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

and mechanisms of HBV reactivation and ALF in patients with occult HBV carrier status receiving chemotherapy or immunosuppressive therapy.

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Figure legends

Fig. 1. Representative clinical courses of patients with reactivation from occult HBV infection.

Serial serum ALT (solid lines), HBV DNA (dashed lines) and HBV serology of four cases that developed HBV reactivation after (cases #1) or during (cases #3, #11 and #14) chemotherapy or immunosuppressive therapy. All cases were treated with entecavir (ETV) immediately after diagnosis of HBV reactivation. BMT, bone marrow transplantation; FK506, tacrolimus; MEL, melphalan; Op, operation; PSL, prednisolone; R-CHOP, rituximab, cyclophosphamide, doxorubicin, vincristine, prednisolone.

Fig. 2. Comparison of viral genetic heterogeneity in patients with reactivation from occult HBV and HBsAg carrier status.

Comparison of viral genetic heterogeneity expressed as the Shannon entropy value among representative patients with reactivation from occult HBV infection (A) and reactivation from HBsAg carriers (B). The total number of different nucleotides from the representative HBV reference sequences (mismatch bases) (C), and the mean Shannon entropy values (D) in both groups. preC-C, pre-core–core; preS, pre-surface; P, polymerase; S, surface.

Fig. 3. Prevalence of G1896A pre-core mutants in the liver of 44 healthy occult HBV carriers.

The ratio of G1896A mutants (red) to wild-type G1896 (yellow) for total reads is shown in the left panel. The number of G1896A mutants, total reads at nucleotide position 1896, and the proportion of G1896A mutants (%) are shown in the right panel.

Fig. 1. Inuzuka et al.

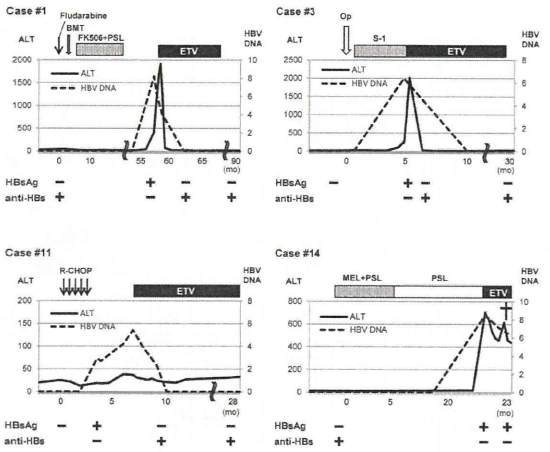

Fig. 2. Inuzuka et al.