Measurement of hepatitis B virus core-related antigen is valuable for identifying patients who are at low risk of lamivudine resistance

Running Head: Prediction of lamivudine resistance

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ABSTRACT

The clinical usefulness of hepatitis B virus core-related antigen (HBVcrAg) assay was compared to that of HBV DNA assay in predicting the occurrence of lamivudine resistance in patients with chronic hepatitis B. Of a total of 81 patients who were treated with lamivudine, 25 (31%) developed lamivudine resistance during a median follow-up period of 19.3 months. The pretreatment positive rate of HBe antigen, or pretreatment levels of HBVcrAg or HBV DNA did not differ between patients with and without lamivudine resistance. Levels of both HBVcrAg and HBV DNA decreased after the initiation of lamivudine administration, however, the level of HBVcrAg decreased significantly more slowly than that of HBV DNA. The occurrence of lamivudine resistance was significantly less frequent in the 56 patients whose HBV DNA level was less than 2.6 log copy/mL at 6 months of treatment than in the remaining 25 patients. The cumulative rate of lamivudine resistance was as high as 70% within two years in the latter group, while it was only 28% in the former group. Lamivudine resistance did not occur during the follow-up period in the 19 patients whose HBVcrAg level was less than 4.6 log U/mL at 6 months of treatment, while it did occur in 50% of the remaining patients within two years. These results suggest that measurement of HBV DNA is valuable for identifying patients who are at high risk of developing lamivudine resistance, and that, conversely, measurement of HBVcrAg is valuable for identifying those who are at low risk of lamivudine resistance.

Key Words: lamivudine resistance, HBV core-related antigen, HBV DNA, chronic hepatitis B
INTRODUCTION

Lamivudine, a nucleoside analogue which inhibits reverse transcriptases, was first developed as an antiviral agent against human immunodeficiency virus (HIV). It was later also found to be effective against hepatitis B virus (HBV) because HBV is a member of the Hepadnaviridae family of viruses, which use reverse transcriptases in their replication process (1, 2). Lamivudine was found to inhibit the replication of HBV, reduce hepatitis, and improve histological findings of the liver in long-term treatment (3-5). Furthermore, it has been shown that lamivudine treatment improves the long-term outcome of patients with chronic hepatitis B (6, 7). However, there are a number of problems with lamivudine therapy, such as relapse of hepatitis due to the appearance of YMDD mutant viruses and the reactivation of hepatitis after discontinuation of the treatment (8-11).

The concentration of HBV DNA in serum decreases and usually becomes undetectable during lamivudine administration, but it rapidly increases when HBV become resistant to lamivudine. Thus, the measurement of HBV DNA is useful for monitoring the anti-viral effects of lamivudine. However, a negative result of HBV DNA in serum does not necessarily indicate a good outcome of lamivudine therapy because lamivudine resistance may occur even if HBV DNA levels remain undetectable during therapy (11-13). Recently, a chemiluminescence enzyme immunoassay (CLEIA) was developed in our laboratory for the detection of hepatitis B virus core-related antigen (HBVcrAg) (14, 15). The assay reflects the viral load of HBV in a similar manner to that used in assays which detect HBV DNA. HBVcrAg consists of HBV core and e antigens; both proteins are transcribed from the precore/core gene and their first 149 amino acids are identical (16-18). The HBVcrAg CLEIA simultaneously measures the serum levels of hepatitis B core (HBc) and e (HBe).
antigens, using monoclonal antibodies which recognize common epitopes of these two denatured antigens. In the present study, we analyzed the clinical significance of the HBVcrAg assay in monitoring the anti-viral effects of lamivudine treatment.

PATIENTS AND METHODS

Patients

A total of 81 patients with chronic hepatitis B who received lamivudine therapy were enrolled in the present study. These were 58 men and 23 women with a median age of 49 years (range 24-79 years). The 81 patients were selected retrospectively from 6 medical institutions in Japan (Shinshu University Hospital, Toranomon Hospital, Nagoya City University Hospital, Kyoto Prefectural University Hospital, Hiroshima University Hospital, National Nagasaki Medical Center). Eight to 25 patients who met the following three criteria were selected consecutively in each institution: the first, a daily dose of 100 mg lamivudine was administered for at least 6 months in a period from 1999 to 2004; the second, histologically confirmed for chronic hepatitis without liver cirrhosis; and the third, serum samples at several time points available for testing. All patients were naïve for lamivudine therapy. Chronic hepatitis B was defined as positive hepatitis B surface (HBs) antigen for more than 6 months with elevated levels of serum transaminases. The HBV genotype was A in 2 patients, B in 3 and C in 76. Serum HBV DNA was detectable in all patients and HBe antigen was positive in 51 (63%) of the 81 patients just before lamivudine administration. The median follow-up period was 19 months with a range from 6 to 50 months. Follow-up of patients ended when lamivudine administration was discontinued. Written informed consent was obtained from each patient.
The occurrence of lamivudine resistance was defined as a rapid increase in serum HBV DNA levels with the appearance of the YMDD mutations during lamivudine administration. Using this criteria, resistance appeared in 27 (33%) of the 81 patients. The median period from the start of lamivudine administration to the occurrence of resistance was 12 months with a range from 4 to 37 months.

**Serological markers for HBV**

HBs antigen, HBe antigen and anti-HBe antibody were tested using commercially available enzyme immunoassay kits (Abbott Japan Co., Ltd., Tokyo, Japan). Six major genotypes (A-F) of HBV can be detected using the method reported by Mizokami et al., in which the surface gene sequence amplified by polymerase chain reaction (PCR) is analyzed by restriction fragment length polymorphism (19). The YMDD motif, that is, lamivudine resistant mutations in the active site of HBV polymerase, was detected with an enzyme-linked mini-sequence assay kit (HBV YMDD Mutation Detection Kit, Genome Science Laboratories Co., Ltd., Tokyo, Japan) (20).

Serum concentration of HBV DNA was determined using Amplicor HBV monitor kit (Roche, Tokyo, Japan) which had quantitative range from 2.6 to 7.6 log copy/mL. Sera containing over 7.0 log copy/mL HBV DNA were diluted 10 or 100 fold with normal human serum and re-tested to obtain the end titer.

Serum concentrations of HBVcrAg were measured using the CLEIA method reported previously (10,11). Briefly, 100 µL serum was mixed with 50 µL pretreatment solution containing 15% sodium dodecylsulfate and 2% Tween 60. After incubation at 70°C for 30 min, 50 µL pretreated serum was added to a well coated with monoclonal antibodies against denatured HBc and HBe antigens (HB44, HB61 and HB114) and
filled with 100 µL assay buffer. The mixture was incubated for 2 hours at room temperature and the wells were then washed with buffer. Alkaline phosphatase-labeled monoclonal antibodies against denatured HBc and HBe antigens (HB91 and HB110) were added to the well, and the mixture was incubated for 1 hr at room temperature. After washing, CDP-Star with Emerald II (Applied Biosystems, Bedford, MA, USA) was added and the plate was incubated for 20 min at room temperature. The relative chemiluminescence intensity was measured, and the HBVcrAg concentration was determined by comparison with a standard curve generated using recombinant pro-HBe antigen (amino acids, 10 to 183 of the precore/core gene product). The HBVcrAg concentration was expressed as units/mL (U/mL) and the immunoreactivity of recombinant pro-HBe antigen at 10 fg/mL was defined as 1 U/mL. In the present study, the cutoff value was tentatively set at 3.0 log U/mL. Sera containing over 7.0 log U/mL HBVcrAg were diluted 10 or 100 fold in normal human serum and re-tested to obtain the end titer.

**Statistical analysis**

The Mann-Whitney U test and Wilcoxon signed-ranks test were utilized to analyze quantitative data, and Fisher's exact test was used for qualitative data. A log-rank test was used to compare the occurrence of lamivudine resistance. Statistical analyses were performed using the SPSS 5.0 statistical software package (SPSS, Inc., Chicago, IL, USA). A $P$ value of less than 0.05 was considered to be statistically significant.

**RESULTS**
Table 1 shows a comparison of the clinical and virological backgrounds of the 27 patients who showed lamivudine resistance and the 54 patients who did not. Median age, gender distribution and median follow-up period did not differ between the two groups, and the positive rate of HBe antigen was similar. Both HBV DNA and HBVcrAg levels at the beginning of lamivudine administration were similar between the two groups, however, both HBV DNA and HBVcrAg levels at 6 months after the start of lamivudine administration were significantly lower in the lamivudine resistance negative group than in the positive group. ALT level was normal at the beginning in 8 (15%) of the 54 patients without lamivudine resistance and in 2 (7%) of the 27 patients with it (P >0.2).

Figure 1 shows changes in HBV DNA and HBVcrAg levels during lamivudine treatment in all patients. The level of HBV DNA decreased rapidly and became undetectable at 3 months after treatment was initiated. On the other hand, although HBVcrAg levels decreased continuously, the median amount of decrease from the base line was significantly lower than that in HBV DNA levels at 1, 3 and 6 months after starting lamivudine administration (Wilcoxon signed-ranks test, P< 0.001 at all analyzed points in time).

Changes in HBV DNA and HBVcrAg levels during lamivudine administration are compared in Fig. 2 between the 27 patients who showed lamivudine resistance and the 54 patients who did not. Serum HBV DNA levels were found to decrease rapidly and become undetectable within 6 months in 45 (83%) of the 54 patients without lamivudine resistance. On the other hand, only 11 (41%) of the 27 patients with lamivudine resistance showed a similar rapid decrease, and the HBV DNA levels of the remaining patients stayed above the detection limit during the follow-up period. HBVcrAg levels decreased but did not reach levels lower than 4.7 log unit/mL (5,000
unit/mL) in the 27 patients with lamivudine resistance. In 19 (35%) of the 54 patients without lamivudine resistance, on the other hand, the levels decreased to levels below 4.7 log unit/mL within 6 months after the start of lamivudine administration. The level of HBVcrAg increased rapidly as did the level of HBV DNA when lamivudine resistance occurred.

The occurrence of lamivudine resistance was significantly less frequent in the 56 patients whose HBV DNA level was less than 2.6 log copy/mL at 6 months after the initiation of treatment than in the remaining 25 patients (Fig. 3). The cumulative occurrence of lamivudine resistance was as high as 70% within two years in the latter group, while it was only 28% in the former group. There was no occurrence of lamivudine resistance during the follow-up period in the 19 patients whose HBVcrAg levels were less than 4.6 log U/mL at 6 months after the initiation of lamivudine therapy (Fig. 3). On the other hand, lamivudine resistance occurred in 50% of the remaining patients within two years.

DISCUSSION

The HBVcrAg assay is a unique assay which measures the amounts of e and core antigens coded by the core gene of the HBV genome with high sensitivity and a wide quantitative range. Serum HBVcrAg levels reflect the viral load in the natural course because these levels correlate linearly with those of HBV DNA (14, 15). On the other hand, the character of HBVcrAg is somewhat different from that of HBV DNA in patients undergoing anti-viral therapies such as lamivudine. That is, HBVcrAg levels decrease significantly more slowly than those of HBV DNA after the initiation of lamivudine administration.
HBV is an enveloped DNA virus containing a relaxed circular DNA genome which is converted into a covalently closed circular DNA (cccDNA) episome in the nucleus of infected cells (18, 21-23). The cccDNA molecules serve as the transcriptional template for the production of viral RNAs that encode viral structural and non-structural proteins. Reverse transcription of the viral pregenomic RNA and second-strand DNA synthesis occur in the cytoplasm within viral capsids formed by the HBV core protein. Because lamivudine, a nucleoside analogue, inhibits reverse transcription of the pregenomic RNA, it directly suppresses the production of HBV virion. Thus, serum HBV DNA levels decrease rapidly after the initiation of lamivudine administration. On the other hand, the production of viral proteins is not suppressed by lamivudine because the production process does not include reverse transcription. Furthermore, it has been reported that the amount of cccDNA which serves as a template for mRNA decreases quite slowly after starting the administration of nucleoside analogues (24-26). Thus, it is reasonable that serum HBVcrAg levels decrease much more slowly than HBV DNA levels after the initiation of lamivudine therapy.

Significant markers that can predict the presence or absence of lamivudine resistance are clinically valuable because the emergence of this resistance and the subsequent recurrence of hepatitis are fundamental problems in lamivudine therapy. Serum markers that reflect the activity of HBV replication have been reported to be associated with the occurrence of lamivudine resistance (11, 12, 27, 28). However, neither the pretreatment existence of HBe antigen nor pretreatment levels of HBV DNA or HBVcrAg were found to be significant markers in the present study. These results may reflect a weak association between the pretreatment activity of HBV replication and the occurrence of lamivudine resistance(13, 29). Changes in HBV
DNA and HBVcrAg levels after starting lamivudine administration clearly differed between patients with and without lamivudine resistance. Thus, HBV DNA and HBVcrAg levels at 6 months after starting lamivudine administration were analyzed to determine whether these levels might serve as predictive markers; both were found to be significantly lower in patients without lamivudine resistance at the tested point in time. Furthermore, patients who showed higher levels of HBV DNA and HBVcrAg at 6 months after the initiation of treatment were significantly more likely to develop lamivudine resistance than those who showed lower levels.

We believe that the measurement of HBV DNA levels is useful to identify patients who are at high risk for lamivudine resistance because as many as 70% of patients who were positive for HBV DNA at 6 months after starting lamivudine administration developed lamivudine resistance within two years. However, a negative result of HBV DNA at 6 months does not necessarily guarantee the absence of lamivudine resistance because nearly 30% of such patients developed resistance within 2 years. On the other hand, HBVcrAg levels of less than 4.7 log unit/mL at 6 months is a useful indicator of patients who are unlikely to develop lamivudine resistance, because no such patients developed resistance during the follow-up period in the present study. Lower serum HBVcrAg levels may reflect lower levels of cccDNA in hepatocytes because the mRNAs of HBVcrAg are transcribed from the cccDNA (18, 22, 23). This possibility may explain our finding that patients whose HBVcrAg levels decreased sufficiently were unlikely to develop lamivudine resistance, because cccDNA provides the templates for viral and pregenomic messenger RNA (18, 22, 23), which may be a source of lamivudine-resistant strains.

In conclusion, our results suggest that measurement not only of HBV DNA but also of HBVcrAg is useful for predicting the occurrence of lamivudine resistance.
DNA measurement is valuable for identifying patients who are at high risk of developing this resistance and HBcrAg measurement is valuable for identifying those who are at low risk.

ACKNOWLEDGMENTS

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REFERENCES


Table 1. Comparison of the clinical and virological backgrounds of patients who showed lamivudine resistance and those who did not.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Appearance of lamivudine resistance</th>
<th>P</th>
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<tbody>
<tr>
<td></td>
<td>negative (n=54)</td>
<td>positive (n=27)</td>
</tr>
<tr>
<td>Age (years) a</td>
<td>47.0 (24-79)</td>
<td>50.6 (34-67)</td>
</tr>
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<td>Gender (male %)</td>
<td>74%</td>
<td>67%</td>
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<tr>
<td>Follow-up period (months) a</td>
<td>16 (6-50)</td>
<td>21 (9-43)</td>
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<tr>
<td>HBV genotype (A/B/C)</td>
<td>2/2/50</td>
<td>0/1/26</td>
</tr>
<tr>
<td>HBe antigen (positive %)</td>
<td>59%</td>
<td>70%</td>
</tr>
<tr>
<td>ALT (IU/mL) a</td>
<td></td>
<td></td>
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<tr>
<td>initial</td>
<td>85 (22-713)</td>
<td>95 (20-1140)</td>
</tr>
<tr>
<td>at 6 months</td>
<td>27 (11-115)</td>
<td>30 (15-92)</td>
</tr>
<tr>
<td>HBV DNA (log copy/mL) a</td>
<td></td>
<td></td>
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<tr>
<td>initial</td>
<td>7.0 (3.5-9.1)</td>
<td>7.3 (4.2-9.2)</td>
</tr>
<tr>
<td>at 6 months</td>
<td>&lt;2.6 (&lt;2.6-4.8)</td>
<td>3.3 (&lt;2.6-6.6)</td>
</tr>
<tr>
<td>HBVcrAg (log U/mL) a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>initial</td>
<td>6.2 (&lt;3.0-8.8)</td>
<td>7.3 (4.4-9.1)</td>
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<tr>
<td>at 6 months</td>
<td>5.2 (&lt;3.0-6.7)</td>
<td>5.8 (4.7-8.4)</td>
</tr>
</tbody>
</table>

a Data are expressed as median (range).

b Mann-Whitney U test.

c Chi-square test.
FIGURE LEGENDS

**Figure 1.** Changes in the median levels of HBVcrAg and HBV DNA during lamivudine administration. The box plots show the 10th, 25th, 50th, 75th, and 90th percentiles, with the open boxes indicating HBV DNA and shaded boxes indicating HBVcrAg. The median amount of decrease from the base line in HBVcrAg levels was significantly smaller (Wilcoxon signed-ranks test) than that in HBV DNA level at 1 (2.80 log copy/mL vs. 0.27 log U/mL, P< 0.001), 3 (3.60 log copy/mL vs. 0.83 log U/mL, P< 0.001), and 6 months (3.90 log copy/mL vs. 1.15 log U/mL, P< 0.001) after the initiation of lamivudine administration.

**Figure 2.** Comparison of changes in serum HBV DNA and serum HBVcrAg levels between patients who showed lamivudine resistance and those who did not. The broken lines indicate the detection limit of each assay.

**Figure 3.** Comparison of the cumulative occurrence of lamivudine resistance between patients who showed HBV DNA levels of less than the detection limit (2.6 log copy/mL) at 6 months after starting lamivudine administration and those who did not (left figure), and similarly between patients who showed HBVcrAg levels of less than 4.7 log U/mL and those who did not (right figure).
Figure 1.

HBV DNA (log copy/mL) vs. HBVcrAg (log U/mL) over 6 months after starting lamivudine administration.
Figure 2.

![Graph showing HBV DNA and HBVcrAg levels over time with and without lamivudine resistance.](image-url)
Figure 3.

Cumulative appearance of lamivudine resistance (%)

HBV DNA

- >2.6 log copy/mL (n=25)
- < 2.6 log copy/mL (n=56)

P< 0.001

HBVcrAg

- >4.7 log U/mL (n=62)
- < 4.7 log U/mL (n=19)

P=0.005

Months after starting lamivudine administration