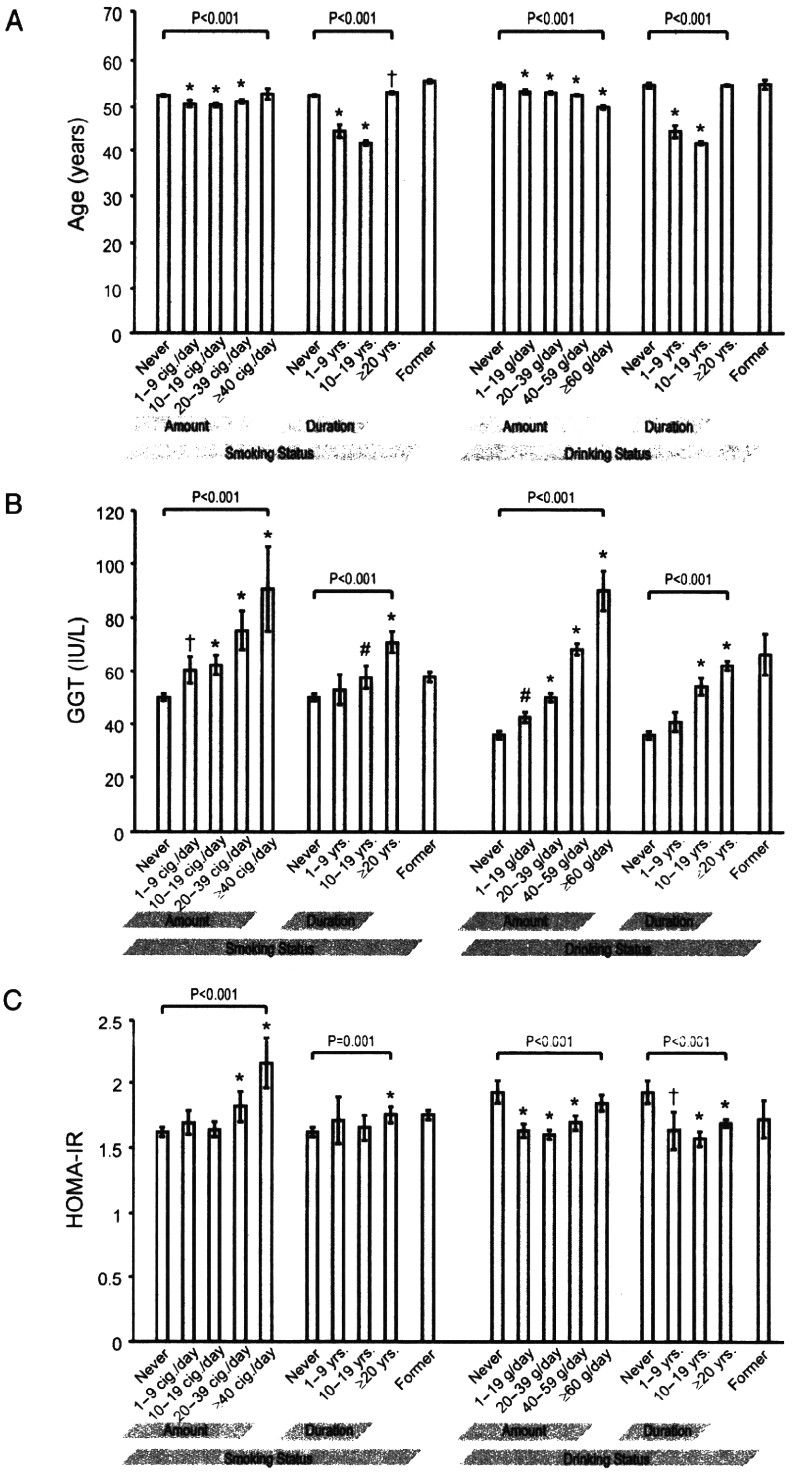

Fig. 1. Age, GGT, and HOMA-IR according to smoking and drinking status.

Bar graphs indicate the mean and 95% CI of age (A), GGT (B), and HOMA-IR. *P* values are for ANOVA trend tests. #, †, and * indicate $p<0.05$, $p<0.01$, and $p<0.001$, respectively, versus never smokers or never drinkers by Dunnett's post-hoc analysis.

Table 2. GGT and HOMA-IR according to smoking and drinking status

Smoking category (cig./day)	Drinking category																									
	#Overall				0 g/day (never drinker)				1-19 g/day				20-39 g/day				40-59 g/day				≥60g/day				†p value
	N	GGT			N	GGT			N	GGT			N	GGT			N	GGT			N	GGT			
		Mean	95%CI			Mean	95%CI			Mean	95%CI			Mean	95%CI			Mean	95%CI			Mean	95%CI		
§Overall	10,482	58.1	56.4	59.8	1,207	35.5	33.9	37.2	1,872	41.7	39.8	43.6	3,062	50.1	48.0	52.1	2,957	68.7	65.9	71.6	1,384	95.2	85.4	104.9	<0.001
										*p= 0.156				*p< 0.001				*p< 0.001				*p< 0.001			
0 (never smoker)	5,535	49.5	48.2	50.9	791	35.6	33.4	37.8	1,222	43.2	40.6	45.8	1,812	47.2	45.3	49.1	1,258	60.7	57.3	64.2	452	69.3	62.0	76.6	<0.001
										*p= 0.004				*p< 0.001				*p< 0.001				*p< 0.001			
1-9	771	59.8	54.7	64.9	48	44.0	32.4	55.6	147	39.0	33.8	44.1	207	51.1	45.4	56.8	253	69.5	57.8	81.2	116	87.1	70.3	103.9	<0.001
										*p= 0.950				*p= 0.845				*p= 0.055				*p= 0.001			
10-19	2,201	61.9	58.3	65.5	196	32.9	30.0	35.7	322	39.0	35.3	42.7	616	55.4	47.7	63.2	758	72.9	66.3	79.6	309	89.9	79.9	100.0	<0.001
										*p= 0.764				*p= 0.003				*p< 0.001				*p< 0.001			
20-39	1,748	75.6	68.1	83.0	151	35.4	32.1	38.7	158	37.0	33.1	40.9	399	55.1	50.3	59.8	623	77.0	71.4	82.6	417	122.1	93.0	151.3	<0.001
										*p= 1.000				*p= 0.413				*p= 0.010				*p< 0.001			
≥40	227	91.1	75.3	107.0	21	39.0	27.0	51.0	23	47.4	36.5	58.4	28	40.9	32.1	49.8	65	93.4	62.1	124.7	90	128.5	97.3	159.6	<0.001
										*p= 0.996				*p= 1.000				*p= 0.171				*p= 0.006			

Smoking category (cig./day)	Drinking category																									
	#Overall				0 g/day (never drinker)				1-19 g/day				20-39 g/day				40-59 g/day				≥60g/day				†p value
	N	HOMA-IR			N	HOMA-IR			N	HOMA-IR			N	HOMA-IR			N	HOMA-IR			N	HOMA-IR			
		Mean	95%CI			Mean	95%CI			Mean	95%CI			Mean	95%CI			Mean	95%CI			Mean	95%CI		
§Overall	10,482	1.67	1.64	1.70	1,207	1.89	1.79	1.99	1,872	1.57	1.51	1.63	3,062	1.5	1.51	1.59	2,957	1.7	1.62	1.77	1,384	1.8	1.78	1.92	<0.001
										*p< 0.001				*p< 0.001				*p= 0.001				*p= 0.878			
0 (never smoker)	5,535	1.62	1.58	1.65	791	1.83	1.70	1.96	1,222	1.58	1.50	1.66	1,812	1.54	1.49	1.59	1,258	1.57	1.51	1.64	452	1.80	1.67	1.94	<0.001
										*p< 0.001				*p< 0.001				*p< 0.001				*p= 0.995			
1-9	771	1.67	1.58	1.77	48	2.20	1.52	2.89	147	1.50	1.31	1.69	207	1.54	1.41	1.67	253	1.68	1.53	1.84	116	1.90	1.64	2.15	0.002
										*p= 0.003				*p= 0.004				*p= 0.026				*p= 0.338			
10-19	2,201	1.64	1.58	1.69	196	1.96	1.77	2.16	322	1.52	1.38	1.66	616	1.43	1.34	1.51	758	1.70	1.60	1.80	309	1.80	1.65	1.96	<0.001
										*p< 0.001				*p< 0.001				*p= 0.030				*p= 0.385			
20-39	1,748	1.82	1.70	1.94	151	2.03	1.74	2.32	158	1.49	1.31	1.66	399	1.74	1.61	1.87	623	1.92	1.62	2.22	417	1.80	1.68	1.92	0.274
≥40	227	2.17	1.97	2.37	21	1.91	1.29	2.52	23	2.71	1.98	3.45	28	1.96	1.46	2.46	65	1.84	1.52	2.16	90	2.39	2.02	2.76	0.068

†*p* values were for ANOVA trend tests. **p* values are versus never drinkers by Dunnett's post-hoc analysis. #Overall indicates all never or current drinkers, and §Overall indicates all never or current smokers. GGT, gamma-glutamyl transpeptidase; HOMA-IR, homeostasis model assessment for insulin resistance.

Table 3. Linear regression analysis using GGT and HOMA-IR as dependent variable

	β	95%CI		Standardized β	p value
Dependent variable: GGT					
Age	−0.57	−1.82	0.68	−0.01	0.372
BMI	2.25	1.79	2.71	0.08	<0.001
Smoking	3.18	2.17	4.19	0.05	<0.001
Alcohol consumption	12.34	11.19	13.49	0.17	<0.001
Dependent variable: HOMA-IR					
Age	0.04	0.01	0.06	0.02	0.001
BMI	0.23	0.22	0.24	0.43	<0.001
Smoking	0.04	0.03	0.06	0.04	<0.001
Alcohol consumption	−0.08	−0.10	−0.06	−0.06	<0.001

For the calculation of β values, age was subdivided into 10-year increments. Alcohol consumption (g/day) corresponding to 0 (never drinker), 1-19, 20-39, 40-59, and ≥60 was coded as 0, 1, 2, 3, and 4, respectively, and smoking (cigarettes/day) corresponding to 1-9, 10-19, 20-39, and ≥40 was coded as 0, 1, 2, 3, and 4, respectively. GGT, gamma-glutamyl transpeptidase; HOMA-IR, homeostasis model assessment for insulin resistance.

tion as independent variables was performed in 10482 individuals (**Table 3**). In this model, alcohol consumption (g/day) corresponding to 0 (never drinker); 1-19, 20-39, 40-59, and 60 or more was coded as 0, 1, 2, 3, and 4, respectively, and smoking (cigarettes/day) corresponding to 1-9, 10-19, 20-39, and 40 or more were defined as 0, 1, 2, 3, and 4, respectively. Alcohol consumption was associated positively with GGT, but negatively with HOMA-IR. On the other hand, smoking was found to be associated positively with both GGT and HOMA-IR. When an interaction term between alcohol consumption and smoking was used as additional independent variable, the interaction term was found to be significantly associated with GGT ($p<0.001$), and showed a borderline significant association with HOMA-IR ($p=0.059$). The variance inflation factor (VIF) scores of all independent variables tested were less than 10 (data not shown).

Association between GGT and HOMA-IR According to Alcohol Consumption

Next, we investigated whether the mode of association between GGT and HOMA-IR differs according to the amount of alcohol consumption. For this purpose, multiple regression analysis was performed in which age, BMI, and GGT were used as independent variables and HOMA-IR was used as a dependent variable after subdividing individuals according to alcohol consumption (**Table 4**). GGT was found be a positive predictive value for HOMA-IR in 19 out of the 25 drinking × smoking categories. In some combinations of drinking and smoking, such as drinking 0 g/day and smoking 1-9 cig./day, GGT was not a statistically significant predictor of HOMA-IR. This may be in part because the number of subjects with specific drinking and smoking conditions was relatively small.

Discussion

In the current study, by analyzing the data of men who underwent general health screening, except former smokers and/or former drinkers, we observed several points: (1) Alcohol consumption showed a graded association with GGT; (2) In individuals who drank 40 g or more per day, smoking 20 cigarettes or more per day further increased GGT levels (**Table 2**); (3) alcohol consumption showed a U-shaped association with HOMA-IR, when the daily number of cigarettes smoked was less than 20 per day; (4) Individuals who smoked 20-39 and ≥40 cigarette per day had higher HOMA-IR than never smokers (**Table 2**); (5) GGT was found be a positive predictive value of HOMA-IR in 19 out of the 25 drinking × smoking categories, and GGT was not a significant negative predictor of HOMA-IR regardless of the drinking or smoking status. These data collectively indicate that, although current drinking may increase GGT and reduce insulin resistance, GGT can be utilized as a marker of insulin resistance regardless of the drinking status.

Many studies have shown that serum GGT is a biomarker of increased alcohol consumption[1-4, 22]; however, GGT is known to be affected by other con-

Table 4. Linear regression analysis using HOMA-IR as dependent variable

		β	95%CI		Standardized β	p value
Current smoking - 0 cig./day (never smoker)						
0 g/day (never drinker)	BMI	0.20	0.16	0.24	0.33	<0.001
	GGT	0.07	0.03	0.11	0.12	0.001
1–19 g/day	BMI	0.22	0.19	0.24	0.42	<0.001
	GGT	0.04	0.02	0.05	0.12	<0.001
20–39 g/day	BMI	0.21	0.19	0.22	0.49	<0.001
	GGT	0.03	0.02	0.05	0.13	<0.001
40–59 g/day	BMI	0.18	0.16	0.20	0.47	<0.001
	GGT	0.04	0.03	0.04	0.20	<0.001
≥60 g/day	BMI	0.26	0.23	0.30	0.57	<0.001
	GGT	0.02	0.01	0.04	0.13	0.001
Current smoking - 1–9 cig./day						
0 g/day (never drinker)	BMI	0.44	0.21	0.67	0.49	<0.001
	GGT	0.13	−0.02	0.28	0.22	0.084
1–19 g/day	BMI	0.24	0.17	0.32	0.47	<0.001
	GGT	0.07	0.01	0.12	0.18	0.016
20–39 g/day	BMI	0.18	0.13	0.23	0.42	<0.001
	GGT	0.01	−0.02	0.04	0.05	0.396
40–59 g/day	BMI	0.23	0.18	0.27	0.50	<0.001
	GGT	0.01	0.00	0.03	0.11	0.049
≥60 g/day	BMI	0.27	0.20	0.34	0.57	<0.001
	GGT	0.04	0.02	0.06	0.26	<0.001
Current smoking - 10–19 cig./day						
0 g/day (never drinker)	BMI	0.16	0.10	0.22	0.34	<0.001
	GGT	0.27	0.19	0.36	0.40	<0.001
1–19 g/day	BMI	0.22	0.18	0.26	0.49	<0.001
	GGT	0.08	0.05	0.12	0.22	<0.001
20–39 g/day	BMI	0.18	0.15	0.21	0.44	<0.001
	GGT	0.00	−0.01	0.01	0.02	0.583
40–59 g/day	BMI	0.25	0.22	0.27	0.57	<0.001
	GGT	0.02	0.01	0.03	0.16	<0.001
≥60 g/day	BMI	0.22	0.18	0.26	0.54	<0.001
	GGT	0.03	0.01	0.04	0.17	<0.001
Current smoking - 20–39 cig./day						
0 g/day (never drinker)	BMI	0.27	0.18	0.37	0.42	<0.001
	GGT	0.17	0.04	0.30	0.20	0.010
1–19 g/day	BMI	0.12	0.06	0.18	0.30	<0.001
	GGT	0.07	0.00	0.14	0.16	0.036
20–39 g/day	BMI	0.22	0.18	0.26	0.47	<0.001
	GGT	0.04	0.02	0.07	0.16	<0.001
40–59 g/day	BMI	0.28	0.18	0.37	0.22	<0.001
	GGT	0.02	−0.02	0.06	0.03	0.395
≥60 g/day	BMI	0.22	0.19	0.26	0.57	<0.001
	GGT	0.01	0.00	0.01	0.15	<0.001
Current smoking - ≥40 cig./day						
0 g/day (never drinker)	BMI	−0.07	−0.29	0.16	−0.12	0.551
	GGT	0.41	0.18	0.64	0.80	0.002
1–19 g/day	BMI	−0.07	−0.29	0.16	−0.12	0.551
	GGT	0.41	0.18	0.64	0.80	0.002
20–39 g/day	BMI	0.15	0.02	0.28	0.42	0.028
	GGT	0.10	−0.15	0.34	0.17	0.425
40–59 g/day	BMI	0.18	0.06	0.30	0.37	0.003
	GGT	0.01	−0.02	0.03	0.07	0.559
≥60 g/day	BMI	0.28	0.18	0.38	0.47	<0.001
	GGT	0.05	0.03	0.07	0.42	<0.001

Standardized β values are estimates resulting from analysis performed on into standardized variables. For the calculation of β values, BMI was subdivided into 1 kg/m^2 increments, and GGT into 10 IU/L increments. Age, BMI, and GGT were used as independent variables. BMI, body mass index; GGT, gamma-glutamyl transpeptidase.

ditions, such as smoking, obesity, and hepatic steatosis[23, 24]. Evidence is accumulating that higher serum GGT levels may be associated with an increased incidence of cardiovascular events[5], metabolic syndrome and diabetes[8, 25, 26]; therefore, more attention has been paid recently to this liver enzyme. It is possible that the association between GGT and various disorders observed in previous studies may be mediated, in part, by enhanced insulin resistance in subjects with increased GGT levels.

Although mild to moderate alcohol consumption may increase GGT, it may improve insulin sensitivity[18, 27], leading to a reduction in the prevalence of metabolic syndrome[17]. This finding is in contrast to the observation that cigarette smoking will not improve insulin resistance, even in light smokers[14]. As alcohol consumption has opposite effects on GGT and insulin resistance, the mode of association between GGT and HOMA-IR might differ according to the drinking status; however, only a few studies have analyzed the relationship between GGT and insulin resistance in various drinking conditions. Yokoyama and colleagues reported that GGT is associated with increased insulin resistance in non-drinkers[28] and light drinkers, but not in heavy drinkers[29], a finding that supports the notion that the mode of association between GGT and HOMA-IR differs according to the drinking status. Yamada *et al.* have reported that HOMA-IR rose with increasing serum GGT in both alcohol consumers and non-consumers, and HOMA-IR values corresponding to all serum GGT levels were lower in alcohol consumers than in non-consumers[30]. A recent study indicated that cigarette smoking may also affect both GGT and insulin resistance independent of the drinking status, and cigarette smoking and alcohol intake may have a synergistic impact on GGT[13]. Smoking status should also be considered when assessing the impact of alcohol intake on the association between GGT and insulin resistance; however, to our knowledge, no previous studies have investigated the relationship between GGT and insulin resistance after stratifying both the drinking status and smoking status, as in the current study.

We found that in 19 of the 25 subgroups divided according to smoking and drinking status, GGT was found to be a positive predictive value of HOMA-IR, which indicates that increased GGT is associated with enhanced insulin resistance regardless of the smoking and drinking status. From this type of cross-sectional study, we cannot conclude whether there is any causal or resultant relationship between GGT and HOMA-IR. A recent study showed that GGT may play a causal role in promoting insulin resistance, presumably by enhancing oxidative stress[31, 32] and hepatic steatosis[33]. Whether a change in HOMA-IR would result in a predicted change in GGT should be investigated in future longitudinal studies.

Our study has some limitations. First, we did not take into account coffee intake, which might affect GGT level[2]. Second, as the prevalence of smokers was low, we did not analyze the data of female subjects. Third, the number of daily cigarettes and alcohol consumption solely reflected the amount that was being consumed at one time, and disregarded the frequency of smoking or drinking consumption. Therefore, this estimation of smoking and drinking quantity was not equal to the mean daily number of cigarettes smoked and the amount of alcohol consumption, except in every-day smokers and drinkers, respectively. We performed such an analysis because the frequency of smoking (or drinking) was reported as a category, two or three times per week, for example; therefore, it was technically difficult to estimate the mean daily number of cigarettes smoked or the alcohol consumption. In the future, however, the frequency of drinking and smoking should also be considered in such an analysis. Fourth, we did not exclude individuals who were taking antihypertensive and/or antidiabetic drugs, which may have affected serum GGT and HOMA-IR values.

In summary, alcohol consumption showed a graded positive association with GGT and a U-shaped negative association with HOMA-IR. Cigarette smoking may further increase GGT levels in individuals who are current drinkers and drink 20 g or more per day. In 19 of the 25 drinking × smoking categories, GGT was found be a positive predictive value of HOMA-IR, and GGT was not a significant negative predictor of HOMA-IR, regardless of the drinking or smoking status. These data indicate a positive association between GGT and insulin resistance also in current drinkers.

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Identification of hepatitis C virus genotype 2a replicon variants with reduced susceptibility to ribavirin

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Drug resistance

ABSTRACT

Ribavirin (RBV), a nucleoside analogue, is used in the treatment of hepatitis C virus (HCV) infection in combination with interferons. However, potential mechanisms of RBV resistance during HCV replication remain poorly understood. Serial passage of cells harboring HCV genotype 2a replicon in the presence of RBV resulted in the reduced susceptibility of the replicon to RBV. Transfection of fresh cells with RNA from RBV-resistant replicon cells demonstrated that the RBV resistance observed is largely replicon-derived. Four major amino acid substitutions: T1134S in NS3, P1969S in NS4B, V2405A in NS5A, and Y2471H in NS5B region, were identified. Site-directed mutagenesis of these mutations into the replicon indicated that Y2471H plays a role in the reduced susceptibility to RBV and leads to decrease in replication fitness. The results, in addition to analysis of sequence database, suggest that HCV variants with reduced susceptibility to RBV identified are preferential to genotype 2a.

1. Introduction

Hepatitis C virus (HCV) is a leading cause of chronic liver diseases, such as chronic hepatitis, cirrhosis and hepatocellular carcinoma, affecting approximately 170 million people worldwide (WHO, 2000). HCV belongs to the genus Hepacivirus of the family Flaviviridae, and its genome is a single-stranded, positive-sense RNA of 9.6 kb. HCV displays marked genetic heterogeneity and is currently classified into 6 major genotypes and more than 50 subtypes. HCV genotypes have regional distribution and, of those, genotypes 1 and 2 are detected worldwide (Simmonds et al., 2000). Current standard therapy for chronic hepatitis C consists of the combination of pegylated interferon alpha (IFN-α) in combination with ribavirin (RBV). However, approximately 50% of treated patients infected with genotype 1 do not respond or show only a partial or transient response and treatment is limited by the adverse effects of both agents (Manns et al., 2001; Fried et al., 2002).

HCV replication is associated with a high rate of mutation that gives rise to a mixed and changing population of mutants, known as quasispecies (Martell et al., 1992; Domingo, 1996). The characteristic of HCV may have important implications concerning viral persistence, pathogenicity and resistance to antiviral agents (Domingo, 1996; Forns et al., 1999; Farci and Purcell, 2000). Most previous studies on the possible relationship between HCV quasispecies and response to chemotherapy have been carried out in HCV genotype 1 patients. In addition, several studies have successfully demonstrated that the HCV subgenomic replicon is derived from genotype 1, which typically contains HCV nonstructural genes placed downstream of the neomycin phosphotransferase gene, in selecting variants resistant to antiviral inhibitors. Two studies have demonstrated the identification of HCV genotype 1 mutants responsible for decreased sensitivity to RBV (Young et al., 2003; Pfeiffer and Kirkegaard, 2005). However, little is known about the generation of genotype 2 isolates resistant to antivirals including RBV, or the molecular mechanisms that confer resistance.

In this study, we report the generation and characterization of HCV genotype 2a replicon variants with reduced susceptibility to RBV. The impacts of major amino acid substitutions observed on RBV susceptibility and viral replication capacity were also examined.

2. Materials and methods

2.1. Compounds

RBV and IFN-α were purchased from MP Biomedicals (Eschwege, Germany) and Dainippon Sumitomo Pharma (Osaka, Japan), respectively.

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Table 1
Primers used for PCR and nucleotide sequencing.

Region	Primer name	Nucleotide sequence	Position[a]	Polarity
NS3–4A–4B region	PCR primers			
	JF1S	GAAAAACACGATGATACCATG	1756–1776	Sense
	JF1AS	AACCCAGTCCCACACGTC	4650–4633	Antisense
	Sequencing primers			
	JF5S	CACTTTCAGTGACAACAGCA	2322–2341	Sense
	JF6S	CGCCACCGACGCCCTCATGA	3003–3022	Sense
	JF4AS	CTGGTCGACAACGGACTGGT	4109–4090	Antisense
NS5A–NS5B region	PCR primers			
	JF2S	TGCTCCGGATCCTGGCTC	4612–4629	Sense
	JF2AS	TACCTAGTGTGTGCCGCTCTA	7786–7806	Antisense
	Sequencing primers			
	JF3S	TGAGGTCCATGCTAACAGA	5209–5228	Sense
	JF4S	TCGAGGGGGAGCCTGGAGAT	5870–5889	Sense
	JF3AS	GAGTGTCTAACTGTTTCCCAG	7220–7200	Antisense

[a] Reference strain: Gene Bank accession no. AB114136.

2.2. Cell culture

The human hepatoma cell line Huh-7 was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with MEM non-essential amino acids (Invitrogen) 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS) at 37 °C in a 5% CO_2 incubator. HCV replicon cells JFH-1/4-1 (Miyamoto et al., 2006), which are Huh-7-derived cells carrying a subgenomic replicon of JFH-1 (Kato et al., 2003) were maintained in the Huh-7 medium as above, supplemented with 1 mg/ml G418 (Nacalai Tesque, Kyoto, Japan).

2.3. Quantification of HCV RNA

Total RNA was isolated from harvested cells using Trizol (Invitrogen). Copy numbers of the viral RNA were determined by real-time RT-PCR involving single-tube reactions and performed using TaqMan EZ RT-PCR Core Reagents (PE Applied Biosystems, Foster City, CA, USA), as described previously (Aizaki et al., 2003; Takeuchi et al., 1999).

2.4. Cell viability assay

Cells were seeded at density of 5×10^4 cells/well in 24-well plates and RBV at various concentrations was added on the next day. Cultures were further incubated for 3 days at 37 °C under a humidified 5% CO_2 atmosphere. Cytotoxicity assay was performed by Cell Titer-GLO™ Luminescent Cell Viability Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Luciferase activities were quantified with LUMAT LB 9501 (Berthold Technologies, Bad Wilbad, Germany).

2.5. Isolation and nucleotide sequencing of HCV nonstructural regions from replicon-containing cells

Total cellular RNA was isolated from replicon cells with or without RBV treatment as described above. cDNA synthesis was carried out by using Super ScriptTM III First-Strand Synthesis System for RT-PCR (Invitrogen) with primer JF1AS for NS34AB region and JF2AS for NS5AB region. Two cDNA fragments, corresponding to NS3–NS4B and NS5A–NS5B regions, were amplified by PCR using Takara EX Taq DNA polymerase (Takara BIO, Kyoto, Japan) and specific primers (Table 1; Date et al., 2004). PCR products were subcloned into pGEM-T vector (Promega) and inserts were sequenced using QIA prep^R Spin Mini Prep kit (QIAGEN, Tokyo, Japan). Nucleotide sequences were analyzed with the 3100 Avant Genetic Analyzer (PE Applied Biosystems).

2.6. Plasmid constructions

pSGR-JFH1/luc, a subgenomic replicon construct with luciferase reporter derived from HCV genotype 2a JFH-1 isolate was reported previously (Miyamoto et al., 2006). Mutant replicons carrying T1134S, P1969S, V2405A, and Y2471H were created by PCR-based site-directed mutagenesis and cDNA fragments containing the above mutations were inserted into the corresponding sites of pSGR-JFH/luc. All plasmids were confirmed by sequencing the entire PCR-generated inserts. Each mutant is referred to by the original amino acid (one letter code) followed by the residue positions within the complete open reading frame of full-length JFH-1 and the substituted amino acid (one letter code).

2.7. RNA synthesis and transient replication assay

The transient replication assay method was described previously (Kato et al., 2005). Briefly, purified plasmids of pSGR-JFH1/Luc, -JFH1/Luc-T1134S, -JFH/Luc-P1969S, -JFH/Luc-V2405A and -JFH/Luc-Y2471H were linearized with XbaI and were treated with proteinase K and SDS, followed by phenol–chloroform extraction. RNA was synthesized with Ampliscribe™ T7 Transcription Kits (Epicentre BIO Technologies, Madison, WI, USA). Each transcribed RNA (5 μg) was electroporated into 2.5×10^6 of Huh7 cells pulsed at 290 mV, 975uFD with Gene pulser II apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Transfected cells were resuspended in growth medium without selection antibiotics and were plated in 24-well plates at 6×10^4 cells per well. Cells were harvested at different time points post-transfection and were lysed in Passive Lysis Buffer (Promega). Luciferase activity in cells was determined using the Luciferase Assay System (Promega).

3. Results

3.1. Selection of replicon variants derived from genotype 2a with reduced susceptibility to RBV

It has been reported that RBV inhibits HCV RNA replication in Huh-7 cells bearing the viral subgenomic replicon RNAs with the EC_{50} (50% effective concentration) values of 15–225 μM (Zhou et al., 2003; Tanaka et al., 2004; Kato et al., 2005; aus dem Siepen et al., 2007). To select for RBV-associated replicon variants, cells bearing a genotype 2a HCV replicon were serially passed in the presence of 200 μM RBV as well as 1 mg/ml G418. After 20-week treatment, variant cells were then tested for RBV resistance. HCV RNA levels were determined after a 72-h incubation with various concentrations of RBV in the absence of G418, and about 5-fold-reduced susceptibility to RBV was observed in the variant replicon

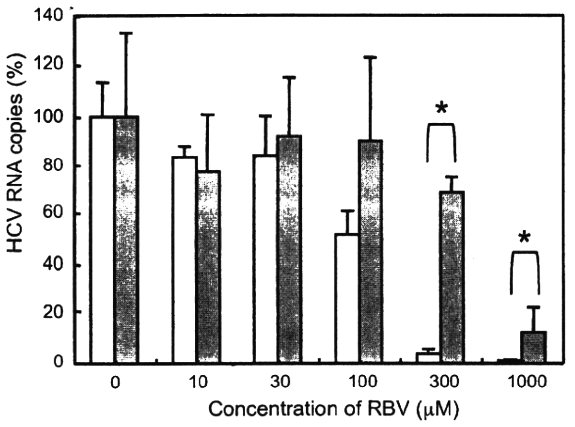

Fig. 1. Inhibitory effect of RBV on HCV RNA levels in genotype 2a replicon cells after long-term treatments with RBV. The replicon cells were serially passaged in 0 or 200 μM RBV for 20 weeks. The cells were then split and incubated with fresh RBV at various concentrations in the absence of G418 for 3 days, followed by the determination of HCV RNA. Clear bars, passage in the absence of RBV; gray bars, passage in the presence of RBV. HCV RNA copies per microgram of total RNA were normalized as percentages of those of untreated (RBV 0 μM). Each data point is presented as the mean of three independent determinations with standard deviation. $^*p < 0.05$.

cells; the EC_{50} values for the variant and wild-type replicon cells were 470 and 102 μM, respectively (Fig. 1). Comparable cytotoxic effects of RBV were observed against wild-type and variant replicon cells, with the CC_{50} (50% cytotoxicity concentration) values of 151 and 156 μM, respectively (data not shown).

3.2. Mapping RBV resistance to cell line or replicon RNA

To test whether reduced susceptibility to RBV in the variant cells observed as above was due to the appearance of mutations within the viral RNA or was cell-derived, total RNAs from the variant and wild-type replicon cells were extracted and used for retransfection of naïve Huh7 cells. Retransfected cells resistant to G418 were established after 4 weeks of cultures in the presence of 1 mg/ml G418 and were assessed for HCV RNA replication sensitivity to RBV (Fig. 2A). HCV RNA levels in the cells obtained from the wild-type replicon were inhibited by 56, 89 and 97% with 100, 300 and 1000 μM RBV, respectively. By contrast, the culture retransfected with RNA derived from the variant replicon cells exhibited inhibition levels of 13, 29 and 89% with the corresponding concentrations of RBV. EC_{50} values were calculated to be 93 and 449 μM, respectively. We confirmed the presence of replicon mutations, as described below, in the cells retransfected with RNA derived from the variant replicon cells.

In order to explore the possibility for cell-derived resistance, both wild-type and variant replicon cells were cured of viral RNAs by IFN treatment; cells were passaged with media containing 100 IU/mL IFN-α in the absence of G418 for 2 months. To compare RBV sensitivity, cured cells were transiently transfected with the wild-type JFH-1 subgenomic replicon RNA and were treated with various concentrations of RBV for 72 h. Similar anti-HCV effects of RBV were observed in the cured cells derived from wild-type and variant replicons, with the EC_{50} values of 147 and 118 μM, respectively (Fig. 2B). Thus, the results suggest that the RBV resistance observed may arise by mutations in the replicon rather than by changes in the cells.

3.3. HCV mutations in replicon variant with reduced susceptibility to RBV

It has been reported that mutations in RNA virus genomes responsible for RBV resistance are mostly present in the coding region for the viral RNA-dependent RNA polymerase (RdRp). On the other hand, it is known that RBV works as an RNA mutagen to generate rapidly mutating viral RNA and that NS5B RdRp and other nonstructural proteins in HCV are involved in the viral replication complex, playing key roles in genome replication. Therefore, we sequenced the coding regions for NS3 through NS5B proteins of the replicon molecules in order to determine whether mutations associated with RBV resistance were generated. As shown in Table 2, there were numerically more synonymous and non-synonymous mutations in the RBV-resistant variant replicon cells (RBV treatment) when compared with untreated replicative conditions (No-treatment) across most regions examined. Mutation frequencies of NS3, NS4B and NS5A regions of RBV treatment were significantly higher than those of No-treatment. The total number of synonymous mutations in the RBV-resistant variant replicon cells was 3 times higher than that under untreated replicative conditions, and the number of non-synonymous mutations in the RBV-resistant variant replicon cells was 1.5 times higher than that under untreated replicative conditions. The number of both synonymous and non-synonymous mutations (NS3, NS4B, NS5A and NS5B regions) in the RBV-resistant replicon cells was greater than that in the control cells. We also found a large number of transition

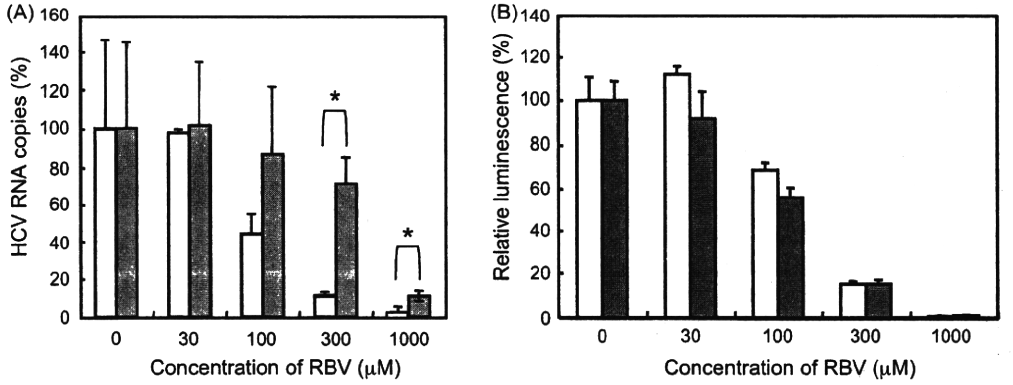

Fig. 2. Testing for replicon-derived resistance (A) or for cell-derived resistance (B). (A) Total RNA from RBV-resistant- or wild-type replicon cells was transfected into naïve Huh7 cells. After selection in 1 mg/ml G418 for 4 weeks, re-established replicon cells, wild-type derived (clear bars) and RBV resistance derived (gray bars), were treated with increasing concentrations of RBV in the absence of G418 for 3 days. HCV RNA copies per microgram total RNA were assessed and the levels from wild-type cells without RBV treatment were set at 100%. Data are indicated as means with standard deviations. $^*p < 0.05$. (B) RBV-resistant- or wild-type replicon cells were cured by passage in IFN-α in the absence of G418. Cured cells were transiently transfected with the replicon RNA derived from pSGR-JFH1/luc. Transient replication assay of transfectants derived from wild-type (clear bars) and RBV resistance (gray bars) was performed after treatment with various concentrations of RBV for 72 h. The values for wild-type-derived cells without RBV treatment were set at 100%. Data are indicated as means with standard deviations.

Table 2
Mutation frequencies in HCV NS regions after 20-weeks culture with or without RBV treatment.

Region	nt length	No-treatment			RBV treatment		
		No. of non-synonymous mutations[a]	No. of synonymous mutations[a]	Mutation frequency (10^{-3})	No. of non-synonymous mutations[a]	No. of synonymous mutations[a]	Mutation frequency (10^{-3})
NS3	1893	1.7 ± 2.1	2.3 ± 1.5	2.1	4.7 ± 2.4	6.5 ± 2.5	5.9[b]
NS4A	165	1.0 ± 1.0	0.3 ± 0.6	8.1	0.3 ± 0.5	0.5 ± 0.9	4.4
NS4B	780	1.3 ± 1.2	0.3 ± 0.6	2.1	2.3 ± 1.5	2.5 ± 1.2	4.7[c]
NS5A	1380	4.0 ± 1.2	2.0 ± 1.2	4.3	5.9 ± 1.2	6.2 ± 2.4	12.2[c]
NS5B	1773	4.5 ± 1.5	2.3 ± 1.5	3.8	4.8 ± 1.8	4.2 ± 1.1	9.0
NS3–NS5B	5991	12.5 ± 2.7	7.3 ± 2.7	-	17.8 ± 4.5	20.1 ± 4.6	-

[a] Values are means ± standard deviations.
[b] $p < 0.05$ relative to No-treatment by the unpaired t-test.
[c] $p < 0.01$ relative to No-treatment by the unpaired t-test.

mutations in RBV-resistant cells, particularly G-to-A and C-to-U transitions, as expected from previous studies. Although mutations were distributed throughout nonstructural regions, four major amino acid substitutions; T1134S in the NS3 region, P1969S in NS4B, V2405A in NS5A, and Y2471H in NS5B, not seen in wild-type cells were observed in most of the subclones among RBV-resistant replicon cells. T1134S, P1969S, V2405A, and Y2471H were present, respectively, in 7 of 11, 6 of 11, 8 of 13, and 7 of 13 PCR subclones sequenced.

3.4. Effects of T1134S, P1969S, V2405A, and Y2471H on RBV susceptibility

To test the possibility that any of the four mutations as identified confer resistance to RBV, we introduced these mutations individually into the JFH-1 subgenomic replicon containing a luciferase reporter gene. Cells transfected with mutant- or wild-type replicon RNA grown in the presence of various concentrations of RBV for 2 or 3 days. As demonstrated in Fig. 3A, the replication levels of all four mutant replicons (SGR-JFH1/Luc-T1134S, -P1969S, -V2405A, and -Y2471H) in the presence of 125 or 500 μM RBV were higher than those of the wild-type replicon. In particular, the Y2471H mutant significantly reduced susceptibility to RBV; replication levels of SGR-JFH1/Luc-Y2471H were 3–5-fold higher when compared to those of wild-type under the present assay conditions.

The relative replication activity of these mutant replicons was further determined in 3-day replication assay without drug treatment (Fig. 3B). All mutant replicons exhibited reduced efficiency relative to the wild-type replicon. Levels of the Y2471H-mutated replicon were approximately 30% of those of the wild-type, thus suggesting that replicon mutants with reduced sensitivity to RBV are associated with decreased replication fitness.

4. Discussion

It is generally accepted that, during chemotherapy against viral infection, high rates of viral replication and high frequencies of mutation lead to generation of drug-resistant mutants. Although several potential mechanisms for the inhibition of HCV replication by RBV have been proposed, the molecular mechanisms involved in the generation of RBV-resistant HCV remain poorly understood.

This study found that long-term treatment of HCV JFH-1-derived replicon cells with RBV leads to selection of preferential mutations in NS3 (T1134S), NS4B (P1969S), NS5A (V2405A) and NS5B (Y2471H) genes. Each mutation only required a single nucleotide change, and P1969S, V2405A and Y2471H are transition mutations, which are known to be commonly caused by incorporated RBV. Site-directed mutagenesis of these mutations into the replicon demonstrated that Y2471H plays a role in reduced susceptibility to RBV.

Crystal structure information revealed that HCV RdRp is organized into an arrangement with palm, fingers, and thumb subdomains (Lesburg et al., 1999). Residue 2471 (the 33rd position of NS5B) is present in the N-terminal loop region that bridges the fingers. Although this site is apparently distant from the active site of the polymerase in the palm region, it has been reported

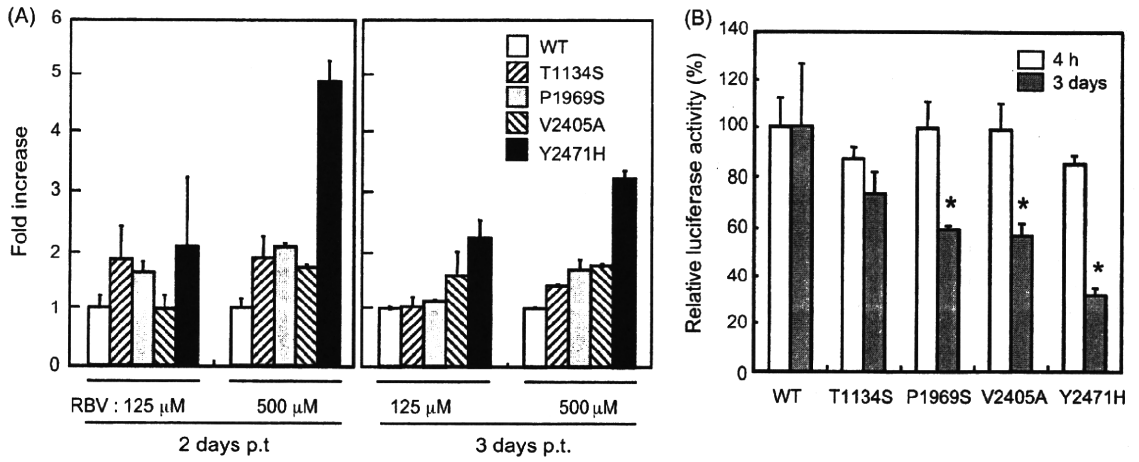

Fig. 3. Impact of major mutations in NS3–NS5B regions on RBV susceptibility (A) and replication capacity (B). Mutated replicons carrying single residue substitutions (T1134S, P1969S, V2405A, and Y2471H) were constructed and used for transient replication assay. Cells were transfected with either wild-type (WT) or with mutant replicon RNA in the absence or presence (125, 500 μM) of RBV. Luciferase activity was assessed at 4 h, 2 days and 3 days post-transfection (p.t.). (A) Luciferase activities of WT were set at 1, and the fold increases in the activities of mutants were plotted. (B) Luciferase activities in the absence of RBV at 4 h and 3 days post-transfection were shown. The activities of mutants were normalized as percentages of the WT activities. Data from triplicate samples were averaged and indicated with standard deviations. *$p < 0.05$ against WT.

that small molecules, such as benzimidazole compounds, are able to specifically bind the fingers-thumb interface and inhibit polymerase activity (Herlihy et al., 2008), thus suggesting that amino acid substitutions in the loop region may affect RNA polymerization. The involvement of tyrosine residue at position 415 of HCV NS5B in RBV resistance has been previously described for patients with genotype 1a infection and for the genotype 1b replicon (Young et al., 2003). Although the mechanism for resistance remains elusive, it has been hypothesized that RBV interacts with RdRp around this residue, which is located in the thumb subdomain, thus affecting RNA polymerization (Young et al., 2003).

Based on analysis of available sequences from Genbank, tyrosine at the 33rd residue of NS5B is conserved in all isolates of genotype 2a, but not in other genotypes. In genotype 1a and 1b isolates, 96% contain histidine and only a small population contains tyrosine or asparagine at the site. All the isolates of genotypes 3, 4, 5 and 6 contain histidine, whereas phenylalanine is conserved for genotype 2b. It should be noted that V2405 and P1969 are also completely conserved for genotype 2a but not for other genotypes. Therefore, it is likely that the identified HCV variants with reduced susceptibility to RBV are genotype-specific. It will be of interest to determine whether HCV genotype 2a is intrinsically more sensitive to RBV when compared with other genotypes.

At present, at least 4 mechanisms of action of RBV are proposed (Lau et al., 2002). They include (1) direct inhibition of the HCV replication machinery, (2) as an RNA mutagen that drives a rapidly mutating RNA virus over the threshold to "error catastrophe", (3) inhibition of the host enzyme inosine monophosphate dehydrogenase (IMPDH), and (4) enhancement of host T-cell-mediated immunity against viral infection. In addition to the direct inhibition, it is also possible that other mechanisms such as error-prone and IMPDH-inhibition are involved in HCV escape from RBV treatment. Further investigation of the interaction of HCV variants with the viral and cellular factors involved in viral resistance may improve understanding of the mechanism(s) of RBV resistance.

In conclusion, RBV encountered resistance from the HCV genotype 2a replicon largely mediated by mutations in the N-terminal region of NS5B. Although whether these mutagenic effects are also demonstrable in IFN-RBV combination therapy will require further studies, the mutations identified in this study represent the first drug-resistant variants belonging to HCV genotype 2a. The drug resistance patterns found in this study may be of benefit in prediction in vivo resistance profiles and the development of next-generation nucleoside analogues as anti-HCV drugs.

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Original Article

Effect of nucleoside analog-interferon sequential therapy on patients with acute exacerbation of chronic hepatitis B

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Aim: Nucleoside analog (NA)-interferon (IFN) sequential therapy may enable the long-term control of chronic hepatitis B (CHB) and the withdrawal of the nucleoside analog. We evaluated the efficacy of NA-IFN sequential therapy for acute exacerbation of CHB.

Methods: A total of 12 patients with acute exacerbation of CHB, nine of whom were positive for hepatitis B e antigen (HBeAg), were enrolled in this study. All the patients were treated with lamivudine 100 mg/day alone for 20 weeks, then with both IFN-α 6 megaunits three times per week and lamivudine for 4 weeks, and lastly, with IFN-α alone for 20 weeks. Patients whose serum alanine aminotransferase (ALT) level was normalized, whose serum hepatitis B virus (HBV) DNA level decreased to less than 5 log copies/mL, and HBeAg level was absent 24 weeks after the end of treatment were defined as having sustained virological response (SVR). The other patients were defined as having no response (NR).

Results: Four out of nine (44.4%) HBeAg-positive and all three HBeAg-negative patients achieved SVR. The levels of serum alanine aminotransferase (ALT), HBV DNA and HBV core-related antigen were similar between SVR and NR patients at baseline. Three of four patients (75.0%) whose serum HBeAg became negative at the end of treatment achieved SVR, while one of five (20.0%) whose serum HBeAg remained positive achieved SVR.

Conclusion: NA-IFN sequential therapy for patients with acute exacerbation of CHB enables the withdrawal of treatment and is particularly effective for patients whose serum HBeAg has become undetectable by the end of the IFN treatment.

Key words: chronic hepatitis B, lamivudine, interferon, sequential therapy.

INTRODUCTION

CHRONIC INFECTION WITH hepatitis B virus (HBV) is a major global health problem, affecting more than 400 million people worldwide.[1] Approximately 15–40% of infected patients develop cirrhosis, liver failure or hepatocellular carcinoma (HCC).[2] An appropriate antiviral treatment to prevent advanced liver disease and reduce the number of HBV-related deaths is thus crucial.[3,4]

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Among the few currently approved agents for the treatment of chronic HBV infection, the most commonly used are interferon (IFN)-α and nucleoside analogs (NA), such as lamivudine, adefovir dipivoxil and entecavir.[5,6]

Interferon-α exerts an antiviral effect by degrading viral mRNA and proteins.[7] Additionally, IFN-α upregulates the immunological response to HBV by enhancing human leukocyte antigen class I expression on hepatocytes.[8] Long-term remission of hepatitis after treatment completion may be expected because the immunological effect of IFN-α continues even after discontinuing the treatment. However, infrequent sustained virological response,[9] several adverse effects[10] and high cost are the problems associated with IFN-α treatment.

Nucleoside analogs cause a rapid and strong antiviral effect.[11–13] Their adverse effects are generally mild.[14]

However, treatment withdrawal is difficult because post-treatment flare with viral proliferation often occurs following the withdrawal.[15] The other problem associated with NA is drug resistance. Prolonged treatment generates a drug-resistant HBV mutant, which often causes breakthrough hepatitis.[16–18] Thus, treatment options that enable long-term remission are necessary.

Nucleotide analog-interferon combination therapy for chronic hepatitis B (CHB) may be more effective than IFN monotherapy because synergistic or additional antiviral effects may be expected. To date, various studies on the combination of IFN and lamivudine have been reported.[19–32]

Sequential therapy switching from lamivudine to IFN may also be effective for the treatment of acute exacerbation of CHB. NA withdrawal is difficult in this situation. IFN treatment overlapping with NA treatment may enable NA withdrawal without generating drug-resistant HBV.[24,25] This treatment is expected to be highly effective because the remission of hepatitis with hepatitis B e (HBe) seroconversion sometimes occurs following the exacerbation of HBV,[33,34] but this needs to be confirmed. We conducted this study to elucidate the efficacy of lamivudine-IFN sequential therapy for those who experienced acute exacerbation of CHB.

METHODS

Patients

A TOTAL OF 12 patients over 20 years old with acute exacerbation of CHB between August 2003 and August 2007, who started a daily dose of 100 mg of lamivudine at St Marianna University School of Medicine, were enrolled in this retrospective cohort study. The diagnosis of acute exacerbation of CHB was made as follows: (i) patients were already diagnosed as having CHB; (ii) patients were positive for hepatitis B surface antigen (HBsAg) and HBV DNA; (iii) other causes of liver damage were excluded; and (iv) serum alanine aminotransferase (ALT) levels in all the patients increased above 300 IU/L within 4 weeks before treatment. As for the serum ALT levels, acute exacerbation of CHB is usually defined as "elevation of ALT over 10 times as upper normal limit".[35] In Japan, 30 IU/L is now regarded as upper normal in many institutions. Therefore, we defined an ALT level above 300 IU/L as exacerbated. The exclusion criteria were as follows: (i) presence of serum antibodies against hepatitis C virus or HIV; (ii) development of liver cirrhosis and/or HCC; (iii) coexistence of other acquired or inherited liver diseases; (iv) coexisting of serious medical or psychiatric illnesses; (v) history of antiviral or immunosuppressive therapy within the preceding 6 months; and (vi) pregnancy.

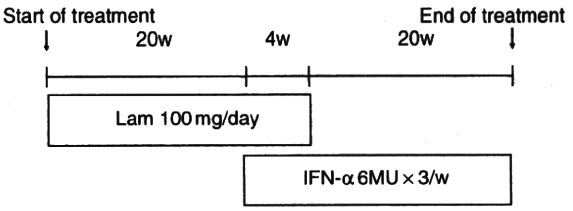

Figure 1 Treatment protocol. All the patients were treated with a daily dose of 100 mg of lamivudine for an initial 20 weeks. Subsequently, 6 megaunits of IFN-α were administrated thrice weekly in combination with lamivudine for 4 weeks, followed by IFN-α monotherapy for an additional 20 weeks. IFN, interferon; Lam, lamivudine; MU, megaunits.

Written informed consent was obtained from all the patients. Our study was approved by the institutional ethics review board of St Marianna University School of Medicine Hospital (no. 1163).

Protocol

All the patients were treated with a daily dose of 100 mg of lamivudine for an initial 20 weeks. Subsequently, 6 megaunits of natural IFN-α (Sumiferon; Dainippon Sumitomo Pharma, Osaka, Japan) were administrated thrice weekly in combination with lamivudine for 4 weeks, followed by IFN-α monotherapy for an additional 20 weeks (Fig. 1). As for the definition of therapeutic effect, antiviral treatment is recommended for the patients whose serum HBV DNA level exceeds 5 log copies/mL because HBV DNA over this level usually accompanies ALT elevation, especially in hepatitis B e-antigen (HBeAg)-positive patients. In addition, one clinical study from Japan adopted this level as a definition of sustained virological response (SVR).[36] Therefore, patients whose serum ALT level was normalized, whose serum HBV DNA level decreased to less than 5 log copies/mL, and whose HBeAg was seroconverted to anti-HBe (in HBeAg-positive cases) 24 weeks after the end of treatment were defined as having SVR. The other patients were defined as having no response (NR).

HBV-related markers

Serum blood samples were frozen at −80°C until use. The HBsAg level was determined using a commercially available chemiluminescence enzyme immunoassay kit (LUMIPULSE II HBsAg; Fujirebio, Tokyo, Japan). The

levels of immunoglobulin (Ig)M anti-hepatitis B core (HBc), HBeAg, and anti-HBe (HBe antibody; HBeAb) were determined using other commercial chemiluminescence immunoassay kits (LUMIPULSE II IgM-HBcAb, LUMIPULSE II HBeAg, LUMIPULSE II HBeAb; Fujirebio). The serum HBV DNA levels were determined using a commercial transcription-mediated amplification kit or commercial polymerase chain reaction kits (DNA probe FR-HBV from Fujirebio; Amplicor HBV monitor or TaqMan PCR from Roche Diagnostics, Tokyo, Japan). The lowest detection limits of those assays were 3.7, 2.6 and 1.8 log copies/mL, respectively. The HBV genotype was determined using a commercial enzyme-linked immunosorbent assay kit (SMITEST HBV genotype detection kit; Genome Science Laboratories, Fukushima, Japan). The serum HBV core-related antigen (HBcrAg) levels were determined using a commercial chemiluminescence enzyme immunoassay (LUMIPULSE HBcrAg; Fujirebio).[37,38] The lowest detection limit of this assay was 3.0 log U/mL. As for the detection of the HBV lamivudine-resistant gene, gene mutation in the YMDD motif was analyzed using a commercial polymerase chain reaction enzyme-linked minisequence assay kit (SMITEST HBV YMDD motif mutation detection kit; Medical & Biological Laboratories, Nagoya, Japan).[39] HBV-related markers (HBeAg, anti-HBe, HBcrAg and HBV DNA) and biochemical tests were examined just before treatment, every 4 weeks during the treatment, and every 4 weeks thereafter for 24 weeks after the end of the treatment. The normal serum ALT level was defined as less than 30 IU/L.

Histological evaluation

Nine of the 12 patients underwent liver biopsies within 1 month before the start of the treatment. Two experienced liver pathologists who had no clinical information except for knowledge of the HBV infection histopathologically evaluated the specimens. The histological appearance of the liver specimens was evaluated using the METAVIR histological score.[40]

Statistical analyses

Quantitative variables were expressed as mean ± standard deviation. The collected data were analyzed using SPSS ver. 15.0J. The distribution of continuous variables was analyzed using Mann–Whitney *U*-test. Differences in categorical data were determined using Fisher's exact test. A two-tailed *P*-value of less than 0.05 was considered to indicate statistical significance.

RESULTS

Baseline characteristics of patients treated with lamivudine and IFN-α

THE BASELINE CHARACTERISTICS of the enrolled patients are shown in Table 1. The mean age of the patients was 32.0 ± 7.8 years, and the patients were 10 men and two women. Of the 12 patients, nine were positive for HBeAg. All the patients were infected with HBV genotype C. The mean platelet count and prothrombin time (activity) were 19.3 ± 4.0 × 10^4/μL and 81.0 ± 13.3%, respectively. The mean serum ALT, total bilirubin and albumin levels were 850.8 ± 664.3 IU/L, 1.2 ± 0.5 mg/dL and 3.8 ± 0.3 g/dL, respectively. The mean levels of serum HBV DNA and HBcrAg were 7.4 ± 1.2 log copies/mL and 6.7 ± 0.4 log U/mL, respectively. Histopathologically, seven patients were diagnosed as having activity stage 2 inflammation and two patients were diagnosed as having activity stage 3 inflammation. As for fibrosis, three patients were diagnosed as having stage 2 fibrosis and six patients were diagnosed as having stage 3 fibrosis.

Response to antiviral treatment

Seven of the 12 (58.3%) patients achieved SVR. The SVR rates among the HBeAg-positive and -negative groups

Table 1 Baseline characteristics of the patients treated with lamivudine-interferon sequential therapy

	All cases
Number of patients	12
Age	32.0 ± 7.8
Sex (male/female)	10/2
Platelet (×10^4/μL)	19.3 ± 4.0
Prothrombin time (%)	81.0 ± 13.3
Albumin (g/dL)	3.8 ± 0.3
Total bilirubin (mg/dL)	1.2 ± 0.5
ALT (IU/L)	850.8 ± 664.3
HBV DNA (log copies/mL)	7.4 ± 1.2
HBcrAg (log U/mL)	6.7 ± 0.4
HBeAg positive/negative	9/3
Activity stage 0/1/2/3 (n = 9)	0/0/7/2
Fibrosis stage 0/1/2/3/4 (n = 9)	0/0/3/6/0

Data are shown as mean ± standard deviation.
Fibrosis and activity stage are evaluated on a scale from 0–4 and 0–3.
ALT, alanine aminotransferase; HBcrAg, hepatitis B virus core-related antigen; HBeAg, hepatitis B virus e antigen; HBV DNA, hepatitis B virus DNA.

Table 2 Comparison of the baseline clinical characteristics between SVR and NR

	SVR	NR	*P*-value
Number of patients	7	5	0.6843
Age	29.7 ± 7.0	35.0 ± 9.2	0.2821
Sex (male/female)	5/2	0/5	0.4697
Platelet ($\times 10^4/\mu L$)	20.1 ± 2.3	18.4 ± 5.7	0.1775
Prothrombin time (%)	75.6 ± 14.1	87.6 ± 9.8	0.1775
Albumin (g/dL)	3.7 ± 0.2	4.0 ± 0.3	0.0519
Total bilirubin (mg/dL)	1.4 ± 0.5	0.9 ± 0.4	0.1190
ALT (IU/L)	931.6 ± 724.9	737.8 ± 570.8	0.8075
HBV DNA (log copies/mL)	7.4 ± 1.4	7.3 ± 1.1	1.0000
HBcrAg (log U/mL)	6.7 ± 0.5	6.8 ± 0.4	0.6905
HBeAg positive/negative	4/3	5/0	0.2045
Activity stage 0/1/2/3 ($n = 9$)	0/0/3/2	0/0/4/0	0.4444
Fibrosis stage 0/1/2/3/4 ($n = 9$)	0/0/2/3/0	0/0/1/3/0	1.0000

Data are shown as mean ± standard deviation.
ALT, alanine aminotransferase; HBcrAg, hepatitis B virus core-related antigen; HBeAg, hepatitis B virus e antigen; HBV DNA, hepatitis B virus DNA; NR, no response; SVR, sustained virological response.

were 44.4% (4/9) and 100% (3/3), respectively. On the other hand, all the NR patients were positive for HBeAg.

Comparison of baseline clinical features between SVR and NR patients

As shown in Table 2, there was no difference in the age, platelet count, prothrombin time, ALT level, albumin level and bilirubin level between SVR and NR patients.

As for the serum HBV DNA and HBcrAg levels, there was little difference between SVR and NR patients (HBV DNA 7.4 ± 1.4 vs 7.3 ± 1.1 log copies/mL; HBcrAg 6.7 ± 0.5 vs 6.8 ± 0.4 log U/mL). HBeAg was detected more often in NR than in SVR patients (100% [5/5] vs 57.1% [4/7]).

Histopathological examination showed that the patients with activity grade 3 are more likely to achieve SVR than those with grade 2 (100% [2/2] in grade 3 and 43.0% [3/7] in grade 2). With regard to the fibrosis stage, no significant difference in SVR was observed between patients with stage 2 and stage 3 (66.7% [2/3] for stage 2 and 50.0% [3/6] for stage 3) (Table 2).

Comparison of SVR rate according to age group

In the Japanese national guidelines, IFN is not recommended as a first line of treatment for patients over 35 years old because it is ineffective. On the basis of the guideline, we evaluated SVR rates according to age group. The SVR rate for all the patients aged under 35 years tended to be higher (66.7% [6/9] of patients achieved SVR) than that for the patients aged 35 years or over (33.3% [1/3]), although the difference was not statistically significant.

Time-dependent change in serum ALT, HBV DNA and HBcrAg levels

The serum ALT levels in all the patients normalized shortly after the treatment and remained within the normal range during the treatment (Fig. 2a). The serum HBV DNA levels decreased below the detection limit shortly after the treatment in most patients. The fluctuation of HBV DNA levels was observed in one SVR and two NR patients (Fig. 2b). As for the serum HBcrAg levels, a continuous decrease during and after the treatment was observed in SVR patients. In contrast, the HBcrAg levels in NR patients did not show a continuous decrease during or after the treatment (Fig. 2c).

HBV-related markers before, during and after treatment

In the HBeAg-positive patients, HBeAg was seroconverted to anti-HBe by the end of the treatment in 75.0% (3/4) of SVR and 40.0% (2/5) of NR patients. One SVR patient achieved HBe seroconversion 24 weeks after treatment. Therefore, HBe seroconversion was observed in all the patients who achieved SVR. On the other hand, one of two NR patients, who once achieved HBe seroconversion during treatment, became HBeAg-positive during IFN-α monotherapy and the other patient became HBeAg-positive after the end of the treatment.

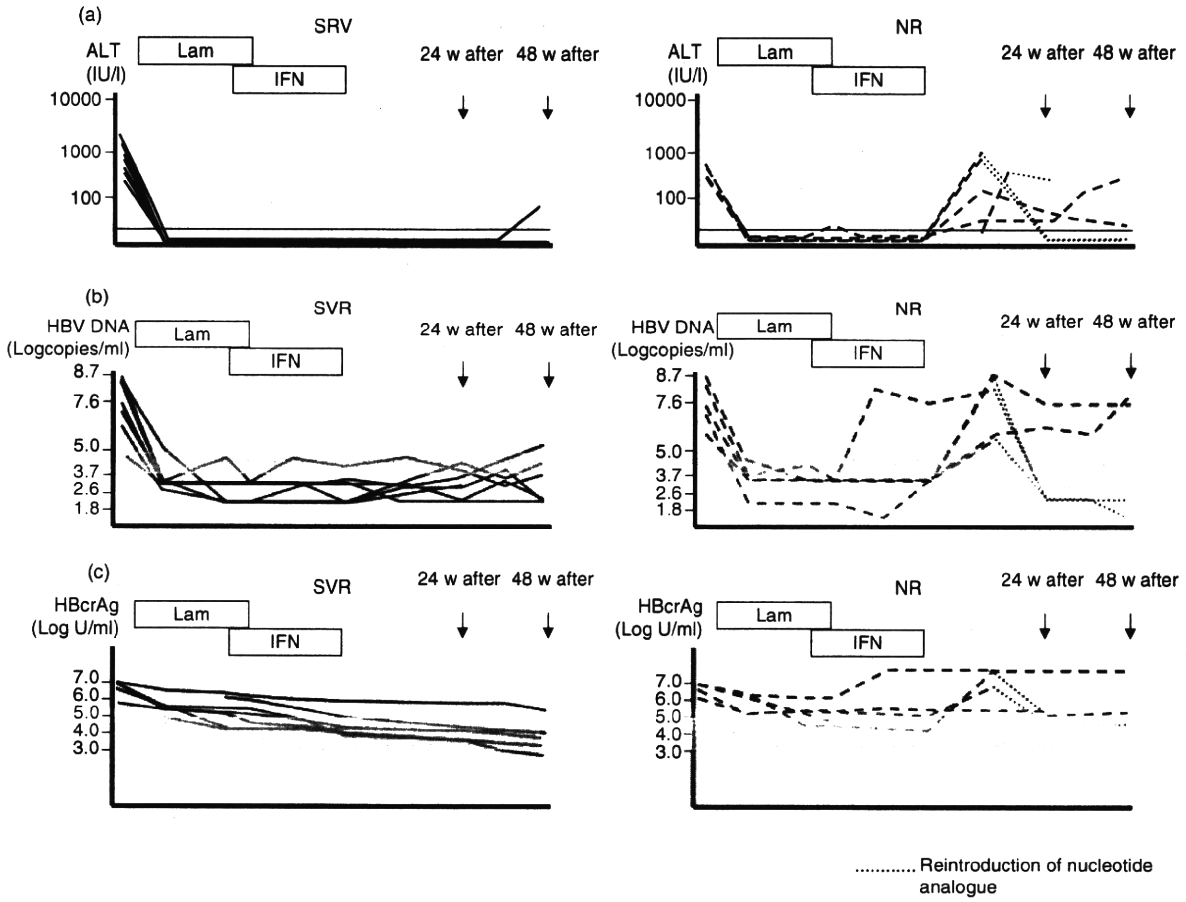

Figure 2 Temporal change in the level of serum ALT and viral markers. The serum ALT levels in all the patients normalized quickly after the start of treatment and remained within the normal range during treatment (a). The serum HBV DNA levels decreased below the detection limit shortly after treatment in most patients. Fluctuation of HBV DNA levels was observed in one SVR and two NR patients (b). The HBcrAg levels decreased continuously during and after treatment in SVR patients. The HBcrAg levels did not decrease continuously in NR patients (c). ALT, alanine aminotransferase; HBV DNA, hepatitis B virus DNA; HBcrAg, HBV core-related antigen; IFN, interferon; Lam, lamivudine; NR, no response; SVR, sustained virological response.

However, all the patients who showed NR remained HBeAg-positive 24 weeks after treatment.

The SVR rate for patients who achieved HBe seroconversion at the end of the treatment was 75.0% (3/4) whereas that for the patients who remained HBeAg-positive was 20.0% (1/5). Hence, HBe seroconversion at the end of the treatment may be predictive of the treatment response. In contrast, the HBV DNA and HBcrAg levels before or at the end of the treatment were not predictive of the outcome (Table 3).

Amino acid sequences of the YMDD motif were determined over time in nine patients. One of the nine patients showed substitution to YIDD during the lamivudine administration period. The substitution was corrected after starting the IFN treatment. The patient showed NR.

Reactivation of CHB was observed in one SVR patient during the observation period of 36 weeks after the end of the treatment, and lamivudine administration was restarted. All the NR patients required retreatment with lamivudine or entecavir for the control of hepatitis. No patient experienced serious side-effects including post-treatment flare after discontinuing the interferon administration.

Table 3 Levels of HBV-related markers before, during and after treatment

Case	HBV DNA (log copies/mL)				HBeAg				HBcrAg (log U/mL)				Outcome
	Before	End of Lam	End of IFN	24 w after Tx.	Before	End of Lam	End of IFN	24 w after Tx.	Before	End of Lam	End of IFN	24 w after Tx.	
1	4.8	<3.7	<3.7	<2.6	(+)	(−)	(−)	(−)	5.8	5.1	4.5	4.2	SVR
2	7.3	<2.6	<2.6	2.7	(+)	(−)	(−)	(−)	>7.0	4.3	4.0	ND	SVR
3	8.5	<3.7	4.1	3.8	(+)	(+)	(+)	(−)	>7.0	6.2	5.9	5.7	SVR
4	8.6	<2.6	<2.6	4.2	(+)	ND	(−)	(−)	ND	5.9	5.0	4.2	SVR
5	7.6	<3.7	<3.7	2.6	(−)	(−)	(−)	(−)	6.9	5.4	4.1	3.6	SVR
6	8.5	<2.6	<2.6	2.9	(−)	(−)	(−)	(−)	ND	4.1	3.9	3.6	SVR
7	6.3	<2.6	3.4	3.4	(−)	(−)	(−)	(−)	6.6	4.6	4.6	4.5	SVR
8	8.7	<3.7	<3.7	7.6>	(+)	(−)	(−)	(+)	>7.0	4.4	4.2	>7.0	NR
9	5.7	<3.7	<3.7	5.3	(+)	(−)	(+)	(+)	6.2	5.1	5.0	6.7	NR
10	7.6	<3.7	7.6	7.7	(+)	(+)	(+)	(+)	>7.0	6.3	>7.0	>7.0	NR
11	7.5	<3.7	<3.7	6.1	(+)	(±)	(+)	(+)	6.6	5.2	5.2	5.1	NR
12	6.8	<2.6	3.5	7.6>	(+)	(+)	(+)	(+)	>7.0	4.7	ND	ND	NR

HBcrAg, hepatitis B virus core-related antigen; HBeAg, hepatitis B virus e antigen; HBV DNA, hepatitis B virus DNA; IFN, interferon; Lam, lamivudine; ND, not determined; NR, no response; SVR, sustained virological response; Tx., treatment; w, weeks.

DISCUSSION

ACUTE EXACERBATION OF CHB may cause liver failure, which results in a high mortality rate.[41] IFN monotherapy is contraindicated in this situation because interferon may upregulate the immune response and cause severe liver injury.[8–10] Hence, the immediate treatment with NA for such patients has become standard.[42] Clinical trials of lamivudine have shown that the acute exacerbation of CHB may result in an increased sustained remission rate.[43,44] Therefore, if the safe withdrawal of NA is possible, acute exacerbation may be a good opportunity for sustained remission. However, post-treatment flare, which may result in liver failure, renders lamivudine withdrawal difficult.[45] Consequently, once treatment is initiated, lifelong HBV suppression is necessary in most patients.

Interferon has both antiviral and immunomodulatory functions.[7,8] Additionally, IFN sometimes elevates liver enzymes by depressing hepatic microsomal cytochrome P-450 levels.[46] However, such effects rarely lead to liver failure in humans.[10] Therefore, lamivudine administration and subsequent IFN treatment may be a safe method of lamivudine withdrawal. Additionally, such treatment may result in a high rate of sustained remission of hepatitis. Indeed, Serfaty *et al.* conducted a lamivudine administration and subsequent IFN treatment and achieved a high rate of sustained response.[22]

Yotsuyanagi *et al.* reported the evaluation of short-term lamivudine administration and its withdrawal for genotype C CHB. Decreased serum ALT and HBV DNA level was observed in four of the 12 patients (33.3%) and three of the 12 patients achieved HBe seroconversion 24 weeks after treatment in HBe-positive patients. As for HBe-negative patients, decreased serum ALT and HBV DNA level was observed in five of six patients (83.3%) 24 weeks after treatment.[45] The sustained response in this study was 58.3% (44.4% in HBe-positive and 100% in HBe-negative patients), which is comparable to that shown in Serfaty's study. Furthermore, all our patients were also infected with genotype C HBV, which is resistant to antiviral treatment.[47,48] Although the number of enrolled patients is small, we may conclude that lamivudine administration and subsequent IFN treatment is an effective treatment for CHB.

Lamivudine-interferon combination therapeutic trials have been conducted to date.[19–32] Some large trials, however, did not show the superiority of combination therapy.[19,26] One of the reasons is that, in many such trials, lamivudine and IFN are administrated simultaneously. Lamivudine monotherapy can upregulate

cytotoxic T-cell response.[49] IFN treatment following viral suppression by lamivudine might induce a stronger immune response than a simultaneous treatment.

Another advantage of sequential therapy over monotherapy is that NA can be safely withdrawn. The long-term safety of NA has not been demonstrated yet. Furthermore, NA can generate drug-resistant mutants.[16–18] Hence, the withdrawal of NA is preferred if possible. Our study demonstrates that the safe withdrawal of lamivudine without generating drug-resistant mutants is possible. However, in one patient, a drug-resistant mutant was found at the end of the lamivudine treatment. NA administration during exacerbation might induce the early emergence of drug-resistant mutants. The change from lamivudine to entecavir, which rarely generates drug-resistant mutants in a short time, may prevent the emergence of drug-resistant mutants, but this should be prospectively studied.

Interestingly, all three anti-HBe-positive patients in this study achieved SVR. Previous studies have shown that anti-HBe-positive patients respond poorly to IFN treatment.[50,51] In contrast, our results suggest that a potent antiviral treatment using lamivudine-IFN combination over a long period may result in high SVR rate even in anti-HBe-positive patients. The antiviral effect caused by the sequential therapy may be sufficiently strong to maintain long-term remission. A larger cohort study should be performed to determine whether sequential therapy is really effective for anti-HBe-positive patients.

Comparison between SVR and NR patients showed that the serum albumin levels were lower in SVR patients than in NR patients. Although the difference was not significant, the prothrombin time was longer and the ALT level was higher in SVR patients. These results showed that SVR patients suffered more severe exacerbation than NR patients. Several studies have shown that a high ALT level is predictive of a favorable response to IFN treatment,[21,52,53] which is compatible with our results.

Age is another important determinant of the response to IFN for Japanese patients, as demonstrated in the Japanese national guideline. Therefore, we studied the effect of age on the response to sequential therapy. Younger patients tended to be more responsive to IFN than older patients. If a larger number of patients is studied, the effect of age on the efficacy of sequential treatment may be clarified.

Table 3 shows the time-dependent change in the level of viral markers. In HBeAg-positive patients, HBeAg at the end of the IFN treatment was predictive of the outcome in seven of nine patients; three of four HBeAg-negative patients achieved SVR and four of five HBeAg-positive patients showed NR. HBV DNA and HBcrAg levels were less predictive of the treatment outcome than HBeAg. A marked reduction or clearance of HBeAg during treatment is also important for SVR in the treatment using pegylated IFN.[28,54] HBeAg might affect the efficacy of long-term, potent IFN-based treatment.

We evaluated the temporal change in the HBcrAg level during IFN treatment for the first time. Although the number of enrolled patients was small, a continuous decrease in the HBcrAg level to less than 5 log U/mL may be predictive of the long-term response to IFN treatment, but this should be further studied.

In conclusion, lamivudine-IFN sequential therapy is effective for the treatment of CHB. This treatment may induce long-term remission of both HBeAg-positive and -negative patients and enable safe withdrawal of NA.

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