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• VIRAL HEPATITIS •

End-of-treatment virologic response does not predict relapse after lamivudine treatment for chronic hepatitis B

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Abstract

AIM: Attaining hepatitis B e antigen (HBeAg) seroconversion during lamivudine treatment is associated with fewer relapses in HBeAg-positive patients. In HBeAg-negative patients, predictors for post-treatment relapse remain largely unknown. We therefore studied whether end-of-treatment virologic response correlated with relapse after lamivudine treatment.

METHODS: We prospectively analyzed 12 HBeAg-negative patients and 14 HBeAg-positive patients with chronic hepatitis B, who received at least 9 mo of lamivudine treatment and were followed up for 12 mo post-treatment. Relapse of hepatitis B activity was defined by an elevation of serum ALT level above twice the upper limit of normal as well as reappearance of serum HBV DNA by the branched DNA assay or HBeAg during the follow-up period. The serum viral loads during and at the end of treatment were further determined by a quantitative real-time polymerase chain reaction assay.

RESULTS: Relapse occurred in 6 (50.0%) HBeAg-negative patients within 12 mo post-treatment. Two relapsers had end-of-treatment serum viral load <1 000 copies/mL, the proportion was not significantly different from that in the 6 non-relapsers (33.3% *vs* 16.7%; $P = 1.00$). Hepatitis B virus (HBV) DNA levels did not correlate with post-treatment relapse in HBeAg-positive patients either. However, genotype C patients tended to have a lower relapse rate than genotype B patients (14.3% *vs* 57.9%, $P = 0.08$).

CONCLUSION: Our results suggest that end-of-treatment virologic response cannot predict post-treatment relapse in patients with HBeAg-negative or -positive chronic hepatitis B. The impact of HBV genotype on the response to lamivudine treatment awaits further studies.

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INTRODUCTION

Infection with hepatitis B virus (HBV) can cause a wide spectrum of liver diseases, such as fulminant or acute hepatitis, chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma[1]. About 350 million people throughout the world suffer from chronic HBV infection. Thus, active treatment of chronic hepatitis B to prevent progression into end-stage liver diseases is urgently needed.

Three drugs have been approved for the treatment of chronic hepatitis B: interferon (IFN) alpha, lamivudine and adefovir dipivoxil[2-4]. IFN alpha is costly and has a narrow range of efficacy and tolerability. Adefovir is potent and has been approved for the treatment of chronic hepatitis B in the United States recently, but is nephrotoxic at doses higher than 10 mg per day and not available in other countries[5]. Lamivudine is cheaper, better tolerated, and has been shown to be effective in patients with hepatitis B e antigen (HBeAg)-positive chronic hepatitis B[6,7]. However, virologic response to lamivudine may not be as durable as that occurred spontaneously or induced by IFN treatment[8,9]. Although Dienstag *et al*. reported that lamivudine-induced HBeAg seroconversion was durable after lamivudine treatment, conflicting data existed. It was reported that lamivudine-induced HBeAg seroconversion was not sustained and cumulative relapse rate within 12 mo was 38% in Korean patients[9]. Thus, it is important to identify predictors for relapse after HBeAg seroconversion.

Lamivudine is also effective for HBeAg-negative chronic hepatitis B[10-14]. The biochemical and virologic responses as well as the accumulated incidence of drug-resistant mutants were comparable to those in HBeAg-positive chronic hepatitis B[11]. Nevertheless, in contrast to the HBeAg seroconversion as an unfavorable predictor for relapse in patients with HBeAg-positive chronic hepatitis B, predictors for post-treatment virologic response could be even more lacking in this special clinical setting, and thus the duration of lamivudine treatment is difficult to define[2-4]. Recently, serum HBV DNA has been considered as a marker of efficacy during therapy for chronic HBV infection[15]. The relationship between HBV DNA level and the post-treatment relapse in HBeAg-positive chronic hepatitis B has also been demonstrated[16,17]. Whether similar situation holds true for patients with HBeAg-negative chronic hepatitis B remains to be clarified. In addition, HBV genotypes have been shown to correlate with the clinical outcomes of HBV infection, the response to IFN treatment, the rate of spontaneous HBeAg seroconversion, and the development of lamivudine resistance[18-29]. It is therefore interesting to know the influence of HBV genotypes on the relapse after cessation of lamivudine treatment.

In this study, traditional polymerase chain reaction (PCR)-based assay was used to monitor and define the response to lamivudine treatment. In addition, we established a real-time PCR assay to quantify serum HBV DNA level in a wider dynamic

range, and compared the relapse rates between HBeAg-negative or -positive patients with detectable serum HBV DNA level and those with undetectable serum HBV DNA level at the end of lamivudine treatment. The impact of HBV genotype on the relapse rate was also analyzed.

MATERIALS AND METHODS

Patients

Between March 1998 and December 1999, 12 patients (10 men and 2 women, median age: 44 years, range: 19 to 61 years) with HBeAg-negative chronic hepatitis B (group I) and 14 patients (13 men and 1 woman, median age: 36 years, range: 20 to 55 years) with HBeAg-positive chronic hepatitis B (group II) who discontinued lamivudine treatment were prospectively enrolled for follow-up studies in the National Taiwan University Hospital. The diagnosis of HBeAg-negative chronic hepatitis B in group I patients was made upon elevated serum alanine aminotransferase (ALT) level at least twice the upper limit of normal (<40 IU/L) for more than 6 mo in the absence of HBeAg, but associated with active HBV replication as reflected by detectable serum HBV DNA by a commercial branched DNA assay (Bayer HBV DNA Assay, Bayer Corporation, Tarrytown, NY). All were positive for antibody to HBeAg (anti-HBe). The diagnosis of HBeAg-positive chronic hepatitis B in group II patients was made upon elevated serum alanine aminotransferase (ALT) level at least twice the upper limit of normal (<40 IU/L) for more than 6 mo in the presence of HBeAg and associated with active HBV replication. None had co-existing hepatitis C virus (HCV) or hepatitis delta virus infection. Other causes of hepatitis, such as alcoholic liver disease and autoimmune hepatitis were excluded. Patients with fulminant hepatitis accompanied with hepatic encephalopathy, concomitant human immunodeficiency virus infection, clinical evidence of pancreatitis, or in pregnancy were excluded.

All of the 12 group I patients received at least 9 mo of lamivudine treatment. During lamivudine therapy, liver biochemical tests were evaluated monthly. After the cessation of lamivudine treatment, liver biochemical tests were followed up monthly for 12 mo. Hepatitis B surface antigen (HBsAg), antibody to HBsAg (anti-HBs), HBeAg, and anti-HBe were examined every 3 mo. Relapse of hepatitis B activity was defined according to recommended criteria[3,4,11], that was an elevation of serum ALT level above twice the upper limit of normal (<40 IU/L) as well as reappearance of serum HBV DNA by the bDNA assay or HBeAg during the follow-up period. Group II patients received the same regimen of lamivudine and follow-up schedule. In these patients, if HBeAg seroconversion (defined as loss of HBeAg and gain of anti-HBe) was attained, additional lamivudine treatment was given for at least 2 mo[30]. For those not attaining HBeAg seroconversion after 9 mo of lamivudine treatment, to avoid the emergence of lamivudine-resistant mutants, the duration of therapy was determined arbitrarily by the attending physician[30]. Again, post-treatment relapse of hepatitis B activity was defined according to recommended criteria[3,4].

After written informed consent was obtained from each patient, serum samples were collected before treatment, at the 12th wk and at the end of lamivudine treatment, and at the time of relapse. Serum samples were stored at -70 ℃ until virologic assays.

Hepatitis virus markers

HBsAg, anti-HBs, HBeAg, anti-HBe, and anti-delta were assayed with commercial kits (AxSYM System®, Abbott Laboratories, North Chicago, IL). Anti-HCV was tested by a second-generation enzyme-linked immunoassay (Abbott Laboratories).

Quantification of HBV DNA level

In this study, we basically used the traditional PCR-based method to monitor and define the response to lamivudine treatment both in HBeAg-positive and -negative patients. Serum HBV DNA levels at baseline, the 12th wk of treatment, the end of treatment, and at the time of post-treatment relapse were determined by a branched DNA assay (Bayer Corporation) according to the manufacturer's instructions. The detection limit of this quantitative assay was 2.5 pg/mL (or 700 000 genome equivalents/mL). The serum HBV DNA levels at the 12th wk and at the end of lamivudine treatment were further determined by the real-time PCR.

Development of real-time PCR assay

To further clarify whether HBV DNA levels in the low range correlated with the response to lamivudine treatment, we developed another real-time PCR assay to detect HBV DNA levels below 2.5 pg/mL.

Extraction of serum HBV DNA

Serum DNA was extracted using a commercially available kit (QIAamp DNA Blood Mini Kit, QIAGEN Inc, Valencia, CA, USA).

Preparation of quantitative standards For preparation of an external standard of the real-time PCR detection assay, HBV DNA (3.2 kb) was cloned into vector pGEM-4Z (Promega, Madison, USA) and transformed into *Escherichia coli* DH5α, and purified using a commercially available kit (GFX™ Micro Plasmid Prep Kit, Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany). The HBV copy number of the plasmid stock solution was determined by measuring the optical density at 260 nm. For each run, serial dilutions of the plasmid stock were prepared freshly from an aliquot stock solution.

Real-time PCR detection assay The real-time PCR assay including the design of primers and probes was performed as previously described[31]. The number of HBV DNA copies was calculated relative to an HBV DNA plasmid 10-fold dilution series (1 pg/mL equals 280 000 copies/mL), which was included in each run. The input of this plasmid series ranged from 10 to 10^{10} copies per reaction mixture. Calibration curves were generated. The calculated r^2 was $\geqslant$0.99 in each run.

In our assay, less than 1 000 copies were not reliably detected by this assay, as judged by the varied detectable rate and the loss of linearity of the input copy number versus the cycle number at threshold crossing plot. For this reason, the lower limit for quantification was set at 1 000 copies/mL serum. In the range from 10^3 to 10^{10} template molecules per reaction, the curves showed good linearity and only minimal run-to-run deviations (data not shown). In 20 HBsAg- and anti-HBs-negative EDTA-plasma samples, none showed positive reactions in duplicate. The HBV DNA levels of the serum samples were then extrapolated using these calibration curves.

Determination of HBV genotype and drug-resistant mutants

HBV genotype was identified by using PCR-restriction fragment length polymorphism[21]. The mutation on tyrosine-methionine-aspartate-aspartate (YMDD) motif of the HBV polymerase gene was identified by PCR and direct sequencing[32].

Ethical considerations

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, and was approved by the Ethical Committee of the National Taiwan University Hospital. The sera were sampled after written or oral informed consent was obtained from the patients.

Statistic analysis

mean±SD or median (range) was used to describe the distribution of continuous variables. Baseline characteristics between relapsers and non-relapsers were compared by Student's *t* test

for continuous variables and by Chi-square test with Yates' correction for categorical variables. Statistical significance of differences in the relapse rate among different subgroups was estimated by using 2×2 contingency table according to serum HBV DNA level. All tests were two-sided and a P value <0.05 was considered statistically significant.

RESULTS

Characteristics of patients

The baseline characteristics of group I and group II patients are shown in Table 1. There was no significant difference with regard to clinical and virologic features. The mean duration of lamivudine treatment in group II patients was 2 mo longer than group I patients, but with no statistical significance.

Seven group II patients attained seroconversion of HBeAg after a median treatment period of 7 mo (range: 4 to 16 mo). Lamivudine was continued for an additional 2 to 12 mo (median: 4 mo in the 7 HBeAg seroconverters. The other 7 non-seroconverters discontinued lamivudine treatment after a median treatment period of 12 mo (range: 9 to 27 mo). The baseline characteristics of the 7 seroconverters and the 7 non-seroconverters were similar (data not shown).

Table 1 Baseline characteristics of HBeAg-negative and -positive patients

	HBeAg-negative	HBeAg-positive	*P* value
Case number	12	14	-
Male-number (%)	10 (83)	13 (93)	0.58
Age (yr)	45.2±15.3	35.3±11.2	0.08
ALT (IU/L)	227 (80-2410)	281 (84-1298)	0.92
Serum total bilirubin (mg/dL)	2.9 (0.9-18.6)	1.1 (0.6-11.7)	0.24
Serum HBV DNA level (pg/mL)	272 (3-3121)	863 (7-3376)	0.65
HBV genotype B/C–number (%)	9/3 (75/25)	10/4 (71/29)	1.00
Duration of lamivudine treatmen-mo	12.3±1.9	14.2±4.9	0.23

Continuous values are expressed as mean±SD or median (range). HBV DNA levels were determined by a branched DNA assay (Bayer Corporation).

Characteristics of relapsers

At the end of lamivudine treatment, all group I and group II patients had normal serum ALT and undetectable serum HBV DNA by the bDNA assay. None developed biochemical or virologic breakthrough during the treatment period. After the end of the treatment, 10 patients relapsed within 6 mo, including 5 group I and 5 group II patients. Another 2 patients relapsed between 6 and 12 mo, including 1 group I and 1 group II patients. There was no significant difference with regard to clinical and virologic features among relapsers and non-relapsers in HBeAg-negative or -positive patients (Table 2, 3). The duration of lamivudine treatment did not differ between them. Only one of the 7 genotype C patients relapsed after the discontinuation of lamivudine treatment, the incidence was lower compared with that in the 19 genotype B patients (14% *vs* 58%, $P = 0.08$).

Among 14 group II patients, the 7 seroconverters tended to have a lower rate of relapse than the 7 non-seroconverters (29% *vs* 57%, $P = 0.30$) (Table 3). The duration of additional lamivudine treatment after attaining HBeAg seroconversion did not correlate with clinical relapse (Table 3). Two relapsers already received an additional 4 and 12 mo of lamivudine treatment, respectively, after HBeAg seroconversion.

Table 2 Characteristics of relapsers and non-relapsers in 12 patients negative for HBeAg before lamivudine treatment

	Relapsers	Non-relapsers	*P* value
Case number	6	6	-
Male-number (%)	6 (100)	4 (66.7)	0.13
Age (yr)	50.7±10.1	39.7±18.5	0.24
ALT (IU/L)	227 (80-452)	320 (98-2410)	0.24
Serum total bilirubin (mg/dL)	1.2 (0.8-6.5)	2.9 (0.6-18.6)	0.21
Serum HBV DNA level (pg/mL)	287 (16-2489)	117 (3-3121)	0.41
Duration of lamivudine treatment (mo)	12.5±2.6	12.2±1.2	0.78
HBV genotype B/C-number (%)	6/0 (100/0)	3/3 (50/50)	0.18

Values are expressed as mean±SD or median (range). HBV DNA levels were determined by a branched DNA assay (Bayer Corporation).

Relation between virologic response at the 12th wk of lamivudine treatment and relapse

At the 12th wk of lamivudine treatment, none of the group I patients had serum HBV DNA level $\geqslant 10^5$ copies/mL. Of the 12 group I patients, 8 (67%) had serum HBV DNA level $\geqslant$1 000 copies/mL. Relapse of hepatitis B activity occurred in 6 (50%) after cessation of lamivudine treatment. All relapsers remained HBeAg-negative and anti-HBe-positive. Three relapsers had serum HBV DNA level <1 000 copies/mL and the other three had serum HBV DNA level $\geqslant$1 000 copies/mL. The distribution of post-treatment relapse was similar between patients with detectable and undetectable serum HBV DNA by the real-time PCR.

Table 3 Characteristics of relapsers and non-relapsers in 14 patients positive for HBeAg before lamivudine treatment

	Relapsers	Non-relapsers	*P* value
Case number	6	8	-
Male-number (%)	5 (83.3)	8 (100)	0.43
Age (yr)	32.5±12.6	37.4±10.4	0.44
ALT (IU/L)	281 (84-1 298)	306 (97-868)	0.34
Serum total bilirubin (mg/dL)	1.1 (0.7-11.1)	1.2 (0.7-3.3)	0.28
Serum HBV DNA level at baseline (pg/mL)	310 (7-1 590)	280 (20-3376)	0.34
Duration of lamivudine treatment (mo)	15.5±6.5	13.3±3.5	0.47
HBeAg seroconversion–number (%)	2 (33.3)	5 (62.5)	0.30
Duration of additional lamivudine treatment after HBeAg seroconversion (mo)	12.4	6.0±4.7	0.75
HBV genotype B/C –number (%)	5/1 (83.3/16.7)	5/3 (62.5/37.5)	0.58

Values are expressed as mean±SD or median (range). HBV DNA levels were determined by a branched DNA assay (Bayer Corporation).

Table 4 Relation between virologic response and relapse after cessation of lamivudine treatment

HBV DNA level (copies / mL)	Total (n = 26)		HBeAg-positive				HBeAg-negative	
			HBeAg seroconverters		HBeAg non-seroconverters			
	Relapser n = 12	Non-relapser n = 14	Relapser n = 2	Non-relapser n = 5	Relapser n = 4	Non-relapser n = 3	Relapser n=6	Non-relapser n=6
<1 000 copies / mL –number (%)	4 (67)	2 (33)	1 (50)	1 (50)	2 (67)	1 (33)	2 (67)	1 (33)
⩾1 000 copies / mL –number (%)	8 (40)	12 (60)	1 (20)	4 (80)	2 (50)	2 (50)	4 (44)	5 (56)
	P^1 = 0.37		P = 1.00		P = 1.00		P = 1.00	

Virologic response was determined at the end of lamivudine treatment. HBV DNA levels were determined by the real-time PCR assay. [1]Two-sided.

Relation between virologic response at the end of lamivudine treatment and relapse

Likewise, none of the group I patients had serum HBV DNA level ⩾10^5 copies/mL at the end of lamivudine treatment. Of the 12 group I patients, 9 (75%) had serum HBV DNA level ⩾1 000 copies/mL and 3 had serum HBV DNA level <1 000 copies/mL. Two of the 6 relapsers had serum HBV DNA level <1 000 copies/mL and the other 4 had serum HBV DNA level ⩾1 000 copies/mL. When compared with the non-relapsers, the proportion of relapsers with undetectable serum HBV DNA by the real-time PCR was similar (P = 1.00, Table 4).

Similar findings were noted in group II patients. Relapse of hepatitis B activity was detected in 6 (43%) of the 14 patients post-treatment. Three of the 6 relapsers had serum HBV DNA level <1 000 copies/mL and the other 3 had serum HBV DNA level ⩾1 000 copies/mL. When compared with non-relapsers, the proportion of relapsers with undetectable serum HBV DNA by the real-time PCR was also similar (50% *vs* 75%, P = 1.00, Table 4). When in-treatment occurrence of HBeAg seroconversion was taken into consideration, serum viral loads in the low range (<10^5 copies/mL) did not correlate with clinical relapse during follow-up, either (Table 3).

At the end of lamivudine treatment, YMDD motif of the HBV polymerase gene was amplified in all patients. PCR products were detectable in 11 and variation at this motif was found in 2 of them, including methionine-to-valine substitution in one (viral load: 32 400 copies/mL; serum ALT: 24 U/L) and methionine-to-isoleucine substitution in the other (viral load: 22 344 copies/mL; serum ALT: 30 U/L). Both developed relapse with wild-type YMDD HBV strain during the follow-up period.

DISCUSSION

In this study, we found that the relapse rate after lamivudine treatment was high and comparable between HBeAg-negative (50%) and -positive (43%) patients, which did not correlate with serum viral load at the end of treatment.

Because we only evaluated relapsers whose serum ALT levels were greater than twice the upper limit of normal, the rate of post-treatment virologic relapse could be even higher. The high relapse rate in our series indicated a poor durability of virologic response to lamivudine treatment in Asian countries when compared with that in Western countries[6,8,9,17,33], and an urgency of identifying an effective means to predict the durability of post-treatment virologic response. Lamivudine possesses potent inhibitory effect on viral replication, and previous studies have shown that after the reduction of viral load by lamivudine, a T cell response over viral replication could be restored[21]. Because serum viral load under anti-viral treatment reflected the control from both drugs and hosts over viral replication, we thus assumed that keeping viral load in the lower range might confer an adequate control over viral replication and therein have a lower relapse rate. Previous studies indeed revealed that viral load at the 12th wk and at the end of lamivudine treatment correlated with a higher HBeAg seroconversion rate and a lower post-treatment relapse rate, respectively[16,17]. In addition, serum HBV DNA level less than 10 000 genomes/mL within 12 wk of starting treatment might predict HBeAg seroconversion[16], and serum HBV DNA level less than 4 700 genomes/mL at the time of HBeAg seroconversion might be associated with a lower relapse rate[17]. However, our results showed that there was no correlation between serum viral load <10^5 copies/mL at the end of lamivudine treatment and post-treatment relapse. Consistently, one recent study demonstrated that the end-of-treatment viremic status by a qualitative PCR assay did not correlate with post-treatment relapse in HBeAg-negative patients receiving lamivudine treatment[34]. This fact suggested that serum viral load might represent the susceptibility of viruses to lamivudine but not reflect the status of host immune control. When suppression pressure from anti-virals was removed, residual viruses within the hepatocytes might actively replicate again and lead to relapse. However, whether an extremely low level of serum viral load can predict a durable anti-viral response post-treatment awaits ultrasensitive HBV quantitative assays. Actually, one recent study demonstrated that in patients with end-of-treatment serum HBV DNA levels of <200 copies/mL, the post-treatment relapse rate was significantly lower than in those with end-of-treatment serum HBV DNA levels of >1 000 copies/mL[35]. This finding supported our speculation.

In HBeAg-positive patients, the serum viral loads at the end of lamivudine treatment were comparable between seroconverters and non-seroconverters. From another aspect, the virologic response was more durable in the seroconverters than in the non-seroconverters during follow-up. Again, this implied that appearance of anti-HBeAg rather than the viremic levels could reflect the ability of host immune control over viral replication. Extended therapy has been suggested to ensure the durability of virologic responses after HBeAg seroconversion was attained[9]. However, whether longer duration of therapy can further enhance the host immunity against HBV needs future immunological assays.

Our results showed that the viremic level at the end of lamivudine treatment could not guarantee a durable post-treatment anti-viral response. Of particular note, HBV genotype C tended to have a lower relapse rate compared with genotype B. Although the molecular virologic mechanisms accounting for clinical outcomes or response to anti-viral treatments remain largely unknown, pathogenic and therapeutic differences do exist among HBV genotypes[18-29,36]. However, in the individualized setting of HBeAg-positive and -negative patients, HBV genotype was not a factor associated with post-treatment relapse (Tables 2, 3). In addition, conflicting data existed in recent reports[34,37]. Since our case number is small, whether HBV genotype serves as a predictor for durability of virologic response to lamivudine treatment remains to be examined.

The major limitation in this pilot study was the relatively small case number in each group. However, our findings highlighted

that virologic response at the end of lamivudine treatment could not predict post-treatment relapse. Further large studies by using more sensitive quantitative assays are needed to address this important issue. Other genetic determinants of the virus and host should be clarified in the future. Since the relapse rate was high in both HBeAg-negative and HBeAg-positive patients in HBV endemic areas and such events were usually preceded by an upsurge of serum viral load[32], frequent monitoring of liver biochemical tests and serum viral loads could be considered to detect early relapse. With the advent of novel anti-viral agents, patients without durable virologic response to current therapy may hopefully benefit from further treatments.

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