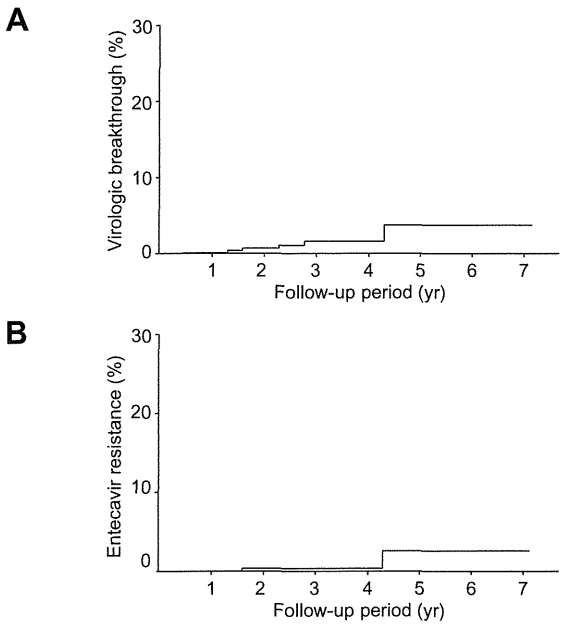


**Fig. 4. Cumulative rates of patients who showed resistance to therapy analyzed with the Kaplan–Meier test.** (A) Virologic breakthrough (VBT) and (B) entecavir-resistance.

DNA after anti-HBe seroconversion. One patient became negative for HBsAg at week 28.

*Monitoring resistance to treatment*

Five patients showed VBT during the treatment period, including two patients (Patient 1 had been reported previously [13]) who developed entecavir-resistant mutations. None of the five patients had mutation(s) for entecavir at baseline. VBT was defined as any increase in serum HBV DNA by >1 $log_{10}$ copies/ml from nadir or redetection of serum HBV DNA at levels 10-fold the lower limit of detection of the HBV DNA assay after having an undetectable result. Table 4 shows the patient baseline demographics, HBV DNA levels, and viral resistance profiles. All patients were positive for HBeAg and had serum levels of HBV DNA >6 $log_{10}$ copies/ml at baseline. The median period until the appearance of the mutation was 120 (68–224) weeks. Two of the 49 (4%) patients who had detectable HBV DNA at the end of the first year subsequently developed resistance to entecavir. Furthermore, 3 of 49 (6%) patients who had detectable HBV DNA at the end of the first year developed VBT. Fig. 4A and B show the cumulative percentages of VBT and entecavir-resistance cases analyzed by the Kaplan–Meier test.

## Discussion

Long-term data are rare for nucleoside-naïve patients treated continuously for more than 4 years with entecavir at the recommended dose of 0.5 mg daily. The only available data [6,8] were generated from follow-up studies of two phase III registration trials [3,4] in which patients showing complete response and non-responders were taken off entecavir. In the rollover studies, entecavir was administered to these patients at 1-mg dose at varying periods after cessation of the initial treatment. This double dose of entecavir was also given to patients showing a partial virological response after 48–96 weeks of entecavir at 0.5 mg daily. The present study has several unique features addressing specific and unanswered questions about entecavir treatment. It provided long-term results with respect to antiviral potency, viral resistance, and clinical safety for treatment-naïve patients who were treated continuously with entecavir at 0.5 mg daily for 4 years. Specifically, we found excellent viral suppression with 96% of patients achieving undetectable HBV DNA levels, only 1.1% (5/475) chance of viral breakthrough, and no clinically serious side effects after 4 years of treatment.

Genotype B was a significant factor associated with undetectable HBV DNA after the first year, although there were no significant differences after subsequent years. Previous studies showed conflicting results on the effect of HBV genotype on the response to lamivudine, with genotypes A, B, and C not affecting the antiviral response to lamivudine [14–16]. However, we have previously found that 47%, 84%, and 76% of patients had undetectable HBV DNA after the third year among patients of genotype

**Table 4. Characteristics of patients with virologic breakthrough.**

| Patient No. | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Age (yr)/gender | 40/M | 28/M | 39/M | 51/F | 64/M |
| At start of entecavir therapy | | | | | |
| HBeAg status | + | + | + | + | + |
| HBV DNA ($log_{10}$ copies/ml) | >7.6 | >7.6 | 7.2 | 7.2 | 6.2 |
| HBV genotype | H | A | C | C | C |
| Viral load at maximum suppression ($log_{10}$ copies/ml) | <2.6 | <2.6 | <2.6 | 3.1 | <2.6 |
| Time of detection of mutation (wk) | 83 | 224 | 120 | 68 | 145 |
| HBV DNA ($log_{10}$ copies/ml), maximum | 6.8 | 7.2 | 7.1 | 7.6 | 7.8 |
| Mutational pattern | L180M+/S202G+/M204V | L180M+/T184I+/S202G+/M204V | L180M+/M204V, L180M+/M204I | A181T | A181S+/T184A+/M204I |

A, B, and C, respectively [17,18]. The difference among these groups was probably due to the younger age of patients of genotype A and that they were often positive for HBeAg compared to those of genotype B or C. However, the genotype was not a significant predictor of HBV DNA loss after >2 years of entecavir therapy in the present study. There was also no difference in HBeAg seronegativity with entecavir among patients infected with genotype A, B, or C virus. These results were consistent with studies on lamivudine therapy [14,18].

In this study, HBeAg positivity was a significant factor associated with detectable HBV DNA at years 1 through 3, and these results were consistent with those reported by Zoutendijk *et al.* [10]. In addition, lower HBV DNA and HBeAg negativity at baseline were associated with enhanced response to lamivudine therapy [18–20]. We have also previously reported that lamivudine induced a better response in HBeAg-negative patients with higher levels of serum ALT [17]. The most important factor of long-term entecavir therapy therefore was low HBV DNA level.

Low HBV DNA level at baseline correlated significantly with HBeAg seroclearance, but not with seroconversion. One of the reasons was that patients who showed HBeAg seroclearance but no seroconversion had lower HBV DNA (median; 6.7 $\log_{10}$ copies/ml) at baseline compared to patients with seroconversion (median; 7.5 $\log_{10}$ copies/ml, $p = 0.005$).

Univariate analysis showed that age (>40 years), serum albumin level (<3.5 g/dl), and platelet count ($<20 \times 10^4/mm^3$) correlated with HBeAg seroconversion rate. We also investigated the correlation between serum albumin and other factors. Serum albumin level correlated significantly with age ($r = -0.378$, $n = 216$, $p < 0.001$), platelet count ($r = 0.262$, $n = 215$, $p < 0.001$), AFP ($r = -0.372$, $n = 161$, $p < 0.001$), cirrhosis ($P < 0.001$) and male sex ($p = 0.004$). Multivariate analysis identified low serum albumin level (<3.5) as the only significant determinant of HBeAg seroconversion. In this regard, Chien *et al.* [21] reported that pre-treatment ALT was the only significant determinant of HBeAg seroconversion during lamivudine therapy. The reasons for the different findings are probably related to the study design. In our study, the age of patients at baseline was higher (47 *vs.* 32 years) and the duration of treatment was longer (2.4 [median] *vs.* 1 year) than in the study of Chien *et al.* [21]. Furthermore, differences in the pharmacodynamics of lamivudine and entecavir could also contribute to the observed differences between the two studies.

On the other hand, resistant mutants and breakthrough hepatitis seemed to be less frequent during long-term therapy with entecavir than with lamivudine [16–19], indicating that entecavir is better than lamivudine for long-term treatment of CHB and cirrhosis patients. Tenney *et al.* [6] reported that 9 out of 663 (1.4%) patients had baseline lamivudine-resistant mutations, and other studies also found only small numbers of preexisting lamivudine-resistant mutations in treatment-naïve patients [22–24]. It is known that the HBV rtM204V (usually with concomitant rt180M) mutation often acquires one of the entecavir signature mutations at rt184, rt202, or rt250 over long-term treatments and patients develop clinical HBV DNA breakthroughs. Although *in vitro* studies showed that rt204I mutations with or without rt180M conferred 3- to 21-fold decrease in entecavir susceptibility [25], in clinical practice, patients with rt204I mutations, even with the entecavir signature mutations, have lower levels of phenotypic resistance to entecavir and can often achieve undetectable HBV DNA levels [6,9,26]. Interestingly, there were three patients in the present study with VBT who had no HBV DNA mutations at rt184, rt202, or rt250 with rt180M and rt204V (entecavir-resistance). The rtM204V/I mutation, lamivudine's signature mutation, is necessary but not sufficient for entecavir-resistance, causing an 8- to 10-fold decrease in susceptibility to entecavir compared with wild-type HBV. Other mutations at positions rtT184, rtS202, and rtM250 confer additional decreases in entecavir susceptibility [25,27,28]. In the present study, two patients (Patients #3 and 5) with mutations at position rtM204V/I, without rtT184, rtS202, or rtM250 mutations, showed emergence of VBT, as did one patient (Patient #4) with an rtA181T mutation, which was first reported in a LAM-treated patient [29]. Although the rtA181T mutation is related to resistance to adefovir dipivoxil, this mutation has not been linked to additional decreases in entecavir susceptibility. Future *in vitro* analyses using replication-competent HBV clones in patients with rtA181T mutations are therefore necessary.

In conclusion, long-term treatment of treatment-naïve CHB patients with 0.5 mg/day entecavir for 4 years suppressed HBV DNA to undetectable levels in more than 90% of patients, regardless of HBeAg status and genotype. Moreover, the drug was very safe and rarely induced resistance mutations. Further studies exploring the therapeutic efficacy over longer durations may be necessary to confirm these findings.

## Conflict of interest

Hiromitsu Kumada has received speaker's honoraria from Bristol-Myers Squibb. All other authors declare no conflict of interest.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

# Determinants of the clinical outcome of patients with severe acute exacerbation of chronic hepatitis B virus infection

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**Abstract**
*Background* Severe acute exacerbation of chronic hepatitis B can sometimes occur and lead to hepatic failure and death. The objective of this study was to elucidate the predictors of progression to hepatic decompensation during severe acute exacerbation.
*Methods* We prospectively analyzed 37 consecutive patients with acute exacerbation of chronic hepatitis B (accompanied by jaundice and coagulopathy) for clinical outcome and factors that influenced the development of severe acute exacerbation, including viral kinetics.
*Results* Fourteen (37.8%) patients progressed to severe acute exacerbation (accompanied by encephalopathy). Multivariate analysis identified serum bilirubin (>5 mg/dl, $P = 0.002$) as a significant determinant of progression to hepatic failure and prothrombin activity (<45%, $P = 0.028$) and as a determinant of liver-related death. The hepatitis B virus (HBV) DNA level before therapy was measured in 25 patients. HBV DNA levels increased or did not change from before commencement of treatment in all 11 patients who progressed to severe acute exacerbation. On the other hand, HBV DNA levels did not change or increased in 8 of 14 patients (57%) with acute exacerbation ($P = 0.02$).
*Conclusions* Serum bilirubin and prothrombin activities were significant predictors of clinical outcome in patients with severe acute exacerbation of chronic hepatitis B. Viral kinetics until commencement of therapy can predict the severity of acute exacerbation of chronic hepatitis B.

**Keywords** Hepatitis B · Acute exacerbation · HBV DNA · Genotype · Encephalopathy

**Abbreviations**

| | |
|---|---|
| AE | Acute exacerbation |
| ALT | Alanine aminotransferase |
| BCP | Basal core promoter |
| CS | Corticosteroid |
| HBV | Hepatitis B virus |
| IFN | Interferon |
| LMV | Lamivudine |
| NA | Nucleos(t)ide analogue |
| PC | Pre-core |
| PT | Prothrombin activity |
| SAE | Severe acute exacerbation |

## Introduction

More than 3 billion people worldwide and approximately 1.5 million people in Japan are chronically infected with hepatitis B virus (HBV), and chronic HBV infection is one of the most common causes of chronic hepatic failure and hepatocellular carcinoma (HCC) [1, 2]. Other complications of HBV infection include fulminant hepatitis and acute liver failure [3, 4]. Acute exacerbation (AE) in HBV carriers occurs either through a natural course [5, 6] or following intensive chemotherapy or immunosuppressive

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therapy [7, 8]. Some abrupt flares may be so severe that decompensation or even fulminant hepatic failure may occur [9–11]. Previous studies have identified pre-existing cirrhosis, high serum bilirubin levels, prolonged prothrombin time, pre-core/core promoter mutants, and high HBV DNA levels as factors associated with hepatic decompensation during AE in HBV carriers, though little is known about the predictive factors [9, 12, 13].

Liver transplantation is suitable therapy for acute hepatic failure, but the rate of liver transplantation has remained about 20% in Japan, where living donor liver transplantation is dominant [14, 15]. Thus, it is necessary to establish other effective therapies for patients with AE apart from liver transplantation. Steroids can rapidly inhibit excessive immune response and inflammatory reactions, and have been reported to be effective in cases of severe and potentially life-threatening exacerbation of chronic HBV (CHB) infection [16]. With the advent of oral nucleos(t)ide analogues (NAs), most guidelines recommend NAs for patients with AE of CHB infection [17–19], and several observational studies reported the use of NAs [9–11, 20, 21]. Timely use of potent anti-HBV agents, such as NAs, interferon (IFN), and steroids [22], during and/or after the development of hepatic decompensation could be potentially effective against various host- and virus-related factors.

The aim of the present study was to investigate the factor(s) that influence the rapid development of hepatic decompensation during AE of CHB.

## Materials and Methods

### Patients

The study subjects were patients with AE admitted to the Department of Hepatology, Toranomon Hospital, Tokyo, between 1984 and 2010. All patients were either followed up at our hospital with clinicopathologically proven CHB infection or were new patients with sudden-onset hepatic flares who visited our hospital outpatient clinic or were referred to our hospital from other clinics/hospitals. The diagnosis of CHB carrier state was established based on either positivity for hepatitis B surface antigen (HBsAg) for at least 6 months prior to the development of AE, or the presence of a high titer of anti-hepatitis B core antibodies (anti-HBcAb), together with negativity or a low titer of IgM anti-HBcAb. Chronic hepatitis and cirrhosis were confirmed by laparoscopy, needle biopsy, or ultrasonography, or treatment for these conditions for 1 year before the development of AE. AE of CHB infection was diagnosed by the following criteria: (1) an abrupt increase in serum alanine aminotransferase (ALT) levels to >300 IU/l in patients with original ALT levels of less than 5× the upper limit of normal or an abrupt two-fold increase in the serum ALT level to greater than 5× the upper limit of normal, (2) hyperbilirubinemia [serum bilirubin (Bil) >3.0 mg/dl], (3) evidence of coagulopathy with plasma prothrombin activity (PT) of <60% during the clinical course, and (4) lack of encephalopathy at admission. We also applied the following exclusion criteria: (1) the presence of viral markers other than HBV (hepatitis A, C, D, E, Epstein-Barr virus, cytomegalovirus, herpes simplex virus), (2) HBV reactivation induced by immunomodulators or chemo-/immunosuppressive therapy, (3) asymptomatic HBV carriers, (4) recent exposure to drugs and chemical agents as well as recent heavy alcohol intake, (5) breakthrough hepatitis caused by NAs, (6) evidence of decompensated liver disease before the onset of exacerbation as characterized previously, (7) HCC diagnosed by ultrasonography or computed tomography, and (8) coexistence of other serious medical conditions and other liver diseases, or metabolic diseases. Progression to severe acute exacerbation (SAE) was diagnosed by the development of hepatic encephalopathy of more than grade 2 within 8 weeks of onset associated with coagulopathy (PT <40%).

HBV DNA levels were measured serially to investigate the effects of HBV kinetics on the prognosis of patients with severe AE. HBV DNA levels were measured before treatment in 25 patients. "Before treatment" represented 1–8 weeks before commencement of treatment. HBV DNA levels were also measured after treatment in 27 patients. "After treatment" was defined as 2 weeks after commencement of therapy. Viral kinetics was assessed using the same assay in all individuals. The Local Ethics Committee of Toranomon Hospital approved the study, and informed consent was obtained from all patients.

### Virological markers

Serial blood samples were obtained during the clinical course of AE and stored at −80°C until used for HBV molecular analysis. Serological tests for HBsAg, HBsAb, hepatitis e antigen (HBeAg), IgM anti-HBcAb, total anti-HBcAb, and anti-HBeAb were conducted using radioimmunoassay kits (Abbot Diagnostics, Chicago, IL, USA) according to the instructions provided by the manufacturer. Precore (PC) mutations were analyzed by PCR enzyme-linked mini-sequence assay (Roche Diagnostics, Tokyo, Japan), and basal core promoter (BCP) mutations were analyzed by PCR specific probe assay (Roche Diagnostics, Tokyo, Japan). HBV DNA was measured by Amplicor monitor assay (dynamic range 2.6–7.6 log copies/ml, Roche Diagnostics, Tokyo, Japan), COBAS TaqMan v.2.0 (dynamic range 2.1–9.0 log copies/ml, Roche Diagnostics), transcription-mediated amplification and hybridization

protect assay (TMA-HPA) (dynamic range 3.7–8.7 LGE/ml, Chugai Diagnostics Science Co., Tokyo) or sandwich hybridization assay with signal amplification using branched DNA (bDNA, dynamic range 0.7–3800 Meq/ml). The major genotype of HBV was determined using enzyme-linked immunosorbent assay (ELISA, Institute of Immunology, Tokyo, Japan) or PCR-invader assay (BML, Inc, Tokyo, Japan) based on the methods described previously [23, 24]. HBVDNA levels assessed by bDNA were re-measured by TaqMan PCR assay using stored serum samples.

### Statistical analysis

Continuous variables were expressed as median (range), and compared by Mann–Whitney *U* test. Categorical variables were compared by $\chi^2$ test or Fisher's exact test, as appropriate. Univariate analysis was applied to determine the relationship between SAE and each of the following factors: sex, age, presence of compensated cirrhosis, and various biological and virological markers as measured at baseline (bilirubin, PT, ALT, albumin, HBeAg, HBV DNA, and HBV genotype, PC and BCP mutations). Each continuous variable was transformed into two categories based on the value with the largest capacity to discriminate between patients for univariate and multivariate analyses. Factors that correlated significantly with SAE were entered into multiple logistic regression analysis, and the odds ratio (OR) with 95% confidence intervals (95% CI) were determined. All analyses were performed using The Statistical Package for Social Sciences (SPSS II v. 11.0, Chicago, IL, USA), and statistical significance was taken as a two-sided *P* value $<0.05$.

## Results

### Clinical features of severe acute exacerbation

A total of 37 patients (30 men and 7 women) fulfilled the criteria of AE and were included in this study. The baseline characteristics at the commencement of therapy of these 37 patients are shown in Table 1. Twenty-two patients were observed at our hospital, and 15 patients were referred from another hospital after the onset of hepatic flares. The majority of patients had genotype C, and 27 patients (72.9%) were HBeAg positive. The PC and BCP mutations were determined in 27 patients; 22 patients had mutations in the PC region, 16 patients had mutations in the BCP region, and 12 patients had mutations in both the PC and BCP regions. During the clinical course, the peak median values were: ALT 713 IU/l (range 307–2857), bilirubin 8.4 mg/dl (3.0–51.4), and PT 47.6% (12.0–60.0).

**Table 1** Baseline characteristics of the 37 patients infected with HBV who developed severe acute exacerbation at the commencement of therapy

| | |
|---|---|
| Number | 37 |
| Sex (male/female) | 30/7 |
| Age (years) | 45 (23–63) |
| Family history (yes/no) | 21/16 |
| Cirrhosis (present/absent) | 7/30 |
| Albumin (g/dl) | 3.4 (2.5–4.6) |
| Bilirubin (mg/dl) | 4.7 (1.0–30.7) |
| AST (IU/l) | 601 (64–2593) |
| ALT (IU/l) | 657 (124–2142) |
| LDH (IU/l) | 297 (106–594) |
| Platelets ($\times 10^4/mm^3$) | 12.3 (6.2–32.0) |
| α-Fetoprotein (μg/ml) | 62.0 (3.0–1600) |
| Prothrombin activity (%) | 53 (26–80) |
| Genotype (A/B/C) | 0/5/32 |
| HBeAg (positive/negative) | 27/10 |
| HBV-DNA ($\log_{10}$ copies/ml) | 8.5 (6.8–8.9) |
| PC (wild/mutant/ND) | 5/22/10 |
| BCP (wild/mutant/ND) | 11/16/10 |

Data are median values (range) or number of patients

*AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *LDH* lactate dehydrogenase, *HBeAg* hepatitis B envelope antigen, *PC* pre core, *BCP* basal core promoter, *ND* not done

### Treatment

NAs were used in 19 patients, IFN in 8, and corticosteroids (CS) in 20 patients. In addition, 7 patients were treated with a combination of NAs and CS; 2 patients were treated with three drugs (NAs, IFN, and CS). At the time of the study, lamivudine (LMV) was not yet available for the treatment of chronic hepatitis B, and thus IFN was used; 6 patients were treated with both IFN and CS. None of the patients underwent liver transplantation.

### Prognosis of severe acute exacerbation and factors associated with progression to hepatic failure

Of the 37 patients admitted with CHB infection and AE, 23 (62.2%) did not develop SAE. The remaining 14 (37.8%) patients developed SAE; 9 (24.3%) patients died of liver-related death, but 5 (13.5%) survived. Further analysis showed that 8 (36.4%) of 22 patients who were observed in our hospital developed AE, and 6 (27.3%) of these patients died, whereas 6 (40.0%) of 15 patients who were referred from other hospitals after the onset of exacerbation developed AE, and 3 (20.0%) of these patients died. There was no significant difference in prognosis by treatment facility before AE. Ten of 37 patients experienced AE before 2000 when LMV was available in Japan, and 19

**Table 2** Biochemical, virological and histological features of patients with severe acute exacerbation at the commencement of therapy

| Case | Age (years)/ sex | Genotype | HBeAg | HBV-DNA (log copies/ ml) | Preexisting cirrhosis | Serum bilirubin (mg/dl) | ALT (IU/l) | PT (%) | Platelets ($\times 10^4$/ $mm^3$) | Therapy | Outcome (time from treatment to death, weeks) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 63/M | B | – | 8.4 | No | 5.8 | 1680 | 43 | 6.2 | LMV + CS | Death (11) |
| 2 | 32/M | B | – | >8.7 | No | 6.9 | 1340 | 41 | 13.4 | CS | Death (1) |
| 3 | 58/M | B | – | 8.6 | No | 7.4 | 1446 | 36 | 7.7 | CS | Death (2) |
| 4 | 29/M | B | – | >8.7 | No | 15.6 | 307 | 26 | 10.0 | LMV | Recovery (alive) |
| 5 | 54/F | C | + | >8.7 | No | 2.4 | 2077 | 79 | 21.0 | LMV + CS | Recovery (alive) |
| 6 | 37/M | C | + | >8.7 | No | 4.1 | 552 | 53 | 8.9 | CS | Recovery (alive) |
| 7 | 62/M | C | + | 7.0 | No | 12.0 | 220 | 53 | 7.1 | LMV + CS + IFN | Recovery (alive) |
| 8 | 33/F | C | + | >8.7 | No | 14.0 | 632 | 39 | 13.1 | CS | Recovery (alive) |
| 9 | 55/M | C | + | >8.7 | Yes | 4.0 | 1089 | 55 | 10.3 | LMV + CS | Death (1) |
| 10 | 37/F | C | + | 7.1 | Yes | 5.8 | 1444 | 34 | 22.0 | LMV + CS + IFN | Death (10) |
| 11 | 49/M | C | + | 8.0 | Yes | 8.8 | 834 | 58 | 9.9 | CS | Death (10) |
| 12 | 33/M | C | + | 8.5 | No | 9.6 | 657 | 26 | 7.4 | LMV + CS | Death (2) |
| 13 | 54/M | C | + | 7.8 | Yes | 12.1 | 364 | 36 | 15.8 | LMV + CS | Death (2) |
| 14 | 55/M | C | + | >8.7 | No | 24.2 | 520 | 44 | 8.3 | CS | Death (5) |

Abbreviations as in Table 1, *PT* prothrombin activity, *LMV* lamivudine, *CS* corticosteroids, *IFN* interferon-α

patients experienced AE after 2000. The other 8 patients experienced AE before 2000, but received LMV through participation in clinical trials or paid for the drug privately. The clinical features at the commencement of therapy of 14 patients who developed SAE are shown in Table 2 (median age 52 years, range 29–63). The mean time period between admission and death of 9 patients who developed SAE was 2 (range 1–11) weeks. Six patients who were admitted before the availability of LMV were treated with CS alone, 5 patients were treated with the combination of LMV and CS, 1 patient was treated with LMV alone, and 2 other patients were treated with LMV, CS, and IFN. Among 8 patients treated with LMV, of those who developed SAE, 5 died, and 2 patients developed complications caused by bacterial infection. Four patients had genotype B, while 10 patients had genotype C. HBeAg status was positive in 10 patients. The mean HBV DNA level was 8.7 (range 7.0–>8.7) log copies/ml, ALT 746 (220–2077) IU/l, serum bilirubin 8.1 (2.4–24.2) mg/dl, PT 42 (26–79)%, and platelet count was 10.0 (62–220) $\times$ $10^4/mm^3$.

Of the 5 patients who were treated successfully after progression to SAE, one later died of severe breakthrough hepatitis caused by emergence of LMV-resistant virus 3 years after SAE (case 7, Table 2). The other four survived (cases 4–6 and 8, Table 2).

Table 3 shows the results of univariate analysis. The following factors showed significant relationship with the development of SAE at the commencement of treatment: serum bilirubin (>5 mg/dl) and PT (<60%). Multivariate analysis identified serum bilirubin as a significant and independent determinant of the development of SAE (Table 3). On the other hand, two parameters showed significant relationships with liver-related death: serum bilirubin (>7 mg/dl, $P = 0.049$) and PT (<45%, $P = 0.003$). Multivariate analysis identified PT (OR 9.50, 95% CI 1.3–71.0, $P = 0.028$) as a significant determinant of death.

## Viral kinetics associated with fulminant hepatic failure

To investigate the relationship between viral kinetics and SAE, HBV DNA levels were measured in 25 patients both before and commencement of treatment and also after treatment in 27 patients. Figure 1 shows the viral load of patients who developed and did not develop SAE at commencement of treatment compared with before treatment. Falls in the HBV DNA level occurred naturally. However, in 11 patients who developed SAE, HBV DNA levels increased in 6 patients and did not change in 5 patients. Among the latter 5, HBV DNA levels of 4 patients were >8.7 log copies/ml. In 14 patients who did not develop SAE, HBV DNA levels increased in 4 patients, were unchanged in 4 patients, and decreased in 6 patients. Hence, the HBV DNA level increased/was unchanged in 8 of 14 (57%) patients who did not develop SAE, compared with 11 of 11 (100%) patients who developed SAE. A significantly higher proportion of patients with SAE showed an increase/was unchanged in viral load compared to those who without SAE ($P = 0.02$). We also examined the viral kinetics in 27 patients by comparing HBV DNA levels at the commencement of treatment to after treatment.

**Table 3** Univariate and multivariate analyses of host and viral factors associated with progression of severe acute exacerbation at commencement of treatment

| Parameter | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|
| | OR (95% CI) | *P* | OR (95% CI) | *P* |
| Sex (female) | 1.30 (0.15–4.11) | 0.76 | | |
| Age (>55 years) | 2.64 (0.57–12.3) | 0.22 | | |
| Cirrhosis (present) | 1.90 (0.39–9.26) | 0.43 | | |
| Albumin (<3.5 g/dl) | 1.75 (0.44–6.97) | 0.85 | | |
| Bilirubin (>5 g/dl) | 17.0 (2.92–99.1) | 0.002 | 11.2 (1.71–73.8) | 0.01 |
| ALT (>800 IU/l) | 1.88 (0.48–7.26) | 0.36 | | |
| AST/ALT ratio (>1) | 1.27 (0.31–5.19) | 0.74 | | |
| Prothrombin activity (<60%) | 11.9 (1.33–106.7) | 0.03 | 8.22 (0.73–92.6) | 0.09 |
| Platelets (<15 × $10^4$/$mm^3$) | 0.81 (0.19–3.58) | 0.89 | | |
| Genotype (B) | 8.82 (0.87–89.1) | 0.06 | | |
| HBeAg (positive) | 0.89 (0.20–3.90) | 0.89 | | |
| HBV-DNA (>8.7 log copies/ml) | 2.34 (0.60–9.20) | 0.70 | | |
| PC mutation | 2.29 (0.22–24.1) | 0.49 | | |
| BCP mutation | 0.19 (0.034–1.08) | 0.06 | | |

Abbreviations as in Tables 1 and 2, *OR* odds ratio, *CI* confidence level

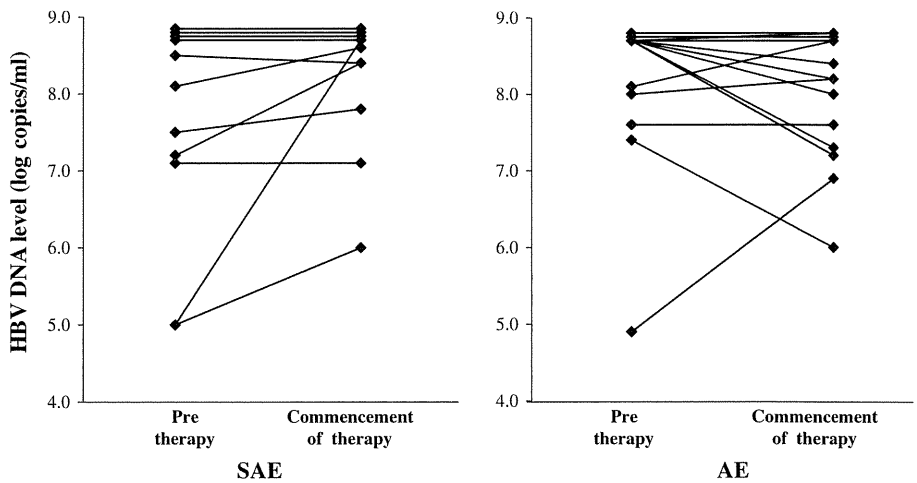


**Fig. 1** Viral kinetics from pre-treatment to commencement of treatment in patients with acute exacerbation. Viral kinetics tended to increase or remained unchanged until treatment in 8 patients with acute exacerbation course ($n = 14$), while the viral load in all patients with severe acute exacerbation ($n = 11$) increased or remained unchanged ($P = 0.02$)

The HBV DNA level decreased more than 1 log copies/ml in 9 of 17 (52.9%) patients who did not develop SAE, compared with 3 of 10 (30.0%) patients who developed SAE, but the difference between the two groups was not significant.

## Discussion

The results of the present study examined the predicting factors of progression to SAE accompanied by coagulopathy and encephalopathy in patients with AE of chronic hepatitis B, as well as the pattern of viral kinetics before and after commencement of therapy. Up to 30% of patients with CHB infection experience reactivation of hepatitis every year [5, 6], while some patients develop acute exacerbation with jaundice and coagulopathy, a severe life-threatening condition with high mortality [9, 12]. It is important to determine the predicting factors of progression to liver decompensation in patients with acute exacerbation. Multivariate analyses in previous studies indicated that pre-existing cirrhosis, a high Child–Pugh score, low albumin level, high serum bilirubin level, prolonged PT, and high HBV DNA levels were associated with the severity or mortality during acute exacerbation [9, 12, 13]. Our results are almost comparable to those of the above studies. Multivariate analysis in the present study identified the serum bilirubin level as a predictor of progression to liver decompensation. Moreover, there were no significant differences in viral load or therapeutic regimen. Genotype B was the predominant HBV strain in patients with SAE compared to patients with variable severity of liver diseases [25]. The frequencies of HBV genotype in patients with chronic hepatitis B admitted to our hospital were 3.0, 12.3, and 84.5%, for genotypes A, B, and C, respectively [26]. In the present study, although patients

with genotype B were only 5 of the total 37 (13.5%), 4 of 14 (28.6%) patients with SAE and 3 of 9 (33.3%) patients who died of liver failure were infected with genotype B. The different HBV genotypes also cause different clinical and epidemiological features. In a study from Japan, a high prevalence of genotype B HBV was found among patients with acute fulminant hepatitis [27]. In two case control studies conducted in Hong Kong, genotype B was the predominant HBV strain among patients with SAE compared to control patients with various severities of liver diseases [25, 28]. In this regard, another study indicated that genotype Bj was associated with high extracellular expression of HBV DNA in vitro [29]. The tendency of genotype Bj to produce high extracellular virion levels would be associated with a more vigorous immune response, leading to a higher risk of hepatic decompensation during the hepatitis flare. Several studies examined the association between specific mutations in the HBV genome and fulminant hepatitis or acute-on-chronic liver failure, especially in the PC (nt 1896) and BCP (nt 1762 and 1764) regions [30–32]. The PC and BCP regions are crucial replications of HBV [33], so alteration of the phenotype by the emergence of mutations in the PC and BCP regions might causes changes in the relationship between the virus and hepatocytes [30], and lead to fulminant hepatitis and acute exacerbation of chronic hepatitis. In the present study, genotype B and PC/BCP mutations were not significant predictors associated with the development of SAE or liver-related death, which is probably related to the small number of cases.

Jeng et al. [13] reported that HBV DNA levels greater than $1.55 \times 10^9$ copies/ml in patients with AE may predict subsequent occurrence of hepatic decompensation. While the overall viral load in our subjects was high (8.5 log copies/ml, Table 1), there was no relationship between viral load and the severity of AE or mortality. In addition, the HBV DNA level could not be estimated correctly when it was above the upper limit. Interestingly, the level of HBV DNA re-measured by TaqMan PCR in stored blood samples was higher than the upper limit (>9.1 log copies/ml) in one-third of the patients. The extremely high HBV DNA levels in patients with AE suggest that the vigorous immune attack on HBV and resultant liver injury will continue and may progress into hepatic decompensation. The present results showed that the decrease of viral load was significantly lower in patients with fulminant hepatic failure than in those with AE. These findings suggest that viral kinetics before the commencement of therapy are an important predictor of hepatic decompensation in patients with CHB infection complicated with AE. Interestingly, there was no significant difference in viral kinetics after the commencement of therapy between the two groups. To our knowledge, this is the first report that identifies viral kinetics before the commencement of therapy as a predictor of prognosis of patients with AE of chronic hepatitis B.

LMV monotherapy does not seem to improve short-term mortality in patients with AE [9], although other studies showed a possible decrease in the mortality rate with earlier administration [21]. In a recent randomized trial designed for the treatment of acute-on-chronic liver failure due to severe reactivation of hepatitis B, the use of tenofovir significantly reduced the mortality rate compared with placebo [11], and the results suggested that rapid suppression of HBV DNA replication with potent antiviral therapy could inhibit the ongoing necroinflammation and permitted hepatic regeneration. Although 8 of 14 patients were treated with LMV in the present study, two patients had to start LMV after the development of SAE because of the rapid exacerbation soon after admission. Five patients developed SAE within a median period of 8 days (range 1–17 days) after the commencement of LMV. The other one patient developed complications caused by bacterial infection and gradually progressed to liver failure over 2 months. Thus, it is thought that most of these patients developed SAE earlier than the available effect of LMV.

The prevailing idea is that AE is the result of a robust quantitative recovery of HBV specific T cells, which directly cause liver injury [34]. Other mechanisms of the effects of CS in AE may be related to the prevention of endotoxin-induced secondary liver injury [35], prevention of cytolysis of ballooned hepatocytes by stabilization of the lysosomal membrane [36], and improvement of the functional activity of the remaining hepatocytes [37]. Other studies showed that the preferential increase in the number of HBV-specific CD8 T and CD4 T cells is associated with viral control rather than liver damage [38, 39]. Whatever the mechanism of AE, a few weeks are needed for sufficient suppression of the production of HBV-related proteins by preventing HBV replication even when NAs are used [40]. Thus, earlier introduction of CS in combination with potent antiviral therapy is a reasonable approach for the initial treatment of AE to prevent excessive immunological reactions and progression of liver cell injury [22, 41]. NA or CS used on its own has limits in the resolution of the serious conditions. Considered together, it is necessary to establish effective standardized strategies, such as the combination of NA and CS. Moreover, to provide cover for NA, especially for the time until NA starts to exert its potent antiviral effect, IFN could be added with NA and CS.

In conclusion, the results of this study suggest that viral kinetics before therapy may influence the clinical course and fate of patients with SAE complicating chronic hepatitis B. Antiviral therapies, including NA and/or IFN with CS, should be started as soon as possible in cases with high serum bilirubin and/or low PT levels, genotype B, and viral

load to prevent progression into hepatic decompensation. Although ethical issues could be an obstacle to randomized trials in such severe cases, more effective strategies are necessary for the treatment of AE associated with chronic hepatitis B.

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**Conflict of interest** The authors declare no conflict of interest.

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***Original Article***

# Prevalence and predictive factors of diabetes in hepatitis virus positive liver cirrhosis with fasting plasma glucose level of <126 mg/dL

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*Aim:* The aim of this study was to evaluate the prevalence and predictive factors of diabetes in hepatitis virus positive liver cirrhotic patients with fasting plasma glucose (FPG) level of <126 mg/dL.

*Methods:* A total of 263 patients with hepatitis C virus (HCV) or hepatitis B virus (HBV) positive liver cirrhosis, FPG level of <126 mg/dL, and had diabetes status evaluated by the use of 75-g oral glucose tolerance test (OGTT), were enrolled in this study. Plasma glucose and insulin levels were analyzed periodically for 3 h after oral glucose loading. Diabetes was defined as a 2-h post-load glucose on the OGTT of ≥200 mg/dL. The prevalence of diabetes by use of OGTT and predictive factors for diabetes were evaluated by the use of the Mann–Whitney *U*-test, Fisher's exact probability test or multivariate analysis by logistic regression. Hypoalbuminemia was defined as serum albumin level of <3.9 g/dL. Elevated indocyanine green retention rate at 15 min (ICG $_R$15) was regarded as ≥ 25%.

*Results:* Out of 263 patients, 44 (16.7%) were diagnosed as having diabetes. Multivariate analysis showed that diabetes occurred when patients had hypoalbuminemia of <3.9 g/dL (odds ratio [OR] 2.33; 95% confidential interval [CI] = 1.04–5.24; $P = 0.040$) and ICG $_R$15 of <25% (OR 2.36; 95%CI = 1.01–5.58).

*Conclusions:* Hypoalbuminemia and elevated ICG $_R$15 in hepatitis virus related cirrhotic patients with FPG level of <126 mg/day enhance diabetes pattern after OGTT with significant difference.

**Key words:** diabetes mellitus, hepatitis virus, liver cirrhosis, oral glucose tolerance test

## INTRODUCTION

HEPATITIS C VIRUS (HCV) is one of the more common causes of chronic liver disease worldwide. Chronic hepatitis C is an insidiously progressive form of liver disease that relentlessly but silently progresses to cirrhosis and/or hepatocellular carcinoma over a period of 10–30 years.[1–8] Lately, it have been reported that chronic HCV infection is associated with type 2 diabetes mellitus (T2DM).[9–11] Moreover, T2DM has been suggested to enhance with the development of HCC and poor prognosis of liver transplantation.[12–15] Thus, early intervention to prevent or improve T2DM is necessary to get good prognosis in HCV patients.

However, the big problem in chronic liver disease is that fasting serum glucose (FPG) often shows normal level. Hence, examination of oral glucose tolerance test (OGTT) is necessary to evaluate diagnosis of precise diabetes in patients with chronic liver disease. With this background, we evaluated the prevalence of abnormal glucose state and predictive factors for diabetes in HCV positive liver cirrhosis patients with fasting plasma glucose level of <126 mg/dL. We investigated the

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prevalence of abnormal glucose state and predictive factors for diabetes in hepatitis B virus (HBV) positive patients with liver cirrhosis and compared with HCV.

## METHODS

### Patients

A TOTAL OF 263 Japanese patients who were diagnosed with liver cirrhosis by laparoscopic and/or histological findings from December 1998 to January 2005 in the Department of Hepatology, Toranomon Hospital, Tokyo, Japan were enrolled. Inclusion criteria were as follows: (i) evidence of liver cirrhosis by laparoscopy and/or histological findings; (ii) FPG level of <126 mg/dL; (iii) evidence of HCV or HBV by serum examination; (iv) negativity for antinuclear antibodies, or antimitochondrial antibodies in serum, as determined by indirect immunofluorescence assay; (v) no evidence of HCC nodules as shown by ultrasonography and/or computed tomography; (vi) no underlying systemic disease, such as systemic lupus erythmatosus, rheumatic arthritis. Patients with any of the following criteria were excluded from the study: (i) advanced liver cirrhosis of encephalopathy, bleeding esophageal varices, or ascites, (ii) a history of diabetes, (iii) taking medicines that may influence on glucose tolerance such as branched chain amino acid (BCAA), thiazide diuretics, and angiotensin receptor antagonist.

A total of 263 patients with FPG of <126 mg/dL undertook a 75-g OGTT. Plasma glucose levels were analyzed periodically for 3 h after oral glucose loading. Impaired glucose tolerance (IGT) were defined as a 2-h post-load glucose on the OGTT of ≥140 mg/dL, but <200 mg/dL. Diabetes was defined as a 2-h post-load glucose on the OGTT of ≥200 mg/dL. T2DM was diagnosed by the use of the 2003 criteria of the American Diabetes Association.[16]

The index of insulin resistance was calculated on the fasting glucose and insulin by the homeostasis model for insulin resistance (HOMA-IR). Insulin secretion was calculated by the insulinogenic index (IGI); IGI = (Ins30-Ins0)/(Glc30-Glc0), Ins0: fasting plasma insulin (mU/L); $Ins_{30}$: insulin 30 min after glucose intake (IU/mL); Glc0: fasting plasma glucose (mg/dL); and Glc30; plasma glucose 30 min after glucose intake (mg/dL).

The physicians in charge explained the purpose and method of the OGTT to each patient. Informed consent was obtained from all patients included in the present study. All of the studies were performed retrospectively by collecting and analyzing data from the patient records. This study had been approved by the Institutional Review Board of our hospital.

### Clinical and laboratory analysis

Anthropometric analysis included height, weight and body mass index (BMI), and the latter was calculated as weight (kg) divided by the square of the height ($m^2$). Laboratory analysis was performed via standard laboratory methods. The biochemical parameters included aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyltransferase (GGT), total cholesterol, high density lipoprotein (HDL) cholesterol, triglyceride, albumin, platelet, fasting plasma glucose (FPG) and fasting insulin. Serum insulin levels were measured with a solid-phase radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA, USA). Hypoalbuminemia was defined as serum albumin level of <3.9 g/dL. Elevated indocyanine green retention rate at 15 min ($ICG_R15$) was regarded as ≥25%.

### Laboratory investigation

Hepatitis B surface antigen (HBsAg) was assayed using commercially available radioimmunoassay kits. Antibody against HCV was detected with a third-generation enzyme-linked immunoassay. HCV-RNA was determined by the Amplicor method (Cobas Amplicor HCV Monitor Test, v2.0, Roche, Tokyo, Japan). HbA1c was measured using a high performance liquid chromatography (HPLC) method. Height and weight were recorded at baseline and the body mass index (BMI) was calculated as weight (in kg)/height (in $m^2$).

### Statistical analysis

The results are presented as means ± standard deviation (SD) or as numbers. Statistical differences in quantitative data were determined using the Mann–Whitney *U*-test, Fisher's exact probability test or multivariate analysis by logistic regression.

Multivariate analysis for diabetes was carried out by logistic regression. The Statistical Program for Social Sciences software package (SPSS 11.5 for Windows, SPSS, Chicago, IL, USA) was used to perform statistical analysis. A $P$-value < 0.05 was considered to be statistically significant.

## RESULTS

### Patients' characteristics

TABLE 1 SHOWS the characteristics at the day of evaluating OGTT in the 263 enrolled patients. The

**Table 1** Clinical characteristics of cirrhotic patients with hepatitis C virus (HCV)†

| Characteristic | |
|---|---|
| *n* | 263 |
| Sex (male/female) | 178/85 |
| Age (years) | 51.6 ± 11.2 |
| Body mass index | 21.8 ± 3.0 |
| HBV/HCV | 96/167 |
| Fasting plasma glucose (mg/dL) | 84 ± 13 |
| Albumin (g/dL) | 4.1 ± 0.5 |
| Total cholesterol (g/dL) | 163 ± 37 |
| HDL cholesterol (g/dL) | 47 ± 13 |
| Triglyceride (mg/dL) | 98 ± 35 |
| Uric acid (mg/dL) | 5.2 ± 1.2 |
| AST (IU/L) | 78 ± 70 |
| ALT (IU/L) | 72 ± 68 |
| GGT (IU/L) | 74 ± 50 |
| Platelet (×104/mm$^3$) | 11.3 ± 4.1 |
| $ICG_R15$ (%) | 25.4 ± 14.0 |

†Data are number of patients or mean ± standard deviation.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, γ-glutamyltransferase; HBV, hepatitis B virus; HDL, high density lipoprotein; $ICG_R15$; indocyanine green retention rate at 15 min.

mean age was 51.6 years and mean FPG level was 84 mg/dL. The serum albumin level was 4.1 ± 0.5 g/dL and $ICG_R15$ was 26.5 ± 14.0%. On the diagnosis of liver cirrhosis, 160 of 263 patients were diagnosed by laparoscopy and liver biopsy; 69 patients were diagnosed by laparoscopy only; 34 patients were diagnosed by biopsy only.

## Prevalence of IGT and diabetes in hepatitis virus positive liver cirrhosis with FPG of <126 mg/dL

Out of 263 patients who had hepatitis virus-related liver cirrhosis with FPG of <126 mg/dL, 44 (16.7%) patients were diagnosed as having DM and 73 (27.8%) patients were diagnosed as having IGT. Table 2 shows the predictive factors for DM pattern by the use of OGTT in hepatitis virus related cirrhotic patients. Multivariate analysis showed that diabetes occurred when patients had hypoalbuminemia of <3.9 g/dL (odds ratio [OR] 2.33; 95% confidential interval [CI] = 1.04–5.24; $P = 0.040$) and $ICG_R15$ of <25% (OR 2.36; 95%CI = 1.01–5.58). Table 3 shows the incidence of diabetes based on serum albumin and $ICG_R15$. The incidence of diabetes in patients with hepatitis virus related liver cirrhosis was 5.8% (7/120) in group A with serum albumin level of ≥3.9 g/dL and $ICG_R15$ of <25%. On the other hand, that was 35.6% (21/59) in group B with hypoalbuminemia of <3.9 g/dL and $ICG_R15$ of ≥25%.

## Changes of glucose state based on difference of serum albumin level

Table 4 shows the glucose and insulin dynamics after OGTT in cirrhotic patients that belonged to group A with serum albumin level of ≥3.9 g/dL and $ICG_R15$ of

**Table 2** Predictive factors for diabetes in cirrhotic patients with hepatitis C virus (HCV)

| Variables | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|
| | OR (95% CI) | *P*-value | OR (95% CI) | *P*-value |
| Age (per 10 years) | 1.50 (1.04–2.16) | 0.031 | | |
| Gender (M/F) | 1.08 (0.52–2.23) | 0.842 | | |
| Body mass index (per 5) | 1.18 (0.59–2.36) | 0.631 | | |
| HCV/HBV | 2.38 (1.05–5.43) | 0.039 | | |
| AST (IU/L, ≥37/<37) | 1.01 (0.45–2.25) | 0.996 | | |
| ALT (IU/L, ≥42/<42) | 0.81 (0.40–1.65) | 0.563 | | |
| GGT (IU/L, ≥109/<109) | 1.89 (0.66–5.45) | 0.238 | | |
| Platelet (×10$^4$/mm$^3$, <10/≥10) | 2.59 (1.26–5.32) | 0.009 | | |
| Albumin (g/dL, <3.9/≥3.9) | 3.40 (1.66–6.94) | 0.001 | 2.33 (1.04–5.24) | 0.040 |
| Triglyceride (mg/dL, ≥150/<150) | 2.26 (0.74–6.90) | 0.152 | | |
| Total cholesterol (mg/dL, ≥180/<180) | 0.69 (0.30–1.60) | 0.387 | | |
| HDL cholesterol (mg/dL, <40/≥40) | 1.09 (0.43–2.73) | 0.857 | | |
| ICGR15 (%, ≥25/<25) | 3.64 (1.67–7.95) | 0.001 | 2.36 (1.01–5.58) | 0.049 |

ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, γ-glutamyltransferase; HBV, hepatitis B virus; HDL, high density lipoprotein; $ICG_R15$, indocyanine green retention rate at 15 min; OR, odds ratio.

**Table 3** Diabetic rate based on serum albumin and ICG $_R$15

| | Albumin; ≥3.9 g/dL | Albumin; <3.9 g/dL | Total |
|---|---|---|---|
| ICG $_R$15 < 25 (%) | 5.8% (7/120) | 15.0% (3/20) | 7.1% (10/140) |
| ICG $_R$15 ≥ 25 (%) | 20.3% (13/64) | 35.6% (21/59) | 27.6% (34/123) |
| Total | 10.9% (20/184) | 30.4% (24/79) | 16.7% (44/263) |

ICG$_R$15, indocyanine green retention rate at 15 min.

<25% or group B with serum albumin level of <3.9 g/dL and ICG $_R$15 of ≥25%. The serum glucose levels at 0, 60, 90, 120, and 180 min after the initiation of OGTT in patients with serum albumin level of <3.9 g/dL and ICG $_R$15 of ≥25% were statistically higher than those in patients with serum albumin level of ≥3.9 g/dL and ICG $_R$15 of <25%. HOMA-IR in patients with serum albumin level of <3.9 g/dL and ICG $_R$15 of ≥25% was higher than that in patients with serum albumin level of ≥3.9 g/dL and ICG $_R$15 of <25%. IGI in patients with serum albumin level of <3.9 g/dL and ICG $_R$15 of ≥25% was lower than that in patients with serum albumin level of ≥3.9 g/dL and ICG $_R$15 of <25%.

## DISCUSSION

WE HAVE DESCRIBED the prevalence of abnormal glucose state and predictive factors for diabetes in HCV or HBV positive liver cirrhosis patients with fasting plasma glucose level of <126 mg/dL in the present study. Enrolled patients had liver cirrhosis diagnosed with laparoscopy and/or histological examination. There are sometimes discrepancies between laparoscopic finding and histological findings in patients with HCV.[17] Thus, in the present study, cirrhotic patients diagnosed by either laparoscopy and/or histological examination were enrolled.

The present study shows several findings with regard to the prevalence of abnormal glucose state in hepatitis virus related cirrhotic patients with FPG level of <126 mg/dL. First, approximately 17% of the cirrhotic patients with FPG level of <126 mg/dL had diabetic pattern by the OGTT. If OGTT was not performed in patients who were diagnosed as having diabetes after OGTT, diabetes would be missed.

Second, multivariate analysis suggested that lower serum albumin level and elevated ICG$_R$15 were independent risk factors of diabetes mellitus. Our result shows that patients with hypoalbuminemia and elevated ICG$_R$15 should pay attention to complication of T2DM even if FPG is in the normal range. In the present study, hypoalbuminemia was defined as serum albumin level of <3.9 g/dL and elevated ICG $_R$15 was regarded as ≥25%. As the serum albumin level (mean ± standard deviation)

**Table 4** Glucose and Insulin dynamics after oral glucose tolerance test (OGTT) in cirrhotic patients

| | Group A (albumin; <3.9 g/dL) (ICG $_R$15; ≥25%) | Group B (albumin; ≥3.9 g/dL) (ICG $_R$15; <25%) | *P*-value |
|---|---|---|---|
| Number | 59 | 120 | |
| HOMA-IR | 3.22 ± 2.24 | 2.14 ± 1.12 | 0.003 |
| IGI | 0.70 ± 0.53 | 0.96 ± 0.83 | 0.042 |
| Glucose (mg/dL) | | | |
| At 0 min | 89.3 ± 12.0 | 82.8 ± 10.3 | 0.034 |
| At 30 min | 170.3 ± 42.1 | 159.4 ± 31.4 | 0.058 |
| At 60 min | 200.6 ± 70.7 | 168.0 ± 49.9 | 0.020 |
| At 90 min | 206.3 ± 64.8 | 168.0 ± 62.0 | 0.002 |
| At 120 min | 179.7 ± 68.5 | 136.3 ± 49.0 | <0.001 |
| At 180 min | 153.4 ± 64.2 | 117.7 ± 59.3 | <0.001 |
| Insulin (mI/L) | | | |
| At 0 min | 14.1 ± 9.4 | 10.4 ± 5.1 | 0.011 |
| At 30 min | 72.1 ± 36.3 | 75.1 ± 31.9 | 0.758 |
| At 120 min | 138.4 ± 76.5 | 102.1 ± 62.8 | 0.004 |

HOMA-IR, homeostasis model for insulin resistance; IGI, insulinogenic index.

of the approximately 70 000 subjects without liver damage and kidney damage in our hospital was 4.5 ± 0.3 g/dL, lower limit of normal albumin level was defined as 3.9 g/dL (=mean−2 × standard deviation). On $ICG_R15$, we divided the patients into two groups based on mean level of 25%. The hypoalbuminemia and elevated $ICG_R15$ indicates the severity of liver cirrhosis. Thus, our results suggest that severity of liver cirrhosis was the most important factor for predicting T2DM. The reported predictive factors of diabetes mellitus in liver cirrhosis were age, male, BMI, and Child–Pugh score.[9,10,18–21] Quintana *et al.* have reported hypoalbuminemia as risk factor of diabetes mellitus in cirrhotic patients.[22] On the other hand, EL-Serag *et al.* have reported that hepatogeneous diabetes is less frequently associated with risk factors such as age, BMI, and family history of diabetes.[23]

Third, patients with serum albumin level of <3.9 g/dL and ICG $_R$15 of ≥25% revealed high insulin resistance and low insulin secretion compared to patients with serum albumin level of ≥3.9 g/dL and ICG $_R$15 of <25%. This result suggests that insulin resistance and insulin secretion are associated with the onset of diabetes in advanced liver cirrhosis.

The precise mechanism of hepatogeneous diabetes is not precisely known. The possible mechanism is the following: (i) insulin resistance of muscle and adipose tissue; and (ii) impairment of the insulin secretion activity of the beta-cells of the pancreas.[24,25] Our results show the elevated insulin resistance and decrease of insulinogenic index. Thus, our results agreed with the possible mechanism of hepatogeneous diabetes.

The limitation of present study is that our cohort contains Japanese patients only. Thus, the result needs to be confirmed in other ethnic groups. Moreover, in patients with chronic liver disease, HbA1c levels have been seen to be apparently lower than real values due to a shortened half-life of erythrocytes originating from hypersplenism.[26] Thus, we could not evaluate the HbA1c in the present study.

In conclusion, our data suggest that physicians in charge of hepatitis virus related cirrhotic patients with hypoalbuminemia and elevated ICG $_R$15 should pay attention to complication of diabetes.

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# Prediction of early HBeAg seroconversion by decreased titers of HBeAg in the serum combined with increased grades of lobular inflammation in the liver

**Authors' Contribution:**
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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## Summary

**Background:** Hepatitis B e antigen (HBeAg) seroconversion is an important hallmark in the natural course of chronic hepatitis B. This study was designed to predict early HBeAg seroconversion within 1 year, by not only biochemical and virological markers, but also pathological parameters in patients with chronic hepatitis B.

**Material/Methods:** In a retrospective cohort study, 234 patients with HBeAg were reviewed for demographic, biochemical, virological and pathological data at the time of liver biopsy. Then, the patients who accomplished HBeAg seroconversion within 1 year thereafter were compared with those who did not, for sorting out factors predictive of early HBeAg seroconversion.

**Results:** Early HBeAg seroconversion occurred in 58 (24.8%) patients. In univariate analysis, factors predictive of early HBeAg seroconversion were: alanine aminotransferase (ALT) (p=0.002), IP-10 (p=0.029), HBsAg (p=0.003), HBeAg (p<0.001), HBV DNA (p=0.001), HBcrAg (p=0.001), core-promoter mutations (p=0.040), fibrosis (p=0.033) and lobular inflammation (p=0.002). In multivariate analysis, only serum HBeAg levels <100 Paul Ehrlich Institute (PEI) U/ml and grades of lobular inflammation ≥2 were independent factors for early HBeAg seroconversion (odds ratio 8.430 [95% confidence interval 4.173–17.032], p<0.001; and 4.330 [2.009–9.331], p<0.001; respectively).

**Conclusions:** HBeAg levels < 100 PEIU/ml combined with grades of lobular inflammation ≥2 are useful for predicting early HBeAg seroconversion. In patients without liver biopsies, high ALT levels (≥200 IU/L) can substitute for lobular inflammation (grades ≥2).

**key words:** **alanine aminotransferase • chronic hepatitis • hepatitis B virus • hepatitis B e antigen • lobular inflammation • seroconversion**

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## Background

Worldwide, an estimated 350 million people are infected with hepatitis B virus (HBV) persistently [1,2]. HBV infection is a major global concern, because up to 40% of patients can develop grave complications, such as decompensated cirrhosis and hepatocellular carcinoma (HCC) [3]. In the natural course of chronic hepatitis B, HBeAg seroconversion, defined by the loss of HBeAg and development of the corresponding antibody (anti-HBe), is an important hallmark, because it is highly correlated with a favorable long-term outcome. Seroconversion is usually followed by sustained suppression of HBV DNA, normalization of alanine aminotransferase (ALT) levels, and clinical remission accompanied by ameliorated necro-inflammatory activities in the liver [4–6].

To date, a number of factors have been found to predispose patients to spontaneous HBeAg seroconversion [7–19]. However, few studies have evaluated pathological factors for predicting early HBeAg seroconversion. In a small series of patients from Spain, the Knodell's index of histological activity was one of the independent predictors of early HBeAg seroconversion [14]. Recently, novel markers of the replication of HBV were introduced, such as levels of HBsAg, HBeAg and HBcrAg (HBV core-related antigen), which can replace HBV DNA levels. These serological markers of HBV replication have been evaluated for sensitive and reliable prediction of early HBeAg seroconversion [20–23]. In the present study, an attempt was made to select factors predictive of early HBeAg seroconversion, from among many biochemical, virological and pathological parameters, based on the data of 234 HBeAg-positive patients with chronic hepatitis B.

## Material and Methods

### Patients and study design

This is a retrospective cohort study with use of stored sera and liver biopsy specimens from patients with chronic hepatitis B who were taken care of in the Hepatology Department, Nagasaki Medical Center, Japan, during 1991 through 2005. The clinical database was reviewed to identify consecutive patients who underwent liver biopsies and had been followed for longer than 1 year. The inclusion criteria were presence of hepatitis B surface antigen (HBsAg) for 6 months or longer, positivity for HBeAg at the time of liver biopsy, and lack of antiviral treatments before receiving liver biopsies. The exclusion criteria were co-infection with hepatitis C virus (HCV) or human immunodeficiency virus type-1, serological markers suggestive of autoimmune disease, daily intake of alcohol >50 g, recent exposure to hepatotoxic drugs, and no stored sera available. They were followed every 3 months or more frequently, if indicated clinically, and their serum samples were monitored for liver biochemistry and serologic markers of HBV infection, including HBsAg, HBeAg, anti-HBe, HBV DNA and HBcrAg. Serum samples had been stored at –20°C until use.

Antiviral therapy was commenced immediately in the patients with: (1) significant fibrosis/cirrhosis detected by liver biopsy; and (2) evidence of decompensation, such as ascites, varices and hepatic encephalopathy.

To identify predictors of early HBeAg seroconversion, clinical, biological, virological and pathological data at the time of liver biopsy were compared between patients who did and who did not achieve early HBeAg seroconversion, within 1 year after receiving liver biopsies, by univariate and multivariate analyses. Further, patients were stratified by independent factors for HBeAg seroconversion, and the cumulative incidence of HBeAg seroconversion was compared between groups using the Kaplan-Meier method. The study protocol complied with the Good Clinical Practice Guidelines and the 1975 Declaration of Helsinki, and was approved by the review board of the institution. Each patient gave a written informed consent before participating in this study.

### Routine laboratory tests for HBV markers

Quantitative measurements of HBsAg and HBeAg were carried out using commercial enzyme-linked immunosorbent assay (ELISA) kits in the ARCHITECT ANALYSER i2000 (Abbott Japan Co., Ltd., Tokyo, Japan) in accordance with the manufactures' instructions in Nagasaki Medical Center. The sensitivity of HBsAg assay ranged from 0.05 to 250 IU/ml. Sera with HBsAg >250 IU/ml were serially diluted 100-fold so as to include them within the dynamic range. HBeAg was quantified by a two-step immunoassay with use of chemiluminescence microparticles. Briefly, undiluted samples were mixed with paramagnetic beads coated with anti-HBe. After a washing step, conjugate and reactants were added for exciting emission of the light that is proportional to the concentration of HBeAg. The result was expressed by the ratio of relative light unit (RLU) of the sample to the cut-off RLU (S/CO). Samples with S/CO values >1.0 were regarded positive for HBeAg. Then, serial dilutions of the reference standard of PE HBeAg (Paul-Ehrlich Institute, Langen, Germany) were used to define the linear range of the assay and create a reference curve for linear regression. The linear range was 0.024–100 PEIU/ml. A standard curve was produced, and linear regression was used to convert assay results into appropriate units (PEIU/ml). For samples that fell outside the linear range of the assay, the assay was performed on serial dilutions to ensure the linearity.

### HBV DNA and HBcrAg

HBV DNA was determined by the COBAS Taqman HBV test (Roche Diagnostics K.K., Tokyo, Japan). Values under or over the detection range were recorded as 2.1 or 9.1 log copies/ml. HBcrAg was measured by the CLEIA HBcrAg assay kit (Fujirebio, Inc., Tokyo, Japan) in a fully automated analyzer (Lumipulse system, Fujirebio, Inc.). Values under or over the detection range were recorded as 3.0 or 7.0 log copies/ml. Assays for HBV DNA and HBcrAg were performed in a commercial clinical laboratory (SRL, Inc., Tokyo, Japan). Sera with values over the detection range were diluted to include them within the dynamic range.

### Interferon-inducible protein 10 (IP-10)

IP-10 was quantified by the Invitrogen Human IP-10 ELISA (Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturer's protocol in Nagasaki Medical Center.

### HBV genotyping

HBV DNA was extracted from serum (100 µl) with use of the SMITEST EX R&D extraction kit (MBL Co., Ltd., Nagoya, Japan). It was amplified for determination of genotypes by

**Table 1.** Histological evaluation of liver biopsy specimens.

| (A) Fibrosis staging | |
|---|---|
| **Stage** | **Fibrosis** |
| 0 | None |
| 1 | Enlarged, fibrotic portal tracts |
| 2 | Periportal or portal-portal septa but intact architecture |
| 3 | Fibrosis with architectural distortion without obvious cirrhosis |
| 4 | Probable or definite cirrhosis |

| (B) Inflammation grading | | | |
|---|---|---|---|
| **Grade** | **Portal/periportal activity** | | **Lobular inflammation** |
| | **Piecemeal necrosis** | **Lymphocyte aggregation** | |
| 0 | None or minimal | None | None |
| 1 | Inflammation only | < 1/3 in portal triad | Inflammation alone |
| 2 | Mild | 1/3–2/3 in portal areas | Focal necrosis or acidphil bodies |
| 3 | Moderate | > 2/3 in portal areas | Severe focal cell damages |
| 4 | Severe | Entire portal triad | Damage with bridging necrosis |

the SMITEST HBV Genotyping Kit (MBL Co., Ltd.) based on hybridization with type-specific probes immobilized on a solid-phase support [24].

### Precore stop codon (G1896A) and core promoter (A1762T/G1764A) mutations

A1896 mutation in the precore (PreC) region was detected by the enzyme-linked minisequence assay (SMITEST HBV PreC ELMA, Roche Diagnostics, Tokyo, Japan), and mutations in the core promoter (CP) region for T1762/A1764 by the enzyme-linked specific probe assay (SMITEST HBV Core Promoter Mutation Detection Kit, Roche Diagnostics K.K.). The results were recorded as "the wild-type" and "mutant types" dominantly expressed by HBV isolates [25].

### Histological examination

Liver biopsy was taken by fine-needle aspiration (16G sonopsy) guided by ultrasonography. Biopsy specimens were fixed in 10% neutral formalin, cut at 3- to 4-μm thickness, and stained with Hematoxyline-Eosin and Azan-Mallory, as well as for silver to visualize reticuline fibers. Tissue sections were examined independently by two senior liver pathologists. For each biopsy specimen, a protocol was filled out for grading necro-inflammation and staging fibrosis by the criteria of Desmet et al. [26] and Scheuer [27] (Table 1). As for the portal activity, not only piecemeal necrosis, but also lymphocytic aggregation was categorized into 5 (0–4) grades in the respective area involved.

### Statistical analysis

Continuous variables were compared between groups by the Mann-Whitney *U* test, and categorical variables by $\chi^2$ and Fisher's exact tests. The cumulative incidence of HBeAg seroconversion was calculated using the Kaplan-Meier method, and the difference was evaluated by the log-rank test. Multiple logistic regression analysis was performed to identify independent factors in significant association with early HBeAg seroconversion. A p value <0.05 was considered significant. Statistical analyses were performed using the SPSS version 17.0 software package (SPSS Inc., Chicago, IL, USA).

## Results

### Baseline characteristics of patients

Among the 673 patients with HBsAg who had received liver biopsies in our hospital during 1991 through 2005, 234 (34.8%) patients who met the inclusion criteria were enrolled in this study. Demographic and laboratory characteristics at the time of liver biopsy are listed in Table 2. They had a median age of 37 years (range: 12–74), and 161 (69%) were men. Of them, 231 (99%) were infected with HBV of genotype C. The median serum ALT level at the baseline was 141 IU/l (range: 13–2644 IU/l), and the median duration of follow-up was 86.5 months (range: 12.0–213.0 months). During the follow-up, 91 (39%) received antiviral treatment, with interferon (IFN) or lamividine, or the combination thereof.

### Comparison of clinical features between patients with and without early HBeAg seroconversion

Early HBeAg seroconversion, within 1 year after receiving liver biopsies, was achieved by 58 of the 234 (24.8%) patients. In univariate analysis, factors predictive of early HBeAg seroconversion were: ALT (p=0.002), IP-10 (p=0.029), HBsAg (p=0.003), HBeAg (p<0.001), HBV DNA (p=0.001), HBcrAg (p<0.001), CP mutations (p=0.040), fibrosis (p=0.033) and lobular inflammation (p=0.002). Other factors including age, albumin, platelets, AFP, PreC mutation, cell infiltration and