Qualitative variables are expressed as the numbers of patients, with percentages in parentheses, and quantitative variables are expressed by the medians, with ranges in parentheses.
ADV, adefovir; ETV, entecavir; HBeAg, hepatitis B e-antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; LAM, lamivudine; LDLT, living donor liver transplantation.

form of arterial chemoembolization, radiofrequency ablation and/or an ethanol injection prior to LDLT. One patient (3.6%) underwent surgical resection of the tumor prior to LDLT.

Of the 28 patients with HCC, three patients (10.7%) received post-LT systemic chemotherapy. Among them, one patient received post-LT chemotherapy for the treatment of combined hepatocellular and cholangiocellular carcinoma. The post-LT chemotherapy regimens included a combination of antimetabolites (5-fluorouracil, gemcitabine), a platinum-based agent (cisplatin), anthracycline (epirubicin) and multikinase inhibitor (sorafenib). Prior to LDLT, 41 patients (91.1%) were treated with antiviral therapy consisting of lamivudine, adefovir, entecavir or a combination thereof.

Overall HBV recurrence

Seven of the 45 patients (15.6%) developed post-LT HBV recurrence. The median interval between LDLT and the development of HBV recurrence was 23.7 months (range, 0.8–35.9). The overall actuarial rates of HBsAg recurrence after LDLT at 1, 3 and 5 years were 6.7%, 17.9% and 17.9%, respectively (Fig. 1). Table 2 shows the results of the univariate analysis of risk factors associated with HBV recurrence after LDLT. The factors significantly associated with HBV recurrence were cessation of HBIG ($P=0.039$) and HCC recurrence ($P=0.021$). According to the multivariate analysis, the same factors were found to be independently associated with a higher risk of HBV recurrence after LDLT: cessation of HBIG (hazard ratio [HR], 20.17; 95% confidence interval [95% CI], 2.091–194.593; $P=0.009$) and HCC recurrence (HR, 30.835; 95% CI, 3.132–303.593; $P=0.003$) (Table 3).

HBIG cessation

Three of the seven patients (42.9%) with HBV recurrence were cases in whom HBIG was suspended during combined prophylaxis after LDLT (Table 4, cases 1–3). In case 1, the HBIG was suspended while the patient received HBV

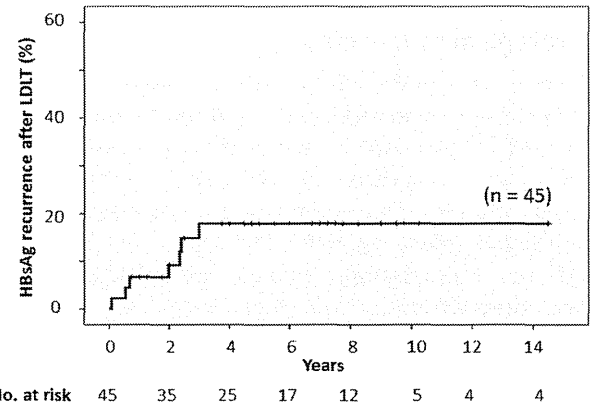

Figure 1 Cumulative rates of hepatitis B surface antigen (HBsAg) recurrence in the 45 patients. LDLT, living donor liver transplantation.

Table 2 Results of the univariate analysis of factors associated with HBV recurrence after LDLT

	HBsAg recurrence ($n = 7$)	HBsAg non-recurrence ($n = 38$)	*P*
Age (years)	53 (46–64)	53.5 (31–67)	0.778
Male sex	4 (57.1%)	24 (63.2%)	0.538
Acute liver failure	1 (14.3%)	11 (43.2%)	0.387
Pre-LT HBeAg positivity	1 (14.3%)	9 (23.7%)	0.506
HBV DNA level at LT >5 log copies/mL ($n = 40$)	2 (33.3%; $n = 6$)	10 (29.4%; $n = 34$)	0.595
HBIG cessation	3 (42.9%)	3 (7.9%)	0.039
Pre-LT HCC	4 (57.1%)	24 (63.2%)	0.468
Beyond Milan criteria by			
preoperative imaging	2 (28.6%)	6 (15.8%)	0.363
explant pathology	4 (57.1%)	11 (28.9%)	0.154
HCC recurrence	3 (42.9%)	2 (5.3%)	0.021
Post-LT systemic chemotherapy	2 (28.6%)	1 (2.6%)	0.059
Duration of corticosteroids (months)	3.7 (1.4–8.4)	2.9 (0.2–23.9)	0.309
Post-LT antiviral therapy	4/2/1	19/5/14	0.402
LAM/LAM + ADV/ETV	(57.1%/28.6%/14.3%)	(50%/13.2%/36.8%)	

Qualitative variables are expressed as the numbers of patients, with percentages in parentheses, and quantitative variables are expressed by the medians, with ranges in parentheses.
ADV, adefovir; ETV, entecavir; HBeAg, hepatitis B e-antigen; HBIG, hepatitis B immunoglobulin; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; LAM, lamivudine; LDLT, living donor liver transplantation.

Table 3 Results of the multivariate analysis of factors associated with HBV recurrence after LDLT

	Hazard ratio	95% CI	*P*
HCC recurrence	30.835	3.132–303.593	0.003
HBIG cessation	20.170	2.091–194.593	0.009
Age (>55)	–	–	0.732
Sex, male	–	–	0.529
Post-LT systemic chemotherapy	–	–	0.863

CI, confidence interval; HBIG, hepatitis B immunoglobulin; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; LDLT, living donor liver transplantation.

vaccination. However, HBsAg and HBV DNA reemerged 2 months after the cessation of HBIG, and the vaccination had failed. Adefovir was subsequently added to his treatment regimen, and the HBV DNA immediately became undetectable. In cases 2 and 3, HBIG was suspended for financial reasons, and HBsAg reemerged 4 and 6 months after the suspension of HBIG, respectively. After HBIG was reintroduced, the HBsAg disappeared and HBsAb reappeared immediately. HBIG was also suspended in three of the 38 patients (7.9%) who did not have HBV recurrence, and these were the cases in whom HBV vaccination was successfully performed. The indication of HBV vaccination was patients who have a normal or near

Table 4 Antiviral therapy administrated and the outcomes of patients with HBV recurrence after LDLT

	Age (years), sex	Primary disease	HBV prophylaxis	Cessation of HBIG	Time to HBV recurrence (months)	Time to HCC recurrence (months)	Outcome
1	53, M	HCC with LC	HBIG + LAM	+	24	–	Alive
2	47, M	ACLF	HBIG + LAM	+	28	–	Alive
3	54, M	LC	HBIG + LAM	+	6	–	Alive
4	46, M	HCC with LC	HBIG + LAM, ADV	–	8	5	Died
5	59, F	HCC with LC	HBIG + LAM, ADV	–	36	15	Died
6	47, F	HCC with LC	HBIG + ETV	–	29	30	Alive
7	64, F	LC	HBIG + LAM	–	1	–	Alive

ACLF, acute-on-chronic liver failure; ADV, adefovir; ETV, entecavir; HBcAb, hepatitis B core antibody; HBIG, hepatitis B immunoglobulin; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; LAM, lamivudine; LC, liver cirrhosis; LDLT, living donor liver transplantation.

normal liver function tests with a low level of immunosuppression, and the follow-up period after LDLT was at least a year. HBIG was suspended 1–4 weeks before starting HBV vaccination.[14]

As shown in Figure 2, the cumulative HBsAg recurrence rates were significantly higher in patients with HBIG cessation than in those receiving combined prophylaxis ($P = 0.013$).

Pretransplant HCC and HCC recurrence

Hepatocellular carcinoma was present in 28 patients (62.2%), and the cumulative HBsAg recurrence rates after LDLT were not significantly higher in patients with HCC than in those without HCC ($P = 0.711$) (Fig. 3a). Between the patients with HCC beyond and within the Milan criteria based on the preoperative imaging, there was no statistically significant difference in the cumulative HBsAg recurrence rates ($P = 0.370$) (Fig. 3b). Meanwhile, in patients with HCC beyond and within the Milan criteria diagnosed by the explant pathology, the cumulative HBsAg recurrence rates were higher (with marginal significance) in the patients with HCC beyond the Milan criteria than in those with HCC within the Milan criteria ($P = 0.068$) (Fig. 3C).

Among these 28 patients with HCC, five patients (17.9%) developed HCC recurrence after LDLT, and HBV recurrence occurred in three of these five patients (60%) (Table 4, cases 4–6). In case 4, the tumors were beyond the Milan criteria at LDLT, and HCC recurred 5 months after transplantation. Despite the use of chemotherapy and radiation, the HCC had grown and the HBsAg reappeared 8 months after transplantation during systemic chemotherapy. In case 5, there were multiple HCC tumors, and

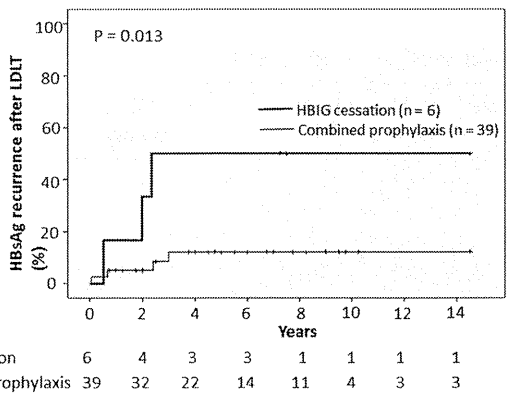

Figure 2 Cumulative rates of hepatitis B surface antigen (HBsAg) recurrence in patients with cessation of hepatitis B immunoglobulin (HBIG) and in those receiving combined prophylaxis (HBIG + antiviral agent). LDLT, living donor liver transplantation.

the disease was beyond the Milan criteria at LDLT. At 15 months after transplantation, HCC recurred as lung metastasis, and HBsAg recurrence was observed at 36 months after LDLT during systemic chemotherapy. In both cases 4 and 5, the HBV DNA increased in spite of combined therapy with entecavir, adefovir and HBIG.[15] Both patients finally died of recurrent HCC, at 12 and 43 months after transplantation, respectively.

In case 6, the HCC was within Milan criteria at LDLT, and HBsAg reemerged 29 months after LDLT, which was 1 month prior to the detection of HCC recurrence. The patient underwent several operations for the treatment of metastasis and, thereafter, the HCC has been under control. She has not received systemic chemotherapy or radiation therapy, and the HBV DNA levels have been undetectable by combined prophylaxis with entecavir and HBIG. In patients with HCC recurrence, the cumulative HBsAg recurrence rate after LDLT was significantly higher than that in patients without HCC recurrence ($P < 0.001$) (Fig. 3d).

The remaining patient with HBV recurrence (Table 4, case 7) was the only case of transplantation from a living donor with positive HBsAg.[16] This living donor was the patient's son whose blood type was identical. He had no history of liver dysfunction, and was referred to as a "healthy carrier". Because no other living donors were available and brain-dead donors are rarely available in Japan, we decided to proceed to LDLT with this donor. To date, the patient has been doing well at 12 years after transplantation and the donor has also been doing well.

Overall survival

The overall survival after LDLT was not significantly reduced for patients with HBV recurrence, with probabilities at 1, 3 and 5 years of 100%, 91.5% and 91.5%, respectively, for patients without HBV recurrence versus 100%, 85.7% and 71.4%, respectively, for patients with HBV recurrence ($P = 0.250$) (Fig. 4a). However, if the six cases of HBIG cessation were excluded ($n = 39$), the cumulative survival rate was significantly reduced for patients with HBV recurrence, with probabilities at 1, 3 and 5 years of 100%, 90.7% and 90.7%, respectively, for patients without HBV recurrence versus 100%, 75% and 50%, respectively, for patients with HBV recurrence ($P = 0.037$) (Fig. 4b).

DISCUSSION

IN OUR STUDY, the demographic, virological, tumor burden, antiviral therapy and immunosuppressive therapy of patients with and without HBC recurrence after LDLT were analyzed to identify the risk factors for HBV

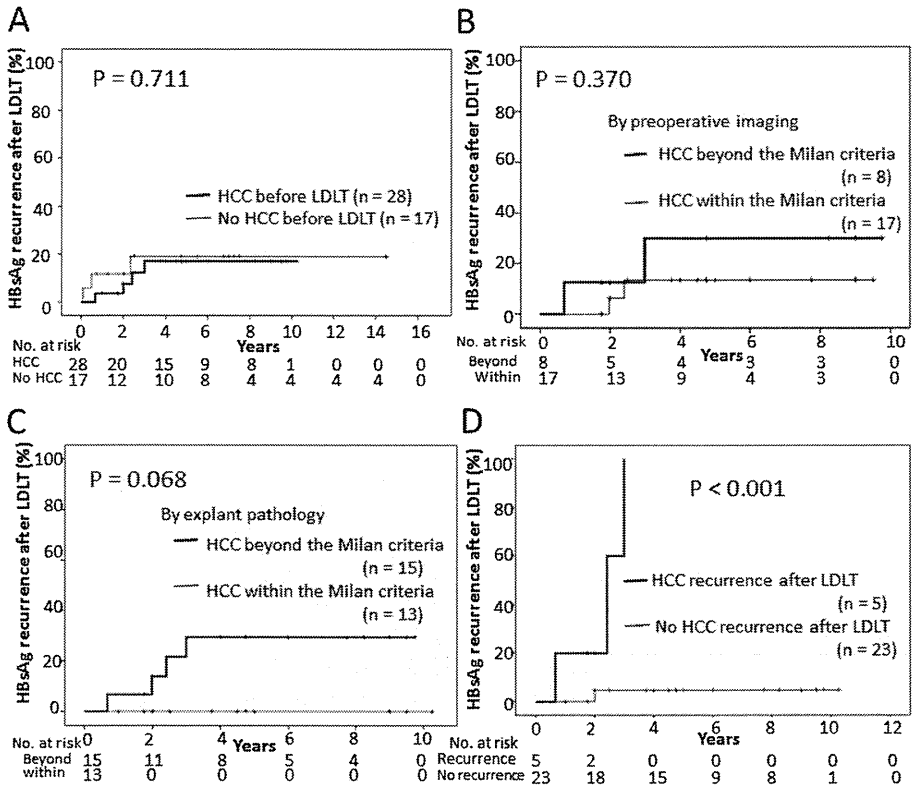

Figure 3 Cumulative rates of hepatitis B surface antigen (HBsAg) recurrence: (a) in patients with and without hepatocellular carcinoma (HCC) before living donor liver transplantation (LDLT); (b) beyond and within the Milan criteria by preoperative imaging; (c) beyond and within the Milan criteria by explant pathology; (d) with and without the recurrence of HCC. LDLT, living donor liver transplantation.

recurrence. We showed that both cessation of HBIG and HCC recurrence were independent risk factors for HBV recurrence after transplantation. Previous studies have demonstrated that pre-LT HBeAg positivity, non-fulminant hepatitis B, immunosuppression from steroids and systemic chemotherapy after LDLT are risk factors for HBV recurrence,[7–9] however, we could not find a statistically significant correlation with those factors and HBV recurrence in the present study. Although a high pre-LT HBV DNA level was also reported to be an independent risk factor for HBV recurrence,[5,6] we also found no statistically significant correlation with this factor. A limitation of our retrospective study is that not all of the HBV DNA levels were known at the time of LDLT. Therefore, the effects of antiviral therapy on the viral load were not available for all patients. However, all patients had undetectable HBsAg and HBV DNA levels after LDLT.

The mechanisms by which HBIG protects the transplanted liver against HBV reinfection are not fully understood. One hypothesis is that HBIG protects naive hepatocytes against the HBV released from extrahepatic sites by blocking a putative HBV receptor.[17,18] Previous studies reported that recurrent hepatitis B during the first 6 months post-LT is usually related to inadequate HBIG doses in patients with a high viral load pre-LT, whereas late recurrence is caused mainly by the selection of immune escape mutants.[19–21] The most common mutation involves a glycine to arginine substitution at codon 145 (G145R) of the HBV S protein. This mutation results in reduced binding to anti-HBs, and such viruses may escape neutralization by HBIG. The cessation of HBIG therapy is accompanied by a reversion to a wild-type sequence, supporting the role of HBIG in the selection of these mutations.

Due to the many drawbacks of HBIG, including its high cost, several trials have attempted to minimize the dose of HBIG in selected patients.[22,23] Recently, Fung *et al.* reported that a HBIG-free regimen of entecavir monotherapy was effective for suppressing HBV after LT.[24] Yi *et al.* also reported the efficacy of sequential entecavir monotherapy after 1-year combination therapy.[25] Compared with lamivudine, entecavir has greater antiviral potency and a

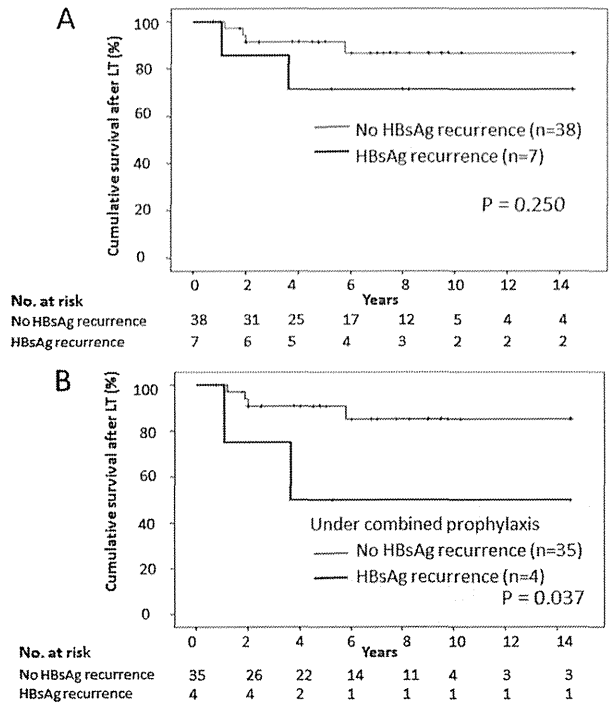

Figure 4 Cumulative survival in liver transplant recipients: (a) with and without hepatitis B surface antigen (HBsAg) recurrence; (b) with and without HBsAg recurrence under combined prophylaxis. LT, liver transplant.

higher genetic barrier to resistance, resulting in lower resistance rates in HBV-related liver disease patients. Further studies are needed to establish an optimal prophylactic regimen.

Except for a few studies that suggested a higher incidence of HBV recurrence in transplanted patients with HCC,[10–12] previous studies had not shown any association between HCC and a higher risk of HBV recurrence.[7,26,5,27,28] In 2008, Faria *et al.* reported an association between HCC recurrence and HBV re-infection.[10] In their study, the presence of HCC at transplantation and HCC recurrence after LT were independent risk factors associated with HBV recurrence. The authors demonstrated the presence of cccDNA in both HCC cells and in non-tumor cells in explanted livers, suggesting that HBV replication may also occur in tumor cells. In 2009, Saab *et al.* reported that pre-LT HCC and HCC recurrence after transplantation were associated with HBV reinfection and with decreased patient survival.[11] HCC recurrence itself is suggested to be a product of any breakthrough of the host immunity, and active cell proliferation due to malignant transformation can induce active replication of the HBV in the liver.[29] In addition, Yi *et al.* reported that chemotherapy and a high corticosteroid dose used for HCC were risk factors for HBV recurrence.[9] From that point of view, the differences in the virological kinetics in our three cases with HCC recurrence (Table 4, cases 4–6) are interesting, and may be explained by the condition of HCC and the use of systemic chemotherapy. These results require confirmation by further investigations.

Saab *et al.* reported decreased cumulative survival for patients with HBV recurrence.[11] Our present study showed no significant effect of HBV recurrence on the overall survival (Fig. 4a). However, if the analysis was limited to the patients with combined prophylaxis, the cumulative survival rates were significantly reduced in the HBV recurrence group compared with the HBV non-recurrence group (Fig. 4b). This result is consistent with previous studies,[11,30] but it should be noted that both of the two death cases in the HBsAg recurrence group (Fig. 4b) were the cases of HCC recurrence (Table 4, cases 4 and 5). The remaining two cases were another case of HCC recurrence and the case from a HBsAg positive donor (Table 4, cases 6 and 7). It is difficult to reach a definite conclusion because of small sample size, but HCC recurrence may be a strong prognostic factor for survival in a HBsAg recurrence group. Whether HBV recurrence itself is truly affecting prognosis or not should be confirmed in further studies.

Although the limitations of this study include its retrospective design and relatively small sample size, our results demonstrated the importance of combined prophylaxis, and confirmed that there is a relationship between HBV and HCC recurrence.

In conclusion, cessation of HBIG and post-LT HCC recurrence were independent risk factors for HBV recurrence in LDLT patients. Despite the improvements achieved in HBV prophylaxis following LDLT, clinicians should remain cautious concerning the risk of HBV recurrence, particularly in these groups.

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