Quantitative Levels of Hepatitis B Virus DNA and Surface Antigen and the Risk of Hepatocellular Carcinoma in Patients with Hepatitis B Receiving Long-Term Nucleos(t)ide Analogue Therapy

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Key Words
HBV DNA · Hepatitis B surface antigen · Hepatitis B virus · Hepatocellular carcinoma · Nucleos(t)ide analogues

Abstract
Background: Serum levels of hepatitis B virus (HBV) DNA are an important predictor of the risk of hepatocellular carcinoma (HCC) in patients with chronic HBV infection. However, little is known about whether high levels of hepatitis B surface antigen (HBsAg) increase the risk for HCC.

Methods: We investigated 167 patients who were treated with nucleos(t)ide analogues (NA) for at least 2 years (median: 5.8 years, range: 2–13.1 years). Relationships between reduced levels of HBsAg and various factors were evaluated. In addition, we evaluated the usefulness of quantitative serum levels of HBV DNA and HBsAg as predictors of HCC development in patients receiving long-term NA therapy.

Results: HCC developed in 9 of the 167 NA-treated patients. In the 9 patients with HCC, HBV DNA was undetectable (<2.1 log copies/mL), but HBsAg levels were ≥2000 C.O.I. in 7 patients. No maternal transmission, long NA treatment period, HBV DNA levels <3.0 log copies/mL, and reduced hepatitis B e antigen levels during the first 24 weeks of treatment were a significant factor of HBsAg levels <2000 C.O.I.

Conclusions: Hepatocarcinogenesis was observed in patients with high HBsAg levels,
Introduction

Globally, approximately 400 million people are infected with the hepatitis B virus (HBV). Among them, half a million people develop cirrhosis or hepatocellular carcinoma (HCC) annually [1, 2]. Approximately 1 million people die annually from hepatitis B-induced HCC, underlining the fact that this is an important problem [3]. In Japan, HBV infection accounts for approximately 6% of HCC cases [4]. For this reason, patients with persistent hepatitis require antiviral interferon (IFN) or nucleos(t)ide analogue (NA) therapy. Anti-HBV therapy aims to suppress hepatitis through the continuous suppression of HBV. Additionally, the ultimate therapeutic goal is to improve vital prognosis through arrested cirrhosis or HCC development.

The risk of progression from chronic hepatitis B to cirrhosis is significantly affected by blood levels of HBV DNA. When the levels of HBV DNA are less than 4.0 log copies/mL, the risk of progression to cirrhosis is low. However, the risk of cirrhosis is reported to increase with an increase in the levels of HBV DNA above 4.0 log copies/mL [5]. Similarly, HBV DNA levels at the start of observation are thought to be associated with hepatocarcinogenesis. Because the risk of hepatocarcinoma increases as the HBV DNA level increases above 4.0 log copies/mL, therapies that control HBV DNA levels are important [6]. NAs are therapeutic agents that strongly control HBV DNA levels and also reduce alanine aminotransferase (ALT) levels. NA therapy is an epoch-making therapy that suppresses both hepatitis [7] and the onset of hepatocarcinoma [7–10].

Recently, the levels of hepatitis B surface antigen (HBsAg), in addition to levels of HBV DNA, were linked with the risk of hepatocarcinogenesis in untreated hepatitis B patients [11]. Quantified HBsAg levels are increasingly recognized as a marker with which to evaluate the host immunological control of HBV replication and infection [12–14]. Low HBsAg levels in patients with HBV genotype B or C are considered to indicate a high likelihood of HBV clearance and lower hepatitis activity [13–15]. Studies of HBV genotype D have also defined patients with less than 1000 IU/mL of HBsAg as inactive virus carriers.

From the clinical perspective, we were interested in whether the incidence of HCC might vary in an HBsAg level-dependent manner in Japanese patients of genotype C in whom HBV DNA is controlled by long-term NA therapy. To address this interesting question, we enrolled 167 hepatitis B patients to whom we had administered NA therapy in our hospital for more than 2 years. This study aimed to identify the predictors of HBsAg level reduction in the context of long-term NA administration. In addition, hepatocarcinogenesis during long-term NA administration about whether related to the amount of HBsAg, we investigated for the first time in Japan.

Patients and Methods

Patients

The subjects were 167 patients who had received NA therapy for more than 2 years and were selected from the hepatitis B patients with ALT levels ≥31 U/L and HBV DNA levels ≥4.0 log copies/mL who visited
Kawasaki Hospital, Kawasaki Medical School, between 1999 and 2010. Seventy-two patients received 0.5 mg/day of entecavir (ETV), 57 patients received 100 mg/day of lamivudine (LMV), and 37 patients received adefovir dipivoxil (ADV) in addition to LMV to treat LMV-resistant virus. The median administration period of NA was 5.8 ± 2.8 years. The average age at the start of therapy, the male to female ratio, and the ratio of subjects with chronic hepatitis to those with cirrhosis were 49.2 ± 11.1 years, 112/55, and 126/41, respectively. The average HBV DNA level at the start of therapy was 6.8 ± 1.3 log copies/mL. Among the subjects, 3, 4, 144, and 16 carried A, B, C, and unknown HBV genotypes, respectively; thus, the majority of infections were of genotype C. Eighty-one patients (50.9%) were positive for the hepatitis B e antigen (HBeAg). The HBsAg levels were below 2000 C.O.I in 8 (4.7%) subjects and above 2000 C.O.I. in 159 (95.3%) subjects (table 1).

Methods

Data were collected to measure the negative conversion rate of HBsAg, the negative conversion and seroconversion rates of HBeAg, the alanine aminotransferase (ALT) normalization (<30 IU/L) rate, and the negative conversion (<3.0 log copies/mL) rate of HBV DNA during the final evaluations of therapeutic effects in 167 patients with hepatitis B who had received NA for more than 2 years. Additionally, we verified the relationships of various factors (age, sex, chronic hepatitis/cirrhosis, HBV DNA level, HBV genotype, initial HBeAg level, initial HBsAg level, initial ALT value, IFN therapy history, the presence or absence of mother-to-child transmission, NA administration period, and HBV DNA and HBsAg levels at 24 weeks after the start of NA administration) with reduced HBsAg levels. Furthermore, we investigated the relationships between HCC incidence and the levels of HBV DNA and HBsAg at the final observation.

HBV Marker Assay

Serum HBsAg and anti-HBs antibody levels were measured by chemiluminescent enzyme immunoassay (CLEIA, Lumipulse System; Fujirebio, Tokyo, Japan). HBeAg and anti-HBe antibody levels were determined using commercially available enzyme-linked immunosorbent assay kits (EIA, Abbot Japan, Tokyo, Japan). HBV DNA was assayed using the COBAS Amplicor HBV Monitor Test (Roche Diagnostics, Tokyo, Japan), which has a dynamic range of 2.6 to 7.6 log copies/mL, or the COBAS TaqMan HBV Test, version 2.0 (Roche Diagnostics, Tokyo, Japan), which has a dynamic range of 2.1 to 9.0 log copies/mL.

Table 1. Characteristics of patients before and after NA treatment

<table>
<thead>
<tr>
<th>NA treatment duration (years), n=167</th>
<th>5.8 ± 2.8</th>
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</thead>
<tbody>
<tr>
<td>ETV, n=72</td>
<td>4.4 ± 1.9</td>
</tr>
<tr>
<td>LMV only, n=57</td>
<td>6.5 ± 3.1</td>
</tr>
<tr>
<td>LMV plus ADV, n=38</td>
<td>7.3 ± 2.8</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>NA treatment duration (years), n=167</th>
<th>Before NA treatment</th>
<th>After NA treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>49.2 ± 11.1</td>
<td>56.7 ± 25.4</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>112/55</td>
<td>—</td>
</tr>
<tr>
<td>Chronic hepatitis/liver cirrhosis</td>
<td>126/41</td>
<td>—</td>
</tr>
<tr>
<td>HBV DNA (log copies/ml)</td>
<td>6.8 ± 1.3</td>
<td>1.6 ± 1.5</td>
</tr>
<tr>
<td>HBV genotype (A/B/C/ND)</td>
<td>3/4/144/16</td>
<td>—</td>
</tr>
<tr>
<td>HBeAg positive</td>
<td>81 (50.9%)</td>
<td>42 (25.6%)</td>
</tr>
<tr>
<td>HBsAg (&lt;2000/≥2000 C.O.I.)</td>
<td>8/159</td>
<td>28/139</td>
</tr>
</tbody>
</table>

Values are mean±standard deviation (SD). ND=no data; C.O.I.=cut off index.
Statistical Analysis
Statistical analyses were performed using the SAS statistical software package, version 9 (Cary, NC, USA). We used the Wilcoxon test, chi-square test, and Fisher’s exact test for univariate analyses. Cumulative HCC incidence rates were analyzed according to the Kaplan–Meier method. We compared the cumulative incidence of HCC using the log-rank test. Significance was defined as p < 0.05 for all two-tailed tests.

Ethical Considerations
Informed consent was obtained from all participants. The study protocol complied with the ethical guidelines of the Declaration of Helsinki of 1975 (2004 revision) and was approved by the Ethics Committee of Kawasaki Hospital, Kawasaki Medical School.

Results

Virological and Biochemical Outcomes after Long-Term NA Therapy
After long-term (median: 5.8 years, range: 2–13.1 years) of NA therapy, the ALT normalization (<30 IU/L) rate was 83% (138/167). The proportion of subjects in whom the HBV DNA level decreased below 3.0 log copies/mL was 86% (143/167). The seroconversion rate was 31% (25/81) (fig. 1a).

Percentage of Patients with Low HBsAg Levels before/after Long-Term NA Therapy
The proportion of patients with low HBsAg levels (<2000 C.O.I.) was 4.7% (8/167) prior to NA administration. However, after long-term NA administration, a significant increase to 16.7% (28/167) in the proportion of patients with low HBsAg levels was observed (p < 0.001) (fig. 1b).

Factors Related to Reduced HBsAg Levels
The 28 patients with low HBsAg values (<2000 C.O.I.) were compared with the 139 patients with high HBsAg values (≥2000 C.O.I.) at the observation after at least 2 years of long-term NA therapy (table 2). A long period of NA therapy (7.0 years vs. 5.5 years, p = 0.010)
and non-mother-to-child transmission (66% vs. 74%, p = 0.042) were extracted as significant factors.

Additionally, an evaluation of viral dynamics during therapy revealed that HBsAg levels decreased significantly both in patients with low HBV DNA levels (<3.0 log copies/mL; 87.5% vs. 66.9%, p = 0.031) and in HBeAg-negative patients (81% vs. 52%, p = 0.005) at 24 weeks after the start of NA therapy. Cirrhosis or a history of IFN therapy was more frequently noted, with marginal significance, in patients with HBsAg levels below 2000 C.O.I. On the other hand, no significant differences were observed with regard to age; sex; genotype; or levels of HBV DNA, HBeAg, or ALT before therapy.

**Factors Related to Liver Carcinogenesis**

Comparison of background factors was conducted for the 9 patients who developed HCC during long-term NA therapy and for the 158 patients who did not develop liver cancer during the treatment period. Among the factors that were present before NA therapy, being male (p = 0.03) and the presence of hepatic cirrhosis (p = 0.04) were significant factors for the development of HCC. However, the ALT level normalization, low HBsAg level, and HBV-DNA negativation at the last observation at the end of the long-term NA therapy were not found to be significant factors for the development of HCC (table 3).
Incidence of HCC in Relation to HBV DNA and HBsAg Levels

HCC was observed in 9 of 167 patients (5.3%) who received long-term NA therapy; of these, 2 patients were positive for HBV DNA (>2.1 log copies/mL) at the onset of HCC. However, the remaining 7 patients developed HCC despite a negative conversion of HBV DNA (<2.1 log copies/mL) and all of these 7 patients had high HBsAg levels (>2000 C.O.I.). Patients who had low levels of both HBV DNA and HBsAg did not develop HCC (figs. 2, 3, 4 and 5).

Discussion

HBV DNA levels have been considered an important factor related to hepatitis B-induced carcinogenesis. The rate of carcinogenesis is known to increase proportionally with increased HBV DNA levels [6]. NAs are epoch-making hepatitis B therapeutic agents because they reduce HBV DNA and ALT levels, resulting in the suppression of hepatitis [7]. Moreover, NAs have been shown to significantly reduce hepatocarcinogenesis [8, 9]. In a matched control study of patients receiving long-term ETV administration versus untreated controls, the 5-year carcinogenic rates were 3.7% and 13.7%, respectively, indicating a significant suppression of carcinogenesis in the ETV group [10].
On the other hand, HBsAg levels, in addition to HBV DNA levels, were recently reported to be related to the carcinogenic risk. Tseng et al. [11] observed the natural courses of 2688 cases of chronic hepatitis B, excluding those with cirrhosis, for an average of 14.7 years and reported that male sex, old age, a high serum ALT level, HBeAg positivity, genotype C, an HBV DNA level ≥ 2000 IU/mL, and an HBsAg level ≥ 1000 IU/mL are significant predictors of HCC.
**Fig. 4.** HCC risk of HBV DNA levels after long-term NA therapy. ——: HBV DNA ≤ 2.1 log copies/mL; – –: HBV DNA > 2.1 log copies/mL.

**Fig. 5.** HCC risk and HBsAg levels in patients with HBV DNA levels ≤ 2.1 log copies/mL after long-term NA therapy. ——: HBsAg ≥ 2000 C.O.I. and HBV DNA ≤ 2.1 log copies/mL; – –: HBsAg < 2000 C.O.I. and HBV DNA ≤ 2.1 log copies/mL.
hepatocarcinogenesis. In an analysis stratified by HBV DNA levels, the HBV DNA level was not extracted as a factor related to carcinogenesis in patients with HBV DNA levels below 2000 IU/mL. On the other hand, the researchers reported the importance of the HBsAg level as a hepatocarcinogenesis-related factor because old age, a high ALT value, and an HBsAg level >1000 IU/mL were significantly related to hepatocarcinogenesis. Additionally, of a total of 5055 hepatitis B cases with natural progression or intervention, only 2 patients of 231 patients with negative conversion of HBsAg developed hepatic carcinoma, indicating that HBsAg could be predictive of hepatocarcinogenesis [16].

HBsAg was first discovered in 1965 as the "Australia antigen" from the sera of Aboriginal people by Blumberg et al. [17]. In 1968, Okochi et al. reported relationships between the Australia antigen and hepatitis [18]. HBsAg is produced via multiple pathways in the lifecycle of HBV and uses cccDNA as a template. Therefore, HBsAg levels are reported to correlate with cccDNA levels [19–22]. Indeed, hepatitis B patients with HBsAg levels below 1–2 × 10^3 IU/mL and HBV DNA levels below 2 × 10^3 IU/mL have a low risk of hepatitis recrudescence. Because the HBsAg level reflects HBV replication, it appears necessary to focus on HBsAg as well as on HBV DNA in hepatocarcinogenesis [23–25]. Additionally, it was reported that HBV DNA levels above 3 log copies/mL at the termination of NA therapy are likely to lead to recrudescence and that, despite the negative conversion of HBV DNA, high HBsAg and HBeAg levels are likely to lead to recrudescence in patients who received NA [26]. Therefore, the HBsAg level is an important indicator of both hepatocarcinogenesis and hepatitis recrudescence.

Moreover, the therapeutic goals for chronic hepatitis B put forth in the guidelines of the American Association for the Study of Liver Diseases, the European Association for the Study of the Liver, and the Asian Pacific Association for the Study of the Liver are improvements in the quality of life and survival rates through the prevention of hepatic cirrhosis, decompensated cirrhosis, end-stage liver diseases, HCC, and progression to death. Furthermore, the guidelines mention that the ideal endpoint is the "disappearance of HBsAg" [27–29].

The disappearance rate of HBsAg during the natural course of infection has been reported in several studies as 0.5–3.0% per year [27–29]. In our previous study, the disappearance rate of HBsAg in cirrhosis patients was 0.9% per year [30]. The significant factors that led to the disappearance of HBsAg within 3 years were reported to be HBsAg levels <2000 IU/mL or a decrease in HBsAg levels at an annual rate of 0.5 log IU/mL [31]. However, because the annual disappearance rate of HBsAg was the lowest in patients infected with HBV genotype C in our present study [32], the HBV subgenotype C2/Ce observed in many Japanese is an independent risk factor for HCC [33]. Therefore, we consider it necessary to reduce HBsAg levels as much as possible through antiviral therapy to suppress hepatocarcinogenesis.

The negative conversion rates of HBsAg after a year of NA therapy were reported to be 2, 0–1, and 0% in ETV-, LAM-, and ADV-treated patients, respectively [7, 34–39]. In our study, HBsAg levels decreased to below 2000 C.O.I. in 16.7% of all patients who received long term NA therapy over 2 years; these proportions were 4.2 and 26.3% in ETV- and LMV (with or without ADV)-treated patients, respectively. However, among all patients, this level of reduction was seen in only four patients (2.3%) with a negative conversion of HBsAg. The significant factors that led to reductions of HBsAg levels below 2000 C.O.I during long-term NA therapy were a long period of NA therapy, non-mother-to-child transmission, a low HBV DNA level at 24 weeks after the start of NA therapy, and HBeAg negativity at 24 weeks after the start of NA therapy. These patients were expected to have reduced HBsAg levels, although they accounted for no more than 16.7% of the total number of patients.

In the present study, hepatocarcinogenesis was observed in 9 of 167 patients (5.3%) who received long-term NA therapy, and 2 of these patients showed a positive conversion of HBV DNA. However, all 7 remaining patients had HBsAg levels above 2000 C.O.I., despite the negative conversion of HBV DNA. This indicates that patients with high HBsAg levels should be
observed for hepatocarcinogenesis even in cases with successful negative conversion of HBV DNA during NA therapy. On the basis of the above results, we consider that both HBV DNA and HBsAg levels are important in hepatocarcinogenesis.

NA are reported low effective in the reduction of HBsAg levels because of difficulties in elimination of cccDNA, although NA strongly reduces HBV DNA levels by inhibiting HBV replication through reverse transcription [19]. On the other hand, after reviewing reductions in HBsAg levels in response to NA therapy and IFN therapy in 11 studies, Liaw reported that IFN therapy reduced HBsAg levels more efficiently than NA therapy did [40]. Furthermore, other reports state that the negative conversion rate of HBsAg after 48 weeks of pegylated-IFN administration is 3–7%, which is higher than the 0–2% rate reported for NA therapy [34, 41]. Moreover, a 5-year follow-up study after the termination of pegylated-IFN therapy showed that the negative conversion rate of HBsAg was further elevated in a time-dependent manner, achieving a rate of 12% at 5 years [42, 43]. Similarly, a study in Japan, where genotype C accounts for the majority of hepatitis B infections, also demonstrated that the negative conversion rate of HBsAg was elevated in a time-dependent manner, although not as markedly in genotype C as in genotypes A and B; this elevation resulted in a negative conversion rate of 11% at 10 years [44].

Our present study revealed that 8 of 9 patients who developed HCC had high HBsAg levels during long-term NA therapy. Therefore, to achieve the suppression of hepatocarcinogenesis, it may be important to reduce HBsAg levels not only by NA therapy, but also by combining NA with IFN. This is the goal of hepatitis B therapy, as the suppressive effects of IFN therapy on carcinogenesis have been previously reported [45, 46].

In conclusion, despite the negative conversion of HBV DNA during long-term NA therapy, hepatocarcinogenesis was observed in patients with high HBsAg levels. Therefore, the control of both HBV DNA and HBsAg levels is important for the suppression of hepatocarcinogenesis.

Conflict of Interest

All authors declare that they have no conflicts of interest.

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