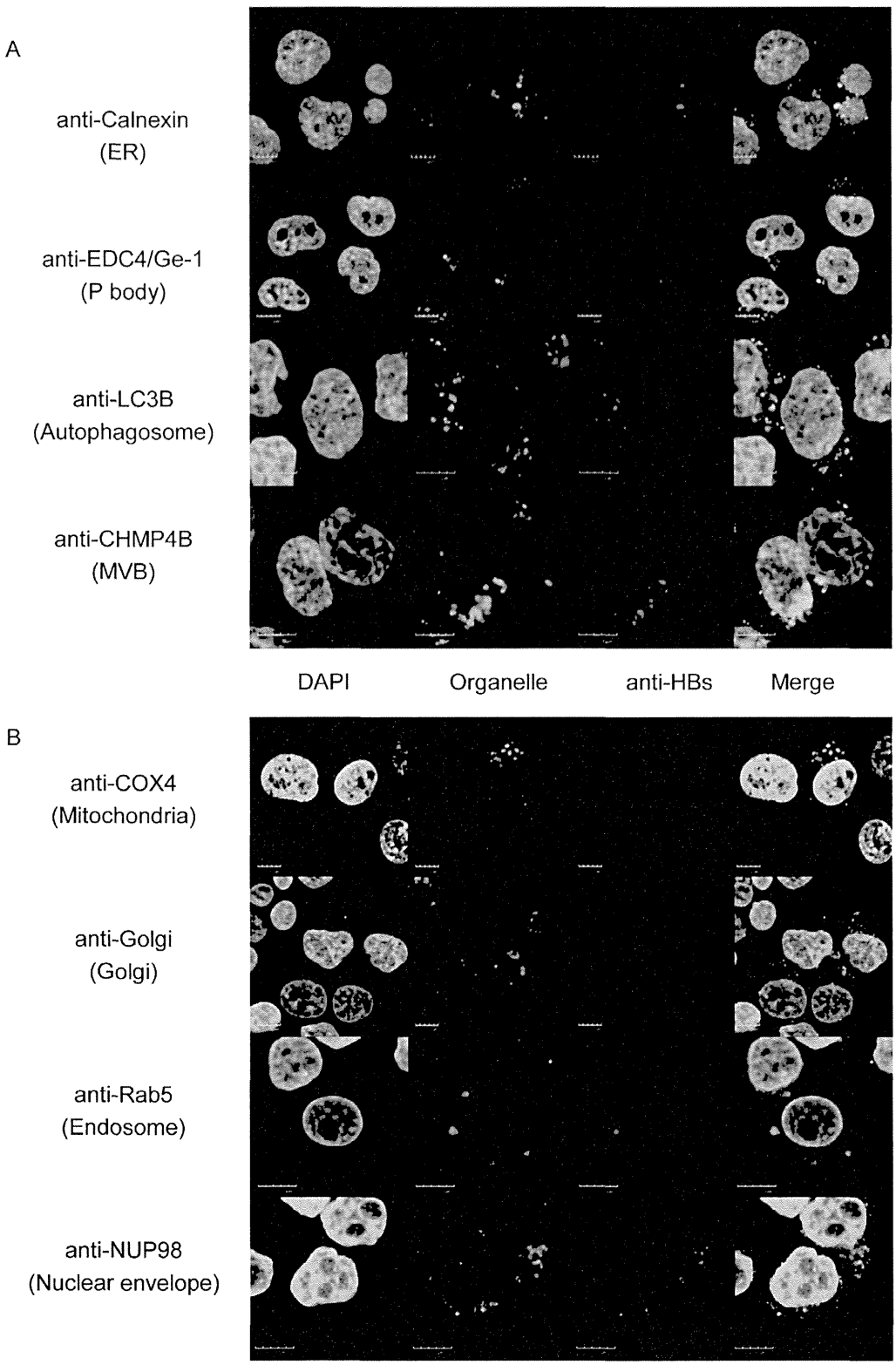
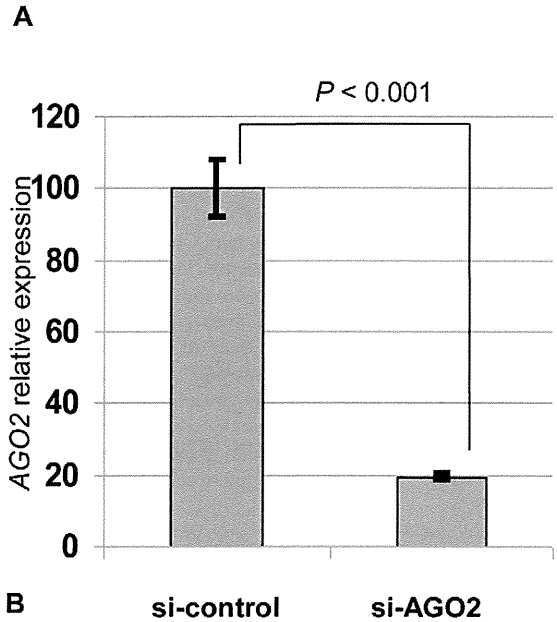
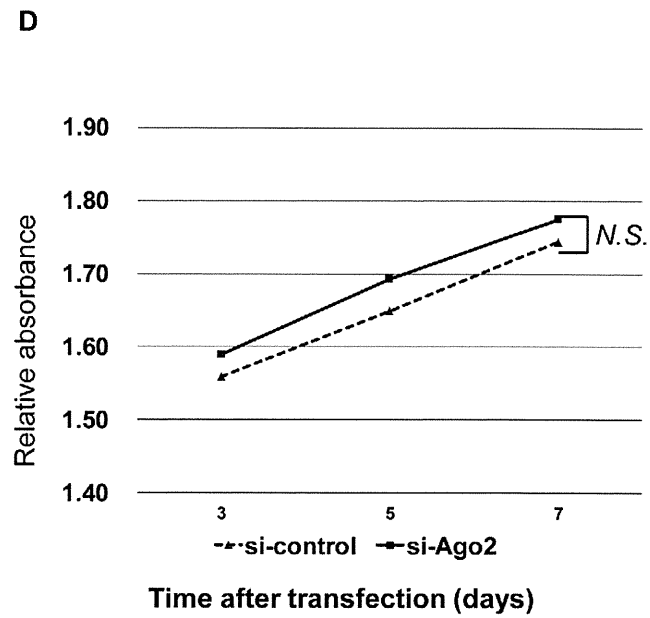
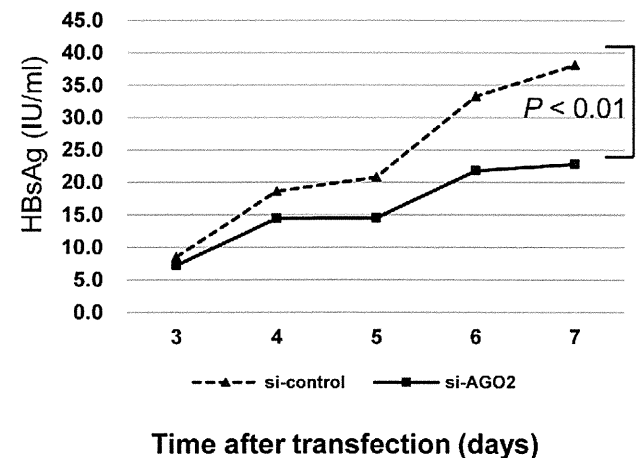


Figure 3. HBsAg localization. A) Co-localization of anti-HBs suggests that HBs localizes in the ER, processing bodies, autophagosomes, and multivesicular bodies, B) and more diffusely in mitochondria, Golgi, endosomes, and at the nuclear envelope.
doi:10.1371/journal.pone.0047490.g003


A
P < 0.001
AGO2 relative expression
120
100
80
60
40
20
0
si-control
si-AGO2


D
Relative absorbance
1.90
1.80
1.70
1.60
1.50
1.40
N.S.
3
5
7
si-control
si-Ago2
Time after transfection (days)


B
HBsAg (IU/ml)
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
P < 0.01
3
4
5
6
7
si-control
si-AGO2
Time after transfection (days)

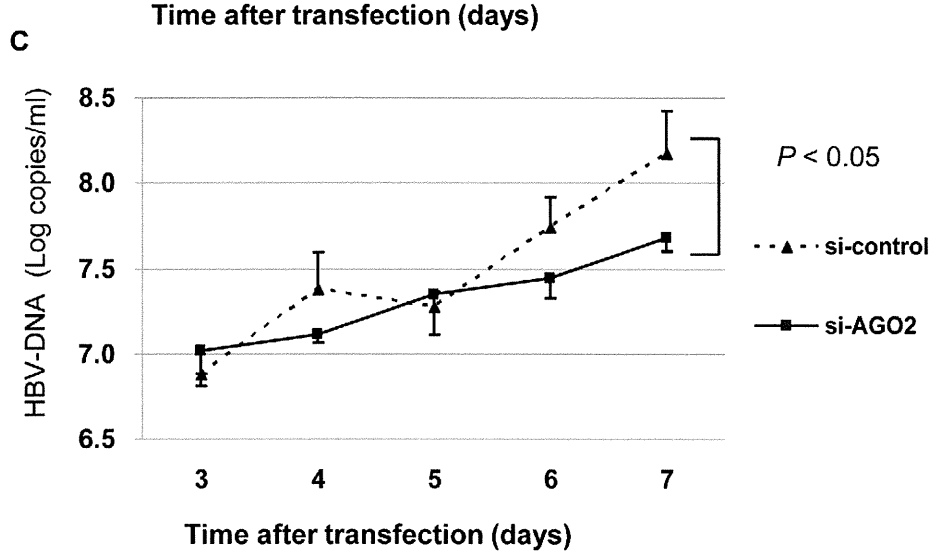

C
HBV-DNA (Log copies/ml)
8.5
8.0
7.5
7.0
6.5
P < 0.05
si-control
si-AGO2
3
4
5
6
7
Time after transfection (days)

Figure 4. siRNA knock down of AGO2 expression. A) Knock down of *AGO2* expression in T23 cells by specific siRNAs for *AGO2* or control siRNAs, confirmed by real-time quantitative RT-PCR analysis. B) Supernatant HBs antigen, and C) HBV-DNA were measured. Both were higher in supernatant of cells transfected with si-control than in cells transfected with si-*AGO2*. D) There was no significant difference in cell viability between cells transfected with si-control compared to those with si-*AGO2*.
doi:10.1371/journal.pone.0047490.g004

controls. Mir-122, miR-22, miR-99a, and miR-125b in particular, were significantly elevated in serum of HBV patients. We also showed that AGO2, an essential component of the RNA silencing complex, co-localizes with both HBc and HBs proteins. HBc and/or HBs localize to several organelles associated with protein synthesis, processing, and degradation, including the ER, Golgi, endosomes, autophagosomes, processing bodies, and multivesicular bodies. Although we expected that depletion of AGO2 would relieve inhibition of HBV replication, we found instead that knockdown of AGO2 appears to inhibit HBV replication, implying that HBV may require AGO2 during its life cycle.

The role of AGO2 is unclear, but viruses have previously been shown to interfere with elements of the RNA-induced gene silencing pathway [17]. HCV core protein and the HIV-1 Tat protein suppress gene silencing by inhibiting Dicer, a cytoplasmic protein that processes pre-microRNA [18]. HBV down-regulates expression of Drosha, the nuclear protein involved in the first step of miRNA processing, which might globally suppress miRNA expression levels [19]. Viruses also influence expression of individual miRNAs [17].

Considering that miR-122 strongly suppresses HBV replication, it is curious that HBV is nonetheless often able to establish chronic infection in the liver [20,21,22]. In the case of HCV, miR-122/AGO2 binding stabilizes the HCV genome and prevents degradation, such that suppression of either miR-122 or AGO2 inhibits HCV replication [23,24,25]. In HBV, we also found that AGO2 knockdown suppresses replication, but Wang et al. demonstrated that anti-sense depletion of miR-122 promoted HBV replication instead of suppressing it [26]. MiR-122 suppresses HBV replication both through direct binding to HBV RNA as well as indirectly through cyclin G1-modulated p53 activity [20,27,28]. HBV might therefore be expected to down-regulate miR-122 levels to evade miR-122 binding and suppression. Wang et al. indeed found that miR-122 levels are significantly decreased in the liver of chronic HBV patient [26], whereas elevated miR-122 levels in the serum have been reported [4,29].

One explanation for the discrepancy between liver and serum miR-122 levels might be that HBV sequesters and expels AGO2-bound miR-122 inside of HBsAg particles, possibly along with other miRNAs that interfere with the viral life cycle. HBV vastly over-produces surface proteins that self-assemble into what were initially thought to be empty particles [30,31], but which may contain miRNAs stably bound to AGO2 [5]. Although HBV is a DNA virus, it relies on reverse transcription via an RNA intermediate in a way similar to retroviruses. Bouttier et al. showed that two unrelated retroviruses, HIV-1 and PFV-1, both require AGO2 interaction with viral RNA for assembly of viral particles. In these viruses, AGO2 is recruited to viral RNA and encapsidated along with it without impairing translation of viral RNA [32]. This suggests that some viruses may take advantage of another function of Argonaute, such as its role in the formation of P-bodies [33], although AGO2 possesses intrinsic exonuclease activity that must be countered. AGO2-mediated gene silencing requires recruitment of GW182 via multiple GW-rich regions [34]. While HIV-1 and PFV-1 encapsidate AGO2, they do not encapsidate GW182, which might provide a means to suppress AGO2 silencing. Some plant viruses use molecular mimicry to inhibit RISC activity by binding to Argonaute proteins through virally encoded WG/GW motifs [35]. Although HBV proteins appear to lack WG/GW motifs, the HBV core protein may use a similar mechanism to disrupt RISC activity while preserving other AGO2 functions. One possibility involves HSP90, a chaperone involved in maintenance of the polymerase/pgRNA complex. HSP90 binds to HBV core protein dimers and is internalized in capsids, but it also binds to the N-terminus of AGO2 and may be required for miRNA loading and targeting to P-bodies [36,37]. Co-localization studies with other proteins and analysis of bound miRNAs may be necessary to elucidate the role of AGO2 in HBV replication, but we speculate that HBV proteins might suppress miRNA activity by binding to and sequestering AGO2 and their bound miRNAs.

Pathway analysis of the predicted targets of the up-regulated serum miRNAs in HBV patients showed that genes involved in phosphatase activity were significantly over-represented. Each of several miRNAs, including miR-122, miR-125b, and miR-99a, was predicted to target a different phosphorylation-associated gene. Regulation of phosphorylation appears to be important in HBV replication, as phosphorylation of the C terminal domain of the HBV core protein is essential for pgRNA packaging and HBV capsid maturation [38]. Phosphorylation also inhibits AGO2 binding of miRNA [39] and is involved in localization to P-bodies [40]. Recent studies have demonstrated that HBV enhances and exploits autophagy via the HBx and small HBs proteins to promote viral DNA replication and envelopment without increasing the rate of protein degradation [41,42]. Sir et al suggested that autophagy may affect dephosphorylation and maturation of the core protein, which protects viral DNA during replication [43]. These reports suggest that HBV exploits multiple cellular pathways in order to establish an intracellular environment conducive to replication.

Although many HBV-associated miRNAs have been reported, the functions of only a few have been examined. MiR-122, miR-125a-5p, miR-199a-3p and miRNA-210 have all been reported to bind to and directly suppress HBV RNA [8,27,44], whereas other miRNAs have been shown to promote or suppress HBV replication indirectly. MiR-1 enhances HBV core promoter activity by up-regulating FXRα, a transcription factor essential for HBV replication [45], whereas miR-141 suppresses HBsAg production in HepG2 cells by down-regulating promoter activity via PPARA [46]. The role of miR-22 and miR-99a in HBV infection is less clear, but both are involved in regulation of cell fate and are implicated in development of HCC. MiR-99a is one of the most highly expressed miRNAs in normal liver tissue and is severely down-regulated in HCC and other cancers, suggesting a role as a tumor suppressor [47]. MiR-99a alters sensitivity to TGF-β activity by suppressing phosphorylation of SMAD3 [48], whereas the HBx protein disrupts TGF-β signaling by shifting from the pSmad3C pathway to the oncogenic pSmad3L pathway [49]. MiR-22 acts as a tumor suppressor by inducing cellular senescence and is down-regulated in several cancer lines [50]. However, over-expression of miR-22 in males is associated with down-regulation of ERα expression, which compromises the protective effect of estrogen and leads to up-regulation of IL-1α in hepatocytes under stress caused by reactive oxygen species, which is another hallmark of HBx interference [51]. Differences in

miRNA levels between hepatic and serum miRNA profiles may reveal miRNAs that play an essential role in the HBV life cycle, with potential application to miRNA-based diagnosis and therapy.

In this study we demonstrated potential interactions between AGO2 and HBc and HBs, but not HBx, in stably transfected HepG2 cells. Suppression of HBV DNA and HBsAg in the supernatant following AGO2 knockdown and the presence of HBV-associated miRNAs in the serum may indicate a dependency on AGO2 during the HBV life cycle.

Supporting Information

Figure S1 Heat map of miRNA expression. Healthy controls and patients with chronic HBV clustered separately based on serum miRNA expression. "Healthy males" and "healthy females" refer to serum mixtures of 12 uninfected males and 10 uninfected females, respectively. "HBV low" and "HBV high" refer to serum mixtures from 10 patients with low (≤42 IU/l) ALT levels and 10 patients with high ALT levels (>42 IU/l), respectively.
(TIF)

Figure S2 Pairwise correlations among pooled serum miRNA samples. Pooled serum samples were collected from 10 healthy males, 10 healthy females, 10 HBV patients with low ALT levels, and 10 HBV patients with high ALT levels. Pairwise correlations in miRNA expression levels among all four pooled samples were strong (>0.90; P<0.001), but correlations were strongest between the healthy male and female samples (0.98) and between the low and high ALT HBV patients (0.98), suggesting that expression of a subset of miRNAs is altered during HBV infection.
(TIF)

Figure S3 Relationship between serum miRNAs and HBsAg levels in chronic HBV patients. Serum levels of several miRNAs were significantly correlated with HBsAg levels in patients with chronic HBV. MiR-99a, miR-122, and miR-125b levels were most strongly correlated with HBsAg levels, with R^2 of 0.69, 0.56, and 0.54, respectively.
(TIF)

Figure S4 Relationship between serum miRNAs and HBV DNA levels in chronic HBV patients. Serum levels of several miRNAs were significantly correlated with HBV DNA levels in patients with chronic HBV. MiR-122, miR-99a, and miR-125b levels were most strongly correlated with HBV DNA levels, with R^2 of 0.44, 0.43, and 0.39, respectively.
(TIF)

Figure S5 Relationship between serum miRNAs and ALT levels in chronic HBV patients. Serum levels of several miRNAs were significantly but somewhat diffusely correlated with ALT levels in patients with chronic HBV. MiR-122 and miR-22 levels were correlated with ALT levels with R^2 of 0.25 and 0.21, respectively.
(TIF)

Figure S6 Relationship between serum miRNAs and presence of HBe antigen in chronic HBV patients. Serum levels of miR-122, miR-99a, miR-720, and miR-125b were significantly elevated in patients positive for the HBe antigen.
(TIF)

Figure S7 Relationship between serum miRNAs and presence of HBe antibody in chronic HBV patients. Serum levels of miR-122, miR-99a, miR-720, and miR-125b were significantly elevated in patients negative for the HBe antibody.
(TIF)

Figure S8 Relationship between individual miRNAs in the liver and serum. Each point represents the level of a specific miRNA in non-cancerous liver tissue relative to serum in the same patient. Red points represent miRNA levels from a patient with chronic HBV, and blue and green points correspond to two different uninfected control subjects. Large red points and labels indicate the subset of miRNAs (Tables 2 and 3) that were significantly elevated in serum of chronic HBV patients. MiRNA expression levels were positively correlated ($R^2 = 0.57$; P<2.1E-16) between liver tissue and serum, suggesting that serum levels broadly reflect miRNA levels in the liver. There appears to be no clear discrepancy between liver and serum miRNA levels in the HBV-infected patient compared to the two uninfected patients.
(TIF)

Figure S9 Subcellular localization of HBx analyzed by immunocytochemistry. HBx localized non-specifically in the nucleus and cytoplasm, but we were unable to verify the subcellular location. Anti-Rab5 staining for endosomes is shown for illustration, but results were similar using antibodies against other compartments.
(TIF)

Table S1 Antibodies used for immunocytochemistry.
(DOC)

Table S2 Significantly up- or down-regulated miRNAs in liver samples from an HBV-infected patient compared to two non-HBV-infected patients.
(DOC)

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Author Contributions

Conceived and designed the experiments: KC CNH SA MT DM HAB HO NH. Performed the experiments: MT DM H. Abe NH MI SY H. Aikata TK YK RA KC. Analyzed the data: CNH SA MT DM HO KC. Contributed reagents/materials/analysis tools: CNH SA MT DM KC. Wrote the paper: CNH SA MT DM KC. Clinical data: KC MT DM HAB NH MI ST HAI TK YK WO. Obtained funding: KC MT DM. Critical review of the manuscript: CNH SA MT DM RA HAB HO NH MI ST HAI TK YK WO KC.

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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

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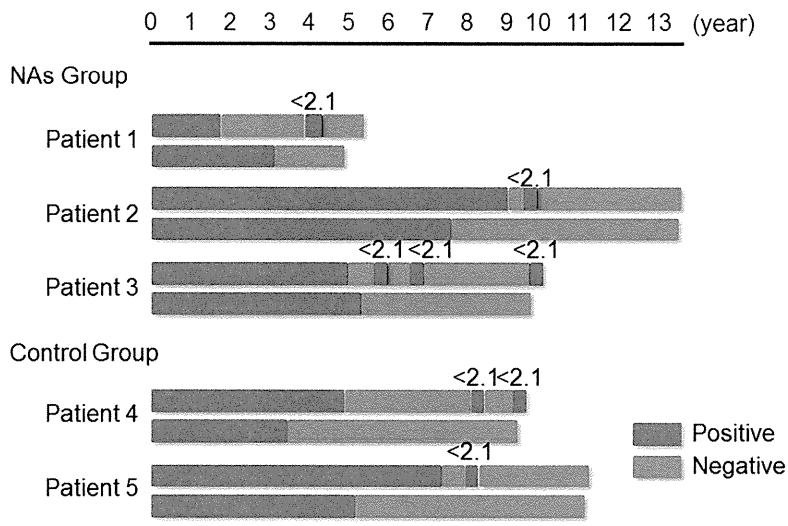
Figure. Patients with reappearance of HBV DNA after HBsAg-seroclearance.

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



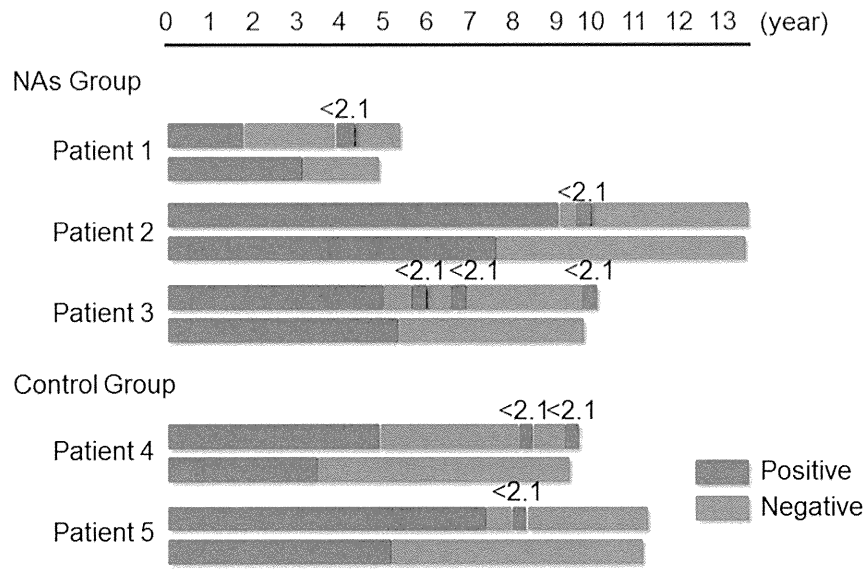


Figure. Patients with reappearance of HBV DNA after HBsAg-seroclearance.
In each patient, upper and lower bars indicate HBV DNA and HBsAg, respectively.
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Virological efficacy of combination therapy with corticosteroid and nucleoside analogue for severe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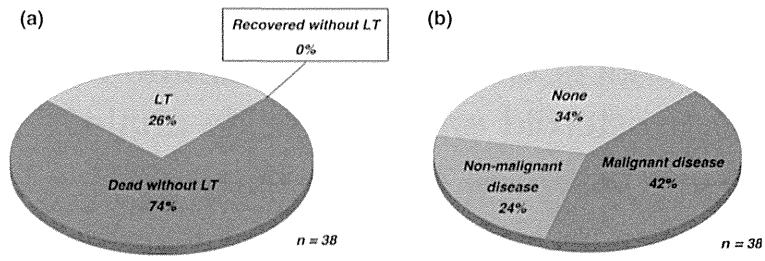

Fig. 1 Outcome **(a)** and primary disease **(b)** of patients with fulminant hepatitis associated with HBV carrier in the Japanese nationwide survey of 2009–2010. None of the patients recovered without liver transplantation (LT), and most patients (66%) had primary disease.

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

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/

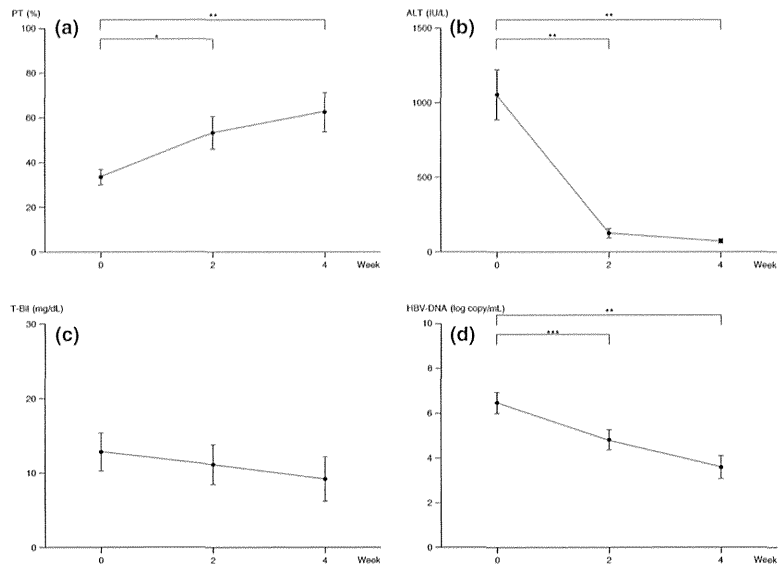

Fig. 2 Changes in prothrombin time (PT) activities **(a)**, alanine aminotransferase (ALT) **(b)**, total bilirubin (T-Bil) **(c)** and HBV DNA level **(d)** in 13 patients with severe acute exacerbation of chronic hepatitis B treated with corticosteroid in combination with nucleoside analogue; $*P =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

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

$P = 0.01$, 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.

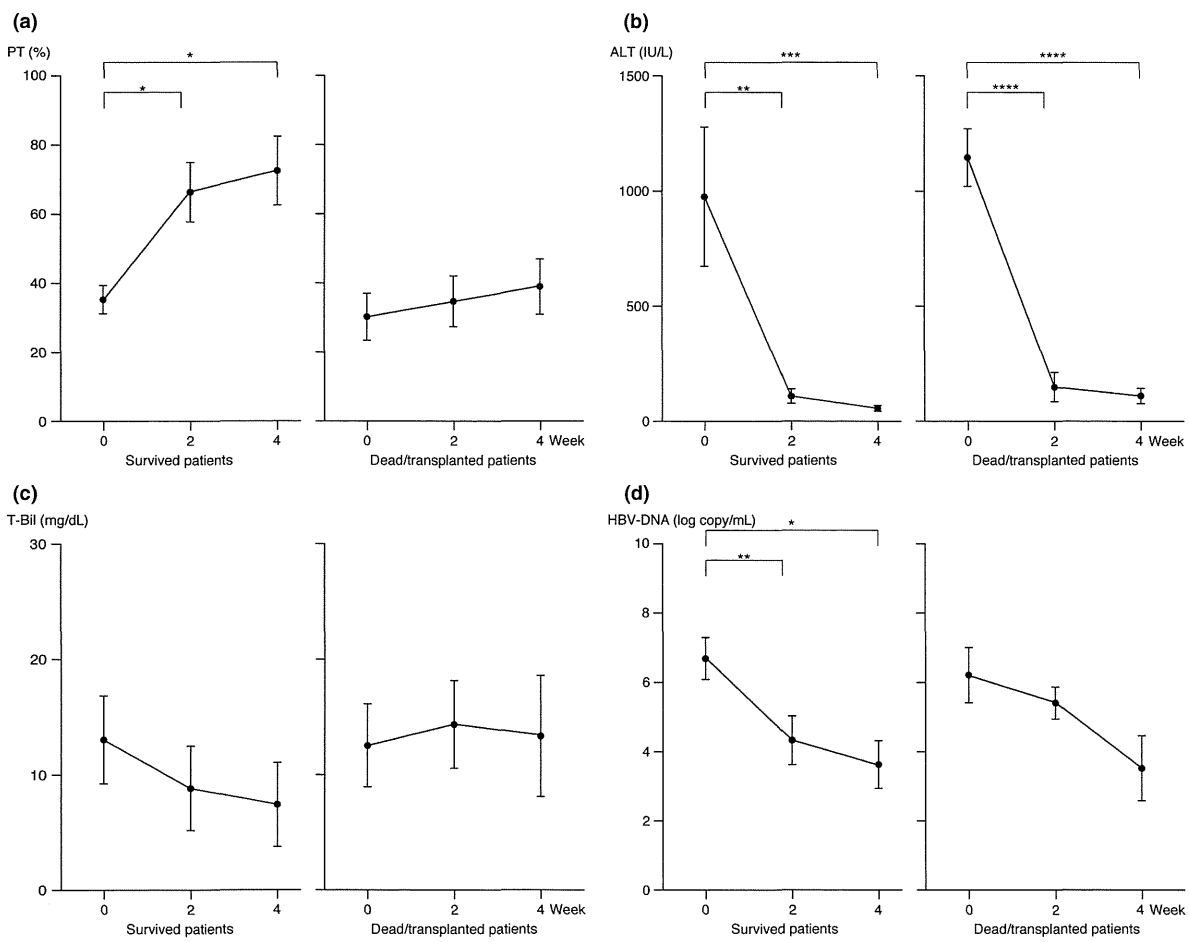

Fig. 3 Changes in prothrombin time (PT) activities **(a)**, alanine aminotransferase (ALT) **(b)**, total bilirubin (T-Bil) **(c)** and HBV DNA level **(d)** in survived and dead/transplanted patients; *$P = 0.01$, **$P = 0.03$, ***$P = 0.02$, ****$P < 0.01$.

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.

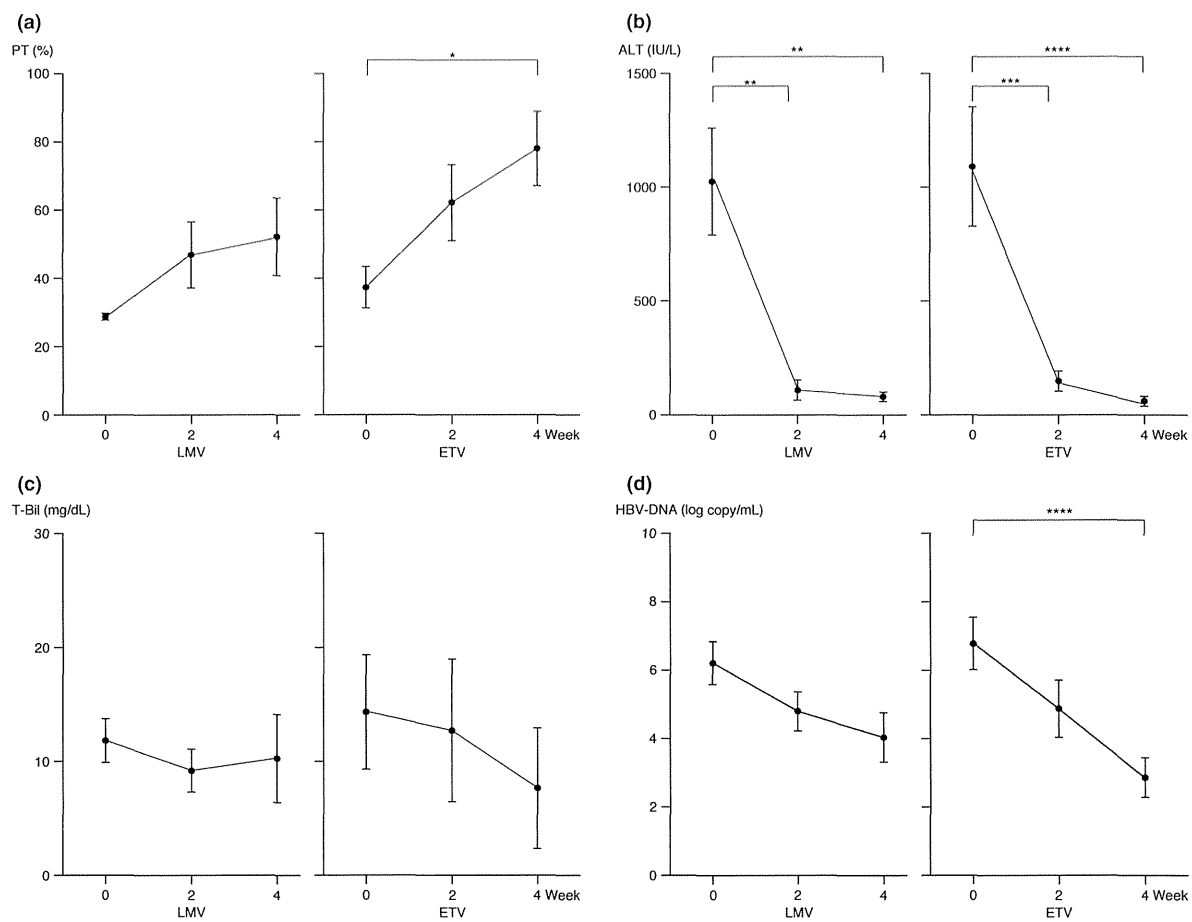

Fig. 4 Changes in prothrombin time (PT) activities **(a)**, alanine aminotransferase (ALT) **(b)**, total bilirubin (T-Bil) **(c)** and HBV DNA level **(d)** in the LMV and ETV groups; *$P = 0.03$, **$P < 0.01$, ***$P = 0.02$, ****$P = 0.01$.

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

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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