recognize human leukocyte antigen (HLA) class I molecules as their ligands; *KIR2DL1* recognizes *HLA*-C group 2 (*HLA-C2*) allotypes having lysine at amino acid position 80, whereas *KIR2DL2* and KIR2DL3 recognize *HLA-C* group 1 (*HLA-C1*) allotypes having asparagine at amino acid position 80 [5]. *KIR2DL2* and *KIR2DL3* also recognize HLA-B*4601 acquiring the-C1 epitope by gene conversion [6]. Furthermore, *KIR3DL1* recognizes subsets of *HLA*-A and *HLA*-B allotypes having the -Bw4 epitope determined by amino acid positions 77-83 [7].

It has been well documented that certain KIR-HLA receptor-ligand combinations are associated with susceptibility to infectious diseases, such as HCV, as well as with disease progression and treatment response [8-15]. Recent reports have also identified a relationship between interleukin (IL) 28B gene polymorphisms and treatment and spontaneous resolution of HCV infection[16-19]. Dring et al. observed that the presence of *IL28B* gene polymorphisms and *KIR* genotypes synergized to increase the risk of chronic HCV infection[20], although this finding is under debate[21]. Suppiah et al. [22] recently reported that genotyping for *IL28B*, *HLA*-C, and *KIR* genes was useful for predicting HCV treatment response in patients of European descent. As these gene associations have not yet been studied in the Japanese population, we evaluated whether HLA-KIR interactions, in addition to an *IL28B* polymorphism, would influence the outcome of pegylated-interferon-α (PEG-IFN) and ribavirin therapy in Japanese patients with chronic hepatitis C.

Materials and Methods

Ethics statement

This study was approved by the ethical 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.

Subjects

One hundred and fifteen consecutive IFN-treatment-naïve patients with chronic hepatitis C were enrolled in this study. All subjects were seen at Shinshu University Hospital or one of its affiliated hospitals. The clinical and demographic characteristics of our cohort are shown in Table 1. Diagnosis of chronic hepatitis C was based on previously reported criteria [23]: 1) presence of serum HCV antibodies and detectable viral RNA; 2) 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 Cobas Amplicor assays (sensitivity: 50 IU/mL; Roche Diagnostic Systems, Tokyo, Japan). HCV genotypes were determined using INNO-LiPA HCV II kits (Innogenetics, Gent, Belgium). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and other relevant biochemical tests were performed using standard methods[24]. Liver fibrosis was assessed using the AST to platelet ratio index (APRI) in this study. APRI has been recognized as a noninvasive test to estimate the degree of liver fibrosis in chronic liver disease with HCV infection[25]. APRI was calculated for all study subjects as follows: AST/upper limit of normal (45 IU/L) × 100/platelet count (10^9/L). Patients received PEG-IFN-α2b (Pegintron; MSD KK, Tokyo, Japan; 1.5 μg/kg of body weight by subcutaneous injection once per week) and ribavirin (Rebetol; MSD KK; 600-1000 grams daily, according to body weight) for 48 weeks, as described previously[26]. Patients achieving a sustained HCV response were defined as those whose serum HCV RNA was undetectable 24 weeks after completing therapy. Patients who did not meet this criterion, who included non-responders and relapsers, were regarded as treatment failures.

Table 1. Clinical features of sustained and non-sustained virological response patients with chronic hepatitis C.

Characteristic	All	SVR	Non-SVR	*P*
	(n = 115)	(n = 56)	(n = 59)	
Age (yr)	60 (24 - 80)	59 (25 - 80)	60 (24 - 75)	0.43
Male	66 (57)	34 (61)	32 (54)	0.48
Alanine aminotransferase (IU/L)	46 (17 - 389)	48 (17 - 389)	45 (17 - 309)	0.81
Aspartate aminotransferase (IU/L)	43 (17 - 246)	42 (17 - 231)	43 (17 - 246)	0.49
White blood cells (/μL)	4410 (2280 - 8240)	4740 (2700 - 8170)	4070 (2280 - 8240)	0.011
Hemoglobin (g/dL)	14.4 (9.2 - 18.2)	15.1 (11.0 - 18.2)	13.9 (9.2 - 17.4)	0.002
Platelet count (10^4/μL)	15.9 (6.7 - 33.6)	16.6 (8.3 - 26.2)	15.6 (6.7 - 33.6)	0.30
APRI	0.89 (0.21 - 5.40)	0.59 (0.22 - 5.40)	0.66 (0.21 - 5.06)	0.41
HCV RNA (log_{10} IU/mL)	6.4 (5.0 - 7.3)	6.1 (5.0 - 6.8)	6.5 (5.0 - 7.3)	< 0.001

Data are expressed as median (range) or n (%) as appropriate. SVR, sustained virological response; HCV, hepatitis C virus

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HLA, KIR, and IL28B (rs8099917) Genotyping

Genomic DNA was isolated from whole blood samples using QuickGene-800 assays (Fujifilm, Tokyo, Japan). We genotyped *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), which is based on PCR sequence-specific oligonucleotide probes[27]. Subjects were identified as having the B/x or A/A genotype as defined previously[28]. Genotypes for the centromeric (*Cen*) and telomeric (*Tel*) parts of the *KIR* locus were determined according to the presence or absence of one or more B haplotype-defining *KIR* genes. Thus, *Cen-A1* and *Tel-A1* were the centromeric and telomeric motifs, respectively, of the canonical *A KIR* haplotype in the present study, *Cen-B1* and *Cen-B2* were alternative centromeric motifs of common *B KIR* haplotypes, and *Tel-B1* was the common telomeric motif of *B* haplotypes[29]. For much of this analysis, *Cen-B1* and *-B2* were grouped together as *Cen-B*, whereas *Cen-A1* was shortened to *Cen-A* and *Tel-A1* to *Tel-A*, as reported

previously[30,31]. Genotyping of an *IL28B* SNP (rs8099917) was performed using a TaqMan 5' exonuclease assay with primers supplied by Applied Biosystems[32]. 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.

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 data of KIR-HLA combinations using the number of comparisons performed by our primary factors of interest in Table 2 (i.e., 8 tests = 4 combinations × 2 comparisons between two groups). 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 each median value. Statistical analyses were performed using SPSS software version 21.0J (IBM, Tokyo, Japan). Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated to determine the reliability of the predictors of therapy response.

Results

Patient Characteristics and Treatment Outcome

All patients in our test cohort were infected with HCV genotype 1b. Of the 115 patients receiving PEG-IFN-α2b and ribavirin therapy, 56 (49%) achieved an SVR. The remaining 59 patients were non-responders, 28 of whom experienced a relapse and 31 who were null responders. The median white blood cell count (P = 0.011) and hemoglobin value (P = 0.002) in the SVR group were significantly higher than those in the non-SVR group prior to treatment. HCV viral load at baseline was significantly associated with treatment outcome (P < 0.001).

Association of HLA and KIR with a Sustained Virological Response

We first determined the frequency of *HLA-Bw* and *HLA-C* alleles in SVR and non-SVR patients (Figure 1). The frequency of *HLA-Bw4Bw6* in responders was significantly higher than that in non-responders (55% [31/56] vs. 36% [21/59]; P = 0.033; OR = 2.24, 95% CI = 1.06 - 4.75). Conversely, patients with the *HLA-Bw6* homozygote had a higher non-SVR rate (32% [18/56] vs. 54% [32/59]; P = 0.017; OR = 0.40, 95% CI = 0.19 - 0.85). Overall, *HLA-Bw4* was associated with an SVR among patients (68% [38/56] vs. 46% [27/59]; P = 0.017; OR = 2.50, 95% CI = 1.17 - 5.35). The frequencies of HLA-C were not statistically significant. We further checked whether particular HLA-Bw or HLA-C alleles were beneficial to treatment outcome. The HLA-B*35:01 allele was more frequently found in patients with an SVR than in those without (13% [15/102] vs. 4% [5/118]; P = 0.014 [Pc = 0.36]; OR = 3.49, 95% CI = 1.23 - 9.97).

Table 2. Frequency of *IL28B* genotype, *KIR3DL1/HLA-Bw4*, and *KIR2DL2/HLA-C1* combinations in 56 patients with a sustained virological response (SVR) and 59 patients with a non-SVR to pegylated interferon and ribavirin therapy of chronic hepatitis C.

KIR3DL1/ HLA-Bw4	*KIR2DL2/HLA-C1*	SVR	Non-SVR	P (Pc)	OR (95% CI)
		(n = 56)	(n = 59)		
+/+	+/+	5 (9%)	7 (12%)	0.61	
+/+	Other	31 (55%)	19 (32%)	0.012 (0.1)	2.61 (1.22 - 5.58)
Other	+/+	1 (2%)	10 (17%)	0.014 (0.12)	0.09 (0.01 - 0.72)
Other	Other	19 (34%)	23 (39%)	0.57	
IL28B	*KIR3DL1/ HLA-Bw4*	SVR	Non-SVR	P (Pc)	OR (95% CI)
		(n = 56)	(n = 59)		
TT	+/+	27 (48%)	13 (22%)	0.003 (0.024)	3.29 (1.47 - 7.39)
TT	Other	17 (30%)	14 (24%)	0.42	
TG/GG	+/+	9 (16%)	13 (22%)	0.42	
TG/GG	Other	3 (5%)	19 (32%)	0.00062 (0.0005)	0.12 (0.03 - 0.43)
IL28B	*KIR2DL2/ HLA-C1*	SVR	Non-SVR	P (Pc)	OR (95% CI)
		(n = 56)	(n = 59)		
TT	Other	38 (68%)	18 (31%)	0.000062 (0.0005)	4.81 (2.19 - 10.58)
TT	+/+	6 (11%)	9 (15%)	0.47	
TG/GG	Other	12 (21%)	24 (41%)	0.026 (0.21)	0.40 (0.17 - 0.91)
TG/GG	+/+	0 (0%)	8 (14%)	0.013 (0.1)	-

Data are expressed as n (%).

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The distribution of *KIR* genes and their association with treatment outcome are shown in Figure 2. No statistically significant differences were found for any allele combination apart from *KIR2DL2* and *KIR2DS2*; patients with these genes had significantly decreased SVR frequencies compared with those without (P = 0.015 [Pc = 0.48]; OR = 0.30, 95% CI = 0.11 - 0.82 and P = 0.025 [Pc = 0.8]; OR = 0.32, 95% CI = 0.12 - 0.90, respectively).

KIR genotype profiles were determined by the presence or absence of each *KIR* locus in patients (Figure 3). Since strong linkage disequilibrium is a prominent feature in the *KIR* region, *KIR* gene profiles were classified based on *Cen* and *Tel* motifs. When we evaluated SVR according to genotype and *Cen* and *Tel* frequencies, we observed that virologic clearance with *Cen-A/A* was significantly higher than that without (54% [50/92] vs.

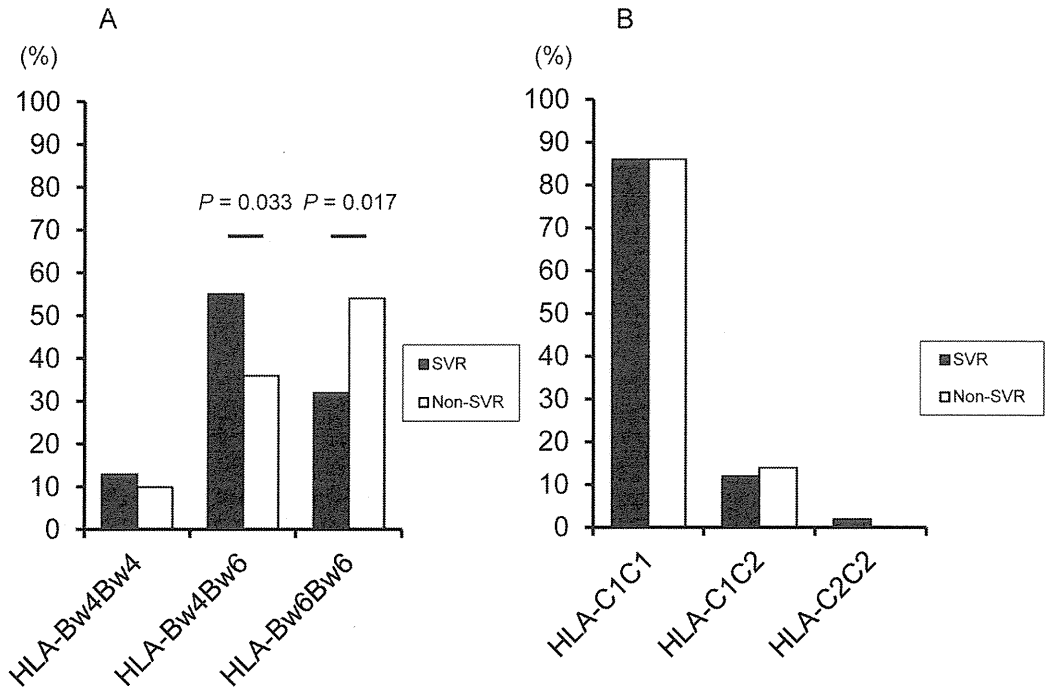

Figure 1. Frequency of *HLA-Bw* and -C alleles in 56 patients with a sustained virological response (SVR) and 59 patients with a non-SVR to pegylated interferon and ribavirin therapy of chronic hepatitis C.

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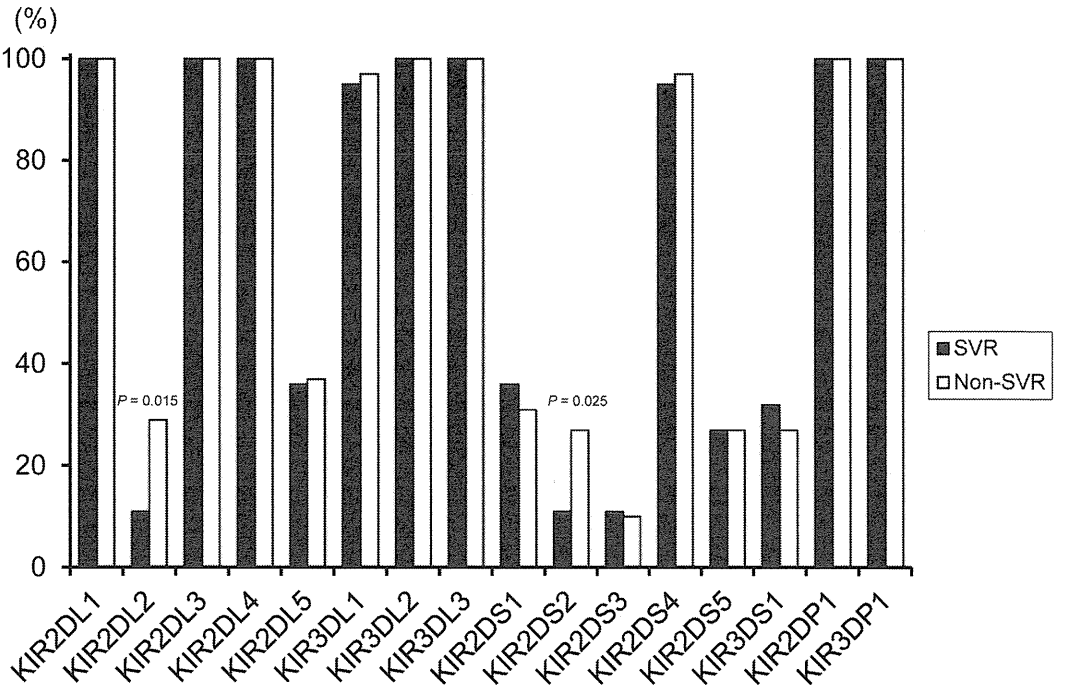

Figure 2. Frequency of each *KIR* gene in 56 patients with a sustained virological response (SVR) and 59 patients with a non-SVR to pegylated interferon and ribavirin therapy of chronic hepatitis C.

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26% [6/23], $P = 0.015$; OR = 3.37, 95% CI = 1.22 - 9.33). There were no significant differences regarding *AA* genotype and *Tel*.

We next analyzed combinations of activation/inhibitory *KIR*s and their *HLA* ligands for possible associations with an SVR. Among the combinations of *KIR3DL1-HLA-Bw4*, *KIR2DL2-HLA-C1*, and KIR2DL1-*HLA*-C2, patients who carried the inhibitory *KIR3DL1* receptor and its ligand *HLA-Bw4* had a significantly higher response rate than those without *KIR3DL1* or *HLA-Bw4* (58% [36/62] vs. 38% [20/53]; $P = 0.030$ [$Pc = 0.12$]; OR = 2.29, 95% CI = 1.08 - 4.84). In contrast, the *KIR2DL2-HLA-C1* combination resulted in a significantly lower SVR rate (26% [6/23] vs. 54% [50/92]; $P = 0.015$ [$Pc = 0.06$]; OR = 0.30, 95% CI = 0.11 - 0.82). Although several studies have found that *KIR2DL3-HLA-C1* carriers are associated with treatment-induced and spontaneous clearance of HCV in Caucasians, no such association was found in our cohort (data not shown).

Patients with *KIR3DL1-HLA-Bw4* but without *KIR2DL2-HLA-C1* had a higher SVR rate (55% [31/56] vs. 32% [19/59]; $P = 0.012$ [$Pc = 0.1$]; OR = 2.61, 95% CI = 1.22 - 5.58) (Table 2). Conversely, the frequency of the *KIR2DL2-HLA-C1* positive, but *KIR3DL1-HLA-Bw4* negative condition was significantly higher in non-responders (17% [10/59] vs. 2% [1/56]; P = 0.014 [$Pc = 0.12$]; OR = 0.09, 95% CI = 0.01 - 0.72).

Prediction of a Sustained Virological Response by KIR-HLA and IL28B

Examination of the *IL28B* rs8099917 SNP in our cohort revealed significant differences in SVR frequencies. The SVR rate in patients with the *IL28B* TT genotype was significantly higher in those with TG or GG genotypes (62% [44/71] vs. 27% [12/44), $P = 0.0003$; OR = 4.35, 95% CI = 1.92 - 9.85). In subjects with *IL28B* TT and *KIR3DL1-HLABw4*, virologic clearance was significantly increased over other combinations (68% [27/40] vs. 39% [29/75]; $P = 0.003$ [$Pc = 0.024$]; OR 3.29, 95% CI = 1.47 - 7.39).

We next evaluated several factors found in association with an SVR to PEG-IFN and ribavirin therapy for independence by logistic regression analysis. Fifty-six responders were compared with 59 non-responders by means of a forward stepwise likelihood ratio logistic regression method; estimated OR coefficients, 95% CI, and *P* values are summarized in Table 3 for the variables that remained in equation at the last step. *IL28B* TT genotype ($P = 0.00009$; OR = 6.87, 95% CI = 2.62 - 18.01), *KIR2DL2-HLA*-C1 ($P = 0.014$; OR = 0.24, 95% CI = 0.08 - 0.75), white blood cell count ≥ 4410/μL ($P = 0.009$; OR = 3.32, 95% CI= 1.35 - 8.16), and *KIR3DL1-HLA-Bw4* ($P = 0.008$; OR = 3.32, 95% CI = 1.37 - 8.05) were all identified as independent parameters that significantly influenced an SVR.

The frequency of the *IL28B* TT genotype with *KIR3DL1-HLA-Bw4* in responders was significantly higher than in non-responders (48% [27/56] vs. 22% [13/59]; $P = 0.003$ [$Pc = 0.024$]; OR = 3.29, 95% CI = 1.47 - 7.39) (Table 2). Patients with the *IL28B* TT genotype without *KIR2DL2-HLA-C1* had a significantly higher SVR rate (68% [38/56] vs. 31% [18/59]; $P = 0.000062$ [$Pc = 0.0005$]; OR = 4.81, 95% CI = 2.19 - 10.58). The frequency of a non-SVR was significantly higher in patients with the *IL28B* non-TT genotype both with and without

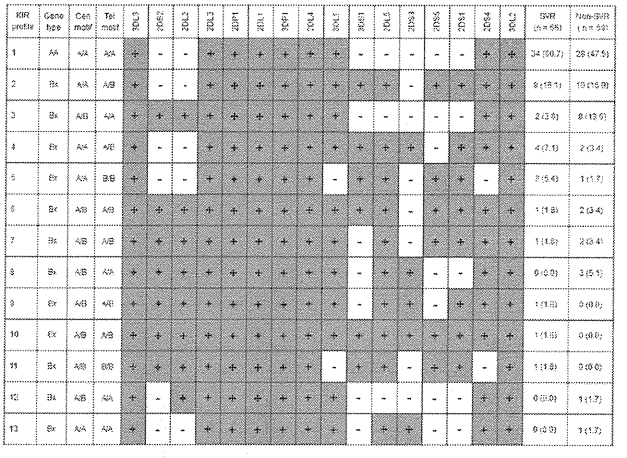

Figure 3. *KIR* gene profile frequencies in 56 patients with a sustained virological response (SVR) and 59 patients with a non-SVR to pegylated interferon and ribavirin therapy of chronic hepatitis C. Numerical data represent the number of individuals (%). The presence of *KIR* genes is indicated by gray shading. Cen, centromeric; Tel, telomeric.

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Table 3. Logistic regression analysis of variables contributing to a sustained virological response to pegylated interferon and ribavirin.

Factor	Odds ratio	95% confidence interval	*P*
IL28B TT genotype	6.87	2.62 - 18.01	0.00009
KIR2DL2/HLA-C1	0.24	0.08 - 0.75	0.014
White blood cells ≥ 4410/μL	3.32	1.35 - 8.16	0.009
KIR3DL1/HLA-Bw4	3.32	1.37 - 8.05	0.008

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

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KIR2DL2-HLA-C1 (14% [8/59] vs. 0% [0/8]; $P = 0.013$ [$Pc = 0.1$] and 41% [24/59] vs. 21% [12/56]; $P = 0.026$ [$Pc = 0.21$]; OR = 0.40, 95% CI = 0.17 - 0.91, respectively). The ability to predict an SVR by *IL28B* genotype and *KIR3DL1-HLA-Bw4* and *KIR2DL2-HLA-C1* was next evaluated. Corresponding values for sensitivity, specificity, PPV, and NPV are listed in Table S1 in File S1. A combination of the *IL28B* TT genotype and *KIR3DL1-HLA-Bw4* demonstrated high predictive specificity (78%), as did the combination of *IL28B* TT genotype and *KIR2DL2-HLA-C1* (86%).

Lastly, we analyzed combinations of the three factors of *IL28B* genotype, *KIR3DL1-HLA-Bw4*, and *KIR2DL2-HLA-C1* for prediction of treatment outcome (Table S2 in File S1). The frequencies of *IL28B* TT, *KIR2DL2-HLA-C1*-negative, with and without *KIR3DL1-HLA-Bw4* were significantly higher among responders (38% [21/56] vs. 19% [11/59]; P = 0.024 [Pc = 0.29]; OR = 2.62, 95% CI = 1.12 - 6.12 and 30% [17/56] vs. 12% [7/59]; P = 0.015 [Pc = 0.18]; OR = 3.24, 95% CI = 1.22 - 8.57, respectively).

Discussion

The present study examined *HLA*, *KIR*, and *IL28B* gene variant associations with an SVR following PEG-IFN and ribavirin therapy in Japanese patients with chronic hepatitis C. We found a significant association of *HLA-Bw* alleles with treatment outcome, although the frequency of *HLA-C* alleles did not differ significantly between responders and non-responders. Functional analyses have demonstrated that NK cells in *HLA-C1C1* subjects exhibit a more rapid and stronger antiviral response that those in *HLA-C2C2* subjects due to differing responses of *HLA-C*-inhibited NK subsets[33]. *HLA-C2C2* homozygousity is strongly associated with treatment failure in HCV patients of European ancestry [11,22], but we could not assess its role in our study because this genotype was found in only 1 of 115 patients.

We uncovered a significant association between the presence of *KIR2DL2* or *KIR2DS2* and lower SVR rates. Several reports have shown that *KIR2DL3-HLAC1* in Caucasians [11,22] and *KIR2DL5* in Brazilians [34] are associated with treatment outcome of antiviral therapy. Since our results showed no such statistical significances, these conflicting interpretations may reflect differences in patient selection, genetic background, sample size, and/or treatment regimen. Further studies are required to clarify this discrepancy in the Japanese population.

A study by Dring et al. examined *KIR* haplotypes in patients with HCV infection and showed that a centromeric *KIR* haplotype was increased in chronic HCV infection as compared with resolved cases [20]. We therefore determined *KIR* haplotypes and *Cen-A/B* and *Tel-A/B* in our patients as well, and found an interesting association between *Cen-A/A* and an SVR to antiviral therapy (P = 0.015; OR 3.37). Since *Cen-A/B* is determined by *KIR2DL3* and *KIR2DS2* and/or *KIR2DL2*, this finding is consistent with our results demonstrating a relationship between *KIR2DS2* and *KIR2DL2* genotypes and treatment failure.

The most significant finding in this study was the association between KIR-HLA receptor-ligand pairings and treatment outcome in chronic hepatitis C. Among the inhibitory KIR-HLA receptor-ligand pairs, patients with *KIR3DL1-HLA-Bw4* exhibited a significantly higher SVR rate when compared to those without this pair (P = 0.03; OR 2.29). Conversely, virologic clearance in patients with *KIR2DL2-HLA-C1* was significantly lower than in those without (P = 0.015; OR = 0.30). Stratification analysis of the 4 groups of *KIR3DL1-HLA-Bw4* (presence or absence) and *KIR2DL2-HLA-C1* (presence or absence) revealed a higher frequency of responders with *KIR3DL1-HLA-Bw4* presence, *KIR2DL2-HLA-C1* absence compared with those possessing *KIR2DL2-HLA-C1* presence, *KIR3DL1-HLA-Bw4* absence (62% vs. 9%; P = 0.0044; OR =16.32). When these KIR-HLA pairs were both either positive or negative, SVR rates were similar at 42% and 45%, respectively. Together with the results of logistic regression analysis, we clearly showed that *KIR3DL1-HLA-Bw4* was positively associated with an SVR (OR = 3.32) and that *KIR2DL2-HLA-C1* had a negative association (OR = 0.24) with treatment outcome. As almost one half of the Japanese population have the functional *KIR3DL1-HLA-Bw4* combination, this inhibitory receptor-ligand interaction 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 [35]. Therefore, these cells might be more weakly controlled by inhibitory signals than other NK cells, more easily activated by viral infection, and more readily promoted for cytolysis and IFN-gamma production.

This study confirmed that the *IL28B* TT genotype is a strong predictor of an SVR in Japanese patients[18,32]. Furthermore, SVR frequencies were positively correlated with a combination of the *IL28B* TT genotype and *KIR3DL1-HLA-Bw4* (P = 0.0019) and negatively associated with the *IL28B* TT genotype and *KIR2DL2-HLA-C1* (P = 0.0067). These combinations were also highly specific for virologic response prediction. In light of these findings, patients with poor expected treatment outcome may be advised to wait for the use of combinations of direct acting antiviral agents[36]. Akuta et al. reported that a combination of amino acid substitutions in the core region of HCV and *IL28B* genotype was a useful predictor of PEG-IFN, ribavirin, and telaprevir therapy results in Japan[37]. Since we could not collect sera before treatment for all patients, we were not able to assess the effect of amino acid substitutions in the HCV core region. Furthermore, interferon-free combinations of direct-acting antiviral agents have become an area of considerable clinical interest. Chu et al. have reported that IL28B genotype appears to affect early viral kinetics in patients with chronic hepatitis C receiving interferon-free treatment [38]. Recently, two groups have discovered *IFN* lambda *4* (*IFNL4*), a new gene that may account for associations of spontaneous and IFN-based treatment clearance of HCV [39,40]. The IFN-λ 4 protein is generated by individuals who carry the ΔG allele of the ss469415590 variant, and the presence of this protein is strongly associated with impaired clearance of HCV. Linkage disequilibrium is strong between the *IFNL4-ΔG* allele and the unfavorable rs12979860-T allele (*IL28B*) in subjects of European or Asian ancestry, whereas this linkage disequilibrium is moderate in individuals of African ancestry [39]. We have confirmed that the linkage disequilibrium between the *IFNL4-ΔG* allele and *IL28B* SNP (rs8099917) is high and that the *IFNL4-ΔG* allele is strongly associated with treatment failure of PEG-IFN and ribavirin therapy in patients with Japanese chronic hepatitis C [41]. Hence, the clinical impacts of HLA-KIR genetic variants, IL28B genotype, and the IFNL4 allele should be explored.

In conclusion, the present study showed significant associations of *KIR3DL1-HLA-Bw4, KIR2DL2-HLA-C1*, and *IL28B* combinations with an SVR to PEG-IFN and ribavirin therapy in Japanese patients with genotype 1 HCV. The clinical significance of *IL28B* genotyping combined with HLA/KIR pairs to predict treatment outcome warrants further validation for triple therapy.

Supporting Information

File S1. Table S1, Sensitivity, specificity, and predictive values of IL28B TT genotype and KIR3DL1/HLA-Bw4 or

KIR2DL2/HLA-C1 for a sustained virological response in 115 patients with chronic hepatitis C. Data are expressed as % (n). PPV, positive predictive value; NPV, negative predictive value. Table **S2**, Frequency of ***IL28B*** genotype and ***KIR3DL1/HLA-Bw4*** and ***KIR2DL2/HLA-C1*** combinations in 56 patients with a sustained virological response (SVR) and 59 patients with a non-SVR to pegylated interferon and ribavirin therapy of chronic hepatitis C. Data are expressed as n (%).
(DOC)

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Author Contributions

Conceived and designed the experiments: YN TU ET MO. Performed the experiments: YN TU YK MO. Analyzed the data: YN TU YK MO. Contributed reagents/materials/analysis tools: YN TU SJ YK SS TK SM MK AM ET. Wrote the manuscript: TU MO.

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Review Article

Guidelines for avoiding risks resulting from discontinuation of nucleoside/nucleotide analogs in patients with chronic hepatitis B

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Nucleoside/nucleotide analogs (NUC) can lead to rapid reduction in hepatitis B virus (HBV) DNA levels in blood and normalization of alanine aminotransferase levels in many patients. They also provide histological improvement which results in a reduction in liver carcinogenesis. However, it is difficult to completely remove viruses even by NUC and there are some problems such as emergence of resistant strains and hepatitis relapse resulting from discontinuation of treatment. One of the reasons for this is that NUC reduce the HBV DNA level in blood but have almost no effects on the HBV cccDNA level in hepatocyte nuclei, which are the origins of HBV replication, and HBV cccDNA remains for a long period. For treatment with NUC in patients with hepatitis B, it is considered that NUC should not be easily discontinued because discontinuation often results in hepatitis relapse. However, it has not been clearly revealed when and how hepatitis relapses after discontinuation. Although some patients do not experience hepatitis relapse after discontinuation of NUC, or experience only mild relapse and finally achieve a stable condition, it has not been established how to identify such patients efficiently. We performed research to investigate characteristics of the course after discontinuation of treatment and definition of hepatitis relapse and estimate the relapse rate. "Guidelines for avoiding risks resulting from discontinuation of NUCs 2012" is summarized based on the study results. Because the guidelines are written in Japanese, we explain them in English as a review article.

Key words: discontinuation of treatment, hepatitis B virus cccDNA, hepatitis B, hepatitis relapse, nucleoside/nucleotide analog

INTRODUCTION

BECAUSE NUCLEOSIDE/NUCLEOTIDE analogs (NUC) that have been recently introduced to treat hepatitis B strongly inhibit proliferation of hepatitis B virus (HBV), they can lead to rapid reduction in HBV DNA levels in blood and normalization of alanine aminotransferase (ALT) levels in many patients.[1] They also provide histological improvement which results in a reduction in liver carcinogenesis[2,3] and can be administrated p.o. with few side-effects, so they are widely used in clinical practice. However, it is difficult to completely remove viruses even by NUC and there are some problems such as emergence of resistant strains and hepatitis relapse resulting from discontinuation of treatment.[4] One of the reasons for this is that NUC reduce the HBV DNA level in blood but have almost no effects on the HBV cccDNA level in hepatocyte nuclei, which are the origins of HBV replication, and HBV cccDNA remains for a long period.[5]

For treatment with NUC in patients with hepatitis B, it is considered that NUC should not be easily discontinued because discontinuation often results in hepatitis relapse. However, it has not been clearly revealed when and how hepatitis relapses occur after discontinuation. Although some patients do not experience hepatitis relapse after discontinuation of NUC, or experience only mild relapse and finally achieve a stable condition, it has not been established how to identify such patients efficiently.

We performed research funded by a Health and Labor Sciences Research Grant to investigate characteristics of the course after discontinuation of treatment, definition of hepatitis relapse and estimation of relapse rate.[6] "Guidelines for avoiding risks resulting from discontinuation of NUCs 2012" is summarized based on the

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study results.[7] The guidelines do not always recommend discontinuation of NUC. We determined them to be referred to if it is necessary to consider discontinuation of NUC due to various reasons.

SERUM MARKERS REFLECTING AMOUNT OF HBV CCCDNA IN HEPATOCYTES

THE REPLICATION PROCESS of HBV in hepatocytes is shown in Figure 1. HBV is an enveloped DNA virus containing a relaxed circular DNA genome converted into a cccDNA episome in the nucleus of infected cells.[8–11] These cccDNA molecules serve as transcriptional templates for production of viral RNA that encode both viral structural and non-structural proteins. Hepatitis B surface antigen (HBsAg) is translated from 2.1-kb and 2.4-kb mRNA. On the other hand, hepatitis B core antigen (HBcAg), p22cr antigen (p22crAg)[12] and hepatitis B e-antigen (HBeAg) are translated from 3.5-kb mRNA which also serves as pregenome RNA. HBeAg is secreted into the blood stream as a secretion protein, and p22crAg forms genome negative core particles. HBcAg forms nucleocapsid particles by incorporating pregenome RNA. Once the pregenome RNA is reverse transcribed to DNA, the particles are enveloped with lipid layer containing HBsAg and then secreted into blood stream as virions.[9,10] When the reverse transcriptation is inhibited by NUC, virus particles with RNA genome are secreted instead of those with DNA genome.[13,14]

Hepatitis B virus cccDNA is a stable molecule like chromosomal DNA which can be barely destroyed by DNase in natural conditions. Because NUC are inhibitors of reverse transcriptase, they have no direct effect on reducing intrahepatic cccDNA levels. Therefore, reactivation of HBV replication which originates from HBV cccDNA and incidental hepatitis relapse occurs when NUC are discontinued.

It is generally considered that HBV cccDNA levels in hepatocytes are well correlated with the proliferative potential of HBV;[5] serum markers reflecting the cccDNA level are suggested to be useful as clinical indicators. Serum level of HBV DNA correlates well with intrahepatic level of HBV cccDNA in the natural course but not under NUC treatment. NUC reduce serum level of HBV DNA rapidly by inhibiting the reverse transcription, but this inhibition does not reduce the cccDNA level.[5] On the other hand, serum levels of HBsAg and hepatitis B core-related antigen (HBcrAg) have been reported as markers reflecting cccDNA levels in hepatocytes even under NUC treatment.[15–18] HBcrAg assay measures all antigens coded by precore/core genome simultaneously which include HBcAg, HBeAg and p22crAg, and has been reported to be useful for predicting clinical outcomes of patients who were treated with NUC.[6,18–23] HBsAg level has received attention recently as a new marker and has been reported to be efficient in prediction of treatment effects by interferon and others.[15,16]

AIMS OF THESE GUIDELINES

THESE GUIDELINES AIM to identify patients with a higher possibility of successful discontinuation or patients who should continue treatments and avoid

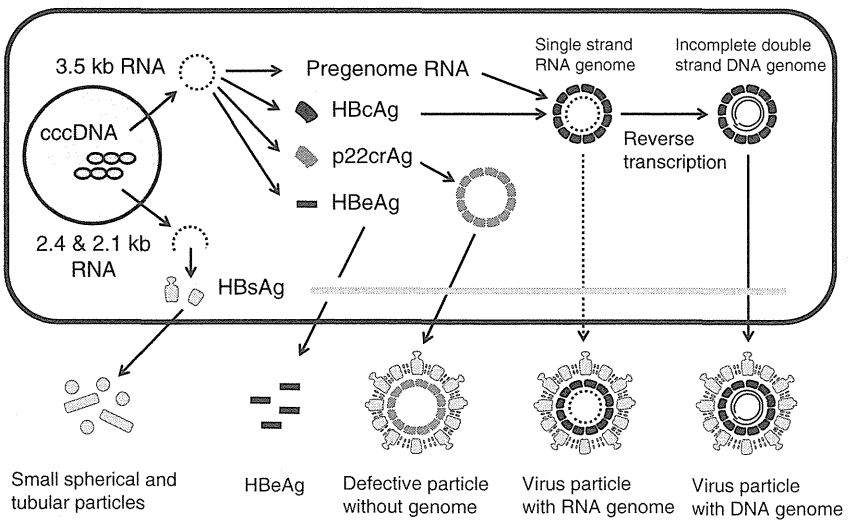

Figure 1 Replication process of hepatitis B virus (HBV) which originates from HBV cccDNA molecules pooled in nucleus of hepatocyte. HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e-antigen; p22crAg, p22cr antigen.

risks resulting from discontinuation of NUC as much as possible by establishing indicators for follow up after discontinuation (Appendix 1-I). Successful discontinuation in the guidelines is defined as final achievement of the inactive carrier state with ALT level of less than 30 IU/L and HBV DNA level in blood of less than 4.0 log copies/mL. These criteria were defined in compliance with the guidelines for treatment of chronic hepatitis B in Japan.[24] It is known that patients in the inactive carrier state show no progression of hepatic diseases and a reduction in the carcinogenic rate[25,26] and the criteria are considered to be appropriate.

REQUIREMENTS TO AVOID RISK OF DEVELOPING SEVERE HEPATITIS RESULTING FROM RELAPSE

WE ARE CURRENTLY unable to predict hepatitis relapse after discontinuation of NUC with sufficient accuracy. Therefore, we reviewed the risk of developing severe hepatitis and established requirements to prevent severe hepatitis (Appendix 1-II).[27] The presence of understanding the risks of hepatitis relapse and severe hepatitis by both doctors and patients as well as the availability of a follow-up system after discontinuation and appropriate treatment for relapse are the basic essential requirements. Considering that patients with hepatic cirrhosis or chronic hepatitis with progressed fibrosis similar to cirrhosis can easily develop severe hepatitis and have higher risks of carcinogenesis in the future, we determined that those patients should not easily discontinue NUC.

ASSESSMENT OF PROLIFERATIVE POTENTIAL OF HBV AND CONDITIONS TO REDUCE THE RELAPSE RISK

IT HAS BEEN experienced that patients with insufficient reduction of HBV DNA level or with HBeAg positive at the time of discontinuation of NUC can develop hepatitis relapse at higher rates after discontinuation. The tendency was also confirmed scientifically in our study.[6] The cut-off value of HBV DNA level to predict hepatitis relapse was 3.0 log copies/mL by receiver operating characteristic (ROC) analysis. Almost all patients with higher HBV DNA levels or were HBeAg positive relapsed within a year while nearly 30% of patients with HBV DNA levels less than 3.0 log copies/mL and without HBeAg were in a stable condition for a long period (Fig. 2). Based on these results, we included sufficient reduction in HBV DNA levels and

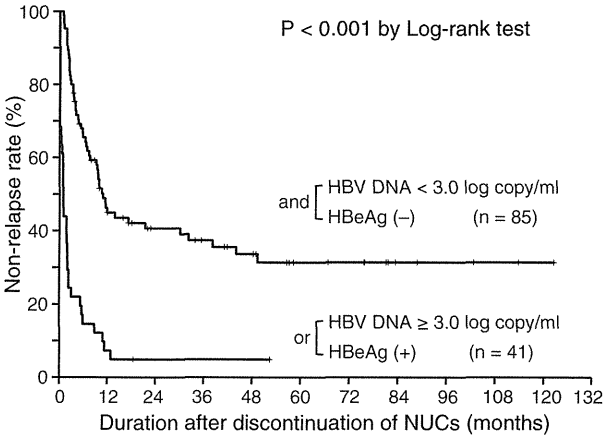

Figure 2 Comparison of non-relapse rates using Kaplan–Meier method between 41 patients with serum hepatitis B virus (HBV) DNA not lower than 3.0 log copies/mL or with hepatitis B e-antigen (HBeAg) and 85 patients with serum HBV DNA lower than 3.0 log copies and without HBeAg at the time of nucleoside/nucleotide analog (NUC) discontinuation.

HBeAg negativity in requirements for discontinuation. We determined the reference range of sufficient reduction in HBV DNA levels in the actual guidelines not to be less than 3.0 log copies/mL but to be negative by real-time polymerase chain reaction (PCR) in consideration of safety.

Factors relating to hepatitis relapse after discontinuation were analyzed in the population except for patients who were obviously predicted to relapse after discontinuation, or those with HBV DNA levels of not less than 3.0 log copies/mL or were HBeAg positive. The following factors were calculated to be significant: duration of treatment period of NUC; HBsAg levels at the time of discontinuation; and HBcrAg levels at the time of discontinuation. Because the cut-off value in duration of treatment period was calculated as 16 months, we overestimated and established that NUC should be discontinued more than 2 years after the initial administration in the guidelines.[6]

Two cut-off values were suggested from the results of the ROC analysis for the HBsAg and HBcrAg levels at the time of discontinuation (Fig. 3): 1.9 and 2.9 log IU/mL for the HBsAg level and 3.0 and 4.0 log U/mL for the HBcrAg level, respectively. Based on this, HBsAg and HBcrAg levels were scored as shown in Appendix 1-III and three groups – low-risk, medium-risk and high-risk – were determined. The percentage of prediction success was 80–90% in the low-risk group, approximately 50% in the medium-risk group and 10–20% in the high-risk

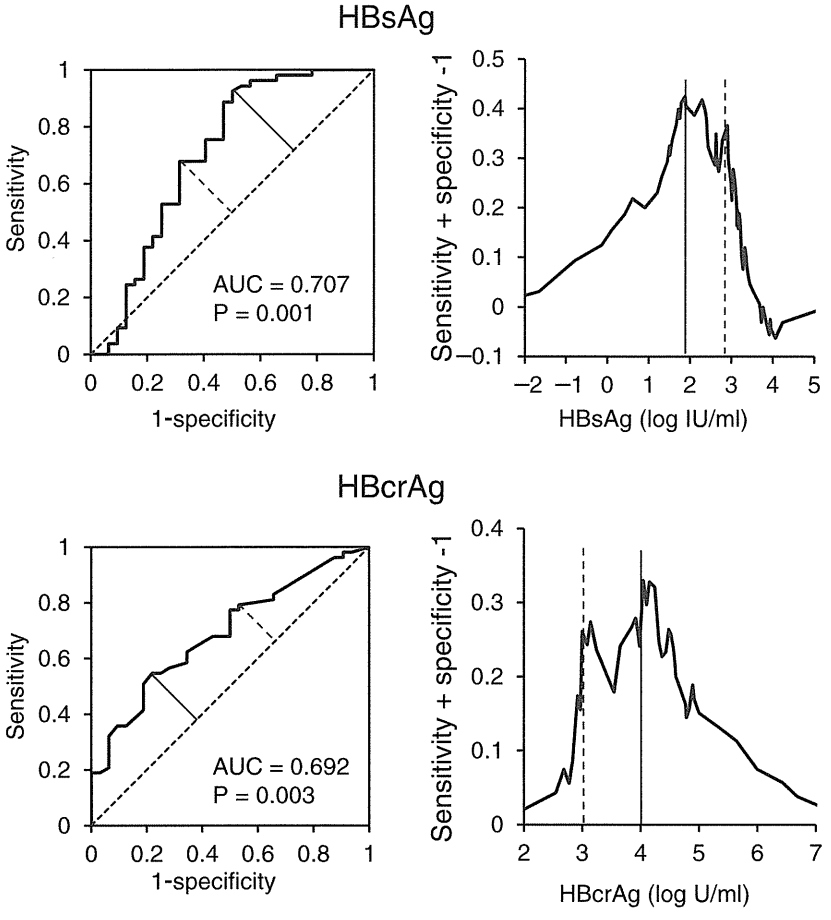

Figure 3 Receiver operating characteristic (ROC) analysis of hepatitis B surface antigen (HBsAg) and HB core-related antigen (HBcrAg) levels to discriminate between patients with and without hepatitis relapse. The existence of two inflection points is suggested for both HBsAg and HBcrAg levels. Short diagonal lines indicate main inflection points and short broken diagonal lines indicate second inflection points. Vertical lines indicate actual values of antigens that correspond to the main inflection points and vertical broken lines indicate actual values of antigens that correspond to the second inflection points. AUC, area under the ROC.

group (Fig. 4). In further investigation of factors relating to hepatitis relapse in each group, no factors were newly found in the low- and medium-risk groups but age was a significant factor in the high-risk group. Although the percentage of prediction success rate is low in the high-risk group (10–20%), it resulted in slightly higher rates of 30–40% with those patients younger than 35 years old.[6] It was interesting to find that the combination of HBsAg and HBcrAg levels were useful in preparing these guidelines for discontinuation. Because productions of HBsAg and HBcrAg are regulated by different promoter and enhance systems of HBV genome, their clinical values vary.

FOLLOW-UP METHOD AFTER DISCONTINUATION AND CONDITIONS FOR RETREATMENT

FOLLOW-UP AFTER DISCONTINUATION of NUC includes periodical measurement of HBV DNA levels (real-time PCR) and ALT levels. This study revealed that

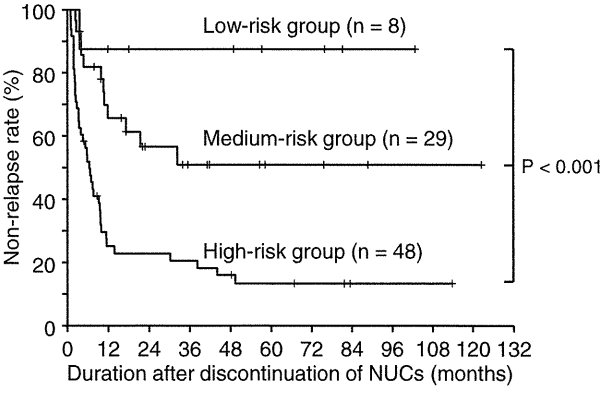

Figure 4 Comparison of non-relapse rates using the Kaplan–Meier method among three groups classified by the sum of the scores of hepatitis B surface antigen and HB core-related antigen levels at the time of nucleoside/nucleotide analog (NUC) discontinuation.

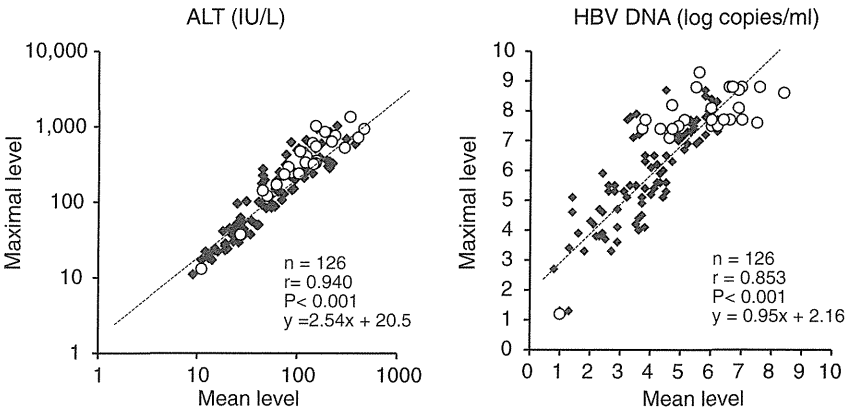

Figure 5 Correlation between maximal and mean levels of alanine aminotransferase (ALT) (left) and hepatitis B virus (HBV) DNA (right) after discontinuation of nucleoside/nucleotide analog (NUC). Open circles indicate patients with detectable hepatitis B e-antigen (HBeAg) and closed squares indicate patients without detectable HBeAg.

relapse after discontinuation occurs mostly within 1 year, gradually decreases after 1 year and rarely occurs after the first 3 years of discontinuation.[6] Therefore, we determined it necessary to pay attention especially to relapse immediately after discontinuation. In particular, we determined that it is desirable to follow up patients by blood tests at every 2 weeks up to 16 weeks after discontinuation and every 4 weeks after 16 weeks.

One of the important points is what the definition of hepatitis relapse is and how to follow up after discontinuation. Transient abnormalities in the ALT level or the HBV DNA level may be observed in approximately two-thirds patients who would finally achieve the inactive carrier state. Therefore, even if the ALT or HBV DNA levels show mild elevations, it is possible to follow up without retreatment. However, no criteria have been identified about when to discontinue follow up and start retreatment. We assessed the transitions of ALT levels and HBV DNA levels after discontinuation of NUC by the mean and maximum values to identify the criteria. From this assessment, a strong correlation was shown between the mean and the maximum value in both (Fig. 5).[6] Results of the ROC analysis revealed that the mean ALT of 30 IU/L corresponded to the maximum ALT of 79 IU/L and the mean HBV DNA of 4.0 log copies/mL corresponded to the maximum HBV DNA of 5.7 log copies/mL. Patients with ALT values of not less than 80 IU/L after discontinuation are highly likely to show a mean value of more than 30 IU/L and not assumed to finally meet the criteria for successful discontinuation. Similarly, patients with HBV DNA value of not less than 5.8 log copies/mL after discontinuation are most likely to show a mean value of more than 4.0 log copies/mL and not assumed to meet the criteria for successful discontinuation. Based on these results, we established the condition that patients with ALT value of not less than 80 IU/L or HBV DNA level of not less than 5.8 log copies/mL are less likely to finally achieve the inactive carrier state and should be considered for retreatment with NUC. It is considered that NUC can be discontinued more efficiently and specifically in this condition. Physicians can use more severe criteria at their own discretion in consideration of safety. Less strict criteria also can be used, but it is recommended that the treatment should be done under a certain policy and do not follow the treatment without any aims.

KEY POINTS AND FUTURE ISSUES

THIS MAY BE the first guideline for discontinuation of NUC. Most of the data used in this guideline are retrospective and some points remain unsolved. Over 90% of the patients enrolled had genotype C and over 90% of cases were treated with lamivudine until discontinuation.[6] Therefore, key points and future issues are summarized in Appendix 1-V. This guideline provides information to support physicians to decide NUC discontinuation timing but physicians should actually consider for each patient whether NUC can be discontinued or not because long-term prognosis after NUC discontinuation is not yet clear enough and patients' wishes and physicians' decision need to be prioritized. When NUC cannot be successfully discontinued, one of the options is re-administration of NUC. However, it has not been investigated whether re-administration of NUC results in the emergence and development of resistant strains. Further, it is not resolved which NUC should be given when re-administration is required. The consent of patients will be necessary on these points.

One of the issues to be investigated in the future is to improve accuracy in predicting hepatitis relapse after discontinuation. Investigations on the following approaches are suggested: higher sensitivity HBV DNA, HBV RNA,[13,14] HBV genotypes and HBV genetic mutations. Because these guidelines were prepared based on retrospective studies, it is necessary to validate them with prospective studies. In addition, how to actively discontinue NUC by sequential treatment with interferon also should be included as an important issue to be investigated.

Three kinds of NUC are available now in Japan. Lamivudine was the first NUC introduced into Japan in 2000. Adefovir dipivoxil is used mainly for patients with lamivudine resistance. Entecavir is now recommended as the first-choice NUC. Over 10 years have passed since the first NUC became available in Japan and this is the first full-scale guideline for NUC discontinuation. Although this guideline may not be completely sufficient and needs further investigations, this is the first step leading to a better one in the future.

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APPENDIX

Guidelines for avoiding risks resulting from discontinuation of nucleoside/nucleotide analogs 2012

I. Aims of these guidelines

IN TREATMENT WITH nucleoside/nucleotide analogs (NUC) in patients with chronic hepatitis B, it is an important treatment goal to aim at drug-free status by discontinuation of NUC. However, discontinuation of NUC often results in hepatitis relapse which may become severe. Sufficient consideration must be given to the risk in case of discontinuation.

Hepatitis B surface antigen (HBsAg) negativity is the goal of treatment with NUC, but it cannot be always achieved easily. Therefore, discontinuation may be considered even if HBsAg remains positive. These guidelines aim to discontinue NUC in such conditions and finally achieve the inactive carrier state (alanine aminotransferase [ALT] <30 IU/L and hepatitis B virus [HBV] DNA level in blood <4.0 log copies/mL).

It is currently unknown which of the two options for NUC, discontinuation or continuation, is effective on life prognosis or liver carcinogenesis. We established these guidelines to be referred in case of considering discontinuation due to various reasons. We aimed to identify patients with a high possibility of successful discontinuation or patients who should inversely continue the treatment and establish indicators for follow up after discontinuation to avoid risks resulting from discontinuation of NUC as much as possible.

II. Requirements to avoid risk of developing severe hepatitis resulting from relapse

The following requirements are determined for discontinuation to previously assume and avoid the risk of developing severe hepatitis.

1. Both the doctor and the patient fully understand the risk of a high frequency of hepatitis relapse that may become severe.
2. It is possible to follow up as well as to treat appropriately in case of relapse. (Involvement of a specialist is recommended.)
3. The patient has mild hepatic fibrosis with good hepatic functional reserve and will not easily develop severe hepatitis in relapse. (NUC should not be discontinued in patients with hepatic cirrhosis or chronic hepatitis with progressed fibrosis similar to cirrhosis.)

III. Assessment of proliferative potential of HBV and conditions to reduce the relapse risk

1. Requirements for discontinuation of nucleoside/nucleotide analogs.
   Almost all patients with high proliferative potential of HBV will relapse after discontinuation. It is essential not to discontinue NUC in these patients and the requirements were determined as follows: (i) HBV DNA level in blood is negative (real-time PCR) at the time of discontinuation; and (ii) hepatitis B e-antigen (HBeAg) level in blood is negative at the time of discontinuation.
2. Condition for duration of treatment period of NUC.
   Because short-term treatment with NUC can easily result in relapse, it is recommended to meet the following condition: more than 2 years after the initial administration of NUC.
3. Assessment of relapse risk by scoring of viral antigen levels.
   For the patients who meet the requirements for discontinuation (HBV DNA negative and HBeAg negative at the time of discontinuation), the HBsAg level and the HBcrAg level at the time of discontinuation can be scored to predict the relapse risk by the following three groups based on the total score. This risk prediction aims to determine whether NUC should be discontinued or not by reference to it to reduce the relapse risk.

HBsAg levels at the time of discontinuation	Scores	Hepatitis B core-related antigen (HBcrAg) levels at the time of discontinuation	Scores
<1.9 log IU/mL (<80 IU/mL)	0	<3.0 log U/mL	0
1.9–2.9 log IU/mL (80–800 IU/mL)	1	3.0–4.0 log U/mL	1
≥2.9 log IU/mL (≥800 IU/mL)	2	≥4.0 log U/mL	2

Relapse risk	Total scores	Percentage of prediction success	Assessment
Low-risk group	0	80–90%	Discontinuation can be considered. It is essential to pay attention to relapse because some patients of low risk may develop hepatitis relapse.
Medium-risk group	1–2	~50%	Discontinuation can be considered depending on the situation. Further consideration is needed about conditions and the way to discontinue in the future.
High-risk group	3–4	10–20%	Continuous treatment is recommended. However, patients under 35 years old show a relatively higher rate of successful discontinuation of 30–40%.

IV. Follow-up method after discontinuation and conditions for retreatment

1. HBV DNA levels (real-time PCR) and ALT levels must be periodically measured after discontinuation of NUC to pay attention to HBV proliferation and hepatitis relapse resulting from proliferation.
2. Relapse after discontinuation is mostly observed within 1 year and then gradually decreases. It is rare to relapse after the first 3 years. Therefore, it is necessary to pay attention to relapse immediately after discontinuation. In particular, patients should be followed up by blood tests every 2 weeks up to 16 weeks after discontinuation and every 4 weeks after 16 weeks.
3. Transient abnormalities in ALT levels or HBV DNA levels may be observed in approximately two-thirds of patients who successfully discontinued NUC and would finally achieve the inactive carrier state. Therefore, even if the ALT level or the HBV DNA level shows mild elevations, it is possible to keep following up without retreatment. However, patients who meet the following condition are less likely to finally achieve the inactive carrier state and should be considered for NUC retreatment.

Condition to consider retreatment with NUC
ALT ≥80 IU/L or HBV DNA ≥5.8 log copies/mL after discontinuation

V. Key points and future issues

1. The status differs in each patient. Objectives and significance also differ by patient. Thus, doctors must determine whether NUC should be discontinued or not in consideration of those conditions. In case of considering discontinuation, it is recommended to consult with a specialist of hepatic diseases.
2. In case of retreatment with NUC due to hepatitis relapse after discontinuation, it is unknown whether it results in higher emergence of strains resistant to NUC or not compared with patients without discontinuation.
3. Because HBV carriers rarely experience hepatitis relapse even in the inactive carrier state (HBV DNA <4.0 log copy/mL and ALT <30 IU/L), they must be followed up after successful discontinuation. Liver carcinogenesis also requires follow up.
4. The followings are included in future issues; improvement of accuracy in the criteria for discontinuation of NUC; investigation of the criteria used in these guidelines in a prospective study; and investigation of the way to actively discontinue NUC using sequential treatment with interferon.

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