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Abstract

Background & Aims

Individuals negative for hepatitis B surface antigen (HBsAg) but positive for antibodies to hepatitis B core antigen (anti-HBc) are at risk of hepatitis B virus (HBV) reactivation under immunosuppressive conditions. We investigated clinical features and viral genetics in patients with reactivation from occult HBV infection triggered by chemotherapy or immunosuppressive therapy.

Methods

Clinical courses of 14 individuals originally HBsAg-negative but anti-HBc-positive that experienced HBV reactivation were examined. Ultra-deep sequencing analysis of the entire HBV genome in serum was conducted. Prevalence of the G1896A variant in latently infected livers was determined among 44 healthy individuals that were HBsAg-negative but anti-HBc-positive.

Results

In 14 cases, HBV reactivation occurred during ($n=7$) and after ($n=7$) termination of immunosuppressive therapy. Ultra-deep sequencing revealed that the genetic heterogeneity of reactivated HBV was significantly lower in patients with reactivation from occult HBV carrier status compared with that in patients from HBsAg carrier status. The reactivated viruses in each case were almost exclusively the wild-type G1896 or G1896A variant. The G1896A variant was detected in 42.9% (6/14) of cases, including two cases with fatal liver failure. The G1896A variant was observed in the liver tissue of 11.4% (5/44) of individuals with occult HBV infection.

Conclusions

Reactivation from occult HBV infection is characterized by low genetic heterogeneity, with the wild-type G1896 or G1896A variant prevalent.

Keywords: G1896A pre-core variant; genetic heterogeneity; immunosuppressive therapy; occult HBV infection; ultra-deep sequencing

Introduction

Clinical features and pathophysiology of hepatitis B virus (HBV) infection are determined by the balance between the host immune response and viral replication. Individuals with persistent HBV infection are at risk of viral reactivation when the host immune system is weakened. HBV reactivation can occur in patients positive for hepatitis B surface antigen (HBsAg) in the serum, under immunosuppressive conditions [1-4]. Evidence has revealed that individuals who are HBsAg-negative but positive for antibodies to hepatitis B core antigen (anti-HBc) can also undergo HBV reactivation, commonly referred to as *de novo* hepatitis B infection, in response to chemotherapy or immunosuppression [5, 6]. HBV persists in the liver after the disappearance of HBsAg in individuals with previous exposure to the virus, retaining the serological footprint of anti-HBc positivity, with such a status defined as an occult HBV infection [7, 8]. Based on viral transmission studies in living-donor liver transplant patients, we previously demonstrated that most healthy individuals with an occult HBV infection were latently infected by the episomal form of HBV, with ongoing viral replication occurring in the liver [9, 10]. Subsequently, we encountered an occult HBV patient with leukemia who developed fatal liver failure caused by viral reactivation [11]. Current guidelines issued by the American Association for the Study of Liver Diseases indicate that immunocompromised patients should undergo testing for HBsAg and anti-HBc before receiving chemotherapy or immunosuppressive therapy; antiviral prophylaxis is recommended for HBV carriers at the onset of chemotherapy or immunosuppression [12]. However, the detailed clinical

features and viral genetics of reactivation from occult HBV carrier status are not yet fully understood because of the low incidence of viral reactivation in HBsAg-negative immunocompromised individuals. For example, Hui et al examined the frequency of *de novo* HBV hepatitis among patients with malignant lymphoma [6]. They reported that 3.3% (8/244) of HBsAg-negative patients receiving rituximab-containing chemotherapy developed HBV reactivation. Moreover, HBV reactivation can also occur only infrequently in HBsAg-negative individuals without hematological malignancies under immunosuppressive conditions [13].

Various mutations in HBV genomes have important implications for sensitivity to antiviral therapy [14, 15], and for the pathophysiology of liver diseases. As an example, acute infection with HBV variants containing point mutations at nucleotide 1896 (G1896A) in the pre-core (pre-C) region represents a high risk for developing acute liver failure (ALF) [16-18]. Similarly, predominant reactivation of G1896A variants is frequently observed in HBsAg-positive carriers who develop fatal viral reactivation under immunosuppressive conditions without antiviral prophylaxis [19]. Recent evidence indicates that reactivation from occult HBV infection is of particular concern because the clinical course and outcome of those patients commonly results in severe liver dysfunction and fatal ALF [6], with most fatal cases predominantly containing G1896A pre-C variants [20]. There are an estimated 3 billion individuals who are positive for anti-HBc worldwide, including 10% of the total population in Europe, 15% in the United States, 20% in Japan, and more than 50% in highly endemic areas such as China and Taiwan [21, 22]. However, little is known about the prevalence of HBV

infection with G1896A pre-C variants among occult HBV carriers, and how reactivation of G1896A pre-C variants leads to fatal consequences.

We examined HBV reactivation in HBsAg-negative and -positive patients. To clarify characteristics of the viral genome and its association with the pathophysiology of HBV reactivation, we used ultra-deep sequencing. This technique allowed for parallel amplification and detection of the full length of the HBV genome for a large number of sequences [23], and assisted in determining the genetic complexity of reactivated viral clones and the prevalence of G1896A pre-C variants.

Patients and Methods

Patients and Samples

Between April 2007 and July 2013, there were 1377 patients negative for HBsAg and positive for anti-HBc testing (220 patients with hematologic malignancies, 790 patients with solid tumors, and 367 patients with noncancerous diseases), prior to initiation of chemotherapy or immunosuppressive therapy at Osaka Red Cross Hospital, Hyogo Prefectural Amagasaki Hospital, Kitano Hospital, and Kyoto University Hospital. Among them, a total of 14 patients were diagnosed with HBV reactivation and their serum samples were available for further analyses (Table 1). All patients were originally HBsAg-negative but anti-HBc-positive before viral reactivation, and lacked any risk factors for external viral transmission, as demonstrated by the absence of blood transfusion, drug abuse, sexual contact, or blood contact with a known hepatitis virus carrier. No patients were co-infected with hepatitis C virus, hepatitis D virus or human immunodeficiency virus. All patients were longitudinally followed up at 0.5–3-month intervals until analysis (July 2013) or death. ALF was defined as the presence of hepatic encephalopathy and deranged blood coagulation (prothrombin time international normalized ratio > 1.5) [24].

Serum samples were obtained at diagnosis of HBV reactivation as demonstrated by the appearance of circulating HBsAg and HBV DNA under immunosuppressive conditions. Serological HBV markers, including HBsAg, antibodies to HBsAg, anti-HBc, hepatitis B e antigen (HBeAg) and

antibodies to HBeAg were measured by chemiluminescent enzyme immunoassay (CLEIA; Fuji Rebio, Tokyo, Japan). Serum HBV DNA titer was analyzed using a commercial polymerase chain reaction (PCR) (COBAS Taqman HBV test; Roche, Branchburg, NJ, USA) with a lower detection limit of 2.1 log copies/mL. The level of HBV DNA was retrospectively quantified in eight samples from five patients with reactivation from occult HBV infection.

To examine the genetic heterogeneity and prevalence of G1896A variants, liver tissues were obtained from 45 consecutive healthy donors negative for HBsAg and positive for anti-HBc who underwent hepatectomy for living-donor liver transplantation at Kyoto University from April 1998 to March 2001. Additionally, we examined the reactivated viruses derived from the serum of six patients who had typical serologic characteristics of the inactive HBsAg carrier state before immunosuppressive therapy. These cases were originally HBsAg-positive, while liver function tests were within the normal range before viral reactivation.

The Kyoto University Ethics Committee approved this study, and written informed consent was obtained from all patients. The study was conducted in accordance with the principles of the Declaration of Helsinki.

PCR and Direct Population Sanger Sequencing

Ultra-Deep Sequencing of the HBV Genome

Sequencing Data Analysis

Statistical Analysis

These processes are described in Supplementary Material.

ACCEPTED MANUSCRIPT

Results

Clinical Features and Outcomes of Reactivation from Occult HBV Infection after Immunosuppression

Baseline clinical and virological characteristics of 14 patients who developed HBV reactivation under immunosuppressive conditions are summarized in Table 1. All patients were originally HBsAg-negative but anti-HBc-positive before viral reactivation, and had no history of liver dysfunction. Pre-reactivation sera from five patients were available for further analysis, and confirmed that serum HBV DNA was undetectable in the repeated high-sensitivity PCR [10]. Among the 14 patients, 12 cases had hematological malignancy and received chemotherapy with steroids (n=12) and/or rituximab (n=7), and with (n=4) or without (n=8) hematopoietic stem cell transplantation (Table 1). One patient was diagnosed with psoriasis and had single-agent cyclosporine therapy for 4 years. Another patient had colon cancer and underwent surgery followed by S-1 (Tegafur/gimeracil/oteracil; Taiho Pharmaceutical Co., Tokyo, Japan) adjuvant chemotherapy.

The median time between initiation of chemotherapy or immunosuppressive therapy and diagnosis of HBV reactivation was 15.6 months (range: 1.0–57.7 months) (Table 1). Viral reactivation in seven of the 14 cases occurred 9.5 months (median; range: 6.4–39.8 months) after termination of chemotherapy or immunosuppressive therapy, while the remaining seven cases developed HBV reactivation during chemotherapy or immunosuppressive therapy. Median serum alanine aminotransferase (ALT) levels and HBV DNA levels at the time of HBV reactivation were

652 IU/mL [range: 15–2028] and 6.6 log copies/mL [range: 5.0–9.0], respectively (Table 2).

All patients except case #5 were treated with entecavir (ETV) (0.5 mg, once daily) immediately after diagnosis of HBV reactivation to suppress viral activity (Table 2). Representative clinical courses of patients with reactivation from occult HBV infection are shown in Fig. 1. Four of 14 patients (cases #2, #6, #9 and #11) got tested for HBV markers at 1-3 months intervals and started the ETV treatment after HBV DNA appearance (Table 2). The remaining ten patients were diagnosed with HBV reactivation when they had elevated levels of serum ALT and ETV was given in these cases (except case #5) after the appearance of liver dysfunction. After administering ETV, serum HBV DNA levels decreased in 11 cases (excluding cases #13 and #14), accompanied by reduced serum ALT levels. Nine (69.2%) of these cases showed loss of HBsAg with the appearance of anti-HBs at a median time of 2.9 months (range: 0.6–13.5 months) following the commencement of ETV treatment (Table 2). After confirming stable HBsAg/anti-HBs seroconversion, ETV was stopped in three of nine cases after 15.2 months (mean; range: 6.8–26.8 months). The four cases without HBsAg disappearance included two cases (#6 and #8) with follow-up of <3 months after ETV administration, and two cases (#13 and #14) that developed fatal ALF before complete disappearance of HBsAg. When the latter two were diagnosed with HBV reactivation, liver function had already deteriorated (serum total bilirubin (T-bil) was 8.0 mg/dL for #13 and 2.3 mg/dL for #14) and they died of liver failure 33 (#13) and 16 days (#14) after ETV administration.

Low Heterogeneity of the Reactivated Viruses in Patients with Reactivation from Occult HBV

Infection

To identify characteristics of viral clones related to HBV reactivation, we determined the entire virus genome sequence using ultra-deep sequencing. We first conducted a control experiment to validate the efficacy and errors in the sequencing platform. We determined two full-length plasmid-derived HBV sequences using expression plasmids encoding wild-type HBV as a template. Sequencing generated 1,229,416 and 2,205,237 filtered reads, corresponding to a mean coverage of 34,026 and 61,504 folds at each nucleotide site. The mean nucleotide mismatch error rate was 0.038% in Control #1 and 0.015% in Control #2, with the distribution of per-nucleotide error rate 0–0.24% and 0–0.16%, respectively; the mean overall error rate was 0.45% and 0.26%, respectively (Supplementary Table 1). This reflected the error introduced by sequencing. We defined the cut-off value in the current platform as 1% to exclude mismatch errors and to detect low-abundance mutations.

We then conducted ultra-deep sequencing on samples from the 14 patients with reactivation from occult HBV infection. A mean of 605,890 reads were mapped onto the reference sequences, and a mean coverage depth of 16,712 bp was achieved for each nucleotide site of HBV sequences (Table 3). The frequency of the overall mismatch mutations, which were nucleotides that did not match to the reference sequences, was 0.015% (15/100,000).

To define the characteristics of the reactivated HBV clones, we compared these clones with

those derived from reactivated viruses in six cases originally positive for HBsAg who developed viral exacerbation triggered by immunosuppressive therapy. There were no significant differences in the maximum levels of elevated serum ALT and HBV DNA during viral exacerbation between the both groups (Table 2). A mean of 630,253 reads for HBV sequences derived from patients with reactivation from HBsAg carriers were mapped onto reference sequences (Table 3). The overall mismatch mutation frequency of total viral genomic sequences was 0.11% (114/100,000), suggesting that viral heterogeneity was significantly lower in the reactivated viruses from occult HBV infection (0.015%) compared with HBsAg carriers ($P < 0.05$) (Fig. 2A-C and Table 3). Viral heterogeneity was also evaluated by calculating Shannon entropy values. The mean overall value of Shannon entropy was 0.00085 (range: 0–0.0022) in patients with reactivation from occult HBV infection, and 0.0051 (range: 0.0006–0.017) in patients with reactivation from HBsAg carriers, indicating that genetic complexity was significantly lower in the reactivated viruses from occult HBV carrier status ($P < 0.05$) (Fig. 2D). These findings suggest that the heterogeneity of reactivated HBV was substantially smaller in originally HBsAg-negative cases than in HBsAg-positive carriers. The levels of heterogeneity were not significantly different between the viral genomic regions, and no significant increase in the population of immune escape variants in both the patients with reactivation from occult HBV and HBsAg carrier status (Fig. 2A, B and Supplementary Fig. 1).

Reactivated Viruses in Each Individual Consisted Almost Exclusively of the Wild-type G1896 or G1896A Variant

The G1896A mutation in the pre-C region is associated with ALF, and is one of the most commonly shared features in patients with HBV reactivation and ALF [16-19]. We found that six of 14 patients, including two fatal ALF cases, had predominant reactivation of variant G1896A pre-C clones. Serologically, all cases with the dominant G1896A pre-C variant were negative for HBeAg and positive for anti-HBe at the time of HBV reactivation (Table 2 and 4). Almost all the reactivated viral clones in the G1896A-dominant cases were G1896A pre-C variant clones (99.4–100%). Very few clones with the wild-type G1896 sequence were detectable by ultra-deep sequencing at the time of HBV reactivation (Table 4). Ultra-deep sequencing also confirmed that patients with reactivation of the wild-type G1896-dominant HBV clones had few or no G1896A pre-C variants in their serum (0–0.9%). These findings indicate that either wild-type G1896 or G1896A pre-C variants were exclusively reactivated in patients with reactivation from occult HBV infection following immunosuppression. We also examined whether the G1896A pre-C variant or the level of heterogeneity was associated with the clinical course. We found no significant association between the ratio of the wild-type/G1896A pre-C mutant or the heterogeneity (represented by the Shannon entropy value) and the levels of peak ALT and peak T-bil (Supplementary Fig. 2). The predominance of A1762T and G1764A variants in the core-promoter region, which are known to be associated with ALF [18, 25], was observed in only two cases (#9 and #11), and was not associated with the two

fatal ALF cases (Table 4).

To clarify the genomic similarity between the viral clones in the liver tissue before reactivation and those in the serum after reactivation, we determined the sequences of HBV genomes in liver tissue before the onset of HBV reactivation in a patient (case #3). The patient was initially negative for HBsAg but positive for anti-HBc, and had colon cancer and liver metastasis. He underwent partial hepatectomy, followed by adjuvant chemotherapy. During cancer treatment, he became seropositive for HBV DNA and HBsAg (Fig. 1). We compared the HBV genome sequences derived from the liver before viral breakthrough (obtained at the time of hepatectomy) with those from his serum at the time of viral reactivation during chemotherapy. We found that 97.9% of the HBV nucleotides derived from his serum at reactivation were identical to those from the liver tissues before viral reactivation. The prevalence of the wild-type G1896 strain was 99.95% in liver prior to reactivation, and 99.94% in serum after reactivation. These results possibly indicate that the viral population in the serum of a patient with reactivation from occult HBV infection was similar to that in the liver tissue during latent infection before viral breakthrough.

Based on those findings, we determined the prevalence of the G1896A variant in the liver of occult HBV carriers that did not experience immunosuppression. We examined the liver tissues of HBsAg-negative but anti-HBc-positive healthy donors used for living-donor liver transplantation. The HBV genome was detectable by PCR in the livers of most (44/45) of the healthy donors that lacked circulating HBV DNA. Ultra-deep sequencing determined viral genome sequences with a

mean 20,503-fold coverage at each nucleotide site for each liver specimen. Sequencing revealed that the viral clones comprised almost exclusively of the wild-type G1896 or G1896A pre-C variant in the livers of occult HBV carriers. Around 11.4% (5/44) of cases had a dominant population of the G1896A pre-C variant, with a frequency of >99.9% for total viral clones (Fig. 3). Approximately 88.6% (39/44) of cases predominantly contained the wild-type G1896 strain, with 38/39 cases (liver #6 was the exception) exhibiting a frequency of >99.9% of total viral clones (Fig. 3). The genetic complexity of viruses in the liver of healthy occult HBV carriers was 0.00080 (mean; range: 0–0.0011), expressed as a Shannon entropy value, and was comparable to that in the serum of patients with reactivation from occult HBV infection (mean: 0.00085; range: 0–0.0022). These findings indicate that occult HBV carriers serologically characterized as HBsAg-negative and anti-HBc-positive are latently infected with HBV clones of low heterogeneity in their livers, and predominantly comprise the wild-type G1896 or G1896A pre-C variants.

Discussion

HBsAg positivity indicates the carrier status of HBV infection and thus reactivation of HBV-related hepatitis can occur in patients carrying HBsAg under certain immunosuppressive conditions [1-4]. Accumulated evidence indicates that HBV infection persists in the liver tissues of individuals tested negative for HBsAg but positive for anti-HBc, and these occult HBV carriers can also develop HBV reactivation and liver dysfunction under certain immunosuppressive conditions [5, 6, 20]. In the present study, we demonstrated the clinical and virological features of patients who experienced viral reactivation under immunosuppressive conditions.

Previous studies demonstrated that immunosuppression in occult HBV carriers with hematological malignancies was at an especially high risk of HBV reactivation [6]. The high risk of viral reactivation in patients with hematological malignancies receiving chemotherapy might be attributable to immunodeficiency caused by underlying primary diseases and strong immunosuppressive therapy. In addition to the patients with hematological malignancy, we observed two patients without hematological malignancies who developed HBV reactivation. One case had colon cancer, with S-1 treatment triggering HBV exacerbation. Another case had psoriasis and received cyclosporine before the onset of HBV reactivation. Previously, we also reported a case of lethal *de novo* HBV hepatitis induced by adalimumab treatment for rheumatoid arthritis [26]. Thus, it is important to note that there is a risk of HBV reactivation in patients not only with hematological malignancies but also with solid tumors or noncancerous diseases undergoing chemotherapy or

immunosuppressive therapy. In addition, it is very important to regularly monitor HBV DNA levels to achieve the early administration of ETV before the onset of ALT elevation, however, the optimum frequency of HBV DNA testing in occult HBV carriers is not yet defined. Recent prospective study suggested that monthly monitoring of HBV DNA levels for lymphoma patients with resolved HBV infection might be a reasonable option during and after rituximab-CHOP chemotherapy [27].

To clarify the virological characteristics of HBV reactivation, we determined the genetic heterogeneity of viruses from patient sera. We found that the genetic complexity of the reactivated viruses in 14 patients with reactivation from occult HBV infection was significantly lower than that in six patients with reactivation from HBsAg carriers. There was no significant difference in circulating HBV DNA levels in serum after reactivation in both groups. The viral population in the sera of patients with reactivation from occult HBV infection was characterized by low heterogeneity, with nearly monoclonal viruses detected. We further examined the genetic complexity of latently infected HBV in the liver of 44 individuals with occult HBV infection. We found that the genetic heterogeneity of latently infected viruses in their livers was also very low. In one case we confirmed that the viral genome detected in serum after viral reactivation was almost identical to that in the latently infected liver before reactivation. These findings possibly suggest that the viral population in latently infected livers of occult HBV carriers is characterized by low heterogeneity, and the predominant viral clone increases in number under immunosuppressive conditions. The reason for the difference in the degree of genetic heterogeneity in the exacerbated viruses between patients with

reactivation from occult infection and those with HBsAg carrier reactivation is unclear. One possibility is that the low levels of viral heterogeneity observed in occult HBV carriers are due to the relatively lower levels of viral replication compared with those of HBsAg carriers. Pollicino et al demonstrated that the host immune system, not viral factors, likely plays a critical role in the strong suppression of viral replication and gene expression [28]. Since we could confirm the genetic homology of HBV DNA in the liver before reactivation and the serum after reactivation in only one case, further studies are required to determine the characteristics of the latent viruses in HBsAg-negative but anti-HBc-positive occult HBV carriers.

In this study, we found that 42.9% of cases that experienced HBV reactivation predominantly contained the G1896A pre-C variant in their sera. Infection with the G1896A variant was predominant in the liver of 11.4% of individuals with occult HBV infection. Patients acutely infected with the HBV G1896A pre-C variant have a high risk of developing ALF [16-18]. The G1896A variant is frequently detected in reactivated viruses in patients with reactivation from occult HBV infection that develop ALF [20]. We revealed that both patients who developed fatal ALF predominantly contained G1896A pre-C variants. The mechanism by which the G1896A mutation triggers the development of ALF remains unknown at present. Previous studies reported that the G1896A variant has increased replication activity compared with the wild-type strain in vitro [18, 29], but we found no significant association between the levels of circulating HBV DNA and the ratios of wild-type/G1896A pre-C mutants in cases with reactivation from occult HBV infection. On

the other hand, it is well recognized that HBeAg/anti-HBe serostatus is closely associated with the ratios of wild-type/G1896A pre-C mutants in patients with chronic HBV infection [30]. Interestingly, accumulating evidence suggests that G1896A mutations abrogating HBeAg synthesis remove the tolerogenic effect of HBeAg, leading to an enhanced immune response that contributes to ALF development [31]. We must also pay attention to the genotype of HBV in cases with viral reactivation. Among the 14 cases with reactivation from occult HBV infection, genotype B and C strains were detected in five and nine patients, respectively. Among them, three of five cases were negative for HBeAg but positive for anti-HBe (60%) in genotype B and three of nine (33.3%) were genotype C-infected patients, and both cases with developing ALF were negative for HBeAg and infected with genotype B. Previous studies demonstrated that HBV genotypes affect the liver disease outcome [32], and genotype B strain is frequently detected in patients developing ALF [18]. Thus, it is possible that the ratios of wild-type/G1896A pre-C mutants and viral genotype influence the pathophysiology of viral reactivation.

In conclusion, our findings suggest that HBV reactivation can occur during and after termination of chemotherapy or immunosuppressive therapy in occult HBV carriers with underlying hematological malignancies, solid tumors or noncancerous diseases. Occult HBV infection and the resulting HBV reactivation is characterized by low genetic heterogeneity. It is unclear whether occult HBV carriers with the G1896A pre-C variant have an increased risk of developing HBV reactivation and fatal ALF. Further analysis with a larger cohort of patients is required to clarify the frequency