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
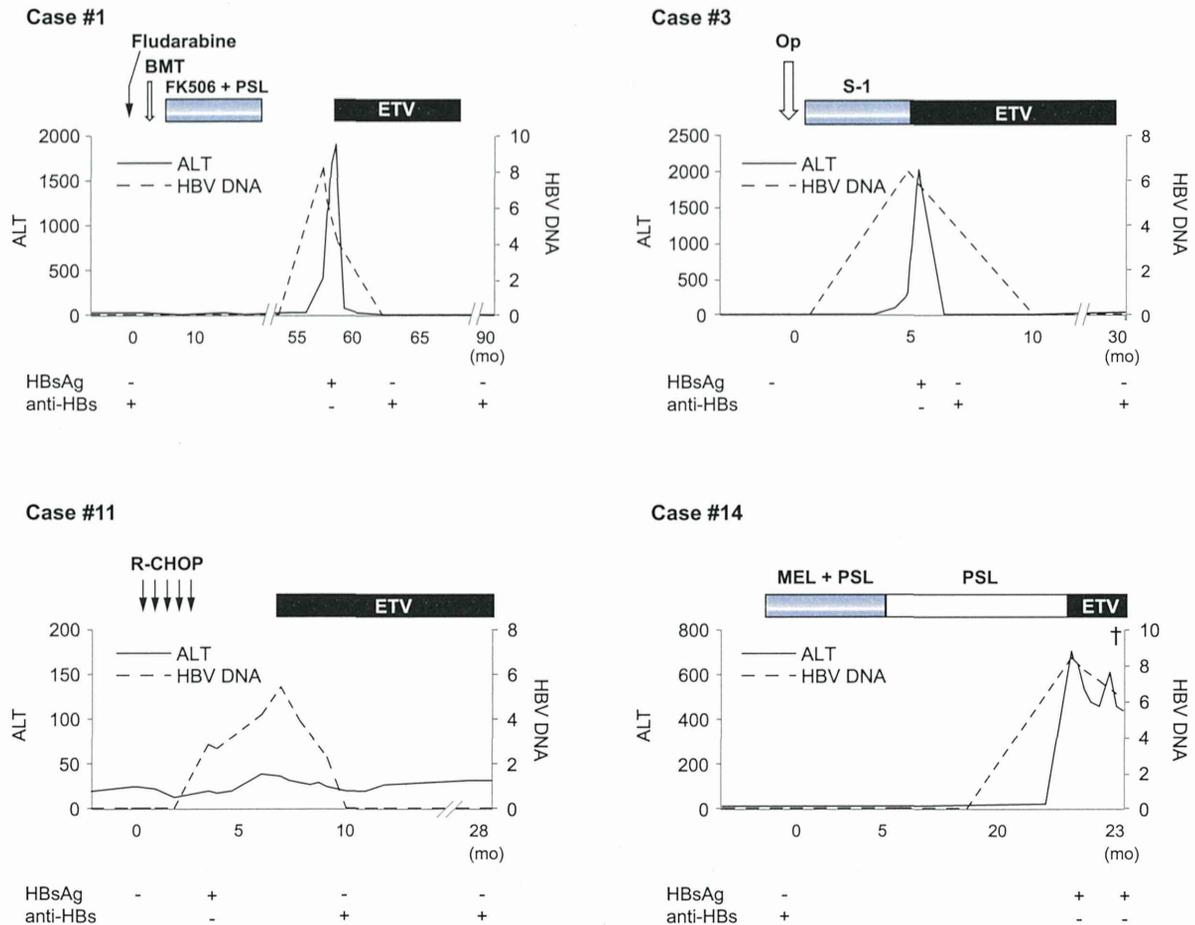
Research Article

Case #1

Fludarabine
BMT
FK506 + PSL
ETV

HBsAg	-		+	-	-
anti-HBs	+		-	+	+

Case #3

Op
S-1
ETV

HBsAg	-	+	-	-
anti-HBs		-	+	+

Case #11

R-CHOP
ETV

HBsAg	-	+	-	-
anti-HBs		-	+	+

Case #14

MEL + PSL   PSL
ETV

HBsAg	-	+	+
anti-HBs	+	-	-

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).

496

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
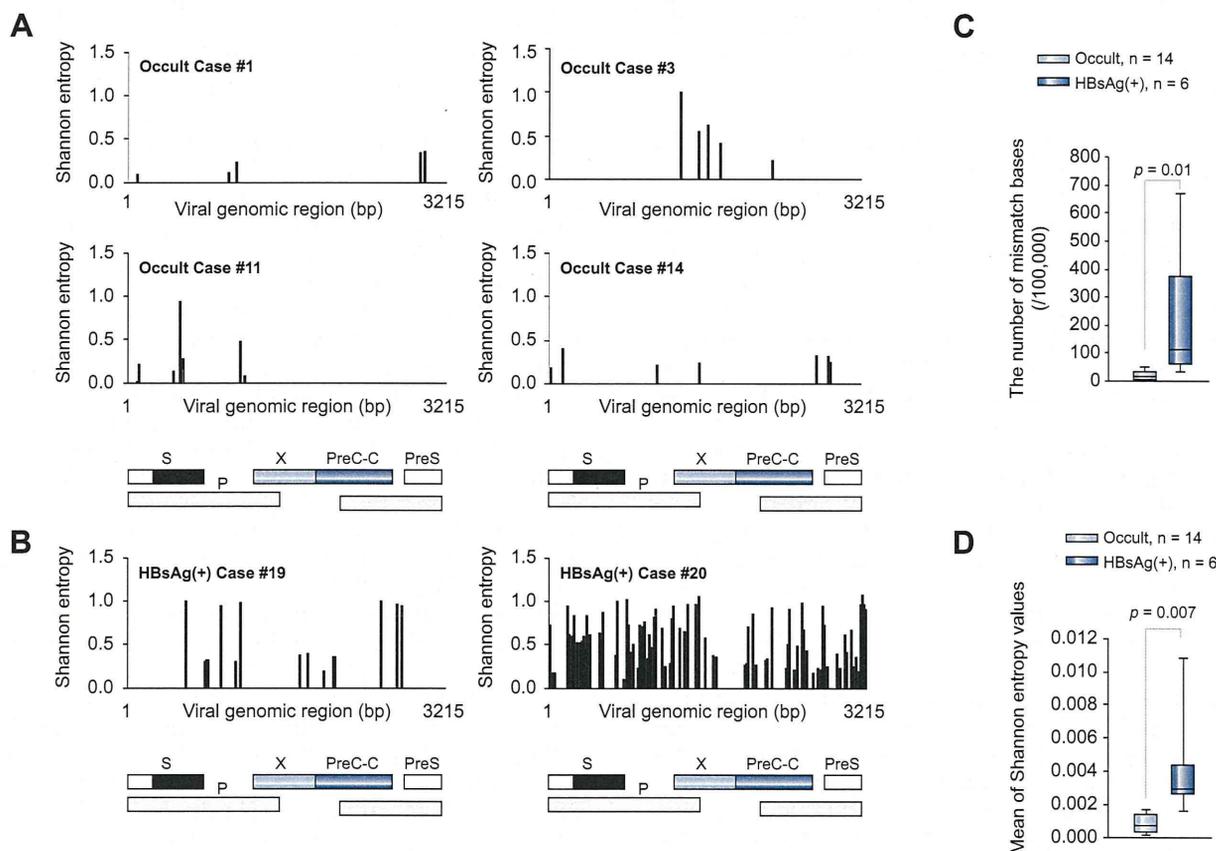

Fig. 2. Comparison of viral genetic heterogeneity in patients with reactivation from occult HBV and HBsAg carrier status. Comparison of viral genetic heterogeneity expressed as the Shannon entropy value among representative patients with reactivation from occult HBV infection (A) and reactivation from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

Research Article

Table 4. Overview of nucleotide 1896, 1762, and 1764 sequencing data with the deep sequencing analyses.

Case	G1896A		A1762T		G1764A	
	Base counts	(%)	Base counts	(%)	Base counts	(%)
Reactivation from occult HBV carrier status						
#1	1/10,833	(0.0)	0/6391	(0.0)	1/6491	(0.0)
#2	1/10,200	(0.0)	0/9213	(0.0)	3/9216	(0.0)
#3	8/27,694	(0.0)	1/16,506	(0.0)	4/16,851	(0.0)
#4	4/13,008	(0.0)	2/12,007	(0.0)	0/11,857	(0.0)
#5	0/6860	(0.0)	0/6175	(0.0)	0/6307	(0.0)
#6	273/31,622	(0.9)	8/29,996	(0.0)	4/30,400	(0.0)
#7	22/12,561	(0.2)	0/3405	(0.0)	1/3492	(0.0)
#8	1/11,500	(0.0)	0/4964	(0.0)	1/5089	(0.0)
#9	12,897/12,904	(100)	11,676/11,677	(100)	11,653/11,659	(100)
#10	11,432/11,444	(100)	1/6153	(0.0)	2/6217	(0.0)
#11	9533/9539	(99.9)	7669/7671	(100)	7681/7685	(99.9)
#12	10,944/10,945	(100)	2/10,874	(0.0)	1/11,325	(0.0)
#13*	9358/9411	(99.4)	2/10,900	(0.0)	0/11,298	(0.0)
#14*	11,174/11,179	(100)	0/6579	(0.0)	2/6773	(0.0)
Reactivation from HBsAg carrier status						
#15	734/12,544	(5.9)	7593/7596	(100)	7556/7570	(99.8)
#16	2/7469	(0.0)	0/6481	(0.0)	2/6618	(0.0)
#17	12,251/12,701	(96.5)	5110/5241	(97.5)	5180/5239	(98.9)
#18	9649/9660	(99.9)	0/10,026	(0.0)	0/10,069	(0.0)
#19	18,402/18,413	(99.9)	1/15,677	(0.0)	3/16,045	(0.0)
#20*	11,158/11,160	(100)	0/6671	(0.0)	3/6929	(0.0)

*Patients who developed fatal acute liver failure.

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 malignances but also with solid tumors or non-cancerous 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. A 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
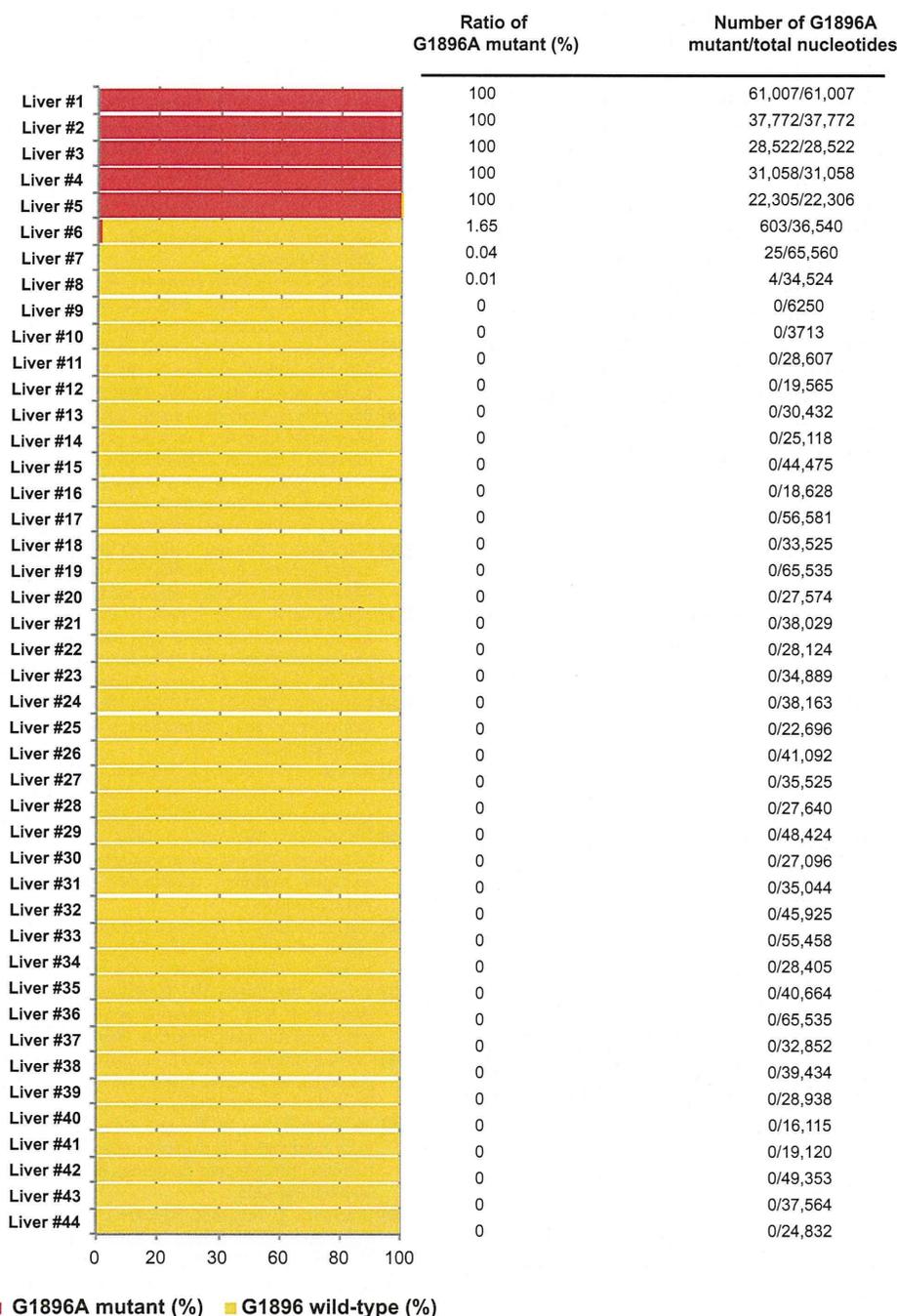
	Ratio of G1896A mutant (%)	Number of G1896A mutant/total nucleotides
Liver #1	100	61,007/61,007
Liver #2	100	37,772/37,772
Liver #3	100	28,522/28,522
Liver #4	100	31,058/31,058
Liver #5	100	22,305/22,306
Liver #6	1.65	603/36,540
Liver #7	0.04	25/65,560
Liver #8	0.01	4/34,524
Liver #9	0	0/6250
Liver #10	0	0/3713
Liver #11	0	0/28,607
Liver #12	0	0/19,565
Liver #13	0	0/30,432
Liver #14	0	0/25,118
Liver #15	0	0/44,475
Liver #16	0	0/18,628
Liver #17	0	0/56,581
Liver #18	0	0/33,525
Liver #19	0	0/65,535
Liver #20	0	0/27,574
Liver #21	0	0/38,029
Liver #22	0	0/28,124
Liver #23	0	0/34,889
Liver #24	0	0/38,163
Liver #25	0	0/22,696
Liver #26	0	0/41,092
Liver #27	0	0/35,525
Liver #28	0	0/27,640
Liver #29	0	0/48,424
Liver #30	0	0/27,096
Liver #31	0	0/35,044
Liver #32	0	0/45,925
Liver #33	0	0/55,458
Liver #34	0	0/28,405
Liver #35	0	0/40,664
Liver #36	0	0/65,535
Liver #37	0	0/32,852
Liver #38	0	0/39,434
Liver #39	0	0/28,938
Liver #40	0	0/16,115
Liver #41	0	0/19,120
Liver #42	0	0/49,353
Liver #43	0	0/37,564
Liver #44	0	0/24,832

■ G1896A mutant (%)   ■ G1896 wild-type (%)

Fig. 3. Prevalence of G1896A pre-core mutants in the liver of 44 healthy occult HBV carriers. The ratio of G1896A mutants to wild-type G1896 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. (This figure appears in colour on the web.)

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.

Research Article

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 non-cancerous 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.

Financial support

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Authors' contribution

Conceived and designed the experiments: TI, HM. Performed the experiments: TI, HM, HM. Analyzed the data: TI, YF, HM. Contributed reagents/materials/analysis tools: TI, YU, MU, TK, YO, SU, HM, TC. Wrote the paper: TI, YU, HM, TC.

Acknowledgments

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2014.04.033.

References

[1] Wands JR, Chura CM, Roll FJ, Maddrey WC. Serial studies of hepatitis-associated antigen and antibody in patients receiving antitumor chemotherapy for myeloproliferative and lymphoproliferative disorders. Gastroenterology 1975;68:105–112.

[2] Galbraith RM, Eddleston AL, Williams R, Zuckerman AJ. Fulminant hepatic failure in leukaemia and choriocarcinoma related to withdrawal of cytotoxic drug therapy. Lancet 1975;2:528–530.

[3] Hoofnagle JH, Dusheiko GM, Schafer DF, Jones EA, Micetich KC, Young RC, et al. Reactivation of chronic hepatitis B virus infection by cancer chemotherapy. Ann Intern Med 1982;96:447–449.

[4] Lok AS, Liang RH, Chiu EK, Wong KL, Chan TK, Todd D. Reactivation of hepatitis B virus replication in patients receiving cytotoxic therapy. Report of a prospective study. Gastroenterology 1991;100:182–188.

[5] Dervite I, Hober D, Morel P. Acute hepatitis B in a patient with antibodies to hepatitis B surface antigen who was receiving rituximab. N Engl J Med 2001;344:68–69.

[6] Hui CK, Cheung WW, Zhang HY, Au WY, Yueng YH, Leung AY, et al. Kinetics and risk of de novo hepatitis B infection in HBsAg-negative patients undergoing cytotoxic chemotherapy. Gastroenterology 2006;131:59–68.

[7] Mason AL, Xu L, Guo L, Kuhns M, Perrillo RP. Molecular basis for persistent hepatitis B virus infection in the liver after clearance of serum hepatitis B surface antigen. Hepatology 1998;27:1736–1742.

[8] Raimondo G, Allain JP, Brunetto MR, Buendia MA, Chen DS, Colombo M, et al. Statements from the Taormina expert meeting on occult hepatitis B virus infection. J Hepatol 2008;49:652–657.

[9] Uemoto S, Sugiyama K, Marusawa H, Inomata Y, Asonuma K, Egawa H, et al. Transmission of hepatitis B virus from hepatitis B core antibody-positive donors in living related liver transplants. Transplantation 1998;65:494–499.

[10] Marusawa H, Uemoto S, Hijikata M, Ueda Y, Tanaka K, Shimotohno K, et al. Latent hepatitis B virus infection in healthy individuals with antibodies to hepatitis B core antigen. Hepatology 2000;31:488–495.

[11] Marusawa H, Imoto S, Ueda Y, Chiba T. Reactivation of latently infected hepatitis B virus in a leukemia patient with antibodies to hepatitis B core antigen. J Gastroenterol 2001;36:633–636.

[12] Lok AS, McMahon BJ. Chronic hepatitis B. Hepatology 2007;45:507–539.

[13] Kusumoto S, Tanaka Y, Mizokami M, Ueda R. Reactivation of hepatitis B virus following systemic chemotherapy for malignant lymphoma. Int J Hematol 2009;90:13–23.

[14] Rodriguez C, Chevaliez S, Bensadoun P, Pawlotsky JM. Characterization of the dynamics of hepatitis B virus resistance to adefovir by ultra-deep pyrosequencing. Hepatology 2013;58:890–901.

[15] Nishijima N, Marusawa H, Ueda Y, Takahashi K, Nasu A, Osaki Y, et al. Dynamics of hepatitis B virus quasispecies in association with nucleos(t)ide analogue treatment determined by ultra-deep sequencing. PLoS One 2012;7:e35052.

[16] Omata M, Ehata T, Yokosuka O, Hosoda K, Ohto M. Mutations in the precore region of hepatitis B virus DNA in patients with fulminant and severe hepatitis. N Engl J Med 1991;324:1699–1704.

[17] Carman WF, Fagan EA, Hadziyannis S, Karayiannis P, Tassopoulos NC, Williams R, et al. Association of a precore genomic variant of hepatitis B virus with fulminant hepatitis. Hepatology 1991;14:219–222.

[18] Ozasa A, Tanaka Y, Orito E, Sugiyama M, Kang JH, Hige S, et al. Influence of genotypes and precore mutations on fulminant or chronic outcome of acute hepatitis B virus infection. Hepatology 2006;44:326–334.

[19] Steinberg JL, Yeo W, Zhong S, Chan JY, Tam JS, Chan PK, et al. Hepatitis B virus reactivation in patients undergoing cytotoxic chemotherapy for solid tumours: precore/core mutations may play an important role. J Med Virol 2000;60:249–255.

[20] Umemura T, Tanaka E, Kiyosawa K, Kumada H. Mortality secondary to fulminant hepatic failure in patients with prior resolution of hepatitis B virus infection in Japan. Clin Infect Dis 2008;47:e52–e56.

[21] Marusawa H, Osaki Y, Kimura T, Ito K, Yamashita Y, Eguchi T, et al. High prevalence of anti-hepatitis B virus serological markers in patients with hepatitis C virus related chronic liver disease in Japan. Gut 1999;45: 284–288.

[22] Cholongitas E, Papatheodoridis GV, Burroughs AK. Liver grafts from anti-hepatitis B core positive donors: a systematic review. J Hepatol 2010;52: 272–279.

[23] Chevaliez S, Rodriguez C, Pawlotsky JM. New virologic tools for management of chronic hepatitis B and C. Gastroenterology 2012;142:e1301.

[24] Polson J, Lee WM. AASLD position paper: the management of acute liver failure. Hepatology 2005;41:1179–1197.

[25] Sato S, Suzuki K, Akahane Y, Akamatsu K, Akiyama K, Yunomura K, et al. Hepatitis B virus strains with mutations in the core promoter in patients with fulminant hepatitis. Ann Intern Med 1995;122:241–248.

[26] Matsumoto T, Marusawa H, Dogaki M, Suginoshita Y, Inokuma T. Adalimumab-induced lethal hepatitis B virus reactivation in an HBsAg-negative patient with clinically resolved hepatitis B virus infection. Liver Int 2010;30:1241–1242.

- 660 -

[27] Hsu C, Tsou HH, Lin SJ, Wang MC, Yao M, Hwang WL, et al. Chemotherapy-induced hepatitis B reactivation in lymphoma patients with resolved HBV infection: a prospective study. Hepatology 2014;59:2092–2100.

[28] Pollicino T, Raffa G, Costantino L, Lisa A, Campello C, Squadrito G, et al. Molecular and functional analysis of occult hepatitis B virus isolates from patients with hepatocellular carcinoma. Hepatology 2007;45:277–285.

[29] Scaglioni PP, Melegari M, Wands JR. Biologic properties of hepatitis B viral genomes with mutations in the precore promoter and precore open reading frame. Virology 1997;233:374–381.

[30] Hadziyannis SJ, Vassilopoulos D. Hepatitis B e antigen-negative chronic hepatitis B. Hepatology 2001;34:617–624.

[31] Milich DR, Chen MK, Hughes JL, Jones JE. The secreted hepatitis B precore antigen can modulate the immune response to the nucleocapsid: a mechanism for persistence. J Immunol 1998;160:2013–2021.

[32] Chu CJ, Lok AS. Clinical significance of hepatitis B virus genotypes. Hepatology 2002;35:1274–1276.

- 661 -

Ultradeep Sequencing Study of Chronic Hepatitis C Virus Genotype 1 Infection in Patients Treated with Daclatasvir, Peginterferon, and Ribavirin

Eisuke Murakami, Michio Imamura, C. Nelson Hayes, Hiromi Abe, Nobuhiko Hiraga, Yoji Honda, Atsushi Ono, Keiichi Kosaka, Tomokazu Kawaoka, Masataka Tsuge, Hiroshi Aikata, Shoichi Takahashi, Daiki Miki, Hidenori Ochi, Hirotaka Matsui, Akinori Kanai, Toshiya Inaba, Fiona McPhee and Kazuaki Chayama

Updated information and services can be found at:
http://aac.asm.org/content/58/4/2105

These include:

REFERENCES
This article cites 35 articles, 6 of which can be accessed free at:
http://aac.asm.org/content/58/4/2105#ref-list-1

CONTENT ALERTS
Receive: RSS Feeds, eTOCs, free email alerts (when new articles cite this article), more»

Journals.ASM.org

Ultradeep Sequencing Study of Chronic Hepatitis C Virus Genotype 1 Infection in Patients Treated with Daclatasvir, Peginterferon, and Ribavirin

Eisuke Murakami,[a,b] Michio Imamura,[a,b] C. Nelson Hayes,[a,b] Hiromi Abe,[a,b] Nobuhiko Hiraga,[a,b] Yoji Honda,[a,b] Atsushi Ono,[a,b] Keiichi Kosaka,[a,b] Tomokazu Kawaoka,[a,b] Masataka Tsuge,[a,b] Hiroshi Aikata,[a,b] Shoichi Takahashi,[a,b] Daiki Miki,[b,c] Hidenori Ochi,[b,c] Hirotaka Matsui,[d] Akinori Kanai,[e] Toshiya Inaba,[d] Fiona McPhee,[f] Kazuaki Chayama[a,b,c]

Department of Gastroenterology and Metabolism, Applied Life Science, Institute of Biomedical & Health Science, Hiroshima University, Hiroshima, Japan[a]; Liver Research Project Center, Hiroshima University, Hiroshima, Japan[b]; Laboratory for Digestive Diseases, Center for Genomic Medicine, The Institute of Physical and Chemical Research (RIKEN), Hiroshima, Japan[c]; Department of Molecular Oncology and Leukemia Program Project, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, Japan[d]; Radiation Research Center for Frontier Science, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, Japan[e]; Bristol-Myers Squibb, Research and Development, Wallingford, Connecticut, USA[f]

Direct-acting antivirals (DAAs) are either part of the current standard of care or are in advanced clinical development for the treatment of patients chronically infected with hepatitis C virus (HCV) genotype 1, but concern exists with respect to the patients who fail these regimens with emergent drug-resistant variants. In the present study, ultradeep sequencing was performed to analyze resistance to daclatasvir (DCV), which is a highly selective nonstructural protein 5A (NS5A) inhibitor. Eight patients with HCV genotype 1b, who were either treatment naive or prior nonresponders to pegylated interferon plus ribavirin (Rebetol; Schering-Plough) (PEG-IFN/RBV) therapy, were treated with DCV combined with PEG-IFN alpha-2b (Pegintron; Schering-Plough, Kenilworth, NJ) and RBV. To identify the cause of viral breakthrough, the preexistence and emergence of DCV-resistant variants at NS5A amino acids were analyzed by ultradeep sequencing. Sustained virological response (SVR) was achieved in 6 of 8 patients (75%), with viral breakthrough occurring in the other 2 patients (25%). DCV-resistant variant Y93H preexisted as a minor population at higher frequencies (0.1% to 0.5%) in patients who achieved SVR. In patients with viral breakthrough, DCV-resistant variant mixtures emerged at NS5A-31 over time that persisted posttreatment with Y93H. Although enrichment of DCV-resistant variants was detected, the preexistence of a minor population of the variant did not appear to be associated with virologic response in patients treated with DCV/PEG-IFN/RBV. Ultradeep sequencing results shed light on the complexity of DCV-resistant quasispecies emerging over time, suggesting that multiple resistance pathways are possible within a patient who does not rapidly respond to a DCV-containing regimen. (This study has been registered at ClinicalTrials.gov under registration no. NCT01016912.)

Chronic hepatitis C virus (HCV) infection is one of the most serious global health problems preceding development of chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) (1, 2, 3, 4, 5). To prevent the development of advanced liver disease, including HCC, pegylated interferon (PEG-IFN)-based therapies have been administered to patients with chronic HCV infection. Eradication of HCV using PEG-IFN combined with ribavirin (Rebetol; Schering-Plough) (PEG-IFN/RBV) has been shown to result in remarkable biochemical and histological improvements in the liver (6, 7). However, patients infected with HCV genotype 1 have experienced a poor response to this therapy as observed by sustained virological response (SVR) rates of only 40% to 50% (8, 9, 10). Recently, new antiviral agents targeting the HCV nonstructural protein 3/4A (NS3/4A) protease activity, telaprevir (TVR) and simeprevir, were approved in several countries as an add-on to PEG-IFN and RBV (triple therapy) for treating patients infected with HCV genotype 1. The triple therapy significantly improved SVR rates in this patient population (11, 12). However, many severe adverse effects such as skin rash, anemia, and renal dysfunction have been reported which often prevent successful continuation of this triple therapy (12).

To improve safety and effectiveness of anti-HCV therapy, a number of selective inhibitors targeting HCV proteins, otherwise known as direct-acting antivirals (DAAs), are currently under de-

velopment. Daclatasvir (DCV; BMS-790052) is a first-in-class, highly selective nonstructural protein 5A (NS5A) inhibitor with picomolar potency and broad genotypic coverage (13, 14, 15). NS5A is an RNA binding multifunctional viral protein and is essential for viral proliferation by interacting with other HCV nonstructural proteins and cellular proteins (16, 17, 18). In a phase 2a study, a higher SVR rate was observed by adding DCV to the PEG-IFN alpha-2a plus RBV regimen (19).

Although DAAs are expected to improve the antiviral effect of PEG-IFN/RBV against HCV genotype 1, drug resistance is still considered a concern. Emergence of drug resistance is often associated with viral rebound and subsequent virologic failure. In the case of the DCV NS5A inhibitor, the emergence of substitutions at the NS5A drug target has been reported (19). In patients infected with HCV genotype 1, one of the most predominant genotypes in

TABLE 1 Clinical characteristics of 8 patients treated with combination therapy with daclatasvir, PEG-IFN alpha-2b, and RBV for 24 weeks against chronic HCV genotype 1b infection[a]

Case	Age (yr)	Sex	Previous interferon treatment	IL28B	HCV RNA (log IU/ml)	No. of platelets ($\times 10^3/\mu$l)	Hepatic fibrosis stage	DCV (mg/day)	Efficacy
1	67	F	Naive	TT	7.1	262	ND	60	SVR
2	42	F	Partial	TG	5.5	146	F2	60	SVR
3	55	F	Naive	TT	5.1	181	F2	60	SVR
4	61	M	Naive	TT	7.1	225	F1	60	SVR
5	39	F	Naive	TG	6.4	207	F1	10	SVR
6	59	M	Partial	TG	7.1	178	F2	10	SVR
7	59	F	Null	TG	7.6	158	ND	10	Breakthrough
8	70	F	Null	GG	7.0	167	F2	60	Breakthrough

[a] IL28B, rs8099917 genotype; DCV, daclatasvir; M, male; F, female; SVR, sustained virological response; Partial, partial responder; Null, null responder; ND, not determined. The hepatic fibrosis stage was determined by liver biopsy analysis according to New Inuyama Classification as follows: F1, fibrous portal expansion; F2, bridging fibrosis.

the world, NS5A amino acid (aa) positions 31 and 93 have been shown to be susceptible to substitution or enrichment (19). Double-amino-acid substitutions in the NS5A region, such as L31M (substitution from leucine to methionine) plus Y93H (from tyrosine to histidine) or L31V (leucine to valine) plus Y93H, conferred high resistance to DCV in an *in vitro* HCV replication system (19).

Recently, ultradeep sequencing has been used as a sensitive technique for characterizing resistance variants (20, 21, 22, 23). In the present study, ultradeep sequencing was performed using sera from 8 Japanese chronic hepatitis C patients who participated in a clinical phase 2a trial using DCV, PEG-IFN alpha-2b (Pegintron; Schering-Plough, Kenilworth, NJ), and RBV to analyze the association between preexisting DCV-resistant variants and clinical antiviral responses.

MATERIALS AND METHODS

Study design. This study was a phase 2a, double-blind, placebo-controlled trial (clinicaltrials.gov identifier NCT01016912) for evaluating the antiviral activity and safety of DCV combined with PEG-IFN alpha-2b and RBV in treatment-naive patients and nonresponders to the standard of care with HCV genotype 1. Written informed consent was obtained from all patients. The study was approved by institutional review boards at each site and conducted in compliance with the Declaration of Helsinki, good clinical practice guidelines, and local regulatory requirements.

Patients. Ten patients who met the following inclusion and exclusion criteria participated in the clinical trial. However, two were excluded from the following analysis because they were assigned to a placebo cohort group and treated with PEG-IFN plus RBV combination therapy without DCV. Inclusion and exclusion criteria for this clinical trial used the following parameters. (i) The patient age was between 20 and 75 years. (ii) The patients had been infected with HCV genotype 1 for at least 6 months, and the serum HCV RNA level was $>10^5$ IU/ml. (iii) Eligible patients had had no evidence of cirrhosis diagnosed by laparoscopy, imaging, or liver biopsy analysis within 2 years. (iv) Eligible patients consisted of three groups: (*a*) treatment-naive patients with no history of anti-HCV therapy, including interferon therapy; (*b*) null responders who had failed to achieve a 2 $\log_{10}$ HCV-RNA decrease in previous interferon therapy lasting 12 weeks or longer; and (*c*) partial responders who had failed to achieved undetectable RNA but had achieved a greater than 2 $\log_{10}$ HCV-RNA decrease in previous interferon therapy lasting 12 weeks or longer. (iv) The patients had no history of hepatocellular carcinoma, coinfection with hepatitis B virus or human immunodeficiency virus, other chronic liver disease, or evidence of hepatic decompensation. (vi) Patients were also excluded if they had other severe or unstable conditions or evidence of organ dysfunction in excess of that consistent with the age of the patient, were unable to tolerate interferon and oral medication or had con-

ditions that could impact absorption of the study drug, or had been exposed to any investigational drug within 4 weeks of study participation or had any previous exposure to inhibitors of NS5A. (vii) Laboratory findings that excluded participation were alanine aminotransferase (ALT) > 5 times the upper limit of normal (ULN); total bilirubin ≥ 2 mg/dl; direct bilirubin $> 1.5\times$ ULN; international normalized ratio of prothrombin time ≥ 1.7; albumin ≤ 3.5 g/dl; hemoglobin < 9.0 g/dl; white blood cells $< 1,500/mm^3$; absolute neutrophil count $< 750/mm^3$; platelets $< 50,000/mm^3$; or creatinine $> 1.8\times$ ULN.

Treatment protocol. All patients received a combination of DCV, PEG-IFN alpha-2b, and RBV for 24 weeks. Patients subcutaneously received PEG-IFN alpha-2b at a dosage of 1.5 mg/kg of body weight/week and were administered ribavirin orally according to their body weight (600 mg for < 60 kg, 800 mg for 60 to 80 kg, 1,000 mg for >80 kg). Patients were randomly assigned to receive DCV at 10 mg or 60 mg once daily for 24 weeks. DCV was provided by Bristol-Myers Squibb, which conducted this clinical trial. When viral breakthrough occurred, treatment was discontinued with the patient's consent.

Determination of IL28B genotypes. The *IL28B* SNP genotype (rs8099917) was determined using TaqMan predesigned single nucleotide polymorphism (SNP) genotyping assays as described previously (24).

Assessment of virological responses. Plasma was collected at baseline and at the following fixed time points: weeks 1, 2, 4, 6, 8, and 12 and then every 4 weeks during treatment. HCV RNA was determined at a central laboratory using a Roche Cobas TaqMan HCV Auto assay (Roche Diagnostics KK, Tokyo, Japan) (lower limit of quantitation [LLOQ], 15 IU/ml). Sustained viral response (SVR) occurred if HCV RNA became continuously undetectable by qualitative PCR assay and ALT levels normalized for 24 weeks after the end of treatment. Viral breakthrough was defined as an increase of $\geq 1 \log_{10}$ IU/ml from nadir at more than one time point or HCV RNA ≥ 15 IU/ml after declining to below that level.

Detection of drug-resistant substitutions by ultradeep sequencing. HCV RNA was extracted from serum samples by Sepa Gene RV-R (Sankojunyaku, Tokyo), and the reverse-transcriptase reaction was performed using a random primer and Moloney murine leukemia virus (MMLV) reverse transcriptase. Briefly, the NS5A region in HCV genome was amplified by nested PCR using the specific primers 5′-TGGCTCCA GTCCAAACTCCT G-3′, 5′-GGGAATGTTCCATGCCACGTG-3′, 5′-T GGAACATTCCCCATCAACGC-3′, and 5′-CCAACCAGGTACTGATT GAGC-3′, and the amplified fragment distributions were assessed using an Agilent BioAnalyzer 2100 platform. The fragments were modified by the use of a Multiplexing Sample Preparation kit (Illumina), and sequence analysis was performed by the use of a Illumina Genome Analyzer. Imaging analysis and base calling were performed using Illumina Pipeline software with default settings as previously reported (20). The N-terminal domain of NS5A, which includes L31 and Y93, was analyzed. This technique revealed an average coverage depth of over 1,000 sequence reads per base pair in the unique regions of the genome. Read mapping to a refer-