passed the quality control filters in the genome-wide association analysis using HBV carriers and healthy controls, and using HBV carriers and HBV-resolved individuals, respectively (Figure 1). All cluster plots for the SNPs showing $P<0.0001$ on association analyses in the allele frequency model were confirmed by visual inspection, and SNPs with ambiguous cluster plots were excluded.

In the following replication stage, we selected a set of 32 SNPs with $P<0.0001$ in the GWAS using HBV carriers and HBV-resolved individuals. SNP genotyping in two independent sets of 256 Japanese HBV carriers, 236 Japanese healthy controls and 150 Japanese HBV-resolved individuals (Table 1, replication-1), and 344 Korean HBV carriers, 151 Korean healthy controls and 106 Korean HBV-resolved individuals (Table 1, replication-2) was completed for the selected 32 SNPs using the DigiTag2 assay [21,22] and custom TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA) on the LightCycler 480 Real-Time PCR System (Roche, Mannheim, Germany).

Statistical Analysis

The observed associations between SNPs and the protective effects on chronic hepatitis B or clearance of hepatitis virus B were assessed by chi-squared test with a two-by-two contingency table in allele frequency model. SNPs on chromosome X were removed because gender was not matched among HBV carriers, healthy controls and HBV-resolved individuals. A total of 597,789 SNPs and 590,278 SNPs passed the quality control filters in the GWAS stage; therefore, significance levels after Bonferroni correction for multiple testing were $P=8.36\times10^{-8}$ (0.05/597,789) and $P=8.47\times10^{-8}$ (0.05/590,278), respectively. For the replication study, 29 of 32 SNPs were successfully genotyped; therefore, we applied $P=0.0017$ (0.05/29) as a significance level, and none of the 29 markers genotyped in the replication stage showed deviations from the Hardy-Weinberg equilibrium in healthy controls ($P>0.01$).

The genetic inflation factor λ was estimated by applying the Cochrane-Armitage test on all SNPs and was found to be 1.056 and 1.030 in the GWAS using HBV carriers and healthy controls, and using HBV carriers and HBV-resolved individuals, respectively (Figure S3). These results suggest that the population substructure should not have any substantial effect on statistical analysis. In addition, the principal component analysis in a total of 550 individuals in the GWAS stage together with the HapMap samples also revealed that the effect of population stratification was negligible (Figure S4).

Based on the genotype data of a total of 1,793 samples including 1,192 Japanese samples and 601 Korean samples in both GWAS and replication stages, haplotype blocks were estimated using the Gabriel's algorithm using the Haploview software (v4.2) (Figure S2). In the logistic regression analysis, two SNPs (rs9276431 and rs7768538) within the HLA-DQ locus were individually involved as a covariate (Table S5). Statistical analyses were performed using the SNP & Variation Suite 7 software (Golden Helix, MT, USA).

Supporting Information

Figure S1 GWAS using samples from HBV carriers with LC or HCC, and HBV carriers without LC and HCC. *P* values were calculated using chi-squared test for allele frequencies.
(PPTX)

Figure S2 Estimation of linkage disequilibrium blocks in HBV patients, HBV resolved individuals and healthy controls in Japanese and Korean. The LD blocks (r^2) were analyzed using the Gabriel's algorithm.
(PPTX)

Figure S3 Quantile-quantile plot for test statistics (allele-based chi-squared tests) for GWAS results. Dots represent P values of each SNP that passed the quality control filters. Inflation factor λ was estimated to be: a) 1.056 in the analysis with HBV carriers and healthy controls; and b) 1.030 with HBV carriers and HBV-resolved individuals.
(PPTX)

Figure S4 Principal component analysis on a total of 550 individuals in GWAS, together with HapMap samples (CEU, YRI and JPT).
(PPTX)

Table S1 Results for 29 SNPs selected in replication study using samples of HBV carriers and healthy controls. [a]*P* values by chi-squared test for allelic model. [b]Odds ratio of minor allele from two-by-two allele frequency table. [c]Meta-analysis was tested using additive, two-tailed CMH fixed-effects model.
(XLSX)

Table S2 Results of meta-analysis for protective effects against persistent HB infection across 6 independent studies, including this study. [a]Minor allele frequency and minor allele in 198 healthy Japanese (ref#19). [b]Odds ratio of minor allele from two-by-two allele frequency table. [c]*P* value of Pearson's chi-squared test for allele model. [d]Heterogeneity was tested using general variance-based method. [e]Meta-analysis was tested using the random effects model.
(XLSX)

Table S3 Results for 29 SNPs selected in replication study using samples from HBV carriers and HBV-resolved individuals. [a]*P* values by chi-squared test for allelic model. [b]Odds ratio of minor allele from two-by-two allele frequency table. [c]Meta-analysis was tested using additive, two-tailed CMH fixed-effects model.
(XLSX)

Table S4 Results of meta-analysis for clearance of HBV across 6 independent studies, including this study. [a]Minor allele frequency and minor allele in 198 healthy Japanese (ref#19). [b]Odds ratio of minor allele from two-by-two allele frequency table. [c]*P* value of Pearson's chi-squared test for allele model. [d]Heterogeneity was tested using general variance-based method. [e]Meta-analysis was tested using the random effects model.
(XLSX)

Table S5 Logistic regression analysis of *HLA-DP* (rs3077 and rs9277542) and *HLA-DQ* (rs9276431 and rs7768538) with susceptibility to CHB and HBV clearance using the *HLA-DQ* genotypes individually as a covariate.
(XLSX)

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Author Contributions

Conceived and designed the experiments: NN HS YT. Performed the experiments: HS Y. Mawatari M. Sageshima YO. Analyzed the data: NN MK AK. Contributed reagents/materials/analysis tools: KM M. Sugiyama SHA JYP SH JHK KS M. Kurosaki YA SM MW ET MH SK EO YI EM AT Y. Murawaki YH IS M. Korenaga KH TI NI KHH YT MM. Wrote the paper: NN M. Kawashima YT KT MM.

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BRIEF ARTICLE

Quantitation of HBsAg predicts response to entecavir therapy in HBV genotype C patients

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Abstract

AIM: To analysis the factors that predict the response to entecavir therapy in chronic hepatitis patients with hepatitis B virus (HBV) genotype C.

METHODS: Fifty patients [hepatitis B e antigen (HBeAg)-negative:HBeAg-positive = 26:24] with HBV genotype C, who received naïve entecavir therapy for > 2 years, were analyzed. Patients who showed HBV DNA levels ≥ 3.0 log viral copies/mL after 2 years of entecavir therapy were designated as slow-responders, while those that showed < 3.0 log copies/mL were termed rapid-responders. Quantitative hepatitis B surface antigen (HBsAg) levels (qHBsAg) were determined by the Architect HBsAg QT immunoassay. Hepatitis B core-related antigen was detected by enzyme immunoassay. Pre-C and Core promoter mutations were determined using by polymerase chain reaction (PCR). Drug-resistance mutations were detected by the PCR-Invader method.

RESULTS: At year 2, HBV DNA levels in all patients in the HBeAg-negative group were < 3.0 log copies/mL. In contrast, in the HBeAg-positive group, 41.7% were slow-responders, while 58.3% were rapid-responders. No entecavir-resistant mutants were detected in the slow-responders. When the pretreatment factors were compared between the slow- and rapid-responders; the median qHBsAg in the slow-responders was 4.57 log IU/mL, compared with 3.63 log IU/mL in the rapid-responders ($P < 0.01$). When the pretreatment factors predictive of HBV DNA-negative status at year 2 in all 50 patients were analyzed, HBeAg-negative status, low HBV DNA levels, and low qHBsAg levels were significant ($P < 0.01$). Multivariate analysis revealed that the low qHBsAg level was the most significant predictive factor ($P = 0.03$).

CONCLUSION: Quantitation of HBsAg could be a useful indicator to predict response to entecavir therapy.

Key words: Chronic hepatitis B; Quantitation of hepatitis B surface antigen; Entecavir; Hepatitis B virus genotype C; Slow-responders; Hepatitis B core-related antigen; Core promoter mutation; Pre-C mutation

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INTRODUCTION

Hepatitis B virus (HBV) is a major causative agent of chronic liver diseases[1]. Various strains of HBV have been isolated all over the world, and have been classified as HBV genotypes from A to J[2]. In Japan, about 85% of patients have HBV genotype C, and about 12% have HBV genotype B[3]. Worldwide, HBV genotypes show specific geographical distributions. HBV genotypes A and D are prevalent in the United States and Europe, while HBV genotypes B and C are prevalent in Asia[4]. Disease progression and prevalence of hepatitis B e antigen (HBeAg)-positive status are often associated with HBV genotypes[5]. Therefore, we analyzed the clinical and virological features of patients with HBV genotype C and homogenous backgrounds, because HBV genotype C is the predominant type in Japan.

Entecavir is widely used as a first-choice nucleot(s)ide analog (NA) for chronic hepatitis B patients, because less than 1% of entecavir-naive patients developed resistant mutants after 5 years of therapy[6-8]. However, in HBeAg-positive patients, the response rate to entecavir therapy is less favorable compared with HBeAg-negative patients[6,7]. In addition, some patients show a slow-response, which indicates that serum HBV DNA levels remain high after long-term entecavir therapy. However, it is unclear which patients become slow-responders. Therefore, the aim of this study is to clarify the virological and clinical characteristics of the slow-responders before and during long-term entecavir therapy for HBV genotype C.

MATERIALS AND METHODS

Patient population

From July 2007, 102 consecutive hepatitis B surface antigen (HBsAg)-positive patients with chronic liver disease were enrolled in a naïve entecavir therapy in our hospital. Ten patients dropped out, 15 patients discontinued therapy, 10 patients received immunosuppressive therapy during entecavir therapy, and 10 patients received entecavir for less than 2 years. Thus, 57 patients were analyzed in this prospective, single center study. The institutional review board of the hospital approved the study. Serum samples were drawn from the patients after obtaining written informed consent.

All the patients received 0.5 mg of entecavir daily. Patients with poor adherence were excluded from the study.

All patients were positive for HBsAg for more than 6 mo, had serum HBV DNA of $\geq$ 3 log viral copies/mL, were negative for anti-HCV, and were negative for anti-human immunodeficiency virus before entecavir therapy. Patients with decompensated cirrhosis, acute hepatitis, or acute exacerbation were excluded. Liver biopsy was not performed in some patients; therefore, the liver disease status was diagnosed by the clinical, laboratory, and imaging tests.

HBeAg-positive patients whose serum HBV DNA levels remained $\geq$ 3.0 log copies/mL after 2 years of entecavir therapy were considered to be slow-responders, while patients with < 3.0 log copies/mL were designated as rapid-responders.

Table 1 Baseline characteristics of the patients with hepatitis B virus genotype C

	HBeAg-negative group	HBeAg-positive group	*P* value
No.	26	24	NS
Age (yr)	57.2 (35-80)	44.2 (35-71)	< 0.01
Gender, M:F	15:16	16:11	NS
Diseases, CH:LC/HCC	21:5	23:1	NS
ALT (IU/mL)	38 (13-950)	102 (812-602)	< 0.01
Platelet counts ($\times 10^4$/mL)	18.6 (3.4-4.9)	18.0 (8.4-26.8)	NS
Albumin (mg/dL)	4.3 (3.4-4.9)	4.2 (2.3-5.0)	NS
Serum HBV DNA level (log copies/mL)	5.1 (3.9-8.8)	7.6 (5.6-8.8)	< 0.01

All data are shown by median (range). HBeAg: Hepatitis B e antigen; HBV: Hepatitis B virus; ALT: Alanine aminotransferase; CH: Chronic hepatitis; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma; NS: Not significant.

Laboratory tests

Quantitation of HBsAg (qHBsAg) was performed using the Architect HBsAg QT immunoassay (Abott Japan, Tokyo, Japan), in accordance with the manufacturer's instructions[9]. The detection range was 0.05 to 250 IU/mL. If HBsAg levels were found to be higher than 250 IU/mL, samples were diluted to 1:500 to 1:20 000. In this study, the results of the quantitative HBsAg levels are shown as the logarithmic value. HBV genotypes were detected by enzyme immunoassay (EIA) (Institute of Immunology, Tokyo, Japan)[10]. The HBV core-related antigen (HBcrAg) was detected by a chemilimunescent EIA method (Fujirebio Inc., Tokyo, Japan)[11]. Pre-C mutation and Core promoter mutations were detected by polymerase chain reaction (PCR) (HBV DNA precore/core promoter mutation decision kit; Roche Diagnostics Japan, Tokyo, Japan). Drug-resistant mutations in HBV against nucleotide analogs (NAs; lamivudine, adefovir and entecavir) were detected by the PCR-Invader method (BML Inc., Tokyo, Japan)[12].

RESULTS

Of the 57 patients, 50 patients were genotype C, three patients were HBV genotype A, one was genotype D, and three were of indeterminate genotype on EIA. Thus, the 50 patients with HBV genotype C were analyzed. Baseline characteristics of the 50 patients are shown in Table 1. The median age of the HBeAg-negative group age was significantly higher, the median alanine aminotransferase (ALT) level was significantly lower, and the median HBV DNA level was significantly lower than those in the HBeAg-positive group.

After 2 years of entecavir therapy, the rates of normalization (< 40 IU/L) of ALT levels were 87.0% in the HBeAg-negative group and 92.5% in the HBeAg-positive group (*P* = Not significant).

In contrast, at year 2, the rates of reduction in HBV

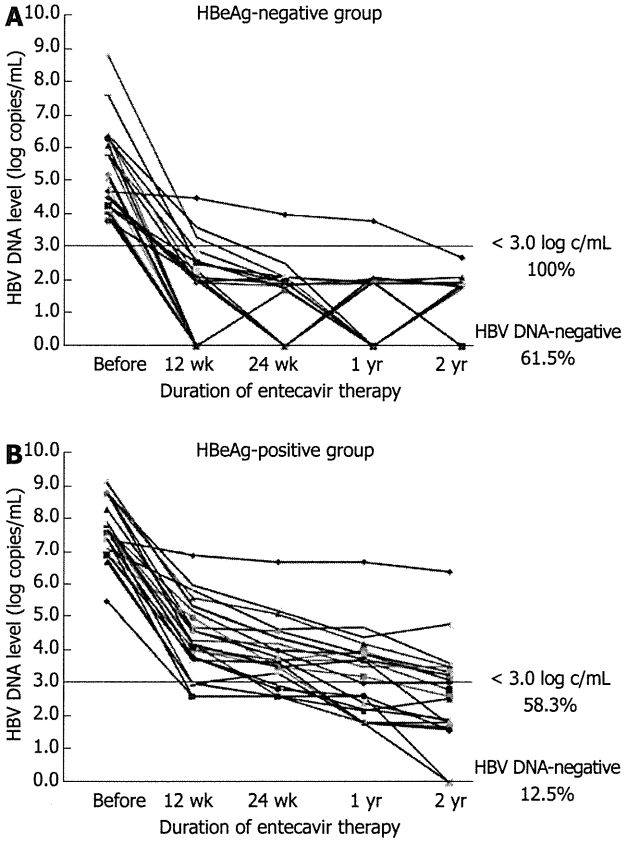

Figure 1 Hepatitis B virus DNA levels before and during entecavir therapy. A: In the hepatitis B e antigen (HBeAg)-negative group, hepatitis B virus (HBV) DNA levels in all patients decreased to < 3.0 log copies/mL at year 2. Of these patients, 61.5% shown to be negative for HBV DNA by the real-time polymerase chain reaction method; B: In the HBeAg-positive group, 58.3% of patients (rapid-responders) showed < 3.0 log copies/mL at year 2, while 41.7% of patients (slow-responders) showed ≥ 3.0 log copies/mL. In addition, at year 2, only 12.5% of the patients were negative for HBV DNA.

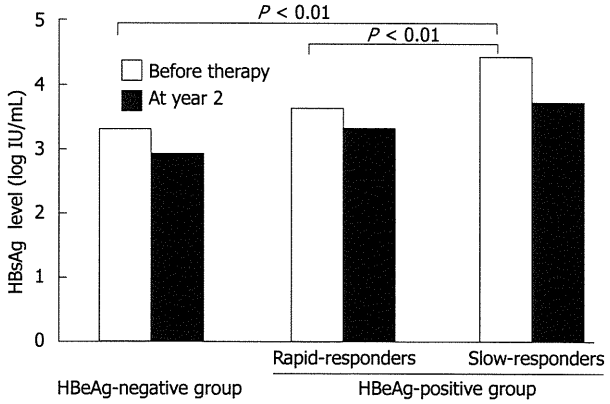

Figure 2 Median quantitative hepatitis B surface antigen levels among patients receiving entecavir therapy. Quantitative hepatitis B surface antigen (HBsAg) levels (qHBsAg) levels in slow-responders in the HBeAg-positive group were significantly higher than those in rapid-responders and the hepatitis B e antigen (HBeAg)-negative group. The median qHBsAg level at year 2 in the slow-responders remained higher than in other groups.

DNA to < 3.0 log copies/mL were 100% in the HBeAg-negative group and 58.3% in the HBeAg-positive group ($P < 0.01$). Thus, in the HBeAg-positive group, 58.3% of patients were designated as rapid-responders, and 41.7% were designated as slow-responders (HBV DNA levels ≥ 3.0 log copies/mL at year 2) (Figure 1). In addition, in the HBeAg-negative group, real-time PCR indicated that 61.5% of the patients were negative for HBV DNA, compared to 12.5% of the HBeAg-positive patients ($P < 0.01$).

Baseline data

When pre-treatment factors were compared between the rapid- and slow-responders (Table 2), age, gender, disease, platelet counts, and albumin were not significantly different. The median ALT level in the rapid-responders group was 131 IU/L compared with 31 IU/L in the slow-responders ($P = 0.02$). The pre-treatment median HBV DNA levels were 7.4 log copies/mL in the rapid-responders, and 8.3 in the slow-responders ($P = 0.06$). The was no difference in the rate of Pre-C and Core promoter mutations between the responder groups. In contrast, the rate of Pre-C mutations in the HBeAg-negative group was 83.3%, compared with 0% in the HBeAg-positive group. Pre-treatment HBcrAg levels did not differ among the three groups. In contrast, the pre-treatment median qHBsAg level was 3.63 log IU/mL in the rapid-responders, compared with 4.57 log IU/mL in the slow-responders ($P < 0.01$).

Data at year 2

At year 2 of therapy, the median qHBsAg level in the rapid-responders was 3.25 log IU/mL, compared with 4.12 log IU/mL in the slow-responders ($P = 0.01$). The median HBcrAg level in the rapid-responders was 5.85 log U/mL, compared with > 6.8 (the upper limit of the detection range) in the slow-responders ($P < 0.01$). In Figure 2, qHBsAg levels before treatment and at year 2 are shown for the HBeAg-negative group, and the rapid-responder and slow-responders in the HBeAg-positive group.

Among all the slow-responders, no entecavir-resistant mutations were found, although three patients showed M204I lamivudine-resistant mutations (Table 3).

Comparison between HBV DNA-negative and -positive patients at year 2 during entecavir therapy in all the patients

At year 2 of entecavir therapy, among 50 patients, real-time PCR showed that 19 (38.0%) were negative for HBV DNA, compared with 31 (62.0%) who were still positive for HBV DNA (Table 4). The pretreatment clinical and virological characteristics between the HBV DNA-negative and -positive groups were compared by univariate analysis. In the HBV DNA-negative group, the median ALT level was significantly lower, the rate of HBeAg-negative status was significantly higher, the median HBV DNA level was lower, and the median qHBsAg level was lower, than those in the HBV DNA-positive group.

However, when multivariate analysis using logistic regression analysis was performed, the median qHBsAg level was the only significant factor that predicted the negative HBV DNA status at year 2 of entecavir therapy

Table 2 Clinical and virological results among the hepatitis B e antigen-negative group, the rapid-responders, and the slow-responders in the hepatitis B e antigen-positive group during 2 years of entecavir therapy

Characteristics	HBeAg-negative group	HBeAg-positive group		*P* value
	(*n* = 26)	RR (*n* = 14)	SR (*n* = 10)	RR *vs* SR
<Baseline data>				
Age	58 (35-80)	45 (34-68)	43 (31-71)	NS
Gender (male:female)	13:13	9:5	6:4	NS
Disease (CH:LC/HCC)	21:5	13:1	6:4	NS
ALT (IU/L)	38 (13-950)	131 (12-602)	31 (13-108)	0.02
Platelet count ($\times 10^4$/mL)	18.6 (3.4-35.1)	17.1 (8.4-22.4)	20.0 (11.0-26.8)	NS
Albumin (mg/dL)	4.3 (3.4-4.9)	4.0 (2.3-5.0)	4.4 (3.7-4.6)	NS
HBV genotype C	100%	100%	100%	NS
HBV DNA (log copies/mL)	5.1 (3.9-8.8)	7.4 (5.6-8.8)	8.3 (7.1-8.8)	NS
qHBsAg (log IU/mL)	3.17 (0.70-4.58)	3.63 (1.68-4.34)	4.57 (4.35-4.76)	< 0.01
HBcrAg (log U/mL)	3.6 (3.0-> 6.8)	> 6.8 (6-> 6.8)	> 6.8 (> 6.8-> 6.8)	NS
Pre-C mutation (%)	83.3	0	0	NS
Core promoter mutation (%)	58.3	57.1	50.0	NS
<At year 2 during therapy>				
HBV DNA (log copies/mL)	0.0 (0.0-2.7)	2.1 (0.0-2.1)	3.5 (3.1-6.9)	-
ALT (IU/L)	18 (9-75)	17.5 (10-31)	23 (13-37)	NS
HBeAg seroconversion	-	23.50%	0%	NS
HBsAg seroclearance	0%	0%	0%	NS
qHBsAg (log IU/mL)	2.91 (0.62-3.9)	3.25 (1.70-3.92)	4.12 (3.23-4.47)	0.01
HBcrAg (log U/mL)	3.0 (3.0-5.4)	5.9 (4.0-> 6.8)	> 6.8 (5.2-> 6.8)	< 0.01
Resistant mutations against entecavir	UDL	UDL	0%	-

HBeAg: Hepatitis B e antigen; ALT: Alanine aminotransferase; CH: Chronic hepatitis; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma; UDL: Under the detection limit; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; qHBsAg: Quantitation of HBsAg level; HBcrAg: HBV core-related antigen; NS: Not significant; RR: Rapid-responder; SR: Slow-responder.

Table 3 Drug resistant mutations in the slow-responders at year 2

Patient	Age (yr)	Gender	Previous therapy	HBV genotype	Drug resistant mutations against						
					Lam	Lam	Lam/Ade	Ade	Ent	Ent	Ent
					L180	M204	A181	N236	T184	S202	M205
1	52	Male	No	C	Wild	Wild	Wild	Wild	Wild	Wild	Wild
2	35	Male	No	C	Wild	Wild	Wild	Wild	Wild	Wild	Wild
3	68	Male	No	C	Wild	Wild	Wild	Wild	Wild	Wild	Wild
4	56	Female	No	C	Wild	M204I	Wild	Wild	Wild	Wild	Wild
5	36	Female	No	C	Wild	M204I	Wild	Wild	Wild	Wild	Wild
6	45	Male	No	C	Wild	M204I	Wild	Wild	Wild	Wild	Wild
7	35	Male	No	C	Wild	Wild	Wild	Wild	Wild	Wild	Wild
8	67	Female	No	C	Wild	Wild	Wild	Wild	Wild	Wild	Wild
9	39	Male	No	C	Wild	Wild	Wild	Wild	Wild	Wild	Wild
10	44	Female	No	C	Wild	Wild	Wild	Wild	Wild	Wild	Wild

Lam: Lamivudine; Ade: Adefovir; Ent: Entecavir; HBV: Hepatitis B virus.

(odds ratio 8.16, 95% CI: 1.28-52.18, $P = 0.03$).

DISCUSSION

In this study, the clinical and virological features of patients with HBV genotype C who received naïve-entecavir therapy were analyzed. After 2 years of entecavir therapy, about 42% of the HBeAg-positive patients showed HBV DNA levels ≥ 3 log copies/mL, while all of the HBeAg-negative patients showed < 3 log copies/mL. Therefore, the factors associated with the slow response to entecavir therapy among the HBeAg-positive group were studied initially. In addition, among the 50 patients, 38% showed HBV DNA-negative status at year 2. Thus, the pretreatment factors that predict the loss of HBV DNA were analyzed in all 50 patients. According to the multivariate analysis, qHBsAg levels are the most important factor for predicting the response to entecavir therapy in patients with HBV genotype C.

In Japan, HBV genotype C is the most prevalent[3]. The response rates to interferon or NA therapy in patients with HBV genotype C, as well as D, are poor when compared to those with HBV genotype B or A[13]. Thus, in this study, only subjects with HBV genotype C were studied.

Recently, a decline in HBsAg levels during PEG-interferon therapy was reported to be significant in the evaluation of the response to therapy[14-17]. In these reports, HBsAg levels were found to be one of the best viral markers for predicting response to anti-viral therapy and viral

Table 4 Pretreatment clinical and virological characteristics between hepatitis B virus DNA-negative and -positive group at year 2 during entecavir therapy

Characteristics	HBV DNA-negative group (n = 19)	HBV DNA-positive group (n = 31)	P value	
			Univariate analysis	Multivariate analysis
Age	51 (31-73)	52 (32-80)	NS	
Gender (male:female)	12:7	20:11	NS	
Disease (CH:LC/HCC)	17:2	27:4	NS	
ALT (IU/L)	36 (12-366)	108 (13-602)	0.03	NS
Platelet counts ($\times 10^4$/mL)	19.0 (8.8-35.1)	17.8 (3.4-26.8)	NS	
Albumin (mg/dL)	4.35 (3.84-4.85)	4.14 (2.28-4.72)	NS	
HBV genotype (B:C:others)	0:19:0	0:31:0	NS	
HBeAg status (positive:negative)	3:16	21:10	< 0.01	NS
HBV DNA (log copies/mL)	5.1 (3.1-7.4)	7.6 (3.7-8.8)	< 0.01	NS
qHBsAg level (log IU/mL)	3.31 (1.90-4.08)	4.20 (3.06-4.87)	< 0.01	0.03
HBcrAg level (log U/mL)	3.45 (3.0-> 6.8)	> 6.8 (3.0-> 6.8)	NS	
Pre-C mutation (%)	75.0	43.3	NS	
Core promoter mutation (%)	37.5	60.0	NS	

CH: Chronic hepatitis; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma; ALT: Alanine aminotransferase; HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen; qHBsAg: Quantitation of hepatitis B surface antigen level; HBcrAg: HBV core-related antigen; NS: Not significant.

activity levels in hepatocytes, correlating with cccDNA levels[14,18]. Although qHBsAg is a predictor of response to entecavir therapy[19], there have been no reports in a homogeneous HBV genotype setting. In this study, qHBsAg was demonstrated to be the most significant predictor of entecavir therapy in patients with HBV genotype C.

HBV DNA levels are also considered an important factor associated with response to anti-viral therapy[17]. In this study, there was a tendency for higher HBV DNA levels in the slow-responders as compared to rapid-responders. The association between HBV DNA levels and response to therapy may be clarified further in a larger number of patients.

HBcrAg levels indicate the serum HB core antigen levels plus HBeAg levels[11]. HBcrAg levels are reported to be associated with cccDNA levels in hepatocytes[20]. However, this study showed no association between HBcrAg levels and slow response. This may be explained by the narrow quantitation range of HBcrAg levels, because HBcrAg levels in most patients in the HBeAg-positive group were greater than the upper level of the detection range of the assay.

The association between Core promoter mutation and response rate to NAs is also interesting, because the replication level of HBV is thought to be high in patients with Core promoter mutations. We reported previously that, in patients with HBV genotype C, the rates of HBeAg-positive status and Core promoter mutations are higher than those in patients with HBV genotype B[5]. In this study, a higher rate of Core promoter mutations was observed in the HBeAg-positive patients with HBV genotype C. In addition, a higher rate of Pre-C mutations was observed in the HBeAg-negative group. However, no association between the mutation rate and response rate to therapy was demonstrated in this study.

Higher ALT levels are considered an important factor in predicting good response to PEG-interferon therapy[21]. However, low ALT levels were observed in the HBV DNA-negative group at year 2 of therapy, because a high proportion of the HBeAg-negative patients had low ALT levels, compared to the HBeAg-positive patients. Thus, we consider that, during entecavir therapy, ALT levels are not associated with treatment response.

In this study, no resistant mutations against entecavir were found during 2 years therapy. As reported previously, resistant mutations against entecavir are rarely developed during 5 years of entecavir therapy[8]. Therefore, slow response was not caused by entecavir-resistant mutants.

In conclusion, we suggest that qHBsAg is a significant and convenient indicator for predicting response to entecavir therapy.

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COMMENTS

Background

Entecavir is a nucleot(s)ide analog that is widely used for the treatment of chronic hepatitis B patients. The efficacy of entecavir is very good for hepatitis B e antigen (HBeAg)-negative patients, but not so good for HBeAg-positive patients. The prognosis and response to anti-viral therapies depend on hepatitis B virus (HBV) genotype. The factors that affect the efficacy of entecavir therapy are still unclear, especially in patients with HBV genotype C.

Research frontiers

As quantitation assay of serum hepatitis B surface antigen (HBsAg) has been recently developed, allowing the serum level of HBsAg to be determined over a very wide range. The upper range of HBsAg levels could be detected to 6.7 log IU/mL by the Architect HBsAg QT immunoassay when samples were diluted to 1:20 000. Thus, the authors could analyze the relationship between the efficacy of entecavir therapy and various HBV markers.

Innovations and breakthroughs

This study showed that the quantitative HBsAg level is a significant factor for predicting the efficacy of entecavir therapy in patients with HBV genotype C. Patients with low levels of HBsAg before entecavir therapy often show HBV DNA levels < 3.0 log copies/mL or are negative for HBV DNA at year 2 during therapy.

Applications

Using the quantitation of HBsAg, the efficacy of various anti-viral therapies can be predicted before treatment. The quantitation of HBsAg could be a useful tool for determining the treatment schedule for chronic hepatitis B patients.

Peer review

Although small in patient numbers, the subject matter is interesting and original. This study adds to the emerging data suggesting HBsAg decline is a predictor of response.

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Serum IP-10 concentrations and *IL28B* genotype associated with responses to pegylated interferon plus ribavirin with and without telaprevir for chronic hepatitis C

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Running title: IP-10 and *IL28B* in chronic hepatitis C

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This manuscript is composed of 234 words of abstract, 4 tables, 5 figures and 2 supplementary files with a total of 2534 words of text.

Abstract

Aim: Several studies have shown that high pretreatment concentrations of serum interferon-gamma inducible protein-10 (IP-10) are correlated with non-response to pegylated interferon (PEG-IFN) plus ribavirin (RBV) for chronic hepatitis C (CHC). However, there are few reports on their effect on the Asian population.

Methods: We enrolled 104 Japanese genotype 1 CHC individuals treated with PEG-IFN/RBV and 45 with PEG-IFN/RBV/telaprevir, and evaluated the impact of pretreatment serum IP-10 concentrations on their virological responses.

Results: The pretreatment serum IP-10 concentrations were not correlated with *IL28B* genotype. The receiver operating characteristic curve analysis determined the cutoff value of IP-10 for predicting a sustained virological response (SVR) as 300 pg/mL. In multivariate analysis, the *IL28B* favorable genotype and IP-10 concentration <300 pg/mL were independent factors for predicting SVR (OR, 17.44; $P<0.001$ and OR, 3.86; $P=0.01$). In a subgroup of patients with the *IL28B* favorable genotype, the SVR rate was higher in the patients with IP-10 <300 than in those with ≥300 pg/mL [69% (24/35) vs. 35% (13/37), $P=0.005$], whereas no patient with the *IL28B* unfavorable genotype and IP-10 ≥300pg/mL achieved SVR. Among the patients treated with PEG-IFN/RBV/telaprevir, low pretreatment concentrations of serum IP-10 were associated with a very rapid virological response, defined

as HCV RNA undetectable at week 2 after the start of therapy.

Conclusion: Pretreatment serum IP-10 concentrations are associated with treatment efficacy in PEG-IFN/RBV and with early viral kinetics of hepatitis C virus in PEG-IFN/RBV/telaprevir therapy.

Key words: *IL28B*, IP-10, hepatitis C, interferon, ribavirin, telaprevir

INTRODUCTION

Chronic infection with hepatitis C virus (HCV) presents a significant health problem worldwide and approximately 170 million people are infected.[1] Over 70% of individuals acutely infected with HCV go on to develop chronic infection and are at significant risk of progressive liver fibrosis and subsequent liver cirrhosis and hepatocellular carcinoma (HCC). Antiviral treatment has been shown to improve liver histology and decrease the incidence of HCC in chronic hepatitis C (CHC).[2, 3] Until 2011, the standard treatment for chronic HCV infection was weekly pegylated interferon (PEG-IFN) doses in association with daily doses of ribavirin (RBV); however, less than 50% of patients infected with HCV genotype 1 treated in this way achieve a sustained virological response (SVR).[4, 5] Newly developed treatments involve direct-acting antivirals (DAAs) and nonstructural (NS) 3/4A protease inhibitors have shown promising outcomes in combination with PEG-IFN/RBV in several clinical trials, more than 70% of patients infected with HCV genotype 1 achieving SVR.[6-8]

Various viral and host factors have been identified as significant determinants of treatment outcome. Among patients with HCV genotype 1 infection, factors associated with a lower rate of SVR include a high baseline viral load, older age, African-American race, insulin resistance, advanced fibrosis, hepatic steatosis and administration of an insufficient dosage of PEG-IFN or RBV.[9, 10] Other viral factors include amino acid substitutions at positions 70 and

91 in the HCV core region,[11] and in the interferon sensitivity-determining region (ISDR) in NS5A.[12] In addition, recent genome-wide association studies (GWAS), including our study of HCV infection,[13] have shown that a single nucleotide polymorphism (SNP) near the interleukin-28B (*IL28B*) gene is strongly associated with response to PEG-IFN/RBV therapy for chronic HCV genotype 1 infection.[13-18]

Chemokines and cytokines regulate immunity and inflammation in HCV infection. They also play critical roles in eradication of HCV during IFN based treatment. Several studies have reported that interferon-gamma-inducible protein 10 kDa (IP-10 or CXCL10) may be a prognostic maker for HCV treatment efficacy in HCV genotype 1 infection: elevated pretreatment serum IP-10 concentrations correlate with nonresponse to PEG-IFN/RBV therapy.[19-23] Darling et al. reported that pretreatment serum IP-10 <600 pg/mL was an independent predictive factor for SVR in PEG-IFN/RBV therapy, especially among patients with the unfavorable *IL28B* genotype; SVR rates differed significantly according to pretreatment serum IP-10 concentrations.[24] Other studies have shown that *IL28B* genotype and pretreatment serum IP-10 concentrations were associated with early viral kinetics of HCV, the first phase decline or rapid virological response (RVR), as well as SVR in PEG-IFN/RBV therapy.[25, 26] However, the impact of serum IP-10 concentrations on virological response to PEG-IFN/RBV/telaprevir therapy has not been elucidated yet.

Furthermore, it has been reported that pretreatment serum IP-10 concentrations tended to be higher in African-American patients infected with HCV than in white patients,[20, 24] suggesting that serum IP-10 concentrations might vary according to race. Until now, there have been few reports concerning the association between pretreatment serum IP-10 concentrations and treatment efficacy in Asian populations infected with HCV.

In this study, we aimed to determine the impact of pretreatment serum IP-10 concentrations on virological responses to PEG-IFN/RBV or PEG-IFN/RBV/telaprevir therapy in a Japanese population infected with HCV genotype 1.

METHODS

Study population and treatment protocol

Serum samples were obtained from 149 patients chronically infected with HCV genotype 1 who were treated at Nagoya City University Hospital and Nagoya Daini Red Cross Hospital. All patients had tested positive for HCV RNA for more than 6 months. Patients chronically infected with hepatitis B virus or human immunodeficiency virus, or with other causes of liver disease such as autoimmune hepatitis and primary biliary cirrhosis, were excluded from this study. Written informed consent was obtained from each patient and the study protocol

conformed to the ethics guidelines of the Declaration of Helsinki and was approved by the appropriate institutional ethics review committees.

One-hundred and four patients were treated with PEG-IFN-α2b (1.5 μg/kg body weight subcutaneously once a week) or PEG-IFN-α2a (180 μg once a week) plus RBV (600-1000 mg daily according to body weight) for 48 weeks. The dosage of PEG-IFN or RBV were reduced according to the recommendations on the package inserts or the clinical condition of individual patients. Forty-five patients received PEG-IFN/RBV/telaprevir therapy: PEG-IFN-α2b (1.5 μg/kg body weight subcutaneously once a week), RBV (600-1000 mg daily according to body weight) and telaprevir (standard dosage of 2250 mg daily three times a day at every 8 hours or a reduced dosage of 1500 mg daily twice a day at every 12 hours) for 12 weeks, followed by an additional 12 weeks of PEG-IFN/RBV. In seven patients, the initial dosage of telaprevir was reduced to 1500 mg daily according to age, body weight, gender, or baseline hemoglobin level, according to the judgment of the physicians. When marked adverse effects: anorexia, anemia, neutropenia, thrombocytopenia, renal dysfunction, skin rash, etc. developed, the dosage of telaprevir was reduced to 1500 mg daily and that of PEG-IFN or RBV was reduced according to the recommendation on the package inserts or the clinical condition of individual patients.

Definition of virological response to treatment

The virological responses were defined as follows: SVR, HCV RNA undetectable at week 24 after the end of therapy; transient virological response (TVR), HCV RNA became undetectable during therapy but reappeared after the end of treatment; non-virological response (NVR), HCV RNA remained detectable during therapy; RVR, HCV RNA undetectable at week 4 after the start of therapy; very rapid virological response (vRVR), HCV RNA undetectable at week 2 after the start of therapy.

Laboratory tests

Blood samples were taken according to protocol from baseline until after the end of treatment and were analyzed by hematologic tests and for blood chemistry. HCV RNA concentrations were measured at baseline, regularly during treatment and at follow-up visits after the end of treatment, using the COBAS TaqMan HCV test (Roche Diagnostics K.K., Tokyo, Japan). The dynamic range of this assay is 1.2-7.8 log IU/mL.

Quantification of serum IP-10 concentrations

Serum IP-10 concentrations were measured in samples collected at baseline (prior to treatment) using the Quantikine human CXCL10/IP-10 immunoassay (R & D System). All

samples were diluted 1:2 and analyzed in duplicate. The linear dynamic range for IP-10 measurement by this assay is 8-500 pg/mL, with a detection limit of 7.8 pg/mL. Samples with IP-10 concentrations above 1,000 pg/mL were diluted 1:5 and reanalyzed.

SNP genotyping

Genetic polymorphisms near the *IL28B* gene (rs8099917) were determined according to the manufacturer's recommendations using TaqMan® SNP Genotyping Assays (Applied Biosystems, Carlsbad, CA) or the DigiTag2 assay.[27]

Statistical Analysis

Categorical variables were compared between groups by the χ^2-test or Fisher's exact test and non-categorical variables by the Mann-Whitney U-test. Pearson's correlation coefficient test was used to evaluate relationships between serum IP-10 concentrations and other variables. Multivariate logistic regression analysis with stepwise forward selection was performed with $P<0.05$ in univariate analysis as the criteria for model inclusion. $P<0.05$ was considered significant. To evaluate the discriminatory ability of IP-10 concentrations to predict SVR, receiver operating characteristic (ROC) curve analysis was conducted and the cutoff value selected. These statistical analyses were carried out using SPSS software package ver.18J

(Chicago, IL, USA).

RESULTS

Demographic characteristics

The clinical characteristics of the study population are described in Table 1.

PEG-IFN/RBV/telaprevir therapy has been known to be poorly tolerated because of adverse effects. In addition, the treatment efficacy varied considerably according to *IL28B* genotype or past IFN-based treatment outcome. Therefore, we examined *IL28B* genotype in all cases before treatment and selected patients for treatment with PEG-IFN/RBV/telaprevir according to age, *IL28B* genotype, past IFN-based treatment response, etc. As a result, the median age was younger and the rate of rs8099917: TT (*IL28B* favorable genotype) was higher in the patients treated with PEG-IFN/RBV/telaprevir. Of the 45 patients treated with PEG-IFN/RBV/telaprevir, 17 were naïve for IFN therapy, 22 were previously treated with PEG-IFN/RBV, 3 with IFN/RBV, and 3 with IFN mono therapy. Of the 28 previously treated patients, 21 and 7 resulted in TVR and NVR, respectively.

Correlations between pretreatment serum IP-10 concentrations and other variables

The median pretreatment serum IP-10 concentration of the 149 patients was 301 pg/mL

(range, 57 to 2053). The pretreatment serum IP-10 concentrations were not correlated with age, gender, *IL28B* genotype and platelet count, but alanine aminotransaminase (ALT), γ-glutamyl transpeptidase (γ-GTP) and HCV RNA concentrations were weakly corrrelated (r=0.330, 0.229, and 0.233, respectively) (Fig. 1 and Fig. S1).

Predictive factors for SVR in PEG-IFN/RBV therapy

We examined the factors associated with SVR in patients treated with PEG-IFN/RBV. By univariate analysis, age, platelet count, γ-GTP, IP-10 and *IL28B* genotype were significantly associated with SVR (Table 2). Dividing the patients into three groups, SVR, TVR and NVR, the IP-10 concentrations were significantly lower in SVR than in TVR and NVR (P=0.017 and P=0.005, respectively) (Fig. 2). ROC analysis of the pretreatment serum IP-10 concentration for predicting SVR revealed that area under the curve (AUC) was 0.68 (Fig. S2). When we set the cutoff value at 300 pg/mL, the sensitivity was 0.67 and the specificity was 0.68. Then, we analyzed predictive factors for SVR in logistic regression models that included the following variables: age (≤58 vs. ≥59 years), platelet count (≥15 vs. $<15 \times 10^4$ /μL), γ-GTP (≤31 vs. ≥32 IU/L), IP-10 concentration (<300 or ≥ 300 pg/mL) and rs8099917 genotype (TT vs. TG/GG). The *IL28B* genotype and IP-10 concentration were independent pridictive factors for SVR (OR, 17.44; P<0.001 and OR, 3.86; P=0.01) (Table 3). Therefore,