sustained virological response (SVR), which is defined as undetectable serum HCV RNA 24 weeks after completion of therapy. Telaprevir (TVR) is an effective HCV non-structural 3/4A protease inhibitor that has recently been approved for the treatment of chronic hepatitis C genotype 1 in Japan. Triple therapy that combines TVR, pegylated-IFN-α2b (PEG-IFN), and ribavirin (RBV) treatment has achieved SVR rates of 70–80% [3,4].

Recent studies have highlighted that polymorphisms in the interleukin (IL) 28B gene are associated with spontaneous and treatment-induced resolution of HCV infection [5–8]. Similarly, killer immunoglobulin-like receptors (KIRs) and their human leukocyte antigen (HLA) class I ligands have also been implicated in spontaneous and treatment-based disease resolution [9–13]. Accordingly, combinations of IL28B polymorphisms and KIR genotypes have been studied by our own laboratory and others with regard to disease treatment and resolution [14–17]. However, it has not yet been elucidated whether polymorphisms of these innate immune genes are associated with virological response to TVR/PEG-IFN/RBV triple therapy. The objective of this study was to clarify whether *KIR-HLA* interactions, in addition to an *IL28B* polymorphism, would influence the outcome of TVR/PEG-IFN/RBV or PRG-IFN/RBV therapy in Japanese patients with chronic hepatitis C.

2. Materials and methods

2.1. Subjects

A total of 200 patients with chronic hepatitis C were enrolled in this study. All subjects were treated at Shinshu University Hospital or one of its affiliated hospitals. The clinical and demographic characteristics of our cohort are shown in Table 1. The diagnosis of chronic hepatitis C was based on previously reported criteria [18] of (1) the presence of serum HCV antibodies and HCV RNA $\geqslant$ 5.0 log IU/mL; (2) the absence of detectable hepatitis B surface antigen and antibody to the human immunodeficiency virus; and (3) exclusion of other causes of chronic liver disease or a history of decompensated cirrhosis or HCC. Serum levels of HCV RNA were determined using the Cobas TaqMan HCV test (Roche Diagnostic Systems, Tokyo, Japan). The linear dynamic range of the assay was 1.2–7.8 log IU/mL, and undetectable samples were defined as negative. All patients in our cohort were infected with HCV genotype 1b as determined by sequence analysis. Alanine aminotransferase, aspartate aminotransferase, and other relevant biochemical tests were performed using standard methods [19].

Ninety-two patients received a 12-week triple therapy regimen that included TVR (Telavic; Mitsubishi Tanabe Pharma, Osaka, Japan; 1500–2250 mg/day), PEG-IFN (Pegintron; MSD KK, Tokyo, Japan; 1.5 μg/kg of body weight weekly by subcutaneous injection), and RBV (Rebetol; MSD KK; 600–1000 mg/day according to body weight) followed by a 12-week course of dual therapy composed of PEG-IFN and RBV. The remaining 108 patients received PEG-IFN and RBV treatment for 48 weeks, as described previously [20]. No patients from our prior study were included in this cohort [21].

Patients achieving a sustained HCV response were identified as those whose serum HCV RNA was undetectable 24 weeks after completing therapy. A rapid virological response (RVR) was defined as undetectable HCV RNA at week 4 of treatment. Patients not attaining an SVR, who included non-responders and relapsers, were regarded as treatment failures.

This study was approved by the ethics committee of Shinshu University School of Medicine, Matsumoto, Japan, and written informed consent was obtained from all participants. The study was conducted in accordance with the principles of the Declaration of Helsinki.

2.2. HLA, KIR, and IL28B (rs8099917) genotyping

Genomic DNA was isolated from whole blood samples using QuickGene-800 assays (Fujifilm, Tokyo, Japan). We genotyped *HLA-A*, *HLA-B*, *HLA-C*, and *KIR* using a Luminex multi-analyzer profiling system with a LAB type® HD and KIR SSO genotyping kit (One Lambda, Inc., Canoga Park, CA) that was based on PCR sequence-specific oligonucleotide probes [22]. *KIR* genes were divided into distinct group A and group B haplotypes based on centromeric as well as telomeric regions of the *KIR* locus. *KIR* genotypes were then identified according to the definition established by Cooley et al. [23]. Briefly, centromeric *AA* genotypes contained *KIR2DL3* but not *KIR2DL2* or *KIR2DS2*, centromeric *AB* genotypes contained *KIR2DL3* with *KIR2DL2* and/or *KIR2DS2*, and centromeric *BB* genotypes contained *KIR2DL2* and/or *KIR2DS2* but not *KIR2DL3*. Meanwhile, telomeric *AA* genotypes contained *KIR3DL1* and *KIR2DS4* but not *KIR3DS1* or *KIR2DS1*, telomeric *AB* genotypes contained *KIR3DL1* and *KIR2DS4* with *KIR3DS1* and/or *KIR2DS1*, and telomeric *BB* genotypes lacked *KIR3DL1* and/or *KIR2DS4*. Genotyping of an *IL28B* SNP (rs8099917) was performed using an ABI TaqMan allelic discrimination kit and the ABI7500 Sequence Detection System (Applied Biosystems, Carlsbad, CA) [24]. Probe fluorescence signals were detected using a TaqMan assay for Real-Time PCR (7500 Real-Time PCR System, Applied Biosystems) according to the manufacturer's instructions.

2.3. Statistical analysis

The Mann–Whitney *U* test was employed to analyze continuous variables. Pearson's chi-squared test was used for the analysis of categorical data. We adopted Fisher's exact test when the number of subjects was less than 5. The Bonferroni correction for multiple testing was applied to our KIR-HLA combination data using the number of comparisons performed on our primary factors of

Table 1
Clinical features of patients with chronic hepatitis C with and without a sustained virological response.

Characteristic	All (n = 200)	SVR (n = 126)	Non-SVR (n = 74)	P
Age (yrs)	61 (53–65)	60 (52–65)	62 (55–67)	0.076
Male	109 (55)	71 (56)	38 (51)	0.493
Triple therapy	92 (46)	74 (59)	18 (24)	2.0×10^{-6}
White blood cells (/μL)	4445 (3788–5578)	4870 (3888–5730)	4275 (3670–5190)	0.022
Hemoglobin (g/dL)	14.4 (13.4–15.4)	14.4 (13.7–15.7)	14.1 (13.1–15.1)	0.010
Platelet count (10^4/μL)	15.9 (13.0–19.3)	16.3 (13.6–19.3)	15.5 (12.1–19.2)	0.163
Serum alanine aminotransferase (IU/L)	44 (29–68)	40 (26–64)	46 (33–70)	0.147
HCV RNA (log IU/mL)	6.5 (6.0–6.8)	6.4 (5.9–6.7)	6.5 (6.2–6.8)	0.027
IL-28 TT genotype	134 (67)	102 (81)	32 (43)	4.4×10^{-8}

Data are expressed as median (interquartile range) or n (%) as appropriate.
SVR, sustained virological response.

Table 2
Frequency of *HLA-Bw* and *-C* alleles in 126 patients with a sustained virological response (SVR) and 74 patients with a non-SVR to antiviral therapy for chronic hepatitis C.

	SVR (n = 126)	Non-SVR (n = 74)	P (Pc)
Genotype			
Bw4/Bw4	15 (12%)	7 (10%)	0.594
Bw4/Bw6	61 (48%)	23 (31%)	0.017 (0.17)
Bw6/Bw6	50 (40%)	44 (60%)	0.011 (0.11)
C1/C1	104 (83%)	65 (88%)	0.318
C1/C2	22 (17%)	9 (12%)	

Data are expressed as n (%).
SVR, sustained virological response.

interest in Table 2 (i.e., 5 combinations × 2 comparisons between two groups = 10 tests). A P value of <0.05 was considered to be statistically significant. Association strength was estimated by calculating the odds ratio (OR) and 95% confidence interval (CI). Our model was checked by regression diagnostic plots to verify normality, linearity of data, and constant variance. Stepwise logistic regression analysis with a forward approach was performed to identify independent factors associated with an SVR after continuous variables were separated into 2 categorical variables by their median value. Statistical analyses were performed using SPSS software version 21.0 J (IBM, Tokyo, Japan). We evaluated synergy between IL28B and KIR-HLA using the method described by Cortina-Borja et al. [25].

3. Results

3.1. Patient characteristics and treatment outcome

Of the 200 patients who received antiviral therapy, 126 (63%) achieved an SVR. The remaining 74 patients were considered to be non-responders: 39 relapsed, 33 were null responders, and 2 experienced viral breakthrough. Before treatment, median white blood cell count (4870 vs. 4275 /μL, P = 0.022) and hemoglobin value (14.4 vs. 14.1 g/dL, P = 0.010) in the SVR group were significantly higher than in the non-SVR group (Table 1). Median HCV RNA level (6.4 vs. 6.5 log IU/mL, P = 0.027) was significantly lower in the SVR group compared with the non-SVR group. Patients who were administered triple therapy had a significantly higher SVR rate (59% [74/126] vs. 24% [18/74], $P = 2.0 \times 10^{-6}$; OR = 4.43, 95% CI = 2.34–8.39). An RVR was also strongly associated with an SVR (66% [83/126] vs. 10% [7/74], $P = 9.7 \times 10^{-15}$; OR = 18.48, 95% CI = 7.81–43.71). The RVR and SVR rates in patients treated with TVR/PEG-IFN/RBV were 78% (72/92) and 80% (74/92), respectively. In contrast, these rates were 17% (18/108) and 48% (52/108), respectively, in patients treated with PEG-IFN/RBV.

3.2. HLA class I allele frequencies and KIR genotypes in patients with chronic hepatitis C

We first examined for associations between *HLA-B* and *-C* alleles and response to antiviral therapy. The frequency of the *HLA-B*51:01* allele in patients with an SVR was higher than in patients with a non-SVR (10% [24/252] vs. 3% [4/148], P = 0.017 [Pc = 0.49]; OR = 3.79, 95% CI = 1.29–11.15). Conversely, the *HLA-B*15:01* allele was less frequently found in responders (5% [12/252] vs. 10% [15/148], P = 0.039 [Pc = 1.13]; OR = 0.44, 95% CI = 0.20–0.98). No specific *HLA-A* or *-C* alleles were detected in our cohort.

Next, we searched for differences in the distribution of *HLA-Bw4* and *HLA-C1* allele frequencies between SVR and non-SVR patients (Table 2). The frequency of *HLA-Bw4Bw6* in responders was higher than in non-responders (48% [61/126] vs. 31% [23/74], P = 0.017 [Pc = 0.17]; OR = 2.08, 95% CI = 1.14–3.81). In contrast, patients with the *HLA-Bw6* homozygote had a higher non-SVR rate (40% [50/126] vs. 59% [44/74]; P = 0.011 [Pc = 0.11]; OR = 0.45, 95% CI = 0.25–0.81). Overall, *HLA-Bw4* was significantly associated with an SVR among patients (60% [76/126] vs. 41% [30/74], P = 0.007, OR = 2.23, 95% CI = 1.24–4.00). No remarkable allelic frequencies were seen for *HLA-C1*.

With respect to *KIR* genes, no associations between the 16 genes examined and treatment outcome were observed (Fig. 1). *KIR* gene profiles were classified based on centromeric and telomeric regions of the *KIR* A and B haplotypes (Cen-A/B and Tel-A/B). When we compared the Cen-A/B and Tel-A/B frequencies between the SVR and non-SVR groups, no significant differences were apparent (Table 3).

3.3. HLA and KIR compound genotypes and antiviral response of HCV

To determine the effect of HLA/KIR genotypes on possible associations with an SVR, we analyzed combinations of activating or inhibitory *KIRs* and their *HLA* ligands. Among the combinations of *KIR2DL1-HLA-C2*, *KIR2DL2-HLA-C1*, *KIR2DL3-HLA-C1*, *KIR3DL1-HLA-Bw4*, and *KIR3DL2-HLA-A3* and *-A11*, only the frequency of the inhibitory *KIR3DL1* receptor and its *HLA-Bw4* ligand was remarkably higher in responders than in non-responders (58% [73/126] vs. 39% [29/74], P = 0.010 [Pc = 0.10]; OR = 2.14, 95% CI = 1.19–3.84) (Table 4). When stratified for patients treated with TVR/PEG-IFN/RBV or PEG-IFN/RBV, although *KIR3DL1-HLA-Bw4* was significantly associated with an SVR in patients treated with PEG-IFN/RBV (69%

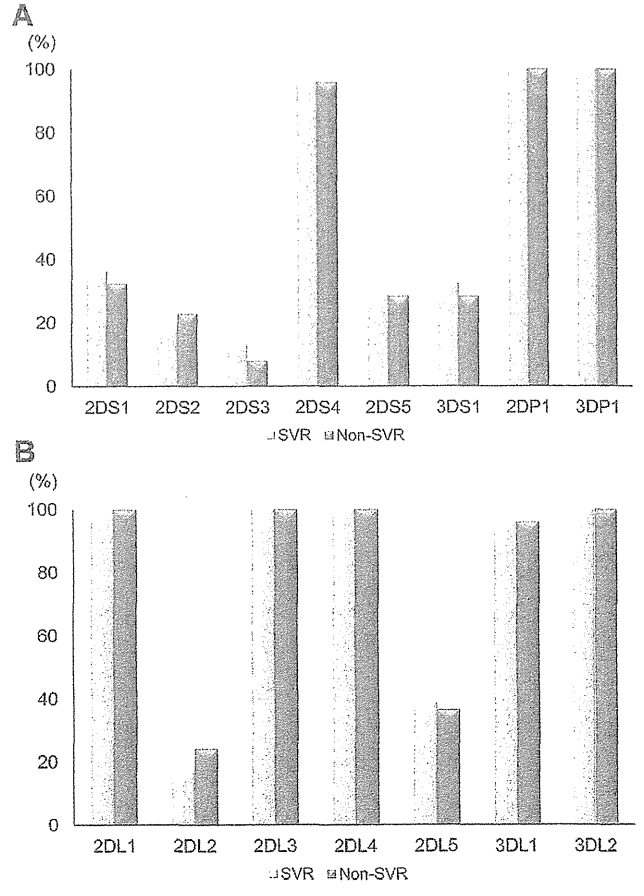

Fig. 1. Frequency of 16 KIR genes in 126 patients with a sustained virological response (SVR) and 74 patients with a non-SVR to antiviral therapy for chronic hepatitis C.

Table 3
Frequencies of centromeric and telomeric KIR genotypes in 126 patients with a sustained virological response (SVR) and 74 patients with a non-SVR to antiviral therapy for chronic hepatitis C.

	SVR (n = 126)	Non-SVR (n = 74)	P
Genotype			
AA	71 (56%)	36 (49%)	0.292
Bx	55 (44%)	38 (51%)	
Centromere motif			
AA	104 (83%)	56 (76%)	0.241
AB	22 (18%)	18 (24%)	
Telomere motif			
AA	80 (64%)	50 (68%)	0.827
AB	41 (33%)	21 (28%)	
BB	5 (4%)	3 (4%)	

Data are expressed as n (%).
SVR, sustained virological response.

Table 4
Frequencies of KIR-HLA receptor-ligand pairs in 126 patients with a sustained virological response (SVR) and 74 patients with a non-SVR to antiviral therapy for chronic hepatitis C.

KIR-HLA receptor-ligand pair	SVR (n = 126)	Non-SVR (n = 74)	P (Pc)
KIR2DL1-HLA-C2	22 (18%)	9 (12%)	0.833
KIR2DL2-HLA-C1	19 (15%)	17 (23%)	0.161
KIR2DL3-HLA-C1	*104 (83%)*	*65 (88%)*	*0.318*
KIR3DL1-HLA-Bw4	73 (58%)	29 (39%)	0.010 (0.10)
KIR3DL2-HLA-A3 and -A11	32 (25%)	20 (27%)	0.800

Data are expressed as n (%).
SVR, sustained virological response.

Table 5
Logistic regression analysis of variables contributing to a sustained virological response to antiviral therapy.

Factor	Odds ratio	95% CI	P
RVR	20.95	7.68–57.11	<0.000001
IL28B TT genotype	5.53	2.30–13.32	0.00014
KIR3DL1-HLA-Bw4	3.42	1.50–7.83	0.004

Only variables achieving statistical significance (P < 0.05) in multivariate logistic regression analysis are shown.

[36/52] vs. 38% [21/56], P = 0.001 [Pc = 0.01]; OR = 3.75, 95% CI = 1.69–8.34), no association between *KIR3DL1-HLA-Bw4* and triple therapy was observed (50% [37/74] vs. 44% [8/18], P = 0.672).

3.4. Association of a sustained virological response with KIR-HLA and IL28B

The SVR rate in patients with the *IL28B* TT genotype was significantly higher than in those with the TG or GG genotype (81% [102/126] vs. 43% [32/74], $P = 4.4 \times 10^{-8}$; OR = 5.58, 95% CI = 2.94–10.58) (Table 1).

We next evaluated several factors found in association with an SVR to antiviral therapy for independence by logistic regression analysis. A total of 126 responders were compared with 74 non-responders by means of a forward stepwise likelihood ratio logistic regression method. An RVR (P < 0.000001; OR = 20.95, 95% CI = 7.68–57.11), the *IL28B* TT genotype (P = 0.00014; OR = 5.53, 95% CI = 2.30–13.32), and *KIR3DL1-HLA-Bw4* (P = 0.004; OR = 3.42, 95% CI = 1.50–7.83) were all identified as independent parameters that significantly influenced an SVR (Table 5).

As the frequency of the *IL28B* TT genotype along with *KIR3DL1-HLA-Bw4* in SVR group was significantly higher than in non-SVR group (46% [58/126] vs. 18% [13/74]; $P = 4.9 \times 10^{-5}$ [$Pc = 3.9 \times 10^{-4}$]; OR = 4.00, 95% CI = 2.00–8.01) (Table 6), we applied a recently described test to evaluate for synergistic effects between these genetic factors [25]. Based on logistic regression analysis, this method evaluated whether the observed ORs of the 2 independent factors were greater combined than separately. We observed an absence of synergy between the 2 favorable factors of *IL28B* TT genotype and *KIR3DL1-HLA-Bw4* in the SVR population (synergy factor = 0.68, 95% CI = 0.19–2.48; $P_{synergy}$ = 0.560), which confirmed that they were indeed independent of each other. Moreover, when stratified for each treatment regimen, patients who achieved an SVR with the PEG-IFN/RBV regimen had a significantly higher frequency of *IL28B* TT and *KIR3DL1-HLA-Bw4* compared with those who did not (50% vs. 18%, P = 0.00040 [Pc = 0.0032], OR = 4.60, 95% CI = 1.92–11.02). For triple therapy, the frequency of the *IL28B* TG/GG genotype without *KIR3DL1-HLA-Bw4* was significantly higher in non-responders (39% vs. 9%, P = 0.0018 [Pc = 0.014], OR = 0.16, 95% CI = 0.05–0.56).

4. Discussion

Natural killer (NK) cells are a subset of lymphocytes that can interact directly with virus-infected cells as well as activate dendritic cells and secrete Th1-type cytokines to augment antiviral cytotoxic T-cell responses. The NK cell response is controlled by multiple activating and inhibitory receptors. It is thought that the net inhibitory or activating signal derived from these receptors determines whether or not the NK cell is activated. KIR molecules are known to interact with their HLA class I ligands to modulate NK cell activity. The ligands for KIR2DL are HLA-C alleles, which are classified as C group 1 (C1) if the amino acid at position 80 is asparagine or C group 2 (C2) if lysine occupies that position. The inhibitory KIR2DL2 and -2DL3 recognize the C1 allotype, while KIR2DL1 recognizes C2 allotypes [26]. KIR3DL1 recognizes HLA-B Bw4 allotypes, particularly those with an isoleucine at position 80 [27].

Our data showed that KIR3DL1 and its HLA-Bw4 ligand were associated with an SVR following antiviral therapy that included TVR/PEG-IFN/RBV triple therapy in Japanese patients with chronic hepatitis C. In combination with a prior study by our group [21], our findings demonstrate a favorable influence of these genes in patients achieving an SVR with IFN-based treatment. As almost one half of the Japanese population have the functional *KIR3DL1-HLA-Bw4* combination, this inhibitory receptor-ligand interaction

Table 6
Frequencies of IL28 genotype and KIR3DL1/HLA-Bw4 combinations in 126 patients with a sustained virological response (SVR) and 74 patients with a non-SVR to antiviral therapy for chronic hepatitis C.

IL28B	KIR3DL1/HLA-Bw4	SVR (n = 126)	Non-SVR (n = 74)	P (Pc)
TT	+/+	58 (46%)	13 (18%)	4.9×10^{-5} (3.9×10^{-4})
TT	Other	44 (35%)	19 (26%)	0.174
TG/GG	+/+	15 (12%)	16 (22%)	0.067
TG/GG	Other	9 (7%)	26 (35%)	4.9×10^{-7} (3.9×10^{-6})

Data are expressed as n (%).
SVR, sustained virological response.

is potentially important in understanding NK-cell diversification. The NK cell surface expression of KIR3DL1 is higher in individuals having Bw4 than in those lacking it [28]. Such cells may be less strongly controlled by inhibitory signals than other NK cells, more easily activated by viral infection, and more readily promoted for cytolysis and IFN-gamma production. On the contrary, although *KIR2DL3-HLAC1* has been associated with treatment-induced and spontaneous HCV eradication in Caucasians [9,11,16], our data showed no association of this gene with the response to treatment for HCV infection.

In multivariate analysis, we witnessed that an RVR, the *IL28B* TT genotype, and *KIR3DL1-HLA-Bw4* were independent factors related to an SVR in patients treated with anti-viral therapy with and without TVR. This confirmed that RVR and IL28B genotype were strong predictors of an SVR to triple therapy in the Japanese population similarly to previous studies of HCV treatment with PEG-IFN/RBV only [7,24]. Furthermore, SVR frequencies were positively correlated with a combination of the *IL28B* TT genotype and *KIR3DL1-HLA-Bw4* ($P = 0.000049$), but we did not observe that they were acting synergistically. The calculation of a synergy factor allows for differentiation between a true synergistic interaction and an apparent one. The synergy factor is designed to be robust for small sizes, even when individual cells are zero. The result of this analysis complemented the findings obtained by logistic regression that the combination of the 2 independent factors had no significant advantage over each factor in isolation for an SVR.

In conclusion, the present study showed significant independent associations of an RVR, the *KIR3DL1-HLA-Bw4* combination, and *IL28B* with an SVR to interferon-based therapy, including TVR triple therapy, in Japanese patients with genotype 1 HCV.

Author contributions

TU and MO conceived and designed the experiments. YK and YN performed the experiments. TU performed the statistical analysis and wrote the first draft. TU, SW, HM, AM, SS, TK, SM, SJ, MK, AM, AK, MK, MT, KY, KK, and ET provided the specimens and clinical data of the patients. All authors contributed to further drafts, and have read and approved the final manuscript.

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References

[1] Kiyosawa K, Sodeyama T, Tanaka E, Gibo Y, Yoshizawa K, Nakano Y, et al. Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. Hepatology 1990;12:671–5.
[2] Umemura T, Ichijo T, Yoshizawa K, Tanaka E, Kiyosawa K. Epidemiology of hepatocellular carcinoma in Japan. J Gastroenterol 2009;44(Suppl. 19):102–7.
[3] Kumada H, Toyota J, Okanoue T, Chayama K, Tsubouchi H, Hayashi N. Telaprevir with peginterferon and ribavirin for treatment-naive patients chronically infected with HCV of genotype 1 in Japan. J Hepatol 2012;56:78–84.
[4] Hayashi N, Okanoue T, Tsubouchi H, Toyota J, Chayama K, Kumada H. Efficacy and safety of telaprevir, a new protease inhibitor, for difficult-to-treat patients with genotype 1 chronic hepatitis C. J Viral Hepat 2012;19:e134–42.
[5] Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 2009;461:399–401.
[6] Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, et al. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet 2009;41:1100–4.
[7] Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, et al. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet 2009;41:1105–9.
[8] Thomas DL, Thio CL, Martin MP, Qi Y, Ge D, O'Huigin C, et al. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. Nature 2009;461:798–801.
[9] Khakoo SI, Thio CL, Martin MP, Brooks CR, Gao X, Astemborski J, et al. HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection. Science 2004;305:872–4.
[10] Romero V, Azocar J, Zuniga J, Clavijo OP, Terreros D, Gu X, et al. Interaction of NK inhibitory receptor genes with HLA-C and MHC class II alleles in hepatitis C virus infection outcome. Mol Immunol 2008;45:2429–36.
[11] Knapp S, Warshow U, Hegazy D, Brackenbury L, Guha IN, Fowell A, et al. Consistent beneficial effects of killer cell immunoglobulin-like receptor 2DL3 and group 1 human leukocyte antigen-C following exposure to hepatitis C virus. Hepatology 2010;51:1168–75.
[12] Vidal-Castineira JR, Lopez-Vazquez A, Diaz-Pena R, Alonso-Arias R, Martinez-Borra J, Perez R, et al. Effect of killer immunoglobulin-like receptors in the response to combined treatment in patients with chronic hepatitis C virus infection. J Virol 2010;84:475–81.
[13] Seich Al Basatena NK, Macnamara A, Vine AM, Thio CL, Astemborski J, Usuku K, et al. KIR2DL2 enhances protective and detrimental HLA class I-mediated immunity in chronic viral infection. PLoS Pathog 2011;7:e1002270.
[14] Dring MM, Morrison MH, McSharry BP, Guinan KJ, Hagan R, O'Farrelly C, et al. Innate immune genes synergize to predict increased risk of chronic disease in hepatitis C virus infection. Proc Natl Acad Sci U S A 2011;108:5736–41.
[15] Knapp S, Warshow U, Ho KM, Hegazy D, Little AM, Fowell A. A polymorphism in IL28B distinguishes exposed, uninfected individuals from spontaneous resolvers of HCV infection. Gastroenterology 2011;141. 320-5, 25. e1–2.
[16] Suppiah V, Gaudieri S, Armstrong NJ, O'Connor KS, Berg T, Weltman M, et al. IL28B, HLA-C, and KIR variants additively predict response to therapy in chronic hepatitis C virus infection in a European Cohort: a cross-sectional study. PLoS Med 2011;8:e1001092.
[17] Rivero-Juarez A, Gonzalez R, Camacho A, Manzanares-Martin B, Caruz A, Martinez-Peinado A, et al. Natural killer KIR3DS1 is closely associated with HCV viral clearance and sustained virological response in HIV/HCV patients. PLoS One 2013;8:e61992.
[18] Umemura T, Alter HJ, Tanaka E, Orii K, Yeo AE, Shih JW, et al. SEN virus: response to interferon alfa and influence on the severity and treatment response of coexistent hepatitis C. Hepatology 2002;35:953–9.
[19] Umemura T, Zen Y, Hamano H, Kawa S, Nakanuma Y, Kiyosawa K. Immunoglobin G4-hepatopathy: association of immunoglobin G4-bearing plasma cells in liver with autoimmune pancreatitis. Hepatology 2007;46:463–71.
[20] Yoneda S, Umemura T, Katsuyama Y, Kamijo A, Joshita S, Komatsu M, et al. Association of serum cytokine levels with treatment response to pegylated interferon and ribavirin therapy in genotype 1 chronic hepatitis C patients. J Infect Dis 2011;203:1087–95.
[21] Nozawa Y, Umemura T, Joshita S, Katsuyama Y, Shibata S, Kimura T, et al. KIR, HLA, and IL28B variant predict response to antiviral therapy in genotype 1 chronic hepatitis C patients in Japan. PLoS One 2013;8:e83381.
[22] Umemura T, Joshita S, Ichijo T, Yoshizawa K, Katsuyama Y, Tanaka E, et al. Human leukocyte antigen class II molecules confer both susceptibility and progression in Japanese patients with primary biliary cirrhosis. Hepatology 2012;55:506–11.
[23] Cooley S, Trachtenberg E, Bergemann TL, Saeteurn K, Klein J, Le CT, et al. Donors with group B KIR haplotypes improve relapse-free survival after unrelated hematopoietic cell transplantation for acute myelogenous leukemia. Blood 2009;113:726–32.
[24] Umemura T, Joshita S, Yoneda S, Katsuyama Y, Ichijo T, Matsumoto A, et al. Serum interleukin (IL)-10 and IL-12 levels and IL28B gene polymorphisms: pretreatment prediction of treatment failure in chronic hepatitis C. Antivir Ther 2011;16:1073–80.
[25] Cortina-Borja M, Smith AD, Combarros O, Lehmann DJ. The synergy factor: a statistic to measure interactions in complex diseases. BMC Res Notes 2009;2:105.
[26] Mandelboim O, Reyburn HT, Vales-Gomez M, Pazmany L, Colonna M, Borsellino G, et al. Protection from lysis by natural killer cells of group 1 and 2 specificity is mediated by residue 80 in human histocompatibility leukocyte antigen C alleles and also occurs with empty major histocompatibility complex molecules. J Exp Med 1996;184:913–22.
[27] Cella M, Longo A, Ferrara GB, Strominger JL, Colonna M. NK3-specific natural killer cells are selectively inhibited by Bw4-positive HLA alleles with isoleucine 80. J Exp Med 1994;180:1235–42.
[28] Yawata M, Yawata N, Draghi M, Little AM, Partheniou F, Parham P. Roles for HLA and KIR polymorphisms in natural killer cell repertoire selection and modulation of effector function. J Exp Med 2006;203:633–45.

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Retrospective Study

Mutations of pre-core and basal core promoter before and after hepatitis B e antigen seroconversion

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Abstract

AIM: To investigate the role of pre-core and basal core promoter (BCP) mutations before and after hepatitis B e antigen (HBeAg) seroconversion.

METHODS: The proportion of pre-core (G1896A) and basal core promoter (A1762T and G1764A) mutant viruses and serum levels of hepatitis B virus (HBV) DNA, hepatitis B surface antigen (HBsAg), and HB core-related antigen were analyzed in chronic hepatitis B patients before and after HBeAg seroconversion (n = 25), in those who were persistently HBeAg positive (n = 18), and in those who were persistently anti-HBe positive (n = 43). All patients were infected with HBV genotype C and were followed for a median of 9 years.

RESULTS: Although the pre-core mutant became predominant (24% to 65%, P = 0.022) in the HBeAg seroconversion group during follow-up, the proportion of the basal core promoter mutation did not change. Median HBV viral markers were significantly higher in patients without the mutations in an HBeAg positive status (HBV DNA: P = 0.003; HBsAg: P < 0.001; HB core-related antigen: P = 0.001). In contrast, HBV DNA (P = 0.012) and HBsAg (P = 0.041) levels were significantly higher in patients with the pre-core mutation in an anti-HBe positive status.

CONCLUSION: There is an opposite association of the pre-core mutation with viral load before and after HBeAg seroconversion in patients with HBV infection.

Key words: Seroconversion; Hepatitis B core-related antigen; Pre-core; Basal core promoter; Mutation; Hepatitis B surface antigen; Hepatitis B virus DNA

Core tip: The exact roles of pre-core (pre-C) and basal core promoter (BCP) mutations remain unclear before and after hepatitis B e antigen (HBeAg) seroconversion.

Here, although the pre-C mutant became predominant in the HBeAg seroconversion group during follow-up, the proportion of the BCP mutation did not change. Hepatitis B virus (HBV) viral markers were significantly higher in patients without the mutations in an HBeAg positive status. HBV DNA and hepatitis B surface antigen levels were higher in patients with the pre-C mutation in an anti-HBe positive status. Taken together, the association of the pre-C mutation on viral load appears to be opposite before and after HBeAg seroconversion in patients with HBV infection.

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INTRODUCTION

Hepatitis B virus (HBV) infection is a major health concern that has an estimated 350 to 400 million carriers worldwide. Chronic infection with HBV can cause chronic hepatitis, which may eventually develop into liver cirrhosis and hepatocellular carcinoma[1-4].

In the natural history of chronic HBV infection, seroconversion from hepatitis B e antigen (HBeAg) to its antibody (anti-HBe) is usually accompanied by a decrease in HBV replication and the remission of hepatitis[5-7]. Thus, HBeAg seroconversion is a favorable sign for patients with chronic hepatitis B. However, there are some patients who persistently exhibit elevated HBV DNA levels in the serum and active liver disease, even after seroconversion[8,9].

Several mutations in the HBV genome have been reported to associate with HBeAg seroconversion. When the pre-core (pre-C) and core genes in the HBV genome are transcribed and translated in tandem, HBeAg is produced and secreted into the circulation[10,11]. The G to A mutation at nucleotide (nt) 1896 in the pre-C region (G1896A), which converts codon 28 for tryptophan to a stop codon, is associated with the loss of detectable HBeAg[12,13]. The double mutations of A1762T and G1764A in the basal core promoter (BCP) of the HBV genome have also been shown to reduce HBeAg synthesis by suppressing the transcription of pre-C mRNA[14-16]. However, the detailed mechanisms of HBeAg seroconversion, including the involvement of mutations that decrease the production of HBeAg, have not been fully clarified. Orito *et al*[17] reported that a predominance of the pre-C mutation was correlated with anti-HBe, while BCP mutations were not associated with either anti-HBe or HBeAg. We previously uncovered that the pre-C and BCP mutations were frequently seen in patients with active replication after HBeAg seroconversion, but not in those with inactive replication[18], which suggested that HBeAg seroconversion was not associated with either mutation in such patients. Since the follow-up duration of these previous reports was limited, this study analyzed the changes in pre-C and BCP mutations among patients who were followed over a longer time course. Furthermore, we assessed the mutations not only in patients who seroconverted from HBeAg to anti-HBe, but also in those whose HBeAg or anti-HBe positive status did not change during follow-up.

MATERIALS AND METHODS

Patients

Three groups of patients with chronic hepatitis B who were categorized according to HBeAg/anti-HBe positive status were enrolled between 1985 and 2000. The subjects were selected retrospectively from a database of patients who had been followed for at least two years, had not received anti-viral therapy, such as nucleos(t)ide analogues, and whose stored serum samples were available from both the start and end of follow-up. We recruited only patients with HBV genotype C since this genotype is predominant in Japan and because the clinical significance of pre-C and BCP mutations differs among genotypes. The first group consisted of 18 patients whose HBeAg was persistently positive throughout the study period. The second group contained 25 patients in whom HBeAg seroconverted to anti-HBe. The third group was made up of 43 patients whose anti-HBe was persistently positive.

Hepatitis B surface antigen (HBsAg) was confirmed to be positive on at least two occasions a minimum of 6 mo apart in all patients before the start of follow-up. Tests for hepatitis C and human immunodeficiency virus antibodies were negative in all subjects. Patients who demonstrated accompanying hepatocellular carcinoma or signs of hepatic failure at the initial follow-up were excluded from the study.

Stored serum samples were kept frozen at -20 ℃ or below until assayed. This study was approved by the Ethics Committee of Shinshu University School of Medicine.

Conventional hepatitis B viral markers

Serological markers for HBV, including HBsAg, HBeAg, and anti-HBe, were tested using commercially available enzyme immunoassay kits (Fujirebio Inc., Tokyo, Japan)[19]. HBsAg was quantified[20] using a chemiluminescence enzyme immunoassay (CLEIA)-based HISCL HBsAg assay manufactured by Sysmex Corporation (Kobe, Japan). The assay had a quantitative range of -1.5 to 3.3 log IU/mL. End titer was determined by diluting samples with normal human serum when initial results exceeded the upper limit of the assay range.

Serum HBV DNA was determined using a COBAS TaqMan HBV kit (Roche, Tokyo, Japan)[21] with a quantitative range of 2.1 to 8.9 log copies/mL. According to the manufacturer's instructions, detection of a positive signal below the quantitative range was described as a positive signal and no signal detection was considered to be a negative signal. Six HBV genotypes (A-F) were

Table 1 Clinical and virological backgrounds among 3 groups of patients classified according to changes in the serum hepatitis B e antigen/anti-hepatitis B e

Characteristic	HBeAg/anti-HBe status			*P* value
	Continuously +/- (*n* = 18)	From +/- to -/+ (*n* = 25)	Continuously -/+ (*n* = 43)	
Age (yr)[1]	44 (24-63)	37 (18-53)	51 (25-77)	< 0.001
Gender (M:F)	11:7	14:11	24:19	> 0.2
Follow-up period (yr)[1]	6.3 (2.1-14.6)	10.8 (2.0-23.7)	8.5 (2.2-16.6)	0.006
Genotype C[2]	18 (100)	25 (100)	43 (100)	1
Viral markers at first follow-up				
HBV DNA (log copies/mL)[1]	8.6 (5.7-> 8.9)	6.1 (< 2.1-> 8.9)	< 2.1 (< 2.1-8.2)	< 0.001
HBsAg (log IU/mL)[1]	4.6 (1.6-5.5)	3.6 (-0.9-4.6)	2.6 (< 0.05-4.3)	< 0.001
HBcrAg (log U/mL)[1]	> 6.8 (5.5->6.8)	6.8 (3.1-> 6.8)	3.0 (< 3.0-6.8)	< 0.001
Viral markers at final follow-up				
HBV DNA (log copies/mL)[1]	7.1 (< 2.1-> 8.9)	3.3 (neg.-6.2)	< 2.1 (neg.-7.0)	< 0.001
HBsAg (log IU/mL)[1]	3.3 (1.0-5.1)	2.8 (< 0.05-2.8)	1.3 (< 0.05-4.2)	< 0.001
HBcrAg (log U/mL)[1]	6.7 (4.4-> 6.8)	< 3.0 (< 3.0-6.2)	< 3.0 (< 3.0-5.3)	< 0.001

[1]Data are expressed as the median (range); [2]Data are expressed as a positive number (%). HBeAg: Hepatitis B e antigen; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; HBcrAg: Hepatitis B core-related antigen.

evaluated according to the restriction patterns of DNA fragments from the method reported by Mizokami *et al*[22]. Serum hepatitis B core-related antigen (HBcrAg) levels were measured using a CLEIA HBcrAg assay kit with a fully automated Lumipulse System analyzer (Fujirebio Inc.) as described previously[23,24]. The HBcrAg assay simultaneously measured all antigens (e, core, and p22cr) encoded by the pre-C/core genes of HBV. The immunoreactivity of pro-HBeAg at 10 fg/mL was defined as 1 U/mL. We expressed HBcrAg in terms of log U/mL with a quantitative range of 3.0 to 6.8 log U/mL.

Determination of pre-C and BCP mutations

The pre-C and BCP mutations were determined using nucleic acid samples extracted from 100 μL of serum with a DNA/RNA extraction kit (Smitest EX-R and D; Genome Science Laboratories Co., Ltd., Tokyo, Japan). The stop codon mutation in the pre-C region (A1896) was detected with an enzyme-linked mini-sequence assay kit (Smitest; Genome Science Laboratories). In principle, G1896 in wild type HBV and A1896 in the mutant were determined by mini-sequence reactions using labeled nucleotides that were complementary to either the wild type or mutant[25]. The results were expressed as percent mutation rates according to the definition by Aritomi *et al*[26] Samples were judged as positive for the pre-C mutation when the mutation rate exceeded 50% in the present study since the mutation rate was found to steadily increase to 100% once surpassing 50%[25].

The double mutation in the BCP was detected using an HBV core promoter detection kit (Smitest; Genome Science Laboratories)[25,26]. This kit detected T1762 and/or A1764 using the polymerase chain reaction (PCR) with primers specific for either wild type or mutant BCP. Results were recorded as wild, mixed, or mutant type. The pre-C and BCP mutations were tested at the start and end of follow-up with kits having manufacturer-established detection limits of 1000 copies/mL.

Full HBV genome sequencing

The nucleotide sequences of full-length HBV genomes were determined by a method reported previously[27]. Briefly, two overlapping fragments of an HBV genome were amplified by PCR, and then eight overlapping HBV DNA fragments were amplified by nested PCR. All necessary precautions to prevent cross-contamination were taken and negative controls were included in each assay. The sequencing reaction was performed according to the manufacturer's instructions (ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kits, Version 3.1; Foster City, CA) with an automated ABI DNA sequencer (Model 3100, Applied Biosystems Carlsbad, CA).

Statistical analyses

The proportions of clinical factors were compared among groups using the χ^2 and Fisher's exact probability tests. Group medians were compared by means of the Mann-Whitney *U* test and Kruskal-Wallis test. The changes in proportions of the pre-C and BCP mutations between the study start and end points were compared using McNemar's test. All tests were performed using the IBM SPSS Statistics Desktop for Japan ver. 19.0 (IBM Japan Inc., Tokyo, Japan). *P* values of less than 0.05 were considered to be statistically significant.

RESULTS

Patients

The clinical and virological backgrounds of the 3 groups are summarized in Table 1. Median age was lowest in patients with seroconversion, intermediate in those with persistent HBeAg, and highest in those with persistent anti-HBe. Gender ratio was similar among the 3 groups. Following our study design, all patients had HBV ge-

notype C.

Changes in pre-C and BCP mutations

The presence of the pre-C mutation could be evaluated in 60 (98%) of 61 HBeAg positive samples and 94 (85%) of 111 HBeAg negative samples. We were able to assess the existence of the BCP mutation in 57 (93%) of 61 HBeAg positive samples and 86 (77%) of 111 HBeAg negative samples.

The changes in the proportion of the pre-C mutation between the start and end of follow-up are shown in Figure 1A. Wild type pre-C accounted for 94% of patients whose HBeAg was continuously positive at study onset and remained constant. Wild type pre-C was also predominant at the start of follow-up (76%, 19/25) in patients who experienced HBeAg seroconversion, but the mutant type had become predominant ($P = 0.022$) by the end of follow-up (65%, 15/23); 11 of 19 wild type pre-C patients converted to mutant type, while 2 of 6 patients with mutant type pre-C reverted to wild type. Mutant type pre-C accounted for 62% of the patients who were continuously positive for anti-HBe at study onset. Such patients with wild type pre-C at the start of follow-up tended to maintain this status (78%), although 22% of initially mutant type pre-C subjects had changed to wild type by the study end point ($P = 0.687$).

Of the 143 samples with determined BCP mutations, 34 (24%) were wild, 11 (8%) were mixed, and 98 (69%) were mutant types. Because few patients with mixed type BCP reverted to wild type in the present and past studies[18], samples were considered to be positive for the BCP mutation when they were either mixed or mutant type.

The changes in the proportion of the BCP mutation between the start and end of follow-up are shown in Figure 1B. Mutant type BCP accounted for 61% of patients whose HBeAg was continuously positive at study onset and remained constant. In patients who experienced HBeAg seroconversion, mutant type BCP was predominant at the start of follow-up (84%, 21/25) and remained so (80%, 16/20) until final follow-up; 3 of 4 patients with wild type BCP and 15 of 16 patients with mutant type BCP maintained their status throughout the study period. Mutant type BCP initially accounted for 82% of patients who were continuously positive for anti-HBe. Both wild (60%) and mutant (84%) types tended to remain constant until the study end point. When all points of measurement were counted for which both pre-C and BCP mutations were evaluated, the prevalence of the pre-C mutation (18%, 9/57) was significantly lower than that of the BCP mutation (82%, 42/57) in patients with persistent HBeAg ($P < 0.001$), as well as in subjects with persistent anti-HBe [62% (53/86) *vs* 78% (67/86), $P = 0.030$], albeit to a lesser degree.

Comparison of viral loads according to pre-C/BCP mutation and HBeAg/anti-HBe positive status

We next compared the serum levels of HBV DNA, HBsAg, and HBcrAg according to pre-C and BCP mutation and HBeAg and anti-HBe positive status (Figure 2). Both pre-C and BCP mutations could be evaluated in 57 (93%) of 61 HBeAg positive samples and 86 (77%) of 111 HBeAg negative samples. HBV DNA levels were significantly higher in an HBeAg positive status than in an anti-HBe positive status ($P < 0.001$) and significantly higher in patients without the mutations than in those with at least one mutation in an HBeAg positive status ($P < 0.01$). On the other hand, HBV DNA levels were significantly lower in patients without the pre-C mutation than in those with it in an anti-HBe positive status ($P = 0.012$).

A similar tendency to HBV DNA levels was observed for HBsAg levels. HBsAg levels were significantly higher in an HBeAg positive status than in an anti-HBe positive status ($P < 0.001$) and significantly higher in patients without the mutations than in those with at least one mutation in an HBeAg positive status ($P < 0.001$). HBsAg levels were significantly higher in patients with the pre-C mutation than in those without it irrespectively of the existence of the BCP mutation ($P = 0.041$).

HBcrAg levels were significantly lower with presence of pre-C and/or BCP mutations in an HBeAg positive status ($P < 0.05$, respectively). HBcrAg levels were uniformly low regardless of the presence of mutations in anti-HBe positive status subjects.

Full genome sequences in patients with and without appearance of the pre-C mutation

Full HBV genome sequences were determined after HBeAg seroconversion in 6 patients who seroconverted without the appearance of the pre-C mutation. All patients were positive for BCP mutations: 1 subject had T1753G and C1766T mutations, although the other mutations reported by Okamoto *et al*[14] were not identified.

DISCUSSION

Although both pre-C and BCP mutations have been associated with HBeAg seroconversion by reducing the production of HBeAg[13-15], their manifestation patterns appear to be different[17]. In the present study, the BCP mutation was already prevalent during the HBeAg positive chronic hepatitis phase and approached 80% around the time of HBeAg seroconversion. On the other hand, the pre-C mutation clearly manifested following the time of seroconversion. These results indicate that the appearance of the pre-C mutation, but not the BCP mutation, is directly associated with seroconversion. It is noteworthy that a considerable number of patients experienced HBeAg seroconversion without evidence of the pre-C G1896A mutation. Furthermore, wild type pre-C remained unchanged in almost all patients whose anti-HBe was continuously positive. Thus, two types of HBeAg seroconversion may exist for chronic HBV in terms of the appearance or absence of the G1896A pre-C mutation. We previously speculated on the possible

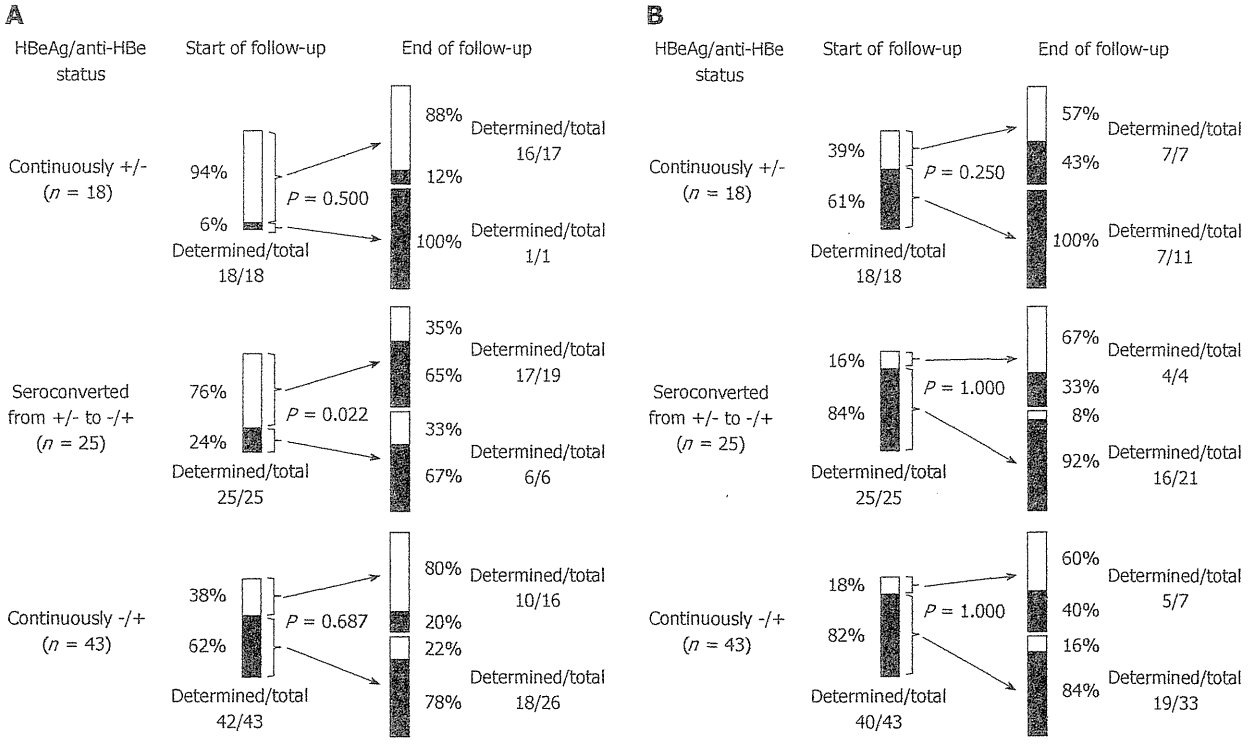

Figure 1 Comparison of changes in pre-core (A) and basal core promoter (B) mutation type among 3 groups of patients classified according to hepatitis B e antigen /anti-hepatitis B e positive status. A: A significant difference was seen in patients with hepatitis B e antigen (HBeAg) seroconversion ($P = 0.022$). One patient whose pre-core (pre-C) mutation was undetermined at the start of follow-up was wild type at the end point; B: Of the 3 patients whose basal core promoter (BCP) mutation was undetermined at the start of follow-up, 2 were wild type and 1 was undetermined at the end point. HBeAg: Hepatitis B e antigen.

existence of two seroconversion types in an analysis of HBV patients who experienced seroconversion[18]. Here, we were able to strengthen this notion by including patients who maintained an HBeAg or anti-HBe positive status in a study of longer duration. It should be noted that the absence of the pre-C G1896A mutation does not necessarily indicate the absence of mutations that halt HBeAg production; several patterns of mutations apart from G1896A have been associated with an HBeAg negative phenotype, such as point mutations in the ATG initiation region and deletion/insertion of nucleotides leading to premature termination[13]. Accordingly, we analyzed full genome sequences in 6 patients who seroconverted without the appearance of the pre-C mutation and uncovered T1753G and C1766T mutations in one subject[14] that might be associated with seroconversion. We observed that several patients reverted from mutant pre-C to wild type in the present report. As this important finding has not been confirmed by sequence analysis, we are planning to determine and compare entire genomic sequences using paired samples before and after HBeAg seroconversion in a future study.

We witnessed that serum HBV DNA was significantly lower in patients with the pre-C and/or BCP mutation in an HBeAg positive phase, which indicated that immune processes from the host to eliminate HBV were stronger in individuals with the mutations than in those without. This also supported the generally held belief that pre-C and BCP mutations appear as a result of host immune pressure[14]. Contrary to the HBeAg positive phase, HBV DNA was significantly higher in subjects with the pre-C mutation in an anti-HBe positive phase. Kawabe *et al*[28] have reported that patients with wild type pre-C demonstrate significantly lower viral loads and ALT levels than those with mutant pre-C among HBeAg negative patients with HBV genotype C infection. Collectively, these results imply that patients with the pre-C mutant have a higher potential to progress to hepa-titis after HBeAg seroconversion. This is consistent with the fact that HBeAg negative hepatitis is usually caused by HBeAg non-producing mutant strains of HBV. Indeed, viral replication seems to be considerably suppressed in patients with wild type HBV after achieving HBeAg seroconversion since this strain has the ability to produce HBeAg when actively replicated.

We adopted serum levels of HBsAg, HBcrAg, and HBV DNA in the present study as markers to estimate HBV replication activity. HBsAg and HBcrAg levels have been reported to reflect HBV cccDNA levels in hepatocytes[20,24,29]. HBsAg has also attracted attention as a useful predictor of treatment outcome by interferon and others[30]. Furthermore, the loss of HBsAg is an important indicator in the treatment of HBV carriers. HBcrAg assays simultaneously measure all antigens encoded by the pre-C/core genome, which include the HB core, e, and p22cr antigens, and have been reported to predict the clinical outcome of patients treated with nucleotide or nucleoside analogues[31]. HBsAg patterns according

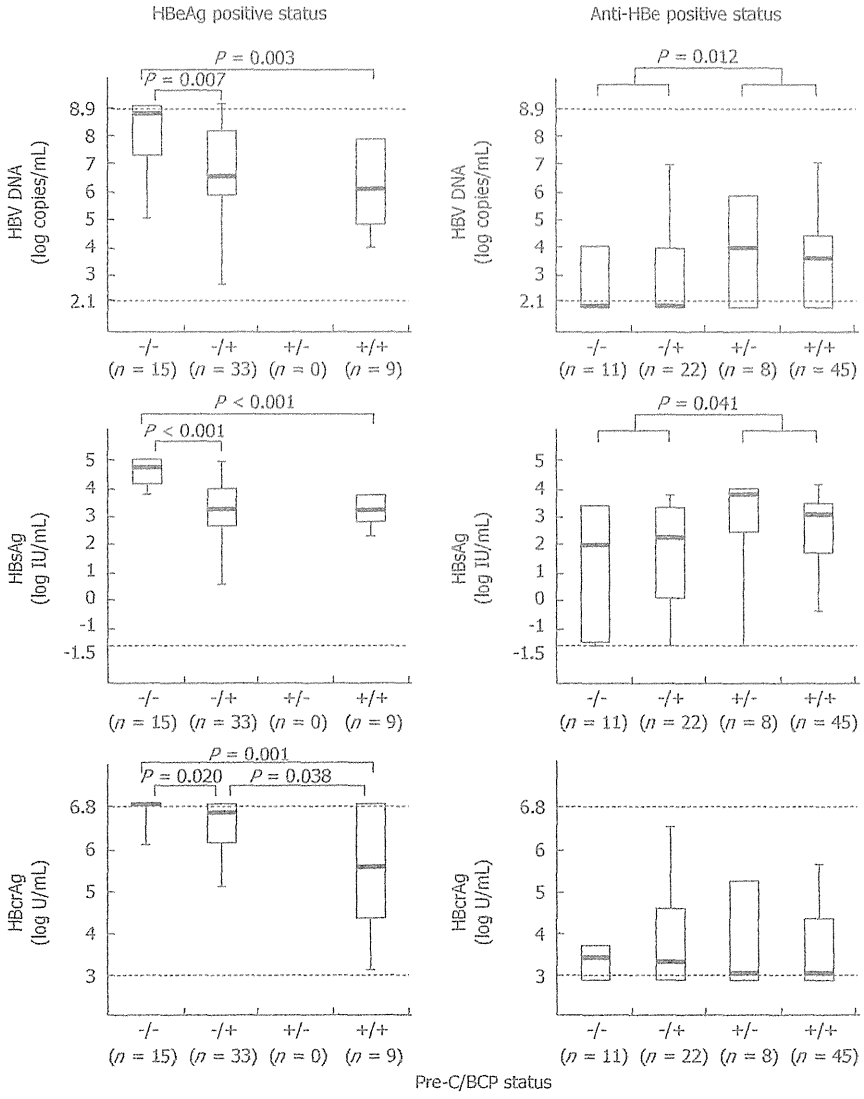

Figure 2 Comparison of serum hepatitis B virus DNA, hepatitis B surface antigen, and hepatitis core-related antigen levels among patients with wild (-/-) and mutant types of the pre-core and basal core promoter mutations. Fifty-seven of 61 samples obtained from HBeAg positive cases and 86 of 111 samples obtained from anti-HBeAg positive cases were eligible for analysis. HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen; HBsAg: Hepatitis B surface antigen; HBcrAg: Hepatitis core-related antigen; pre-C: Pre-core; BCP: Basal core promoter.

to HBeAg/anti-HBe and pre-C/BCP status were similar to HBV DNA patterns both in HBeAg and anti-HBe positive states; HBsAg was significantly lower in patients with pre-C and/or BCP mutations than in those with wild type pre-C but was significantly higher in patients with the pre-C mutation than in those without it in an anti-HBe positive state. These results confirmed that the pre-C mutation was oppositely associated with viral load in patients before and after HBeAg seroconversion. Since elevated levels of HBV DNA and HBsAg are related to a higher rate of hepatocarcinogenesis, pre-C mutation patterns appear to be clinically important, at least in the context of HBV genotype C patients. We witnessed that the patterns of HBcrAg were similar to those of HBV DNA in the HBeAg positive state but different in the anti-HBe positive state. This difference may reflect the fact that the main antigen measured by the HBcrAg assay is HBeAg.

In conclusion, our findings indicate that the association of the pre-C G1896A mutation on viral load is opposite before and after HBeAg seroconversion in patients with HBV infection in that its presence results in a higher viral load after seroconversion. These observations may shed light on the pathology and treatment of chronic hepatitis B, especially that of an anti-HBe positive status.

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COMMENTS

Background

Although pre-core (pre-C) and/or basal core promoter (BCP) mutations in the hepatitis B virus (HBV) genome have been reported to associate with hepatitis

B e antigen (HBeAg) seroconversion, the detailed mechanisms have not been fully clarified.

Research frontiers

In this study, the authors show that the association of the pre-C mutation on viral load is opposite before and after HBeAg seroconversion in patients with HBV infection in that its presence results in a higher viral load after seroconversion.

Innovations and breakthroughs

Recent reports have highlighted the importance of pre-C and BCP mutations of the HBV genome in association with HBeAg seroconversion. This study analyzed the changes in pre-C and BCP mutations in patients over a long follow-up period. The authors demonstrate that the association of the pre-C mutation on viral load is opposite before and after HBeAg seroconversion in patients with HBV infection.

Applications

This study may shed light on the pathology and treatment of chronic hepatitis B, especially that of an anti-HBe positive status.

Terminology

In the natural history of chronic HBV infection, seroconversion from HBeAg to anti-HBe is usually accompanied by a decrease in HBV replication and the remission of hepatitis. Thus, HBeAg seroconversion is a favorable sign for patients with chronic hepatitis B. However, there are some patients who persistently exhibit elevated HBV DNA levels in the serum and active liver disease, even after seroconversion.

Peer review

The authors investigated the pre-C and/or BCP mutations before and after HBeAg seroconversion. They found that the association of the pre-C mutation on viral load is opposite in patients before and after HBeAg seroconversion. It is an interesting report. However there are several concerns.

REFERENCES

1 **Hoofnagle JH**, Doo E, Liang TJ, Fleischer R, Lok AS. Management of hepatitis B: summary of a clinical research workshop. *Hepatology* 2007; **45**: 1056-1075 [PMID: 17393513 DOI: 10.1002/hep.21627]

2 **Lok AS**, McMahon BJ. Chronic hepatitis B. *Hepatology* 2007; **45**: 507-539 [PMID: 17256718 DOI: 10.1002/hep.21513]

3 **Lee WM**. Hepatitis B virus infection. *N Engl J Med* 1997; **337**: 1733-1745 [PMID: 9392700 DOI: 10.1056/NEJM199712113372406]

4 **Umemura T**, Ichijo T, Yoshizawa K, Tanaka E, Kiyosawa K. Epidemiology of hepatocellular carcinoma in Japan. *J Gastroenterol* 2009; **44** Suppl 19: 102-107 [PMID: 19148802 DOI: 10.1007/s00535-008-2251-0]

5 **Hoofnagle JH**, Dusheiko GM, Seeff LB, Jones EA, Waggoner JG, Bales ZB. Seroconversion from hepatitis B e antigen to antibody in chronic type B hepatitis. *Ann Intern Med* 1981; **94**: 744-748 [PMID: 7235415 DOI: 10.7326/0003-4819-94-6-744]

6 **Liaw YF**, Chu CM, Su IJ, Huang MJ, Lin DY, Chang-Chien CS. Clinical and histological events preceding hepatitis B e antigen seroconversion in chronic type B hepatitis. *Gastroenterology* 1983; **84**: 216-219 [PMID: 6848402]

7 **Realdi G**, Alberti A, Rugge M, Bortolotti F, Rigoli AM, Tremolada F, Ruol A. Seroconversion from hepatitis B e antigen to anti-HBe in chronic hepatitis B virus infection. *Gastroenterology* 1980; **79**: 195-199 [PMID: 7399226]

8 **Bonino F**, Rosina F, Rizzetto M, Rizzi R, Chiaberge E, Tardanico R, Callea F, Verme G. Chronic hepatitis in HBsAg carriers with serum HBV-DNA and anti-HBe. *Gastroenterology* 1986; **90**: 1268-1273 [PMID: 3956945]

9 **Hsu YS**, Chien RN, Yeh CT, Sheen IS, Chiou HY, Chu CM, Liaw YF. Long-term outcome after spontaneous HBeAg seroconversion in patients with chronic hepatitis B. *Hepatology* 2002; **35**: 1522-1527 [PMID: 12029639 DOI: 10.1053/jhep.2002.33638]

10 **Bruss V**, Gerlich WH. Formation of transmembraneous hepatitis B e-antigen by cotranslational in vitro processing of the viral precore protein. *Virology* 1988; **163**: 268-275 [PMID: 3354197 DOI: 10.1016/0042-6822(88)90266-8]

11 **Garcia PD**, Ou JH, Rutter WJ, Walter P. Targeting of the hepatitis B virus precore protein to the endoplasmic reticulum membrane: after signal peptide cleavage translocation can be aborted and the product released into the cytoplasm. *J Cell Biol* 1988; **106**: 1093-1104 [PMID: 3283145 DOI: 10.1083/jcb.106.4.1093]

12 **Carman WF**, Jacyna MR, Hadziyannis S, Karayiannis P, McGarvey MJ, Makris A, Thomas HC. Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. *Lancet* 1989; **2**: 588-591 [PMID: 2570285 DOI: 10.1016/S0140-6736(89)90713-7]

13 **Okamoto H**, Yotsumoto S, Akahane Y, Yamanaka T, Miyazaki Y, Sugai Y, Tsuda F, Tanaka T, Miyakawa Y, Mayumi M. Hepatitis B viruses with precore region defects prevail in persistently infected hosts along with seroconversion to the antibody against e antigen. *J Virol* 1990; **64**: 1298-1303 [PMID: 2304145]

14 **Okamoto H**, Tsuda F, Akahane Y, Sugai Y, Yoshiba M, Moriyama K, Tanaka T, Miyakawa Y, Mayumi M. Hepatitis B virus with mutations in the core promoter for an e antigen-negative phenotype in carriers with antibody to e antigen. *J Virol* 1994; **68**: 8102-8110 [PMID: 7966600]

15 **Buckwold VE**, Xu Z, Chen M, Yen TS, Ou JH. Effects of a naturally occurring mutation in the hepatitis B virus basal core promoter on precore gene expression and viral replication. *J Virol* 1996; **70**: 5845-5851 [PMID: 8709203]

16 **Takahashi K**, Aoyama K, Ohno N, Iwata K, Akahane Y, Baba K, Yoshizawa H, Mishiro S. The precore/core promoter mutant (T1762A1764) of hepatitis B virus: clinical significance and an easy method for detection. *J Gen Virol* 1995; **76** (Pt 12): 3159-3164 [PMID: 8847524 DOI: 10.1099/0022-1317-76-12-3159]

17 **Orito E**, Mizokami M, Sakugawa H, Michitaka K, Ishikawa K, Ichida T, Okanoue T, Yotsuyanagi H, Iino S. A case-control study for clinical and molecular biological differences between hepatitis B viruses of genotypes B and C. Japan HBV Genotype Research Group. *Hepatology* 2001; **33**: 218-223 [PMID: 11124839 DOI: 10.1053/jhep.2001.20532]

18 **Misawa N**, Matsumoto A, Tanaka E, Rokuhara A, Yoshizawa K, Umemura T, Maki N, Kimura T, Kiyosawa K. Patients with and without loss of hepatitis B virus DNA after hepatitis B e antigen seroconversion have different virological characteristics. *J Med Virol* 2006; **78**: 68-73 [PMID: 16299733]

19 **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 [PMID: 18643758 DOI: 10.1086/590968]

20 **Matsumoto A**, Tanaka E, Morita S, Yoshizawa K, Umemura T, Joshita S. Changes in the serum level of hepatitis B virus (HBV) surface antigen over the natural course of HBV infection. *J Gastroenterol* 2012; **47**: 1006-1013 [PMID: 22370816 DOI: 10.1007/s00535-012-0559-2]

21 **Ronsin C**, Pillet A, Bali C, Denoyel GA. Evaluation of the COBAS AmpliPrep-total nucleic acid isolation-COBAS TaqMan hepatitis B virus (HBV) quantitative test and com-parison to the VERSANT HBV DNA 3.0 assay. *J Clin Microbiol* 2006; **44**: 1390-1399 [PMID: 16597867]

22 **Mizokami M**, Nakano T, Orito E, Tanaka Y, Sakugawa H, Mukaide M, Robertson BH. Hepatitis B virus genotype assignment using restriction fragment length polymorphism patterns. *FEBS Lett* 1999; **450**: 66-71 [PMID: 10350059]

23 **Kimura T**, Rokuhara A, Sakamoto Y, Yagi S, Tanaka E, Kiyosawa K, Maki N. Sensitive enzyme immunoassay for hepatitis B virus core-related antigens and their correlation to virus load. *J Clin Microbiol* 2002; **40**: 439-445 [PMID: 11825954]

24 **Suzuki F**, Miyakoshi H, Kobayashi M, Kumada H. Correlation between serum hepatitis B virus core-related antigen and

intrahepatic covalently closed circular DNA in chronic hepatitis B patients. *J Med Virol* 2009; **81**: 27-33 [PMID: 19031469 DOI: 10.1002/jmv.21339]

25 **Yamaura T**, Tanaka E, Matsumoto A, Rokuhara A, Orii K, Yoshizawa K, Miyakawa Y, Kiyosawa K. A case-control study for early prediction of hepatitis B e antigen seroconversion by hepatitis B virus DNA levels and mutations in the precore region and core promoter. *J Med Virol* 2003; **70**: 545-552 [PMID: 12794716 DOI: 10.1002/jmv.10429]

26 **Aritomi T**, Yatsuhashi H, Fujino T, Yamasaki K, Inoue O, Koga M, Kato Y, Yano M. Association of mutations in the core promoter and precore region of hepatitis virus with fulminant and severe acute hepatitis in Japan. *J Gastroenterol Hepatol* 1998; **13**: 1125-1132 [PMID: 9870800]

27 **Sugauchi F**, Mizokami M, Orito E, Ohno T, Kato H, Suzuki S, Kimura Y, Ueda R, Butterworth LA, Cooksley WG. A novel variant genotype C of hepatitis B virus identified in isolates from Australian Aborigines: complete genome sequence and phylogenetic relatedness. *J Gen Virol* 2001; **82**: 883-892 [PMID: 11257194]

28 **Kawabe N**, Hashimoto S, Harata M, Nitta Y, Murao M, Nakano T, Shimazaki H, Arima Y, Komura N, Kobayashi K, Yoshioka K. The loss of HBeAg without precore mutation results in lower HBV DNA levels and ALT levels in chronic hepatitis B virus infection. *J Gastroenterol* 2009; **44**: 751-756 [PMID: 19430716 DOI: 10.1007/s00535-009-0061-7]

29 **Chan HL**, Wong VW, Tse AM, Tse CH, Chim AM, Chan HY, Wong GL, Sung JJ. Serum hepatitis B surface antigen quantitation can reflect hepatitis B virus in the liver and predict treatment response. *Clin Gastroenterol Hepatol* 2007; **5**: 1462-1468 [PMID: 18054753 DOI: 10.1016/j.cgh.2007.09.005]

30 **Li WC**, Wang MR, Kong LB, Ren WG, Zhang YG, Nan YM. Peginterferon alpha-based therapy for chronic hepatitis B focusing on HBsAg clearance or seroconversion: a meta-analysis of controlled clinical trials. *BMC Infect Dis* 2011; **11**: 165 [PMID: 21651820 DOI: 10.1186/1471-2334-11-165]

31 **Tanaka E**, Matsumoto A. Guidelines for avoiding risks resulting from discontinuation of nucleoside/nucleotide analogs in patients with chronic hepatitis B. *Hepatol Res* 2014; **44**: 1-8 [PMID: 23607862 DOI: 10.1111/hepr.12108]

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Original Article

Factors associated with the effect of interferon-α sequential therapy in order to discontinue nucleoside/nucleotide analog treatment in patients with chronic hepatitis B

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Aim: The factors associated with the outcome of sequential therapy with interferon-α (IFN-α) in order to halt nucleoside/nucleotide analog (NUC) maintenance treatment for chronic hepatitis B were analyzed.

Methods: A total of 50 patients with chronic hepatitis B who underwent IFN-α sequential therapy for cessation of NUC were enrolled retrospectively. The subjects received NUC plus IFN-α for 4 weeks followed by IFN-α alone for 20 weeks. Natural IFN-α of 6-MU doses was administrated three times a week. A successful response to NUC/IFN-α sequential therapy was defined as serum hepatitis B virus (HBV) DNA below 4.0 log copies/mL, serum alanine aminotransferase (ALT) below 30 IU/L, and hepatitis B e-antigen negativity at 24 months after completing the treatment.

Results: Multivariate analysis revealed that hepatitis B surface antigen (HBsAg) of 3.0 log U/mL or more ($P < 0.002$) and hepatitis B core-related antigen (hepatitis B core-related antigen [HBcrAg]) of 4.5 log U/mL or more ($P < 0.003$) at the start of IFN-α administration were significant factors associated with a 24-month non-response. Maximal levels of ALT and HBV DNA during the follow-up period after completing IFN-α therapy were significantly related ($P < 0.001$), and receiver operating characteristic analysis showed that both maximal ALT ($P < 0.001$) and HBV DNA ($P < 0.001$) were significantly related to the final 24-month response.

Conclusion: The combinational use of HBsAg and HBcrAg levels may be useful to predict the 24-month outcome of NUC/IFN-α sequential therapy. Maximal levels of ALT and HBV DNA during post-treatment follow-up may also help monitor responses to IFN-α sequential therapy.

Key words: hepatitis B core-related antigen, hepatitis B surface antigen, interferon-α, nucleoside/nucleotide analogs, sequential therapy

INTRODUCTION

HEPATITIS B VIRUS (HBV) infection is a widespread health problem with an estimated 350–400 million carriers worldwide. Prolonged infection with HBV can cause chronic hepatitis, which may eventually develop into liver cirrhosis and hepatocellular carcinoma (HCC).[1-3] Currently available antiviral treatments for hepatitis B include nucleoside/nucleotide analogs (NUC) and interferon-α (IFN-α).[4] NUC are p.o. administrated and are associated with low rates of adverse effects. Although treatment with NUC, such as lamivudine (LVD), adefovir dipivoxil and entecavir (ETV), induces virological and biochemical responses in most patients, NUC therapy also carries the risk of drug resistance. Furthermore, patients with hepatitis B are required to undergo extended

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treatment with NUC because early discontinuance often leads to relapse.[5,6] In contrast, the remission of chronic hepatitis B by IFN-α is prolonged, but is achieved only in a small percentage of patients.

Serfaty *et al.*[7] conducted a pilot study on sequential therapy using LVD and IFN-α and concluded that this treatment could induce a sustained virological response in patients with chronic hepatitis B who did not respond to IFN-α alone. However, ensuing reports[8-12] were unable to confirm such a cooperative effect. Because the clinical backgrounds of the enrolled patients also differed among the above reports, it has become necessary to clarify the factors associated with the outcome of IFN-α sequential therapy in order to estimate its clinical significance.

We previously analyzed patients with chronic hepatitis B who ceased NUC therapy and showed that lower hepatitis B surface antigen (HBsAg) and hepatitis B core-related antigen (HBcrAg) levels were associated with a favorable clinical outcome in subjects negative for hepatitis B e-antigen (HBeAg) and HBV DNA at NUC discontinuation.[13,14] Although we identified patients in whom NUC could be safely halted with high reliance, such patients accounted for a relatively minor percentage. Therefore, we conducted the present study to analyze the effect of IFN-α sequential therapy on successfully stopping NUC.

This report retrospectively analyzes the factors associated with outcome of IFN-α sequential therapy following NUC treatment. As the subjects were followed long term, treatment responses at 24 months after stopping IFN-α were evaluated and compared with those at 6 and 12 months.

METHODS

Patients

A TOTAL OF 50 patients with chronic hepatitis B who underwent IFN-α sequential therapy in order to halt NUC therapy between May 2002 and September 2010 were enrolled. Subjects received NUC plus IFN-α for 4 weeks followed by IFN-α alone for 20 weeks (Fig. 1). Natural IFN-α (Sumiferon; Sumitomo Dainippon Pharma, Tokyo, USA) at a dose of 6 MU was administrated three times a week. Doses were reduced to 3 MU during exceptional circumstances, such as side-effects. All patients completed 24 weeks of IFN-α administration and received over 80% of the scheduled dose. Patients were recruited retrospectively from eight hospitals across Japan (Shinshu University Hospital, National Hospital Organization Nagasaki Medical Center, Toranomon Hospital, Hiroshima University Hospital, Chiba University Hospital, The Hospital of Hyogo College of Medicine, Kumamoto Shinto General Hospital,and Teine Keijinkai Hospital). The demographic data of the subjects are presented in Table 1. The median age at NUC cessation was 35 years. Approximately three-fourths of the patients were men. Genotype C HBV was predominant as has earlier been reported for Japan.[15] Eighty-six percent of patients began NUC therapy with LVD and 14% did so with ETV. The duration of NUC administration ranged from 4 to 121 months. The follow-up period was defined as the point of stopping IFN-α administration up until the last visit or to when NUC were re-administrated due to reactivation of hepatitis B. NUC were recommenced in 25 (50%) of the 50 patients enrolled. Among them, 17 were treated before judgment of the 24-month response to sequential therapy. All patients requiring re-administration

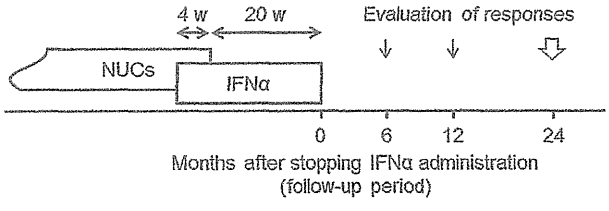

Figure 1 Experimental design of the present study. IFN, interferon; NUC, nucleoside/nucleotide analog; w, weeks.

Table 1 Demographic data of 50 enrolled patients

Characteristic	Value
Age at start of NUC administration (years)†	34 (21–57)
Age at end of NUC administration (years)†	35 (22–62)
Sex (male : female)	38:12
Genotype (B : C : undetermined)	3:36:11
NUC at start (LVD : ETV)	43:7
NUC at end (LVD : ETV : LAM + ADV : ETV + ADV)	40:8:1:1
Duration of NUC administration (months)†	6 (4–121)
HBeAg positivity at start of NUC‡	70% (35/50)
HBeAg positivity at end of NUC‡	42% (21/50)
Follow-up period after stopping IFN-α administration (months)†	28 (2–102)
Patients requiring re-administration of NUC‡	50% (25/50)
Patients developing HCC‡	0% (0/50)

†Data are expressed as the median (range).
‡Data are expressed as a positive percentage (positive number/total number).
ADV, adefovir dipivoxil; ETV, entecavir; HBeAg, hepatitis B e-antigen; HCC, hepatocellular carcinoma; IFN, interferon; LAM, lamivudine; LVD, lamivudine; NUC, nucleoside/nucleotide analog.

of NUC possessed alanine aminotransferase (ALT) levels of over 80 IU/L and HBV DNA levels of over 5.8 log copies/mL at or just before the point of NUC re-continuation, which fulfilled the established requirements for restarting NUC.[13,14,16]

Hepatitis B surface antigen was confirmed to be positive on at least two occasions at least 6 months apart in all patients before NUC treatment. Tests for hepatitis C virus and HIV antibodies were all negative. Patients complicated with HCC or signs of hepatic failure at the cessation of NUC administration were excluded from the study. No such complications were observed during follow up.

With few exceptions, patients were seen at least once a month during the first year of follow up, at least once every 3 months during the second year and at least once every 6 months afterwards. No patient developed HCC or hepatic failure during the follow-up period. Stored serum samples were kept frozen at −20 °C or below until assayed. This study was approved by the ethics committees of all participating institutions (approval reference 1117 for Shinshu University Hospital, 24085 for National Hospital Organization Nagasaki Medical Center, 758 for Toranomon Hospital, 321 for Hiroshima University Hospital, 934 and 977 for Chiba University Hospital, 779 for The Hospital of Hyogo College of Medicine, 411 for Kumamoto Shinto General Hospital, and "Analysis of efficacy of IFN- to stop NUC in patients with chronic hepatitis B" for Teine Keijinkai Hospital).

Hepatitis B viral markers

Serological markers for HBV, including HBsAg, HBeAg and antibody to HBeAg, were tested using commercially available enzyme immunoassay kits (Abbott Japan, Tokyo, Japan; Fujirebio, Tokyo, Japan; and/or Sysmex, Kobe, Japan) at each hospital. Quantitative measurement of HBsAg[17] was performed using a chemiluminescence enzyme immunoassay (CLEIA)-based HISCL HBsAg assay manufactured by Sysmex (Kobe, Japan). The assay had a quantitative range of -1.5 to 3.3 log IU/mL. End titer was determined by diluting samples with normal human serum when initial results exceeded the upper limit of the assay range.

Serum HBV DNA was determined using a COBAS TaqMan HBV kit (Roche, Tokyo, Japan)[18] with a quantitative range of 2.1–9.0 log copies/mL. According to the manufacturer's instructions, detection of a positive signal below the quantitative range was described as a positive signal, and no signal detection was regarded as a negative signal. Six HBV genotypes (A–F) were evaluated according to the restriction patterns of DNA fragments from the method reported by Mizokami *et al.*[19]

Serum HBcrAg levels were measured using a CLEIA HBcrAg assay kit with a fully automated Lumipulse System analyzer (Fujirebio) as described previously.[20,21] The HBcrAg assay measures all antigens transcribed and translated from the precore and core genes of the HBV genome, which include hepatitis B e, core and p22cr antigens.[14,20] HBcrAg concentration was calculated based on a standard curve generated using recombinant pro-HBeAg. The immunoreactivity of pro-HBeAg at 10 fg/mL was defined as 1 U/mL. We expressed HBcrAg in terms of log U/mL, with a quantitative range set at 3.0–6.8 log U/mL.

Evaluation of response to NUC/IFN-α sequential therapy

The clinical conditions of a successful response to NUC/IFN-α sequential therapy were set at serum HBV DNA below 4.0 log copies/mL, serum ALT below 30 IU/L and negative HBeAg, according to established Japanese guidelines in which patients who meet these conditions are not recommended to start antiviral therapy.[22] We assessed the final response at approximately 24 months after completing IFN-α sequential therapy and compared results to those at 6 and 12 months after the treatment.

Statistical analyses

Fisher's exact and Pearson's χ^2-tests were adopted to test for differences between subgroups of patients. The Mann–Whitney *U*-test was employed to compare continuous data. Each cut-off value was decided using receiver operating characteristic (ROC) analysis, and results were evaluated by measuring the area under the ROC (AUC). Multivariate analysis was performed using a logistic model for the 24-month response to NUC/IFN-α sequential therapy. Correlations between maximal values of ALT and HBV DNA were calculated using Spearman's rank correlation coefficient test. The non-relapse rate was analyzed by the Kaplan–Meier method.

All tests were performed using the IBM SPSS Statistics Desktop for Japan version 19.0 (IBM Japan, Tokyo, Japan). $P < 0.05$ was considered to be statistically significant.

RESULTS

Factors associated with the 24-month response to NUC/IFN-α sequential therapy

OF THE 50 patients enrolled, 18 were judged as responders at 24 months after completing IFN-α sequential therapy (i.e. 24-month responders), while the

remaining 32 were classified as 24-month non-responders. The clinical backgrounds of both groups are compared in Table 2. The median age at NUC commencement and sex distribution did not differ remarkably between the groups. Genotype C was similarly predominant. The types of NUC administrated at the start and end of treatment were comparable between the groups, but the duration of NUC administration was significantly longer in responders. Re-administration of NUC due to aggravation of hepatitis B before judgment of the 24-month response was observed in approximately half of the 32 non-responders. After the final evaluation at 24 months, re-continuation of NUC was seen in only one of the 18 responders versus roughly half of the 15 non-responders who had previously not required it. The follow-up period was significantly longer in responders because observation was discontinued when NUC were re-administrated.

Biochemical and virological markers were compared between 24-month responders and non-responders at the start of NUC, at the start of IFN-α and at the end of IFN-α (Table 3). Positivity for the HBeAg was significantly lower in responders at all time points. HBsAg and HBcrAg levels did not differ between the groups at the start of NUC, but became significantly lower in responders at the start and end-points of IFN-α administration. A significant difference in HBV DNA level was seen between the groups at the end of IFN-α administration only. ALT levels did not differ between the groups at any point.

Multivariate analysis revealed that HBsAg and HBcrAg levels of 3.0 or more and 4.5 log U/mL or more, respectively, at the start of IFN-α administration were significant factors associated with a 24-month non-response to NUC/IFN-α sequential therapy (Table 4). The factors adopted for this logistic model were as follows: age at end of NUC of 37 years or more, duration of NUC administration of 18 months or more, sex, type of NUC at start, HBV genotype, HBeAg positivity at the start of IFN-α, HBsAg level at the start of IFN-α of 3.0 log IU/mL or more, and HBcrAg level at the start of IFN-α of 4.5 log U/mL or more. The corresponding cut-off values for each factor were determined by ROC analysis.

Of the 50 patients enrolled, 23 (46%) had HBsAg of 3.0 log IU/mL or more and HBcrAg of 4.5 log U/mL or more, 27 (54%) had HBsAg of less than 3.0 log IU/mL or HBcrAg of less than 4.5 log U/mL, and none had HBsAg of less than 3.0 log IU/mL and HBcrAg of less than 4.5 log U/mL at the start of IFN-α administration. Whereas none of the 23 patients with the highest HBsAg and HBcrAg levels were responders, 18 (67%) of the remaining 27 patients responded to NUC/IFN-α sequential therapy ($P = 0.005$).

Comparison of responses to NUC/IFN-α sequential therapy at different time points

We assessed the responses to NUC/IFN-α sequential therapy at 6 and 12 months after completing IFN-α administration using the same criteria as those for determining the 24-month outcome. Responses were in 78% agreement ($P < 0.001$) between 6 and 24 months and 80% agreement ($P < 0.001$) between 12 and 24 months.

Prediction of response to NUC/IFN-α sequential therapy using maximal levels of ALT and HBV DNA

The maximal levels of ALT and HBV DNA during follow up were found to be significantly related ($r = 0.777$, $P < 0.001$). ROC analysis showed that both maximal ALT

Table 2 Comparison of clinical backgrounds between 24-month responders and non-responders

Clinical background	24-month responders ($n = 18$)	24-month non-responders ($n = 32$)	*P*
Age at start of NUC (years)†	36 (21–56)	34 (21–57)	0.486
Sex (male : female)	15:3	23:9	0.497
Genotype (B:C:undetermined)	1:16:1	2:20:10	0.101
NUC at start (LVD : ETV)	16:2	27:5	1.000
NUC at end (LVD : ETV : LAM + ADV : ETV + ADV)	16:2:0:0	24:6:1:1	0.610
Duration of NUC administration (months)†	51 (5–121)	5 (4–72)	0.001
Follow-up period after stopping IFN-α administration (months)†	30 (23–102)	22 (2–81)	0.014
Re-administration of NUC before judging 24-month response‡	0% (0/18)	53% (17/32)	<0.001
Re-administration of NUC after judging 24-month response‡	6% (1/18)	47% (7/15)	0.012

†Data are expressed as the median (range).
‡Data are expressed as a positive percentage (positive number/total number).
ADV, adefovir dipivoxil; ETV, entecavir; HBeAg, hepatitis B e-antigen; HCC, hepatocellular carcinoma; IFN, interferon; LAM, lamivudine; LVD, lamivudine; NUC, nucleoside/nucleotide analog.

Table 3 Comparison of ALT level and viral markers between 24-month responders and non-responders at the time points of starting NUC administration, starting IFN-α administration and stopping IFN-α administration

ALT/viral marker	24-month responders ($n = 18$)	24-month non-responders ($n = 32$)	*P*
At start of NUC administration			
ALT (IU/L)†	242 (32–2274)	281 (22–1044)	0.872
HBeAg‡	44% (8/18)	84% (27/32)	0.008
HBV DNA (log copies/mL)†	8.0 (<2.1–>9.0)	7.8 (<2.1–>9.0)	0.866
HBsAg (log IU/mL)†	3.5 (1.8–4.9)	3.5 (2.5–4.4)	1.000
HBcrAg (log U/mL)†	>6.8 (3.7–>6.8)	>6.8 (<3.0–>6.8)	0.121
At start of IFN-α administration			
ALT (IU/L)†	29 (12–103)	29 (12–111)	0.779
HBeAg‡	11% (2/18)	59% (19/32)	0.001
HBV DNA (log copies/mL)†	<2.1 (neg.–3.9)	<2.1 (neg.–4.8)	0.142
HBsAg (log IU/mL)†	2.9 (1.5–4.1)	3.7 (2.5–4.3)	0.028
HBcrAg (log U/mL)†	3.6 (<3.0–5.9)	5.6 (<3.0–>6.8)	0.002
At end of IFN-α administration			
ALT (IU/L)†	25 (10–48)	28 (12–134)	0.384
HBeAg‡	6% (1/18)	59% (19/32)	<0.001
HBV DNA (log copies/mL)†	<2.1 (neg.–4.1)	4.6 (<2.1–>9.0)	<0.001
HBsAg (log IU/mL)†	2.8 (1.9–4.0)	3.6 (2.6–4.7)	0.007
HBcrAg (log U/mL)†	3.4 (<3.0–5.5)	5.5 (<3.0–>6.8)	0.017

†Data are expressed as the median (range).
‡Data are expressed as a positive percentage (positive number/total number).
ALT, alanine aminotransferase; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e-antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; IFN, interferon; neg., negative; NUC, nucleoside/nucleotide analog.

Table 4 Multivariate analysis of factors associated with 24-month non-responders to NUC/IFN-α sequential therapy

Selected factor	Odds ratio	95% CI	*P*
HBsAg ≥3.0 log IU/mL at start of IFN-α	17.7	2.9–108.2	0.002
HBcrAg ≥4.5 log U/mL at start of IFN-α	15.0	2.5–88.6	0.003

CI, confidence interval; HBcrAg, hepatitis B core-related antigen; HBsAg, hepatitis B surface antigen; IFN, interferon; neg., negative; NUC, nucleoside/nucleotide analog.

and HBV DNA levels were significantly associated with the treatment response (Fig. 2), with an AUC for each parameter of over 0.8. The cut-off values providing the highest significance in ROC analysis were 128 IU/L for ALT and 4.5 log copies/mL for HBV DNA. The existence of a second cut-off value was also identified for HBV DNA (6.0 log copies/mL) to discriminate between 24-month responders and non-responders. These results indicated that patients reaching a maximal ALT level of over 128 IU/L or maximal HBV DNA level of over 6.0 log copies/mL during post-treatment follow up were likely to be non-responders.

Lastly, we analyzed the changes in cumulative non-relapse rate of hepatitis B during and after IFN-α administration by tentatively defining relapse as ALT level exceeding 128 IU/L during follow up. We selected maximal ALT instead of maximal HBV DNA because: (i) the inflection point to distinguish a response was clear for maximal ALT but ambiguous for maximal HBV DNA; (ii) the value for "sensitivity + specificity − 1" as calculated by ROC analysis was larger for maximal ALT (7.5 vs 6.5); and (iii) the maximal levels of ALT and HBV DNA were closely associated, and thus ALT values were considered to represent those of HBV DNA. The cumulative non-relapse rate decreased rapidly after completely halting NUC until just prior to 6 months after stopping IFN-α and then was seen to plateau until the study end-point (Fig. 3). This suggests that the recurrence of hepatitis associated with a 24-month non-response can be expected to occur primarily during the first 6 months after stopping IFN-α administration.

DISCUSSION

THE COOPERATIVE EFFECT of NUC/IFN-α sequential therapy has been controversial.[7-12] Enomoto *et al.*[10] first analyzed the results of ETV/IFN-α sequential therapy in patients with HBeAg positive chronic hepatitis B and detected several differences. Although their results were negative, they witnessed that patients who had achieved HBeAg

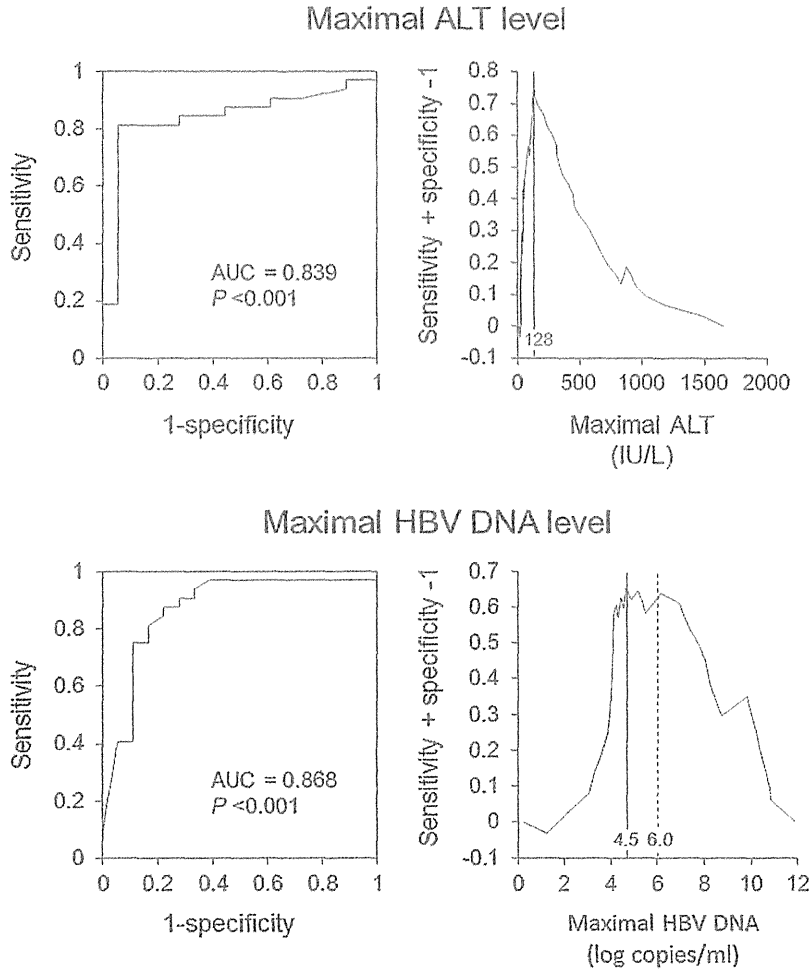

Figure 2 Receiver operating characteristic analysis of maximal alanine aminotransferase (ALT) and hepatitis B virus (HBV) DNA levels to discriminate between 24-month responders and non-responders. Vertical solid lines indicate the actual values of markers corresponding to main inflection points and the vertical broken line indicates the actual value of the marker corresponding to a second inflection point; AUC, area under the receiver operating characteristic curve.

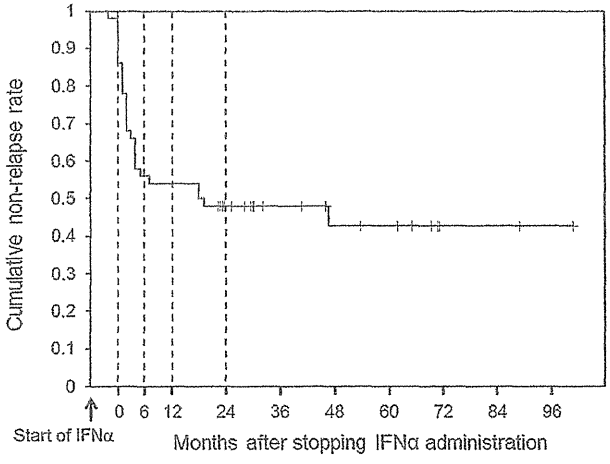

Figure 3 Kaplan–Meier analysis of the non-relapse rate after stopping interferon (IFN)-α administration by defining relapse of hepatitis B as alanine aminotransferase (ALT) level exceeding 128 IU/L.

seroconversion by the time of IFN-α commencement experienced a significantly higher sustained virological response rate than those in whom the HBeAg persisted. Thus, it appeared beneficial to further clarify the factors associated with the response to NUC/IFN-α sequential therapy.

The present study analyzed the factors associated with a long-term response to IFN-α sequential therapy in order to safely discontinue NUC therapy. All patients were treated with natural IFN-α for 6 months and followed for at least 24 months after completing the sequential therapy, with the exception of those who required re-administration of NUC due to aggravation of hepatitis B. The type and duration of NUC administration were not fixed in this study because IFN-α sequential therapy was implemented to discontinue NUC in patients who were undergoing maintenance treatment. Although a prospective study would have been ideal to elucidate the factors associated with

IFN-α sequential therapy outcome, we undertook this retrospective trial because no variables have been sufficiently analyzed to date. Furthermore, we were able to address the long-term response to IFN-α sequential therapy in relation to the results of earlier retrospective studies. It has been reported that pegylated IFN-α (PEG IFN-α) provides a higher HBV response rate than does conventional IFN-α.[23] Therefore, additional prospective studies of sequential therapy using PEG IFN-α are needed as well.

Both HBsAg and HBcrAg levels at the time of NUC cessation were factors significantly associated with the response to NUC/IFN-α sequential therapy. HBsAg has been closely linked with PEG IFN-α therapy outcome.[24-27] Moucari *et al.*[26] analyzed HBeAg negative hepatitis B patients who had been treated with PEG IFN-α for 48 weeks and concluded that an early serum HBsAg drop was strongly predictive of a sustained virological response. Sonneveld *et al.*[24] assessed HBeAg positive hepatitis B patients who had received PEG IFN-α with or without LVD for 52 weeks and observed that patients who experienced no decline in HBsAg level from baseline at week 12 had little chance of achieving a sustained response and no possibility of HBsAg loss. HBcrAg includes antigens that are transcribed and translated from precore and core genes of the HBV genome, and HBeAg is a primary component of these antigens. Thus, our results were consistent with those described by Enomoto *et al.*[10] that the proportion of patients losing HBeAg positivity during ETV treatment was significantly higher in responders to ETV/IFN-α sequential therapy than in non-responders.

Hepatitis B surface antigen and HBcrAg levels have both been associated with intrahepatic HBV cccDNA, which is a key molecule in HBV replication whose value is closely related to HBV replication activity.[21,27,28] Several reports[27,29,30] have shown that HBV cccDNA level is associated with the response to antiviral therapy, such as with PEG IFN-α and NUC. Sung *et al.*[29] analyzed HBeAg positive hepatitis B patients who had been treated with either LVD monotherapy or a combination of PEG IFN-α and LVD and concluded that intrahepatic HBV cccDNA level at the end of therapy was superior to serum HBV DNA in predicting a sustained virological response. Serum HBV DNA is associated with intrahepatic HBV cccDNA and is widely used as a marker for HBV replication activity. However, such associations may be incompatible with antiviral therapies, and especially NUC treatment, because NUC directly hamper production of the HBV virion by inhibiting reverse transcription of pre-genomic RNA without affecting HBV cccDNA directly. As serum levels of HBsAg and HBcrAg are easier to measure than intrahepatic HBV cccDNA, these two antigen assays may be more suitable as surrogate markers for HBV replication activity in patients undergoing antiviral therapy. We previously reported that the combinational use of HBsAg and HBcrAg was beneficial to forecast the risk of hepatitis relapse after discontinuation of NUC.[13,14] The present study confirms this notion; it is possible that HBsAg and HBcrAg have complimentary roles in monitoring antiviral effects because the production of these two antigens is regulated by alternative enhancer–promoter systems in the HBV genome.

It is noteworthy that ROC analysis revealed maximal levels of ALT and HBV DNA to be closely associated with the 24-month response to NUC/IFN-α sequential therapy. We observed that patients with ALT higher than 128 IU/mL or HBV DNA higher than 6.0 log copies/mL during follow up were likely to be non-responders. When a relapse of hepatitis B was tentatively defined as ALT exceeding 128 IU/L during observation, relapses occurred frequently during the first 6 months after ceasing IFN-α and then became more sporadic afterwards. The timing of judgment of a virological response to NUC/IFN-α sequential therapy is critical when evaluating treatment efficacy. As this period is usually set at 6 months after completing therapy, our results confirm that 6 months is indeed appropriate. Our findings also suggest that maximal levels of ALT and HBV DNA are useful for monitoring the results of NUC/IFN-α sequential therapy. Accordingly, patients who are likely to be non-responders can now be identified as early as 24 weeks in advance and alternative strategies for treatment may be considered in a more timely fashion.

In conclusion, the combinational use of HBsAg and HBcrAg levels may be useful to predict the response to NUC/IFN-α sequential therapy. Maximal levels of ALT and HBV DNA during follow up may also be employed for monitoring the results of IFN-α sequential therapy.

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REFERENCES

1 Hoofnagle JH, Doo E, Liang TJ, Fleischer R, Lok AS. Management of hepatitis B: summary of a clinical research workshop. *Hepatology* 2007; 45: 1056–75.

2 Lok AS, McMahon BJ. Chronic hepatitis B. *Hepatology* 2007; 45: 507–39.
3 Lee WM. Hepatitis B virus infection. *N Engl J Med* 1997; 337: 1733–45.
4 Ghany M, Liang TJ. Drug targets and molecular mechanisms of drug resistance in chronic hepatitis B. *Gastroenterology* 2007; 132: 1574–85.
5 Honkoop P, de Man RA, Niesters HG, Zondervan PE, Schalm SW. Acute exacerbation of chronic hepatitis B virus infection after withdrawal of lamivudine therapy. *Hepatology* 2000; 32: 635–9.
6 Honkoop P, de Man RA, Heijtink RA, Schalm SW. Hepatitis B reactivation after lamivudine. *Lancet* 1995; 346: 1156–7.
7 Serfaty L, Thabut D, Zoulim F *et al.* Sequential treatment with lamivudine and interferon monotherapies in patients with chronic hepatitis B not responding to interferon alone: results of a pilot study. *Hepatology* 2001; 34: 573–7.
8 Shi M, Wang RS, Zhang H *et al.* Sequential treatment with lamivudine and interferon-alpha monotherapies in hepatitis B e antigen-negative Chinese patients and its suppression of lamivudine-resistant mutations. *J Antimicrob Chemother* 2006; 58: 1031–5.
9 Manesis EK, Papatheodoridis GV, Hadziyannis SJ. A partially overlapping treatment course with lamivudine and interferon in hepatitis B e antigen-negative chronic hepatitis B. *Aliment Pharmacol Ther* 2006; 23: 99–106.
10 Enomoto M, Nishiguchi S, Tamori A *et al.* Entecavir and interferon-alpha sequential therapy in Japanese patients with hepatitis B e antigen-positive chronic hepatitis B. *J Gastroenterol* 2013; 48: 397–404.
11 Enomoto M, Tamori A, Nishiguchi S, Kawada N. Combination therapy with a nucleos(t)ide analogue and interferon for chronic hepatitis B: simultaneous or sequential. *J Gastroenterol* 2013; 48: 999–1005.
12 Minami M, Okanoue T. Management of HBV infection in Japan. *Hepatol Res* 2007; 37: S79–82.
13 Matsumoto A, Tanaka E, Suzuki Y *et al.* Combination of hepatitis B viral antigens and DNA for prediction of relapse after discontinuation of nucleos(t)ide analogs in patients with chronic hepatitis B. *Hepatol Res* 2012; 42: 139–49.
14 Tanaka E, Matsumoto A. Guidelines for avoiding risks resulting from discontinuation of nucleoside/nucleotide analogs in patients with chronic hepatitis B. *Hepatol Res* 2014; 44: 1–8.
15 Orito E, Ichida T, Sakugawa H *et al.* Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. *Hepatology* 2001; 34: 590–4.
16 JSH Guidelines for the Management of Hepatitis B Virus Infection. *Hepatol Res* 2014; 44 (Suppl S1): 1–58.
17 Schuttler CG, Wend UC, Faupel FM, Lelie PN, Gerlich WH. Antigenic and physicochemical characterization of the 2nd International Standard for hepatitis B virus surface antigen (HBsAg). *J Clin Virol* 2010; 47: 238–42.
18 Ronsin C, Pillet A, Bali C, Denoyel GA. Evaluation of the COBAS AmpliPrep-total nucleic acid isolation-COBAS TaqMan hepatitis B virus (HBV) quantitative test and comparison to the VERSANT HBV DNA 3.0 assay. *J Clin Microbiol* 2006; 44: 1390–9.
19 Mizokami M, Nakano T, Orito E *et al.* Hepatitis B virus genotype assignment using restriction fragment length polymorphism patterns. *FEBS Lett* 1999; 450: 66–71.
20 Kimura T, Rokuhara A, Sakamoto Y *et al.* Sensitive enzyme immunoassay for hepatitis B virus core-related antigens and their correlation to virus load. *J Clin Microbiol* 2002; 40: 439–45.
21 Suzuki F, Miyakoshi H, Kobayashi M, Kumada H. Correlation between serum hepatitis B virus core-related antigen and intrahepatic covalently closed circular DNA in chronic hepatitis B patients. *J Med Virol* 2009; 81: 27–33.
22 Kumada H, Okanoue T, Onji M *et al.* Guidelines for the treatment of chronic hepatitis and cirrhosis due to hepatitis B virus infection for the fiscal year 2008 in Japan. *Hepatol Res* 2010; 40: 1–7.
23 Cooksley WG, Piratvisuth T, Lee SD *et al.* Peginterferon alpha-2a (40 kDa): an advance in the treatment of hepatitis B e antigen-positive chronic hepatitis B. *J Viral Hepat* 2003; 10: 298–305.
24 Sonneveld MJ, Rijckborst V, Boucher CA, Hansen BE, Janssen HL. Prediction of sustained response to peginterferon alfa-2b for hepatitis B e antigen-positive chronic hepatitis B using on-treatment hepatitis B surface antigen decline. *Hepatology* 2010; 52: 1251–7.
25 Brunetto MR, Moriconi F, Bonino F *et al.* Hepatitis B virus surface antigen levels: a guide to sustained response to peginterferon alfa-2a in HBeAg-negative chronic hepatitis B. *Hepatology* 2009; 49: 1141–50.
26 Moucari R, Mackiewicz V, Lada O *et al.* Early serum HBsAg drop: a strong predictor of sustained virological response to pegylated interferon alfa-2a in HBeAg-negative patients. *Hepatology* 2009; 49: 1151–7.
27 Chan HL, Wong VW, Tse AM *et al.* Serum hepatitis B surface antigen quantitation can reflect hepatitis B virus in the liver and predict treatment response. *Clin Gastroenterol Hepatol* 2007; 5: 1462–8.
28 Wong DK, Tanaka Y, Lai CL, Mizokami M, Fung J, Yuen MF. Hepatitis B virus core-related antigens as markers for monitoring chronic hepatitis B infection. *J Clin Microbiol* 2007; 45: 3942–7.
29 Sung JJ, Wong ML, Bowden S *et al.* Intrahepatic hepatitis B virus covalently closed circular DNA can be a predictor of sustained response to therapy. *Gastroenterology* 2005; 128: 1890–7.
30 Wursthorn K, Lutgehetmann M, Dandri M *et al.* Peginterferon alpha-2b plus adefovir induce strong cccDNA decline and HBsAg reduction in patients with chronic hepatitis B. *Hepatology* 2006; 44: 675–84.