Table 1 Baseline clinical characteristics of patients treated with combination therapy

	All patients (n = 1280)	Older patients (n = 254)	Younger patients (n = 1026)	P-value
Gender (male/female)	668/612	123/131	545/481	0.180
Age (years)	54.2 ± 12.1	68.1 ± 2.8	50.7 ± 11.0	< 0.0001
AST (IU/L)	55.5 ± 44.0	59.0 ± 40.1	54.5 ± 44.9	0.2332
ALT (IU/L)	67.3 ± 64.3	59.9 ± 45.6	69.2 ± 68.0	0.0387
GGT (IU/L)	55.4 ± 71.2	47.7 ± 51.5	57.3 ± 75.1	0.0589
WBC (/μL)	5177.8 ± 1566.8	4889.2 ± 1313.7	5249.2 ± 1615.9	0.0010
Hemoglobin (g/dL)	14.0 ± 1.4	13.6 ± 1.3	14.1 ± 1.5	< 0.0001
Platelets (× 10^4/μL)	17.5 ± 6.3	15.4 ± 4.7	18.0 ± 6.6	< 0.0001
HCV-RNA (logIU/mL)	6.1 ± 0.7	6.0 ± 0.7	6.2 ± 0.7	0.0359
Genotype (1/2)	867/413	177/77	690/336	0.458
Activity (A0/A1/A2/A3)	48/491/338/32	10/97/77/10	38/394/261/22	0.3868
Fibrosis (F0–F1/F2/F3/F4)	567/213/109/17	94/56/36/5	473/157/73/12	0.0001

Patients were defined as two age groups: (i) older patients ≥ 65 years old and (ii) younger patients < 65 years old.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyltranspeptidase; HCV-RNA, hepatitis C virus RNA; WBC, white blood cell.

Table 2 Efficacy of combination therapy

	All patients (n = 1280)	Older patients (n = 254)	Younger patients (n = 1026)	P-value
SVR rate (ITT)	54.8 (701/1280)	38.2 (97/254)	58.9 (604/1026)	< 0.0001
SVR rate (PP)	62.9 (687/1092)	46.7 (92/197)	66.5 (595/895)	< 0.0001
Discontinuation rate of treatment	14.7 (188/1280)	22.4 (57/254)	12.8 (131/1026)	< 0.0001

ITT, intention-to-treat; PP, per-protocol; SVR, sustained virological response.

those who did not achieve SVR was higher than of those who achieved SVR, but this difference was not significant (Fig. 2a). However, in older patients with GGT ≥ 44 IU/L who achieved SVR, there was a marked reduction in the development of HCC compared with the older patients with GGT ≥ 44 IU/L who did not achieve SVR (older patients with GGT < 44 IU/L, $P = 0.265$; older patients with GGT ≥ 44 IU/L, $P = 0.020$, log–rank test) (Fig. 2b).

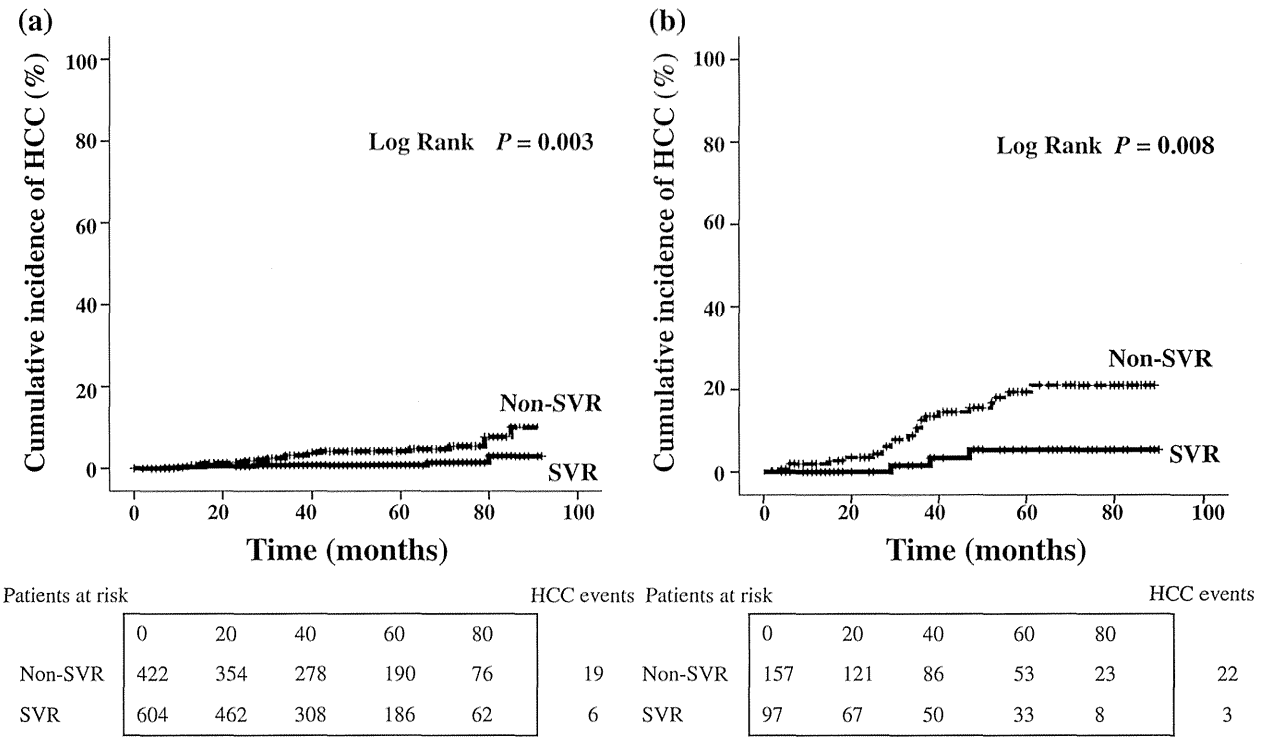
Next, we analyzed which older patients were more likely to achieve SVR. At first, we identified factors associated with SVR by univariate analysis. Among older patients, the ratio of males who achieved SVR was higher than among those who did not ($P = 0.0017$). The baseline HCV viral load in patients who achieved SVR was significantly lower than that in patients who did not achieve SVR ($P < 0.0001$). There was a higher proportion of genotype 2 patients who achieved an SVR than genotype 2 patients who did not ($P = 0.0003$) (Table 4). Factors associated with SVR in combination therapy were determined by multivariate analysis. HCV-RNA, gender, and genotype were significantly associated with SVR in older patients (Table 4).

Discussion

IFN-based therapy, including combination therapy with ribavirin, has improved the SVR rate in patients with CH-C and liver cirrhosis. However, there have been several reports of HCC occurring in patients despite achieving SVR, so the need for long-term follow-up remains among patients who achieve SVR.[35–37] HCC remains life-threatening in patients with eradicated HCV. Achieving SVR is important to reducing hepatic inflammation and histological improvement. However, the ultimate goal of treatment for patients with CH-C is to prevent the development of liver cirrhosis and HCC. It is important to evaluate indications for treatment in terms of HCC prevention in CH-C patients. This study provides data on HCC incidence after combination therapy consisting of peginterferon and ribavirin in CH-C patients, including older patients, who have a high incidence of HCC.

Due to the high incidence of HCC in older patients, the cumulative incidence of HCC in older patients showed significant reduction among older patients who achieved SVR compared with those who did not achieve SVR, and this decrease was more distinct than in younger patients in this study. Previous reports have shown that IFN monotherapy reduces the risk of HCC development,[38,39] even in patients ≥ 60 years if they achieve SVR.[20–22] These are all treated with IFN monotherapy. Given the current aging trend in CH-C patients, older patients are often defined as patients ≥ 65 years of age. However, there are no reports on the effects of peginterferon and ribavirin on the development of HCC in patients over 65 years. This is the first report that peginterferon alfa-2b plus ribavirin was associated with a significant reduction in the development of HCC in patients ≥ 65 years if they achieved SVR.

In this cohort, factors associated with HCC development among older patients included GGT and non-SVR status. GGT is a heterodimeric glycoprotein that catalyzes the transpeptidation and hydrolysis of the gamma-glutamyl group of glutathione and



Patients at risk	0	20	40	60	80	HCC events
Non-SVR	422	354	278	190	76	19
SVR	604	462	308	186	62	6

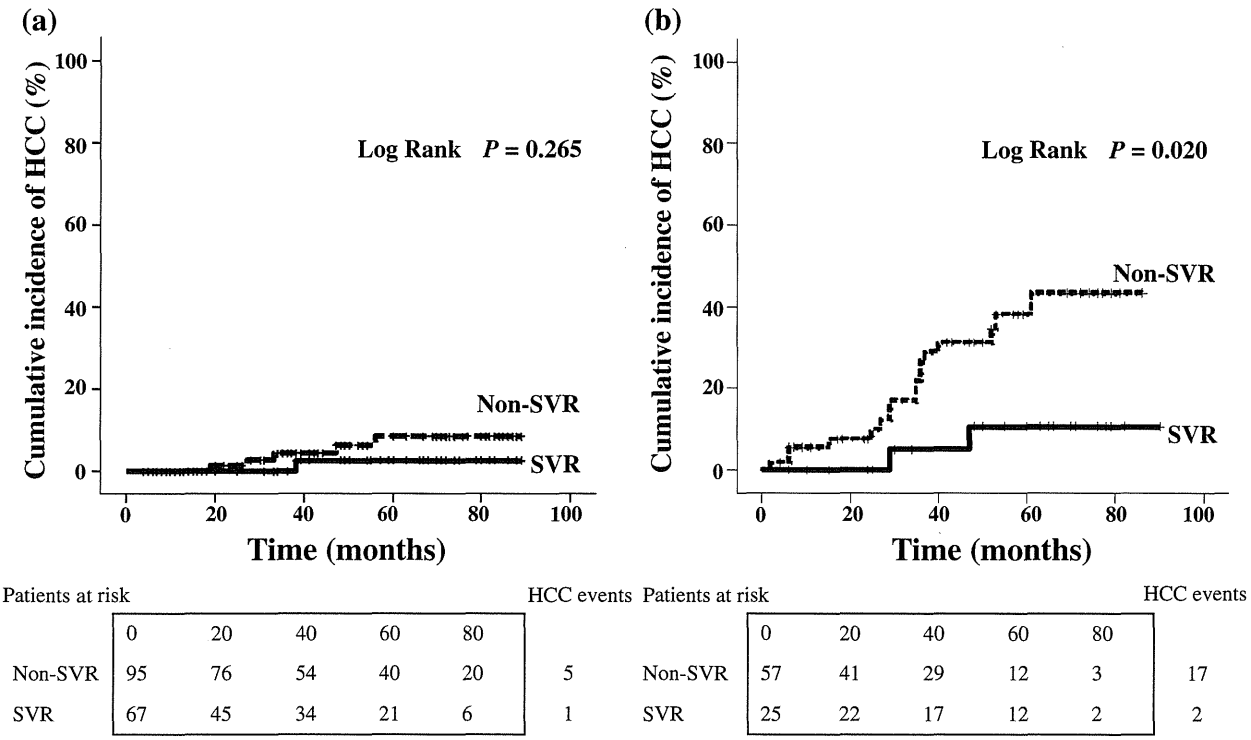
Patients at risk	0	20	40	60	80	HCC events
Non-SVR	157	121	86	53	23	22
SVR	97	67	50	33	8	3

Figure 1 Cumulative incidence of HCC after peginterferon alfa-2b and ribavirin in patients who achieved SVR (solid line) or did not achieve SVR (dashed line) in younger patients < 65 years old (a) and older patients ≥ 65years old (b). The number of patients at risk and HCC events at each time point are shown below the graphs. HCC, hepatocellular carcinoma; SVR, sustained virological response.

Table 3 Factors associated with development of HCC

All patients				
Variable	Category	Hazard ratio	95% CI	*P*-value
Age	Younger	1	1.927–6.369	< 0.0001
	Older	3.504		
Advanced fibrosis	Non-advanced fibrosis	1	1.601–5.308	< 0.0001
	Advanced fibrosis	2.915		
Treatment efficacy	SVR	1	2.209–9.942	< 0.0001
	Non-SVR	4.686		
Gender	Female	1	1.283–4.495	0.006
	Male	2.402		
Older patients				
Variable	Category	Hazard ratio	95% CI	*P*-value
GGT	< 44	1	2.869–20.269	< 0.0001
	≥ 44	7.626		
Treatment efficacy	SVR	1	1.175–14.882	0.027
	Non-SVR	4.181		

CI, confidence interval; GGT, gamma-glutamyltranspeptidase; HCC, hepatocellular carcinoma; SVR, sustained virological response.



Patients at risk	0	20	40	60	80	HCC events
Non-SVR	95	76	54	40	20	5
SVR	67	45	34	21	6	1

Patients at risk	0	20	40	60	80	HCC events
Non-SVR	57	41	29	12	3	17
SVR	25	22	17	12	2	2

Figure 2 Cumulative incidence of HCC after peginterferon alfa-2b and ribavirin in older patients who achieved SVR (solid line) or did not achieve SVR (dashed line), among those with GGT < 44 IU/L (a) and GGT ≥ 44 IU/L (b). HCC, hepatocellular carcinoma; GGT, gamma-glutamyltranspeptidase; SVR, sustained virological response.

related compounds. It has been observed that the intake of certain xenobiotics, including carcinogens and drugs, induces hepatic expression of this enzyme.[40] GGT was one of the significant factors associated with non-SVR in CH-C patients treated with IFN-based therapy.[41–44] In terms of mechanism, a previous report showed that GGT levels may act as a surrogate marker of tumor necrosis factor-alpha expression in the liver, and this may explain the importance of serum GGT levels in predicting treatment outcome.[45] However, the mechanisms through which GGT significantly affects HCC development among older patients remain unclear.

Platelet counts correlate with advanced fibrosis in the histological examination of the liver in patients with CH-C. Decreased platelet counts and fibrosis were reported to be factors associated with the development of HCC.[17–22]

SVR, a treatment factor, is also reported to be one of the most important factors in reducing the risk of HCC in patients with CH-C.[17–19] Factors associated with development of HCC in older patients are similar to those in previous reports on IFN-based treatment;[20–22] however, this is the first report demonstrating factors associated with development of HCC in CH-C patients 65 years or older treated with only combination therapy between peginterferon alfa-2b and ribavirin therapy.

We examined which CH-C patients at high risk for HCC may especially benefit from combination therapy. We determined factors associated with SVR using a univariate analysis, followed by a multivariate analysis of factors associated with SVR in older patients who underwent combination therapy. In addition, we compared the cumulative incidence of HCC between older patients who did and did not achieve SVR. In older patients, low HCV-RNA, male gender, and genotype 2 were associated with SVR. These were the same factors identified in our previous report,[15] and gender was reported to be a factor associated with SVR in older patients in another report.[46]

This study has several limitations. This is a retrospective cohort study, so selection bias cannot be excluded. We cannot exclude the possibility that older patients selected for treatment in the outpatient department were more likely to be better overall candidates than younger patients. However, regardless of potential bias, older patients had low WBC counts and hemoglobin levels, and more advanced fibrosis. Especially on older patients, as life expectancy is shorter than with younger patients, the health economics evaluation of costs of treatment *versus* savings of reducing HCC incidence would be extremely valuable, so further investigation, including cost–benefit, would be needed.

In conclusion, older patients who received combination peginterferon alfa-2b and ribavirin therapy had low hemoglobin levels, low WBC and platelet counts, and advanced fibrosis. Older patients had higher treatment discontinuation rates, lower SVR rates, and higher rate of HCC development than younger patients. However, older patients who achieved SVR had a marked reduction in the development of HCC compared with older patients who did not achieve SVR, especially among older patients with GGT > 44 IU/L. Low HCV-RNA, male gender, and genotype 2

Table 4 Factors associated with SVR in older patients

Univariate analysis			
Variable	SVR (n = 97)	Non-SVR (n = 157)	P-value
Sex (male/female)	59/38	64/93	0.0017
Age (years)	67.6 ± 2.5	68.3 ± 2.9	0.0473
AST (IU/L)	60.1 ± 39.7	58.4 ± 41.5	0.7807
ALT (IU/L)	63.1 ± 45.7	57.9 ± 45.5	0.3714
GGT (IU/L)	50.2 ± 70.0	46.5 ± 36.8	0.5911
WBC (/μL)	4856.4 ± 1268.6	4909.6 ± 1344.7	0.7550
Hemoglobin (g/dL)	13.8 ± 1.1	13.6 ± 1.4	0.2345
Platelets (× 10^4/μL)	15.8 ± 4.4	15.1 ± 4.8	0.2646
HCV-RNA (logIU/mL)	5.8 ± 0.9	6.2 ± 0.6	< 0.0001
Genotype (1/2)	55/42	122/35	0.0003
Liver activity (A0/A1/A2/A3)	6/38/24/4	4/59/53/6	0.3123
Fibrosis (F0–1/F2/F3/F4)	42/20/9/0	52/36/27/5	0.0560

Multivariate analysis			
Variable	Category	Odds ratio (95% CI)	P-value
HCV-RNA	≥ 5.8	1	0.002
	< 5.8	2.604 (1.408–4808)	
Gender	Female	1	0.004
	Male	2.312 (1.309–4.085)	
Genotype	1	1	0.011
	2	2.203 (1.202–4.038)	

ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyltranspeptidase; HCV-RNA, hepatitis C virus RNA; SVR, sustained virological response; WBC, white blood cell.

were factors associated with SVR in older patients. Older patients who have these factors should be considered for treatment in hopes of achieving SVR to prevent HCC.

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INTERNAL MEDICINE

□ CASE REPORT □

Hepatitis B e Antigen and Hepatitis B Surface Antigen Seroclearance with the Emergence of Lamivudine-associated and Core Mutations Following CD4 Elevation in a Patient with Hepatitis B and HIV

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Abstract

Obtaining a better understanding of the mechanisms associated with hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) loss in patients with hepatitis B virus (HBV) is important for treating patients with chronic hepatitis B. We herein describe the case of a patient with HBV and human immunodeficiency virus whose chronic hepatitis was stabilized due to HBe and HBs seroconversion with the emergence of lamivudine-associated and core mutations after CD4 elevation. A full-length HBV DNA analysis indicated that HBsAg had been lost after the development of the rtS143T mutation, which corresponded to the emergence of the sF134L and core mutations. The details of this case shed some light on the mechanisms associated with HBsAg and HBeAg clearance.

Key words: hepatitis B virus, human immunodeficiency virus, lamivudine, mutation, nucleotide analogue

(Intern Med 54: 585-590, 2015)
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Introduction

There are several nucleotide analogues available for treating hepatitis B. However, the administration of long-term antiviral therapy directed towards the hepatitis B virus (HBV) reverse transcriptase (RT) can lead to the selection of drug resistant mutations (1-3), and the efficacy of lamivudine in cases of chronic hepatitis B is limited by the emergence of drug-resistant mutants (1). Mutations conferring resistance to lamivudine are primarily located in the YMDD motif, i.e., M204V or M204I, in the C-terminal domain of RT and may be associated with compensatory mutations in the C-terminal domain, i.e., V173L or L180M (2). Entecavir and tenofovir are highly effective against hepatitis B and less frequently associated with the development of resistant mutants than lamivudine; however, in patients coinfected with HIV, the use of entecavir monotherapy has been reported to result in the accumulation of HIV-1 variants with the lamivudine-resistant mutation M184V (3), and tenofovir monotherapy may induce the accumulation of HIV-1 variants. The introduction of tenofovir and emtricitabine into the HBV armamentarium for HBV/HIV-coinfected patients provides another treatment option in such cases (4). However, the challenge is to increase the number of antiviral drugs available for controlling the emergence of additional mutations.

Patients coinfected with HBV and HIV exhibit more rapid progression of liver fibrosis and a higher rate of decompensated cirrhosis (5). Among these cases, hepatitis B e antigen (HBeAg) and/or hepatitis B surface antigen (HBsAg) seroclearance has been observed in a minority of patients starting antiretroviral therapy (ART) comprising an antiviral drug for HBV (6) and correlates with a sustained HIV response

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Table. **HIV Treatment**

Regimen	Date	HIV RNA copies/mL	CD4 cell count	CD4/CD8
	Jun-02	100,000 <	463.7	0.7
AZT+ddI+IDV	Jul-02	n/a	n/a	n/a
	Aug-02	7,900	478.8	1
	Sep-02	2,900	530.6	1.3
	Oct-02	1,100	434.5	1.3
	Dec-02	800	501.7	1.4
	Feb-03	1,500	446.5	1.3
	Mar-03	3,400	431.9	1.2
d4T+3TC+EFV	Apr-03	2,300	451.4	1.3
	May-03	undetectable	595	1.3
	-	-	-	-
	Jun-05	undetectable	637.8	1.2
	Oct-05	620	582.6	0.9
	Nov-05	691	n/a	n/a
LPV+RTV+ddI	Dec-06	1,700	n/a	n/a
	Jan-06	undetectable	n/a	n/a
	Feb-06	undetectable	765.6	1.2

n/a: not applicable

to antiretroviral therapy consisting mostly of ART regimens that include lamivudine (7).

Selection for both HBV polymerase gene mutations and S gene mutations has been observed during antiviral therapy for HBV in HBV/HIV-coinfected patients (8). These mutations sometimes alter the antigenicity of HBsAg to such an extent that the antigens can no longer be recognized by many monoclonal antibodies (9). As a result, these mutations are very similar to vaccine-escape mutations selected for by HBV vaccination. Moreover, some mutations associated with lamivudine resistance may cause premature stop codons in the S gene, resulting in the impaired secretion of HBsAg, according to a previous report (10).

Obtaining a better understanding of the mechanisms associated with HBsAg and HBeAg loss is important for treating patients with chronic hepatitis B. The following report describes the case of a HBV/HIV-coinfected patient with HBeAg and HBsAg seroclearance and undetectable HBV DNA following the emergence of lamivudine-associated and core mutations, without the addition of any new antiviral drugs.

Case Report

A 35-year-old man with a past history of acute hepatitis B consequently advanced to chronic hepatitis B and was followed up at another hospital. He was subsequently admitted to Nagoya University Hospital due to an elevated level of alanine transaminase (ALT). He had been found to be infected with HIV, and an ART regimen [azidothymidine (AZT) + didanosine (ddI) + indinavir (IDV)] had been initiated in mid-July 2002 (Table). At that time, the laboratory data were as follows: HBsAg= positive, HBsAb= negative, HBeAg= positive, AST= 66 IU/L and ALT= 62 IU/L. However, during follow-up, the viral load did not decrease to an undetectable level; therefore, the medication regimen was changed to stavudine (d4T) + lamivudine (3TC) + efavirenz (EFV) at the end of May in 2003. The HIV RNA viral load transiently became undetectable, although viral mutations conferring resistance to all antiretroviral drugs, including lamivudine, emerged. The previous antiviral drugs were replaced with another regimen [lopinavir (LPV) + ritonavir (RTV) + ddI]. After stopping the dose of lamivudine, the patient's ALT level increased to 1,161 IU/L then spontaneously decreased to approximately 140 IU/L without any elevation in the total bilirubin level (Fig. 1A). The HIV viral load subsequently became undetectable, and the CD4 cell count recovered to 750 cells/mm^3. He was then admitted due to pneumonia and, during hospitalization, was referred to our group at the beginning of April in 2006. At that time, the laboratory data included the following findings: AST=74 IU/L, ALT=91 IU/L, platelet count=369,000/μL, HBeAb= negative, HBV DNA load=8.7 log copies/mL. A liver biopsy was performed, which showed a moderate activity and portal fibrosis without septa (A2/F1), according to the Metavir scoring system (11). After the patient recovered from the pneumonia, he was followed up as an outpatient. During follow-up, the ALT level increased to 502 IU/L and the HBV DNA load increased to 9.1 log copies/mL; therefore, he was admitted to our hospital. The laboratory data on admission were as follows: ALT=613 IU/L, platelet count= 199,000/μL, HBeAg= positive. A sequence analysis revealed a wild-type HBV polymerase pattern and genotype A. At the beginning of July 2006, antiviral therapy with lamivudine was restarted, as it was unlikely that the high HBV viral load could be controlled with interferon and, at that time, adefovir and tenofovir were not available under the health insurance system in Japan. The patient's HBV viral load subsequently decreased to 3.8 log copies/mL, and HBeAg seroconversion began. However, the HBV viral load rebounded to 5.4 log copies/mL and an rt M204V mutation was detected. Since March 2008, the HBeAb titer has demonstrated approximately 100% inhibition, with complete seroconversion. The ALT level then normalized, and the HBV viral load gradually decreased to below 5 log copies/mL. Since July 2010, the HBV viral load has remained below 4.0 log copies/mL and the ALT level has remained below 35 IU/mL. Finally, the HBV DNA and HBsAg titers became negative in April 2012, continuing to July 2013. The HBcrAg titer also gradually decreased to approximately 3.0 logU/mL in correlation with the decline in the HBV DNA titer (Fig. 1B). The HBV DNA titer was not measured and no stored serum was available before referral to our liver unit at the beginning of April 2006.

Serum samples exhibiting a peak in the fluctuating HBV viral load were collected at five time points (Fig. 1A). The levels of HBsAg (Architect HBsAg QT, Abbott Laboratories, Park, USA), HBeAg (Architect HBeAg, Abbott Laboratories), antibodies to HBeAg (anti-HBe) (Architect HBeAb,

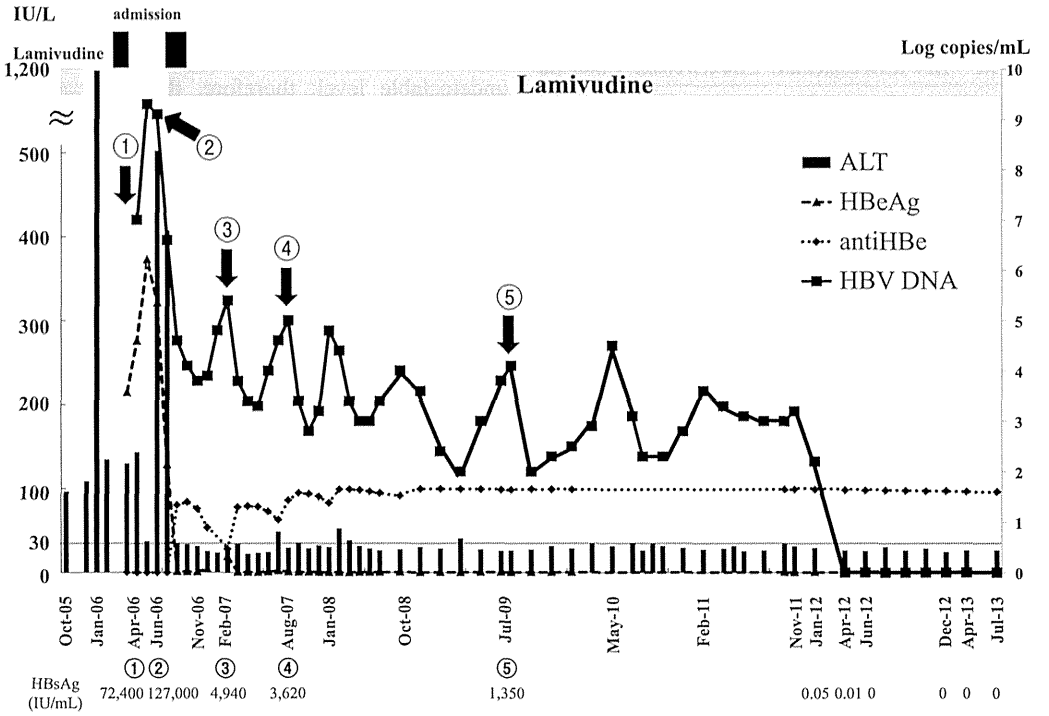

Figure 1A. Overview of the major events in the patient's clinical course. The vertical bars represent the AST level; the triangles (▲) represent the HBeAg titer; the diamonds (♦) represent the anti-HBe titer, the squares (■) represent the HBV DNA level. The numbers from 1 to 5 indicate the time points for which full length sequence analyses were performed. The timing of hospitalizations and lamivudine therapy are highlighted in the graph. The levels of HBsAg at the time points of the full length sequence analyses are indicated at the bottom.

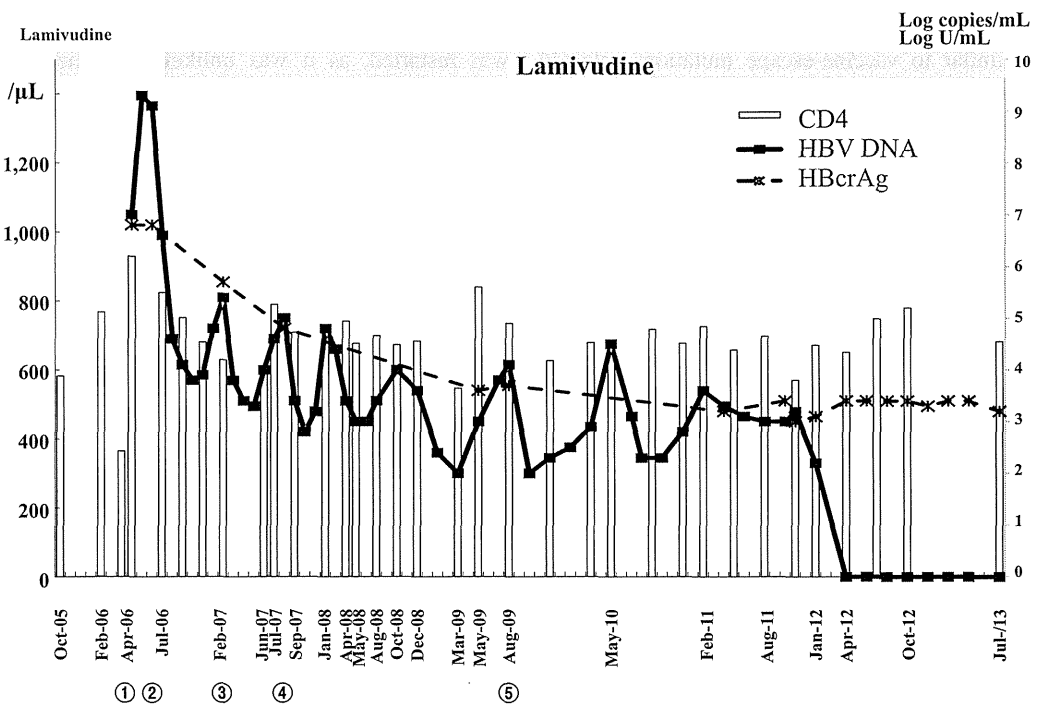

Figure 1B. Additional overview of the major events in the patient's clinical course. The vertical bars represent the CD4 level; the squares (■) represent the HBV DNA level, the asterisks (*) represent the HBcrAg titer. The timing of lamivudine therapy is highlighted in the graph.

a

	367	436	556	667	739	748	1091	2048	2129	2345	2458	3086	3193
1	T	A	T	T	A	G	T	C	G	G	G	G	G
2	T	A	T	T	A	G	T	C	G	G	G	G	G
3	**G**	A	**A**	**A**	A	G	**A**	C	G	G	**A**	G	G
4	T	**G**	**A**	**A**	**G**	G	T	**G**	**C**	G	**A**	**A**	**A**
5	T	A	**A**	**A**	**G**	**T**	T	**G**	**C**	**A**	**A**	G	G

b

	80	103	143	180	204	207	321
1	L	I	S	L	M(V)	V	F
2	L	I	S	L	M	V	F
3	**V**	I	**T**	**M**	M(V)	V	**Y**
4	L	**V**	**T**	**M**	**V**	V	F
5	L	I	**T**	**M**	**V**	**L**	F

c

	134	195	198
1	F	I	M
2	F	I	M
3	**L**	I	M
4	**L**	**M**	M
5	**L**	**M**	**I**

d

	50	77	149
1	P	E	V
2	P	E	V
3	P	E	V
4	**A**	**Q**	V
5	**A**	**Q**	**I**

Figure 2. Differences in nucleotides and amino acids from baseline. The numbers 1 to 5 refer to the time points at which serum samples were collected, as detailed in Figs. 1 and 2. Nucleotides and amino acids that differed from baseline are shown in bold type. a: Nucleotides that differed from baseline are highlighted in the full-length analysis. b: Amino acids that differed from baseline in the RT region are highlighted. Minor clones seen as a second peaked wave are indicated in parentheses. c: Amino acids that differed from baseline in the S region are highlighted. d: Amino acids that differed from baseline in the core region are highlighted. The dashed lines indicate corresponding changes.

Abbott Laboratories) and hepatitis B core-related antigens (HBcrAg) (Lumipulse® HBcrAg, Fujirebio, Tokyo, Japan) were examined using chemiluminescent or enzyme immunoassays. HBV DNA was detected quantitatively using a PCR-based assay (COBAS TaqMan HBV auto v2.0, Roche Molecular, Pleasanton, USA; detection limit from 2.1 to 9.0 log copies/mL). The predominant nucleotide (nt) sequences of full-length HBV DNA at each time point were determined according to the PCR direct sequencing method. Briefly, HBV DNA was extracted from 200 μL of serum stored at -20℃ using the QIAamp® DNA Mini kit according to the manufacturer's instructions (Qiagen, Hiden, Germany). The first round of PCR used the following primers, which were previously designed by Gunther et al. (12): FullS (5'-CCG GAA AGC TTG AGC TCT TCT TTT TCA CCT CTG CCT AAT CA-3', nt 1821 to 1841); FullAS (5'-CCG GAA AGC TTG AGC TCT TCA AAA AGT TGC ATG GTG CTG G-3', nt 1823 to 1806). The second round of PCR reactions used three sets of primers to obtain mutually overlapping HBV DNA fragments. The following primers previously designed by Kanada et al. (13) were used: BF 1s (5'-TTT TTC ACC TCT GCC TAA TCA-3', nt 1821 to 1841), BR5 (5'-AAC TGG AGC CAC CAG CAG GA-3', nt 74-55), BF4 (5'-GTC ACC ATA TTC TTG GGA AC-3', nt 2816 to 2835), BR7 (5'-GGG TTC AAA TGT ATA CCC AA-3', nt 839-820), BF7 (5'-TAT TGG GGG CCA AGT CTG TA-3', nt 752 to 771) and BR1s (5'-AAA AAG TTG CAT GGT GCT GG-3', nt 1825 to 1806). All PCR reactions were performed using the TaKaRa Ex Taq™ Hot Start Version system (Takara Bio, Shiga, Japan). After purification, the DNA fragments were directly sequenced using the GenomeLab™ Dye Terminator Cycle Sequencing with the Quick Start Kit and the CEQ8000 DNA sequencer (Beckman Coulter, Tokyo, Japan). The cycle sequencing reaction was conducted using primers designed in a previous report (13): BF2 (5'-CAG ACA ACT ATT GTG GTT TC-3', nt 2191 to 2210), BF3 (5'-TCT TTA ATC CTG AGT GGC AA-3', nt 2512 to 2531), BF5 (5'-AAG AGA CAG TCA TCC TCA GG-3' nt 3183 to 3202), BF6 (5'-CCT CCA ATT TGT CCT GGC TA-3', nt 350 to 369), BF8 (5'-TTT ACC CCG TTG CCC GGC A-3', nt 1142 to 1160), BR2 (5'-CAG AAT AGC TTG CCT GAG TG-3', nt 2080 to 2061), BR3 (5'-TTC CCG AGA TTG AGA TCT TC-3', nt 2440 to 2421), BR4 (5'-GAC CAA ATG CTC CCG CTC CT-3', nt 3040 to 3021) and BR6 (5'-GAG CAG GGG TCC TAG GAA TC-3' nt 193 to 174), as well as the above-mentioned primers, BF1s, BF4, BF7, BR5, BR7 and BR1s. This sequence-based method allowed for the detection of the mutation at an HBV concentration of approximately 9 to 3 log copies/mL. We compared five fully sequenced nucleo-

tide sequences obtained at different time points and determined which nucleotides differed from that observed baseline Fig. 2. We subsequently translated the nucleotide sequence to the amino acid sequence in the polymerase region, including the RT, S, core and X regions; the differences from baseline are presented in Fig. 2. Mutations were seen in 13 nucleotides (Fig. 2a), with seven mutations in the RT region (Fig. 2b). The lamivudine mutation rtL180M was noted starting at time point 3, and a direct sequence analysis indicated the presence of the lamivudine mutation rtM204V as a second peaked wave at time points 1 and 3 and as the main peaked wave at time points 4 and 5. Meanwhile, three mutations were detected in the S region (Fig. 2c). The mutation sI195M in the S region was identified corresponding to the lamivudine resistance rtM204V mutation in the RT region at time points 1 and 3 and as the main peaked wave at time points 4 and 5, whereas the sF134L mutation in the S region was noted starting at time point 3 in the 'a' determinant region. In the core region, three mutations of cP50A, cE77Q and cV149I were observed (Fig. 2d). HBeAg seroclearance subsequently occurred after fluctuations in the HBV viral load. However, there were no precore (G1896A) or basal core promoter (A1762T, G1764A) mutations from points 1 to 5.

The data were retrospectively obtained from the patients' records, and informed consent was obtained for this study. The study protocol was carried out in accordance with the Declaration of Helsinki.

Discussion

We herein experienced a patient whose chronic hepatitis B virus stabilized due to HBeAg and HBsAg seroclearance without treatment involving any additional nucleotide analogues. We searched for viral mutations starting before the second episode of acute exacerbation and at several time points at which the viral load was elevated. Consequently, the lamivudine mutation rtL204V was recognized as a second peaked wave starting at time point 1. Furthermore, the rtL180M mutation emerged at time point 3 in correlation with the rtS143T mutation, which corresponds to the S gene sF134L mutation. Because the S gene is overlapped by the polymerase gene, whenever a nucleotide mutation occurs in the polymerase gene, a concomitant nucleotide mutation develops in the S gene (14). The mutation in sF134L/rtS143T emerged based on the following two main possible explanations: the sF134L mutation induced by host immune pressure forced the development of the rtS143T mutation and the lamivudine-resistant rtS143T mutation forced the development of the sF134L mutation. In this case, the former possibility is that lamivudine reduced the viral load of HBV DNA, HBs antigens and HBc antigens and consequently activated host immune responses to HBV, thus allowing preexisting M204V mutations to become dominant. The sF134L mutation may be induced under immune pressure (15). Finally, the patient's immune responses inducing the core mutations resulted in HBs seroclearance. As to the latter scenario, there were no S gene mutations initially and the rtS 143T mutations emerged at time point 3 concomitantly with the rtL180M mutation, a lamivudine mutation; thus, the rtS 143T mutation associated with lamivudine may have forced the development of the sF134L mutation. Although we cannot deny the possibility that both amino acid changes were significant and not due to viral persistence, the sF134L mutation and core mutation are key mutations in this case. However, further in vitro studies are needed to elucidate the role of the sF134L mutation.

In addition, although we did not detect sG145R in this case, Sheldon et al. reported that some mutations associated with lamivudine resistance may cause premature stop codons in the S gene, resulting in the impaired secretion of HBsAg (10). The sF134L mutation exists in the 'a' determinant (amino acids 124 to 147), which is the major target of neutralizing antibodies. This mutation, which exists in the 'a' determinant, may also impair the secretion of HBsAg. The level of HBsAg decreased dramatically after the emergence of the sF134L mutation and subsequently remained low Fig. 1A, bottom), while the level of HBcrAg decreased in correlation with the decline in the HBV DNA titer. The HBcrAg titer reflects the amount of HBcAg, HBeAg and p22cr translated from mRNA transcribed from intrahepatic covalently closed circular DNA. These results indicate that both the impaired secretion of HBsAg and immune reaction were associated with the reduction in HBV DNA. Moreover, from the time point of 4, core mutations occurring in the cP50A [T helper T1 (C1-C20), T2 (C50-C69), T3 (C117-C131), B cell (C130-C138) and T-cytotoxic: CTL (C18-C27)] epitope associated with HBV clearance have been reported (16), and the cP50A mutation is present in the helper T2 epitope. This mutation may also have contributed to the patient's viral clearance in this case. Furthermore, in the current case, the core mutation may have induced the increased clearance of HBV due to the immune reaction. Following the suppression of HIV RNA with successful treatment for HIV, the CD4 level became elevated and the HBV DNA titer increased. Therefore, the patient's immune reaction and impaired secretion of HBsAg may account for the dramatic decrease in the HBsAg level and ultimate clearance of HBsAg. Moreover, there is a paper in which a mutation near the 'a' determinant and a core mutation were found to interact with respect to HBV virion secretion (17). Further investigation is therefore required to clarify whether immune pressure on the core region and various mutations in the 'a' determinate region, such as sF134L or both, increases the probability of HBsAg or HBeAg seroclearance in a large number of chronic hepatitis B patients with lamivudine resistance. In addition, the further accumulation of longitudinal data as well as virological analyses of the surface and core region and T-cell proliferation analyses are also needed.

We were consulted on this case prior to the patient's second episode of acute exacerbation, at which point we decided to restart the dose of lamivudine in order to control

the wild-type HBV infection. Since the liver biopsy showed relatively mild fibrosis (A2/F1), guidelines for treating HBV/HIV coinfected patients state that drugs active against HIV, such as emtricitabine, lamivudine, tenofovir and entecavir, were not suitable for HIV treatment in this case (18); rather, adefovir and telbivudine, which have not been proven to exhibit an antiviral activity against HIV, are preferred (19). However, at that time, we were unable to use adefovir and tenofovir due to health insurance restrictions in Japan. Moreover, since the administration of interferon-based treatment was unlikely to be successful due to the patient's high HBV viral load at the time, we restarted the treatment with lamivudine in order to suppress the wild-type HBV infection.

Resistance to lamivudine develops in the majority of HBV/HIV-coinfected patients after prolonged use. Adding tenofovir to the treatment regimen is one of the most promising strategies in such cases, as the antiviral efficacy of tenofovir is not influenced by the presence of lamivudine resistance (20). The introduction of tenofovir and emtricitabine into the HBV armamentarium for HBV/HIV-coinfected patients provides another treatment option (4). However, since HBsAg seroclearance is rare, long-term maintenance therapy and regimens containing multiple drugs may be required in patients with HBV and more so in those with HBV/HIV coinfection. If the rate of HBV replication is reduced and the ALT level is within the normal range, it is preferable not to use unnecessary antiviral agents in order to prevent the potential for additional mutations in both HBV and HIV. The details of the present case shed light on some of the mechanisms associated with HBsAg and HBeAg seroclearance.

The authors state that they have no Conflict of Interest (COI).

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Endoscopic treatment of esophageal varices in hemophiliac patients with liver cirrhosis

To the Editor:

Hemophilia is a rare X-linked congenital bleeding disorder caused by a deficiency of clotting factor VIII (hemophilia A) or IX (hemophilia B). Although many hemophiliac patients have both been infected by hepatitis C virus through receiving virus-inactivated clotting factors and experienced esophageal varices (EV) caused by liver cirrhosis, their treatment during endoscopic treatment of EV remains poorly known. We previously reported the first case of endoscopic variceal ligation (EVL) of EV in a cirrhotic patient with severe hemophilia A.[1] In that report, bleeding from a post-EVL ulcer was seen after the first EVL, and at the next treatment, bleeding was prevented by the administration of a proton pump inhibitor (PPI) and continuation of clotting factor replacement therapy until ulcer healing was confirmed. After that, we performed 9 EVL procedures for EV in 2 other cirrhotic patients with severe hemophilia A. In all of these cases, the administration of PPI was continued during and after EVL; however, in the first 3 treatments, 2 post-EVL bleedings from an unhealed ulcer occurred (Fig. 1A and B). In these bleeding cases, replacement therapy was discontinued without checking post-EVL ulcer healing on day 7 and day 9 after the last EVL session, respectively, in response to the patients' requests for early discharge. Because a deficiency of coagulation factors has been reported to cause delayed healing,[2] after the 2 episodes of bleeding, we adopted a rule to continue replacement therapy until the white coating had started to become thin and the regenerating epithelium was extending into the ulcer base (Fig. 1C). After the adoption of this rule, no rebleeding was observed at the subsequent 6 consecutive EVL procedures. In conclusion, with sufficient duration of coagulation factor replacement therapy, EVL of EV can be performed in cirrhotic patients with severe hemophilia A.

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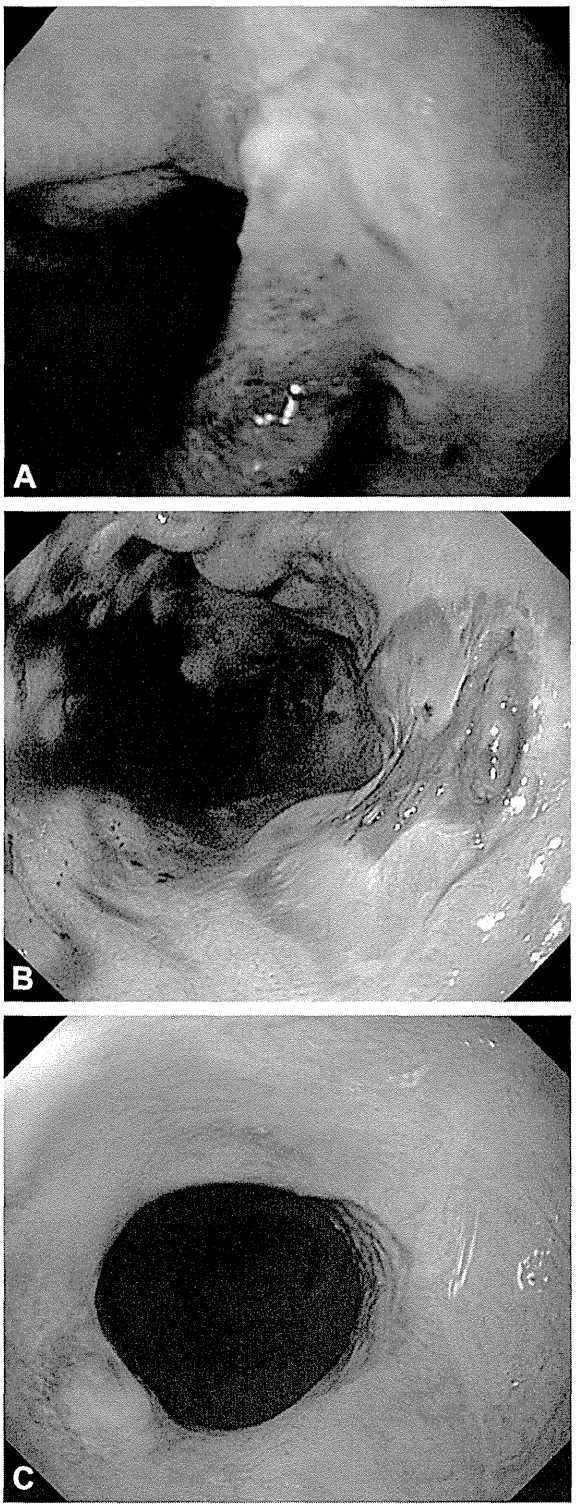

Figure 1. A, B. Endoscopic image showing bleeding from an ulcer after endoscopic variceal ligation. **C,** Endoscopic image showing a healed ulcer after endoscopic variceal ligation.

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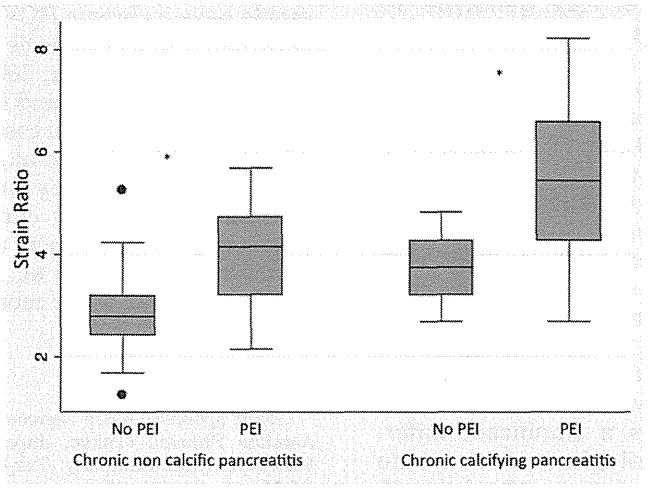
ERRATA

Reference 21 in "Quality indicators for ERCP" (Gastrointest Endosc 2015;81:54-66) should be as follows: Cotton PB, Durkalski V, Romagnuolo J, et al. Effect of endoscopic sphincterotomy for suspected sphincter of Oddi dysfunction on pain-related disability following cholecystectomy – the EPISOD randomized clinical trial. JAMA 2014;311;2101-9.

Figure 4 in "EUS elastography to predict pancreatic exocrine insufficiency in patients with chronic pancreatitis" (Gastrointest Endosc 2015;81:136-142) is incorrect. The corrected figure appears below.



In the March 2015 issue of Gastrointestinal Endoscopy, in the reply to the letter by Dr. Douglas K. Rex (Gastrointest Endosc 2015;81:778-9), the name of Dr. Morris Barocas was misspelled.

Journal of Medical Virology

Associations Between Responses to Interferon Therapy and Genetic Variation in Interleukin-28B and the Core Region of Hepatitis C Virus Genotype 3a

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The single-nucleotide polymorphism (SNP) of interleukin-28B (IL-28B) and mutations in the core region of hepatitis C virus (HCV) genotype 1b have been associated with response to interferon (IFN) therapy. However, whether this IL-28B SNP affects responses to INF therapy for HCV genotype 3a is not known. The aim of this study is to investigate whether this IL-28B SNP (rs8099917) and specific missense mutations in the HCV core region affect the response to IFN therapy for HCV genotype 3a. Patients (n = 19; median age 44.5) infected with HCV genotype 3a who received IFN therapy were studied. Of the 19 patients, 12 (63.1%) achieved sustained virological response. Of those 12 patients, 11 had the TT genotype (11/16; 68.7%), and one had the TG genotype (1/3; 33.3%). The difference in the sustained virological response rate between IL-28B genotype groups was not significant ($P = 0.5232$). HCV core region was well conserved; however, polymorphisms at position 72 were identified. Of the 19 HCV samples; 15 carried a glutamic acid at position 72, and these were defined as E type; the others (4/19) were defined as non-E type. Notably, there was a significant difference in the sustained virological response rate between E type and non-E-type; 12 of the 15 patients with E-type achieved sustained virological response, but none of the four patients with non-E-type achieved sustained virological response ($P = 0.009$). A glutamic acid at position 72 in the core region of HCV genotype 3a was associated with a good response to IFN therapy. ***J. Med. Virol.***

KEY WORDS: genotype 3a; core region; amino acid substitution; sustained virological response

INTRODUCTION

Hepatitis C virus (HCV) infection remains a serious health problem worldwide. World Health Organization estimates that about 169.7 million people, approximately 2.9% of the world's population, are seropositive for HCV antibody. The prevalence of chronic HCV infection worldwide is 2.2%; approximately 127 million people have a chronic HCV infection and are at risk of developing liver cirrhosis and hepatocellular carcinoma (HCC) [Ray Kim, 2002]. Cirrhosis and HCC are each potentially fatal, and it is estimated that 27% and 25%, respectively, of all cases worldwide occur in HCV-infected people [Perz et al., 2006]. HCVs are classified into six major genotypes that have different geographic distributions, different clinical courses, and different responses to interferon (IFN) therapy [Simmonds et al., 2005; Ghany et al., 2009]. Of these genotypes, HCV genotype 1 is the most resistant to IFN therapy.

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Recent standard-of-care treatment for genotype 1 is a triple combination therapy comprising pegylated-interferon (PEG-IFN), ribavirin (RBV), and a protease inhibitor (telaprevir or boceprevir), the sustained virological response rate with this treatment is approximately 70% [Poordad et al., 2011; Sherman et al., 2011]. Genotype 2 and 3 each exhibit better IFN responsiveness than genotype 1, and a double combination therapy comprising PEG-IFN and RBV results in sustained virological response rates that approach 80% [Shiffman et al., 2007; Tapper and Afdhal, 2013]. HCV genotype is known to be a major viral factor that affects sustained virological response rates with IFN therapy. Other viral factors might, like HCV genotype, affect IFN responsiveness; for example, amino acid substitutions in the HCV core region, NS5A variants, 5' untranslated region (UTR) variants, or some combination thereof may be associated with sustained virological response rates with INF therapy [Enomoto et al., 1996; Akuta et al., 2005; Katano et al., 2007]. Reportedly, patients infected with HCV genotype 1b, which has arginine at position 70 in the HCV core or leucine at position 91, respond well to IFN therapy [Akuta et al., 2005]. However, the HCV core region, including positions 70 and 91, is well conserved among HCV genotype 1a isolates, and no significant mutations were found in this region, which is associated with IFN responsiveness [Hayashi et al., 2012]. Genetic variation in the core region of HCV genotype 3a isolates has not been examined thoroughly, nor has the relationship between such variation and clinical responses to IFN therapy. Genome-wide association studies have revealed that single nucleotide polymorphisms (SNPs) of interleukin-28B (IL-28B) are significantly associated with variation in outcomes of IFN therapy for treatment of HCV genotype 1 infections [Ge et al., 2009; Tanaka et al., 2009]. However, only a few studies have examined effects of IL-28B variation on IFN responsiveness for HCV genotype 3 infections [Mangia et al., 2010; Moghaddam et al., 2011; Bucci et al., 2013].

Consequently, whether IL-28B variants are associated with variation in responses to IFN therapy remains unclear for patients infected with HCV genotype 3a. The utility of determining IL-28B genotype and amino acid substitutions in the core region for predicting IFN responsiveness has been investigated for HCV genotype 1b but not for genotype 3a [Hayashi et al., 2011; Hiraga et al., 2011]. The aim of this study was to evaluate the impact of one IL-28B SNP and mutations in the core region of HCV on responses to IFN therapy among patients infected with HCV genotype 3a.

MATERIALS AND METHODS

Study Patients

The study subjects were 19 patients with HCV genotype 3a. Of these 19 patients, 15 were Japanese individuals with hemophilia, two were Pakistani, one was Indonesian, and one was Chinese. None of the patients had a history of chronic alcohol abuse, autoimmune disease, or metabolic disease. Patients with active intravenous drug use or positive for hepatitis B surface antigen had been excluded from the study patients. Of the 19 patients, four were co-infected with human immunodeficiency virus (HIV) that was well controlled by highly active antiretroviral therapy (HAART). The clinical characteristics of the study patients are summarized in Table I.

Informed consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

Schedule of IFN Therapy

One patient received 300 mega international unit of IFN beta via intravenous injection three times a week for 24 weeks. Another eight patients received 600 mega unit of IFN alpha 2b via subcutaneous injection daily for 2 weeks and then three times a week for 22 weeks; each of these eight patients also received oral RBV at a dose of 600–800 mg daily during the IFN therapy. The other 10 patients received PEG-IFN alpha 2b at a dose of 1.5 μg per kilogram of body weight via subcutaneous injection once a week for 48 weeks; each of these 10 patients also received oral RBV at a dose of 600–1000 mg daily during the IFN therapy. After the end of each treatment regime, each patient was followed for a further 24 weeks. Patients who persistently tested negative for serum HCV-RNA during the 24-week follow-up after withdrawal of IFN treatment were considered to exhibit a sustained virological response, and all other patients were considered to exhibit a non-sustained virological response.

Virological Tests

A commercial kit (Abbott Japan, Tokyo, Japan) was used to test serum from each patient for anti-HCV antibodies. Additionally, HCV RNA viral load was measured via a commercial assay [Kawai et al., 2002]. Anti-HIV antibodies were detected with a commercial kit (Abbott Japan, Tokyo,

TABLE I. Clinical Characteristics

	N = 19
Age (years)	45 (25–64)
Sex (male/female)	18/1
hemophilia (yes/no)	15/4
AST (IU/L)	56.0 (28–190)
ALT (IU/L)	76.0 (21–238)
Platelet count ($\times 10^4/\mu l$)	15.0 (5.6–32.9)
HCV RNA (log IU/ml)	5.8 (4.6–7.0)
HIV co-infection (yes/no)	4/15

AST, aspartate aminotransferase; ALT, alanine aminotransferase; HCV, hepatitis C virus; HIV, human immunodeficiency virus.
Data are expressed as median (range).

Japan). HCV genotyping was performed by direct sequencing of the 5′-UTR and E1 regions as described previously [Otagiri et al., 2002; Hayashi et al., 2003]. The genotypes were classified according to Simmonds' nomenclature [Ghany et al., 2009]. Direct sequencing of the HCV core region was performed as described previously [Hayashi et al., 2012]. Briefly, the QIAamp Viral RNA kit (Qiagen, Valencia, California, USA) was used according to manufacturer's protocols to extract RNA from each 140-μl serum sample; extracted RNA was dissolved in 50 μl diethylpyrocarbonate-treated water. Oligonucleotide primers, random hexamer primers, and the iScript cDNA Synthesis Kit (BIO-RAD, Hercules, California) were used to reverse transcribe target sequences from 10 ng of template RNA.

Nested PCR was then performed to amplify the HCV core region. In brief, each 50-μl PCR contained 100 nM of each primer, 1 ng of template cDNA, 5 μl of GeneAmp 10× PCR buffer, 2 μl of dNTPs, and 1.25 U AmpliTaq Gold (Applied Biosystems, Foster City, California). The sense and antisense primers for the core region were 5′-GGGAGGTCTCGTAGACCGTG-CACCATG-3′ and 5′ -GAGMGGKATRTACCCCAT-GAGRTCGGC-3′, respectively. The amplification conditions were 10 min at 94°C, followed by 40 cycles of 94°C for 10 sec, 55°C for 30 sec, and 72°C for 30 sec in a thermal cycler (GeneAmp PCR System 9700, Applied Biosystems, Foster City, California). The second nested amplification was performed in the same reaction buffer with the first-round PCR products as template; sense and antisense primers were 5′-AGACCGTGCACCATGAGCAC-3′ and 5′-TACGCCGGGGGTCAKTRGGGCCCCA-3′, respectively. The second-round PCR products were separated by electrophoresis on 2% agarose gels, stained with ethidium bromide, and visualized under ultraviolet light. These PCR products were then purified, and the second-round PCR primers, a dye terminator sequencing kit (BigDye Terminator vl.l Cycle Sequencing Kit, Applied Biosystems, Foster City, California) and an ABI 310 DNA Sequencer (Applied Biosystems, Foster City, California). A mutation mixture was defined as viral mutants that constituted 50% or more of the total viral population.

Genomic Analysis

A real-time PCR system was used as previously reported to detect the relevant IL-28B SNP, rs8099917 [Hayashi et al., 2012]. In brief, QIAamp DNA Blood mini Kits (Qiagen, Valencia, California) were used to extract genomic DNA from each 150-μl sample of whole blood. Extracted DNA was dissolved in 50 μl diethylpyrocarbonate-treated water. The purified genomic DNA (10 ng), Taqman SNP Genotyping Assay kits (Applied Biosystems, Foster City, California), and included primers and probes were used to amplify IL-28B sequences. The IL-28B region containing the rs8099917 SNP was amplified, and a real-time PCR in a thermal cycler (7300 Real-time PCR System, Applied Biosystems, Foster City, California) was used to analyze the results.

Statistical Analysis

Data are expressed as medians and the corresponding ranges. The paired *t*-test, the Chi-square test, and Fisher's exact test were used as appropriate to analyze differences in variables. A value of $P < 0.05$ was considered statistically significant. SPSS software (SPSS Incorporated., Chicago, Illinois) was used for all statistical calculations.

RESULTS

The amino acid sequence of the HCV core region (position 5–105), IL-28B rs8099917 genotype, IFN regimen, and treatment outcome for each patient are listed in Figure 1. Sequences from HCV-1a (GenBank EU862841) were used as the reference sequences for genotype 1a, HCV-J (GenBank S70788) sequences were used for genotype 1b, MD2a-7 (GenBank AF238485) sequences were used for genotype 2a, ZS260 (GenBank KC844048) sequences were used for genotype 2b, and NZL1 (GenBank D17763) sequences were used for genotype 3a. When compared with the NZL1 sequence, HCV isolates from six patients had the same core region sequence; in contrast, HCV isolates from the other 13 patients had more than one amino acid difference from the NZL1 sequence. The core region was well conserved, but mutations were most common at positions 16 (n = 5) and 72 (n = 4). The amino acids found at position 16 were isoleucine (n = 14), threonine (n = 1), leucine (n = 1), asparagine (n = 1), and valine (n = 2). For position 16, isoleucine was defined as I type, and the others are defined as non-I type. The amino acids found at position 72 of the HCV core region were glutamic acid (n = 15), asparatic acid (n = 2), glycine (n = 1), and glutamic acid/glycine (n = 1). For position 72, glutamic acid was defined as E type and all others were defined as non-E type. Of the 19 patient genotypes at the IL-28B SNP (rs8099917), 16 were homozygous for the major allele (TT), none were homozygous for the minor allele (GG), and three were heterozygous (TG). The overall sustained virological response rate was 63.1% (12 out of 19 patients). The results of statistical analysis of associations between the response to IFN therapy and clinical characteristics, including mutations in the core region and the SNP of IL-28B, are shown in Table II. Sustained virological response occurred for 12 of the 15 patients (80%) with E type, and none of the four patients with non-E type. The sustained virological response rate was significantly higher among patients in the E type group than among those in the non-E type group ($P = 0.009$). Each of the other clinical factors, including IL-28B rs8099917

genotype	strain	5					10						16				20										30										40		
1a	HCV-1a	P	K	P	Q	R	K	T	K	R	N	T	N	R	R	P	Q	D	V	K	F	P	G	G	G	Q	I	V	G	G	V	Y	L	L	P	R	R	G	P
1b	HCV-J	P	K	P	Q	R	K	T	K	R	N	T	N	R	R	P	Q	D	V	K	F	P	G	G	G	Q	I	V	G	G	V	Y	L	L	P	R	R	G	P
2a	MD2a-7	P	K	P	Q	R	K	T	K	R	N	T	N	R	R	P	Q	D	V	K	F	P	G	G	G	Q	I	V	G	G	V	Y	L	L	P	R	R	G	P
2b	ZS260	P	K	P	Q	R	K	T	K	R	N	T	N	R	R	P	Q	D	V	K	F	P	G	G	G	Q	I	V	G	G	V	Y	L	L	P	R	R	G	P
3a	NZL1	P	K	P	Q	R	K	T	K	R	N	T	I	R	R	P	Q	D	V	K	F	P	G	G	G	Q	I	V	G	G	V	Y	V	L	P	R	R	G	P
	case 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	case 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	case 3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	case 4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	case 5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	case 6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	case 7	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	case 8	-	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	case 9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	case 10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	case 11	-	-	-	-	-	-	-	-	-	-	-	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	case 12	-	-	-	-	-	-	-	-	-	-	-	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	case 13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	case 14	-	-	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	case 15	-	-	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	case 16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	case 17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	case 18	-	-	-	-	-	-	-	-	-	-	-	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	case 19	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

strain								50										60										70		72								80			
HCV-1a	R	L	G	V	R	A	T	R	K	T	S	E	R	S	Q	P	R	G	R	R	Q	P	I	P	K	A	R	R	L	E	G	R	T	W	A	Q	P	G	Y	P	W
HCV-J	R	L	G	V	R	A	T	R	K	T	S	E	R	S	Q	P	R	G	R	R	Q	P	I	P	K	D	R	R	P	E	G	R	A	W	A	Q	P	G	Y	P	W
MD2a-7	R	L	G	V	R	T	T	R	K	T	S	E	R	S	Q	P	R	G	R	R	Q	P	I	P	K	D	R	R	S	T	G	K	S	W	G	K	P	G	Y	P	W
ZS260	R	L	G	V	R	A	T	R	K	T	S	E	R	S	Q	P	R	G	R	R	Q	P	I	P	K	D	R	R	S	T	G	K	S	W	G	K	P	G	Y	P	W
NZL1	R	L	G	V	R	A	T	R	K	T	S	E	R	S	Q	P	R	G	R	R	Q	P	I	P	K	A	R	R	S	E	G	R	S	W	A	Q	P	G	Y	P	W
case 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
case 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
case 3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
case 4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
case 5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
case 6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
case 7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
case 8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
case 9	-	-	-	-	-	-	-	-	-	-	-	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
case 10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
case 11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
case 12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Y	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
case 13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
case 14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
case 15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
case 16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-	-	-	-	-	D	-	-	-	-	-	-	-	-	-	-	-
case 17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	D	-	-	-	-	-	-	-	-	-	-	-
case 18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	E/G	-	-	-	-	-	-	-	-	-	-	-
case 19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	G	-	-	-	-	-	-

strain							90																IL28B	treatment	outcome
HCV-1a	P	L	Y	G	N	E	G	C	G	W	A	G	W	L	L	S	P	R	G	S	R	P			
HCV-J	P	L	Y	G	N	E	G	M	G	W	A	G	W	L	L	S	P	R	G	S	R	P			
MD2a-7	P	L	Y	G	N	E	G	L	G	W	A	G	W	L	L	S	P	R	G	S	R	P			
ZS260	P	L	Y	G	N	E	G	C	G	W	A	G	W	L	L	S	P	R	G	S	R	P			
NZL1	P	L	Y	G	N	E	G	C	G	W	A	G	W	L	L	S	P	R	G	S	R	P			
case 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	TT	IFN-α/RBV	sustained virological response
case 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	TG	PEG-IFN/RBV	sustained virological response
case 3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	TT	PEG-IFN/RBV	sustained virological response
case 4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	TT	PEG-IFN/RBV	sustained virological response
case 5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	TT	IFN-α/RBV	sustained virological response
case 6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	TT	PEG-IFN/RBV	non-sustained virological response
case 7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	TT	PEG-IFN/RBV	non-sustained virological response
case 8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	TT	IFN-β	sustained virological response
case 9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	TT	PEG-IFN/RBV	sustained virological response
case 10	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	TT	IFN-α/RBV	sustained virological response
case 11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	TT	IFN-α/RBV	sustained virological response
case 12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	TT	PEG-IFN/RBV	sustained virological response
case 13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	TT	PEG-IFN/RBV	sustained virological response
case 14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	TG	PEG-IFN/RBV	non-sustained virological response
case 15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	TT	IFN-α/RBV	sustained virological response
case 16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	TG	PEG-IFN/RBV	non-sustained virological response
case 17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	TT	IFN-α/RBV	non-sustained virological response
case 18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	TT	IFN-α/RBV	non-sustained virological response
case 19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	TT	PEG-IFN/RBV	non-sustained virological response

Fig. 1. Sequences of amino acids 5-105 in the core region, IL-28B rs8099917 genotype, IFN regimen, and treatment outcome for each patient. Upper 5 sequences are the reference sequences for genotype 1b, genotype 1a, genotype 2a, genotype 2b, and genotype 3a. Dashes indicate amino acids identical to the reference sequence of genotype 3a, and substituted amino acids are shown by standard single-letter codes. Mutations were most common at position 16 (n=5) and 72 (n=4). None of patients with mutations at position 72 achieved sustained virological response.

TABLE II. Univariate Analysis: Factors Predictive of Sustained Virological Response

	Sustained virological response (n = 12)	Non-sustained virological response (n = 7)	*P*-value
Age (years)	44.5 (36.5–54.8)	46.0 (37.0–49.5)	0.9281
hemophilia (yes/no)	10/2	5/2	0.6027
AST (IU/L)	47.0 (38.0–64.5)	99.0 (64.0–105.0)	0.0565
ALT (IU/L)	63.0 (21.0–167.0)	119.0 (105.0–168.0)	0.1101
Platelet count ($\times 10^4/\mu l$)	16.4 (13.2–19.6)	12.5 (10.9–20.8)	0.8747
HCV RNA (log IU/ml)	5.77 (5.42–6.38)	5.94 (5.63–6.60)	0.3440
HIV co-infection: yes/no	3/9	1/6	1
IL28B(rs8099917): TT/TG	11/1	5/2	0.5232
core 16: I/non-I	10/2	4/3	0.3047
core72: E/non-E	12/0	3/4	0.0090

AST, aspartate aminotransferase; ALT, alanine aminotransferase; HCV, hepatitis C virus; HIV, human immunodeficiency virus; IL28B, interleukin 28 B; I, Isoleucine; E, Glutamic acid.
Data are expressed as median (range).

genotype, was not associated with sustained virological response rate. The amino acid at position 72 of the HCV core region was the only significantly predictive factor for sustained virological response. Potential associations between clinical factors and the amino acids at position 72 of the HCV core region were then assessed. There were no significant differences between each factor and amino acid substitution of core 72 (Table III).

DISCUSSION

HCV isolates can be divided into six genotypes and these HCV genotypes show unique geographic distributions. HCV genotypes 1b, 2a, and 2b are predominant in Japan [Otagiri et al., 2002; Hayashi et al., 2003]. The HCV genotype 3a is predominant in West Asia and uncommon in Japan [Ebeling, 1998]. The results of this study have confirmed that the majority of cases with genotype 3a involve Japanese hemophiliacs who have received imported clotting factors or immigrants. The IFN response to HCV infection is determined by both host and viral factors. Although the IL-28B SNP rs8099917 is strongly associated with responsiveness to IFN therapy for treatment of HCV genotype1b infections [Tanaka et al., 2009], there was not a significant relation between this IL-28B SNP and the sustained virological response rate among patients infected with HCV genotype 3a [Mangia et al., 2010; Moghaddam et al., 2011; Bucci et al., 2013]. The results of this study also confirmed previous findings that there is no association between this IL-28B SNP and the sustained virological response rate. The *IL-28B* gene encodes IFN-λ, and mediates HCV clearance [Kotenko et al., 2003; Robek et al., 2005]. It is reasonable to speculate that IL-28B genotype could affect the sustained virological response rate and responsiveness to IFN-based treatments of HCV genotype 3a infections. A possible explanation for the difference between the 1b and 3a HCV genotypes with regard to this IL-28B SNP is that the association between the SNP and HCV infections might be masked for genotype 3a infections because the genotype 3a infections have better IFN responsiveness overall. This IL-28B SNP has utility for predicting non- sustained virological response, but not for predicting sustained virological response. The effects of IL-28B may be more evident with the low sustained virological response rates, such as those seen with genotype 1 patients, but these effects may not be evident with the high sustained virological response rates seen among genotype 3a patients. However, a limitation of this study is that it included small number of patients.

The amino acid at position 72 of the HCV core region was apparently associated with the sustained

TABLE III. Clinical Characteristics According to the Amino Acid Substitution of Core 72

	E (n = 15)	non-E (n = 4)	*P*-value
Age (years)	45 (28–60)	42.5 (25–64)	0.9116
hemophilia (yes/no)	12/3	3/1	1
AST (IU/L)	47.0 (28–111)	91.0 (44–190)	0.2198
ALT (IU/L)	63.0 (21–238)	69.5 (43–175)	0.4999
Platelet count ($\times 10^4/\mu l$)	16.6 (5.6–25.2)	11.8(10.7–32.9)	0.9167
HCV RNA (log IU/ml)	5.8 (4.6–6.8)	5.63 (5.36–7.0)	0.2883
HIV co-infection: yes/no	3/12	1/3	1
IL28B(rs8099917): TT/TG	13/2	3/1	0.5300
core 16: I / non-I	11/4	3/1	0.7278

E, Glutamic acid; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HCV, hepatitis C virus; HIV, human immunodeficiency virus; IL28B, interleukin 28 B; I, Isoleucine.
Data are expressed as median (range).

virological response rate among patients with HCV genotype 3a infections. Reportedly, the HCV core protein directly or indirectly induces suppressors of cytokine signaling-3 and thereby inhibits the IFN signal transduction that is mediated by Janus family tyrosine kinases/STAT signaling [Bode et al., 2003]. Notably, amino acid substitutions at positions 70 and 91 of the HCV core region are associated with the IFN responsiveness to HCV genotype 1b [Akuta et al., 2005]. Among the genotype 3a HCVs isolated in this study, there were no identified amino acid substitutions at position 70 or 91 of the HCV core region. This finding was similar to previous findings with genotype 1a isolates [Hayashi et al., 2012]. However, amino acid variations at positions 16 and 72 of the HCV core were identified, and the sustained virological response rate was significantly higher among patients with glutamic acid at the HCV core 72; these HCVs were defined as E-type viruses. The amino acid at position 72 of the HCV core region is glutamic acid for genotypes 1a and 1b and it is threonine for genotypes 2a and 2b. GenBank was used to further explore sequences of the HCV core region, and it was confirmed that position 72 was well conserved among genotype 1a, 1b, 2a, and 2b isolates. Therefore, it was proper that the mutation at position 72 of the HCV core region was specific to HCV genotype 3a. Notably, HCV genotype 1 infections respond poorly to IFN therapy; in contrast, genotype 2 infections respond well to IFN therapy. However, the amino acid at position 72 of the HCV core region is glutamic acid for most genotype 1 isolates and threonine for most genotype 2 isolates. There was a discrepancy between IFN responsiveness and HCV genotypes with amino acid substitutions at the core 72. The factor associated with amino acid variation at position 72 of the HCV core region was not found (Table III). The mechanisms mediating the effects of variation at position 72 of the core region were not identified, and further studies are needed to clarify this issue. HCV genotype 3a is related to liver steatosis [Castera et al., 2005; Jackel-Cram et al., 2010]. Liver steatosis has some effects on IFN response rate among genotype 3a [Shah et al., 2011; Restivo et al., 2012]. Reportedly, amino acid variation in the HCV core region among genotype 1b isolates are related to liver steatosis and hepatic oxidative stress [Tachi et al., 2010]. The histological change affected by amino acid variation in the HCV core region is very interesting phenomenon. However most patients participating in this study are hemophiliacs who have a coagulation disorder; therefore, it was difficult to perform liver biopsy with these individuals. The lack of liver biopsies was another limitation of this study.

The high sustained virological response rates were achieved among patients with HCV genotype 1 infections who were treated with either of two combinations of direct-acting antivirals (DAAs) [Chayama et al., 2012; Afdhal et al., 2014].

The standard of care for genotype 1 infections may change from IFN-based therapy to IFN-free therapy in near future, but development of IFN-free therapies for genotype 3a infections has been delayed because HCV genotype 3a is evidently resistant to DAAs [Gallo et al., 2010; Alves et al., 2013; Palanisamy et al., 2013; Li et al., 2014]. Although IFN-free treatments for genotype 3a infections may become available within a few years [Zeuzem et al., 2014], determination of the amino acids at position 72 of the HCV core region may be useful for developing the optimal treatment strategy for patients with genotype 3a infections.

In conclusion, the amino acids at position 72 of the HCV core region may be a predictive factor of response to IFN therapy among patients with HCV genotype 3a infections.

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