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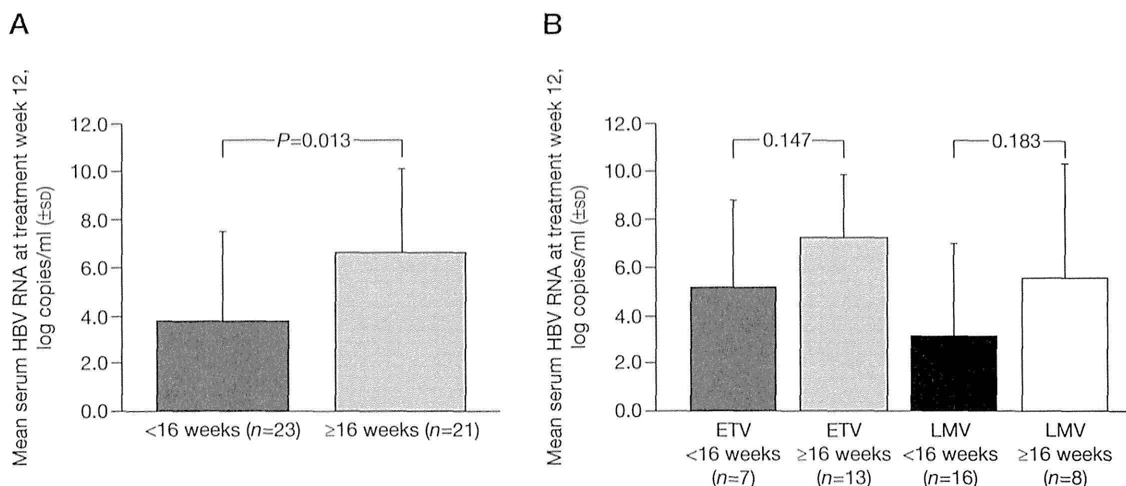
Figure 2. Serum HBV RNA levels at week 12 with intervals from detectable to undetectable serum HBV DNA level <16 weeks versus ≥16 weeks



(A) Serum HBV RNA levels at week 12 in chronic hepatitis B patients with intervals from detectable to undetectable serum HBV DNA level <16 weeks was significantly lower than those with interval ≥16 weeks (3.8 ±3.8 versus 6.6 ±3.5 log copies/ml, P=0.013). (B) Serum HBV RNA level at week 12 in chronic hepatitis B patients based on entecavir (ETV) and lamivudine (LMV) therapy with intervals from detectable to undetectable serum HBV DNA level <16 weeks was comparable to those with interval ≥16 weeks.

serum qHBsAg level at week 12 of therapy in Cox regression analysis (Table 3).

Discussion

In this study, we focused on on-treatment predictors of initial virological response and found serum HBV RNA at week 12 of therapy as a novel predictor, independent of serum HBV DNA level at week 12, qHBsAg level at week 12 or pretreatment serum ALT level. In the Cox regression models of on-treatment predictors, we included on-treatment HBV DNA and qHBsAg instead of their pre-treatment counterparts. Furthermore, we avoided concomitant inclusion of both pre- and on-treatment week 12 HBV DNA and qHBsAg levels due to the issue of multicollinearity, which may generate inaccurate individual predictors.

CHB patients with interval from detectable to undetectable serum HBV DNA level <16 weeks had a significantly lower serum HBV RNA level at week 12 of NA therapy than those with interval ≥16 weeks (Figure 2A). Furthermore, a low serum HBV RNA level at week 12 independently predicted a shorter interval to undetectable HBV DNA level (Table 3). Apart from serum HBV DNA level, serum HBV RNA was the only independent on-treatment predictor of initial virological response in such patients.

The AASLD guidelines for lamivudine-treated CHB patients recommend measurement of serum HBV DNA every 3–6 months (12–24 weeks) [12]. With a roadmap approach, primary non-response in NA-treated CHB patients was assessed at week 12 of therapy [16]. In addition, primary treatment failure is defined by changes in serum HBV DNA levels at week 12 on monitoring for the development of resistance [18]. Furthermore, our previous study suggested serum HBV RNA at week 12 of lamivudine therapy could predict early emergence of YMDD mutation [8]. This present study showed serum HBV RNA level at treatment week 12 predicted time to undetectable serum HBV DNA, supporting the usefulness of on-treatment week 12 monitoring of NA-treated patients.

Serum HBV RNA levels tend to correlate better with serum qHBsAg than with serum HBV DNA levels (Figure 1). Serum qHBsAg poorly predicts NA treatment outcomes; however, HBeAg-positive patients with elevated ALT are likely to experience a decrease in qHBsAg during NA therapy [15]. This decrease is parallel with the gradual decrease in serum HBV RNA during NA therapy as we previously reported [9]. By contrast, serum HBV DNA usually displays a more rapid decrease and thus does not correlate as well with serum HBV RNA.

[AU:OK] OK

This present study showed that the amount and detectability of serum HBV RNA were higher in entecavir as compared with lamivudine-treated patients, which is consistent with our previous report [9]. Entecavir is more potent than lamivudine in the inhibition of

Table 3. On-treatment predictors of initial virological response[a] during nucleoside/nucleotide analogue therapy by Cox regression analysis[b]

Variable	Adjusted hazard ratios	95% CI	*P*-value
Serum HBV RNA level at week 12	0.908	0.829, 0.993	0.035
Serum HBV DNA level at week 12	0.717	0.563, 0.913	0.007
Quantitative HBsAg level at week 12	1.524	0.981, 2.368	0.061
Pre-treatment ALT level	1.820	0.919, 3.606	0.086

[a]Duration to undetectable HBV DNA. [b]P=0.048. All variables were logarithm transformed before included into the analysis.

serum HBV DNA [12]. Thus, as compared with lamivudine, entecavir may potently inhibit reverse transcriptase more, leading to a higher level of serum HBV RNA. By contrast, entecavir or lamivudine does not have direct effect on serum qHBsAg as reflected by the poor predictive value of serum qHBsAg levels in therapeutic outcomes of NA treatments [16] and the comparable serum qHBsAg levels between entecavir- or lamivudine-treated patients as shown in this study. These findings confirm that serum HBV RNA level, but not qHBsAg, may reflect the antiviral potency of NAs. Furthermore, serum HBV RNA, but not qHBsAg, independently predicts initial virological response in both entecavir- and lamivudine-treated patients.

In contrast to a rapid decrease in serum HBV RNA observed in individuals treated with combination of NA and interferon [9], our previous study showed a gradual decrease of serum HBV RNA in NA-treated patients. Thus, the inhibitory effect of interferon on HBV RNA replicative intermediates may potentiate the suppression of HBV replication [9]. The findings presented in this study suggest that low on-treatment serum HBV RNA could predict earlier HBV suppression and response to NA therapy. Taken together, serum HBV RNA might be useful for optimizing treatment outcomes in patients with CHB, including a shift to more effective oral antiviral drugs or to immunomodulatory interferon.

[AU: OK?]

OK

Randomized double-blind trials have shown that the mean log HBV DNA difference between lamivudine and entecavir therapy was approximately 0.5 to 0.8 copies/ml at treatment weeks 12 and 24 [19,20]. In the present study, the mean log HBV RNA difference between lamivudine and entecavir therapy was 2.7 and 3.3 copies/ml at treatment weeks 12 and 24, respectively. This difference could not merely be explained by the stronger suppression of HBV DNA by entecavir as compared to lamivudine, instead, suggesting the presence of higher level of serum HBV RNA under entecavir therapy.

[AU:Please reword for clarity]

OK

The specific presence of serum HBV RNA in CHB patients treated with NA was validated in our previous study using ribonuclease digestion [8]. We have also previously reported persistently detectable serum HBV RNA during NA therapy, although it was inhibited under sequential lamivudine and interferon therapy [9]. Rokuhara *et al.* [21] have shown that HBV RNA was detectable before lamivudine therapy in serum samples of 24 patients; however, the detection rate was not specified. Their results of sucrose density gradient fractionation studies indicated that viral particles containing HBV DNA were dominant at the start of treatment, whereas those containing HBV RNA became more prevalent after 1 and 2 months of treatment. They also suggested that under untreated conditions, viral particles containing HBV RNA accounted for only approximately 1% of total HBV virions. These specific particles became the major component under lamivudine treatment [7]. Furthermore, Rokuhara *et al.* [21] reported a more significant decrease of serum HBV DNA than HBV RNA levels during lamivudine therapy, which support our findings on the poor immediate inhibition of serum viral particles containing HBV RNA by NAs [9].

There were several limitations in this study. First, the enrolled number of patients was relatively small; however, we were able to report that serum HBV RNA is a suitable independent on-treatment predictor. In daily clinical practice, complete collection of samples at several time points (pre- and on-treatment) and maintenance of good quality easily degradable RNA samples by timely handling as well as storage in -80°C remain a daunting challenge. Second, the predictive role of serum HBV RNA in long-term outcomes of these NA-treated patients was unclear. The evaluation of long-term outcomes of such patients was difficult due to the variable duration of NA therapy and the shift to interferon therapy in some.

In conclusion, on-treatment low serum HBV RNA level at treatment week 12 independently predicts initial virological response in NA-treated patients with CHB and further large studies are needed to confirm these observations.

Acknowledgements

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Disclosure statement

Y-WH has served as a speaker for GlaxoSmithKline and Bristol–Myers Squibb. KC has served as a speaker and a received grant from Bristol–Myers Squibb. D-SC, S-SY and J-HK have served as a speaker, a consultant and an advisory board member for GlaxoSmithKline and Bristol–Myers Squibb. All other authors declare no competing interests.

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CORRESPONDENCE

Reappearance of serum HBV DNA in patients with hepatitis B surface antigen seroclearance

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Key words: HBV DNA; reactivation; corticosteroid; CD20; HBsAg.

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To the Editor:

We read with great interest the article by Di Bisceglie et al.[1] In this report from the United States, the recommendation that all patients undergoing chemotherapy, immunosuppressive therapy, hematopoietic stem cell transplantation or solid organ transplantation be screened for active or prior HBV infection by testing for HBsAg and anti-HBc in serum. This problem is also serious in Japan, where no universal vaccination programs against HBV exist, and HBV infections are still viewed as important issues.[2,3]

The elimination of HBsAg is one of the goals in the treatment of HBV infection. We examined the incidence of the reappearance of HBV DNA in chronic hepatitis B patients. We compared the backgrounds of 9 patients who achieved HBsAg seroclearance treated by nucleoside analogues (NAs group) with those of 13 patients in whom natural HBsAg seroclearance occurred (control group). We also evaluated HBV DNA levels at 4-12-month intervals after the disappearance of HBsAg. HBV DNA reappearance was defined as the detection of serum HBV DNA after the disappearance of both of HBV DNA and HBsAg. Age at HBsAg seroclearance in the NAs group and in the control group were 62±14 and 59±6.3 years, respectively. In the NAs group, 5 patients (56%) were treated with immunosuppressive agents (3, antibodies to CD20

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[rituximab]; 1, corticosteroid; and 1, tacrolimus; p = 0.011, compared with the control group [0%]). After HBsAg seroclearance, HBV DNA reappeared in 3 (33%) and 2 (15%) individuals in the NAs and control groups, respectively (Figure). Of interest, patients in whom HBV DNA reappeared after HBsAg seroclearance did not exhibit any elevation in ALT or the reappearance of HBsAg. There was no significant difference in patients' characteristics between patients with and patients without the reappearance of HBV DNA in either the NAs or control group.

HBV DNA reappearance was occasionally observed in chronic hepatitis B patients with HBsAg seroclearance, suggesting that reactivation occurs in patients who have recovered from hepatitis B and have anti-HBc but no detectable serum HBsAg.[1] Measurement of HBV DNA after HBsAg seroclearance may thus also be important in such patients receiving chemotherapy. Our data strongly support their recommendations.[1] Further understanding of the mechanism of HBV reactivation with or without immunosuppressant or anti-cancer drug treatment is needed.[1,3]

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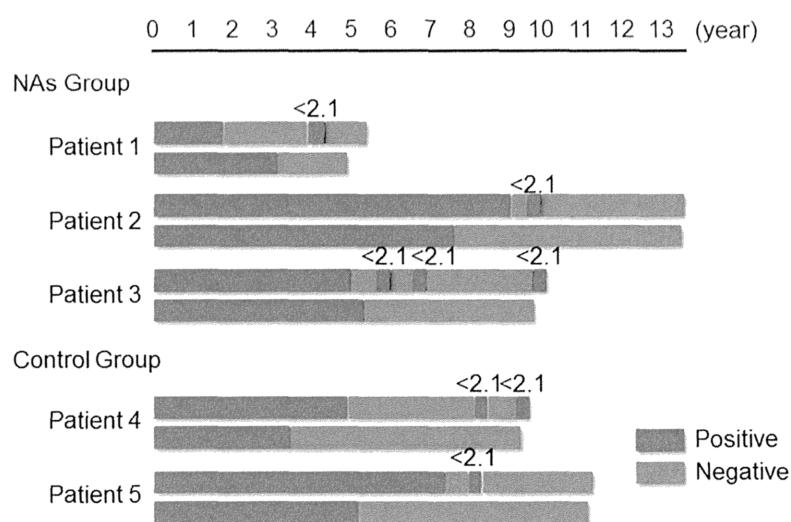
Figure. Patients with reappearance of HBV DNA after HBsAg-seroclearance.

In each patient, upper and lower bars indicate HBV DNA and HBsAg, respectively.



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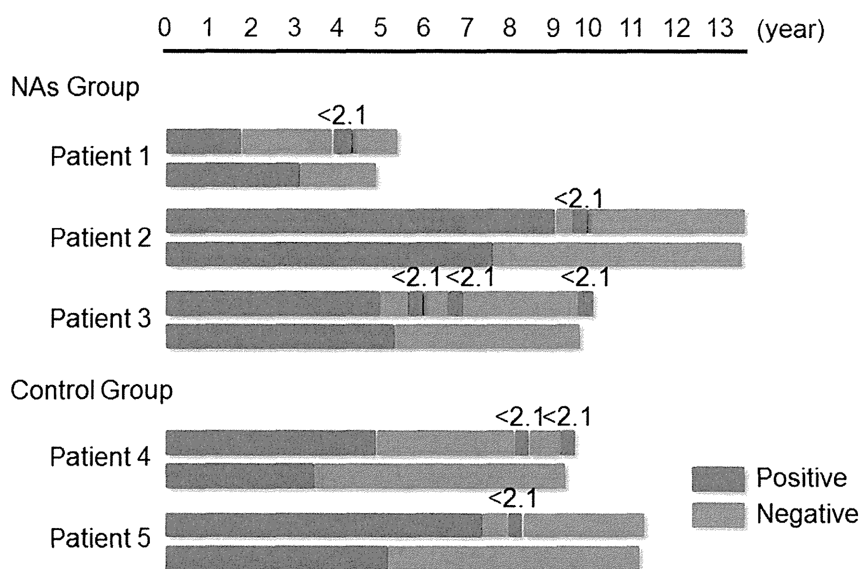

Figure. Patients with reappearance of HBV DNA after HBsAg-seroclearance.
In each patient, upper and lower bars indicate HBV DNA and HBsAg, respectively.
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Virological efficacy of combination therapy with corticosteroid and nucleoside analogue for severe acute exacerbation of chronic hepatitis B

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SUMMARY. The short-term prognosis of patients with severe acute exacerbation of chronic hepatitis B (CHB) leading to acute liver failure is extremely poor. We have reported the efficacy of corticosteroid in combination with nucleoside analogue in the early stages, but virological efficacy has not been documented. Our aim was to elucidate the virological efficacy of this approach. Thirteen patients defined as severe acute exacerbation of CHB by our uniform criteria were prospectively examined for virological responses to treatment. Nucleoside analogue and sufficient dose of corticosteroids were introduced as soon as possible after the diagnosis of severe disease. Of the 13 patients, 7 (54%) survived, 5 (38%) died and 1 (8%) received liver transplantation. The decline of HBV DNA was significant between the first 2 weeks ($P = 0.02$) and 4 weeks ($P < 0.01$). Mean reduction in HBV DNA during the first 2 weeks was 1.7 ± 0.9 log copies per mL in overall patients, 2.1 ± 0.8 in survived patients and 1.2 ± 0.9 in dead/transplanted patients. The decline of HBV DNA was significant between the first 2 weeks ($P = 0.03$) and 4 weeks ($P = 0.02$) in survived patients, but not in dead/transplanted patients. Our study shows that corticosteroid treatment in combination with nucleotide analogue has sufficient virological effect against severe acute exacerbation of CHB, and a rapid decline of HBV DNA is conspicuous in survived patients.

Keywords: chronic hepatitis B, corticosteroid, nucleoside analogue, severe acute exacerbation, viral reduction.

INTRODUCTION

An estimated 350 million persons worldwide are chronically infected with hepatitis B virus (HBV) [1]. Reactivation of HBV is a well-characterized syndrome marked by the abrupt reappearance or rise of HBV DNA in the serum of a patient with previously inactive or resolved HBV infection. Reactivation is often spontaneous, but can also be triggered by cancer chemotherapy, immune suppression or alteration in immune function. Acute exacerbation, which is characterized by a high alanine aminotransferase (ALT) level and jaundice, sometimes occurs and may progress to acute liver failure (ALF) and death. The short-term prognosis of patients with severe acute exacerbation of chronic hepatitis B (CHB) leading to ALF is extremely poor [2–4]. Liver transplantation has been the only definitive therapy available to salvage this group of patients. However, the problem of a shortage of donor livers still remains in Japan. Moreover, according to the most recent Japanese nationwide survey of 2009–2010, 'none' of the patients with fulminant liver failure among HBV carriers recovered without liver transplantation, and most patients had no indication for liver transplantation because of complicating malignant or nonmalignant diseases, and old age (Fig. 1) [5,6]. Thus, therapies other than transplantation must be further investigated.

In HBV infection, liver injury is considered to be induced mainly by cytotoxic T-lymphocyte-mediated cytolytic pathways in HBV-infected hepatocytes [7], and it was suggested that treating CHB patients with corticosteroid (CS) to inhibit an excessive immune response and prevent cytolysis of infected hepatocytes would be reasonable, if the HBV could be controlled [8].

Nucleoside analogues (NA), such as lamivudine (LMV), entecavir (ETV) and tenofovir (TDF), have therapeutic effects on CHB. They can markedly suppress HBV

Abbreviations: ACLF, acute-on-chronic liver failure; ALF, acute liver failure; CHB, chronic hepatitis B; CS, corticosteroid; ETV, entecavir; HBV, hepatitis B virus; LMV, lamivudine; TDF, tenofovir.

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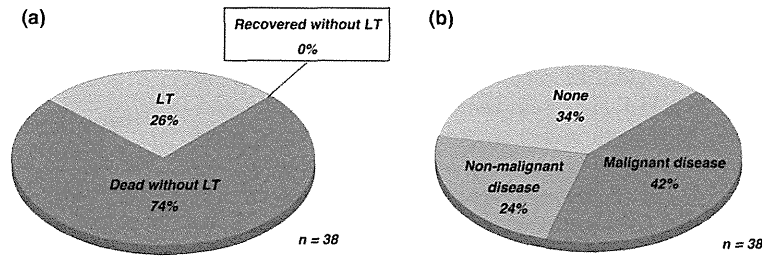

Fig. 1 Outcome **(a)** and primary disease **(b)** of patients with fulminant hepatitis associated with HBV carrier in the Japanese nationwide survey of 2009–2010. None of the patients recovered without liver transplantation (LT), and most patients (66%) had primary disease.

replication by suppression of HBV polymerase activity. In recent studies, it has been reported that the rapid reduction in HBV DNA is a good predictor for the survival of patients of acute-on-chronic liver failure (ACLF) associated with HBV treated with NA monotherapy [9,10].

In our previous studies, we reported that the introduction of high-dose CS and NA could significantly reverse deterioration in patients with 'clinically severe, life-threatening' exacerbation of CHB compared with historical controls, when used in the early stage of illness and for more than a few weeks [11–13]. But the virological efficacy of the combination therapy with CS and NA is unknown.

In this study, we analysed patients with clinically severe acute exacerbation of CHB treated by the initiation of sufficient dosages and durations of CS and NA, to clarify the virological efficacy of the treatment.

MATERIALS AND METHODS

Patients

Thirteen patients with severe acute exacerbation of CHB admitted to our liver unit (Chiba University Hospital) between 2000 and 2012 were studied. The diagnosis of a CHB viral carrier state was made based on either the positivity of hepatitis B surface antigen (HBsAg) for at least 6 months before entry or, in patients with follow-up periods less than 6 months before entry, it was based on the positivity of HBsAg, the presence of antihepatitis B core antibody (HBcAb) at a high titre and negativity or a low titre of IgM antihepatitis B core antibody (IgM-HBc). Patients fulfilling all the following three criteria during the course were defined as having severe exacerbation: prothrombin time (PT) activity ≤60% of normal control, total bilirubin (T-Bil) ≥3.0 mg/dL and alanine transaminase (ALT) ≥300 IU/L during the course. Patients with PT activity ≤40% of control and hepatic encephalopathy were defined as having fulminant hepatitis. Patients with pre-existing liver cirrhosis were excluded. All patients were in poor general condition, including general malaise, fatigue, jaundice, oedema, ascites and encephalopathy.

The work described in this manuscript was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). Informed consent was obtained from all patients or appropriate family members.

All patients were negative for IgM anti-HAV antibody, anti-HCV antibody, HCV RNA, IgM anti-Epstein-Barr virus antibody (IgM-EBV), IgM antiherpes simplex antibody (IgM-HSV), IgM anticytomegalovirus antibody (IgM-CMV), antinuclear antibody, antismooth muscle antibody, liver kidney microsomal antibody and antimitochondrial antibody (AMA). Patients with recent exposure to drugs and chemical agents as well as those with recent heavy alcohol intake were ruled out. One patient was HIV positive but had no clinical evidence of acquired immune deficiency syndrome.

Treatment protocols

All patients treated were examined prospectively. Patients were treated with NA – LMV before 2007, ETV from 2007 and CS. Early introduction of CS was defined as follows: 40 mg or more of prednisolone (PSL) daily was administered within 10 days after the diagnosis of severe disease, using the above-mentioned criteria. This dosage was maintained for a minimum of 4 days. When the patient showed a trend towards of PT, the dosage was reduced by 10 mg at least every 4 days and tapered off. Patients for whom more than 10 days had already passed after the diagnosis were treated with delayed introduction of CS (delayed CS). Patients with marked prolongation of PT were treated with 1000 mg of methylprednisolone (MPSL) daily for 3 days followed by the same PSL therapy as that described above.

Lamivudine was administered at a daily dose of 100–300 mg (LMV group). ETV was administered at a daily dose of 0.5–1.0 mg (ETV group). Patients were also treated with intravenous glycyrrhizin, an aqueous extract of licorice root, at a daily use of 60–100 mL. This agent is reported to have anti-inflammatory activity and has been used for the treatment of acute and chronic liver injuries in Japan [14,15].

Serological markers

HBsAg, hepatitis B envelope antigen (HBeAg), anti-HBe antibody (HBeAb), HBcAb, IgM-HBc and IgM anti-HAV antibody were detected by commercial radioimmunoassay (Abbott Laboratories, Chicago, IL, USA), and second- or

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third-generation anti-HCV antibody was measured by enzyme immunoassay (Ortho Diagnostics, Tokyo, Japan). IgM-EBV, IgM-CMV and IgM-HSV were examined by enzyme-linked immunosorbent assays. Antinuclear antibody, antismooth muscle antibody and AMA were examined by a fluorescent antibody method, and AMA-M2 was examined by chemiluminescent enzyme immunoassay. The HBV DNA level was measured by Amplicor monitor assay (dynamic range 2.6–7.6 logcopies per mL, Roche Diagnostics, Tokyo, Japan) or COBAS TaqMan v.2.0 (dynamic range 2.1–9.0 logcopies per mL, Roche Diagnostics).

Statistical analysis

Differences in proportions among groups were compared by Fisher's exact probability test, Student's *t*-test and Welch's test.

RESULTS

Clinical features of patients with severe acute exacerbation at admission

Of the 13 patients, nine were men and four women. Mean age at the time of diagnosis was 48.9 ± 11.6 years. Five patients had primary disease and conditions (two rheumatoid arthritis, one gastrointestinal stromal tumour, one Non-Hodgkin lymphoma and one HIV positive without immunodeficiency), and four had been treated with immunosuppressive or cytotoxic drugs, suffering exacerbations after their withdrawal. Six patients were diagnosed with fulminant hepatitis on admission.

At admission to our unit, mean PT activity was 33 ± 11%, mean ALT was 968 ± 552 IU/L, and mean T-BIL was 12.6 ± 8.9 mg/dL. HBeAg/HBeAb status was +/− in 4, −/+ in 6 and +/+ in 3. Mean HBV DNA was 6.4 ± 1.7 logcopy per mL, mean alfa-fetoprotein (AFP) was 225 ± 272 ng/mL, and mean hepatocyte growth factor (HGF) was 6.5 ± 9.7 ng/mL. HBV genotype was examined in five patients, and three and two were genotype C and B, respectively. Precore/core promoter mutation was examined in nine patients, and two were wild/mutant, two mutant/wild, one mixed/wild and four mixed/mutant.

Type of therapies

As initial CS, six patients received 1000 mg of MPSL, one received 500 mg of MPSL, five received 60 mg of PSL, and one received 40 mg of PSL. Mean duration between the diagnosis of severe disease and introduction of CS was 5.2 ± 4.6 days, and mean duration of CS therapy was 53.5 ± 53.1 days. Eleven patients were treated with early CS and two with delayed CS. As NA, LMV was administered to seven patients and ETV to 6. In the six patients with fulminant hepatitis, artificial liver support (plasma exchange, hemodiafiltration and transfusion of fresh frozen plasma) was performed.

Outcome

Of the 13 patients, 7 (54%) survived and 5 (38%) died. The remaining one (8%), whose liver function did not recover with the combination therapy of CS and NA, underwent a liver transplantation and survived. Of the five dead patients, 4 (30%) were liver-related deaths and 1 (8%) was complication-related.

Biochemical responses to therapy

Changes in PT activities, ALT levels, T-Bil levels and HBV DNA levels after the introduction of combination therapy are shown in Fig. 2.

Mean PT activity was 31 ± 9% before initiation of the combination therapy (week 0), 50 ± 24% at 2 weeks after starting (week 2) and 58 ± 25% at 4 weeks (week 4). The improvement in PT activity was significant between week 0 and 2 and between week 0 and 4 ($P = 0.03$ and $P < 0.01$, respectively). The mean ALT level was 1055 ± 606 IU/L at week 0, 112 ± 101 at week 2 and 76 ± 48 at week 4. The decline in ALT was significant between week 0 and 2 and between week 0 and 4 ($P < 0.01$, respectively). The mean T-Bil level was 13.7 ± 8.7 mg/dL at week 0, 12.0 ± 9.1 at week 2 and 10.1 ± 9.5 at week 4, changes not reaching statistical significance in the 4 weeks.

Virological responses to therapy

Mean HBV DNA was 6.5 ± 1.7 log copies per mL at week 0, 4.8 ± 1.5 at week 2 and 3.6 ± 1.5 at week 4. The decline in HBV DNA was significant between week 0 and 2 and between week 0 and 4 ($P = 0.02$ and $P < 0.01$, respectively). The mean reduction in HBV DNA was 1.7 ± 0.9 log copies per mL between week 0 and 2, and 1.6 ± 1.3 log copies per mL between week 2 and 4.

Complication of combination therapy

After the start of treatment for severe acute exacerbation of CHB, three patients had additional complications, one with pneumonia due to pneumocystis and cytomegalovirus, one with pneumonia due to pneumocystis and one with enteritis due to methicillin-resistant staphylococcus aureus (MRSA).

Comparison between survived and dead/transplanted patients

Baseline differences in mean age, sex, ALT level, T-Bil level, PT activity, AFP, HGF and HBV DNA level were not statistically significant between survived patients and dead/

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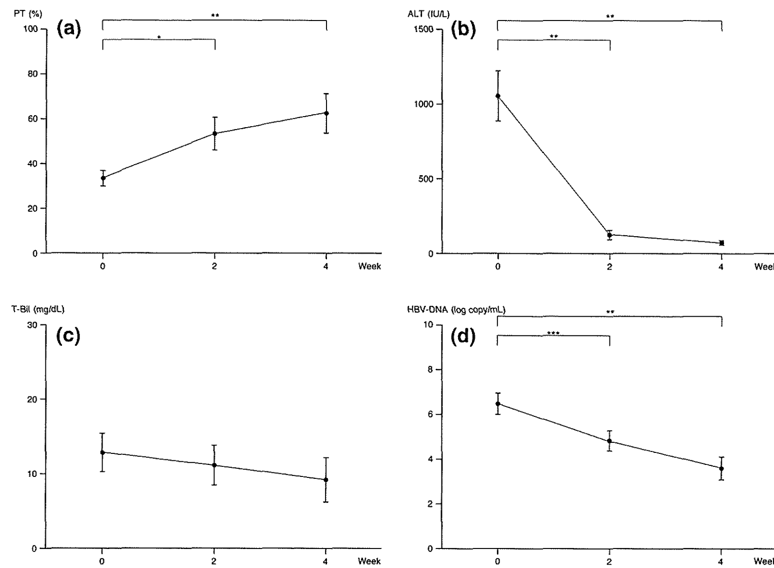

Fig. 2 Changes in prothrombin time (PT) activities **(a)**, alanine aminotransferase (ALT) **(b)**, total bilirubin (T-Bil) **(c)** and HBV DNA level **(d)** in 13 patients with severe acute exacerbation of chronic hepatitis B treated with corticosteroid in combination with nucleoside analogue; *P = 0.03, **P < 0.01, ***P = 0.02.

Table 1 Comparison of characteristics between survived and dead/transplanted patients

	Survived n = 7	Dead/transplanted n = 6	P
Age (years)	45.3 ± 10.3	53.2 ± 12.6	0.25
Sex (M/F)	6/1	3/3	0.27
Fulminant hepatitis on admission	1	5	0.03
LMV/ETV	4/3	3/3	1.00
PT (%)	36 ± 11	29 ± 11	0.28
ALT (IU/L)	1048 ± 628	875 ± 488	0.59
T-Bil (mg/dL)	12.7 ± 10.7	12.4 ± 7.1	0.97
AFP (ng/mL)	134 ± 234	351 ± 296	0.21
HGF (ng/mL)	1.9 ± 1.2	12.5 ± 12.3	0.13
HBV DNA (log copies per mL)	6.7 ± 1.6	6.0 ± 1.9	0.49
HBV DNA reduction (log copies per mL)			
Week 0–2	−2.1 ± 0.8	−1.2 ± 0.9	0.16
Week 2–4	−1.4 ± 0.8	−1.8 ± 1.8	0.72

LMV, lamivudine; ETV, entecavir; PT, prothrombin time; ALT, alanine aminotransferase; T-BIL, total bilirubin; AFP, alfa-fetoprotein; HGF, hepatocyte growth factor.

transplanted patients. The proportion of fulminant hepatitis was higher in dead/transplanted patients (P = 0.03) (Table 1).

Changes in PT activities, ALT levels, T-Bil levels and HBV DNA levels of both groups after the introduction of combination therapy are shown in Fig. 3. The improvement in PT activity was significant between week 0 and 2 and between week 0 and 4 (P = 0.01, respectively) in survived patients, but not significant in dead/transplanted patients. The decline of ALT was significant between week 0 and 2 and between week 0 and 4 in both groups (P = 0.03 and P = 0.02 in survived patients and P < 0.01 in dead/transplanted patients, respectively). In both groups, the changes in mean T-Bil levels were not significant at 4 weeks. The decline of HBV DNA was significant between week 0 and 2 and between week 0 and 4 (P = 0.03 and

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$P = 0.01$, respectively) in survived patients, but was not significant in dead/transplanted patients. The mean reduction in HBV DNA was 2.1 ± 0.8 log copies per mL between week 0 and 2 and 1.4 ± 0.8 log copies per mL between week 2 and 4 in survived patients, and 1.2 ± 0.9 log copies per mL between week 0 and 2 and 1.8 ± 1.8 log copies per mL between week 2 and 4 in dead/transplanted patients. The reduction in HBV DNA was not different between week 0 and 2 in both groups.

Comparison between LMV and ETV groups

Baseline differences in mean age, sex, the proportion of fulminant hepatitis, ALT level, T-Bil level, PT activity and HBV DNA level were not statistically significant between the LMV and ETV groups (Table 2).

Changes in PT activities, ALT levels, T-Bil levels and HBV DNA levels of both groups after the introduction of combination therapy are shown in Fig. 4. The improvement in PT activity was significant between week 0 and 4 ($P = 0.03$) in the ETV group, but was not significant in the LMV group. The decline in ALT was significant between week 0 and 2 and between week 0 and 4 in both groups (both $P < 0.01$ in the LMV group, $P = 0.02$ and $P = 0.01$ in the ETV group, respectively). In both groups, the changes in mean T-Bil levels were not significant at 4 weeks. The decline in HBV DNA was significant between week 0 and 4 ($P = 0.01$) in the ETV group, but was not significant in the LMV group. The mean reduction in HBV DNA was 1.4 ± 1.0 log copies per mL between week 0 and 2 and 1.3 ± 0.7 log copies per mL between week 2 and 4 in the LMV group, and 2.1 ± 0.7 log copies per mL between week 0 and 2 and 2.3 ± 2.3 log copies per mL between week 2 and 4 in the ETV group. The differences in reduction in HBV DNA were not significant between the two groups.

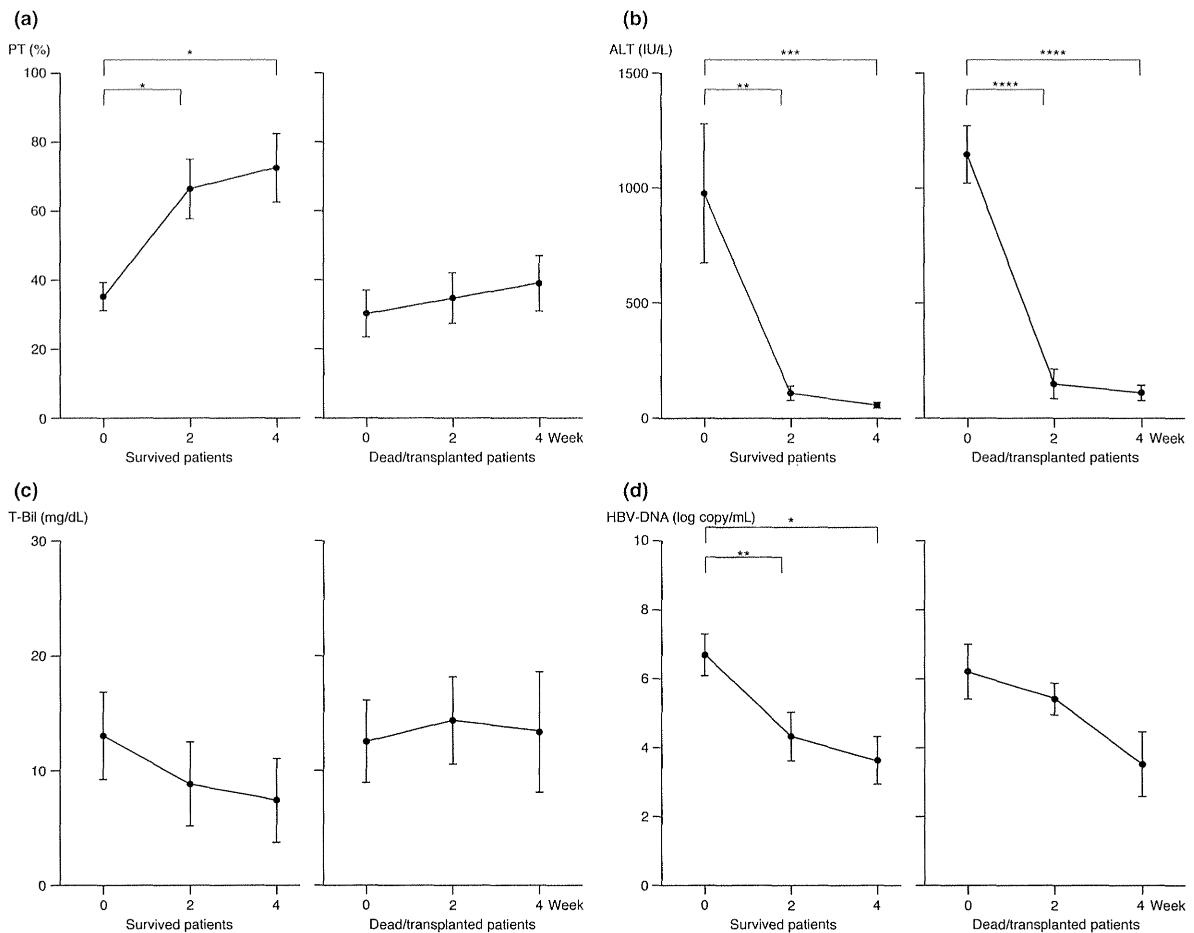

Fig. 3 Changes in prothrombin time (PT) activities **(a)**, alanine aminotransferase (ALT) **(b)**, total bilirubin (T-Bil) **(c)** and HBV DNA level **(d)** in survived and dead/transplanted patients; $*P = 0.01$, $**P = 0.03$, $***P = 0.02$, $****P < 0.01$.

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Table 2 Comparison of characteristics and outcome between LMV and ETV groups

	LMV group $n = 7$	ETV group $n = 6$	P
Age (years)	47.6 ± 13.7	50.5 ± 9.9	0.67
Sex (M/F)	6/1	3/3	0.27
Fulminant hepatitis on admission	4	2	0.59
PT (%)	28 ± 4	38 ± 15	0.17
ALT (IU/L)	833 ± 656	1126 ± 399	0.37
T-Bil (mg/dL)	11.5 ± 3.6	13.9 ± 13.0	0.63
HBV DNA (log copies per mL)	6.1 ± 1.7	6.8 ± 1.9	0.47
HBV DNA reduction (log copies per mL)			
Week 0–2	−1.4 ± 1.0	−2.1 ± 0.7	0.25
Week 2–4	−1.3 ± 0.7	−2.3 ± 2.3	0.22
Outcome			
Survived	4	3	1.00
Dead/transplanted	3	3	

LMV, lamivudine; ETV, entecavir; PT, prothrombin time; ALT, alanine aminotransferase; T-BIL, total bilirubin.

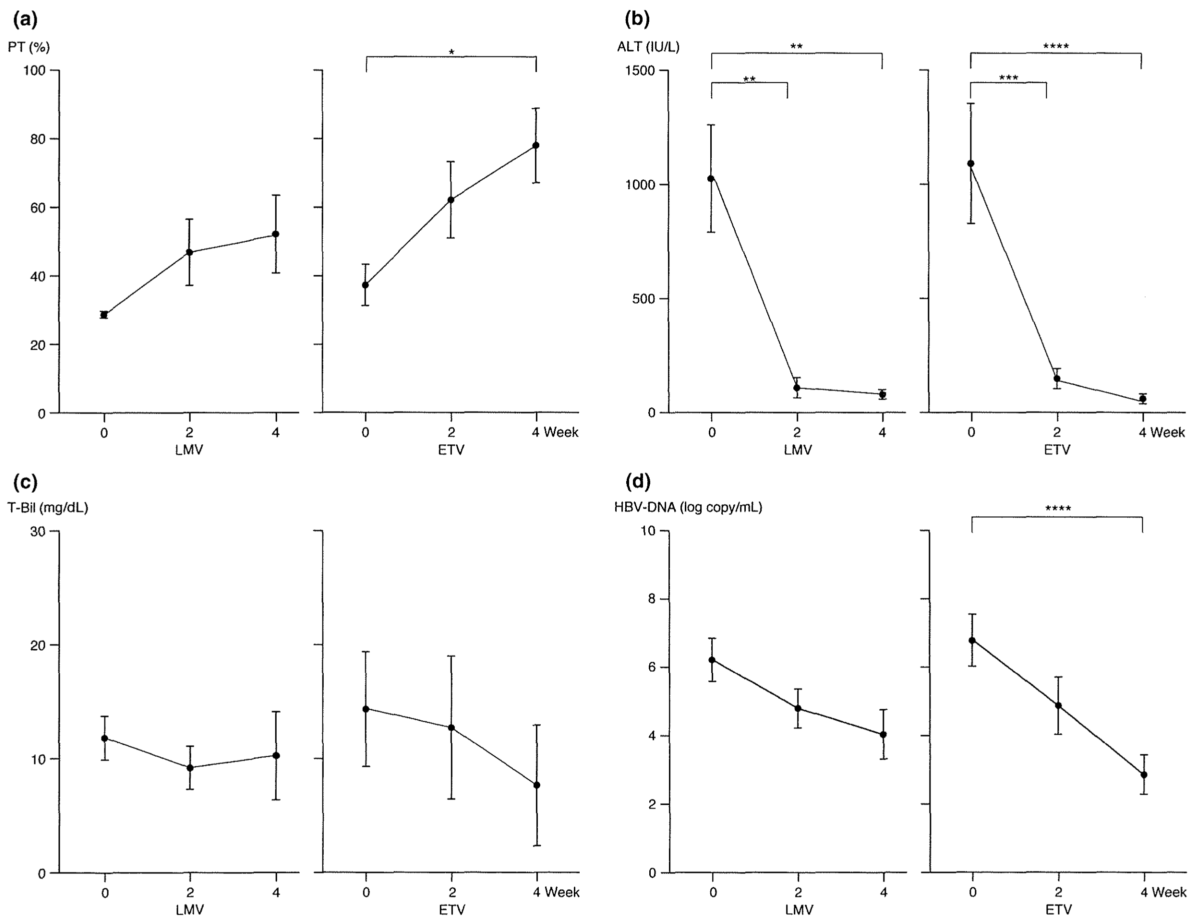

Fig. 4 Changes in prothrombin time (PT) activities **(a)**, alanine aminotransferase (ALT) **(b)**, total bilirubin (T-Bil) **(c)** and HBV DNA level **(d)** in the LMV and ETV groups; $^{*}P = 0.03$, $^{**}P < 0.01$, $^{***}P = 0.02$, $^{****}P = 0.01$.

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DISCUSSION

This study shows that CS treatment in combination with NA has the sufficient virological effect against severe acute exacerbation of CHB, and the rapid decline of HBV DNA is conspicuous in survived patients.

In this study, the combination therapy with CS and NA showed a rapid decline of HBV DNA especially in survived patients. In a recent randomized controlled study that evaluated the efficacy of TDF and determine the predictor of spontaneous reactivation of CHB with ACLF, more than 2 log reduction in HBV DNA levels at 2 weeks was found to be an independent predictor of survival, with the authors concluding that the reduction in HBV DNA level at 2 weeks is a desirable goal [10]. In another retrospective study that evaluated the efficacy of LMV and determined the predictor of CHB with ACLF, >2-log reduction at 4 weeks was found to be a good predictor of outcome [9]. The rapid decline of HBV DNA is one of the important factors for recovery. In our present study, the combination therapy with CS and NA achieved a desirable goal at week 2 in survived patients.

On the other hand, in a recent study from China that evaluated the efficacy of NA treatment with HBV-associated ACLF, LMV and ETV achieved significant viral suppression after 3 months, but did not improve survival [16]. Recently, NAs have been administered in severe reactivation of CHB. In one initial case series from Japan, three patients with cirrhosis who presented with severe acute exacerbation and hepatic encephalopathy responded dramatically to LMV treatment [17]. However, later studies did not demonstrate any benefit of LMV treatment for survival [3,4]. In the management of severe acute exacerbation of CHB, the rapid decline of HBV DNA is one of the important goals, but it is not sufficient to improve survival. It is reported that HBV DNA decreases rapidly with the administration of NAs, but improvements in liver function and liver regeneration are delayed by a few weeks to a few months [11,18,19]. During this time-lag phase, excessive immunological reaction may continue, liver cell injury may progress and liver regeneration may be impaired. Therefore, it is understood that additional rapid cessation of ongoing necro-inflammation is essential for the achievement of liver regeneration.

We have used CSs for the rapid cessation of necro-inflammation. In severe acute exacerbation of CHB, liver injury is considered to be induced mainly by cytotoxic T-lymphocyte-mediated cytolytic pathways of infected hepatocytes [7], and it has been suggested that treating CHB patients with CSs to inhibit an excessive immune response and prevent cytolysis of infected hepatocytes would be reasonable, if the HBV could be controlled [8]. Our present study showed that HBV is controllable in patients treated with immunosuppressive therapy. In a recent study from China that evaluated the combination therapy with short-term dexamethasone and LMV for pre-ACLF patients, no significant differences in HBV DNA levels were observed between the dexamethasone group and control group during the observation period [20]. In our previous studies, we reported that the early introduction of high-dose CS improve survival [11], the combination therapy with high-dose CS and NA could reverse deterioration of severe acute exacerbation of CHB [12] and that more than a few weeks of CS treatment in combination with NAs is required [13]. Additionally, we recently reported that the introduction of high-dose CS in the early stage of viral ALF suppressed the destruction of hepatocytes [21]. In another study from Japan evaluating the predictors of progression to hepatic decompensation during severe acute exacerbation of CHB, the authors concluded that antiviral therapies with CS should be started as soon as possible in cases with high T-Bil level and/or low PT levels [22]. Moreover, in a recent meta-analysis evaluating the safety, efficacy and side effects of glucocorticoid therapy for severe viral hepatitis B, treatment with glucocorticoids significantly increased the survival rate of patients with severe viral hepatitis B [23,24]. We believe that both rapid decline of HBV DNA and cessation of necro-inflammation are necessary to improve the survival of severe acute exacerbation of CHB, and the combination therapy of CS and NA is a reasonable strategy.

The decline of HBV DNA could be brought not only by NA but also the host immune response. In the randomized study of ACLF patients described above [10], the nine of fourteen patients had >2 log reduction in the HBV DNA level in the TDF group, otherwise none of nine patients had >2 log reduction in the placebo group at day 15. In another study [16], patients treated with NAs had significant reduced HBV DNA levels at weeks 2, 4, 6, 8, 10 and 12 compared with patients without NAs. Thus, NAs bring the decline of HBV DNA more effectively than the host immune response alone.

In a study from Hong Kong, ETV was associated with increased short-term mortality compared with LMV although the patients treated with ETV had superior virological response compared to those on LMV [25]. The cause of increased short-term mortality was unknown. In the present study, the recovery rate of patients treated with ETV was not different from that with LMV, and ETV-treated patients had significant reduction in HBV DNA at week 4. ETV is a potent HBV inhibitor with a high barrier to resistance and can therefore be confidently used as a first-line monotherapy for CHB [26].

The prognosis of patients with severe acute exacerbation of CHB leading to ALF is extremely poor. In the recent studies, Cui, *et al.* [16] reported that the survival at 3 months of HBV-associated ACLF was 49.25% for patients with NA treatment and 40.54% without NA treatment, and Garg, *et al.* [10] reported that the survival at 3 months of severe spontaneous reactivation of CHB presenting as ACLF was 57% for patients with TDF treatment and 15% with placebo. In the present study, overall survival of our patients

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was 54%, which is equal to those with NA treatment studies described above, but the proportion of fulminant liver failure at admission was 46% in our study which was higher than those in two studies described above (21% ($P = 0.08$) and 7% ($P = 0.03$), respectively). This clearly means that our patients had severer disease than those in two studies. Therefore, we suppose that the survival by combination therapy with CS and NA is not inferior to that by NA monotherapy, although we could not include placebo-controlled patients, considering the current knowledge of the poor prognosis of the patients. Almost our 'dead/transplanted' patients had already developed fulminant liver failure at admission to our units, the state of impaired liver regeneration. Therefore, we have administered combination therapy with early CS and NA according to the appropriate definition of severe disease before the development into fulminant liver failure.

Regarding adverse events, opportunistic infections occurred in three of our patients with combination therapy. These opportunistic infections seem to be specific complications of immunosuppressive therapy and the immunodeficient status of ALF. Therefore, the appropriate definition of severe acute exacerbation of CHB is required to decide the indication for combination therapy.

Our study had a few limitations. First, the number of patients in our study was small. Second, this was not randomized study. Severe acute exacerbation of CHB is an uncommon but potentially life-threatening condition. Ethical issues obviously prevent a randomized control study with such life-threatened patients. Further multicenter studies are necessary.

In summary, our study showed that early introduction of CS treatment in combination with NA has sufficient virological effect against severe acute exacerbation of CHB, and the rapid decline of HBV DNA is distinct in survived patients. The survival in our patients by combination therapy with CS and NA is not inferior to that in recent reports by NA monotherapy considering the severity of our patients. We believe that both rapid decline of HBV DNA and cessation of necro-inflammation are necessary to improve the survival of severe acute exacerbation of CHB. For this purpose, 'high-dose' CS in combination with NA should be administered 'as soon as possible' according to the 'appropriate definition' of severe disease.

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DISCLOSURES

All authors have nothing to disclose.

CONFLICT OF INTERESTS

The authors have no conflict of interest to declare.

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Expression of microRNA-155 correlates positively with the expression of Toll-like receptor 7 and modulates hepatitis B virus via C/EBP-β in hepatocytes

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SUMMARY. Effective recognition of viral infection and successive activation of antiviral innate immune responses are vital for host antiviral defence, which largely depends on multiple regulators, including Toll-like receptors (TLRs) and microRNAs. Several early reports suggest that specific TLR-mediated immune responses can control hepatitis B virus (HBV) replication and express differentially with disease outcome. Considering the versatile function of miR-155 in the TLR-mediated innate immune response, we aimed to study the association between miR-155 and TLRs and their subsequent impact on HBV replication using both a HBV-replicating stable cell line (HepG2.2.15) and HBV-infected liver biopsy and serum samples. Our results showed that miR-155 was suppressed during HBV infection and a subsequent positive correlation of miR-155 with TLR7 activation was noted. Further, ectopic expression of miR-155 *in vitro* reduced HBV load as evidenced from reduced viral DNA, mRNA and subsequently reduced level of secreted viral antigens (HBsAg and HBeAg). Our results further suggested that CCAAT/enhancer-binding protein-β (C/EBP-β), a positive regulator of HBV transcription, was inhibited by miR-155. Taken together, our study established a correlation between miR-155 and TLR7 during HBV infection and also demonstrated *in vitro* that increased miR-155 level could help to reduce HBV viral load by targeting C/EBP-β.

Keywords: C/EBP-β, chronic, hepatitis B virus, miRNA, Toll-like receptors.

INTRODUCTION

Despite the presence of an effective vaccine and several agents to combat hepatitis B virus (HBV), it continues to be a serious threat to human life globally affecting more than 350 million people across the world. HBV carriers might also develop progressive liver diseases, such as liver failure, liver cirrhosis and hepatocellular carcinoma, which result in more than 500 000 deaths per year [1]. The outcome of patients with HBV infection is closely related to their innate as well as adaptive immune response [2]. While the adaptive arm of the immune system against HBV has been studied extensively [3], reports relating to the role of innate immunity in HBV infection are limited.

The innate immune response is the first line of defence activated by the host against invading pathogens. Recent data reveal that activation of the innate immune system may contribute to controlling HBV infection in hepatocytes [4]. Toll-like receptors (TLRs) play a central role in initiation of the innate immune response. They are conserved pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) and mediate the innate immune responses against invading pathogens

Abbreviations: BIC, B-cell integration cluster; EBP, enhancer-binding protein; HBeAg, HBV e antigen; HBsAg, HBV surface antigen; HBV, hepatitis B virus; miRNAs, microRNAs; PAMPs, pathogen-associated molecular patterns; PDTC, pyrrolidine dithiocarbamate; PRRs, pattern recognition receptors; TLR, Toll-like receptor; UTR, untranslated region.

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[5]. Early reports indicate that viral infection alters the expression of TLRs as well as the downstream signalling pathways [6,7]. Reports also suggested that specific Toll-like receptor (TLR)-mediated immune response can control HBV replication and express differentially with disease outcome [8–10].

Recently, microRNAs (miRNAs) have emerged as important controllers of TLR signalling [11]. Several miRNAs are induced by TLR activation [11]. miRNAs are a class of evolutionary conserved short noncoding RNAs of 19–25 nt, synthesized from larger transcripts of the eukaryotic genome by RNA polymerase II [12]. They bind to the 3′-untranslated region (UTR) of target messenger RNA to down-regulate gene expression [13]. Multiple miRNAs are induced during the innate immune response with a consensus emerging that miR-155, miR-146 and miR-21 are predominantly ubiquitous [11]. Among them, miR-21 and miR-155 are particularly important in the context of viral infection [14–17]. However, although the contribution of miR-21 in HBV pathogenesis has been studied well [14,18], reports pertaining to the role of miR-155 in HBV infection are limited. MiR-155 was first found within the B-cell integration cluster (BIC) on chromosome 21 in the human genome [19]. It was previously reported that miR-155 plays a vital role in the regulation of host antiviral immunity [11]. It regulates the production of type I interferon and thus participates in innate antiviral immune responses [20]. An earlier report in this field reveals that miR-155 activates interferons against HBV via the JAK–STAT pathway [13]. Studies have also conveyed that miR-155 is required for effector CD8+ T-cell response to viral infection [21]. Moreover, Swaminathan *et al.* [22] have clearly established that miR-155 significantly diminishes the HIV load. Therefore, possibly miR-155 might as well be effective against HBV. Thus, considering the contribution of miR-155 in antiviral defence and its versatile function in the TLR-mediated innate immune response, we hypothesize that the expression level of TLRs might modulate the expression of miR-155 in viral pathogenesis. Therefore, we aimed to study the association between miR-155 and TLRs during HBV infection and the subsequent impact of this association on HBV replication.

MATERIALS AND METHODS

Study subjects

Liver biopsy specimens were procured from 12 patients with chronic HBV infection. Biopsy specimens from six individuals with steatosis but with no history of HBV, HCV or HIV infections served as disease controls. These specimens were obtained from Kalinga Gastroenterology Foundation, (Cuttack, Orissa, India). Sera specimens from 14 patients infected with HBV were also procured from Kalinga Gastroenterology Foundation, (Cuttack, Orissa, India). Seven Control serum samples were obtained from healthy voluntary donors tested negative for HIV, HBV and HCV. Signed informed consent was obtained from the patients, and the institutional ethical committee approved the study. The diagnosis of patients with CHB was conformed according to the AASLD guidelines 2009 [23].

Construction of plasmids

pCDNA3.1.BIC

Plasmid pCDNA3.1.BIC expressing miR-155 was constructed as described earlier [24].

psiCHECK2.C/EBPβ reporter construct

Total RNA was extracted from HepG2 cells with Trizol (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed into cDNA by Revert Aid first-strand cDNA synthesis kit (MBI Fermentas, Vilnius, Lithuania), and a 180-bp region of the 3′UTR of C/EBP-*β*, encompassing the miR-155 seed sequence CAAGACTTA, was cloned downstream of the Renilla luciferase gene, of the dual luciferase reporter plasmid psiCHECK-2. The construct was confirmed by DNA sequencing.

Cell culture, stimulation and transfection

The hepatoma cell lines HepG2 and HepG2.2.15, respectively, were maintained in DMEM and RPMI 1640 medium, with 10% foetal bovine serum (Sigma-Aldrich, Munich, Germany) at 37 °C in a humidified atmosphere with 5% CO_2.

HepG2 or HepG2.2.15 cells (5×10^5 cells) were transfected with 1 μg of pUC19-HBV1.3 plasmid or 1 μg of pCDNA3.1.BIC plasmid, respectively, into a six-well plate, using Lipofectamine™ 2000 (Invitrogen). HepG2 cells transfected with 1 μg of blank pUC19 and HepG2.2.15 cells transfected with 1 μg of blank pCDNA3.1 served as controls, respectively. Total RNA was extracted from transfected cells after 24, 48, 72 and 96 h of transfection.

For pathway screening, HepG2 cells were stimulated with 5 μg/mL of R837 (Invivogen, San Diego, CA, USA) alone or after pretreatment with either 10 μM pyrrolidine dithiocarbamate (PDTC) (NF-*κ*B inhibitor), 2 μM LY294002 (PI3K inhibitor), 25 μg/mL SP600125 (a JNK inhibitor) or 10 μM SB203580 (p38 inhibitor) from Sigma (St. Louis, MO, USA) [20]. Relative miR-155 levels were analysed within 24 h of stimulation.

RNA isolation, cDNA synthesis and quantitative real-time PCR

Total RNA was extracted using Trizol reagent (Invitrogen) from 5×10^5 cells or from liver biopsy specimens. Total RNA from patient sera was isolated by miR-vana™ microRNA isolation kit (Ambion, Austin, USA). RNA quality and quantity were accessed by spectrophotometer and additional visualization in agarose gels.

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