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Original Article

Recommendation of lamivudine-to-entecavir switching treatment in chronic hepatitis B responders: Randomized controlled trial

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Aim: In the 2007–2008 guidelines of the study group (Ministry of Health, Labor and Welfare of Japan), lamivudine (LAM)-continuous treatment was recommended in patients treated with LAM for more than 3 years who maintained hepatitis B virus (HBV) DNA less than 2.6 log copies/mL, because in these patients LAM resistance might exist and switching treatment to entecavir (ETV) might cause ETV resistance. However, there was no evidence on whether switching treatment to ETV- or LAM-continuous treatment was better in those patients. In the present study, we performed a randomized controlled trial of LAM-to-ETV switching treatment.

Methods: Twenty-seven patients treated with LAM for more than 3 years whose HBV DNA levels were less than 2.6 log copies/mL were enrolled and randomly divided into two groups, LAM-continued group or switching to ETV group. Then, we examined incidence of virological breakthrough (VBT) and breakthrough hepatitis (BTH) in each group.

Results: There was no BTH in any of the patients. VBT was observed in six patients of the LAM group (6/15, 40%), and no patient of the ETV group (0/11, 0%) ($P = 0.02$). The differences of the proportion of cumulated VBT using a log–rank test with Kaplan–Meier analysis were significant between the LAM and ETV groups ($P = 0.025$).

Conclusion: In patients treated with LAM for more than 3 years maintaining HBV DNA less than 2.6 log copies/mL, switching treatment to ETV is recommended at least during the 2 years' follow-up period.

Key words: chronic hepatitis B, entecavir, lamivudine, lamivudine resistance, randomized controlled trial, switching treatment

INTRODUCTION

OVER THE PAST two decades, treatment of chronic hepatitis B (CHB) has greatly improved with the availability of nucleos(t)ide analogs (NA), including lamivudine (LAM), adefovir dipivoxil (ADV), entecavir (ETV), telbivudine, clevudine and tenofovir. NA target

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the reverse transcriptase of hepatitis B virus (HBV), and are highly effective in suppressing HBV replication and clinical progression to liver cirrhosis and hepatocellular carcinoma in CHB patients.[1–4]

Lamivudine, ADV and ETV are commonly available in Japan. LAM, the first approved NA, has been shown to provide benefit for CHB patients with respect to the reduction of HBV DNA, normalization of alanine aminotransferase (ALT) and improvement of liver histology.[5,6] However, a serious problem of LAM is the high incidence of drug resistance during long-term treatment. The detection rate of LAM resistance has been reported to be 24% at 1 year and 70% after 5 years of treatment.[7–10] Even when the HBV DNA level was maintained at less than 2.6 log copies/mL, the accumulated incidence of LAM resistance reached 65% in patients treated with LAM for a long period (3 to ~10 years).[11] LAM resistance is caused by amino acid substitution(s) at rtM204V/I within the reverse transcriptase domain of the HBV polymerase gene.[12–14] The emergence of a LAM-resistant strain leads to virological breakthrough (VBT) and breakthrough hepatitis (BTH).

Recently, ETV has been demonstrated to exert antiviral efficacy in both NA-naïve and LAM-resistant CHB patients.[15–17] The frequency of ETV resistance has been reported to be 1.2% after 5 years of treatment in NA-naïve CHB patients.[18,19] On the other hand, in switching treatment to ETV for LAM-resistant CHB patients, the cumulative probability of ETV resistance increases.[17,20] After 5 years of treatment, 51% of LAM-refractory patients treated with ETV showed genotypic ETV resistance.[21]

The 2007–2008 guidelines of the study group (Ministry of Health, Labor and Welfare of Japan) for patients on LAM therapy are summarized in Table 1.[22] Regardless of duration of LAM administration, in cases where HBV DNA is more than 2.6 log copies/mL with BTH, ADV add-on treatment was recommended. In patients treated with LAM for less than 3 years who maintained HBV DNA of less than 2.6 log copies/mL or HBV DNA of 2.6 log copies/mL or more without BTH, switching to ETV was recommended. On the other hand, in patients treated with LAM for more than 3 years who maintained HBV DNA of less than 2.6 log copies/mL or HBV DNA of 2.6 log copies/mL or more without BTH, LAM-continuous treatment was recommended because in these patients LAM resistance might exist, and switching treatment to ETV might cause ETV resistance. However, there is insufficient evidence on whether switching treatment to ETV- or LAM-continuous treatment is better for CHB patients treated with LAM for more than 3 years with HBV DNA of less than 2.6 log copies/mL.

In the present study, we performed a randomized controlled trial of LAM-to-ETV switching treatment in CHB patients treated with LAM for more than 3 years who maintained HBV DNA of less than 2.6 log copies/mL.

METHODS

Patients

A TOTAL OF 27 CHB patients (mean age 55 ± 9 years, 17 men) from 11 institutions all over Japan (Hokkaido University Hospital, Tohoku University Hospital, Akita City Hospital, Kuramitsu Clinic, Juntendo University Hospital, Chukyo Hospital, Nagoya City University Hospital, Okayama University Hospital, Kawasaki Medical University Hospital, Ehime University Hospital, Shin-Kokura Hospital) were enrolled from April 2008. All the patients were followed at least 6 months after they were diagnosed with CHB. Their characteristics are shown in Table 2. They were treated with LAM (100 mg/day) for more than 3 years (median 50 months, range 36–106 months). Before starting LAM administration, all patients were positive for hepatitis B surface antigen (HBsAg) in serum, abnormal for ALT, detectable for HBV DNA, and were not

Table 1 2007–2008 guidelines of the study group (Ministry of Health, Labor and Welfare of Japan) for patients on lamivudine treatment

Duration of lamivudine treatment HBV DNA		<3 years	≥3 years
<2.6 log copies/mL, persistently		May be switched to ETV 0.5 mg/day	LAM 100 mg/day
≥2.6 log copies/mL	No BTH†	May be switched to ETV 0.5 mg/day	LAM 100 mg/day
	With BTH	Add on ADV 10 mg/day	Add on ADV 10 mg/day

†After checking for absence of LAM resistance.
ADV, adefovir; BTH, breakthrough hepatitis; ETV, entecavir; HBV, hepatitis B virus; LAM, lamivudine.

Table 2 Characteristics of LAM continuous group and ETV switch group at baseline

	LAM ($n = 15$)	ETV ($n = 11$)	*P*-value
Male	10	6	NS
Age	53 ± 7	57 ± 7	NS
Duration of LAM administration (month)	59 ± 23	55 ± 18	NS
ALT (IU/L)	33 ± 29	28 ± 22	NS
HbeAg positive	1	1	NS

ALT, alanine aminotransferase; ETV, entecavir; HBeAg, hepatitis B e-antigen; LAM, lamivudine; NS, not significant.

infected with hepatitis C virus and HIV. Patients diagnosed with alcoholism, primary biliary cirrhosis or autoimmune hepatitis were excluded.

Study design

The overview of this study design is shown in Figure 1. Twenty-seven patients treated with LAM for more than 3 years were enrolled, who showed HBV DNA of less than 2.6 log copies/mL at entry. They were randomly divided into two groups by each institution, the LAM-continued group (LAM group) or switching to the ETV group (ETV group). The primary end-points were the incidences of VBT and BTH in each group. VBT was defined as having more than 1 log copies/mL increase of HBV DNA level from the nadir on at least two occasions after initial virological response. BTH was defined as showing abnormal ALT level due to LAM or ETV resistance. All subjects were monitored at least every 3-month intervals. At every visit, routine examination with biochemical (ALT, bilirubin, albumin) and virological (HBV DNA level, hepatitis B e-antigen [HBeAg], anti-HBe) assessments took place. The mean follow-up period was 24 ± 3 months.

This study was registered in the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR) on 4 April 2008 as "A randomized trial of lamivudine continuous therapy and entecavir switching therapy for chronic hepatitis B patients treated with lamivudine monotherapy" (no. UMIN000001120).

The study protocol conformed to the Declaration of Helsinki, and was approved by the Committee for Ethics of Medical Experiments on Human Subjects of all the institutions, and written informed consent was obtained from every participant.

Serological and virological markers of HBV

Hepatitis B surface antigen, antibody against HBsAg (anti-HBs), HBeAg and antibody against HBeAg (anti-HBe) were determined using commercially available enzyme immunoassays. HBV DNA was determined by an Amplicor HBV Monitor (Roche Molecular Systems, Branchburg, NJ, USA; detection limit 2.6 log copies/mL)

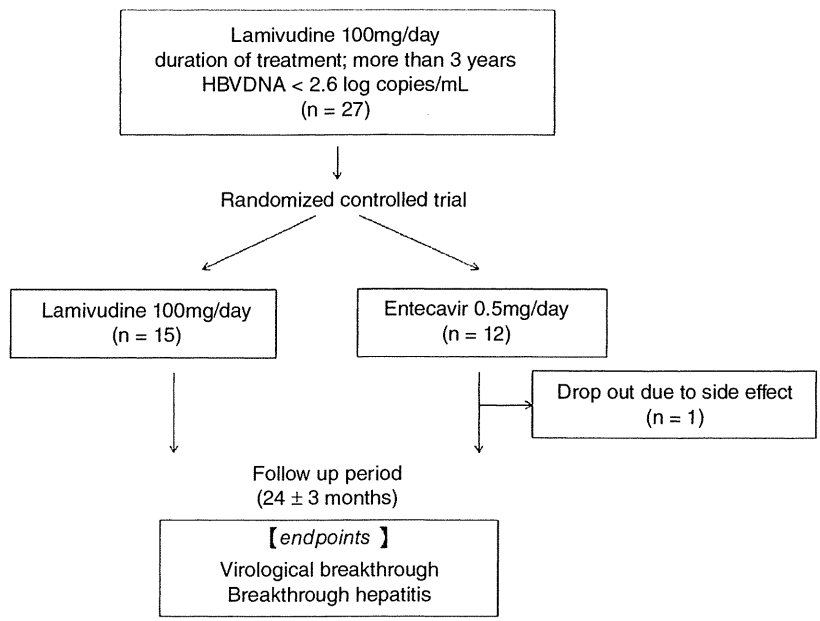

Figure 1 Overview of this study design. Twenty-seven patients treated with lamivudine for more than 3 years whose hepatitis B virus (HBV) DNA was maintained at <2.6 log copies/mL were enrolled. They were randomly divided into two groups by each institution, lamivudine-continued group or switching to entecavir group. We examined the incidence of virological breakthrough and breakthrough hepatitis in each group.

or COBAS AmpliPrep-COBAS TaqMan HBV test (Roche Molecular Systems; detection limit 2.1 log copies/mL). Positive results (signals) below the quantitative HBV DNA concentrations are referred to as "detected" and negative signals are "not detected" when registered by COBAS AmpliPrep-COBAS TaqMan HBV test. The presence of LAM-resistant rtM204V/I and rtL180M substitutions was analyzed by direct sequencing of the HBV DNA polymerase reverse transcriptase site.

Retrospective analysis

Using a conserved serum sample, we examined the existence of LAM-resistant rtM204V/I or rtL180M at baseline in patients with VBT. We also measured HBV DNA by COBAS AmpliPrep-COBAS TaqMan HBV test, and we evaluated the subsequent occurrence of VBT according to the DNA level (not detected/detected/2.1 to <2.6 log copies/mL).

Statistical analysis

Categorial variables were compared between groups by the χ^2-test or Fisher's exact test, and non-categorical variables by Mann–Whitney's *U*-test. The cumulated VBT rate was compared between each group using a log–rank test with Kaplan–Meier analysis. All data were analyzed using SPSS ver. 15.0J software. $P < 0.05$ was considered statistically significant.

RESULTS

Baseline characteristics of the patients

BASED ON THIS randomized controlled trial, 12 patients were placed in an ETV group and 15 in a LAM group. One patient in the ETV group dropped out because of skin rash by ETV. The baseline characteristics of the patients are described in Table 2. At the entry, one patient was positive for HBeAg in each group. There was no difference in sex, age, duration of LAM administration and ALT level between the two groups.

Incidence of VBT and BTH

There was no BTH in any of the patients. The incidence of VBT was six patients out of 15 (40%) in the LAM group, and no patient in the ETV group ($P = 0.02$). The Kaplan–Meier curve for the proportion of cumulated VBT is shown in Figure 2. The differences in the rates of VBT were significant between the LAM and ETV groups (log–rank test $P = 0.025$).

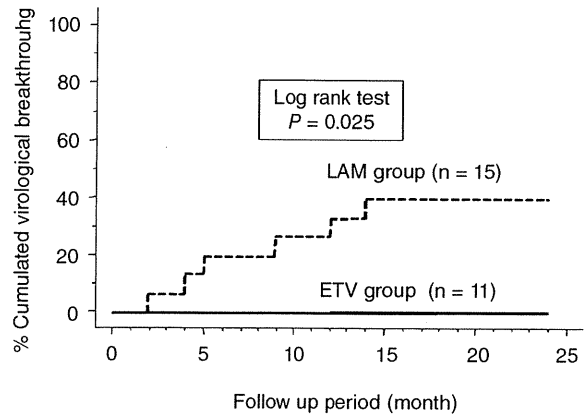

Figure 2 Proportion of cumulated virological breakthrough in lamivudine (LAM) and entecavir (ETV) group. The cumulated rate of virological breakthrough was higher in patients treated with LAM than those with ETV (40% vs 0%, $P = 0.025$ by log–rank test).

Characteristics of patients with VBT in LAM group

Details of the six VBT cases in the LAM group are described in Table 3. Assessment of LAM-resistant mutations at the time of VBT showed that both rtM204V and rtL180M were observed in all cases. For five of the six cases, HBV DNA was detected by COBAS AmpliPrep-COBAS TaqMan HBV test at baseline, although the HBV DNA level was very low. With respect to LAM-resistant mutation at baseline, rtM204V and rtL180M were observed in one of six cases. In contrast, no LAM-resistant mutations were observed in 20 non-VBT cases at baseline.

Incidence of VBT based on the HBV DNA level by COBAS AmpliPrep-COBAS TaqMan HBV test

Incidence of VBT based on the HBV DNA level according to COBAS AmpliPrep-COBAS TaqMan HBV test at baseline is shown in Figure 3. HBV DNA levels were less than 2.6 log copies/mL by Amplicor HBV Monitor in all cases. However, HBV DNA levels in the LAM group were "not detected" in five cases, "detected" in eight cases and 2.1 log copies/mL or more in two cases by COBAS AmpliPrep-COBAS TaqMan HBV test. VBT was observed in five of the 10 cases whose results were either "detected" or 2.1 log copies/mL or more and in one of the five "not detected" cases. On the other hand, although HBV DNA levels in the ETV group were

Table 3 Characteristics of patients with virological breakthrough in LAM group

At baseline						At virological breakthrough		
Age	Sex	Duration of LAM administration (month)	HBeAg	HBV DNA by TaqMan HBV (log copies/mL)	Mutant of LAM resistance	Period of VBT (months)	HBV DNA (log copies/mL)	Mutant of LAM resistance
49	M	37	Negative	Detected	None	14	4.9	L180M/M204V
54	F	106	Negative	Detected	None	5	2.8	L180M/M204V
63	F	81	Negative	Not detected	None	9	4.5	L180M/M204V
57	F	43	Negative	Detected	None	10	3	L180M/M204V
55	M	84	Negative	Detected	None	12	2.8	L180M/M204V
57	M	36	Negative	2.3	L180M/M204V	2	4	L180M/M204V

ALT, alanine aminotransferase; ETV, entecavir; HBeAg, hepatitis B e-antigen; HBV, hepatitis B virus; LAM, lamivudine; VBT, virological breakthrough.

"detected" in six cases by COBAS AmpliPrep-COBAS TaqMan HBV test, there was no incidence of VBT: HBV DNA levels of five patients were undetectable and that of one patient was "detected" at the last follow-up point after switching to ETV.

DISCUSSION

AT PRESENT, LAM, ADV and ETV are only approved for treatment of CHB patients in Japan. ETV has become the first-line treatment for NA-naïve patients, because the ETV resistance is much less frequent than LAM-resistance.[8,23,24] On the other hand, in switching treatment to ETV for LAM-resistant CHB patients, the frequency of ETV resistance was increased.[17,20,25–27] It has also been reported that ADV add-on treatment suppressed HBV replication more effectively than ETV or ADV monotherapy in patients with LAM-resistant CHB.[25,28] Therefore, it is desirable to examine LAM-resistant mutants before switching to ETV in patients treated with LAM. However, as the assay for the LAM-resistant mutants is not covered by the Japanese health insurance system at present, the Japanese guidelines for CHB management after LAM therapy were based on HBV DNA, duration of LAM administration and incidence of BTH (Table 1).[22] In patients treated with LAM for more than 3 years, maintaining HBV DNA of less than 2.6 log copies/mL or HBV DNA of 2.6 log copies/mL or more without BTH, LAM-continuous treatment was recommended because in these patients, LAM-resistance might exist, and switching treatment to ETV might cause ETV-resistance. It was reported that although LAM-resistant strains were detected in 34% cases treated with LAM for more than 3 years and whose HBV DNA level was suppressed to less than 2.6 log copies/mL, switching to ETV maintained undetectable HBV DNA level over 2 years.[29] In addition, Kurashige *et al.* reported that LAM-to-ETV switching treatment maintained an undetectable HBV DNA level in patients with baseline HBV DNA of less than 2.6 and 2.6 to less than 4.0 log copies/mL for a period of ETV treatment ranging 10–23 (median 20) months.[30] In the present study, randomized controlled trial evidenced that switching treatment to ETV or LAM-continuous treatment would be recommended in CHB patients treated with LAM for more than 3 years and maintained HBV DNA of less than 2.6 log copies/mL. Interestingly, even though HBV DNA had been suppressed to less than 2.6 log copies/mL, a high rate of VBT was observed in the LAM group, whereas no VBT over 24 months was observed in the ETV group. Of the six patients with VBT,

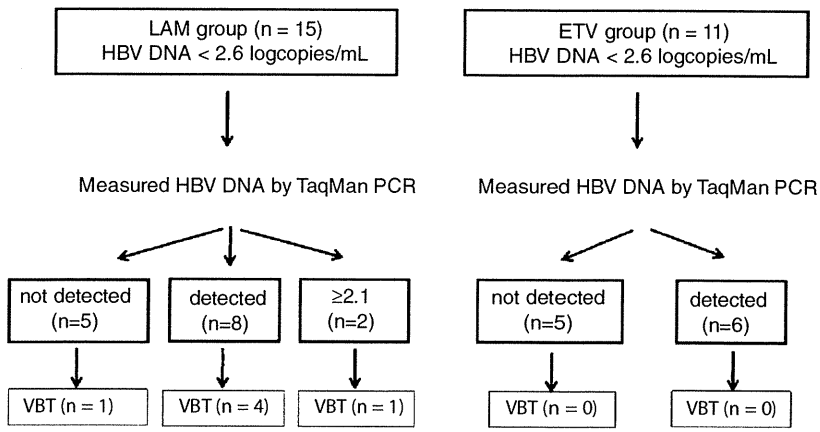

Figure 3 Incidence of virological breakthrough (VBT) based on the hepatitis B virus (HBV) DNA level at baseline by COBAS AmpliPrep-COBAS TaqMan HBV test (TaqMan PCR). The subsequent occurrence of VBT according to the DNA level by TaqMan PCR (not detected/detected/2.1 to <2.6 log copies/mL) was evaluated. In the lamivudine (LAM) group, VBT was observed in five of the 10 cases in which the results were either "detected" or ≥2.1 log copies/mL and in one of the five "not detected" cases. On the other hand, HBV DNA levels in the entecavir (ETV) group were "detected" in six, but there was no incidence of VBT.

five had no LAM resistance at baseline. However, the LAM resistance of rtM204V and rtL180M were found in all the patients with VBT in the LAM group. Moreover, a retrospective assessment by COBAS AmpliPrep-COBAS TaqMan HBV test showed that HBV DNA was detectable in 10 patients in the LAM group and six patients in the ETV group. Only five of the 10 patients in the LAM group had VBT, but none in the ETV group. In addition, one patient had VBT in the LAM group even though DNA was not detected by the TaqMan test, suggesting that switching to ETV was preferable. Hence, our data supported the 2010 Japanese guidelines which recommend switching to ETV in patients whose HBV DNA levels are less than 2.1 log copies/mL by TaqMan PCR.

A potential limitation of the present study is that the number of the cases was small. Nevertheless, our randomized controlled trial indicated significant difference in the incidence of VBT between the LAM and ETV groups. Therefore, this study is valuable for the purpose of verifying the 2007–2008 guidelines in Japan. In the present study, although no LAM-resistant mutant was observed in the ETV group at baseline, a very low level of LAM-resistant mutants may derive ETV resistance for long-term therapy. The results of switching to ETV in the present study were favorable during the 24-month observation period, but we have to be careful of possible emergence of ETV-resistant mutants in long-term follow up.

In conclusion, in patients treated with LAM for more than 3 years maintaining HBV DNA of less than 2.6 log copies/mL, switching treatment to ETV is recommended in at least a 2-year follow-up period.

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Antiviral activity of novel 2′-fluoro-6′-methylene-carbocyclic adenosine against wild-type and drug-resistant hepatitis B virus mutants

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ABSTRACT

Novel 2′-fluoro-6′-methylene-carbocyclic adenosine (**9**) was synthesized and evaluated its anti-HBV activity. The titled compound demonstrated significant antiviral activity against wild-type as well as lamivudine, adefovir and double lamivudine/entecavir resistant mutants. Molecular modeling study indicate that the 2′-fluoro moiety by a hydrogen bond, as well as the van der Waals interaction of the carbocyclic ring with the phenylalanine moiety of the polymerase promote the positive binding, even in the drug resistant mutants.

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Chronic hepatitis B virus (HBV) infection is one of the leading causes of morbidity and mortality worldwide. Chronic infection with HBV occurs in approximately 350 million of the world population, including 1.7 million in the USA.[1] HBV infection can persist for the life of the host, often leading to severe consequences such as liver failure, cirrhosis and eventually hepatocellular carcinoma, resulting in annually 0.5–1.2 million deaths worldwide.[2] HBV is an incomplete double-stranded DNA virus. Its DNA replication is unique because it includes a reverse transcription step. The HBV DNA polymerase/reverse transcriptase is an essential and multifunctional enzyme, which operates as a DNA polymerase/reverse transcriptase, an RNAse H, through coordinating the assembly of viral nucleocapsids, as well as catalyzing the generation of DNA primers.[3] Nucleoside analogues can suppress HBV replication by inhibiting the viral polymerase/reverse transcriptase. The pivotal role of nucleoside/nucleotide analogues such as lamivudine, adefovir, telbivudine, entecavir, clevudine, and tenofovir has been demonstrated by their therapeutic efficacy in clinical practice. However, long-term therapy with these drugs is often associated with viral resistance, which significantly compromises the clinical application of these agents. For example, the extensive use of lamivudine resulted in the emergence of mutants that are resistant to the anti-HBV activity; 24% after a 1-year therapy, increasing to over 70% after 4 years of therapy. Adefovir has been used for the patients, who develop lamivudine-resistant mutants, however, a significant number of patients (29% after 5 years of use) also develop the adefovir resistant mutant (N236T).

Entecavir is a carbocyclic 2′-deoxyguanosine analog that demonstrates potent anti-HBV activity[4] and is recommended for patients with the wild-type strain as well as for those patients harboring lamivudine-resistant strains.[5] However, a recent study by Tanaka and his co-workers suggest that the viral breakthrough was observed in the lamivudine-refractory group in 4.9% of patients at baseline and increase to 14.6%, 24% and 44.8% at weeks 48, 96 and 144, respectively.[6]

In view of the fact that currently adefovir and entecavir are the most prescribed drugs for the treatment of chronic HBV infection, it is critical to discover the agents that do not confer cross-resistance with the adefovir and lamivudine/entecavir-mutants for the future treatment of drug resistant patients. In this report we try to demonstrate that our newly discovered compound **9** may potentially play a significant role for that purpose.

Carbocyclic nucleosides are an interesting class of compounds in which the methylene group replaces the oxygen atom of a furanose ring. As a consequence, the glycosidic bond is resistant to nucleoside phosphorylase as well as nucleoside hydrolase, which makes the carbocyclic nucleosides more stable towards metabolic

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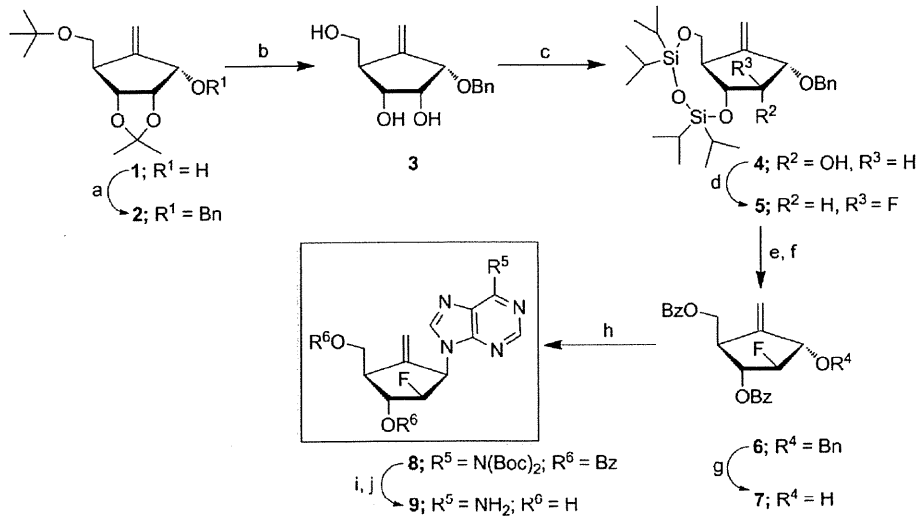

Scheme 1. Synthesis of target compound **9**. Reagents and conditions: (a) NaH, BnBr, DMF, 0 °C; (b) TFA/H_2O (2:1), 50 °C; (c) TIDPSCl_2/imidazole, DMF, 0 °C; (d) DAST, CH_2Cl_2, rt; (e) TBAF/AcOH, THF, rt; (f) BzCl, pyridine, rt; (g) BCl_3, CH_2Cl_2, −78 °C; (h) *N,N*-dibocprotected adenine, DIAD, Ph_3P, THF, 0 °C; (i) TFA, CH_2Cl_2, rt; (j) DIBAL-H, CH_2Cl_2, −78 °C.

degradation.[7] Due to these features, carbocyclic nucleosides have received much attention as potential chemotherapeutic agents.[8] Carbovir and entecavir are examples of results of these efforts.

It is also well known that incorporation of a fluorine atom at the 2′-position of nucleosides can increase the stability of the glycosyl bond towards chemical and metabolic degradation.[9,10] A fluorine substitution on the carbocyclic sugar moiety has been proven to be useful in producing effective antiviral agents as demonstrated by our group in 2′-fluoro-5-methyl-β-L-arabinofuranosyluracil (L-FMAU or clevudine)[11] as well as in clofarabine.[12]

In view of the 2′-F substitution[9,10] as well the introduction of an exocyclic double bond to carbocyclic nucleosides,[4] which have been beneficial for anti-HBV activity such as in entecavir, 2′-fluoro-6′-methylene-carbocyclic adenosine or (+)-9-[(1*R*,2*R*,3*R*,4*R*)-2-fluoro-3-hydroxy-4-(hydroxymethyl)-5-methylenecyclopentan-1-yl] adenine **9** was synthesized and evaluated for its antiviral activity against wild-type HBV as well as adefovir, lamivudine and lamivudine/entecavir (double)-resistant mutants in vitro.

The synthesis of the target nucleoside **9** commenced with compound **7** as the key intermediate (Scheme 1). Compound **1** was synthesized according to the reported procedure from our group.[13] The allylic hydroxyl group of **1** was protected with a benzyl group and subsequent deprotection of the acetonide and the *t*-butyl group of compound **2** gave **3** in 86% yield. The 3, 5-hydroxy groups of **3** were selectively protected with 1,3-dichloro-1,1,2,2-tetraisopropyl disilazine to give **4** in 95% yield. Transformation of the 2-β-hydroxyl group to 2-α-fluoro was accomplished by treating the alcohol **4** with DAST to give 47% yield of compound **5**. However, debenzylation of **5** was unsuccessful under the Birch reduction or the Lewis acid (BCl_3) conditions. Therefore, the silyl group of **5** was removed by using tetrabutyl ammonium fluoride (TBAF/HOAc) to yield 82% of a diol, which was re-protected by benzoyl chloride in pyridine to give the fully protected intermediate **6** in 86% yield. The compound **6** was then treated with BCl_3 at −78 °C to obtain the key intermediate **7** in 76% yield. *N,N*-diboc protected adenine was synthesized according to the reported protocol in literature[14] and condensed with **7** to obtain **8** in 51% yield. The deprotection of the Boc group was carried out by TFA to afford 82% yield. Eventually, the treatment of DIBAL-H gave the target compound **9**[15] in 76% yield.

The synthesized nucleoside **9** was evaluated for its antiviral activity against wild-type HBV as well as adefovir, lamivudine and lamivudine/entecavir-drug resistant mutants in vitro,[16] and the results are summarized in Table 1. As the compound **9** is a derivative of an adenine analog, we directly compared its antiviral activity to that of adefovir instead of entecavir (a guanine analog) although the carbocyclic moiety is similar to that of entecavir. Furthermore, compound **9**, an adenine analogue, can interact with the thymidine moiety in the DNA template–primer site while entecavir interacts with the cytosine moiety at the same site in the active site. Thus, the base moiety is the major deciding factor, not the sugar moiety in determining the mode of action.

The target compound **9** demonstrated a significant antiviral in vitro activity against wild-type (WT) HBV with an EC_{50} value of 1.5 μM. The antiviral potency was similar to that of adefovir, while being 7-fold less potent than lamivudine. However, the concentration of the compound **9** required to inhibit 90% (EC_{90}) of wild-type HBV is 4.5 μM, which is 1.5-fold more potent than adefovir (EC_{50} 7.1 μM; Table 1).

The compound **9** also showed excellent activity against both lamivudine and adefovir resistant HBV mutants.[17] Particularly, the compound **9** showed a 4.5-fold enhanced potency of EC_{50} (1.7 μM) and a 7.8-fold more favorable EC_{90} (4.6 μM) against adefovir mutant rtN236T. For lamivudine mutants, rtM204V and rtM204I, the compound **9** showed an EC_{50} value of 1.8 versus 1.6 μM for adefovir, and 1.0 versus 1.9 μM for compound **9** and adefovir, respectively, while in the EC_{90} value, compound **9** demonstrated more favorable anti-HBV activity for both mutants, rtM204 V (4.7 vs 7.0 μM) and rtM204I (5.0 vs 8.0 μM). For mutant rtL180M, the antiviral activity of compound **9** was similar to that of lamivudine in the EC_{50} 2.1 versus 1.5 μM, while the compound **9** exhibited a 4.3-fold increased antiviral activity in the EC_{90} value (5.1 vs 22.0 μM).

Compound **9** was also evaluated against the lamivudine double mutant, rtL180M/rtM204V, and it exhibited the EC_{50} 2.2 μM that was equal to the adefovir, while the EC_{90} value of 5.5 μM of compound **9** was more effective than that of adefovir (8.5 μM). In addition, deamination studies with adenosine deaminase from calf thymus indicated that the compound **9** was completely stable.[18]

In preliminary studies, compound **9** was also evaluated against lamivudine/entecavir double resistant clone (L180M + S202I + M202V), in which compound **9** demonstrated significant anti-HBV activity (EC_{50} 0.67 μM) against the mutant. In the case of lamivudine and entecavir, there are significant decrease in their

Table 1
In vitro anti-HBV activity against adefovir, lamivudine and entecavir drug-resistant mutants in the intracellular HBV DNA replication assay[16,17]

Strains	Compound **9** (μM)				Adefovir (μM)			Lamivudine (μM)			Entecavir (μM)		
	EC_{50}[b]	EC_{90}[c]	CC_{50}[d,e]	Fold resistance[f] (EC_{90})	EC_{50}	EC_{90}	Fold resistance (EC_{90})	EC_{50}	EC_{90}	Fold resitance (EC_{90})	EC_{50}	EC_{90}	CC_{50}
Wild Type	1.5	4.5	>100	—	1.3	7.1	—	0.2	0.6	—	0.008	0.033	28
rtM204V	1.8	4.7	>100	1.0	1.6	7.0	1.0	>100	>100	>166	NT[h]	NT	NT
rtM204I	1.0	5.0	>100	1.1	1.9	8.0	1.1	>100	>100	>166	NT	NT	NT
rtL180M	2.1	5.1	>100	1.1	5.5	7.7	1.1	1.5	22.0	36.7	NT	NT	NT
rtLM/rtMV[a]	2.2	5.5	>100	1.2	2.1	8.5	1.2	>100	>100	>166	NT	NT	NT
rtN236T	1.7	4.6	>100	1.0	7.8	36.0	5.1	0.2	0.9	1.5	NT	NT	NT
rtLM/rtMV/rtSG[g]	0.67	NT	NT	—	NT	NT	—	>500[i]	NT	—	1.20[j]	NT	NT

[a] rtLM/rtMV = rtL180M/rtM204V double mutant.
[b] Effective concentration required to inhibit 50% of HBV-DNA.
[c] Concentration required to reduce infectious virus titer by 90%.
[d] The > sign indicates that the 50% inhibition was not reached at the highest concentration tested.
[e] The drug concentration required to reduce the cellular viability by 50% as assayed by an MTT assay.
[f] Fold resistance = (mutant EC_{90})/(wt EC_{90}).
[g] rtLM/rtMV/rtSG = rtL180M/rtM204V/rtS202G.
[h] NT = not tested.
[i] Ref. 19.
[j] Ref. 20.

antiviral potency (EC_{50} > 500 and 1.2 μM, respectively) as shown in Table 1.[19,20]

It was of interest to know how the compound **9** demonstrated the favorable anti-HBV activity in comparison to that of adefovir. Therefore, molecular modeling studies were conducted to obtain the insight of the molecular mechanism of compound **9** by using the Schrodinger suite.[21] The homology model of HBV RT was constructed based on the published X-ray crystal structure of HIV reverse transcriptase (PDB code: 1RTD),[22] which was previously used for molecular mechanism studies of several anti-HBV nucleosides.[23] In the homology model of HBV polymerase, the relative position of α-, β- and γ-phosphates of compound **9** with respect to the catalytic triad were assumed to occupy the similar position to the dNTP in the crystal structure of the HIV-1 RT-DNA-dNTP complex. The molecular docking[24] of compound **9** shows that the triphosphate forms all the network of hydrogen bonds with the active site residues, S85, A86, A87, R41, K32 (Fig. 1a). The γ-phosphate of compound **9** retains a critical H-bonding with the OH of S85 with connection of hydrogen bonds between S85 and N236. Generally, the N236T mutant loses the hydrogen bond to S85, which results in destabilization of the S85 to γ-phosphate interaction, thus causes resistance. However, compound **9** (as its triphosphate) maintains a critical H-bonding with S85 (Fig. 1b) similar to that as observed in wild type HBV (Fig. 1a).

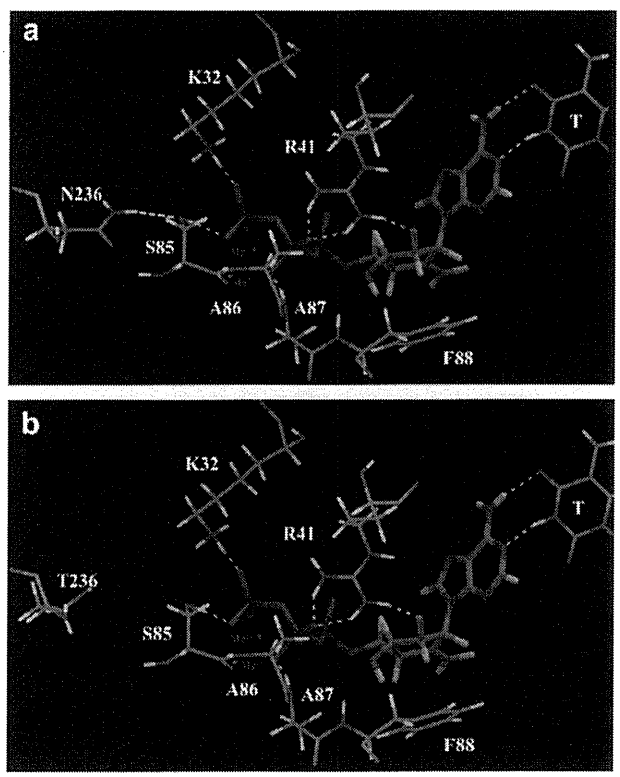

Figure 1. Binding mode and van der Waals interaction of compound **9** (a) in wild-type HBV and (b) in N236T adefovir mutant HBV. Yellow dotted lines are hydrogen bonding interactions (<2.5 Å).

The carbocyclic ring with an exocyclic alkene of compound **9** occupies the hydrophobic pocket (residues F88, L180 and M204) and makes the favorable van der Waals interaction with F88 (Fig. 1a and b). The 2′-fluorine substituent in the carbocyclic ring of compound **9** appears to promote an additional binding with R41 as shown in Figure 1a and b, which corroborates with the antiviral activity of compound **9** shown in Table 1. Overall, the modeling studies can qualitatively explain the favorable anti-HBV activity of the newly discovered compound **9** in WT (Fig. 1a) as well as against adefovir resistant mutant, N236T (Fig. 1b). These modeling studies are qualitative, and therefore, more quantitative calculation is warranted in the future.

In summary, a novel carbocyclic adenosine derivative **9** was synthesized, and evaluated for its anti-HBV activity. From these studies, the target nucleoside demonstrated significant anti-HBV activity against both the wild-type as well as the major nucleoside-resistant HBV mutants (adefovir and lamivudine), including the lamivudine/entecavir double mutant. In view of these promising anti-HBV activities, further biological and biochemical studies of the nucleoside **9** is warranted to assess the full potential as an anti-HBV agent.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.08.113.

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Cross-species transmission of gibbon and orangutan hepatitis B virus to uPA/SCID mice with human hepatocytes

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ABSTRACT

To investigate the potential of cross-species transmission of non-human primate HBV to humans, severe combined immunodeficiency mice transgenic for urokinase-type plasminogen activator, in which the mouse liver has been engrafted with human hepatocytes, were inoculated with non-human primate HBV. HBV-DNA positive serum samples from a gibbon or orangutan were inoculated into 6 chimeric mice. HBV-DNA, hepatitis B surface antigen (HBsAg), and HB core-related antigen in sera and HBV cccDNA in liver were detectable in 2 of 3 mice each from the gibbon and orangutan. Likewise, applying immunofluorescence HBV core protein was only found in human hepatocytes expressing human albumin. The HBV sequences from mouse sera were identical to those from orangutan and gibbon sera determined prior to inoculation. In conclusion, human hepatocytes have been infected with gibbon/orangutan HBV.

1. Introduction

Hepatitis B is caused by hepatitis B virus (HBV), a hepatotropic virus of the family *Hepadnaviridae*. This family comprises two genera, Avihepadnavirus and Orthohepadnavirus which can infect birds and mammals, respectively (Mason et al., 2005). As for humans, approximately 350 million chronic carriers have been infected by HBV worldwide (Lavanchy, 2004) and 15–40 percent have developed liver cirrhosis and hepatocellular carcinoma (Lee, 1997; McQuillan et al., 1989; Sharma et al., 2005). In addition to humans, HBV also infects higher non-human primates (apes) such as orangutans (*Pongo pygmaeus*), gibbons (*Hylobates* sp. and *Nomascus* sp.), gorillas (*Gorilla gorilla*), and chimpanzees (*Pan troglodytes*) (Grethe et al., 2000; MacDonald et al., 2000; Makuwa et al., 2003; Noppornpanth et al., 2003; Sall et al., 2005; Sa-nguanmoo et al., 2008; Starkman et al., 2003; Warren et al., 1998). In comparison with human HBV, non-human primate HBVs contain a 33 nucleotide deletion in the *PreS1* gene and all non-human primate HBVs cluster within their respective group separate from each human HBV genotype (Grethe et al., 2000; Kramvis et al., 2005; Robertson, 2001; Takahashi et al., 2000).

Several experiments have been conducted to study cross-species transmission of human HBV to non-human primates. Human HBsAg positive sera were intravenously inoculated into chimpanzees. In all experiments, inoculated chimpanzees displayed HBsAg in their sera (Kim et al., 2008; Tabor et al., 1980). In 1977, Bancroft et al. inoculated pooled saliva collected from 5 human carriers into gibbons. Gibbons which received subcutaneous injections of the pooled saliva developed serological markers of HBV infection. In contrast, gibbons infected via either the nasal or oral route did not show evidence of HBV infection (Bancroft et al., 1977). However, the negative results in this study are probably attributable to the lack of a sufficiently sensitive test available at that time. Alter et al. transmitted semen and saliva of carrier patients to chimpanzees. Chimpanzees developed HBsAg and elevated ALT after inoculation (Alter et al., 1977). In 1980, Scott et al. inoculated semen donated by HBsAg and HBeAg positive patients

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into gibbons via the subcutaneous and vaginal route. Moreover, saliva of carrier patients was pooled and inoculated into gibbons via the subcutaneous and oral route. The results showed that semen and saliva from carrier patients cause asymptomatic disease in gibbons when transmitted via the subcutaneous or vaginal route, yet not via the oral route (Scott et al., 1980).

In addition to these experiments, Mimms et al. performed studies by infecting a chimpanzee with gibbon HBV. The HBV-DNA sequence from this chimpanzee was similar to that of gibbon HBV (Mimms et al., 1993). In conclusion, human HBV can be transmitted to non-human primates and cross-species transmission of non-human primate HBV can occur among various non-human primate species. However, cross-species transmission of non-human primate HBV to humans has not yet been supported by scientific evidence. To avoid performing experiments in humans, severe combined immunodeficiency mice transgenic for urokinase-type plasminogen activator, with the liver replaced with human hepatocytes (chimeric mice) serve as a suitable model for studies on human liver-specific pathogens such as HCV and HBV, human hepatic metabolism of pharmaceutical agents, and human hepatic toxicity of candidate anti-proliferative agents (Kneteman and Mercer, 2005). The mice present evidence that more fully characterizes the repopulation of the mouse liver with human hepatocytes (Meuleman et al., 2005). Histological studies have revealed that chimeric mice show evidence of human hepatocyte replace integration with infiltration into mouse liver. Moreover, human albumin and 21 other human specific proteins can be detected in mouse sera (Dandri et al., 2001; Mercer et al., 2001). Subsequently, these mice were used to support woodchuck and human hepatocyte culture and were supported infection with woodchuck hepatitis virus (WHV) and HBV (Meuleman et al., 2005; Petersen et al., 1998; Tabuchi et al., 2008).

The aim of this study has been to demonstrate that non-human primate HBV can be replicated in human hepatocytes in order to consider preventive measures in case of potential HBV transmission from non-human primates to humans.

2. Materials and methods

The study was approved by the Faculty of Veterinary Science, Animal Care and Use Committee, Mahidol University. All experiments were performed in a biosafety level 2 laboratory.

2.1. Gibbon and orangutan HBsAg-positive serum

To study cross-species transmission of non-human primate HBV to humans, the HBsAg and HBV-DNA positive sera of white-cheeked gibbon (*Nomascus leucogenys*) and orangutan (*P. pygmaeus*) were collected from Dusit zoo, Bangkok and Khao Pratub Chang Wildlife Breeding Center, Ratchaburi, Thailand, respectively. These sera constitute the stored surplus sera from a previous study (Sa-nguanmoo et al., 2008).

2.2. Chimeric mice inoculation

Twelve-week-old SCID mice transgenic for urokinase-type plasminogen activator with human hepatocytes (PhoenixBio Co, Ltd., Hiroshima, Japan) were used in this study (Tateno et al., 2004). Real-time PCR was employed to detect non-human primate HBV DNA concentration in gibbon and orangutan serum. This detection method has been shown elsewhere (Abe et al., 1999).

The minimum infectious dose of pre-acute and late acute HBV for HBV transmission to chimeric mice with human hepatocyte repopulation is approximately 10^0 and 10^2 copies (Tabuchi et al., 2008). In this study, 10^4 gibbon or orangutan HBV genome equivalents were intravenously inoculated into 3 chimeric mice of each group. However, none of the chimeric mice showed evidence of HBV markers until week 4 after inoculation. Then, all chimeric mice were re-inoculated with 10^5 genome equivalents.

2.3. Serum collection and HBV DNA extraction

Twenty microliter serum samples were collected once a week after inoculation. HBV DNA was extracted from 5 μl mouse sera by using the QIAamp® DNA Mini kit (QIAGEN, QIAGEN Sciences Inc., MD) following the manufacturer's recommendation.

2.4. HBV DNA quantitative method

HBV DNA quantity was determined by real-time PCR (ABI 7500 Fast Real-time PCR, Applied Biosystems, Foster City, CA). To that end, the small S region was amplified as previously described (Abe et al., 1999). Briefly, 5 μl of DNA were subjected to quantitative HBV DNA analysis by ABI 7500 Fast Real-time PCR (Applied Biosystems, Foster City, CA). The reaction mixture comprised 12.5 μl TaqMan® Universal PCR MasterMix (Applied Biosystems, Foster City, CA), 0.5 μl of 10 μM forward primer (HBSF2: 5′-CTTCATCCTGCTGCTATGCCT-3′), 0.5 μl of 10 μM reverse primer (HBSR2: 5′-AAAGCCCAGGATGATGGGAT-3′), 0.5 μl of 10 μM probe (HBSP2G: FAM-ATGTTGCC CGTTTGTCCTCTAATTCCAG-TAMRA) and 6 μl distilled water. The real-time PCR was performed under the following conditions: 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 30 s, and 4 °C for the holding step. The HBV viral load in unknown samples was calculated by comparison with the standard curve. The detection limit in this study was 1000 copies/ml due to the small sample volume.

2.5. DNA extraction from mouse liver tissue and cccDNA detection in liver and sera of infected chimeric SCID mice

Mouse liver tissues from one HBV-DNA positive mouse each from the gibbon and orangutan HBV inoculation group were collected at week 15 after inoculation. To extract DNA from mouse liver tissue, 25 mg of liver tissue were extracted by using the DNeasy® Blood & Tissue kit (QIAGEN, QIAGEN Sciences Inc., MD) and eluted in 200 μl of elution buffer. HBV cccDNA was detected by conventional PCR (GeneAmp® PCR System 9700, Applied Biosystems, Foster City, CA). Primer sequences have been previously published (Suzuki et al., 2009). Partially double-stranded HBV DNA could not be amplified by these primers. The details have been previously described (Mason et al., 1998). Briefly, 5 μl of DNA were subjected to amplification by GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA). The reaction mixture comprised 1 U of Ampli Taq Gold® (Applied Biosystems, Foster City, CA), 2.5 μl of 10× PCR buffer containing 15 mM $MgCl_2$, 2 μl of GeneAmp® dNTP Mix (Applied Biosystems, Washington, UK), 1 μl of 10 μM forward primer (cccF2: 5′-CGTCTGTGCCTTCTCATCTGA-3′), 1 μl of 10 μM reverse primer (cccR4: 5′-GCACAGCTTGGAGGCTTGAA-3′), and 13.3 μl distilled water. The PCR was performed under the following conditions: 96 °C for 10 min, followed by 45 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s, and 4 °C for the holding step.

2.6. Entire genome sequencing and phylogenetic analysis

Mouse serum samples positive for HBV DNA were subjected to further studies by sequencing the entire genome sequences. To amplify the entire genome, 1 μl of DNA re-suspended solution was used as template for round I PCR. The entire genome was distinguished into two segments (fragment A and fragment B). Fragment A was amplified by 10 μM forward primer (HBV17F-SARU: 5′-CAAACTCTGCAAGATCCCAGAG-3′) and 10 μM reverse

primer (HBV1799R-SARU 5′-GACCAATTTATGCCTACAGCCTC-3′). Fragment B was amplified by 10 μM forward primer (HBV1595F-SARU: 5′-CTTCACCTCTGCACGTTGCATGG-3′) and 10 μM reverse primer (HBV262R-SARU: 5′-CCACCACGAGTCTAGACTCTGTGG-3′). Both fragment A and fragment B used the same reaction mixture as follows: 5 μl of 2.5 mM dNTP, 2 μl of 10 μM forward primer, 2 μl of 10 μM forward primer, 0.33 μl of LA-Taq (TaKaRa BIO INC, Shiga, Japan), and 29.67 μl distilled water. The amplification method was performed on GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA). The thermal cycle was continued as follows: 95 °C for 2 min (pre-denaturation) and followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 2 min, and 72 °C for 15 min (final extension).

For the second round PCR, 2 μl of round I PCR was used as template. Round I PCR product of fragment A was nested by HBV47F-SARU forward primer (5′-CTGTATTTTCCTGC-TGGTGGCTCCAG-3′) and HBV1760R-SARU reverse primer (5′-TAACCTCGTCTCCGCCCCAAACTC-3′). The first round I PCR product of fragment B was nested by HBV1608F-SARU forward primer (5′-GCATGGAGACCACCGTGAACG-3′) and HBV201R-SARU reverse primer (5′-TGTAACACGAGCAGGGGTCCTAGG-3′). Both fragment A and fragment B used reaction mixtures as round I PCR except increasing in the first round PCR template to 2 μl and adjusting distilled water to 28.67 μl. The amplification program was performed as follows: 95 °C for 2 min (pre-denaturation) and followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 2 min, and 72 °C for 20 min (final extension).

The second round PCR products were segregated by electrophoresis on 1% agarose gel stained with ethidium bromide. The bands of PCR products were purified using the QIAquick Gel Extraction kit (QIAGEN GmbH, Hilden, Germany). Purified products were further analyzed by ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

The genome was sequenced using the 8 primer sets previously published (Sugauchi et al., 2001). Cycle sequencing was performed using the BigDye Terminator 3.1V cycle sequencing kit (Applied Biosystems, Foster City, CA) according to the manufacturer's recommendations. The conditions for sequencing were programmed into the GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA) as previously reported (Sugauchi et al., 2001). Nucleotide sequences were edited and assembled using SEQMAN 4.00 (LASERGENE program package, DNASTAR, DNASTAR Inc., Madison, WI). All complete HBV genomes isolated from mouse sera were compared to nucleotide sequences available at the GenBank database by using the Blast program (NCBI, Bethesda, MD). Moreover, the HBV sequences obtained from mouse sera were compared with gibbon and orangutan HBV strains determined prior to inoculation and also compared with other non-human primate HBVs and each human genotype from the GenBank database (NCBI, Bethesda, MD). Genetic comparison was performed by Clustal X program version 2.0.10 (European Bioinformatics Institute, Cambridge, UK). Subsequently, the phylogenetic tree was constructed using the Tamura – 3 parameter neighbor-joining method by Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 (The Biodesign Institute, Tempe, AZ).

2.7. HBsAg, HBcrAg, and human albumin measurement in mouse sera

Mouse sera were diluted (1:10) and subjected to chemiluminescence enzyme immunoassay (CLEIA) (Fujirebio Diagnostic, Inc., Tokyo, Japan) to detect HBV surface antigen (HBsAg) and HBV core – related antigen: – the antigen which includes both the HBV pre-core/core proteins (HBcrAg) (Kimura et al., 2005; Shinkai et al., 2006). HBcrAg measurement by this assay implies detection of pre-core/core proteins, including core protein and HBeAg (Kimura et al., 2002, 2005; Rokuhara et al., 2003; Wong et al., 2007). HBcrAg also showed a good correlation with HBV DNA levels in Asian patients (Kimura et al., 2002; Rokuhara et al., 2003, 2005) and intrahepatic parameters, including fibrosis scores, intrahepatic HBV, cccDNA and nuclear HBcAg (Wong et al., 2007). To expose the core protein and HBeAg, the diluted serum was fist incubated with the solution that contains sodium dodecylsulfate. Subsequently, the lysate was added to the plate coated with primary antibody to HBcAg and HBeAg. After incubation, the plate was washed to discard excess primary antibody and the second antibody labeled with alkaline phosphatase was added. Upon addition of substrate solution, the incubated reaction was measured by chemiluminescent enzyme immunoassay (CLEIA). Fully automated analysis was performed using the Lumipulse® System (Fujirebio Diagnostic, Inc., Tokyo, Japan). Human serum albumin (h-Alb) levels were determined applying a commercial enzyme linked immunosorbent (ELISA) test kit (Bethyl Laboratories Inc., Montgomery, TX).

2.8. Immunohistofluorescence assay

To detect HBcAg and human albumin, thick mouse liver tissue was prepared by cutting the frozen mouse liver with a Leica CM1900 Cryostat-microtome (Meyer Instruments, Inc., Houston, TX) and mounting the slices on glass slides. Histological analysis was performed by immunofluorescence assay as previously reported (Sugiyama et al., 2006). Briefly, mouse liver tissue was blocked by DakoCytomation antibody diluent (Dako North America, Inc., Carpinteria, CA) for 10 min at room temperature. After drying by air, the tissue was incubated in the dark with 50 μg/ml of polyclonal rabbit anti-hepatitis B virus core antigen (HBcAg) (Dako North America, Inc., Carpinteria, CA) for 1 h at 37 °C. After washing 5 times with 1× phosphate buffered saline (PBS) (GIBCO, Invitrogen Corporation, Carlsbad, CA) the tissue was incubated with 50 μg/ml of Cy3® goat anti-rabbit IgG (H + L) (Invitrogen Molecular Probes, Eugene, OR) or 5 μg/ml of goat anti-human albumin FITC (Bethyl Laboratories, Inc., Montgomery, TX) in the dark at 37 °C for 1 h. After washing 5 times with 1× PBS, the tissue was mounted by VECTASHIELD mounting medium with DAPI (Vector Laboratories, Inc., Birmingham, CA). The stained mouse tissue was examined under a Nikon Microscope ECLIPSE E800 (Nikon Instruments, Inc., Melville, NY).

3. Results

3.1. Serum HBV DNA, HBsAg, HBcrAg and human albumin level quantitation

Upon first inoculation with serum containing 10^4 copies of gibbon or orangutan HBV, none of the mice could be infected. Then, chimeric mice were re-inoculated with 10^5 copies. One mouse died before re-inoculation. After re-inoculation, mouse sera were collected once a week. Samples were subjected to quantitative HBV DNA analysis by real-time PCR while HBsAg and HBcrAg were quantitatively determined by CLIEA. Four of 5 mice could be infected with gibbon or orangutan HBV. Two mice each from the gibbon and orangutan groups showed levels of HBV DNA, HBsAg, and HBcrAg with the remaining mouse not displaying any of these markers. In detail, HBV DNA and HBcrAg could be detected in serum samples from two mice of the gibbon group (code 101 and 103) and two mice of the orangutan group (code 201 and 202) 4 weeks after inoculation. HBsAg was present in the orangutan group 4 weeks and in the gibbon groups 6 weeks after inoculation, respectively.

In this experiment, the expected HBV markers HBV DNA, HBsAg and HBcrAg could be detected in mouse serum around 4–5 weeks after inoculation. This finding matched previous studies that had

inoculated human HBV genotypes A2, C2, B1 and J into chimeric SCID mice (Sugiyama et al., 2009; Tatematsu et al., 2009). The time appearance and progression of non-human primate HBV markers presented as same as with human HBV markers (Ganem and Prince, 2004). Human albumin (h-Alb) was measured by ELISA as a quality control. Serum h-Alb levels prior to inoculation of all mice in this study exceeded 7 mg/ml indicating a human hepatocyte replacement index (RI) of over 70 percent (PhoenixBio Co, Ltd., Hiroshima, Japan) and were stable during the experiment (Fig. 1). Mean alanine aminotransferase (ALT) levels were around 200 IU/L in the uPA/SCID mouse sera. After non-human primate HBV inoculation, ALT levels slightly increased in this study (data not shown).

3.2. Intrahepatic cccDNA detection in liver tissue and mouse sera

Using the specific primers that amplify only cccDNA (Suzuki et al., 2009), HBV cccDNA was detected in mouse liver tissue from those mice that had been infected with gibbon and orangutan HBV (Fig. 2A). Moreover, cccDNA was found in the sera of mice infected with gibbon HBV (Fig. 2B).

3.3. Phylogenetic analysis of the entire HBV genome from mouse sera

HBV-DNA from all four mice was amplified and subjected to sequencing of the entire genome. The sequences from mouse sera were identical to HBV from gibbon or orangutan serum determined prior to inoculation (gibbon code GD14, GenBank ID: HQ603061; orangutan code OS25, GenBank ID: EU155824) (Fig. 3). Comparison between the complete HBV sequences from mouse sera and gibbon or orangutan sera prior to inoculation showed 99.9% and 100% similarity, respectively.

3.4. HBcAg and human albumin detection in mouse liver tissue

The mouse liver was also tested for HBcAg by staining with polyclonal rabbit anti-HBcAg and goat anti-rabbit IgG labeled with Cy3 (Fig. 4A). To locate the human hepatocyte area in chimeric mouse liver, the tissue was examined for human albumin. The same mouse liver tissue was stained with goat anti-human albumin conjugated with FITC (Fig. 4B). The study confirmed that HBcAg was found in the same area of human hepatocytes (Fig. 4C).

4. Discussion

In a previous study, Hu et al. (2000) constructed a phylogenetic tree and found that the S gene sequence from two chimpanzees clustered with human HBV genotypes A and C which could suggest possible virus transmission from human to chimpanzee. Currently, there is no evidence indicating natural infection of humans with non-human primate HBV (Noppornpanth et al., 2003). However, non-human primate HBV would be transmitted to humans because the respective HBV genomes are largely similar.

In this study, cross-species transmission was performed using chimeric mice containing human hepatocytes. The results showed that HBV-DNA, HBsAg and HBcrAg can be detected in sera of mice inoculated with HBV-DNA positive sera from orangutan or gibbon carriers. Detection of HBV cccDNA in liver as well as immune staining data have provided the evidence that gibbon and orangutan HBV can be replicated in human hepatocytes of the chimeric mice sero-positive for HBV DNA. HBsAg and HBV DNA concentrations could increase over time following inoculation. Interestingly, based on phylogenetic analysis, all strains of HBV sequences obtained from mouse sera inoculated with gibbon or orangutan HBV carrier sera grouped with HBV from gibbon and orangutan sera determined prior to inoculation. Nucleotide comparison between HBV in mouse sera and the HBV strain used for inoculation showed 100% identity.

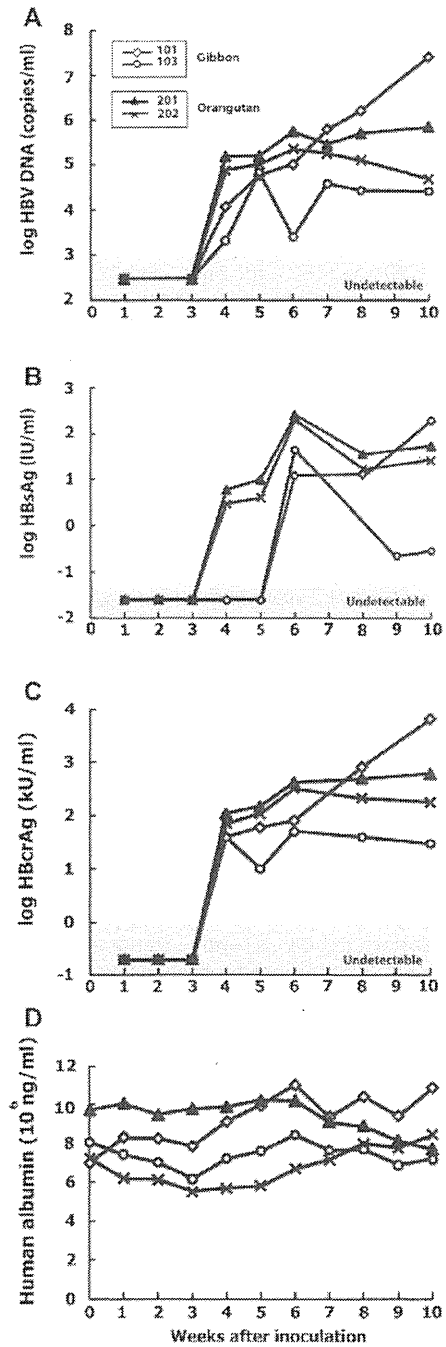

Fig. 1. HBV DNA, HBsAg, HBcrAg, and human albumin concentration in inoculated mouse sera on secondary inoculation. (A) Serum HBV DNA level. Gray zone indicates an area below the minimum sensitivity of real-time PCR ($<10^3$ copies/ml) (B) HBsAg concentration. The limitation of the test is 0.05 IU/ml. (C) HBcrAg level with the limited sensitivity at 1 kU/ml and (D) h-Alb concentration.

HBV infection depends on the infectious doses of HBV inoculums and host factors. In our experiment, one SCID mouse with human hapatocytes could not be infected with non-human primate HBV. This mouse lacks T- and B-lymphocytes as a protection from viral infection but still, it remains clear from viral infection. Some

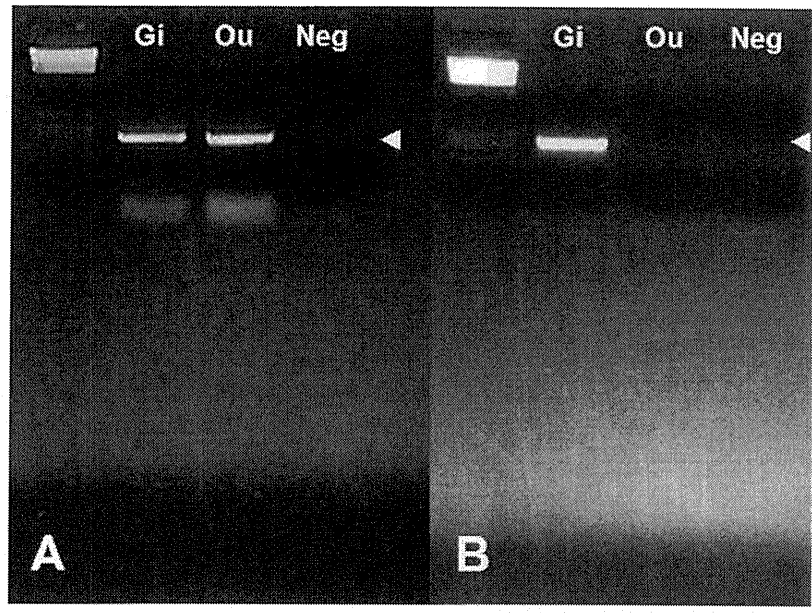

Fig. 2. cccDNA detection in liver (A) and sera (B) of mice that infected with gibbon HBV (Gi) or orangutan HBV (Ou). Neg represents negative PCR control (lacking DNA template). Arrow represents the target cccDNA PCR product.

researchers have attributed this to innate immunity of SCID mice (Lin et al., 1998). SCID mice have a normal innate immune system such as monocytes and macrophages (Ansell and Bancroft, 1989) which probably plays an important role in these mice. Moreover, infection of human hepatocytes with non-human primate HBV may be difficult due to the higher infectious dose required. Moreover, research on the early step of non-human primate HBV attachment to human hepatocytes has not been performed and the pathway of non-human primate HBV infection is still unclear. In comparison with human HBV, it might not be easy for non-human primate HBV to infect human hepatocytes.

Notably, a previous study has reported a new human HBV genotype (HBV-J) isolated from a Japanese patient with hepatocellular carcinoma (Tatematsu et al., 2009). The first HBV strain of interspecies HBV genotype J was closely related to gibbon and orangutan HBV strains and had a deletion of 33 nucleotides at the *preS1* region identical to non-human primate strains. Interestingly, this patient used to live in Borneo—a gibbon and orangutan habitat and hence, an endemic area (Tatematsu et al., 2009). He may have been infected with non-human primate HBV either by close contact or by eating raw meat of non-human primate HBV carriers (personal communication). However, infection of humans with non-human

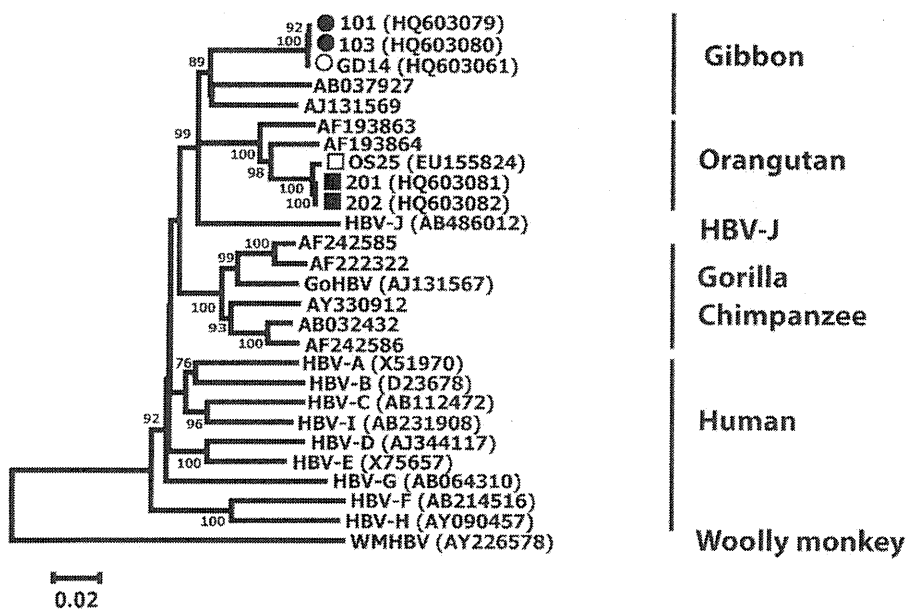

Fig. 3. Phylogenetic analysis of the entire HBV sequence obtained from mouse sera and available sequences of non-human primate HBV strains from GenBank database. Support of each branch as determined from 1000 bootstrap samples. Only 75% bootstrap values are indicated at each node. The scale bar at the bottom represents the genetic distance. Non-human primate HBV sequences obtained from our study are indicated by symbol (gibbon, ○; orangutan, □). HBV sequences obtained from mouse sera were ● and ■ for mice inoculated with gibbon and orangutan sera, respectively.

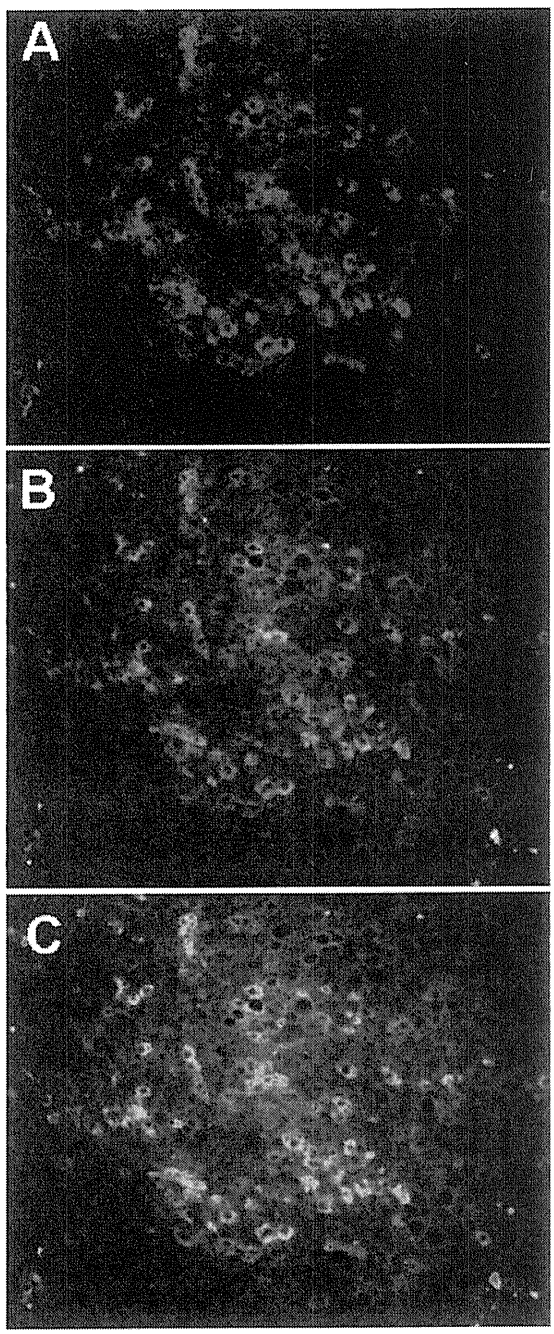

Fig. 4. Immunohistofluorescence of SCID mice infected with gibbon HBsAg-positive serum. Mouse liver tissue incubated for HBcAg (A), human albumin (B), and co-localization of HBcAg and human albumin (C).

primate HBV by eating raw meat or close contact with non-human primate HBV carriers would be hypothesis.

Yet, it has been reported that chimeric SCID mice with human hepatocytes can be infected by inoculation with HBV positive chimpanzee sera (Tabuchi et al., 2008) similar to what has been found in this study. In that previous study, human hepatocyte transplanted chimeric mice were used to study the HBV infectious titer in sera of pre-acute and late acute phase patients. These mice were inoculated with HBV infected chimpanzee sera. The chimeric mice also displayed HBV infection markers such as HBsAg, anti-HBc and anti-HBs as has been shown in this research. But the HBV in chimpanzee sera used to inoculate chimeric mice was human HBV, in contrast to the non-human primate HBV used in this study. Thus, this study is the first scientific evidence to prove and confirm that non-human primate such as gibbon and orangutan HBV can infect and replicate in human hepatocytes. Moreover, this finding can support the discovery of the HBV-J genotype which was found in the human and the assumption that humans can be infected with non-human primate HBV strains is still hypothesis.

Even though uPA-SCID mice with human hepatocytes constitute a useful animal model to study cross-species transmission, this model does not mirror the humoral and cellular immune response of the natural host. In real life, humans may be infected with non-human primate HBV and may clear this virus by their immune system. However, the results of this study indicated that human hepatocytes of chimeric mice have been infected with HBV from gibbon, orangutan and also with human HBV from infected chimpanzee sera as previously reported (Tabuchi et al., 2008). Previous studies have demonstrated cross-species transmission of human HBV to non-human primates, of non-human primate HBV to other species of non-human primates, and this study has demonstrated that non-human primate HBV can replicate in human hepatocytes. As non-human primates represent various virus reservoirs, not only of HBV but also lymphocryptovirus (LCV), Epstein-Barr virus (EBV), or simian foamy virus (SFV), people in close contact with animal HBV carriers should be aware and protect themselves from animal bites or exposure to infected blood or body fluids of non-human primates.

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REVIEW

Reactivation of hepatitis B virus following rituximab-plus-steroid combination chemotherapy

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Abstract Reactivation of hepatitis B virus (HBV) has been reported as a fatal complication following systemic chemotherapy or other immunosuppressive therapy. The risk of HBV reactivation differs according to both the patient's HBV infection status prior to systemic chemotherapy and the degree of immunosuppression due to chemotherapy. For establishing an optimal strategy for hepatitis prevention and treatment, it is necessary to understand the characteristics, the clinical course and the risk factors for HBV reactivation and to recognize the difference between hepatitis B surface antigen (HBsAg)-positive and -negative patients with HBV reactivation. Among the important viral risk factors, HBV-DNA level and HBV-related serum markers have been reported to be associated with HBV reactivation in addition to cccDNA, genotypes and gene mutations. Rituximab-plus-steroid combination chemotherapy has recently been identified as a host risk factor for HBV reactivation in hepatitis B core antibody (anti-HBc)-positive and/or hepatitis B surface antibody (anti-HBs) positive—but nonetheless HBsAg-negative—lymphoma patients. For these patients with resolved hepatitis B, preemptive therapy guided by serial HBV-DNA monitoring is a reasonable strategy to enable early diagnosis of HBV reactivation and initiation of antiviral therapy. In this review, we summarize the characteristics of HBV reactivation following rituximab-plus-steroid combination chemotherapy, mainly in HBsAg-negative lymphoma patients, and propose a strategy for managing HBV reactivation.

Keywords Reactivation · HBV · Rituximab

Abbreviations

Anti-HBc	Hepatitis B core antibody
Anti-HBs	Hepatitis B surface antibody
cccDNA	Covalently closed circular DNA
CHOP	Cyclophosphamide, doxorubicin, vincristine, prednisolone
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HSCT	Hematopoietic stem cell transplantation
R-CHOP	Rituximab, cyclophosphamide, doxorubicin, vincristine, prednisolone
RTD-PCR	Real-time detection PCR

Introduction

Reactivation of hepatitis B virus (HBV) has been reported not only in hepatitis B surface antigen (HBsAg)-positive patients following systemic chemotherapy, but also in a proportion of HBsAg-negative patients with anti-hepatitis B core antibody (anti-HBc) positivity and/or anti-hepatitis

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