mutations that direct-sequencing by the Sanger method could not reveal, existing in their probe regions using ultra-deep sequencing. Ultra-deep sequencing is useful for revealing the resistant strain(s) of HCV before DAA treatment as well as mixed infection with different genotypes or subgenotypes of HCV.

Materials and Methods

Patients

Sera from 80 consecutive treatment-naïve chronic hepatitis C genotype 1 patients with well-characterized clinical follow-up were selected from a chronic hepatitis C database at Chiba University Hospital, Chiba, Japan. All patients were negative for hepatitis B and HIV antibodies. Sera from patients were stored at -20°C until analysis. This study was approved by the Ethics Committee, Chiba University, Graduate School of Medicine, Chiba, Japan (permission numbers 1462 and 282), and conformed to the Helsinki Declaration. Written informed consent was obtained from all patients before enrollment in this study.

RNA extraction

Total RNA was extracted from 140 µL of each serum sample using a QIAamp Viral RNA Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. RNA was eluted in 60 µL of elution buffer and quantified using a NanoDrop Lite spectrophotometer (Thermo Scientific, Madison, WI, USA).

HCV subgenotyping by real-time PCR-based methods in the HCV NS5B region

Reactions were performed in a final volume of 50 µL using the Superscript™ III Platinum one-step quantitative RT-PCR system (Invitrogen, Carlsbad, CA, USA) as previously described [12]. The designed primers and MGB probes shown in Table 5 and the real-time PCR HCV subgenotyping protocol were used according to the previous description [12]. The reaction mixture was prepared in two fractions. The first fraction (F1) consisted of 18.5 µL of 2X reaction mix (6 mM $MgSO_4$, and 0.4 mM of each dNTP), 1.0 µL of *Taq* mix (Superscript™III RT, Platinum *Taq* Mix, Invitrogen), 200 nM of reverse primer (R56_1), and 0.5 µL of RNase Out (Invitrogen). Twenty-one microliters of F1 was added to optical tubes. The second reaction mix fraction (F2) consisted of 4.4 µL of 2X reaction mix, 200 nM of forward primer (F56_1), 1.5 µL of 50 mM $MgSO_4$, 0.1 µL of ROX (25 µM), and 200 nM of each probe (against either genotype 1a or 1b). A final F2 volume of 10 µL was transferred to another optical tube. After the addition of 19 µL of RNA, F1 was carefully added to F2. Reverse transcription was carried out in a TakaRa PCR Thermal Cycler (TaKaRa, Ohtsu, Shiga, Japan) at 55°C for 35 min with the thermocycler cover open. Following this, the tubes were briefly centrifuged and real-time PCR was carried out in a 7300 Real-Time PCR System (Applied Biosystems, Foster, CA, USA) using the following cycling parameters: 50°C for 2 min; 95°C for 10 min; and 40 cycles at 95°C for 15 sec, 50°C for 30 sec, and 60°C for 1 min. The total time required for performing this assay was only 2 hours, as previously described [12].

Table 5. Probes and primers used for real-time PCR-based method.

Name	Fluorophore	Sequence	Quencher
1a66	6-FAM	5'-CAGCTTGAACAGGC-3' MGB	NFQ-MGB
1b266	VIC	5'-CAACTTGAAAAAGC-3' MGB	NFQ-MGB
F56_1	-	5'-CACACTCCAGTYAAYTCCTGG -3'	-
R56_1	-	5'-CWMCTGGAGAGTAACTGTGGAG -3'	-

Probes and primers used for HCV subgenotypes 1a and 1b in HCV subgenotyping assay by real-time PCR-based method in the HCV NS5B region [12]. Probes 1a66 and 1b266 were used for the detection of HCV subgenotypes 1a and 1b, respectively.

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Table 6. Probes and primers for sequencing used in the present study.

Name	Sequence	Location*
Pr1 (sense)	5'-GTATGATACCCGCTGCTTTGA-3'	nt. 8253-8272
Pr2 (sense)	5'-TTCACGGAGGCTATGAC-3'	nt. 8613-8629
Pr2 (antisense)	5'-GTCATAGCCTCCGTGAA-3'	nt. 8629-8613
R56_1 (antisense)	5'-CTGGAGAGTAACTGTGGAG-3'	nt. 9036-9018

Primers used in direct-sequencing by Sanger method and ultra-deep sequencing of HCV NS5B region. * The location of primers corresponding to Con1.

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cDNA synthesis and amplification by PCR for ultra-deep sequencing

To perform ultra-deep sequencing of the MGB probe region in HCV NS5B, we used the HPLC-purified specific primers shown in Table 6. cDNA was synthesized with R56_1 (antisense) for 1 cycle at 55°C for 30 min and 85°C for 5 min using a Transcript high-fidelity cDNA synthesis kit (Roche, Tokyo, Japan). Then amplification was performed with Pr1 (sense) and R56_1 (antisense) for 35 cycles at 95°C for 30 sec, 55°C for 30 sec, and 72°C for 60 sec using a FastStart high-fidelity PCR system, dNTPack kit (Roche).

Then, the first PCR product was further amplified with two inner primer sets using the FastStart high-fidelity PCR system, dNTPack kit (Roche). Set one was Pr2 (sense primer) and R56_1 (antisense primer), and the other was Pr1 (sense primer) and Pr2 (antisense primer). Considering the MGB probe locations at nt 8913-8926(1a) in reference sequence HCV subgenotype 1a, strain H77 (AF009606.1), and nt8910-8923(1b) in reference sequence HCV subgenotype 1b, strain Con1 (AJ238799.1), primer set one was used for PCR. The PCR conditions were as follows: 35 cycles at 95°C for 30 sec, 55°C for 30 sec, and 72°C for 60 sec. Amplified products were separated by agarose gel electrophoresis and purified using a high pure PCR clean-up micro kit (Roche). Each amplicon was quantified using a NanoDrop Lite spectrophotometer (Thermo Scientific), and all amplicons from

a single viral genome were pooled together at equimolar ratios. Each pool was then quantitated, and approximately 500 ng of each was used in a fragmentation reaction mix using a GS FLX Titanium Rapid Library Preparation Kit (Roche). Final libraries representing each genome were characterized for average size by using an Agilent High Sensitivity DNA kit on Agilent 2100 Bioanalyzer (Agilent Technologies, Loveland, CO, USA). 4 x 10^7 molecules of these final DNA libraries were then subjected to emulsion PCR, and enriched DNA beads were loaded onto a picotiter plate and pyrosequenced with a Roche/454 GS Junior sequencer using Titanium chemistry (454 Life Sciences Corp., Branfold, CT, USA). GS Amplicon Variant Analizer Version 2.7 (Roche) was used for read mapping and calculating variant frequencies at each nucleotide position according to reference sequence HCV subgenotype 1a, strain H77 or HCV subgenotype 1b, strain Con1.

Direct-sequencing by Sanger method

Then, we amplified the first PCR product using primer set one and TaKaRa Ex Taq (TaKaRa). The PCR conditions were as follows: 40 cycles at 98°C for 10 sec, 55°C for 30 sec, and 72°C for 60 sec; the last cycle followed at 72°C for 7 min. Sanger sequencing was performed using a BigDye(R) Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Tokyo, Japan). Sequences were detected using Applied Biosystems 3730xl. Nucleotide sequences were analyzed by GENETYX 10 (GENETYX Corp., Tokyo, Japan) and imported to MEGA version 4 [28], which was used to align the sequences, according to reference sequences.

Construction of phylogenetic trees

A fragment of about 424-bp length containing the HCV NS5B region was used for phylogenetic analysis. Phylogenetic trees were inferred using the Neighbor-Joining (NJ) model and robustness of the tree branches was tested using bootstrap analysis (500 replicates) with MEGA version 4 [28].

Nucleotide sequence accession number

All sequence reads have been deposited in the DNA Data Bank of Japan (DDBJ) Sequence Read Archive under accession number DRA001077.

Statistical analysis

Data were expressed as mean ± standard deviation. We used univariative analyses, applying Student's t-test or Chi-square test as appropriate. *P 0.05* was considered statistically significant.

Author Contributions

Conceived and designed the experiments: SW TK SN. Performed the experiments: SW TK SN XJ TM OY. Analyzed the data: SW TK SN. Contributed reagents/materials/analysis tools: SW TK SN SMN SKO ATN TG. Wrote the manuscript: SW TK.

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Research Paper

Efficacy of Lamivudine or Entecavir against Virological Rebound after Achieving HBV DNA Negativity in Chronic Hepatitis B Patients

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Abstract

Nucleos(t)ide analogues (NAs) lead to viral suppression and undetectable hepatitis B virus (HBV) DNA in some individuals infected with HBV, but the rate of virological rebound has been unknown in such patients. We examined the prevalence of virological rebound of HBV DNA among NA-treated patients with undetectable HBV DNA. We retrospectively analyzed 303 consecutive patients [158 entecavir (ETV)- and 145 lamivudine (LAM)-treated] who achieved HBV DNA negativity, defined as HBV DNA < 3.7 log IU/mL for at least 3 months. They were followed up and their features, including their rates of viral breakthrough, were determined. Viral rebound after HBV DNA negativity was not observed in the ETV-group. Viral rebound after HBV DNA negativity occurred in 38.7% of 62 HBe antigen-positive patients in the LAM-group. On multivariate analysis, age was an independent factor for viral breakthrough among these patients (P = 0.035). Viral rebound after HBV DNA negativity occurred in 29.1% of 79 HBe antigen-negative patients in the LAM-group. Differently from LAM, ETV could inhibit HBV replication once HBV DNA negativity was achieved. In contrast, LAM could not inhibit HBV replication even if HBV negativity was achieved in the early phase. Attention should be paid to these features in clinical practice.

Key words: Entecavir, HBeAg, HBV DNA, Lamivudine, Virological rebound.

INTRODUCTION

Hepatitis B virus (HBV) infection remains a major health problem and one of the risk factors for the development of hepatocellular carcinoma (HCC) worldwide [1,2]. Chronic HBV infection has been linked epidemiologically to the development of HCC for more than 30 years [3]. To date, the mechanism of HBV-related hepatocarcinogenesis is not clear. Although effective vaccine exists for preventing HBV

infection [4], acute liver failure due to HBV or acute exacerbation of chronic hepatitis B is also a life-threatening disease [5,6].

Positivity for hepatitis B e antigen (HBeAg), which in serum indicates active viral replication in hepatocytes, is associated with an increased risk of HCC [7]. Chronic HBV carriers with high-titer viremia are also at increased risk for HCC [8]. The risk for cirrhosis and that for HCC increase significantly with increasing HBV DNA levels [9, 10]. Thus, it cannot be overstated that HBV DNA should be directly suppressed to prevent the development of HCC.

There are several nucleos(t)ide analogues (NAs) for the treatment of chronic hepatitis B [11]. Currently, the Japanese national health insurance system approves lamivudine (LAM) and entecavir (ETV) as first-line therapy for treatment-naïve patients with chronic hepatitis B, although some patients are treated with standard interferon-alfa or peginterferon-alfa-2a [6,12]. In general, LAM, the first oral NA available for the treatment of chronic hepatitis B, is associated with high rates of drug-resistance, with ~76% after 8 years of treatment [13,14]. ETV is found to be superior to LAM from the point of view that ETV is stronger than LAM and that resistance to ETV is rare, about 1.2% after 5 years of ETV treatment [14,15].

The aim of this study was to determine the efficacy and the rates of virological rebound after achieving HBV DNA negativity in the use of ETV or LAM in clinical practice. Our study showed that ETV could inhibit HBV replication if HBV DNA negativity had been achieved, but LAM was unable to inhibit HBV replication even if HBV negativity was achieved in the early phase.

MATERIALS AND METHODS

Patients and Study Design

This was a retrospective analysis comparing the rates of virological rebound in patients treated with ETV versus those in patients treated with LAM. A total of 303 patients were examined from Chiba University Hospital, Chiba, Japan, and 4 affiliated hospitals between the period of January 2000 and December 2011. NAs-naïve chronic hepatitis B patients daily receiving 0.5 mg of ETV (ETV group, N=158) or receiving 100 mg of LAM (LAM group, N=145) with undetectable HBV DNA (< 3.7 log IU/mL) for three months were enrolled. Some of the included patients had been previously reported [12, 16]. All patients had serum hepatitis B surface antigen (HBsAg) detectable for at least 6 months, regardless of their HBeAg status. They were negative for hepatitis C virus and human immunodeficiency virus antibodies. This study was approved by the Ethics Committee of Chiba University, Graduate School of Medicine (No. 977).

Definition of Virological Rebound of HBV

We defined virological rebound as $\geq$ 3.7 log IU/mL for at least 3 months after achieving undetectable HBV DNA.

Monitoring of HBV DNA, Serum Liver Function Tests and Hematological Tests

The primary outcome of this study was the virological rebound. Patients were followed up at least every 3 months to examine physical status and to monitor liver biochemistry and virology. All clinical laboratory tests including hematological data, biochemical data, and HBV serologies were performed at the Central Laboratory of Chiba University Hospital. HBsAg, HBeAg and anti-HBe antibody were determined by ELISA (Abbott, Chicago, IL, USA) or CLEIA (Fujirebio, Tokyo, Japan) [17]. HBV genotype was determined from patients' sera by ELISA (Institute of Immunology, Tokyo, Japan) as reported by Usuda et al [18]. HBV DNA was measured by transcription-mediated amplification (TMA) assay, COBAS Amplicor HBV Monitor assay, or COBAS TaqMan (Roche Diagnostics, Branchburg, NJ, USA). The clinical efficacy of NAs was assessed as the proportion of patients achieving HBV DNA negativity, defined as an HBV DNA level of < 3.7 log IU/mL.

Statistical analysis

Data were expressed as mean $\pm$ standard deviation (SD). Differences were evaluated by Student's *t*-test, chi-square test, or Fisher's exact test. $P < 0.05$ was considered statistically significant. Variables with $P < 0.05$ at univariate analysis were retained for multivariate logistic-regression analysis. For all tests, two-sided P-values were calculated and the results were considered statistically significant at $P < 0.05$. Statistical analysis was performed using the Excel-statistics program for Windows, version 7 (SSRI, Tokyo, Japan).

RESULTS

A total 303 patients were recruited into either the ETV group (n = 158) or the LAM group (n = 145), with a follow-up period of 33.7 $\pm$ 11.3 months (28.6 $\pm$ 11.3 months or 39.3 $\pm$ 31.4 months, respectively). Baseline demographic and laboratory data are summarized in Table 1. There were no differences in age, gender, HBV DNA, alanine aminotransferase (ALT) levels, ultrasound findings/presence of cirrhosis, and periods from the initial administration of ETV or LAM to

undetectable HBV DNA, between the ETV and LAM groups, although the proportion of HBeAg-positive patients in the ETV group (55%) tended to be higher than that in the LAM group (44%).

Virological Rebound

The patient flow and outcome are summarized in Figure 1. We excluded 9 patients, whose HBeAg status at baseline was unknown, from this analysis. When comparing the baseline characteristics of patients according to HBeAg status, HBeAg-positive patients were younger, had higher ALT levels and HBV DNA levels, and less cirrhotic findings by ultrasound than HBeAg-negative patients (Table 2). The period from the initial administration of ETV or LAM to the determination of undetectable HBV DNA in the HBeAg-negative group tended to be shorter than that in the HBeAg-positive group (Table 2).

In the ETV group, none of the patients had virological rebound during the follow-up periods. In the LAM group, 24 and 23 patients of 62 HBeAg-positive and 79 HBeAg-negative patients at baseline, respectively, developed evidence of virological rebound. In the 24 HBeAg-positive patients at baseline with virological rebound, 9, 8, 3, 1, 2, and 1 had virological rebound at ≤ 1, $1 \sim \leq 2$, $2 \sim \leq 3$, $3 \sim \leq 4$, $4 \sim \leq 5$, and details unknown, respectively. In the 23 HBeAg-negative patients at baseline with virological rebound, 10, 8, 3, 0, 1, and 1 had virological rebound at ≤ 1, $1 \sim \leq 2$, $2 \sim \leq 3$, $3 \sim \leq 4$, $4 \sim \leq 5$ and details unknown, respectively. Baseline characteristics of patients treated with ETV or LAM according to HBeAg status are shown in Table 3. In the ETV group, the period from the initial administration of ETV to the determination of undetectable HBV DNA in the HBeAg-negative group was the same as that in the HBeAg-positive group (Table 3). In the LAM group, the period from the initial administration of LAM to undetectable HBV DNA in the HBeAg-negative group was shorter than that in the HBeAg-positive group (Table 3). In the HBeAg-positive patients, the period from the initial administration to undetectable HBV DNA in the ETV group was shorter than that in the LAM group (Table 3).

Predictors of Virological Rebound in Patients treated with LAM

To clarify the predictors of virological rebound in patients treated with LAM, we compared the pretreatment factors between patients with and without virological rebound according to HBeAg status (Table 4A & 4B). Univariative analysis showed that age, HBV DNA, ALT levels and the period from the initial administration of LAM to the determination of undetectable HBV DNA in HBeAg-positive patients contributed to the occurrence of virological rebound (Table 4A). Factors significantly associated with virological rebound in HBeAg-positive patients treated with LAM by univariate analysis were also analyzed by multivariate logistic regression analysis. Virological rebound was attained independently of age in HBeAg-positive patients treated with LAM (Table 4C). In HBeAg-negative patients, no significant factors contributing to virological rebound could be found (Table 4B).

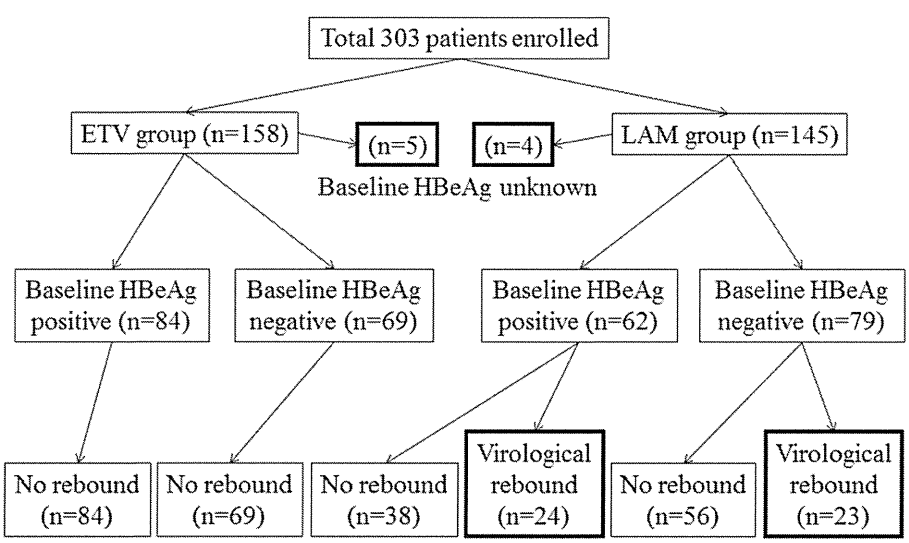

Figure 1. Study design and patient flow for both groups.

Table 1. Baseline characteristics of patients treated with entecavir (ETV) or lamivudine (LAM).

	Total	ETV group	LAM group	*P*-values
Number	303	158	145	
Age (years)	51 ± 12	51 ± 12	50 ± 12	*N.S.*
Gender (male)	205	101	104	*N.S.*
HBeAg (+)	146	84	62	*0.079*
HBV DNA (log IU/mL)	6.5 ± 1.5	6.6 ± 1.7	6.4 ± 1.3	*N.S.*
ALT (IU/L)	203 ± 280	187 ± 290	220 ± 266	*N.S.*
US: Cirrhosis (+)	113	56	57	*N.S.*
Periods to undetectable HBV DNA (months)	10.0 ± 18.2	8.5 ± 11.9	11.8 ± 23.3	*N.S.*

Data are expressed as mean ± SD. ETV group, patients receiving 0.5 mg of ETV daily; LAM group, patients receiving 100 mg of LAM daily; *P*-values, *P*-values between ETV and LAM groups; HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; US, ultrasound findings; *N.S.*, no statistically significant difference.

Table 2. Baseline characteristics of patients according to HBeAg status.

HBeAg	Positive group	Negative group	*P*-values
Number	146	148	
Age (years)	46 ± 12	55 ± 11	*< 0.001*
Gender (male)	101	97	*N.S.*
HBV DNA (log IU/mL)	7.2 ± 1.1	5.8 ± 1.4	*< 0.001*
ALT (IU/L)	257 ± 332	156 ± 211	*0.002*
US: Cirrhosis (+)	41	70	*< 0.001*
Periods to undetectable HBV DNA (months)	11.0 ± 18.1	7.4 ± 14.4	*0.063*

Data are expressed as mean ± SD. *P*-values, *P*-values between HBeAg-positive and HBeAg-negative groups; HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; US, ultrasound findings; *N.S.*, no statistically significant difference.

Table 3. Baseline characteristics of patients treated with entecavir (ETV) or lamivudine (LAM) according to HBeAg status.

	ETV group		LAM group	
HBeAg	Positive	Negative	Positive	Negative
Number	84	69	62	79
Age (years)	48 ± 12	56 ± 11*	44 ± 11 ##	54 ± 11**
Gender (male)	53	45	48	52**
HBV DNA (log IU/mL)	7.5 ± 1.1	5.7 ± 1.5*	6.9 ± 1.1$	5.9 ± 1.3**
ALT (IU/L)	219 ± 325	159 ± 246	309 ± 334	154 ± 174**
US: Cirrhosis (+)	25	29	16	41
Periods to undetectable HBV DNA (months)	8.3 ± 10.5	7.3 ± 11.0	15.0 ± 24.7$$	7.5 ± 16.9#

Data are expressed as mean ± SD. HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; US, ultrasound findings; *$P < 0.001$, compared to HBeAg-positive of ETV group; **$P < 0.001$ and #$P = 0.034$, compared to HBeAg-positive of LAM group; ##$P = 0.041$, $P = 0.001$ and $$P = 0.027$, compared to HBeAg-positive of ETV group.

Table 4A. Predictors of virological rebound in patients treated with lamivudine (LAM). (A) Comparison of HBeAg-positive patients with or without virological rebound by univariate analysis.

Virological rebound	No	Yes	*P-values*
Number	38	23	
Age (years)	42 ± 11	49 ± 11	*0.019*
Gender (male)	30	17	*N.S.*
HBV DNA (log IU/mL)	6.9 ± 1.2	6.8 ± 0.9	*N.S.*
ALT (IU/L)	379 ± 377	196 ± 205	*0.037*
US: Cirrhosis (+)	7	9	*N.S.*
Periods to undetectable HBV DNA (months)	20.6 ± 29.1	4.1 ± 3.1	0.009

Data are expressed as mean ± SD. *P*-values, *P*-values between patients with or without virological rebound groups; HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; US, ultrasound findings; *N.S.*, no statistically significant difference.

Table 4B. (B) Comparison of HBeAg-negative patients with or without virological rebound by univariate analysis.

Virological rebound	No	Yes	*P-values*
Number	56	22	
Age (years)	54 ± 11	54 ± 10	*N.S.*
Gender (male)	40	12	*N.S.*
HBV DNA (log IU/mL)	5.9 ± 1.4	5.9 ± 1.0	*N.S.*
ALT (IU/L)	163 ± 179	137 ± 163	*N.S.*
US: Cirrhosis (+)	30	11	*N.S.*
Periods to undetectable HBV DNA (months)	7.3 ± 14.8	3.1 ± 2.1	*N.S.*

Data are expressed as mean ± SD. *P*-values, *P*-values between patients with or without virological rebound groups; HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; US, ultrasound findings; *N.S.*, no statistically significant difference.

Table 4C. (C) Factor associated with virological rebound among HBeAg-positive patients treated with LAM by multivariate analysis.

Factor	Category	Odds ratio	95% CI	*P-value*
Age ≤ 44.5 (years)	(+/-)	0.222	0.0547-0.9023	0.0354

DISCUSSION

To date, there is not much data regarding virological rebound after achieving HBV DNA negativity in the use of ETV or LAM. A recent report supported the merit of the change from LAM to ETV [14]. This study concluded that prior optimal viral suppression with ETV did not confer any significant advantage for patients who switched to LAM.

The present study revealed that ETV could suppress HBV replication after achieving HBV DNA negativity, although additional longer follow-up studies will be needed. On the other hand, LAM could not suppress HBV replication even after achieving HBV DNA negativity (Figure 1), although most cases with virological rebound were observed within 2 years of the start of LAM medication. We could not check the emergence of YMDD motif mutations [19] in all of the cases because the present study was performed as part of regular clinical practice. Of 2 of the HBeAg-positive patients at baseline with virological rebound, one showed YVDD motif (50%). In 4 of the HBeAg-negative patients at baseline with virological rebound, one YVDD motif (25%) and three YIDD motifs (75%) were seen. Virological rebound may not mean the emergence of NA-resistance mutations [12].

We do not know the reason why virological rebound was attained independently of age in HBeAg-positive patients treated with LAM. HBeAg to anti-HBe antibody seroconversions were found in 20 and 11 patients with and without virological rebound, that is, the HBeAg to anti-HBe antibody seroconversion rates were similar in the two groups (data not shown), although the number of study patients seemed small in the present study. Further studies might be needed. In any event, it might be important to consider the LAM-to-ETV switch in HBeAg-positive patients treated with LAM, although some of our patients in the LAM group remained HBV-negative throughout the observation period.

In the present study, 95.3% (122 of 128), 82.3% (14 of 17) and 89.2% (25 of 28) had an adherence rate >90% [16] in ETV-treated, LAM-treated with virological rebound and LAM-treated patients without virological rebound, respectively. These results supported our previous study that viral breakthrough associated with poor adherence could be a more important issue in the treatment with especially stronger NAs, such as ETV [12,16], although we cannot ensure durable HBV negativity after NAs are discontinued. We and others reported that HBeAg could impair both innate and adaptive immune responses to promote chronic HBV infection [16,20,21]. Of interest, the virological rebound with the use of LAM seemed unrelated to the HBeAg status, suggesting that it was dependent on resistant mutation.

Recently, other effective antiviral therapies such as peginterferon [22,23] and tenofovir [24,25] were reported to be useful for the control of HBV infection. These drugs might also be candidates for treating virological rebound. Fung et al. [14] reported that prior optimal viral suppression with ETV did not confer any significant advantage for patients who switched to LAM. Our results also supported the previous studies that ETV was much more efficient than LAM [26-29]. In conclusion, ETV could inhibit HBV replication if HBV DNA negativity had been achieved. In contrast, LAM could not inhibit HBV replication even if HBV negativity was achieved in the early phase. Attention should be paid to these features in clinical

practice.

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CONFLICT OF INTEREST

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ABBREVIATIONS

ALT: alanine aminotransferase; ETV: Entecavir; HBeAg: Hepatitis B e antigen; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; LAM: lamivudine; NA: nucleos(t)ide analogue.

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Research Paper

Adherence to Medication Is a More Important Contributor to Viral Breakthrough in Chronic Hepatitis B Patients Treated with Entecavir Than in Those with Lamivudine

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Abstract

Viral breakthrough is related to poor adherence to medication in some chronic hepatitis B patients treated with nucleos(t)ide analogues (NAs). Our study aimed to examine how adherence to medication is associated with viral breakthrough in patients treated with NAs. A total of 203 patients (135 ETV and 68 LAM) were analyzed in this retrospective analysis. Physical examination, serum liver enzyme tests, and hepatitis B virus marker tests were performed at least every 3 months. We reviewed medical records and performed medical interviews regarding to patients' adherence to medication. Adherence rates <90% were defined as poor adherence in the present study. Cumulative viral breakthrough rates were lower in the ETV-treated patients than in the LAM-treated patients ($P<0.001$). Seven ETV-treated (5.1%) and 6 LAM-treated patients (8.8%) revealed poor adherence to medication ($P=0.48$). Among ETV-treated patients, 4 (3.1%) of 128 patients without poor adherence experienced viral breakthrough and 3 (42.8%) of 7 patients with poor adherence experienced viral breakthrough ($P<0.001$). Only 3 of 38 (7.8%) LAM-treated patients with viral breakthrough had poor adherence, a lower rate than the ETV-treated patients ($P=0.039$). Nucleoside analogue resistance mutations were observed in 50.0% of ETV- and 94.1% of LAM-treated patients with viral breakthrough ($P=0.047$). Viral breakthrough associated with poor adherence could be a more important issue in the treatment with especially stronger NAs, such as ETV.

Key words: Adherence, Entecavir, Lamivudine, Hepatitis B, Viral Breakthrough.

INTRODUCTION

Two billion people have been exposed to hepatitis B virus (HBV), and 350-400 million people remain chronically infected worldwide. In Japan, the prevalence of HBV carriers is estimated at ~1% of the pop-

ulation, but HBV is a major health issue because it causes acute hepatitis, chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) [1, 2].

Lamivudine (LAM) is a reverse-transcriptase inhibitor of HBV DNA polymerase that possesses excellent profile of safety and tolerability and causes inhibition of viral replication. LAM was the first nucleos(t)ide analogue (NA) to be approved for antiviral treatment of hepatitis B patients [3, 4]. Entecavir (ETV), a deoxyguanosine analogue, is a potent and selective inhibitor of HBV replication. The in vitro potency of ETV is 100- to 1,000-fold greater than that of LAM, and it has a selectivity index (concentration of drug required to reduce viable cell number by 50% [CC_{50}] / concentration of drug required to reduce viral replication by 50% [EC_{50}]) of approximately 8,000 [5, 6]. LAM (until 2005) and ETV (from 2006) have been used as first-line NAs for most patients with chronic hepatitis B in Japan. Most patients with chronic hepatitis B have been undergoing treatment for longer durations, and prolonged treatment is associated with increasing rates of viral breakthrough [7]. It has been reported that not all cases are associated with resistance mutations [8, 9]. We have also reported that some cases of viral breakthrough during ETV treatment were related to poor adherence to medication [10].

Adherence rates are usually lower in patients with long-term treatment regimens, such as for hypertension, than in patients with short-term regimens, such as for gastric ulcers [11]. It has been reported that 74.8% of patients with hypertension were determined to have an adherence rate ≥80% [12], and that 55.3% of patients with chronic hepatitis B had an adherence rate >90% [8].

In the present study, we aimed to investigate whether drug adherence is related to viral breakthrough in chronic hepatitis B patients treated with LAM or ETV. We also investigated the pattern of poor adherence and suggested how adherence to medication could be improved.

MATERIALS AND METHODS

Patients

Two hundred seventy-five NA-treated naïve patients (185 ETV- and 90 LAM-treated patients), who were admitted to Chiba University Hospital between April 2000 and September 2011, were enrolled (Figure 1). Some of these patients had already been included in a previous report [10]. Between November 2011 and April 2012, doctors performed medical interviews of those patients to determine their adherence to medication. Seventy-two patients (50 ETV- and 22 LAM-treated patients) were excluded from this retrospective analysis, because their adherence to medication could not be confirmed. One hundred thirty-five patients were administered 0.5 mg of ETV daily and 68 patients were administered 100 mg of LAM daily (Table 1). In all patients, serum hepatitis B surface antigen (HBsAg) and HBV DNA were positive. All patients had negative results for hepatitis C virus or human immunodeficiency virus antibodies. Physical examinations, serum liver enzyme tests, and HBV marker tests were performed at least every 3 months. The study was carried out in accordance with the Helsinki Declaration, and was approved by the Ethics Committee of Chiba University, Graduate School of Medicine (No. 977).

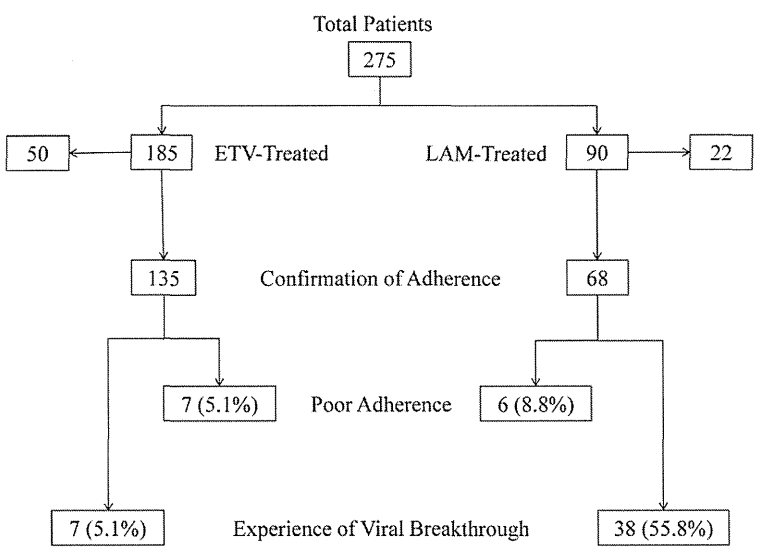


Figure 1. Patients, adherence rates, and the prevalence of viral breakthrough in this study. ETV, entecavir; LAM, lamivudine.

Table 1. Baseline characteristics of patients.

	ETV	LAM	P-values
Number of cases	135	68	
Age (years)	51.7 ± 11.7	45.5 ± 12.1	<0.001
Gender (male/female)	83/52	49/19	0.135
HBeAg (+/-)	64/71	45/23	0.011
Genotype (A/B/C/unknown)	0/11/78/46	1/6/57/4	0.427
HBV DNA (log IU/mL) (≤5.0/> 5.0/unknown)	27/108/0	3/55/10	0.009
ALT (IU/L)	161 ± 195	353 ± 394	<0.001
Platelets ($\times 10^4/mm^3$)	16.3 ± 5.9	16.9 ± 7.0	0.556
APRI	2.49 ± 4.19	6.52 ± 6.98	<0.001
Follow-up period (months)	26.9 ± 21.6	49.0 ± 39.7	<0.001

ETV, entecavir; LAM, lamivudine; HBeAg, hepatitis B e antigen; N.D., not determined; HBV DNA, hepatitis B virus deoxyribonucleic acid; ALT, alanine aminotransferase; APRI, aspartate aminotransferase platelet ratio index. Continuous variables are expressed as mean ± standard deviation.

Blood examinations

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels, and platelet counts were reviewed in the present study. We also calculated the aspartate aminotransferase platelet ratio index [APRI: AST (IU/L)/ 35/platelet count ($10^3/\mu L$) x 100], which is significantly correlated with the staging of liver fibrosis, with a higher correlation coefficient than platelet count or AST level alone [13].

Detection of HBV markers

HBsAg, hepatitis B e antigen (HBeAg) and anti-HBe antibody were determined by ELISA (Abbott, Chicago, IL, USA) or CLEIA (Fujirebio, Tokyo, Japan)[14]. HBV genotype was determined by ELISA (Institute of Immunology, Tokyo, Japan) [15]. HBV DNA was measured by Roche Amplicor PCR assay (detection limits: 2.6 log IU/mL; Roche Diagnostics, Tokyo, Japan).

Follow-up period

The follow-up period ended when the NA was switched to another NA or another NA was added, or it was discontinued for various reasons.

Definition of adherence to medication

To obtain information regarding adherence to medication, we reviewed medical records. We also interviewed patients about their adherence to medication. We expressed the rate of adherence to medication as a percentage calculated by the number of days of taking a pill divided by the follow-up period (days). Adherence rates <90% were defined as poor adherence in the present study.

Definition of viral breakthrough

Viral breakthrough was defined as an increase of ≥ 1 log IU/mL in serum HBV DNA level from nadir.

Sequence analysis of HBV DNA

The YMDD motif was analyzed by PCR-ELMA in sera of patients who had experienced viral breakthrough, as reported by Kobayashi et al [16]. HBV polymerase/reverse transcriptase (RT) substitutions were also analyzed in sera of ETV-treated patients who had experienced viral breakthrough. Briefly, HBV DNA was extracted from 100 μL of sera using SepaGene (Sanko Junyaku, Tokyo, Japan). Nested PCR was performed using LA Taq polymerase (Takara Bio, Otsu, Shiga, Japan) under the following conditions: 5-min denaturation at 94°C, 35 cycles with denaturation at 94°C for 40 s, annealing at 58°C for 1 min, and extension at 68°C for 1.5 min [2]. An 862 base-pair fragment (nt 242-1103) containing the polymerase RT domain was amplified on the PCR Thermal Cycler Dice Model TP600 (Takara Bio). The primers for the first PCR were 5′-CAG AGT CTA GAC TCG TGG-3′ (sense, nt 242-258) and 5′-GGC GAG AAA GTG AAAGCC-3′ (antisense, nt 1103-1086). The PCR product was sequenced using the primers: 5′-TGG CTC AGT TTA CTAGTG CC -3′ (nt 668-687) and 5′-GGC ACT AGT AAA CTGAGC CA-3′ (nt 687-668), and these primers were also used for the second PCR. To prepare the sequence template, PCR products were treated with ExoSAP-ITR (Affymetrix, Inc., Santa Clara, CA, USA), and then sequenced using the BigDye(R) Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Tokyo, Japan). Sequences were performed with Applied Biosystems 3730xl (Life Technologies) [17].

Statistical analysis

Statistical analyses were performed using SAS 9.3 Software (SAS Institute, Cary, NC, USA). Continuous variables were expressed as mean ± standard deviation and were compared by Student's t-test or

Welch's t-test. Categorical variables were compared by chi-square test or Fisher's exact probability test. The Kaplan-Meier method was used to calculate viral breakthrough rates. Baseline was taken as the date when the first dose of LAM or ETV was taken. Statistical significance was considered at a *P-value* < *0.05*.

RESULTS

Baseline characteristics of patients

Baseline characteristics of patients are shown in Table 1. In ETV-treated patients, the age was higher, the prevalence of HBeAg-negative patients was higher, HBV DNA was lower, ALT levels were lower, and APRI was lower (ie., liver fibrosis was milder) than in LAM-treated patients. HBV genotype C was dominant in both groups. The follow-up period in ETV-treated patients was shorter than that in LAM-treated patients, based on the fact that ETV was a newer drug and many ETV-treated patients had started treatment more recently.

Adherence to medication, and viral breakthrough between ETV- and LAM-treated patients

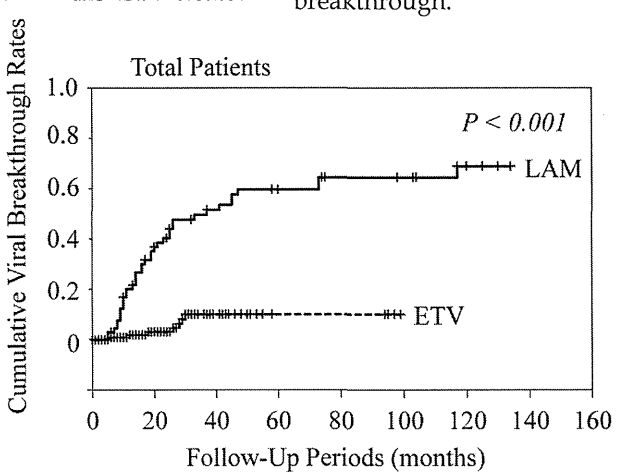
Most patients presented good adherence to medication in the present study. Seven ETV-treated (5.1%) and 6 LAM-treated patients (8.8%) had poor adherence (Figure 1). The number of patients with poor adherence was not significantly different between the ETV- and LAM-treated groups (*P=0.48*). The characteristics of the 13 patients with poor adherence are shown in Table 2. Cumulative viral breakthrough rates were lower in the ETV-treated patients than in the LAM-treated patients (*P<0.001*) (Figure 2).

Viral breakthrough in HBeAg-positive and -negative patients

Among the LAM-treated patients, cumulative viral breakthrough rates in HBeAg-positive patients at baseline (n=45; 25.0% at 1 year, 55.1% at 3 years, and 67.0% at 5 years) were similar to those in HBeAg-negative patients at baseline (n=23; 9.5% at 1 year, 38.2% at 3 years, and 44.4% at 5 years; *P=0.16*). Among the ETV-treated patients, cumulative viral breakthrough rates in HBeAg-positive patients at baseline (n=64; 2.2% at 1 year, 18.1% at 3 years, and 18.1% at 5 years) were also similar to those in HBeAg-negative patients at baseline (n=71; 1.6% at 1 year, 1.6% at 3 years, and 1.6% at 5 years; *P=0.050*).

Among the LAM-treated patients who were HBeAg-positive at baseline, cumulative viral breakthrough rates in patients who converted to HBeAg-seronegative were lower than those in patients who maintained HBeAg seropositivity (*P<0.001*) (Figure 3). All LAM-treated patients who did not become HBeAg-seronegative experienced viral breakthrough. Among the ETV-treated patients who were positive for HBeAg at baseline, conversion to HBeAg seronegativity did not affect the rate of viral breakthrough (*data not shown*).

There were no differences in HBV viral loads at study entry between HBeAg-positive patients with and without viral breakthrough. There were also no differences in HBV viral loads between HBeAg-negative patients with and without viral breakthrough.



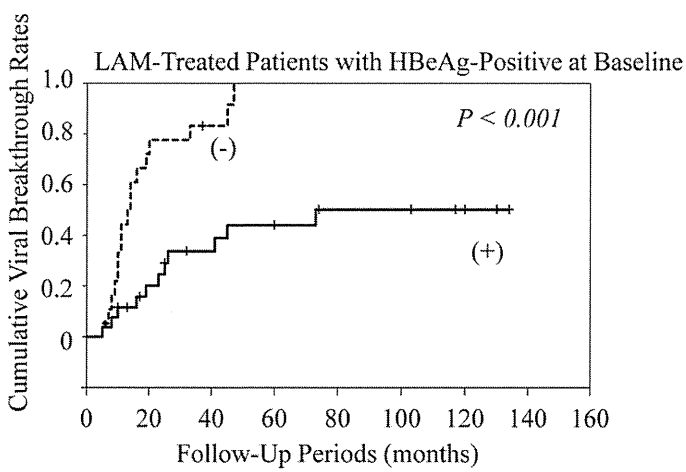
	Drug	Baseline	12	24	36	48	60
Number of Patients	LAM	68	52	34	27	20	19
	ETV	135	95	69	34	19	5

Figure 2. Cumulative viral breakthrough rates. ETV, entecavir; LAM, lamivudine.

Table 2. Patients with poor adherence to medication.

Case	Drug	Adherence rate (%)	Age (years)	Gender	Genotype	HBeAg	HBV DNA (log IU/mL)	ALT (IU/L)	APRI	HBeAg-seronegative	HBV DNA negativity	VT	Duration of treatment before VT (months)	Resistance mutations	Treatment after VT	Clinical outcome
1	ETV	50	55	F	B	-	3.8	16	0.33	N.A.	+	+	6	-	ETV	good
2	ETV	75	49	M	C	+	7.3	107	1.60	+	+	+	28	+	LAM+ADV	good
3	ETV	85	38	M	C	+	6.9	59	2.80	-	+	+	29	N.D.	ETV	good
4	ETV	80	39	M	C	+	5.8	51	0.63	+	+	-	N.A.	N.A.	ETV	good
5	ETV	85	37	F	C	+	6.9	160	2.25	+	+	-	N.A.	N.A.	ETV	good
6	ETV	85	66	M	N.D.	+	7.7	68	0.95	-	-	-	N.A.	N.A.	ETV	good
7	ETV	85	38	M	C	+	6.5	478	7.94	-	+	-	N.A.	N.A.	ETV	good
8	LAM	50	47	F	C	+	6.5	455	2.54	+	+	+	45	-	LAM	good
9	LAM	80	36	M	C	+	7.0	110	4.25	+	+	+	41	+	LAM+ADV	good
10	LAM	85	23	M	C	+	>7.6	161	3.53	-	+	+	11	-	cessation	flare
11	LAM	85	32	M	C	+	>7.6	343	1.30	+	+	-	N.A.	N.A.	LAM	good
12	LAM	85	54	F	C	-	4.1	196	2.68	N.A.	+	-	N.A.	N.A.	LAM	good
13	LAM	85	36	M	C	+	6.7	1576	15.78	+	+	-	N.A.	N.A.	LAM	good

Cases 2 and 3 had already been included in a previous report.[10] HBeAg, hepatitis B e antigen; HBV DNA, hepatitis B virus deoxyribonucleic acid, ALT, alanine aminotransferase; APRI, aspartate aminotransferase platelet ratio index; VT, viral breakthrough; ETV, entecavir; LAM, lamivudine; ADV, adefovir; F, female; M, male; N.D., not determined; N.A., not available; HBeAg-seronegative, conversion to HBeAg-seronegative after administration of a nucleoside analogue; HBV DNA negativity, achieving HBV DNA negativity after administration of a nucleoside analogue; flare, fluctuating ALT after treatment after VT.



HBeAg-Seronegative	Baseline	12	24	36	48	60
Number of Patients (-)	19	10	4	3	0	0
Number of Patients (+)	26	22	17	13	11	11

Figure 3. Cumulative viral breakthrough rates in lamivudine (LAM)-treated patients with HBe antigen (HBeAg)-positive at baseline. (-), maintaining HBeAg seropositivity; (+), conversion to HBeAg-seronegative.

Viral breakthrough in patients who achieved, and did not achieve HBV DNA negativity

Among the LAM-treated patients, cumulative viral breakthrough rates in patients who did not achieve HBV DNA negativity were higher than in those who achieved HBV DNA negativity ($P<0.001$) (Figure 4). All patients who did not achieve HBV DNA negativity experienced viral breakthrough. In

contrast, among the ETV-treated patients, cumulative viral breakthrough rates in patients who did not achieve HBV DNA negativity were similar to the rates in those who achieved HBV DNA negativity (*data not shown*).

Correlation between adherence to medication and viral breakthrough

We also compared viral breakthrough rates according to adherence to medication. Among 62 LAM-treated patients who did not have poor adherence, 35 patients (56.4%) experienced viral breakthrough (Figure 5). Among 6 LAM-treated patients with poor adherence, 3 patients (50.0%) experienced viral breakthrough. In LAM treatment, poor adherence did not contribute to viral breakthrough (*P=0.89*). However, among 128 ETV-treated patients who did not have poor adherence, 4 patients (3.1%) experienced viral breakthrough. Among 7 ETV-treated patients with poor adherence, 3 patients (42.8%) experienced viral breakthrough. In the treatment with ETV, poor adherence contributed to viral breakthrough (*P<0.001*).

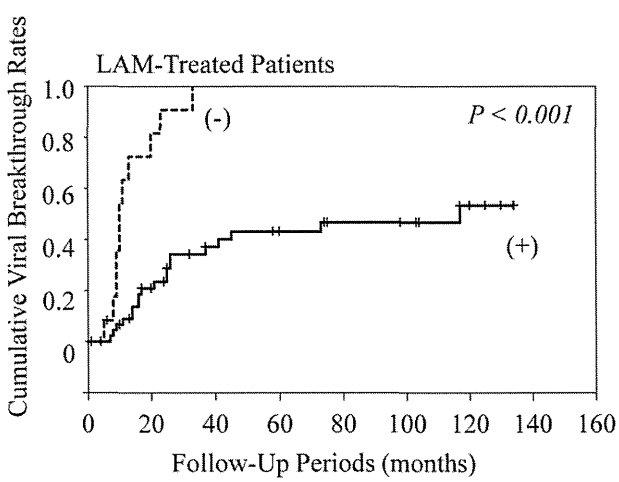
Resistance mutations

Resistance mutations were analyzed in some patients who experienced viral breakthrough. They were analyzed in 34 LAM-treated patients and 4 ETV-treated patients (Table 3). Thirty-two LAM-resistant patients had 10 YVDD, 17 YIDD, and 5 YV/IDD motifs, and 2 ETV-resistant patients had two YVDD motifs. Resistance mutations were not observed in 2 LAM-treated patients (5.8%) and 2 ETV-treated patients (50.0%) (*P=0.047*).

Table 3. Patients with viral breakthrough.

	ETV		LAM	
Adherence rate	≥90%	<90%	≥90%	<90%
Resistance mutation (+)	1	1	31	1
L180M	1	1	N.D.	N.D.
T184A	1	0	N.D.	N.D.
S202G	0	1	N.D.	N.D.
M204V	1	1	9	1
M204I	0	0	17	0
M204V/I	0	0	5	0
M250V	0	0	N.D.	N.D.
Resistance mutation (-)	1	1	0	2

ETV, entecavir; LAM, lamivudine; N.D., not determined. Numbers of amino acid positions were according to Refs. 2 and 10.



HBV DNA Negativity	Baseline	12	24	36	48	60
Number of Patients (-)	12	4	1	0	0	0
Number of Patients (+)	47	39	30	23	19	18

Figure 4. Cumulative viral breakthrough rates in lamivudine (LAM)-treated patients who achieved HBV DNA negativity and those who did not. (-), maintaining HBV DNA positivity; (+), achieving HBV DNA negativity. HBV DNA negativity was unknown in 9 patients because of lack of data.

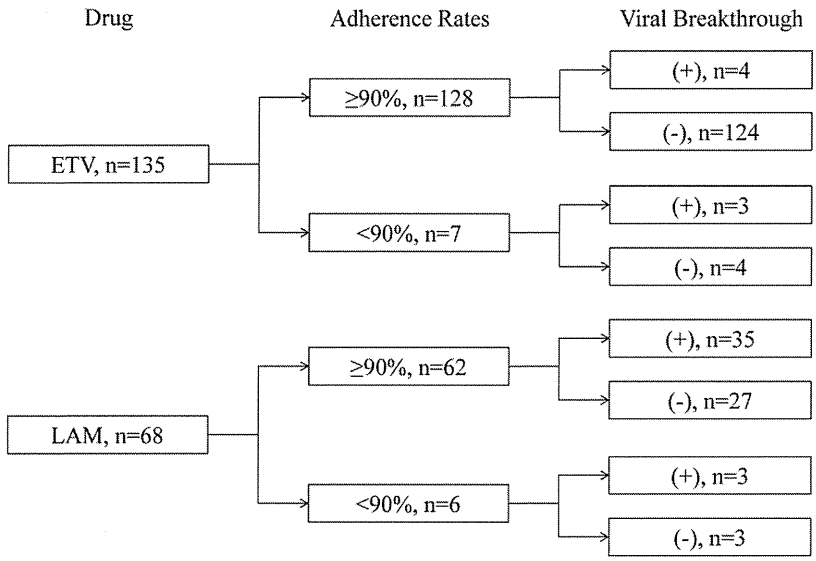


Figure 5. Association between adherence to medication and viral breakthrough.

DISCUSSION

The current study found that ETV-treated patients were not likely to acquire any resistance mutations and experience an ALT flare. Therefore, patients with poor liver residual function, such as liver cirrhosis, were likely to be administered ETV rather than LAM. Unexpectedly, HBsAg loss was observed in 3 of 28 LAM-treated patients without viral breakthrough (10.7%) and in 3 of 118 ETV-treated patients without viral breakthrough (2.5%). Long-term treatment with these drugs might result in HBsAg loss, although several reports have stated that one-year treatment with peg-interferon led to more HBsAg loss than these drugs [18-25].

In the current study, adherence to medication of most patients was excellent. The reasons for this might be as follows: (1) Our setting was a University Hospital, and this may have strengthened their will to succeeded with the treatment; (2) some patients with poor adherence might have been excluded because they did not see a doctor during the interview period; and (3) the rate of adherence to medication was based on patient self-assessment. A previous report showed that adherence might be underestimated by the Medication Event Monitoring System, a system that automatically records whenever a drug bottle is opened, and might be overestimated by pill counting and at interviews [26]. We classified the adherence rate as good at 90% or more, and as poor at less than 90%. However, we could not prove any significant influence of this classification on viral breakthrough as well as resistance mutation.

In the 13 patients with poor adherence (Table 2), we examined the reasons for their failure to take the pills. All 13 patients displayed some carelessness about taking pills. Two ETV-treated patients did not see a doctor and could not take pills continuously for a certain period of time, which particularly appeared to affect their viral breakthrough.

In LAM-treated patients, conversion of HBeAg to seronegative and achieving HBV DNA negativity was one of the important factors for successful treatment (Figures 3 & 4). In contrast, among ETV-treated patients, maintaining HBeAg seropositivity or HBV DNA positivity was not associated with viral breakthrough in the present study. Because of the stronger effect of ETV, it has been reported that long-term ETV treatment leads to a viral response in the vast majority of patients with detectable HBV DNA after 48 weeks [27]. Moreover, in the current study, poor adherence to medication was a major factor of viral breakthrough in the ETV-treated patients, but not in the LAM-treated patients. Ha et al. [9] also reported that medication non-adherence is likely to be a more important contributor to treatment failure than antiviral resistance, especially with new anti-HBV agents such as ETV and tenofovir. In LAM-treated or ETV-treated patients, viral breakthrough without resistance mutations might occur to some degree because of poor adherence to medication. In the present study, in LAM-treated patients, emergence of viral breakthrough with resistance mutations was common. Therefore, viral breakthrough due to poor adherence to LAM might not be important, compared with ETV-treated patients. However, in ETV-treated pa-

tients, viral breakthrough with resistance mutations was rare, and therefore, viral breakthrough due to poor adherence to ETV might be important.

In conclusion, viral breakthrough associated with poor adherence could be an important issue in the treatment with strong nucleoside analogues, such as ETV.

ABBREVIATIONS

ALT: alanine aminotransferase
ETV: entecavir
HBeAg: hepatitis B e antigen
HBsAg: hepatitis B surface antigen
HBV: hepatitis B virus
HCC: hepatocellular carcinoma
NA: nucleos(t)ide analogue
LAM: lamivudine

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Contributors

HK, TK, FI, and OY designed the study. HK, TK, MA, TC, HM, KF, FK, FI, FN and OY saw patients and conducted the interview. HK, TK, WS, and SN analyzed the data. HK and TK drafted the paper and all authors approved the paper.

COMPETING INTERESTS

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Regulation of miRNA by HBV infection and their possible association with control of innate immunity

Jiang X *et al.* HBV and miRNA

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Abstract

Hepatitis B virus (HBV) chronically infects more than 350 million people worldwide. HBV causes acute and chronic hepatitis, and is one of the major causes of cirrhosis and hepatocellular carcinoma. There exist complex interactions between HBV and the immune system including adaptive and innate immunity. Toll-like receptors (TLRs) and TLR-signaling pathways are important parts of the innate immune response in HBV infections. It is well known that TLR-ligands could suppress HBV replication and that TLRs play important roles in anti-viral defense. Previous immunological studies demonstrated that HBV e antigen (HBeAg) is more efficient at eliciting T-cell tolerance, including production of specific cytokines IL-2 and IFNγ, than HBV core antigen. HBeAg downregulates cytokine production in hepatocytes by the inhibition of MAPK or NF-κB activation through the interaction with receptor-interacting serine/threonine protein kinase (RIPK/RIP). MicroRNAs (miRNAs) are also able to regulate various biological processes such as the innate immune response. When the expressions of ~1,000 miRNAs were compared between human hepatoma cells HepG2 and HepG2.2.15, which could produce HBV virion that infects chimpanzees, using real-time RT-PCR, we observed several different expression levels in miRNAs related to TLRs. Although we and others have shown that HBV modulates the host immune response, several of the miRNAs seem to be involved in the TLR signaling pathways. The possibility that alteration of these miRNAs during HBV infection might play a critical role in innate immunity against HBV infection should be considered. This article is intended to comprehensively review the association between HBV and innate immunity, and to discuss the role of miRNAs in the innate immune response to HBV infection.

Keywords: Hepatitis B virus; HepG2.2.15; Innate immunity; MicroRNA; Persistent infection; Toll-like receptor

Core tip: Hepatitis B virus (HBV) is the leading cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma in the world. HBV could interact with the host's innate and adaptive immune responses to establish chronic infection. HBV also interacts with Toll-like receptors (TLRs) and TLR signaling pathways, and regulates host immune responses through the regulation of miRNAs to some extent. This article focuses on the involvement of miRNA in the association between HBV and TLR signaling pathways and reviews the miRNAs involved in HBV infection.

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