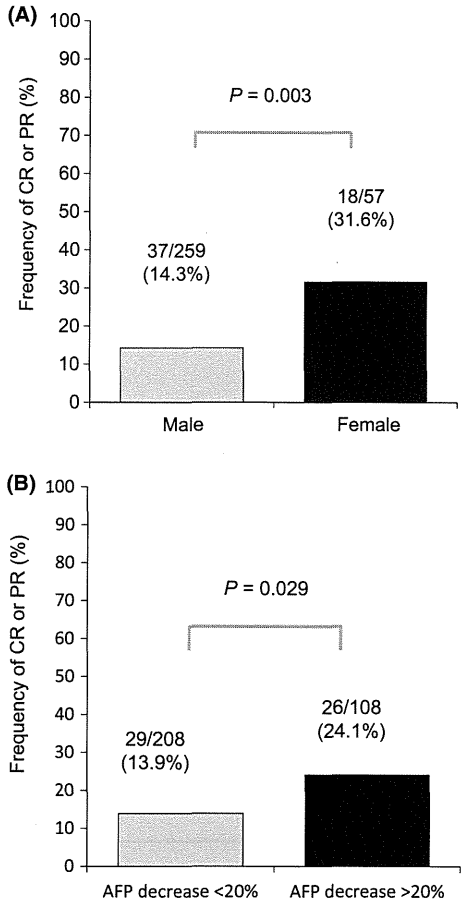

Fig. 3. Objective response rate (ORR) according to (A) gender and (B) presence or absence of a >20% decrease in the serum alpha-foetoprotein (AFP) level. The ORR was significantly higher in females than in males ($P = 0.003$) and in patients with a >20% decrease in the serum AFP level than in those without ($P = 0.029$). CR, complete response; PR, partial response.

significantly associated with high ORR in multivariate analysis ($P = 0.013$, OR = 3.785, 95% CI: 1.270–11.281). In patients with BCLC-C patients, ORR and DCR were 17% (32/193) and 54% (105/193), and female tended to achieve PR ($P = 0.066$, OR = 2.431, 95% CI: 0.944–6.260). DCR in both subgroups are a little higher than the results of SHARP trial (16).

As almost all patients included in SHARP trial had liver function of Child-Pugh A, we performed subgroup analysis concerning clinical outcome of Child-Pugh A patients. Their ORR was 18% (49/271). The result of DCR [62% (168/271)] was not so far from that of SHARP trial (4, 16). In this subgroup, only female gender also correlated with responders in multivariate analysis ($P = 0.002$, OR = 3.286, 95% CI: 1.550–6.970). Because of relatively small number of Child-Pugh B patients, we could not perform multivariate analysis in them. Their ORR [13% (6/45)] and DCR [51% (23/45)] were lower than those in Child-Pugh A patients.

Discussion

For clinicians, it is of great importance to identify factors that predict the tumour response to sorafenib therapy in patients with unresectable HCC. In this study of the factors predicting a radiological response to sorafenib therapy, multivariate analysis identified a >20% decrease in the serum AFP level and female gender as independent predictors of a higher ORR. A decrease in the serum AFP level after sorafenib therapy has previously been reported to be associated with a radiological response (19, 30). Indeed, AFP decrease is not a pre-treatment predictive marker. Some patients, however, achieve CR or PR several months after sorafenib initiation, and AFP decrease in the first month was associated with the best radiological response throughout the treatment with sorafenib in this study. Thus, in the early stage of sorafenib therapy, we can predict the best radiological response. This seems to be critical issue for the management of HCC patients treated with sorafenib. Although the usefulness of the serum AFP level for assessing the response to treatment for HCC remains controversial, our results are consistent with previously reported findings (30).

In this study, females had a significantly higher ORR than males. Although the reason for this result is unclear, several previous studies also reported an association between female gender and a good response to MTT, such as gefitinib therapy for lung cancer (30, 31). In lung cancer, clinicopathological factors such as female gender, non-smoking, Asian ethnicity and adenocarcinoma were reported to be associated with a good response to gefitinib therapy (30). Genomic analysis also showed that epidermal growth factor receptor (EGFR) mutation was associated with the response to gefitinib therapy (31). The latest National Comprehensive Cancer Network guidelines for unresectable lung cancer recommend using gefitinib monotherapy only in patients with EGFR mutation, indicating that EGFR mutation is considered to be a useful biomarker. This is one of the very precious topics in considering the predictive factors to MTT including sorafenib.

Several possible reasons for the higher ORR in females in this study should be considered. As females generally have a lower body weight, they may receive a higher drug dose per kilogram than males. However, the females in this study were significantly older and had lower body weight and worse ECOG-PS than males, resulting in frequent reduction in the initial dose of sorafenib. The initial dose of sorafenib per kilogram was significantly lower in females than in males (Table 4). Potential differences in compliance should be considered. Females are more sensitive to hand-foot syndrome and skin rashes, and may be more likely to perform watchful skin care, resulting in a lower risk of discontinuation of sorafenib therapy because of adverse events. However, the rate of hand-foot syndrome was not significantly different between males and females in this

Table 3. Factors predicting a good response to sorafenib therapy

Variable	Number of patients	Number of responders	Univariate *P*	Multivariate analysis		
				Odds ratio	95% CI	*P**
Age (years), >70/<70	160/156	23/32	0.182	1.746	0.882–3.459	0.110
Gender, female/male	57/259	18/37	0.003	2.876	1.350–6.123	0.001
HCV infection, yes/no	180/136	32/23	0.882			
HBV infection, yes/no	52/264	9/46	1.000			
ECOG-PS 0, yes/no	251/65	42/13	0.663			
BCLC stage, A or B/C or D	123/193	23/32	0.650			
Tumour invasion of the portal vein, present/absent	69/247	9/46	0.369			
Tumour occupation rate in the liver, >50/<50 (%)	31/285	3/52	0.320			
Lung metastasis, present/absent	64/252	12/43	0.716			
Bone metastasis, present/absent	39/277	7/48	1.000			
Ascites, present/absent	26/290	2/53	0.277			
Hepatic resection prior to sorafenib, yes/no	65/251	10/45	0.716			
TACE prior to sorafenib, yes/no	276/40	47/8	0.656			
Pretreatment AST, >50/<50 (IU/L)	168/148	26/29	0.374			
Pretreatment ALT, >50/<50 (IU/L)	141/175	23/32	0.658			
Pretreatment total bilirubin, >1.0/<1.0 (mg/dL)	91/225	10/45	0.071	1.448	0.602–3.487	0.409
Pretreatment albumin, >3.5/<3.5 (g/dl)	164/152	32/23	0.373			
Pretreatment ALP, >350/<350 (IU/L)	154/162	25/30	0.657			
Pretreatment LDH, >240/<240 (IU/L)	127/173	16/38	0.047	1.627	0.773–3.425	0.200
Pretreatment γGTP, >80/<80 (IU/L)	157/155	21/34	0.054	1.068	0.534–2.136	0.852
Pretreatment cholinesterase, >150/<150 (IU/L)	142/143	21/31	0.167	1.526	0.760–3.064	0.235
Pretreatment platelet, >10/<10 (10^4/μl)	107/209	16/39	0.438			
Pretreatment haemoglobin, >12/<12 (g/dl)	209/107	33/22	0.347			
Pretreatment serum AFP, >1000/<1000 (ng/ml)	100/216	18/37	0.874			
Pretreatment serum DCP, >500/<500 (mAU/ml)	161/155	20/35	0.018	1.526	0.781–2.982	0.216
AFP decrease >20%, yes/no	108/208	26/29	0.029	1.982	1.026–3.829	0.042
Initial dose of sorafenib, 800 mg/reduced dose	130/186	23/32	1.000			

*Multivariate logistic regression analysis.

AFP, alpha-foetoprotein; ALT, alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; BCLC, Barcelona Clinic Liver Cancer; DCP, des-gamma-carboxy prothrombin; ECOG-PS, Eastern Cooperative Oncology Group performance status; γGTP, gamma-glutamyl transpeptidase; LDH, lactate dehydrogenase; TACE, transcatheter arterial chemoembolization.

study, and the median duration of sorafenib therapy was almost the same in males and females (Table 4). Another possibility is that the response is associated with genomic alterations such as mutation or amplification, similar to the association between EGFR mutation and Asian female lung cancer patients. Although no biomarkers for predicting therapeutic effectiveness have been established, some genomic changes have recently been reported to be associated with a favourable therapeutic response to sorafenib (23), and new biomarkers are expected to be detected in the near future. In addition, it remains unclear whether sorafenib may have a relevant interaction with a female hormone such as oestrogen. This possibility should be further investigated.

Although the degree of tumour differentiation was not associated with ORR, the DCR was significantly higher in patients with well-differentiated HCC than in other patients. These results may reflect the generally less aggressive nature of more differentiated tumours. Because of the small sample size, definitive conclusions regarding the association between tumour differentiation and the response to sorafenib therapy cannot be reached. Further investigation is needed to confirm these results.

Comparison of our results with those of previous studies, (4–7) shows that the ORR was relatively high in this study. This may be because we assessed the radiological response using mRECIST rather than the conventional RECIST, or because we excluded patients in whom the tumour response could not be assessed because of early discontinuation of sorafenib therapy or an extremely poor prognosis. However, patients who received a reduced initial dose of sorafenib achieved treatment efficacy comparable with those who received an initial dose of 800 mg/day. It is also notable that all four patients who achieved a CR received a reduced initial dose of sorafenib. Although analysis of the impact of a reduced initial dose of sorafenib on the response to treatment was beyond the scope of this study, our results suggest that a reduced initial dose can be considered in some patients.

In the four patients who have achieved a CR, the time to normalization of the serum AFP level was 2–4 months. Although the target lesions initially enlarged in Cases 1 and 4, continued administration of sorafenib eventually resulted in a CR (11). As sorafenib is a molecular agent targeting vascular endothelial growth factor

Table 4. Clinical features according to gender

Variable		Males (n = 259)	Females (n = 57)	P
Age (years)	Mean ± SD	68.9 ± 10.2	72.9 ± 9.1	0.007*
Body weight (kg)	Mean ± SD	61.5 ± 11.6	50.4 ± 11.4	<0.001*
HCV infection	Yes/No	138/121	42/15	0.005†
HBV infection	Yes/No	47/212	5/52	0.113†
BCLC stage	A or B/C or D	158/101	35/22	1.000†
ECOG-PS	0/1 or 2	211/48	40/17	0.070†
Child-Pugh classification	A/B/C	221/36/2	50/7/0	0.739†
Pretreatment serum albumin (g/dl)	Mean ± SD	3.54 ± 0.50	3.58 ± 0.43	0.519*
Pretreatment serum cholinesterase (IU/L)	Mean ± SD	168.3 ± 73.7	161.1 ± 63.4	0.516*
Pretreatment serum AST (IU/L)	Mean ± SD	64.4 ± 55.7	65.0 ± 36.1	0.948*
Pretreatment serum ALT (IU/L)	Mean ± SD	47.7 ± 37.7	46.0 ± 30.8	0.748*
Pretreatment AFP (ng/ml)	Median (range)	193.4 (1.9–30 300)	139.5 (1.9–270 300)	0.267*
Pretreatment DCP (mAU/ml)	Median (range)	637 (9–847 000)	280 (10–123 000)	0.232*
Previous therapies for HCC				
TACE	Yes/No	223/36	53/4	0.191†
RFA or PEI	Yes/No	130/129	35/22	0.144†
Surgery	Yes/No	52/207	13/44	0.717†
Initial dose of sorafenib (mg)	Mean ± SD	568.3 ± 205.5	515.8 ± 203.4	0.081*
Initial dose per kg (mg/kg)	Mean ± SD	8.55 ± 3.74	7.23 ± 3.05	0.013*
HFS during sorafenib therapy	Yes/No	144/115	33/24	0.770†

*Unpaired t test.

†Fisher's exact test.

AFP, alpha-foetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BCLC, Barcelona Clinic Liver Cancer; DCP, des-gamma-carboxy prothrombin; ECOG-PS, Eastern Cooperative Oncology Group performance status; HBV, hepatitis B virus; HCV, hepatitis C virus; HFS, hand-foot syndrome; SD, standard deviation.

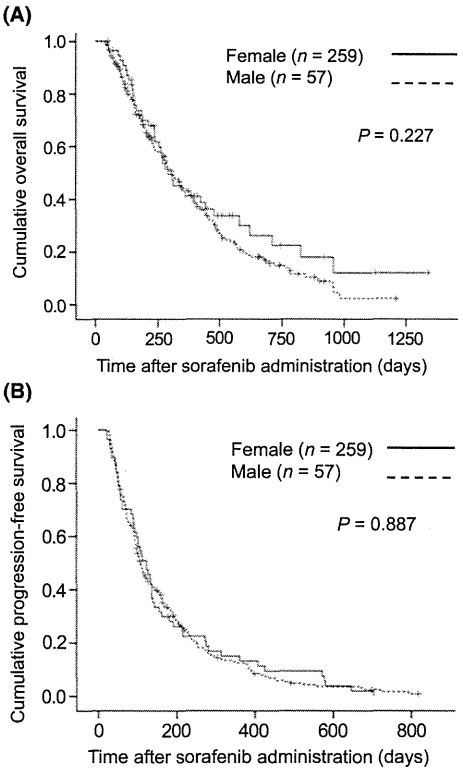

Fig. 4. Kaplan–Meier curves for (A) OS and (B) PFS according to gender. The median OS time was 307 days in males and 292 days in females (P = 0.227). The median PFS time was 107 days in males and 123 days in females (P = 0.887).

receptor, the initial clinical course may differ from the course after administration of conventional cytotoxic chemotherapeutic agents.

The present study has several limitations. First the design is retrospective. Second, the initial dose of sorafenib was variable, which could have led to bias. Third, more than 100 patients with insufficient available data were excluded to enable more precise assessment of the tumour response to sorafenib (Fig. 1), which could also have led to bias. Fourth, various treatments were administered after discontinuation of sorafenib therapy, potentially leading to bias in the analysis of OS. Finally, our study cohort was limited to Japanese patients, who have a relatively low body weight compared with patients in Western countries. Our results therefore cannot be extended to other racial cohorts. Despite these limitations, this multicenter study analysed data from a large number of patients, and the background characteristics of included patients did not differ remarkably compared with previously reported studies. Our findings may reflect the response to sorafenib therapy in patients with unresectable HCC in Japan.

In conclusion, this large multicenter study found that a >20% decrease in the serum AFP level was an independent predictor of a favourable radiological response to sorafenib therapy for unresectable HCC. Our results also suggest that gender differences may be associated with the radiological response to MTT, as reported in patients with lung cancer. If clinicopathological biomarkers that predict the response to treatment can be

identified, sorafenib administration can be limited to patients with a potentially favourable response, leading to personalized treatment plans for unresectable HCC.

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Research Article

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Reactivation from occult HBV carrier status is characterized by low genetic heterogeneity with the wild-type or G1896A variant prevalence

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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.

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Abbreviations: ALF, acute liver failure; ALT, alanine aminotransferase; anti-HBc, antibodies to hepatitis B core antigen; ETV, entecavir; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; PCR, polymerase chain reaction; pre-C, pre-core; T-bil, total bilirubin.

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 tissue was 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.

Sequencing

PCR and direct population Sanger sequencing, ultra-deep sequencing of the HBV genome, sequencing data analysis, and statistical analysis are described in the Supplementary materials and methods.

Data deposition

Sequence reads with Genome Analyzer were deposited in the DNA Data Bank of Japan Sequence Read Archive (http://trace.ddbj.nig.ac.jp/dra/index_e.shtml) under accession number DRA001211.

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

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Table 1. Clinical characteristics of patients with reactivation from occult HBV and HBsAg carrier status BEFORE viral exacerbation.

Case	Age/ sex	Anti-HBs	Primary disease	Treatment	Use of steroids	HSCT	Period between HBV reactivation and start of treatment (months)	Period between HBV reactivation and end of treatment (months)
Reactivation from occult HBV carrier status								
#1	48M	+	ML	Fludarabine	+	+	57.7	39.8
#2	25M	-	AML	IDA + AraC	+	+	27.0	19.2
#3	59M	Unknown	Colon cancer	S-1	-	-	3.6	During treatment
#4	61M	Unknown	ML	R-CHASE	+	+	13.8	9.5
#5	64M	-	MM	MP→CAD	+	+	13.6	6.4
#6	72M	-	ML	MTX + AraC →Rituximab	+	-	10.9	During treatment
#7	78M	Unknown	ML	R-CVP	+	-	34.7	34.2
#8	66M	Unknown	MM	MP	+	-	49.1	6.6
#9	61F	-	ML	R-FND	+	-	1.0	During treatment
#10	66M	Unknown	Psoriasis	Cyclosporine	-	-	37.8	During treatment
#11	79F	Unknown	ML	R-CHOP	+	-	3.7	During treatment
#12	81F	-	ML	R-CVP	+	-	11.2	7.6
#13	84F	Unknown	ML	R-CHOP	+	-	17.4	During treatment
#14	87F	+	MM	MP	+	-	23.1	During treatment
							median: 15.6	**median: 9.5**
Reactivation from HBsAg carrier status								
#15	32F	-	Sjögren synd.	PSL	+	-	15.1	During treatment
#16	63F	-	Raynaud's dis.	PSL	+	-	20.4	During treatment
#17	42F	-	Aortitis synd.	PSL	+	-	122.2	During treatment
#18	59M	-	Lung cancer	Chemotherapy[a]	+	-	17.9	During treatment
#19	54M	-	RA	MTX + PSL	+	-	11.5	During treatment
#20	72M	-	RA	Bucillamine	-	-	6.7	During treatment
							median: 16.5	

[a]Carboplatin, paclitaxel → docetaxel → gemcitabine, vinorelbine → cisplatin, irinotecan.
AML, acute myeloid leukemia; AraC, cytarabine; dis, disease; CAD, cyclophosphamide, doxorubicin, dexamethasone; F, female; HBsAg, hepatitis B surface antigen; HSCT, hematopoietic stem cell transplantation; IDA, idarubicin; M, male; ML, malignant lymphoma; MM, multiple myeloma; MP, melphalan, prednisolone; MTX, methotrexate; PSL, prednisolone; RA, rheumatoid arthritis; R-CHASE, rituximab, cyclophosphamide, cytosine arabinoside, etoposide, dexamethasone; R-CHOP, rituximab, cyclophosphamide, doxorubicin, vincristine, prednisolone; R-CVP, rituximab, cyclophosphamide, doxorubicin, prednisolone; synd, syndrome; R-FND, rituximab, fludarabine, mitoxantrone, dexamethasone.

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 fold 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

Table 2. Clinical courses of patients with reactivation from occult HBV and HBsAg carrier status AFTER viral exacerbation.

Case	At diagnosis of HBV reactivation				ETV treatment*	Period to HBsAg disappearance** (months)
	HBV genotype	HBeAg/ anti-HBe	HBV DNA level (log_{10} copies/ml)	ALT[a] level (IU/ml)		
Reactivation from occult HBV carrier status						
#1	C	+/-	8.2	1915	+	13.3
#2	C	+/-	6.2	24	+	2.8
#3	C	+/-	6.4	2019	+	0.6
#4	C	+/-	8.3	720	+	3.1
#5	C	+/-	5.4	681	n.t.	-
#6	C	+/-	8.4	15	+	-
#7	B	+/-	7.7	1983	+	2.9
#8	B	+/-	6.2	97	+	-
#9	C	-/+	5.0	18	+	1.7
#10	C	-/+	6.6	2028	+	0.9
#11	C	-/+	5.4	38	+	13.5
#12	B	-/+	9.0	503	+	10.5
#13	B	-/+	6.5	623	+	-
#14	B	-/+	8.5	705	+	-
			median: 6.6	**median: 652**		**median: 2.9**
Reactivation from HBsAg carrier status						
#15	C	+/-	8.8	499	+	-
#16	C	+/-	7.1	1740	+	-
#17	C	-/+	7.8	628	+	-
#18	C	-/+	5.5	1674	+	-
#19	B	-/+	5.8	619	+	-
#20	C	-/+	8.8	813	+	0.4
			median: 7.5	**median: 716**		

ALT, alanine aminotransferase; anti-HBe, antibodies to hepatitis B e antigen; ETV, entecavir; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; n.t., not treated.

*All patients except case #5 were treated with ETV immediately after diagnosis of HBV reactivation to suppress viral activity.

**Period (months) between ETV administration and HBsAg disappearance a normal range 10–42 IU/L.

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 and 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 (Tables 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

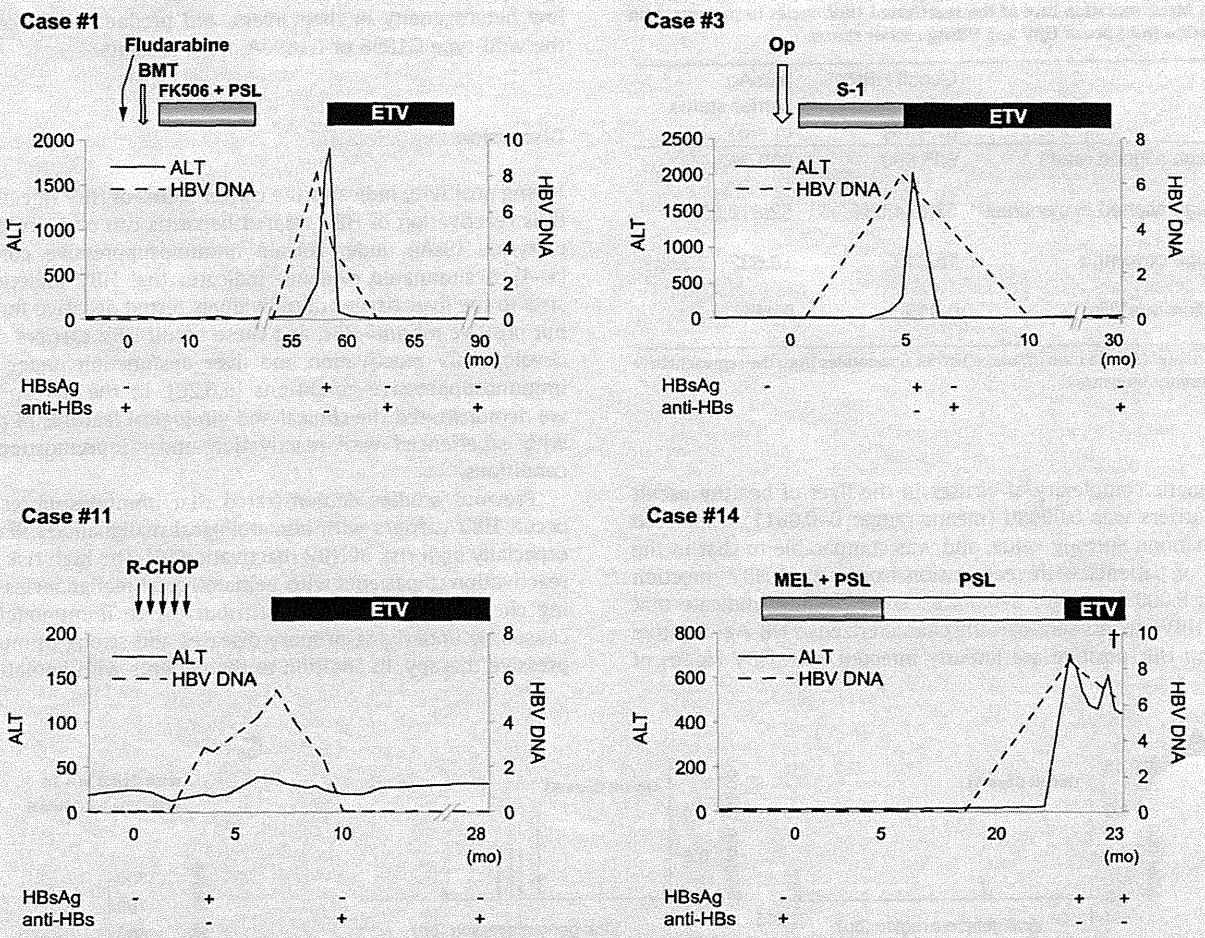

Fig. 1. Representative clinical courses of patients with reactivation from occult HBV infection. Serial serum ALT, HBV DNA and HBV serology of four cases that developed HBV reactivation after (cases #1) or during (cases #3, #11 and #14) chemotherapy or immunosuppressive therapy. All cases were treated 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.

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).

Table 3. Mean mutation rate of the reactivated HBV clones in patients with reactivation from occult HBV and HBsAg carrier status.

	Occult HBV carrier status (n = 14)	HBsAg carrier status (n = 6)
Average aligned reads	605,890	630,253
Average aligned nucleotides	52,814,651	52,812,297
Average coverage	16,712	16,632
Mutation rate* (%)	0.015	0.114

Mutation rate* (%): the ratio of total different nucleotides from the representative HBV reference sequences.

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

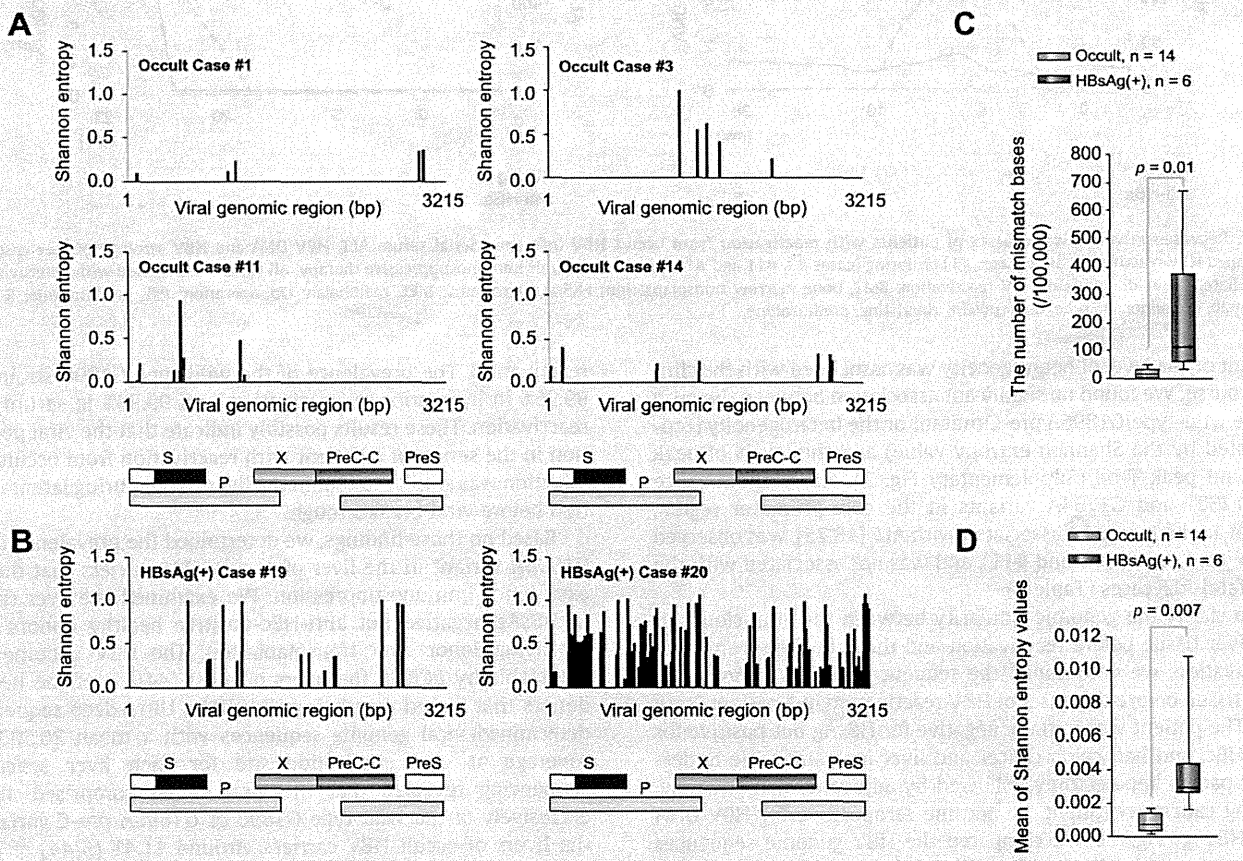

Fig. 2. Comparison of viral genetic heterogeneity in patients with reactivation from occult HBV and HBsAg carrier status. Comparison of viral genetic heterogeneity expressed as the Shannon entropy value among representative patients with reactivation from occult HBV infection (A) and reactivation from 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.