(range: 1.1–1.8 μg/kg) once a week. Ribavirin was administered at a median dose of 8.3 mg/kg body weight (range: 5.8–13.2 mg/kg) twice a day every 12 hr. Each drug was discontinued or its dose reduced, as required upon judgment of the attending physician, in response to a fall in hemoglobin level, leukocyte count, neutrophil count or platelet count, or the appearance of side effects. The triple therapy was discontinued when the leukocyte count decreased to $<1{,}000/mm^3$, neutrophil count $<500/mm^3$, or platelet count $<5 \times 10^4/mm^3$, or when hemoglobin decreased to <8.5 g/dl.

### Measurement of HCV RNA

The virological response was assessed using the COBAS TaqMan HCV test. The linear dynamics range of this assay is 1.2–7.8 $\log_{10}$ IU/ml and samples with undetectable HCV RNA were defined as negative. The response to treatment was divided into the following: sustained virological response-12 (negative HCV RNA at 12 weeks after completion of therapy), which is relevant to sustained virological response-24 defined by Martinot-Peignoux et al. [2010] and Mauss et al. [2012], relapse (rise in viral load after the end of treatment, even when HCV RNA was negative at the end of treatment), and viral breakthrough (rise in viral load before the end of treatment, even when HCV RNA was temporarily negative during treatment).

### Detection of Amino Acid Substitutions in Core and NS5A Regions of HCV-1b

With the use of HCV-J (accession no. D90208) as a reference [Kato et al., 1990], the sequence of 1–191 amino acids (aa) in the core protein of HCV-1b was determined and then compared with the consensus sequence constructed in a previous study to detect substitutions at aa 70 of arginine (Arg70) or glutamine/histidine (Gln70/His70) and aa 91 of leucine (Leu91) or methionine (Met91) [Akuta et al., 2005]. The sequence of 2,209–2,248 aa in the NS5A of HCV-1b (ISDR) reported by Enomoto et al. [1996] was determined and the numbers of aa substitutions in the ISDR were defined as wild-type (0, 1) or non wild-type (≥2), compared with HCV-J. In the present study, aa substitutions of the core region and NS5A-ISDR of HCV-1b were analyzed by direct sequencing.

### Determination of IL-28B Genotype

IL-28B (rs8099917) was genotyped by the Invader assay, Taq Manassay, or direct sequencing, as described previously [Ohnishi et al., 2001; Suzuki et al., 2003].

### Statistical Analysis

The $\chi^2$ test, Fisher's exact probability test, and Mann–Whitney's *U*-test were used to compare the background characteristics of the groups. All *P* values were two-tailed, and $P < 0.05$ was considered statistically significant. Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS, Inc., Chicago, IL).

## RESULTS

### Efficacy of Triple Therapy

Figure 1a illustrates the negative rates of HCV RNA at different time points. The disappearance rate of HCV RNA during treatment was 11% (2/18), 33% (6/18), 83% (15/18), 89% (16/18), 78% (14/18), 78% (14/18), 67% (12/18), and 67% (12/18) at 1, 2, 4, 8, 12, 16, 20, and 24 weeks, respectively. Furthermore, 50% (9/18) of elderly patients achieved sustained virological response-12.

Four of the 18 patients discontinued triple therapy because of side effects, but in the remaining 14 patients, HCV RNA level was below the detection limit of the test during treatment. Two patients experienced viral breakthrough at 20 weeks after the commencement of treatment and three patients experienced relapse. Four patients discontinued the triple therapy due to the appearance of side effects [two developed skin disease (at 4th and 10th week), one developed anemia (at second week), and one patient discontinued due to poor appetite (at the 11th week)]. Figure 1b shows changes in hemoglobin level in patients who received the triple therapy. During the administration of telaprevir to 12 weeks, hemoglobin decreased steadily, with a maximum of 3.7 g/dl (mean value) at 12 week. However, hemoglobin tended to increase after the end of telaprevir medication, during treatment with peginterferon and ribavirin. Figure 1c shows changes in serum creatinine level in patients who received the triple therapy. During administration of telaprevir to 12 weeks, creatinine increased steadily, with a maximum of 0.14 g/dl (mean value) at 8 week. Similar to the pattern described above for hemoglobin, serum creatinine tended to decrease after the end of telaprevir medication, during treatment with peginterferon and ribavirin.

### Response to Treatment as a Function of Telaprevir Dose

Table II summarizes the profiles and laboratory data of the 18 patients according to the dose of telaprevir. At baseline, leukocyte count in patients treated with telaprevir 1,500 mg/day was lower than in those treated with 2,250 mg/day. None of the female patients received telaprevir at 2,250 mg/day. The HCV RNA clearance rate was similar in the 2,250 and 1,500 mg/day groups (Fig. 2a). Both doses of telaprevir resulted in fall in hemoglobin, but the falls in the 2,250 mg/day group at 2, 4, 8, weeks after the start of treatment were significantly more profound compared with the 1,500 mg/day group (Fig. 2b). Furthermore, both doses of telaprevir

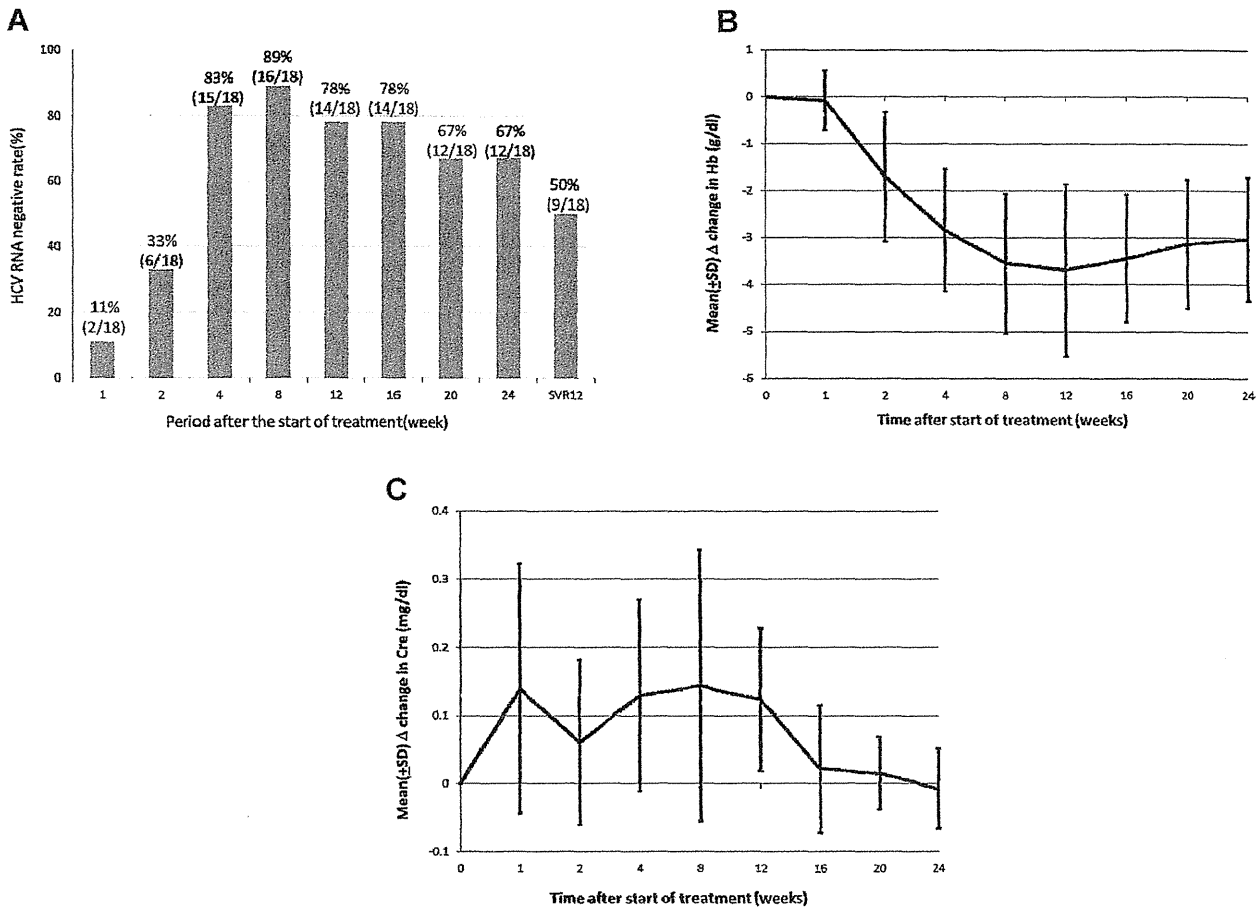


Fig. 1. **a**: HCV RNA clearance rate at different time points after the start of triple therapy of telaprevir with peginterferon and ribavirin. The sustained virological response-12 rate was 50% and the end-of-treatment response rate was 67%. **b**: Fall in hemoglobin in patients who received triple therapy of telaprevir, with peginterferon and ribavirin. **c**: Rise in creatinine in patients who received triple therapy of telaprevir, with peginterferon and ribavirin.

induced a rise in serum creatinine, but the rises in the 2,250 mg/day group at 12, 16, 24, weeks after the start of treatment were significantly more profound compared with the 1,500 mg/day group (Fig. 2c).

### Relation between Loss of HCV RNA and IL-28B (rs8099917) Genotype TT

Figure 3a illustrates the negative rates of HCV RNA in patients with the rs8099917 genotype TT/non TT at different time points. The HCV RNA disappearance rate in patients with the rs8099917 genotype TT during treatment was 9% (1/11), 36% (4/11), 82% (9/11), 100% (11/11), 91% (10/11), 91% (10/11), 73% (8/11), and 73% (8/11) at 1, 2, 4, 8, 12, 16, 20, and 24 weeks, respectively. Furthermore, 64% (7/11) of the elderly patients achieved sustained virological response-12.

Figure 3b illustrates the HCV RNA clearance rates in patients with the rs8099917 genotype TT/non TT during treatment with telaprevir 1,500 mg. The HCV RNA clearance rates in patients with the rs8099917 genotype TT during treatment was 11% (1/9), 44% (4/9), 89% (8/9), 100% (9/9), 100% (9/9), 100% (9/9), 78% (7/9), and 78% (7/9) at 1, 2, 4, 8, 12, 16, 20, and 24 weeks, respectively. Furthermore, 67% (6/9) of the elderly patients achieved sustained virological response-12. These results highlight the safety and efficacy of telaprevir 1,500 mg, peginterferon, and ribavirin in elderly patients with the rs8099917 genotype TT.

## DISCUSSION

With the aging society in Japan, it is important to evaluate the efficacy of interferon therapy in elderly patients with chronic HCV infection. This is important especially due to the lack of information on the safety and efficacy of triple therapy of telaprevir, peginterferon, and ribavirin. In the study of Suzuki

TABLE II. Characteristics of Patients at Baseline According to Telaprevir Dose and Adherence to Each Drug

| Characteristics | Telaprevir dose | | *P*-value |
|---|---|---|---|
| | 2,250 mg | 1,500 mg | |
| Number of patients | 4 | 14 | |
| Age (years)* | 67 (66–68) | 69 (66–73) | 0.079 |
| Male/female | 4/0 | 10/4 | 0.023 |
| Body mass index (kg/m$^2$)* | 23.1 (22.3–24.1) | 22.6 (18.9–26.3) | NS |
| Viral load of HCV ($\log_{10}$ IU/ml) | 5.9 (5.3–7.0) | 6.5 (5.1–7.3) | NS |
| Serum aspartate aminotransferase (IU/L) | 43 (37–48) | 27 (11–95) | NS |
| Serum alanine aminotransferase (IU/L) | 36 (23–44) | 36 (19–80) | NS |
| Serum albumin (g/dl) | 3.9 (3.6–4.0) | 3.7 (3.3–4.1) | NS |
| Gamma-glutamyl transpeptidase (IU/L) | 31 (19–62) | 22 (10–61) | NS |
| Leukocyte count (/mm$^3$) | 5,400 (4,000–7,300) | 3,900 (2,500–5,300) | 0.035 |
| Hemoglobin (g/dl) | 14.4 (13.5–16.1) | 13.9 (12.5–14.9) | NS |
| Platelet count ($\times 10^4$/mm$^3$) | 16.9 (15.1–21.0) | 14.9 (9.6–21.4) | NS |
| Alpha-fetoprotein (μg/L) | 6 (5–7) | 3 (1–18) | NS |
| Treatment | | | |
| Peginterferon α-2b dose (μg/kg)* | 1.4 (1.3–1.6) | 1.5 (1.0–1.8) | |
| Ribavirin dose (mg/kg)* | 12.4 (11.6–13.2) | 7.0 (5.8–12.9) | 0.005 |
| Amino acid substitutions in the HCV genotype 1b | | | NS |
| Core aa 70 (arginine/glutamine) | 1/3 | 9/5 | NS |
| Core aa 91 (leucine/methionine) | 2/2 | 9/5 | NS |
| ISDR of NS5A (wild-type/non wild-type/ND) | 4/0/0 | 13/0/1 | NS |
| Genetic variation near IL28B gene rs8099917 genotype (TT/TG/GG) | 2/1/1 | 9/5/0 | NS |
| Past history of interferon therapy Treatment-naïve/relapsers to previous treatment/nonresponders to previous treatment | 2/2/0 | 1/8/5 | 0.087 |
| PegIFN adherence (%) | 78.7 (55.6–100) | 80.0 (8.3–100) | NS |
| RBV adherence (%) | 33.9 (17.7–68.8) | 50.0 (6.7–79.2) | NS |
| TVR adherence (%) | 68.4 (36.7–100) | 66.7 (11.2–66.7) | NS |
| Comorbidities[a] | | | |
| Diabetes mellitus | 1 (25%) | 2 (14%) | NS |
| Hypertension | 2 (50%) | 7 (50%) | NS |

Date are number (percentage) of patients, except those denoted by *, which represent the median (range) values.
[a]All patients were not on medications.

et al. [2012], 20 patients with chronic HCV infection and high viral load of genotype 1b were randomly assigned to two telaprevir-based regimens of 2,250 and 1,500 mg/day in combination with peginterferon and ribavirin for 12 weeks. The sustained virological response rates were not different between the 1,500 and 2,250 mg groups, while serum creatinine increased more extensively in the 2,250 mg group than in the 1,500 mg group. However, their patients were <65 years old and treated for only 12 weeks. In the present study, the response to triple therapy with telaprevir for 12 weeks, peginterferon, and ribavirin for 24 weeks was examined in a pilot study that included 18 elderly patients infected with HCV-1b with high viral loads. Four of the 18 patients were treated with telaprevir 2,250 mg/day and the other 14 patients were treated with telaprevir 1,500 mg/day. The results showed no tolerance to the triple therapy in 4 of 18 (22%) patients due to skin rashes, anemia, and poor appetite. However, 9 of 18 (50%) elderly patients who received the triple therapy were able to achieve sustained virological response-12. Furthermore, even when treated for 24 weeks, elderly patients of the 1,500 mg group showed reduction in the elevated serum creatinine that was similar to that seen in patients aged <65 years.

The IL-28B genotype is identified as a pretreatment predictor of virological response to 48-week peginterferon plus ribavirin combination therapy in individuals infected with HCV-1 [Ge et al., 2009; Tanaka et al., 2009; Suppiah et al., 2009], and also as a predictor of response to triple therapy with telaprevir, peginterferon, and ribavirin in Japanese patients infected with HCV-1 [Akuta et al., 2010, 2012; Chayama et al., 2011]. In the present study, among patients with the rs8099917 genotype TT who were treated with telaprevir 1,500 mg, 6 of 9 (67%) could achieve sustained virological response-12, and none discontinued the triple therapy because of side effects. Thus, for elderly patients with the rs8099917 genotype TT, triple therapy with telaprevir 1,500 mg, peginterferon, and ribavirin was safe and efficacious, especially in patients with the rs8099917 genotype TT.

Iwasaki et al. [2006] and Honda et al. [2010] reported that the sustained virological response rates at the completion of the 48-week interferon and ribavirin combination therapy for elderly patients were only 16% and 31%, respectively. However, in the present study, of 18 elderly patients, 12 (67%) were negative for HCV RNA at the end of the triple therapy, and sustained virological response-12 was

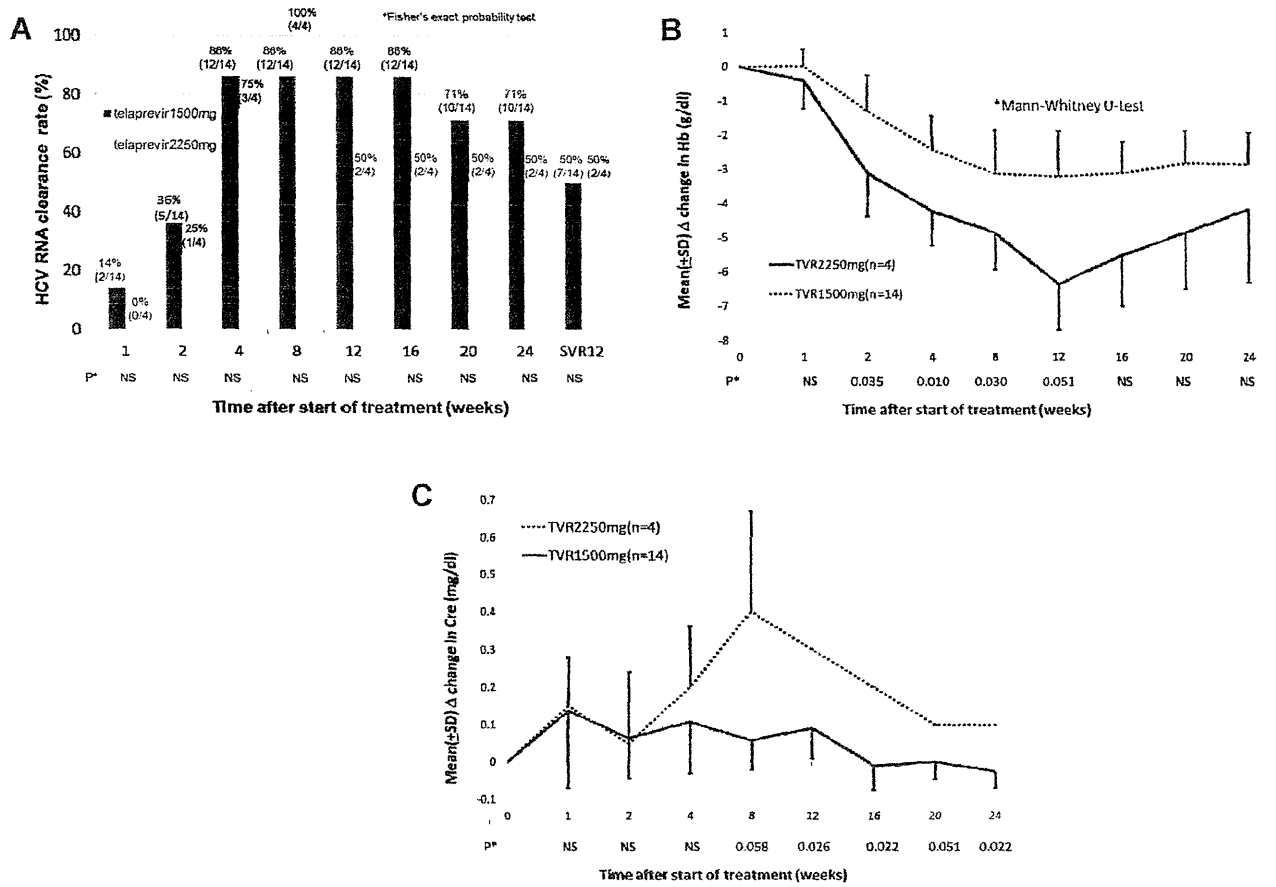


Fig. 2. **a**: HCV RNA clearance rate according to the dose of telaprevir (1,500 and 2,250 mg/day), combined with peginterferon and ribavirin. The sustained virological response-12 rate was 50% in both dose groups and the end-of-treatment response rates were 71% and 50%, respectively. **b**: Fall in hemoglobin according to the dose of telaprevir (1,500 and 2,250 mg/day), in combination with peginterferon and ribavirin. The fall was more profound in the 2,250 mg/day group at 2, 4, and 8 weeks compared with the 1,500 mg/day group. **c**: Rise in serum creatinine according to the dose of telaprevir (1,500 and 2,250 mg/day), in combination with peginterferon and ribavirin. The rise was more profound in the 2,250 mg/day group at 12, 16, and 24 weeks compared with the 1,500 mg/day group.

achieved by 9 patients (50%). Analysis of the data of the 14 elderly patients showed sustained virological response-12 was achieved in seven (50%) patients who received triple therapy with telaprevir 1,500 mg, peginterferon, and ribavirin, seven (50%). These results indicate that triple therapy with telaprevir 1,500 mg, peginterferon, and ribavirin, is safe and efficacious. Further studies are needed to determine if such treatment can be shortened to 24 weeks.

This study is not without limitations. The number of patients who received triple therapy was small and the study failed to show statistical significance in any comparison of various factors, especially between telaprevir 1,500 mg and telaprevir 2,250 mg treatment groups. This study is retrospective in nature; therefore, selection bias may have affected the results. We did not estimate sustained virological response-24 in the present study. Martinot-Peignoux et al. [2010] and Mauss et al. [2012] reported sustained virological response-12 as endpoint for future trials because HCV relapse usually occurs within the first 12 weeks after the end of treatment. Accordingly, in this study, we estimated sustained virological response-12. To generalize medical treatment for elderly patients with chronic HCV infection, further large scale randomized control clinical trials for telaprevir 1,500 mg and 2,250 mg are necessary to investigate the sustained virological response-24.

In conclusion, triple therapy with telaprevir 1,500 mg, peginterferon, and ribavirin, is safe and efficacious in elderly patients with chronic HCV infection. The triple therapy could be selected as

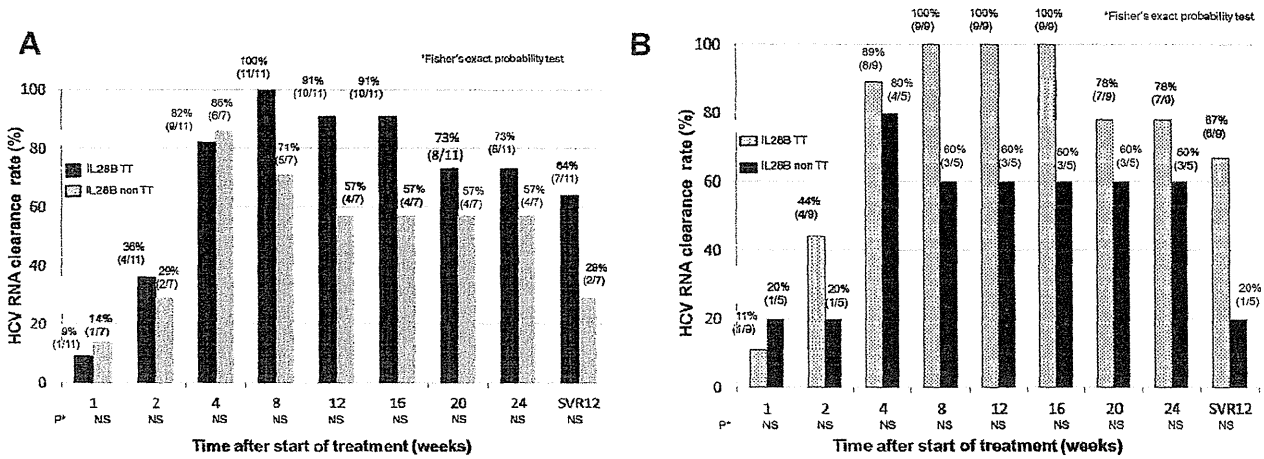


Fig. 3. **a**: HCV RNA clearance rate according to rs8099917 genotype TT. The sustained virological response-12 rate was 64% and the end-of-treatment response rate was 73% in patients with rs8099917 genotype TT. **b**: HCV RNA clearance rate according to rs8099917 genotype TT after the start of triple therapy of telaprevir 1,500 mg with peginterferon and ribavirin. The sustained virological response-12 rate was 78% and the end-of-treatment response rate was 67%.

potentially suitable therapy for elderly Japanese patients aged >66 years with chronic HCV of genotype 1b.

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ORIGINAL ARTICLE

# Factors associated with early cancer-related death after curative hepatectomy for solitary small hepatocellular carcinoma without macroscopic vascular invasion

**Kazunari Sasaki · Masamichi Matsuda · Yu Ohkura · Yusuke Kawamura · Masafumi Inoue · Masaji Hashimoto · Kenji Ikeda · Hiromitsu Kumada · Goro Watanabe**



**Abstract**
*Background* Unexpected early cancer-related death (ECRD) within 2 years due to recurrence after curative hepatectomy for solitary small (<5 cm) hepatocellular carcinoma without macroscopic vascular invasion (SSHCC) is occasionally observed.
*Method* A total of 415 patients were enrolled (19 patients with ECRD and 396 patients with non-ECRD) to elucidate the risk factors of ECRD after curative hepatectomy for SSHCC. They were initially compared by limiting variables to preoperative factors to reveal predictors that could enable the modification of primary treatment. Subsequently, the same analysis was performed with all variables, including perioperative and histological factors.
*Results* In the preoperative factors, tumor size > 3 cm and elevation of tumor marker level were independent predictors of ECRD. In the analysis with all variables, excessive intraoperative blood loss, poor differentiation, and microscopic vascular invasion were predictors of ECRD. In the recurrence patterns, 79% of ECRD presented as advanced (four or more lesions) or extra-hepatic recurrence, whereas these accounted for 18% in the non-ECRD.
*Conclusion* Excessive blood loss during the operation and histopathological findings of microscopic vascular invasion and poor differentiation are predictive factors of cancer-related death within 2 years of a hepatectomy for SSHCC.

**Keywords** Early cancer-related death · Hepatectomy · Hepatocellular carcinoma

K. Sasaki (✉) · M. Matsuda · Y. Ohkura · M. Hashimoto · G. Watanabe
Department of Digestive Surgery, Hepato Pancreato Biliary Surgery Unit, Toranomon Hospital, Tokyo, Japan
e-mail: sasaki-kz@toranomon.gr.jp

Y. Kawamura · K. Ikeda · H. Kumada
Department of Hepatology, Toranomon Hospital, Tokyo, Japan

M. Inoue
Department of Pathology, Toranomon Hospital, Tokyo, Japan

## Introduction

Hepatectomy for hepatocellular carcinoma (HCC) is considered to be the most reliable treatment and a potential curative treatment; nevertheless, HCC recurrence is observed in up to 70–80% of patients within 5 years of curative resection [1, 2]. The 5-year overall survival rates after curative resection are 50% or more in high-volume centers. However, approximately 20% of patients experience cancer-related death within 2 years after resection [3–5].

A solitary HCC smaller than 5 cm and without macroscopic vascular invasion (SSHCC) in preserved liver function patients is considered the best candidate for hepatectomy in many treatment algorithms, and SSHCC has been reported to have a better prognosis [6–10].

To date, there have been several studies regarding predictors of early cancer-related death (ECRD) among subjects, including patients with multiple HCC and/or macroscopic vascular invasion; however, the predictors of ECRD in SSHCC are still unclear. Although ECRD after curative hepatectomy for SSHCC is uncommon, it has significant clinical relevance because the percentage of these patients has been increasing with advances in imaging modalities and the establishment of screening programs for high-risk patients [6, 7]. This study aimed to reveal the predictors of ECRD in SSHCC to clarify which patients require treatment modification and additional treatment.

## Methods

From June 1994 to December 2010, 528 patients received a primary curative hepatectomy (R0 or R1 resection). Of those patients, 425 with a pathologically confirmed solitary HCC smaller than 5 cm that did not have macroscopic tumor invasion were included. The clinical findings, including gender, age, hepatoviral infection status, liver function status, pre-operative tumor marker levels, tumor marker elevation pattern, maximal tumor diameter on preoperative imaging examination, intraoperative blood loss, presence of intra-operative red blood cell transfusion, type of resection, complications, histopathologic results, and prognosis, were retrospectively collected. The histological variables were defined according to the General Rule for the Clinical and Pathological Study of Primary Liver Cancer by the Liver Cancer Study Group of Japan [11]. The macroscopic appearance of the HCC was divided into two groups according to classification by the aforementioned criteria; one was the boundary type, which included the vaguely nodular type and single nodular type, and the other was the non-boundary type, which included the single nodular type with extranodular growth, the confluent multinodular type, and the invasive type. An elevation in the levels of both tumor markers was defined as patients with an alpha-fetoprotein (AFP) > 20 ng/ml and a des-gamma-carboxyprothrombin (DCP) > 40 AU/L simultaneously. Because the current analysis was focused on the oncologic outcome after hepatectomy for HCC, we excluded 10 patients from the current study for the following reasons: four patients with hospital deaths related to the hepatectomy (mortality rate of 1.0%), two patients who died from causes unrelated to liver disease within 2 years, and four patients who were lost to follow-up within 2 years of the primary operation. Eventually, 415 patients with SSHCC were included in the analysis and followed until death or at least 2 years after the primary operation. The median follow-up period for the patients was 75.7 (6.4–218.4) months. None of these patients received adjuvant chemotherapy or radiation treatment.

The 415 patients were divided into two groups: 19 patients with cancer-related death within 2 years of the operation (ECRD) and 396 patients who survived more than 2 years (non-early cancer-related death; NECD). These two groups were compared with respect to preoperative, operative, and histopathological factors and recurrence patterns to reveal the predictors of ECRD after curative hepatectomy for SSHCC. The two groups were first compared by limiting the variables to preoperative factors to reveal predictors that could enable the modification of primary treatment. Subsequently, the same analysis was performed with all variables, including preoperative, operative, and histological factors, to reveal perioperative predictors of ECRD.

The patients were followed-up via tumor marker analysis every month, ultrasonography every 3 months, and dynamic computed tomography (CT) scan or magnetic resonance imaging (MRI) every 6 months during the first 2 years after the operation. Subsequently, the follow-up period was determined according to the likelihood of recurrence. The number of recurrent tumors was determined with dynamic CT/MRI and/or CT angiography. Extra-hepatic recurrence was detected with CT, MRI, and scintigraphy. The site and pattern of the initial recurrence were defined as follows: (1) solitary or oligonodular (two or three tumor nodules) recurrence; (2) advanced recurrence, defined as recurrence with four or more lesions, portal vein invasion, or both; and (3) recurrence at an extra-hepatic site regardless of simultaneous intra-hepatic recurrence. Treatment for the initial recurrence was determined according to the recurrence type and hepatic function reserve. A second curative resection and local ablation therapy were actively indicated for solitary and oligonodular recurrences, including extra-hepatic recurrence considered to be controlled, as reported previously [12]. Trans-arterial chemoembolization (TACE) and trans-arterial chemoinfusion were indicated for patients who were not indicated for curative treatment.

The data were analyzed with SPSS software, ver. 19 (SPSS, Chicago, IL, USA).

All clinical and pathological features depicted in Table 1 were selected for potential relation to ECRD based on previous studies or our own clinical experience [13–19].

They were categorized as continuous or categorical variables. Continuous variables were summarized as medians and ranges. Categorical variables were summarized as frequencies and percentages. Categorical data were analyzed using a $\chi^2$ test or Fisher's exact test as appropriate. A Mann–Whitney *U*-test was used to compare continuous variables between the two groups. Differences in the clinicopathological variables between the two groups were identified with multivariate logistic regression analysis. In the multivariate analysis including prognostic factors, all factors that were $P < 0.15$ in univariate analysis were included. A two-tailed *P*-value of $< 0.05$ was considered statistically significant.

This study was approved by the Human Ethics Review Committee of Toranomon Hospital.

## Results

The patient characteristics, treatments and histopathological characteristics of the ECRD and NECD groups are shown in Table 1. The results of the univariate analysis of the two groups are also shown in Table 1. The results of the multivariate analysis of the differences in preoperative factors between the two groups are shown in

**Table 1** Comparison of the patients with early cancer-related deaths and non-early deaths

| | ECRD ($n = 19$) | NECD ($n = 396$) | $P$ |
|---|---|---|---|
| Preoperative characteristics | | | |
| Gender (male/female) | 16/3 | 296/100 | 0.43 |
| Age (years) | 64 (28–79) | 62 (30–87) | 0.93 |
| >65 years (yes/no) | 9/10 | 157/239 | 0.63 |
| HBV infection (yes/no) | 8/11 | 121/275 | 0.31 |
| HCV infection (yes/no) | 11/8 | 233/163 | 1.00 |
| Platelet count (μL) (>$10^5$/<$10^5$) | 4/15 | 125/271 | 0.45 |
| ICG > 30 % (yes/no) | 5/14 | 73/323 | 0.37 |
| Child–Pugh grade (A/B) | 17/2 | 355/41 | 1.00 |
| Elevation in the levels of both tumor markers[a,b] (yes/no) | 4/15 | 48/341 | <0.01 |
| AFP > 400 ng/ml (yes/no) | 4/15 | 42/354 | 0.15 |
| DCP > 100 AU/L (yes/no)[b] | 5/14 | 72/317 | 0.38 |
| Tumor size (cm) | 2.9 (1.3–5.0) | 2.1 (0.6–5.0) | <0.01 |
| >3 cm (yes/no) | 9/10 | 74/322 | <0.01 |
| Operative characteristics | | | |
| Anatomical resection (yes/no) | 3/16 | 76/320 | 1.00 |
| Intraoperative blood loss (ml) | 368 (20–2268) | 195 (5–5367) | <0.01 |
| >1,000 ml (yes/no) | 6/13 | 10/386 | <0.01 |
| Intraoperative RBC transfusion (yes/no) | 5/14 | 12/384 | <0.01 |
| Histopathological characteristics | | | |
| Macroscopic tumor appearance (boundary/non-boundary) | 6/13 | 248/148 | <0.01 |
| Tumor differentiation grade (poorly/non-poorly) | 11/8 | 67/329 | <0.01 |
| Capsule formation (yes/no) | 16/3 | 286/110 | 0.30 |
| Vascular invasion (yes/no) | 10/9 | 80/316 | <0.01 |
| Surgical margin (R0/R1) | 16/3 | 345/51 | 0.73 |
| Liver cirrhosis (yes/no) | 14/5 | 221/175 | 0.16 |

*AFP* alpha-fetoprotein, *DCP* des-gamma-carboxyprothrombin, *ECD* early cancer-related death, *HBV* hepatitis B virus, *HCV* hepatitis C virus, *ICG15* indocyanine green 15-minute retention rate, *NED* non-early death, *RBC* red blood cell

[a] AFP > 20 ng/ml and DCP > 40 AU/L simultaneously

[b] DCP was not measured in seven patients

Table 2. In the multivariate analysis limited to preoperative factors, tumor size > 3 cm and an elevation in the levels of both tumor markers were independent preoperative predictors of ECRD. The results of the multivariate analysis including preoperative, operative, and histopathological variables are also shown in Table 2. Intraoperative blood loss > 1000 ml, poor histological differentiation grade, and microscopic vascular invasion were significantly frequent in the ECRD group. The preoperative factors tumor size and elevation in the levels of both tumor markers were no longer significant in the multivariate model with all variables.

The recurrence patterns and secondary treatments are summarized in Table 3. Advanced recurrence and extra-hepatic recurrence were more prevalent in the ECRD group and mostly occurred within one year of the primary operation. Advanced recurrence and extra-hepatic recurrence accounted for 58% and 21% of the recurrences in the ECRD group, respectively, but only accounted for 16% and 2% in the NECD group, respectively. The median duration between the primary operation and initial recurrence in the ECRD and NECD groups was 7.3 and 26.0 months, respectively ($P < 0.01$). In the secondary treatment after recurrence, only 16% of the ECRD group received a potential curative treatment, such as a second hepatectomy or local ablation therapy, whereas more than half of the NECD group received potentially curative secondary treatment. Other secondary treatments included two cases of radiation therapy and one case of no treatment due to massive tumor progression in the ECRD group and two cases of radiation therapy, two cases of systemic chemotherapy, and eight cases of treatment refusal in the NECD group.

## Discussion

The current study examined the risk factors of cancer-related death within 2 years after curative hepatectomy for

**Table 2** Comparison of the clinicopathological characteristics of the patients with early cancer-related death (ECRD) and non-early cancer-related death (NECD)

| | Multivariate analysis | | | | |
|---|---|---|---|---|---|
| | ECRD | NECD | P | HR | 95% CI |
| a) Analysis limited to preoperative factors | | | | | |
| Elevation in the levels of both tumor markers[a,b] (yes/no) | 7/12 | 48/341 | ***0.02*** | ***3.38*** | ***1.23–9.25*** |
| AFP > 400 ng/ml (yes/no) | 4/15 | 42/354 | 0.96 | 0.97 | 0.26–3.61 |
| Tumor size > 3 cm (yes/no) | 9/10 | 74/322 | ***0.02*** | ***3.28*** | ***1.26–8.57*** |
| b) Analysis with all perioperative factors | | | | | |
| Elevation in the levels of both tumor markers[a,b] (yes/no) | 7/12 | 48/341 | 0.07 | 2.77 | 0.91–8.40 |
| AFP > 400 ng/ml (yes/no) | 4/15 | 42/354 | 0.97 | 1.03 | 0.25–4.28 |
| Tumor size > 3 cm (yes/no) | 9/10 | 74/322 | 0.19 | 2.06 | 0.70–6.08 |
| Intraoperative blood loss > 1000 ml (yes/no) | 6/13 | 10/386 | ***<0.01*** | ***11.84*** | ***3.34–41.93*** |
| Intraoperative RBC transfusion (yes/no) | 5/14 | 12/384 | 0.34 | 2.32 | 0.41–13.06 |
| Tumor differentiation grade (poorly/non-poorly) | 11/8 | 67/329 | ***<0.01*** | ***4.63*** | ***1.67–12.88*** |
| Macroscopic tumor appearance (boundary/non-boundary) | 6/13 | 248/148 | 0.20 | 2.05 | 0.69–6.08 |
| Vascular invasion (yes/no) | 10/9 | 80/316 | ***0.045*** | ***2.86*** | ***1.03–7.96*** |

*CI* confidence interval, *HR* hazard ratio

[a] Alpha-fetoprotein (AFP) > 20 ng/ml and des-gamma-carboxyprothrombin > 40 AU/L simultaneously

[b] Des-gamma-carboxyprothrombin was not measured in seven patients

**Table 3** Comparison of recurrence patterns and secondary treatment types

| | ECRD ($n$ = 19) | NECD ($n$ = 396) | $P$ |
|---|---|---|---|
| Total recurrence (%) | 19 (100%) | 236 (60%) | |
| Initial recurrence pattern | | | <0.01 |
| Solitary or oligonodular | 4 (21%) | 193 (82%) | |
| Advanced[a] | 11 (58%) | 38 (16%) | |
| Extrahepatic | 4 (21%) | 5 (2%) | |
| Initial recurrence pattern and duration until recurrence | | | |
| Recurrence within one year from operation | 17 (89%) | 50 (21%) | <0.01 |
| Advanced recurrence within one year | 10 (53%) | 14 (6%) | <0.01 |
| Extra-hepatic recurrence within one year | 4 (21%) | 2 (1%) | <0.01 |
| Second treatment type | | | <0.01 |
| Second hepatectomy | 1 (5%) | 36 (15%) | |
| Local ablation therapy[b] | 2 (11%) | 101 (43%) | |
| TACE | 13 (68%) | 87 (37%) | |
| Others | 3 (16%) | 12 (5%) | |

*ECRD* early cancer-related death, *NECD* non-early cancer-related death, *TACE* trans-arterial chemo embolization

[a] Advanced recurrence was defined as recurrence with four or more lesion, portal vein invasion, or both

[b] Including local ablation therapy with TACE

SSHCC so that future studies can focus on providing better therapies to this group of patients. In the statistical analysis limited to preoperative factors, tumor size larger than 3 cm and elevation of the levels of both tumor markers were revealed as independent prognostic factors of ECRD. In the analysis using all variables, including perioperative, operative and histopathological factors, none of the preoperative factors were predictors of ECRD. However, one operative factor (excessive intraoperative blood loss) and two histopathological factors (poorly differentiated HCC and the presence of vascular invasion) were found to be predictors of ECRD.

This study focused on SSHCC, which has good survival and a lower recurrence rate. However, even when curative

resection was performed for SSHCC, some patients developed early postoperative recurrence and experienced subsequent cancer-related death. The occurrence of ECRD in patients with SSHCC is relatively uncommon compared to those with large tumors, multiple tumors and/or macroscopic vascular invasion, while the prevalence of hepatectomy for SSHCC has been increasing due to medical innovations. In fact, 72.8% of patients who underwent curative resection had an HCC smaller than 5 cm, and 73.9% of them had a solitary HCC in a nationwide Japanese report including 13772 patients [7]. Therefore, the occurrence of ECRD in SSHCC is uncommon, but the total number of patients is not small.

Although the recurrence of HCC is determined by both recurrence of the resected tumor and multicentric carcinogenesis due to the damaged liver, the resected tumor is considered the main cause of recurrence in the early period after the operation. The recurrence patterns in this study revealed that advanced recurrence and extra-hepatic recurrence were more frequent in the ECRD group, supporting the hypothesis that the aggressive recurrence of the resected tumor is the main cause of ECRD. Therefore, the prognosis of potential ECRD patients may be improved with perioperative treatment modification or additional treatments to effectively control the resected tumor. The main objective of this study was to identify patients who required treatment modification and additional treatments as quickly as possible.

To predict potential ECRD, identifying important preoperative factors would provide the most useful information because it would permit the early customization of the treatment strategy to potentially have the best impact on prognosis. Therefore, we initially performed a statistical analysis limited to preoperative factors and found that tumor size larger than 3 cm and elevation in the levels of both tumor markers were preoperative predictors of ECRD. However, in the multivariate analysis using all factors, those preoperative factors were not significant. The tumor size and elevation in the levels of both tumor markers were considered to be confounded by microscopic vascular invasion and/or poor differentiation in the multivariate analysis. HCC develops in a multistep fashion, and the frequencies of poor differentiation grades and vascular invasion increase as the tumor size increases [20, 21]. Similarly, the elevation in the levels of both tumor markers was previously reported to be an indicator of advanced recurrence, microscopic vascular invasion and poor histological differentiation [13, 22]. Considering the abovementioned observations, the confounding effect among these variables appears to be rational.

We found that poor histological differentiation and the presence of microscopic vascular invasion were significant predictors of ECRD in SSHCC. Those factors have been reported to be predictors of early recurrence and ECRD in previous reports, although the backgrounds of the included patients were different in each of the studies [13–19]. Furthermore, Endo et al. previously reported that reduced E-cadherin expression was correlated with poorly differentiated HCC and that E-cadherin underexpression may reduce the adhesiveness of HCC cells, thereby potentiating invasion and metastasis. Because early cancer-related death appears to be related to the malignancy of the resected tumor, factors that indicate cancer cell invasion into the peripheral circulating blood are reasonable predictors of ECRD [23].

Interestingly, operative blood loss was the most powerful predictor of ECRD in this study. Although excessive blood loss during the operation was known to be an important predictor of the postoperative prognosis, the relationship between excessive blood loss and poor oncologic outcomes has not been clearly identified [24]. In a previous study by Katz et al., the potential reasons for the correlation between excessive blood loss and poor prognosis included tumor spillage and hematogenous spread during the operation, hypoperfusion and impaired oxygen delivery to vital organs, and the introduction of some cytokines by hemorrhagic shock [24].

In addition to the abovementioned possible explanations, we suggest that excessive blood loss is potentially correlated with tumor location, such as near the hepatic hilar and/or major vasculature, poor background liver function, including hemostatic functions, and an insufficient operation due to the massive bleeding. In any case, the surgeon should avoid excessive blood loss for not only operative safety but also oncological benefits.

Because nearly 80% of the initial recurrence in the ECRD group presented as advanced or extra-hepatic recurrence, we propose a correlation between ECRD and tumor dissemination from the resected tumor, although whether these sources of recurrence already existed at the time of operation or subsequently is uncertain. The systemic or hepatic artery infusion of neo-adjuvant and adjuvant chemotherapy appears to be a hopeful treatment for those patients. However, there is currently insufficient evidence to show that those therapies increase the survival rate of HCC, although there is limited evidence suggesting that those therapies may be useful for disease-free survival [25].

Our study had a possible limitation. Because the statistical analyses were performed with only 19 patients who had an event, our findings must be confirmed in a larger prospective study. As mentioned above, the occurrence of ECRD in SSHCC patients is not large, but the detection of those patients and improvement of the subsequent treatment approaches would enable further understanding of tumor control.

In conclusion, excessive blood loss during the operation and histopathological findings of microscopic vascular invasion and poor differentiation are predictive factors of cancer-related death within 2 years of a hepatectomy for SSHCC. The predictors revealed in this study and recurrence patterns of the ECRD group suggest a correlation between ECRD and tumor spillage and/or dissemination from the resected tumor. Reducing the intraoperative blood loss and additional perioperative treatments targeting disseminated tumors are expected to prevent ECRD.

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**Conflict of interest** None declared.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

# Seroclearance rate of hepatitis B surface antigen in 2,112 patients with chronic hepatitis in Japan during long-term follow-up

**Mariko Kobayashi · Tetsuya Hosaka · Fumitaka Suzuki · Norio Akuta · Hitomi Sezaki · Yoshiyuki Suzuki · Yusuke Kawamura · Masahiro Kobayashi · Satoshi Saitoh · Yasuji Arase · Kenji Ikeda · Yuzo Miyakawa · Hiromitsu Kumada**

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**Abstract**
*Background* Rate of hepatitis B surface antigen (HBsAg) seroclearance was determined in 2,112 Japanese patients with chronic hepatitis B who were followed up for at least 15 years.
*Methods* Patients had a median age of 37 years and included 1,431 (67.8 %) men. Median values were AST/ALT, 43/62 IU/L; platelet counts, 182 × $10^3$/$mm^3$; HBsAg, 3,400 IU/mL; and hepatitis B virus (HBV) DNA, 6.2 log copies/mL. Factors influencing HBsAg seroclearance were evaluated by the Cox proportional model and annual rate of HBsAg seroclearance by the Kaplan–Meier life table method.
*Results* The overall annual rate of HBsAg seroclearance was 1.75 % in 2,112 patients; it was 1.65 % in 1,130 untreated and 2.05 % in 982 treated patients ($p = 0.289$). In untreated patients, seroclearance was influenced by age, no HBV infections in third-degree or closer relatives, and HBsAg levels in univariate analysis. Seroclearance was influenced by a median age ≥50 years [relative risk (RR) 1.61 ($p = 0.018$)] and HBsAg ≤2,000 IU/mL [RR 1.77 ($p = 0.014$)] in multivariate analysis. In treated patients, age, male gender, no HBV infections in third-degree or closer relatives, interferon therapy, chronic hepatitis, high AST and γ-GTP levels, low platelet counts, hepatitis B e antigen (HBeAg)-negative status, low HBsAg levels and the wild-type precore sequence significantly influenced HBsAg seroclearance. In multivariate analysis, no family history [RR 2.22 ($p = 0.006$)], interferon treatment [RR 3.15 ($p < 0.001$)], and HBeAg-negative status [RR 3.75 ($p < 0.001$)] significantly influenced HBsAg seroclearance.
*Conclusions* In this retrospective cohort study, the annual rate of HBsAg seroclearance was 1.65 % in untreated patients and 2.05 % in treated patients.

**Keywords** Seroclearance · Hepatitis B surface antigen · Hepatitis B virus · Chronic hepatitis B

**Abbreviations**

| | |
|---|---|
| ALT | Alanine aminotransferase |
| AST | Aspartate aminotransferase |
| ETV | Entecavir |
| HBeAg | Hepatitis B e antigen |
| HBcrAg | Hepatitis B core-related antigen |
| HBV | Hepatitis B virus |
| HBV DNA | Hepatitis B virus DNA |
| HBsAg | Hepatitis B surface antigen |
| IFN | Interferon |
| LAM | Lamivudine |

## Introduction

Worldwide, an estimated 400 million people are infected with hepatitis B virus (HBV) persistently. HBV infection is a common disease that can induce a chronic carrier state

Mariko Kobayashi (✉)
Research Institute for Hepatology, Toranomon Hospital, 1-3-1 Kajigaya, Takatsu-ku, Kawasaki 213-8587, Japan
e-mail: vj7m-kbys@asahi-net.or.jp

T. Hosaka · F. Suzuki · N. Akuta · H. Sezaki · Y. Suzuki · Y. Kawamura · Masahiro Kobayashi · S. Saitoh · Y. Arase · K. Ikeda · H. Kumada
Department of Hepatology, Toranomon Hospital, 1-3-1 Kajigaya, Takatsu-ku, Kawasaki 213-8587, Japan

Y. Miyakawa
Miyakawa Memorial Research Foundation, Tokyo 107-0062, Japan

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and is associated with the risk of developing progressive disease and hepatocellular carcinoma (HCC) [1–5]. In regions highly endemic for HBV, such as Asia and Africa, the persistent carrier state is established by perinatal transmission or early in infancy. Carriers serve as the reservoir of HBV in the community and can spread the infection to susceptible individuals. The incidence of HCC is decreased extremely by eradicating HBV from the circulation that is responsible for liver damage [6–9]. In Japan, interferon (IFN) was introduced for the treatment of persistent HBV infections, and long-term IFN increased seroclearance of hepatitis B surface antigen (HBsAg) [10]. Since 2000, the effect of long-term nucleot(s)ide analogues, such as lamivudine [11, 12] and entecavir [13], on HBsAg seroclearance has been monitored in Japan.

In the current study, we followed untreated or treated patients for at least 15 years. We evaluated the seroclearance of HBsAg, achieved in both groups of patients, by using highly sensitive assays. Our aim was to determine factors that can lead to HBsAg seroclearance and to elucidate the factors associated with its success.

## Patients and methods

### Patients

During at least 15 years from 1968, 2,112 consecutive patients, chronically mono-infected with HBV (confirmed by HBsAg-positivity for at least 6 months) were followed at the Department of Hepatology, Toranomon Hospital, in Metropolitan Tokyo. Patients met the following inclusion and exclusion criteria: (1) negativity for hepatitis C antibody and/or hepatitis C virus RNA by polymerase chain reaction (PCR) in the serum; (2) no history of HCC; and (3) no history of autoimmune hepatitis, alcohol liver disease, hemochromatosis, or chronic liver disease other than chronic hepatitis B. Thus, the 2,112 patients were enrolled in this cohort study. A written informed consent was obtained from each patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved a priori by the institution's human research committee.

### Treatment

Nine hundred and eighty-two patients received antiviral treatments. Of them, 156 patients received prednisolone (PSL) 40 mg daily for 1 week, 30 mg daily for 1 week, 20 mg daily for 1 week, and then 10 mg daily for 1 week until it was abruptly withdrawn (total 700 mg). A total of 428 patients received 100 mg lamivudine (LAM) daily as an initial therapy. In total, 333 patients received 3–12 MU of IFN-α or IFN-β. The durations and regimens of treatment were as follows: daily for 2 or 4 weeks and then 2 or 3 times per week for 26–104 weeks. The median duration of treatment was 26 weeks (range 4–981). There were 190 (57 %) patients who received multiple treatments of IFN.

LAM treatment was continued as a rule; median duration of LAM treatment was 75 months (55–102). LAM-resistant rtM204I/V mutants developed in 151 (35 %) of the 428 patients, and they were provided with adefovir dipivoxil (10 mg) added on LAM, as a rescue therapy. The remaining patients continued to receive LAM monotherapy. In addition, 65 patients received 0.5 mg entecavir (ETV) daily as an initial therapy. ETV treatment was continued as a rule, and median duration of ETV treatment was 45 months (1.0–104).

### Markers of HBV infection

Serum HBsAg titers were determined annually using ARCHITECT HBsAg QT assay kits (Abbott Laboratories, Tokyo, Japan), which have a lower limit of detection of 0.05 IU/mL and an upper limit of detection of 250 IU/mL. To expand the upper limit from 250 to 125,000 IU/mL, serum samples going off the scale were diluted stepwise to 1:20 and 1:500 with ARCHITECT diluents following instructions from the manufacturer.

Hepatitis B e antigen (HBeAg) was determined by enzyme-linked immunosorbent assay with a commercial kit (HBeAg EIA; Institute of Immunology, Tokyo, Japan). HBV DNA was quantified using the Amplicor monitor assay (Roche Diagnostics, Tokyo, Japan) with a dynamic range of 2.6–7.6 log copies/mL, or COBAS TaqMan HBV v.2.0 (Roche Diagnostics, Tokyo, Japan) with a dynamic range of 2.1–9.0 log copies/mL. Hepatitis B core-related antigen (HBcrAg) was determined by chemiluminescence enzyme immunoassay (CLEIA) with the HBcrAg assay kit (Fujirebio Inc., Tokyo, Japan). A commercial kit (HBV Genotype EIA; Institute of Immunology, Tokyo, Japan) was used to serologically determine HBV genotypes by the combination of epitopes expressed on the pre-S2 region product, which is specific for each of the 7 major genotypes (A–G).

### Statistical analysis

Baseline data were obtained on the day of the first visit in untreated patients. In patients who received antivirals, baseline data were obtained at the start of the first day of treatment. Categorical data were compared between groups by chi-squared or Fisher's exact tests. Continuous variables with a nonparametric distribution were analyzed by Mann–Whitney $U$ tests, whereas those with a parametric distribution were analyzed by the Student's $t$ test. Cox

regression analyses were used to assess variables that were significantly associated with HBsAg seroclearance. All baseline factors that were found to be significantly associated with HBsAg seroclearance by univariate analysis were entered into a multivariate analysis. Independent baseline factors associated with the seroclearance of HBsAg were evaluated using a stepwise Cox regression analysis. We then performed a time-dependent Cox regression to analyze independent factors associated with HBsAg seroclearance while on-treatment factors and independent baseline factors had been adjusted.

Cumulative HBsAg seroclearance rates were analyzed using the Kaplan–Meier method; differences in the resulting curves were evaluated using log-rank tests. Significance was defined as $p < 0.05$ for all two-tailed tests. Data analysis was performed with the SPSS software package version 11.0.1 J (SPSS Inc., Chicago, IL, USA).

## Results

### Baseline characteristics in the 2,112 patients

The baseline characteristics of studied patients are shown in Table 1. They had a median age of 37 years (range 1–81), included 1,431 (67.8 %) men, and 2,031 (96.2 %) of them had chronic hepatitis. Their baseline values were AST/ALT, 43 (3–2,192)/62 (2–3,020 IU/L); γ-GTP, 27 (4–1,494) IU/L; platelet counts, 182 (40–483) × $10^3$/$mm^3$; and HBV markers were HBsAg, 3,400 (0.06–27,700) IU/mL; and HBV DNA, 6.2 (<2.1 to >9.1) log copies/mL. HBeAg was not detectable in 5.4 % of studied patients, and the distribution of genotypes A/B/C/others was 4.5:15.6:79.6:0.3 %.

The HBsAg seroclearance rate analyzed by the Kaplan–Meier method was 9 % in 5 years, 17 % in 10 years, 27 % in 15 years, 35 % in 20 years, 44 % in 25 years, and 54 % in 30 years. The annual rate of HBsAg seroclearance was 1.75 % during 20 years (Fig. 1).

In the 2,112 patients, factors influencing HBsAg seroclearance in univariate analysis by the Cox regression analyses were cirrhosis [relative risk (RR) 2.40 ($p = 0.014$)]; HBeAg negative [RR 3.01 ($p = 0.001$)]; and HBsAg ≤2,000 IU/mL [RR 2.13 ($p = 0.004$)]. In multivariate analyses, only 2 factors contributed to HBsAg seroclearance: HBeAg negative [RR 1.81 ($p < 0.001$)]; and HBsAg ≤2,000 IU/mL [RR 2.60 ($p < 0.001$)] (Table 2).

### Untreated patients and treated patients

Differences in the baseline characteristics between 1,130 untreated and 982 treated patients are shown in Table 3: age [31 years vs. 36 ($p < 0.001$)]; male gender [62.4 vs. 71.9 % ($p < 0.001$)]; AST [median 27 vs. 56 IU/L ($p < 0.001$)]; ALT [median 28 vs. 96 IU/L ($p < 0.001$)]; γ-GTP [median 20 vs. 45 IU/L ($p < 0.001$)]; total bilirubin

**Table 1** Baseline characteristics 2,112 patients infected with HBV followed for longer than 15 years

| Features at the baseline | Patients ($n = 2,112$) |
|---|---|
| Demographic data | |
| Age (years) | 37 (1–81) |
| Men | 1,431 (67.8 %) |
| Liver disease | |
| Chronic hepatitis | 2,031 (96.2 %) |
| Cirrhosis | 81 (3.8 %) |
| Laboratory data | |
| AST (IU/L) | 43 (3–2,192) |
| ALT (IU/L) | 62 (2–3,020) |
| γ-GTP (IU/L) | 27 (4–1,494) |
| Total bilirubin (mg/dL) | 0.7 (0.1–21.2) |
| Albumin (g/dL) | 4.3 (1.1–5.8) |
| Platelets (×$10^3$/$mm^3$) | 182 (40–483) |
| α-Fetoprotein (μg/L) | 4 (1–2,060) |
| HBV markers | |
| HBeAg-negative status | 1,169 (55.4 %) |
| HBsAg (IU/mL) | 3,400 (0.06–277,000) |
| HBcrAg (log U/mL) | 5.4 (<3.0 to >6.8) |
| Genotypes (A/B/C/others) | 4.5 %/15.6 %/79.6 %/0.3 % |
| HBV DNA (log copies/mL) | 6.2 (<2.1 to >9.1) |

Median values with the range in parentheses or numbers with the percentage in parentheses are given

*HBV* hepatitis B virus, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *γ-GTP* γ-guanosine triphosphate, *HBeAg* hepatitis B e antigen, *HBsAg* hepatitis B surface antigen, *HBcrAg* hepatitis B core-related antigen

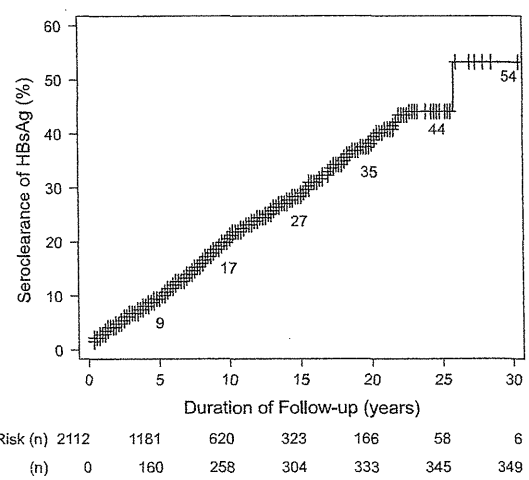


**Fig. 1** Seroclearance of HBsAg in the 2,112 patients studied. Numbers of patients at risk and those of patients who lost HBsAg are indicated below each time point

**Table 2** Factors influencing the seroclearance of HBsAg in 2,112 patients evaluated by time-dependent uni- and multivariate analyses

| Factors | Univariate analysis HBsAg clearance Relative risk (95 % CI) | *p* value | Multivariate analysis HBsAg clearance Relative risk (95 % CI) | *p* value |
|---|---|---|---|---|
| Age ≥50 years | 1.06 (0.64–1.76) | 0.824 | | |
| Male gender | 1.15 (0.69–1.90) | 0.594 | | |
| No HBV infection in family | 1.55 (0.93–2.57) | 0.092 | | |
| Treatment | 1.26 (0.72–2.19) | 0.413 | | |
| Cirrhosis | 2.40 (1.20–4.83) | 0.014 | | |
| AST ≥50 IU/L | 1.30 (0.66–2.57) | 0.454 | | |
| ALT ≥50 IU/L | 1.81 (0.89–3.70) | 0.104 | | |
| γ-GTP ≥20 IU/L | 1.26 (0.72–2.23) | 0.418 | | |
| Total bilirubin ≥1 mg/dL | 1.39 (0.69–2.79) | 0.358 | | |
| Albumin ≥4 g/dL | 1.03 (0.58–1.81) | 0.927 | | |
| Platelets >150 × $10^3$/mm$^3$ | 1.22 (0.68–2.18) | 0.501 | | |
| α-Fetoprotein ≤10 μg/L | 1.06 (0.59–1.89) | 0.845 | | |
| Genotype A or B, C | 1.55 (0.86–2.76) | 0.142 | | |
| HBeAg-negative status | 3.01 (0.79–2.07) | 0.001 | 1.81 (1.30–2.77) | <0.001 |
| HBV DNA ≥5 log copies/mL | 1.17 (0.64–2.15) | 0.612 | | |
| HBsAg ≤2,000 IU/mL | 2.13 (1.27–3.56) | 0.004 | 2.60 (1.94–3.50) | <0.001 |
| HBcrAg ≥4 log U/mL | 1.11 (0.61–2.03) | 0.731 | | |
| Wild-type precore sequence | 0.98 (0.59–1.53) | 0.964 | | |
| Wild-type core promoter sequence | 2.74 (0.80–9.30) | 0.104 | | |

Wild-type precore sequence, G1896; wild-type core promoter sequence, A1762/G1764

*AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *γ-GTP* γ-guanosine triphosphate, *HBeAg* hepatitis B e antigen, *HBsAg* hepatitis B surface antigen, *HBcrAg* hepatitis B core-related antigen

[median 0.5 vs. 0.7 mg/dL ($p < 0.001$)]; albumin [median 4.4 vs. 4.3 g/dL ($p < 0.001$)]; platelets [median 202 vs. 181 × $10^3$/mm$^3$ ($p < 0.001$)]; α-fetoprotein [median 4 vs. 4 μg/L ($p < 0.001$)]; HBeAg-negative status [75.8 vs. 31.8 % ($p < 0.001$)]; HBsAg levels [median 2,240 vs. 5,270 IU/mL ($p < 0.001$)]; HBcrAg [median 3.6 vs. >6.8 log U/mL ($p < 0.001$)]; distribution of genotypes A/B/C/others (5.7/20.0/72.6/1.7 vs. 3.4/11.1/84.9/0.5 %, $p < 0.001$); and HBV DNA [median 4.7 vs. 8.0 log copies/mL ($p < 0.001$)].

The rate of HBsAg seroclearance in treated patients was 8 % in 5 years, 20 % in 10 years, 28 % in 15 years, 41 % in 20 years, 49 % in 25 years, and 49 % in 30 years, with an annual HBsAg seroclearance rate of 2.05 % (Fig. 2). The rate in untreated patients was 9 % in 5 years, 18 % in 10 years, 26 % in 15 years, 33 % in 20 years, 42 % in 25 years, and 56 % in 30 years, with an annual HBsAg seroclearance rate of 1.65 %. No differences in the annual HBsAg seroclearance rate were noted between treated and untreated patients ($p = 0.289$).

## HBsAg seroclearance in untreated patients

In the 1,130 untreated patients, HBsAg persisted in 930 (82.3 %), whereas HBsAg seroclearance occurred in 200 (17.7 %). In the baseline characteristics, significant differences were found for age ($p < 0.001$), male gender ($p = 0.003$), chronic hepatitis ($p = 0.020$), γ-GTP ($p < 0.001$), albumin ($p = 0.004$), HBV genotypes ($p < 0.001$), HBeAg-negative status ($p < 0.001$), HBV DNA ($p < 0.001$), HBsAg level ($p < 0.001$), HBcrAg ($p < 0.001$), precore wild-type ($p < 0.001$), and core promoter wild-type ($p = 0.001$) (Table 4).

## Factors contributing to HBsAg seroclearance in untreated patients

In the 1,130 untreated patients, factors influencing HBsAg seroclearance in univariate analysis by the Cox regression analyses were age ≥50 [RR 1.63 ($p = 0.002$)]; no family history in third-degree or closer relatives [RR 1.38 ($p = 0.037$)]; and HBsAg ≤2,000 IU/mL [RR 1.87 ($p < 0.006$)].

In multivariate analyses, only 2 factors contributed to HBsAg seroclearance: age ≥50 [RR 1.61 ($p = 0.018$)] and HBsAg ≤2,000 IU/mL [RR 1.77 ($p = 0.014$)] (Table 5).

## HBsAg seroclearance in treated patients

In the 982 treated patients, HBsAg persisted in 833 (84.8 %). HBsAg seroclearance occurred in 149 (15.2 %). In the baseline characteristics, significant difference were found for male gender ($p = 0.004$), no family history in third-degree or closer relatives ($p = 0.010$), chronic hepatitis ($p = 0.001$), AST ($p = 0.010$), γ-GTP ($p = 0.023$), platelet counts ($p < 0.001$), HBeAg-negative status

**Table 3** Baseline characteristics in untreated and treated patients

| Features at the baseline | Untreated ($n$ = 1,130) | Treated ($n$ = 982) | Differences $p$ value |
|---|---|---|---|
| Age (years) | 31 (1–81) | 36 (6–75) | <0.001 |
| Men | 705 (62.4 %) | 726 (71.9 %) | <0.001 |
| Chronic hepatitis | 1,094 (96.8 %) | 937 (96.4 %) | 0.079 |
| Cirrhosis | 36 (3.2 %) | 45 (3.6 %) | |
| AST (IU/L) | 27 (3–1,776) | 56 (6–2,192) | <0.001 |
| ALT (IU/L) | 28 (2–3,020) | 96 (8–2,740) | <0.001 |
| γ-GTP (IU/L) | 20 (4–1,494) | 45 (4–1,278) | <0.001 |
| Total bilirubin (mg/dL) | 0.5 (0.1–20.1) | 0.7 (0.2–21.2) | <0.001 |
| Albumin (g/dL) | 4.4 (2.2–5.8) | 4.3 (1.1–5.4) | <0.001 |
| Platelets ($\times 10^3$/mm$^3$) | 202 (40–443) | 181 (40–483) | <0.001 |
| α-Fetoprotein (μg/L) | 4 (1–2,060) | 4 (1–1,610) | <0.001 |
| HBeAg-negative status | 857 (75.8 %) | 312 (31.8 %) | <0.001 |
| HBsAg (IU/mL) | 2,240 (0.06–141,000) | 5,270 (0.09–277,000) | <0.001 |
| HBcrAg (log U/mL) | 3.6 (<3.0 to >6.8) | > 6.8 (<3.0 to >6.8) | <0.001 |
| Genotypes [A/B/C/others (%)] | 5.7/20.0/72.6/1.7 | 3.4/11.1/84.9/0.5 | <0.001 |
| HBV DNA (log copies/mL) | 4.7 (<2.1 to >9.1) | 8.0 (<2.1 to >9.1) | <0.001 |

Median values with the range in parentheses or numbers with the percentage in parentheses are given

*AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *γ-GTP* γ-guanosine triphosphate, *HBeAg* hepatitis B e antigen, *HBsAg* hepatitis B surface antigen, *HBcrAg* hepatitis B core-related antigen

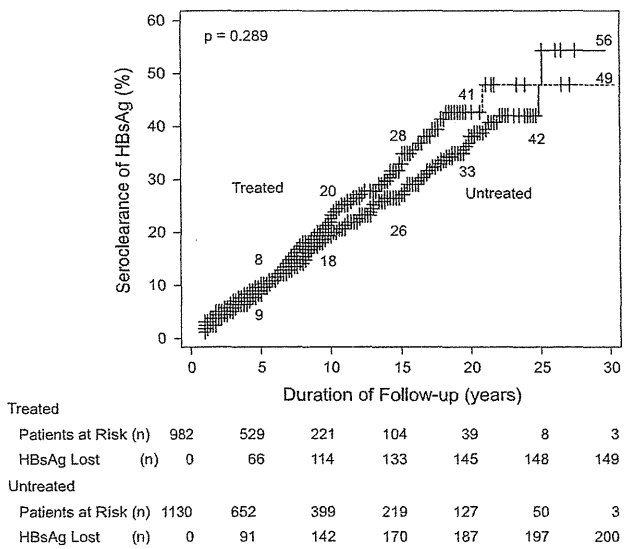


**Fig. 2** Comparison of HBsAg seroclearance rates between 982 treated and 1,130 untreated patients. Numbers of patients at risk and those of patients who lost HBsAg are indicated below each time point

($p < 0.001$), HBV DNA ($p = 0.002$), HBsAg ($p < 0.001$), HBcrAg ($p = 0.003$), and precore wild-type ($p = 0.013$) (Table 6).

### Factors contributing to HBsAg seroclearance in treated patients

In the 982 treated patients, factors influencing HBsAg seroclearance in univariate analysis by the Cox regression analyses were age ≥50 [RR 1.91 ($p = 0.001$)]; male gender [RR 2.14 ($p = 0.001$)], no family history in third-degree or closer relatives [RR 1.58 ($p = 0.005$)]; previous treatment with interferon [RR 2.13 ($p < 0.001$)]; chronic hepatitis [RR 3.12 ($p < 0.001$)]; AST ≥50 IU/L [RR 1.47 ($p = 0.031$)]; γ-GTP ≥20 IU/L [RR 1.87 ($p = 0.001$)]; platelets ≤150 × $10^3$/mm$^3$ [RR 2.10 ($p < 0.001$)]; HBeAg-negative status [RR 2.53 ($p < 0.00$)]; HBV DNA ≤5 log copies/mL [RR 2.07 ($p = 0.001$)]; HBsAg ≤2,000 IU/mL [RR 2.29 ($p < 0.001$)]; HBcrAg ≤4 log U/mL [RR 2.28 ($p = 0.003$)]; and the wild-type precore sequence [RR 2.04 ($p = 0.011$)].

In multivariate analysis, only 3 factors contributed to HBsAg seroclearance: no family history in third-degree or closer relatives [RR 2.22 ($p = 0.006$)]; previous treatments with interferon [RR 3.15 ($p < 0.001$)]; and HBeAg-negative status [RR 3.75 ($p < 0.001$)] (Table 7).

## Discussion

In Japan, perinatal materno-fetal transmission was the main route of HBV infection, but this transmission has been prevented since 1986 by the national campaign to prevent it by immunoprophylaxis with combined passive-active immunization of babies born to HBeAg-positive carrier mothers. However, HCC develops in about 10 % of the patients who have established chronic HBV infection by materno-fetal infection or through child-to-child transmission. Hence, HBsAg seroclearance is crucially required for preventing the development of cirrhosis followed by HCC.

In the present study, we analyzed 2,112 patients with persistent HBV infection to establish the factors

**Table 4** Differences between the baseline characteristics of 917 untreated patients in whom HBsAg persisted and 213 those who lost HBsAg

| Features at the baseline | HBsAg persisted ($n = 917$) | HBsAg lost ($n = 213$) | Differences $p$ value |
|---|---|---|---|
| Age (years) | 37 (1–81) | 44 (0–80) | <0.001 |
| Men | 553 (60.3 %) | 152 (71.4 %) | 0.003 |
| HBV in family members | 349 (38.1 %) | 76 (35.7 %) | 0.509 |
| Chronic hepatitis | 893 (97.4 %) | 201 (94.4 %) | 0.020 |
| AST (IU/L) | 27 (3–1,144) | 25 (6–1,776) | 0.283 |
| ALT (IU/L) | 28 (6–1,960) | 27 (6–3,020) | 0.389 |
| γ-GTP (IU/L) | 22 (1–1,494) | 29 (4–1,092) | <0.001 |
| Total bilirubin (mg/dL) | 0.6 (0.2–20.1) | 0.7 (0.1–4.0) | 0.257 |
| Albumin (g/dL) | 4.3 (2.0–5.3) | 4.4 (1.6–5.7) | 0.004 |
| Platelets ($\times 10^3$/mm$^3$) | 203 (40–443) | 203 (33–417) | 0.473 |
| α-Fetoprotein (μg/L) | 3 (1–2,060) | 1 (1–478) | 0.373 |
| Genotypes [A/B/C/others (%)] | 5.7/19.0/73.3/1.9 | 5.5/24.7/69.2/0.7 | <0.001 |
| HBeAg-negative status | 663 (72.3 %) | 194 (91.1 %) | <0.001 |
| HBV DNA (log copies/mL) | 4.9 (<2.1 to >9.1) | 3.8 (<2.1 to >9.1) | <0.001 |
| HBsAg (IU/mL) | 3,100 (1.94–141,000) | 149 (0.06–88,800) | <0.001 |
| HBcrAg (log U/mL) | 3.9 (<3.0 to >6.8) | 2.9 (<3.0 to >6.8) | <0.001 |
| Wild-type precore sequence | 441 (48.1 %) | 160 (75.0 %) | <0.001 |
| Wild-type core promoter sequence | 320 (34.9 %) | 47 (22.0 %) | 0.001 |

Wild-type precore sequence, G1896; wild-type core promoter sequence, A1762/G1764

*AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *γ-GTP* γ-guanosine triphosphate, *HBeAg* hepatitis B e antigen, *HBsAg* hepatitis B surface antigen, *HBcrAg* hepatitis B core-related antigen

**Table 5** Factors influencing the seroclearance of HBsAg in untreated patients evaluated by time-dependent uni- and multivariate analyses

| Factors | Univariate analysis HBsAg clearance Relative risk (95 % CI) | $p$ value | Multivariate analysis HBsAg clearance Relative risk (95 % CI) | $p$ value |
|---|---|---|---|---|
| Age ≥50 years | 1.63 (1.19–2.23) | 0.002 | 1.61 (1.09–2.37) | 0.018 |
| Male gender | 1.08 (0.79–1.48) | 0.618 | | |
| No HBV infection in family | 1.38 (1.02–1.86) | 0.037 | | |
| Cirrhosis | 1.19 (0.73–1.93) | 0.484 | | |
| AST ≥50 IU/L | 1.01 (0.70–1.45) | 0.979 | | |
| ALT ≥50 IU/L | 0.93 (0.68–1.27) | 0.633 | | |
| γ-GTP ≥20 IU/L | 1.17 (0.85–1.61) | 0.330 | | |
| Total bilirubin ≥1 mg/dL | 1.41 (0.80–2.49) | 0.239 | | |
| Albumin ≥4 g/dL | 0.78 (0.51–1.18) | 0.239 | | |
| Platelets >150 × $10^3$/mm$^3$ | 0.99 (0.67–1.46) | 0.946 | | |
| α-Fetoprotein ≤10 μg/L | 0.84 (0.48–1.47) | 0.543 | | |
| Genotype A or B | 1.17 (0.81–1.69) | 0.410 | | |
| HBeAg-negative status | 0.78 (0.79–2.07) | 0.314 | | |
| HBV DNA ≥5 log copies/mL | 0.84 (0.58–1.24) | 0.383 | | |
| HBsAg ≤2,000 IU/mL | 1.87 (1.19–2.91) | 0.006 | 1.77 (1.12–2.77) | 0.014 |
| HBcrAg ≥4 log U/mL | 0.85 (0.50–1.45) | 0.555 | | |
| Wild-type precore sequence | 0.99 (0.60–1.52) | 0.967 | | |
| Wild-type core promoter sequence | 0.78 (0.35–1.73) | 0.538 | | |

Wild-type precore sequence, G1896; wild-type core promoter sequence, A1762/G1764

*AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *γ-GTP* γ-guanosine triphosphate, *HBeAg* hepatitis B e antigen, *HBsAg* hepatitis B surface antigen, *HBcrAg* hepatitis B core-related antigen

contributing to HBsAg seroclearance. The overall rate of HBsAg seroclearance was 1.75 % annually. The annual seroclearance rates of HBsAg are reported to be 1.7 % in Korea [14] and 1.6 % in Taiwan [15–17], as well as 2.5 % in Goto Islands of Japan, where HBV infections are very prevalent [18]. In 1,271 natives in Alaska, the rate of HBsAg seroclearance was 0.7 % annually [19]. These differences could be ascribed, in part, to HBV genotypes distinct among Asian countries and Alaska. Since treatment with IFN and/or nucleot(s)ide analogues has suppressive effects on the development of HCC [6, 20], they may influence HBsAg seroclearance.

**Table 6** Differences in baseline characteristics between the 833 treated patients in whom HBsAg persisted and 149 those who lost HBsAg

| Features at the baseline | HBsAg persisted ($n$ = 833) | HBsAg lost ($n$ = 149) | Differences $p$ value |
|---|---|---|---|
| Age (years) | 41 (13–88) | 43 (17–71) | 0.285 |
| Men | 601 (72.2 %) | 124 (83.2 %) | 0.004 |
| HBV in family members | 496 (59.6 %) | 72 (48.3 %) | 0.010 |
| Chronic hepatitis | 802 (96.3 %) | 134 (89.9 %) | 0.001 |
| AST (IU/L) | 54 (6–2,192) | 78 (7–888) | 0.010 |
| ALT (IU/L) | 93 (8–2,740) | 118 (8–1,700) | 0.117 |
| γ-GTP (IU/L) | 44 (4–1,278) | 46 (4–1,278) | 0.023 |
| Total bilirubin (mg/dL) | 0.7 (0.2–21.2) | 0.7 (0.3–8.4) | 0.273 |
| Albumin (g/dL) | 4.3 (1.1–5.4) | 4.5 (1.4–5.3) | 0.281 |
| Platelets ($\times 10^3$/mm$^3$) | 182 (40–483) | 171 (50–391) | <0.001 |
| α-Fetoprotein (μg/L) | 4 (1–1,610) | 4 (1–765) | 0.682 |
| Genotypes [A/B/C/others (%)] | 3.2/10.7/85.1/1.0 | 5.1/12.4/81.6/0.9 | 0.565 |
| HBeAg-negative status | 230 (27.6 %) | 79 (53.0 %) | <0.001 |
| HBV DNA (log copies/mL) | 7.8 (<2.1 to >9.1) | 8.3 (<2.1 to >9.1) | 0.002 |
| HBsAg (IU/mL) | 7,880 (0.04–277,000) | 1,380 (0.04–188,000) | <0.001 |
| HBcrAg (log U/mL) | 6.9 (<3.0 to >6.8) | 5.9 (<3.0 to >6.8) | 0.003 |
| Wild-type precore sequence | 554 (66.6 %) | 61 (41.2 %) | 0.013 |
| Wild-type core promoter sequence | 274 (32.9 %) | 67 (45.0 %) | 0.836 |

Wild-type precore sequence, G1896; wild-type core promoter sequence, A1762/G1764

*AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *γ-GTP* γ-guanosine triphosphate, *HBeAg* hepatitis B e antigen, *HBsAg* hepatitis B surface antigen, *HBcrAg* hepatitis B core-related antigen

**Table 7** Factors influencing the seroclearance of HBsAg in treated patients evaluated by time-dependent uni- and multivariate analyses

| Factors | Univariate analysis HBsAg clearance Relative risk (95 % CI) | $p$ value | Multivariate analysis HBsAg clearance Relative risk (95 % CI) | $p$ value |
|---|---|---|---|---|
| Age ≥50 years | 1.91 (1.32–2.77) | 0.001 | | |
| Male gender | 2.14 (1.37–3.33) | 0.001 | | |
| No HBV infection in family | 1.58 (1.15–2.19) | 0.005 | 2.22 (2.32–3.94) | 0.006 |
| Treatments (interferon vs. others) | 2.13 (1.53–2.98) | <0.001 | 3.15 (1.69–5.87) | <0.001 |
| Chronic hepatitis | 3.12 (2.05–4.74) | <0.001 | | |
| AST ≥50 IU/L | 1.47 (1.04–2.09) | 0.031 | | |
| ALT ≥50 IU/L | 1.29 (0.82–1.92) | 0.201 | | |
| γ-GTP ≥20 IU/L | 1.87 (1.30–2.70) | 0.001 | | |
| Total bilirubin ≥1 mg/dL | 1.35 (0.87–2.08) | 0.179 | | |
| Albumin ≥4 g/dL | 1.11 (0.66–1.86) | 0.688 | | |
| Platelets ≤150 × $10^3$/mm$^3$ | 2.10 (1.49–2.96) | <0.001 | | |
| α-Fetoprotein ≤10 μg/L | 1.33 (0.92–1.92) | 0.136 | | |
| Genotype A or B vs. others | 1.16 (0.74–1.82) | 0.529 | | |
| HBeAg-negative status | 2.53 (1.83–3.50) | <0.001 | 3.75 (2.09–6.74) | <0.001 |
| HBV DNA ≤5 log copies/mL | 2.07 (1.37–3.13) | 0.001 | | |
| HBsAg ≤2,000 IU/mL | 2.29 (1.52–3.47) | <0.001 | | |
| HBcrAg ≤4 log U/mL | 2.28 (1.31–3.97) | 0.003 | | |
| Wild-type precore sequence | 2.04 (1.18–3.55) | 0.011 | | |
| Wild-type core promoter sequence | 1.18 (0.63–2.21) | 0.608 | | |

Wild-type precore sequence, G1896; wild-type core promoter sequence, A176.2/G1764

*AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *γ-GTP* γ-guanosine triphosphate, *HBeAg* hepatitis B e antigen, *HBsAg* hepatitis B surface antigen, *HBcrAg* hepatitis B core-related antigen

Therefore, we went on to extend our analysis to untreated patients and those treated with IFN or nucleotide analogues separately. Criteria for upper or lower levels of each parameter were set, taking into consideration the median value or a cutoff value with the lowest $p$ value of the entire 2,112-patient cohort (Table 1), and unified for untreated and treated patients (Tables 5, 7).

Firstly, in the univariate analysis, age, no family history of HBV infection in third-degree or closer relatives, and decreased HBsAg levels lowered the annual rate of HBsAg seroclearance significantly. In multivariate analysis, age $\geq$50 years (RR 1.61, $p = 0.018$) and HBsAg $\leq$2,000 IU/mL (RR 1.77, $p = 0.014$) decreased the annual rate of HBsAg seroclearance significantly. Kato et al. [18] reported high HBsAg seroclearance rates in patients over 40 or over 50 years; in our patients, also, age $\geq$50 years increased RR to 1.61 ($p = 0.018$). As for HBsAg and HBV DNA, low HBsAg and HBV DNA levels increased the HBsAg seroclearance rate to 37.7 %, and therefore, low HBsAg levels are an important factor. In actuality, HBsAg levels $\leq$2,000 IU/mL increased the rate of HBsAg seroclearance with RR 1.77 ($p = 0.014$).

In treated patients, by contrast, age, the male gender, no HBV infections in third-degree or closer relatives, treatment with IFN, chronic hepatitis, high AST levels, high $\gamma$-GTP levels, low platelet counts, HBeAg-negative status, low HBsAg levels, low HBcrAg levels and the wild-type precore sequence were significant factors in univariate analysis. In multivariate analysis, no HBV infections in third-degree or closer relatives (RR 2.22, $p = 0.006$), interferon treatments (RR 3.15, $p < 0.001$), and HBeAg-negative status (RR 3.75, $p < 0.001$) were significant factors.

Thus, there were differences in factors predictive of the HBsAg loss between untreated and treated patients. Remarkably, age and HBsAg titer were independent factors in untreated patients, whereas family history and negative HBeAg were independent factors in treated patients. Since this work studied patients who were followed for a long time (>15 years), age and HBsAg titer were factors for clearance of HBsAg in untreated patients. Treated patients, in contrast, would have included more patients with HBeAg, with a good response to antiviral treatment, as well as those without family history who would have been infected with HBV with a sorter duration than those with family history. In other words, most untreated patients were those with favorable clinical course, in whom HBsAg titer gradually decreased and eventually lost it with time. In fact, there would be many such patients, the majority of whom do not visit hospitals and are unaware of HBV infection, who may have unapparent liver disease. Treated patients, on the other hand, would have had higher risks for cirrhosis and HCC, owing to elevated ALT/AST levels; this risk is especially high for patients with a family history of HBV [21]. Therefore, patients with family history would not be able to easily lose HBsAg.

In treated patients, IFN led to HBsAg loss more effectively than other treatments [RR 2.13, $p < 0.001$ (Table 7)]. The immunomodulatory activity of IFN, which is not shared by nucleot(s)ide analogues, would have accelerated the immune response to HBV required for the seroclearance of HBsAg. Of the 333 patients who received IFN, 190 (57 %) were treated with IFN multiply. In them, seroclearance of HBsAg was achieved in 49 of the 190 (26 %) patients with multiple IFN treatments in comparison with 41 of the 143 (29 %) with single IFN treatment. Owing to indications for IFN, patients who received IFN tended to be younger, without previous treatments and higher HBV DNA as well as ALT levels. They might have increased the rate of HBsAg loss that was higher with IFN than other treatments.

Since this is a retrospective cohort study of patients visiting our hospital for more than 15 years, and there has been so much innovation in the treatment of chronic hepatitis B during that period, treated and untreated patients have different backgrounds at the baseline. Hence, treated patients had higher ALT and HBV DNA levels with severer liver disease than untreated patients (Table 3). This might have been responsible, at least in part, for the failure in finding differences in the rate of HBsAg loss between untreated and treated patients (Fig. 2). Future studies will be aimed at analyzing contributing factors in treated and matched controls. This will allow us to analyze factors contributing to HBsAg seroclearance in the treatment of patients with chronic hepatitis B.

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