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Original Article

Chronic hepatitis C in patients co-infected with human immunodeficiency virus in Japan: a retrospective multicenter analysis

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Aim: A nationwide survey in Japan revealed that nearly one-fifth of human immunodeficiency virus (HIV)-positive patients are co-infected with hepatitis C virus (HCV). We conducted a study to further analyze the features of liver disease in HIV–HCV co-infected patients.

Methods: We analyzed 297 patients from eight hospitals belonging to the HIV/AIDS Network of Japan.

Results: HCV genotypes 1, 2, 3, 4 and mixed genotypes were detected in 55.2, 13.7, 18.9, 0.9 and 11.3% of patients, respectively, in contrast to the fact that only genotypes 1 and 2 are detected in HCV mono-infected patients in Japan. This is compatible with the transmission of HCV through imported blood products contaminated by HCV. Sixteen of 297 HIV–HCV co-infected patients had advanced liver disease accompanied by ascites, hepatic encephalopathy or hepatocellular carcinoma. The average age of such patients was 41.1 ± 14.0 years, which was much younger than that of HCV mono-infected patients with the same complications. The progression speed of liver disease estimated from the changes in the levels of serum albumin, bilirubin, or platelet was slower in patients who achieved sustained virological response with interferon treatment than in those who did not receive it. The overall sustained virological response rate to interferon treatment was 43.3%.

Conclusions: Our findings suggest that liver disease is more advanced in HIV–HCV co-infected patients than in HCV mono-infected patients, and interferon treatment may retard the progression of liver disease in such patients.

Key words: acquired immunodeficiency syndrome, chronic liver disease, genotype, interferon therapy

INTRODUCTION

THE PROGNOSIS OF human immunodeficiency virus (HIV) infection has markedly improved since the introduction of hyperactive anti-retroviral therapy (HAART).[1,2] Opportunistic infection has been prevented or properly managed, resulting in lower mortality rates. Liver disease, in particular related to hepatitis C virus (HCV) infection, has now become the main cause of mortality among HIV-infected patients on HAART in Western countries.[3,4] A national survey among Japanese HIV-infected patients with coagulation disorders has shown that the mortality rate related to HCV-related liver disease after 1997 was twofold that before 1997.[5] In Japan, therefore, HCV infection may also be a major cause of death in HIV–HCV co-infected patients. However, there has been no extensive analysis of liver disease in HIV–HCV co-infected patients in Japan.

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Interferon (IFN) treatment in combination with ribavirin administration, which is now the first choice for HCV mono-infected patients,[6] is also a standard treatment for chronic hepatitis in HIV–HCV co-infected patients. Eradication of HCV is assumed to improve liver function, and normalization of serum aminotransferase (ALT) levels by IFN treatment may retard the progression of liver disease in HIV–HCV co-infected patients, even if they are on HAART. However, in general, the response rate to IFN treatment is lower in HIV–HCV co-infected patients than in HCV mono-infected patients.[7] The effects of IFN treatment on liver function and prognosis in HIV–HCV co-infected patients in Japan are yet undefined.

In 2004, we conducted a nationwide survey to determine the prevalence of HCV infection in HIV-infected patients by distributing a questionnaire to the hospitals in the HIV/AIDS Network of Japan, which revealed that 935 (19.2%) of 4877 HIV-positive patients were also positive for anti-HCV antibody.[8] In this study, we analyzed the progression of liver diseases and the impact of IFN treatment on the parameters of liver function in HIV–HCV co-infected patients in a multicenter retrospective study.

METHODS

Registry of patients with HIV–HCV co-infection

THE QUESTIONNAIRE REGARDING the current state of HIV–HCV co-infection was sent to the 366 hospitals in the HIV/AIDS Network of Japan in 2004, sponsored by the Japanese Ministry of Health, Labour and Welfare. One hundred seventy-six hospitals (48.1%) responded. The results, already published,[8] showed that HIV–HCV co-infected patients are concentrated in particular hospitals in big cities around Japan. Among these hospitals, we chose three hospitals in the Tokyo metropolitan area, and one each in the Hokkaido, Chubu, Osaka, Chugoku and Kyushu areas. These eight hospitals belong to the HIV/AIDS Network and had more HIV–HCV co-infected patients than other hospitals.

In the study, the following information was obtained from the hospitals regarding each HIV–HCV co-infected patient who visited the hospitals at least once between January and December in 2004: (1) age and sex of HIV-positive patients with anti-HCV; (2) possible transmission routes of HIV; (3) history of habitual alcohol intake; (4) date of the first and last visits; (5) counts of white blood cells, CD4-positive lymphocytes and platelets at the first and last visits; (6) levels of serum albumin and bilirubin at the first and last visits; (7) levels of HIV-RNA and HCV-RNA at the first and last visits; (8) history of IFN treatment with or without ribavirin; (9) history of HAART; and (10) history of jaundice, ascites, hepatic encephalopathy and hepatocellular carcinoma (HCC). The study sheets were completed by the physicians in charge and sent to the Department of Internal Medicine, University of Tokyo.

Ethical issues

The protocol of the current survey was approved by the ethical committee of each institution, and written informed consent was obtained from each patient.

Statistical analysis

The collected data were analyzed using Mann–Whitney's *U*-test whenever appropriate. *P*-values less than 0.05 were regarded as statistically significant.

RESULTS

Clinical backgrounds of registered patients

FROM THE EIGHT hospitals, 297 patients were registered. The number, age, sex, estimated transmission routes and history of habitual alcohol intake are shown in Table 1. Two hundred and ninety (97.6%) were male patients. The mean age of the patients was 37.9 ± 10.3.

HCV genotype was determined in 212 patients. One hundred seventeen (55.2%) patients were infected by genotype 1 HCV. Infection by genotypes 2, 3 or 4 HCV was found in 29 (13.7%), 40 (18.9%) and 2 (0.9%) patients, respectively. Twenty-four (11.3%) patients were infected by HCV of mixed genotypes. In the remaining 85 patients, the genotype was indeterminable or undetermined. The mean ages of patients infected by different HCV genotypes were similar (Table 1).

In 259 (87.2%) of 297 registered patients, HIV was most probably transmitted through the administration of blood products. Other transmission routes were sexual contacts among men who have sex with men (MSM) (4.0%), heterosexual contacts (3.0%) and intravenous drug use (IDU) (0.3%). Habitual alcohol consumption was noted in only one patient with genotype 1 HCV (0.6%).

Outcomes of IFN treatment in HIV–HCV co-infected patients

Serum HCV-RNA levels were available both at the first visit and registry to the study (i.e. the end of observa-

Table 1 Demography, transmission route and HCV genotypes in HIV–HCV co-infected patients

HCV genotype	Number (%)	HCV sub-genotypes	Viral load† (High : Low)	Age	Sex (Male : Female)	Transmission route				
						Transfusion	MSM	Hetero-sexual	IDU	Others
1	117 (55.2)	1a 31, 1b 43, 1a+1b 31, undetermined 12	31:11	38.3 ± 10.4	114:3	102	7	1	0	7
2	29 (13.7)	2a 16, 2b 11, undetermined 2	5:5	39.8 ± 9.5	29:0	24	1	1	0	3
3	40 (18.9)	3a 40	12:2	36.1 ± 8.9	40:0	38	0	0	0	2
4	2 (0.9)	4a 2	2:0	38.5 ± 2.1	2:0	2	0	0	0	0
Mixed	24 (11.3)	2a+3a 6, 1b+3a 3, others 15	11:0	38.7 ± 8.7	24:0	24	0	0	0	0
Others	85	Undetermined 85	6:1	36.2 ± 11.5	81:4	69	4	7	1	4
Total	297		67:19	37.9 ± 10.3	290:7	259 (87.2%)	12 (4.0%)	9 (3.0%)	1 (0.3%)	16 (5.5%)

†Viral loads are available in only a subset of patients. High viral load: more than 1 Meq/mL by branched DNA-probe assay or more than 100 KIU/mL by Amplicor monitor assay.
HCV, hepatitis C virus; HIV, human immunodeficiency virus; IDU, intravenous drug users; MSM, men who have sex with men.

tion) in 158 patients. Of these 158, 60 patients (38.0%) received IFN treatment for HCV, and 35 of these 60 patients did it in combination with ribavirin. Those who did not complete the scheduled treatment were excluded from the current analysis.

As shown in Table 2, 26 (43.3%), 11 (18.4%) and 23 (38.3%) of the treated patients achieved sustained virological response (SVR), end-of-treatment virological response (ETR) and no virological response (NR), respectively. The SVR rate in patients with each genotype is shown in Table 2. The SVR rate in the patients who underwent IFN treatment in combination with ribavirin was 31.4% in total. The SVR rate in patients with each genotype who underwent IFN/ribavirin combination therapy is shown in Table 2.

All of the 26 patients who achieved SVR remained negative for serum HCV-RNA in the further follow-up periods. In contrast, none of the patients with ETR or NR became negative for serum HCV-RNA in the follow-up periods. In five patients who did not receive IFN treatment, HCV-RNA was negative at the end of the observation period, although it was positive at least twice before the registry. The profiles of the five patients are shown in Table 3.

Changes in liver function and associated complications (Table 4)

As mentioned above, the data on liver function and serum HCV-RNA positivity were available both at the first visit and registry (end of observation) in 158 of the 297 registered patients. The mean observation period was 9.5 ± 5.0 and 8.2 ± 8.2 years in the IFN-treated and IFN-untreated patients, respectively. Unfortunately, few, if any, patients underwent liver biopsy, because most HIV–HCV co-infected patients had coagulation disorders.

The annual change in the serum albumin concentration was +0.05 ± 0.42 g/dL in the IFN-treated patients, and −0.80 ± 0.82 g/dL in the non-IFN-treated patients. The annual change in the serum bilirubin concentration was +0.08 ± 0.38 mg/dL in the IFN-treated patients, while it was +0.15 ± 0.15 mg/dL in the non-IFN-treated patients. Among the IFN-treated patients, the serum bilirubin concentration decreased by 0.02 ± 0.08 mg/dL in the patients who achieved SVR, which was significantly larger than that in the non-IFN-treated patients at the end of the observation ($P < 0.05$). The annual changes in platelet counts were +0.06 ± 1.13 ($\times 10^4/\mu l$) in the IFN-treated patients and −0.94 ± 0.95 ($\times 10^4/\mu l$) in the non-IFN-treated patients. The change in platelet

Table 2 Virological response to interferon treatment in HIV–HCV co-infected patients

Genotype	Viral load (High : Low)†	Response			Total
		SVR	ETR	NR	
(a) Response to interferon treatment in total (with or without ribavirin)					
1	9:6	7 (33.3%)	1	13	21
2	5:3	4 (40.0%)	2	4	10
3	5:1	5 (62.5%)	1	2	8
4	1:0	0	1	0	1
Mixed	5:1	2 (33.3%)	3	1	6
Others	6:2	8 (57.1%)	3	3	14
Total	31:13	26 (43.4%)	11	23	60
(b) Response to ribavirin/interferon combination therapy including peginterferon					
1	8:2	2 (15.3%)	0	11	13
2	1:2	1 (25.0%)	0	3	4
3	4:1	4 (66.7%)	1	1	6
4	1:0	0	1	0	1
Mixed	4:1	1 (20.0%)	3	1	5
Others	3:0	3 (50.0%)	1	2	6
Total	21:6	11 (31.4%)	6	18	35

†Viral loads are available in only a subset of patients. High viral load: more than 1 Meq/mL by Branched DNA-probe assay or more than 100 KIU/mL by Amplicor monitor assay.
ETR, end of treatment virological response; NR, no virological response; SVR, sustained virological response.

counts in the patients who achieved SVR was significantly larger than that in the non-IFN-treated patients ($P < 0.05$, Table 4).

No symptoms of hepatic failure (ascites or hepatic encephalopathy) were observed in the 60 IFN-treated patients while they were observed in six of the 98 non-IFN-treated patients. HCC was found in one IFN-treated patient after SVR, while it was found in two non-IFN-treated patients (Table 4).

Impact of HAART on liver function and associated complications (Table 5)

Information on HAART was available in 292 patients. The mean observation periods were 8.4 ± 4.2 years in 234 patients on HAART, and 9.8 ± 6.0 years in 58 patients not on HAART. Changes in the levels of albumin, bilirubin or platelet were similar between the two groups (statistically not significant). The morbidities of hepatic decompensation symptoms (ascites and hepatic encephalopathy) and HCC were not significantly different between the two groups. In total, nine patients had hepatic decompensation and seven had HCC, and the average age of such patients was 41.1 ± 14.0 years, which was much younger than that of HCV mono-infected patients with the same complications.[9]

DISCUSSION

IN THE CURRENT study, the features of liver disease in HIV–HCV co-infected patients in Japan were analyzed. The determination of HCV genotypes revealed that genotype 3 or 4, which is rarely seen in HCV mono-infected patients in Japan,[10] was found in a substantial fraction of HIV-infected patients. In addition, some of these patients were infected with HCV of mixed genotypes. These results are compatible with the fact that HCV is transmitted through imported blood products that were contaminated by HCV, as is the case with HIV infection.[11] Infection by HCV of mixed genotypes may reflect frequent administrations of blood products of different lots.

We evaluated the response rate to IFN treatment in HIV–HCV co-infected patients in Japan. Because the IFN treatment protocol varied between facilities, it was not easy to evaluate the effects of the treatments including IFN in this cohort. However, the regimen of ribavirin/IFN combination therapy was similar between the hospitals: the treatment period was 24 weeks in patients with HCV genotypes 2 and 3, and 48 weeks in those with HCV of other genotypes when either pegylated or standard IFN in combination with ribavirin was used.[12] Therefore, it may be possible to estimate the effect

Table 3 Clinical backgrounds of patients who spontaneously cleared HCV in HIV-infected patients

Patient no.	Age	Sex	Transmission route	Observation period (years)	HCV-RNA (KIU/mL)	HCV genotype	HIV-RNA ($\times10^2$/mL)	WBC (/μL)	CD4+T cells (/μL)	Platelets ($\times10^4$/mL)	ALT (U/l)	HAART
1	33	M	Transfusion	8.8	290	ND	200 000	4500	5	26.3	21	Yes
2	31	M	MSM	2.3	Positive†	ND	13 000	5760	931	22.7	29	Yes
3	27	M	Transfusion	9.3	>850	3a	180 000	4000	51	10.1	84	Yes
4	53	M	Transfusion	4.5	Positive†	1a	20 000	4800	296	35.4	24	No
5	22	M	Transfusion	7.8	220	ND	990	5500	125	33.1	44	Yes

†Positive: HCV-RNA was positive by qualitative PCR, but was not quantitatively determined.
ALT, aminotransferase; HAART, highly active anti-retroviral therapy; HCV, hepatitis C virus; HIV, human immunodeficiency virus; MSM, men who have sex with men; ND, not determined; WBC, white blood cells.

Table 4 Changes in clinical parameters and IFN treatment in HIV–HCV co-infected patients

	Outcome of IFN treatment	Number	Observation period (years)	⊿Albumin†	⊿Bilirubin‡	⊿Platelet§	Ascites/ encephalopathy	HCC
IFN-treated patients		60	9.5 ± 5.0	0.05 ± 0.42	0.08 ± 0.38*	0.06 ± 1.13	0	1
	SVR	26	9.1 ± 4.4	0.13 ± 0.59	(−) 0.02 ± 0.08*	0.14 ± 0.76*	0	1
	ETR	11	14.6 ± 7.0	(−) 0.07 ± 0.14	0.51 ± 1.04	0.07 ± 1.50	0	0
	NR	23	7.4 ± 2.0	0.01 ± 0.30	0.09 ± 0.30	(−) 0.18 ± 0.32	0	0
Non-IFN-treated patients		98	8.2 ± 8.2	(−) 0.80 ± 0.82	0.15 ± 0.15	(−) 0.94 ± 0.95	6	2
All		158	8.7 ± 4.7	(−) 0.45 ± 2.93	0.13 ± 0.52	(−) 0.59 ± 3.78	6	3

*$P < 0.05$ versus patients without IFN treatment.
†⊿Albumin: changes in albumin concentration (g/dL)/observation period (years).
‡⊿Bilirubin: changes in bilirubin concentration (mg/dL)/observation period (years).
§⊿Platelet: changes in platelet count ($\times10^4$/μL)/observation period (years).
ETR, end of treatment virological response; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HIV, human immunodeficiency virus; IFN, interferon; NR, no virological response; SVR, sustained virological response.

Table 5 Changes in clinical parameters and HAART in HIV–HCV co-infected patients

	Number	Age	Sex (M : F)	Observation period (years)	⊿Albumin†	⊿Bilirubin‡	⊿Platelet§	IFN	Ascites/ encephalopathy	HCC
HAART (+)	234	37.8 ± 10.4	227:7	8.4 ± 4.2	(−) 0.002 ± 0.18	0.13 ± 0.53	(−) 0.40 ± 3.71	143 (61.1%)	6	5
HAART (−)	58	38.1 ± 10.5	58:0	9.8 ± 6.0	(−) 0.14 ± 0.18	0.03 ± 0.25	(−) 1.40 ± 3.30	30 (51.7%)	3	2

†⊿Albumin: changes in albumin concentration (g/dL)/observation period (years).
‡⊿Bilirubin: changes in bilirubin concentration (mg/dL)/observation period (years).
§⊿Platelet: changes in platelet count ($\times 10^4$/(L)/observation period (years).
HAART, highly active anti-retroviral therapy; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HIV, human immunodeficiency virus.

of ribavirin/IFN combination therapy in HIV–HCV co-infected patients in this study.

The response rate to ribavirin/IFN combination therapy was 31.4% in total, and 15.3% in patients with HCV genotype 1, which are comparable rates to those achieved in previous studies on HIV–HCV co-infected patients in Western countries.[7] The low response rate in HIV–HCV co-infected patients compared with HCV mono-infected patients[12] may be attributed to several factors: impaired immune response, high HCV loads and viral quasi-species caused by frequent chances of transmission. Of these, high viral loads may be essential, because Table 2 shows that patients with genotype 1 HCV achieved SVR even by IFN monotherapy if their viral loads were low. In the era of IFN monotherapy, patients with favorable conditions were treated first of all: pretreatment viral loads in patients who received IFN monotherapy were lower than those who received PEG-IFN–ribavirin combination therapy. This may be the reason why the efficacy of PEG-IFN–ribavirin combination therapy was lower than that with IFN monotherapy in this study.

The serum bilirubin concentrations and platelet counts were improved in the patients who achieved SVR by IFN treatment. Although the response rate to IFN treatment is lower in HIV–HCV co-infected patients than in HCV mono-infected patients, the overall benefit of IFN treatment on liver function may be similarly expected in the patients who achieved SVR. HAART showed no impact on the liver function in HIV–HCV co-infected patients. Improvement of liver function can be expected only in IFN-treated patients, although there is a possibility that only patients with preserved liver function were able to receive IFN treatment. Given that liver disease is the major life-threatening factor in HIV-infected patients, IFN treatment should be considered in the early stage of HIV–HCV co-infection.

It should be noted that nine patients had hepatic decompensation and seven had HCC, and the average age of such patients was much younger than that of HCV mono-infected patients with the same complications.[9] This finding is compatible with reports from Western countries showing a faster progression of fibrosis[13] and earlier development of HCC.[14] A possibly interesting finding is that five patients (approximately 3% of patients whose serum HCV-RNA level was serially determined) cleared HCV-RNA from the serum without IFN treatment. Previous reports showed that some HIV-infected patients could spontaneously clear HCV-RNA.[15–17] The clearance of HCV among patients with chronic HCV infection is rare, although it has been

reported in Japan.[18] Three of the five patients had high HCV loads and low CD4$^+$ T-lymphocyte counts, which are generally thought to be unfavorable for spontaneous HCV clearance. A difference in immune status of HIV-infected patients from HCV mono-infected patients may be involved in such an observation, although further studies are awaited.

In summary, our study demonstrated that approximately 20% of HIV-infected patients are co-infected with HCV. Some of the HIV–HCV co-infected patients had advanced liver disease such as ascites, encephalopathy or HCC at a younger age than HCV mono-infected patients, suggesting that the progression of liver disease may be more rapid in HIV–HCV co-infected patients than in HCV-mono-infected ones. Treatments with regimens including IFN, which may improve liver function and decrease liver-related death, should be considered in HIV–HCV co-infected patients.

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Original Article

Short-term prolongation of pegylated interferon and ribavirin therapy for genotype 1b chronic hepatitis C patients with early viral response

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Aim: We tailored extended treatments using pegylated interferon (PEG IFN) and ribavirin (RBV) to viral responses after initiation of therapy and investigated the efficacy and safety of its therapy for chronic hepatitis C (CHC) patients.

Methods: Eighty-two genotype 1b CHC patients were enrolled in the present study. All patients received PEG IFN-α-2b and weight-based RBV therapy. We defined a viral response in which serum HCV-RNA is undetectable at week 4 as rapid viral response (RVR), detectable at week 4 and undetectable by week 12 as early viral response (EVR), and detectable at week 12 and undetectable by week 24 as late viral response (LVR). We set the treatment duration depending on viral response; 48 weeks for RVR patients and 72 weeks for LVR. Furthermore, EVR patients received a short-term extension of treatment duration to 52–60 weeks. We prospectively investigated sustained viral response (SVR) rates of these groups.

Results: Overall SVR rate for the total patient group was 57.3%. SVR rates of the RVR, EVR and LVR patients were 100%, 80.5% and 40.0%, respectively. Nine patients could not complete this treatment protocol. Baseline platelet count and mutation in the interferon sensitivity-determining region of NS5A were significant independent predictors of SVR, and amino acid substitution of the core region was a significant independent predictor of non-viral response by multivariate logistic regression analyses.

Conclusion: The results indicate that short-treatment extension of PEG IFN plus RBV treatment protocols in EVR patients can improve overall SVR rates.

Key words: chronic hepatitis C, extended treatment, pegylated interferon, ribavirin

INTRODUCTION

SUSTAINED VIRAL RESPONSE (SVR) rates have improved with the development of combined pegylated interferon (PEG IFN) plus ribavirin (RBV) therapy for patients with chronic hepatitis C (CHC). However, the SVR rate for genotype 1 CHC patients is around 50% when this treatment is used for 48 weeks, which is the current standard duration of treatment.[1,2] Various drugs are now undergoing clinical trials to improve the SVR rate.[3,4] However, these drugs will not be available for a few years. As a result, modification of current therapeutic protocols is being attempted worldwide.

The SVR rate varies depending on the viral response after initiating treatment and patients with late viral response display the lower therapeutic efficacy.[5]

Pearlman *et al.* reported that extended treatment from 48 to 72 weeks increases SVR rates among patients in whom serum HCV-RNA is detected at week 12 and becomes undetectable by week 24.[6] Additionally, Sanchez-Tapias *et al.* compared SVR rates at 48 weeks

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with those at 72 weeks of treatment in patients with serum HCV-RNA detected at week 4.[7] Their results suggested that extended treatment to 72 weeks is also useful for increasing the SVR rate in CHC. However, extended treatment for all patients in whom serum HCV-RNA is detected at week 4 is difficult, given the associated decrease in medication tolerability and the increased medical expense.

In the present study, serum HCV-RNA was measured every 4 weeks and a rapid viral response (RVR) was defined by a viral response in which serum HCV-RNA was undetectable at week 4, an early viral response (EVR) was defined by a viral response in which serum HCV-RNA was detectable at week 4 and undetectable by week 12, and a late viral response (LVR) was defined by a viral response in which serum HCV-RNA was detectable at week 12 and undetectable by week 24. We set the treatment duration depending on the viral response; 48 weeks for RVR patients and 72 weeks for LVR patients. Furthermore, EVR patients received a short-term extension of treatment duration to 52–60 weeks, as EVR patients with PEG IFN plus RBV therapy have made up about 50% of subjects in several Japanese trials.[8,9]

The aim of the present study was to prospectively investigate the efficacy and safety of extended treatment using PEG IFN and RBV depending on the week in which serum HCV-RNA became undetectable, and to clarify whether a short-term extension of treatment in EVR patients would increase overall SVR for Japanese genotype 1 CHC patients. In addition, we investigated refractory factors for extended treatment to clarify prediction of treatment efficacy.

METHODS

Patients

SUBJECTS COMPRISED OF 82 consecutive Japanese genotype 1b CHC patients treated with PEG IFN-α-2b and RBV between December 2004 and March 2006 (Table 1). All patients were positive for anti-HCV antibodies, genotype 1b and high viral load (> 100 KIU/mL) according to HCV-RNA level. We excluded patients with other causes of liver disease, co-infection with HIV or hepatitis B virus, evidence of decompensated liver disease, or a history of hepatocellular carcinoma.

Treatment

All patients received PEG IFN-α-2b (1.5 μg/kg/week) and weight-based RBV (600 mg for < 60 kg; 800 mg for > 60 kg and < 80 kg; 1000 mg for > 80 kg). Duration of treatment was prospectively determined by the time at which serum HCV-RNA became undetectable. The detailed treatment protocol is shown in Figure 1. Concerning the patient group with undetectable HCV-RNA at 12 weeks treatment, the initial study design, which was to assign those patients to two subgroups randomly with treatment duration of 56 weeks or 60 weeks, was later changed to assign them to a single treatment duration of 60 weeks. Patients with positive serum HCV-RNA at 24 weeks finished treatment without prolongation of PEG IFN plus RBV therapy. We defined this group as non-viral response (NR). Serum HCV-RNA was estimated every 4 weeks, defining response by the time at which serum HCV-RNA became undetectable, as follows: RVR, by 4 weeks; EVR by 8–12 weeks; and LVR, by 16–24 weeks. SVR rates were prospectively investigated in these groups.

Table 1 Characteristics of patients at baseline

Sex (male/female)	45/37
Age (years)	57.1 ± 10.6
Height (cm)	164.7 ± 9.3
Bodyweight (kg)	59.2 ± 9.7
Body mass index (kg/m^2)	22.3 ± 2.7
History of treatment with interferon (naïve/retreatment)	60/22
Laboratory data	
ALT (IU/L)	64.5 ± 30.2
GGT (IU/L)	30.8 ± 24
LDL cholesterol (mg/dL)	58.2 ± 13.0
Hyaluronic acid (ng/mL)	94.6 ± 116
Leukocytes (mm^3)	4350 ± 1090
Hemoglobin (g/dL)	14.1 ± 1.3
Platelets (10^4/mm^3)	14.6 ± 4.1
HCV RNA level (KIU/L)	2032 ± 1590
Histological findings	
Activity (1/2/3/ND)	18/16/2/46
Staging (1/2/3/4/ND)	12/14/9/1/46
Amino acid substitutions in HCV gene	
ISDR in the NS5A, mutant-type/non-mutant-type/ND	14/62/6
Aa 70 and/or Aa91 in the core region, Double-wild/Non-double-wild/ND	38/40/4

Continuous variables are mean ± standard deviation.
ALT, alanine aminotransferase; GGT, γ-glutamyl transpeptide; ISDR, interferon sensitivity-determining region; LDL, low density lipoprotein; ND, not determined.

Evaluation of serum HCV-RNA

The HCV-RNA level was measured quantitatively by polymerase chain reaction (PCR) before and during

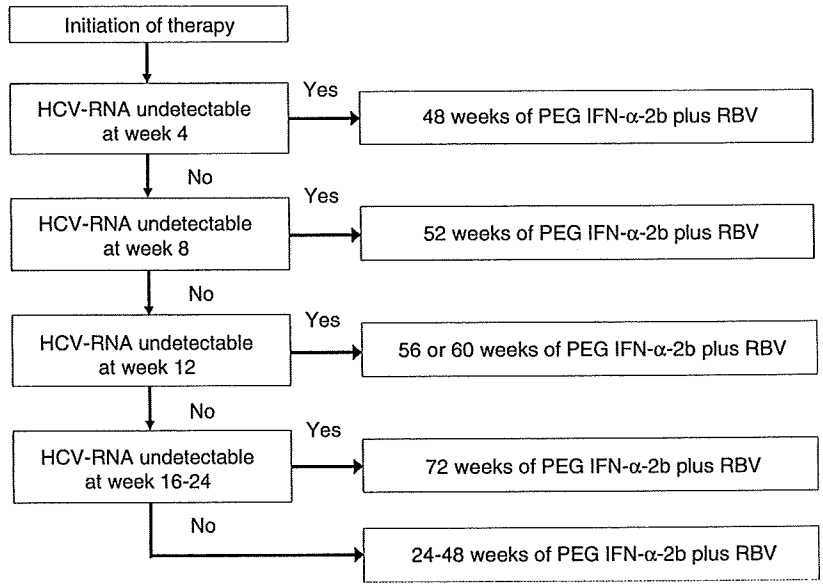

Figure 1 Treatment protocol. Serum hepatitis C virus ribonucleic acid (HCV-RNA) was measured every 4 weeks and treatment duration was set according to viral response. PEG IFN, pegylated interferon; RBV, ribavirin.

therapy (Cobas Amplicor HCV monitor v2.0 using the 10-fold dilution method; Roche Diagnostics, Branchburg, NJ, USA). The lower limit of the assay was 5×10^3 IU/mL. When HCV-RNA was quantitatively undetectable, HCV-RNA levels were also checked by qualitative PCR assay with a limit of 50 IU/mL. Qualitative PCR assay was used to decide the time at which serum HCV-RNA became undetectable.

Evaluation of amino acid substitutions in interferon sensitivity-determining region of NS5A and core region

Part of the amino acid sequence for NS5A in HCV genotype 1b, the interferon sensitivity-determining region (ISDR), reportedly correlates with the response to IFN therapy in Japanese patients.[10] Amino acid substitution patterns in the core region of HCV genotype 1b have also recently been correlated with the response to interferon therapy in Japanese patients.[11,12] We examined amino acid substitutions in the ISDR of NS5A and at aa71 and aa90 in the core region. Whether these factors are predictive of SVR or NR was analyzed. The present study split amino acid substitutions in the ISDR of NS5A into two patterns: mutant type (> 4 mutations) and non-mutant type (0–3 mutations), according to the number of amino acid substitutions. Wild-type pattern at both aa71 and aa90 was evaluated as double wild-type, whereas all other patterns were evaluated as non-double wild-type.

Statistical analysis

SVR was analyzed on an intention-to-treat basis. Uni- and multivariate logistic regression analyses were used to determine predictive factors for SVR and NR. We also calculated odds ratios and 95% confidence intervals (95% CI). Variables that achieved statistical significance ($P < 0.05$) or marginal significance ($P < 0.10$) on univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors. Potential predictive factors associated with SVR included the following variables: sex; age; body mass index; history of treatment with interferon; alanine aminotransferase; gamma-glutamyl transferase; low-density lipoprotein cholesterol; hyaluronic acid; leukocytes; hemoglobin; platelet count; HCV-RNA level; and amino acid substitutions at the ISDR of NS5A and aa71 and aa90 of the HCV core region. We evaluated predictive factors of SVR for patients with undetectable HCV-RNA by 24 weeks after initiation of therapy.

RESULTS

Tolerability of treatment

THE TREATMENT COURSE was completed in 73 of the 82 patients, with 43 patients completing treatment without requiring any reduction in drug dose. Five patients completed treatment with reduced dosages of

PEG IFN-α-2b, while 29 patients completed treatment with reduced dosages of RBV. Nine patients withdrew from the trial.

Time at which HCV-RNA became undetectable

Figure 2 shows the numbers of patients who achieved RVR, EVR, LVR and NR. Patients with RVR, EVR and LVR accounted for 9.8%, 50.0% and 18.3%, respectively. Patients with NR accounted for 22.0%. Patients with EVR accounted for half of all patients.

Response to therapy

Overall SVR rate for the total patient group was 57.3%. SVR rates for RVR, EVR and LVR patients were 100% (8/8), 80.5% (33/41) and 40.0% (6/15), respectively. Numbers of patients with SVR according to the time at which HCV-RNA became undetectable are shown in Figure 3.

Prediction of SVR and NR at baseline

Univariate analysis identified four parameters adversely influencing SVR: platelet count; HCV-RNA, mutant-type amino acid substitution at ISDR; and age. Multivariate analysis using the above variables identified two parameters independently influencing SVR: platelet count and mutant-type amino acid substitution at ISDR (Table 2).

Univariate analysis identified one parameter influencing NR: amino acid substitution at aa71 and aa90 in the core region. Multivariate analysis using this variable identified the same parameter as independently influencing NR (Table 3).

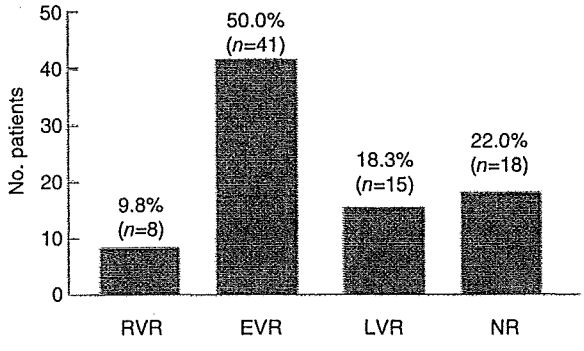

Figure 2 Time at which hepatitis C virus ribonucleic acid became undetectable. EVR, early viral response; LVR, late viral response; RVR, rapid viral response; NR, non-viral response.

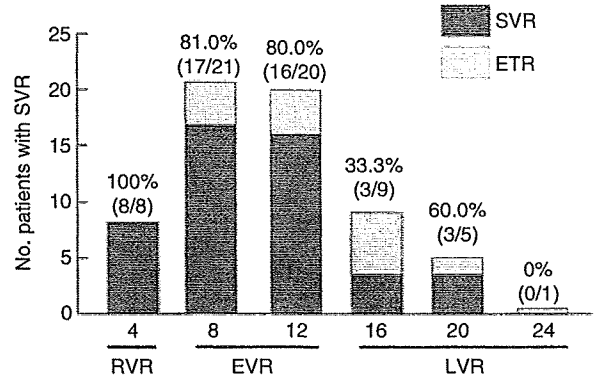

Figure 3 Number of patients with sustained viral response (SVR) and end-of-treatment reponse (ETR) according to the time when hepatitis C virus ribonucleic acid (HCV-RNA) became undetectable. EVR, early viral response; LVR, late viral response; RVR, rapid viral response; NR, non-viral response.

Prediction of SVR for patients with undetectable HCV-RNA by 24 weeks after commencement of therapy

In 64 patients, HCV-RNA was undetectable by 24 weeks after initiation of therapy. Of these, 17 patients experienced relapse (26.6%). Univariate analysis identified three parameters influencing SVR: platelet count, age and HCV-RNA. Multivariate analysis using these variables identified two parameters independently influencing SVR: age and HCV-RNA (Table 4).

Evaluation of predictive factors for SVR according to each treatment duration

We evaluated predictive factors for SVR according to each of the treatment durations by uni- and multivariate analysis. No parameters were significant in all treatment duration.

DISCUSSION

TREATMENT DURATION WAS based on the time at which HCV-RNA became undetectable under PEG IFN plus RBV therapy for genotype 1b and high viral load CHC patients. SVR rate of our response guided therapy was also investigated.

It is presently accepted that treatment duration of PEG IFN plus RBV therapy for LVR patients can be extended from 48 weeks to 72 weeks.[6] In RVR patients, standard 48-week therapy reached nearly 100% SVR rate.[13] EVR patients also achieved a high SVR rate by 48-week

Table 2 Predicting factors of sustained viral response by multivariate analysis

Factor	Category	Odds ratio (95% CI)	*P*
Platelets (10^4/mm^3)	0: < 13	1	0.018
	1: > 13	9.84 (2.34–41.3)	
Amino acid substitutions in the ISDR of NS5A	0: non mutant type	1	0.029
	1: mutant type	7.69 (1.23–48.0)	

CI, confidence intervals; ISDR, interferon sensitivity-determining region.

Table 3 Predicting factors of non-viral response by multivariate analysis

Factor	Category	Odds ratio (95% CI)	*P*
Amino acid substitutions in aa 70 and/or aa91 of the core region	0: Non double-wild	1	0.049
	1: Double-wild	0.315 (0.1–0.994)	

CI, confidence intervals.

therapy.[14] Interestingly, it has been observed that the SVR rate decreases in a stepwise manner among EVR patients whose HCV-RNA became undetectable at different times. Therefore, it was hypothesized in the present study that it would be effective to add stepwise short-time treatment duration, and we set treatment duration to 52 weeks for EVR patients with HCV-RNA undetected at week 8 and 56–60 weeks for EVR patients with HCV-RNA undetected at week 12.

Mangia *et al.* reported that the SVR rates of standard 48-week therapy for RVR patients, EVR patients whose HCV-RNA became undetectable at week 8, and EVR patients whose HCV-RNA became undetectable at week 12 were 87%, 70.3% and 38.1%, respectively.[14]

In contrast, the results of the present study showed that the SVR rate of our protocol in RVR patients, EVR patients whose HCV-RNA became undetectable at week 8 and EVR patients with HCV-RNA undetected at week 12 were 100%, 81.0% and 80.0%, respectively. The SVR rate of EVR patients with HCV-RNA undetected at week 12 was even higher than the SVR rate of the 72-week therapy that Mangia *et al.* reported. Only two cases with HCV-RNA undetected at 12 weeks had the treatment duration of 56 weeks. Since these two cases reached SVR, we concluded that even if we had a standardized 60-week therapy for patients with HCV-RNA undetected at 12 weeks, it would not have made any difference to our result. In contrast, the availability of the 56-week therapy for patients with HCV-RNA undetected at 12 weeks remains unclear. Thus, further investigation is clearly required to establish a more appropriately designed treatment protocol.

Table 4 Predicting factors of sustained viral response for patients with undetectable HCV-RNA by 24 weeks after start of therapy by multivariate analysis

Factor	Category	Odds ratio (95% CI)	*P*
Age (years)	0: < 55	1	0.0284
	1: > 55	0.079 (0.01–0.76)	
HCV-RNA (KIU/mL)	0: < 4000	1	0.029
	1: > 4000	0.123 (0.03–0.62)	

CI, confidence intervals.

This treatment extension protocol was well tolerated, with a total frequency of treatment discontinuation of only 11.0%. Dose reduction following adverse events was used for 25 patients with a reduction in RBV, one patient with a reduction in PEG IFN and four patients with reductions in both drugs. Reductions in PEG IFN or RBV have previously been suggested as a reason for lower SVR rates.[15] Conversely, in the present study, a factor contributing to higher SVR rates was that a large number of patients were treated with sufficient volumes of both drugs. Evaluation of the time at which HCV-RNA became undetectable showed EVR in 50.0% of patients. This EVR rate is similar to results from other reports in Japan, including clinical trials, showing EVR in about half of patients who receive PEG IFN plus RBV therapy. Conversely, SVR rates have been about 70% in EVR patients from other Japanese clinical trials.[8] If we achieve a 10% increase in the SVR rate for patients with EVR, this represents a 5% advantage in the overall SVR rate. Increasing SVR among EVR patients with short-term extended therapy thus offers an important contribution to increases in overall SVR.

Baseline factors that might be useful to predict SVR and NR were evaluated in the present study. One patient factor associated with SVR was a higher platelet count on pretreatment blood tests. Decreased platelet counts are known to be correlated with more advanced hepatic fibrosis. Decreased platelet count in conjunction with advanced hepatic fibrosis seems to represent a factor associated with treatment resistance. However, no histological factors were identified, but only a limited number of biopsies were carried out. A viral factor associated with SVR was ISDR amino acid mutations in the NS5A region. Enomoto *et al.* reported ISDR mutations as predictive of treatment response to IFN monotherapy[10] and IFN plus RBV therapy.[16] Based on the present findings, ISDR mutations are also useful in predicting response to PEG IFN plus RBV therapy. Zuezem *et al.* reported no correlation between ISDR mutations and treatment response, whereas another study reported a correlation that was characteristic of a Japanese-specific subtype of HCV genotype 1b.[17] This issue clearly warrants further investigation. Viral factors associated with NR were amino acid substitutions at aa70 and aa91. Akuta *et al.* have also reported ISDR region mutations as contributing factors to SVR and core region mutations as contributing factors to NR.[12] The present findings are in agreement with those results. In contrast, no patient factors associated with NR were identified.

Patients with end-of-treatment response (ETR) were also evaluated. ETR patients displayed a low relapse rate of 26.6%. Analysis of ETR identified age and viral load as factors contributing to SVR. In other words, treatment protocols (including further treatment extension) must be considered for older patients and high baseline HCV-RNA levels. In Japan, sex has also recently attracted interest as a treatment resistance factor. In the present study, 11 of 17 patients with relapse were female. Although sex was not identified as a significant factor in the present study, we suggest this is because of the small size of this study.

The present study could not identify any significant predictive factors for SVR according to each of the treatment durations. So, we conclude that a larger subject population might identify this as a significant factor in treatment resistance.

The TaqMan PCR assay, now widely used in clinical practice, is more sensitive for detecting serum HCV than the Amplicor Monitor assay. In the present study, the time at which HCV-RNA became undetectable was based on 50 IU/mL as the lower limit of detection using the Amplicor Monitor assay. However, the TaqMan PCR assay, with a lower limit of detection of 10 IU/mL, may allow a more accurate evaluation of viral response.[18,19] Thus, in some relapsing patients, TaqMan PCR assay might have allowed viral detection for a longer period of time. This might be more suitable to evaluate the effects of a short-term extension of treatment.

In conclusion, the present study showed a higher SVR rate than previously reported, although the number of patients was small. To more accurately evaluate the usefulness of short-term extensions to the treatment protocol, a large-scale, multicenter, standard-controlled study is desirable. New drugs for the treatment of refractory CHC are being developed at a rapid pace, but it will be several years before such drugs can be applied in clinical practice. In the meantime, the CHC patient population in Japan is aging, disease severity is progressing, and the pharmacotherapeutic options remain limited. Modification of treatment protocols is one possible option to reduce relapse rates. The results of the present study indicate that short-treatment extension of PEG IFN plus RBV treatment protocols in EVR patients can improve SVR rates.

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Enhanced intracellular retention of a hepatitis B virus strain associated with fulminant hepatitis

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ABSTRACT

A plasmid carrying 1.3-fold HBV genome was constructed from a HBV strain that caused five consecutive cases of fulminant hepatitis (pBFH2), and HepG2 cells were transfected with pBFH2 or its variants. The pBFH2 construct with A1762T/G1764A, G1862T, and G1896A showed the largest amount of core particle-associated intracellular HBV DNA, but no significant increase of extracellular HBV DNA in comparison with the wild construct, suggesting that these mutations might work together for retention of the replicative intermediates in the cells. The retention might relate to the localization of hepatitis B core antigen (HBcAg) in the nucleus of HepG2, which was observed by confocal fluorescence microscopy. HBcAg immunohistochemical examination of liver tissue samples obtained from the consecutive fulminant hepatitis patients showed stronger staining in the nucleus than acute hepatitis patients. In conclusion, the fulminant HBV strain caused retention of the core particles and the core particle-associated HBV DNA in the cells.

Introduction

Hepatitis B virus (HBV) causes a spectrum of liver diseases such as acute self-limiting or fulminant hepatitis, chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. Fulminant hepatitis, which is lethal during a short period in many cases, occurs in approximately 1% of patients with acute HBV infection (Lee, 1993). The pathogenesis that leads to fulminant hepatitis B is considered to result from enhanced replication of the virus (Baumert et al., 1996; Hasegawa et al., 1994) and the exuberant immune response of the host (Rivero et al., 2002), but it is not fully understood.

HBV contains a small (3.2 kb), circular, partially double-stranded DNA genome. This genome includes four, partly overlapping open reading frames: the precore/core gene encoding for the hepatitis B e antigen (HBeAg) and the core antigen (HBcAg), the polymerase gene encoding for the polymerase protein, the preS/S gene encoding for the hepatitis B surface antigen (HBsAg), and the X gene encoding for the X protein. In the course of replication, four kinds of viral RNAs, 3.5, 2.4, 2.1, and 0.8 kb in size, are transcribed (Ganem and Varmus, 1987). There are two types of 3.5 kb RNAs whose 5′ termini and functions are different: the pregenomic RNA (pgRNA) and the precore mRNA. The pgRNA, which is encapsidated as the template for reverse transcription to generate viral DNA, serves as the mRNA for HBcAg and the polymerase protein (Summers and Mason, 1982). The precore mRNA is translated into the precore/core fusion protein that is post-translationally modified to HBeAg (Roossinck et al., 1986; Standring et al., 1988). The transcription of the pgRNA and the precore mRNA are regulated by the core promoter corresponding to nucleotide (nt) 1613–1849 (Kramvis and Kew, 1999). The double core promoter mutations of A1762T/G1764A, which were frequently observed in HBeAg-negative chronic hepatitis patients (Okamoto et al., 1994) and fulminant hepatitis patients (Sato et al., 1995), were reported to reduce the production of HBeAg and enhance the replication of HBV in an in vitro study (Buckwold et al., 1996; Moriyama et al., 1996). It was also documented that the precore mutation of G1896A, which makes a stop codon and abrogates HBeAg (Carman et al., 1989), was associated with fulminant hepatitis (Liang et al., 1991; Omata et al., 1991), and the enhanced replication of HBV with G1896A in vitro has been described (Ozasa et al., 2006; Scaglioni et al., 1997).

Recently, we reported five consecutive cases of fulminant hepatitis B that were caused by the same strain of HBV (Nagasaki et al., 2008). The full-length sequences of HBV obtained from them

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were 99.8–100% identical to each other. This strain belonged to genotype B2 (Ba), which was reported to cause fulminant hepatitis less frequently than genotypes A, C, D, and other subgenotypes of genotype B (Ozasa et al., 2006). The fulminant strain was found to have several mutations in the core promoter and precore regions. The aim of this study is to investigate the significance of these mutations in the pathogenesis of fulminant hepatitis, using an in vitro culture system.

Results

Mutations in the fulminant strain of HBV

In the fulminant HBV strain FH-2, many mutations throughout the genome were observed. The full-length sequence (accession number: AB302943) (Nagasaki et al., 2008) was compared with a genotype B2 consensus sequence that was deduced from 52 full-length sequences deposited on GenBank/EMBL/DDBJ. A total of 45 nucleotide mutations, including the double core promoter mutations of A1762T/G1764A and the precore stop mutation of G1896A, were found. In the precore region, a distinctive mutation of G1862T located within the bulge of the ε signal as an RNA structure (Fig. 1C) was also found. Other nucleotide mutations were not found in the core promoter and precore regions except for G1632C. Because nt 1632 varies in the known genotype B2 strains, the nucleotide was considered not to have an important role in the pathogenesis of fulminant hepatitis.

As for amino acid (aa), 17 mutations in the polymerase gene, 7 mutations in the preS/S gene, 6 mutations in the precore/core gene, and 6 mutations in the X gene were observed (Table 1). Focusing on the core promoter and precore regions, an amino acid mutation of residue 17 of Val to Phe (V17F) in the precore region was found besides the precore stop mutation and the double core promoter mutations (corresponding to aa 130 and 131 in the X gene). V17F in the precore region corresponds to the G1862T mutation. We suspected that the nonsynonymous mutations of G1862T might have some effects on the development of fulminant hepatitis, besides A1762T/G1764A and G1896A.

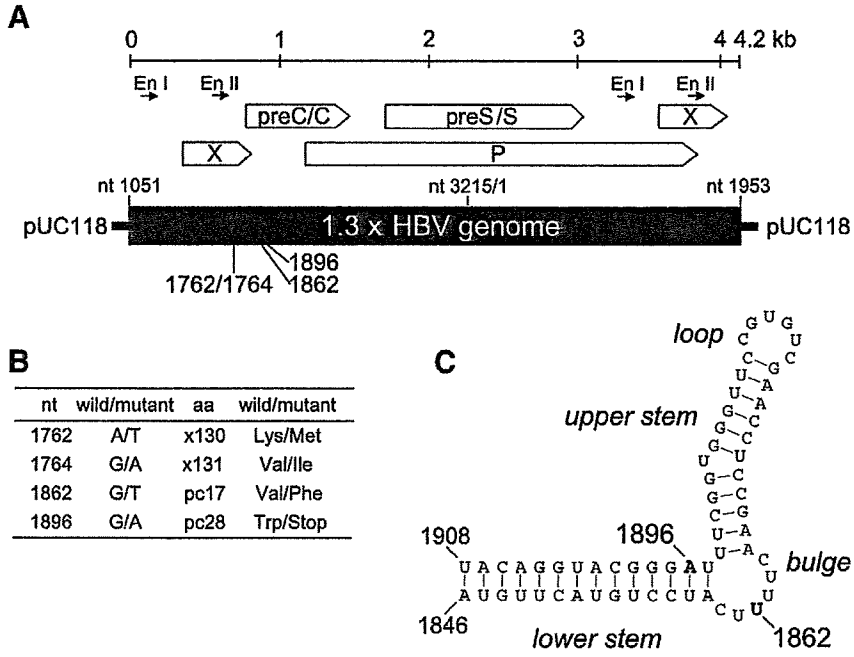
Validation of the replication capacity and the transfection efficiency

A plasmid containing 1.3-fold genome of the FH-2 strain, named pBFH2, was transfected to HepG2 or Huh7 cells, and the replication capacity of the strain was compared in these cell lines. Because the amount of HBsAg and HBV DNA in the culture medium was significantly larger in HepG2 than Huh7 (Fig. 2A), HepG2 was used in the following experiments.

To investigate the significance of the mutations of A1762T/G1764A, G1862T, and G1896A in the FH-2 strain, pBFH2 and its variants were transfected to HepG2 cells and compared. First, the efficiency of transfection was validated using the SEAP reporter system. It was shown that the differences in the activity of SEAP between pBFH2 and its variants coincided with that of expressed HBsAg (Fig. 2B). Therefore, the assay of HBsAg was thought to be suitable for the validation of the transfection efficiency in this system.

Intracellular replicative intermediates of HBV

Southern blotting analysis was performed using HepG2 collected 3 days after transfection. Intracellular core particle-associated HBV DNA was shown as replicative intermediates such as relaxed circular DNA, double-stranded linear DNA, and single-stranded DNA (Fig. 3A). The amount of intracellular HBV DNA was measured by densitometry (Fig. 3B). When compared with the all-wild construct (A1762/G1762, G1862, and G1896), the intracellular HBV DNA was decreased significantly in the construct with the single mutation of G1862T. This finding was in agreement with a previous report (Guarnieri et al., 2006). However, the suppression of HBV replication was overcome by the coexisting mutation of A1762T/G1764A or G1896A. Moreover, only the all-mutant original construct, pBFH2, showed significantly larger amounts of intracellular HBV DNA than the all-wild construct. The double mutations of



nt	wild/mutant	aa	wild/mutant
1762	A/T	x130	Lys/Met
1764	G/A	x131	Val/Ile
1862	G/T	pc17	Val/Phe
1896	G/A	pc28	Trp/Stop

Fig. 1. Construction of a plasmid (pBFH2) and mutations in the strain. (A) Schema of the plasmid containing 1.3-fold HBV genome and the positions of nucleotide mutations found in the core promoter and the precore region. (B) Nucleotide mutations and corresponding amino acid mutations in the core promoter and the precore region in pBFH2. (C) The structure of the ε signal as an RNA secondary structure. It consists of two base-paired segments (lower and upper stems), a bulge, and a loop. nt 1862 is within the bulge and nt 1896 is within the lower stem. En, enhancer; preC/C, precore/core; P, polymerase.

Table 1
Differences of amino acids between the B2 consensus sequence and the FH-2 strain.

Gene	aa position	B2 consensus[a]	FH-2
Polymerase	50	Ile	Thr
	93	Lys	Glu
	104	Asn	Thr
	118	Asn	Lys
	150	Thr	Ile
	212	Pro	Ser
	261	Gly	Asp
	266	His	Asn
	301	Ala	Thr
	464	Asn	Asp
	470	Asn	His
	474	Thr	Asn
	480	Asn	Asp
	566	Leu	Ile
	678	Ser	Arg
	679	Lys	Asn
	809	Thr	Ser (Phe)[b]
preS/S	81	Ala	Thr
	120	Met	Ile
	132	Gln	His
	214	Asn	Ser
	250	Cys	Tyr
	294	Pro	Thr
	358	Val	Ala
Precore/core	17	Val	Phe
	28	Trp	Stop
	106	Glu	Gln
	108	Pro	Gln
	113	Leu	Ala
	210	Ser	Pro
X	37	Leu	Val
	44	Val	Ala
	48	Asp	Ile
	87	Gly	Arg
	130	Lys	Met
	131	Val	Ile

[a] Deduced from 52 full-length sequences of genotype B2 HBV registered on GenBank/EMBL/DDBJ.
[b] Found in the other isolates obtained from consecutive cases of fulminant hepatitis.

A1762T/G1764A, or the triple mutations of A1762T/G1764A and G1896A, had a tendency to increase the replicative intermediates, but not significantly. The single G1896A mutation did not seem to increase them.

Released HBV virions and HBeAg

The amount of the yielded HBV virions in the culture supernatant of pBFH2-transfected HepG2 was assayed by real-time PCR and compared with pBFH2-variant constructs (Fig. 4). The single mutation of G1862T reduced it significantly whereas the double mutation of A1762T/G1764A increased it. Notably, the amount was not increased significantly for pBFH2, in contrast to the result of intracellular HBV DNA. These data suggested that pBFH2 might cause accumulation of the replicative intermediates in the cells due to the mutations in the core promoter and precore region.

The secreted HBeAg was reduced in the pBFH2-variant constructs with A1762T/G1764A and/or G1896A expectedly (Fig. 4), but the effect of A1762T/G1764A was limited in this study. Consistent with a previous report (Guarnieri et al., 2006), G1862T did not seem to affect the expression of HBeAg.

Distribution of HBcAg in HepG2 cells

To investigate the mechanism of the retention of viral replicative intermediates in the cells, the relationship between the distribution of HBcAg in the cells and the mutations of HBV was analyzed using confocal microscopy (Fig. 5). The all-wild construct showed weak HBcAg staining mainly in the nucleus. The construct with only A1762T/G1764A showed the predominant distribution of HBcAg in the cytoplasm, which was concordant with a previous report (Kawai et al., 2003; Liu et al., 2009). The predominant distribution of HBcAg in the nucleus was observed in the construct with the single mutation of G1896A. Although the construct with only G1862T showed HBcAg staining mainly in the cytoplasm, strong staining of HBcAg was observed in the nucleus of the transfected cells of the all-mutant construct, pBFH2. The relationship between the predominance of the HBcAg distribution and the amount of intracellular/extracellular HBV DNA is shown in Table 2. The constructs demonstrating predominant HBcAg distribution in the cytoplasm, such as the construct having only A1762T/G1764A, had a tendency to yield a large amount of extracellular HBV DNA in comparison with intracellular HBV DNA. In contrast, pBFH2, which produced the largest amount of intracellular HBV DNA, showed HBcAg distribution in the nucleus. Whereas the HBcAg distribution in the cytoplasm might lead to the efficient release of the HBV particles, its distribution in the nucleus might be related to the retention of replicative intermediates in the cells. The same results were obtained also using a mouse monoclonal anti-HBcAg antibody (Hyb-3120; Institute of Immunology, Tokyo, Japan), which recognizes a capsid conformation-specific epitope and not HBeAg (Conway et al., 2003) (data not shown). Taken together, the presence of all of the mutations of A1762T/G1764A, G1896A, and G1862T was considered to work together to accumulate HBV DNA in the cells via retention of HBV core particles in the nucleus.

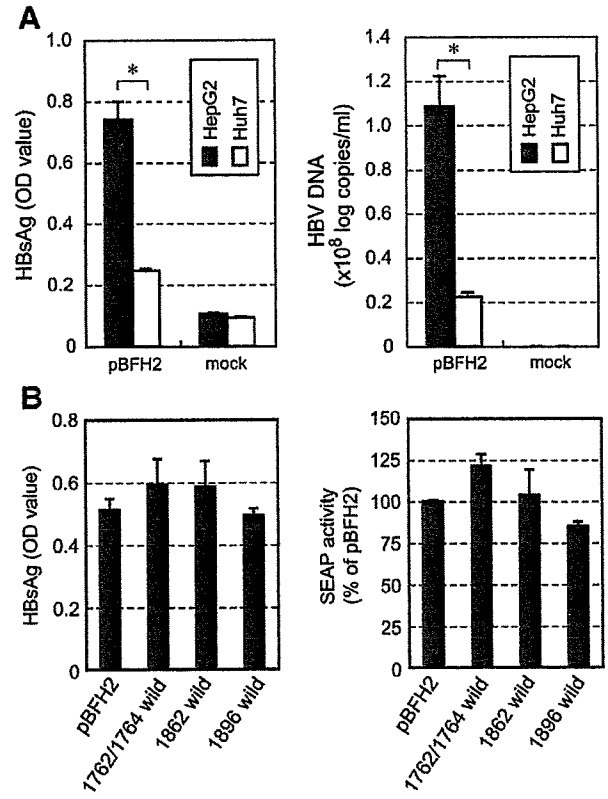


Fig. 2. (A) Comparison of replicative capacity of the FH-2 strain in HepG2 and Huh7. HBsAg and HBV DNA in the culture medium were assayed by ELISA and real-time PCR, respectively. *, $P<0.05$. (B) Validation of transfection efficiency using the SEAP reporter system. The SEAP activity in the culture supernatant was compared with HBsAg, and it was evaluated to determine whether the HBsAg assay was appropriate for the validation of transfection efficiency. Values represent means of triplicate experiments ± standard deviation.

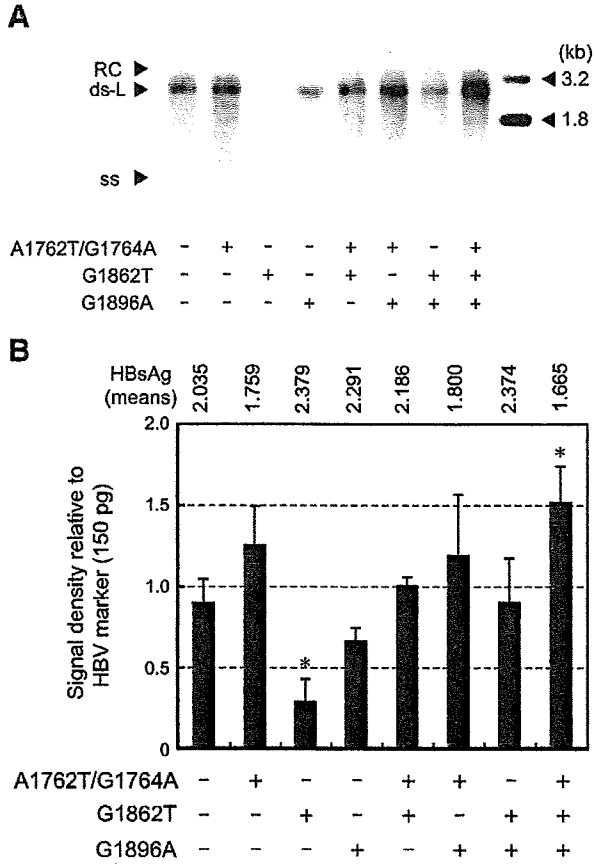


Fig. 3. Detection of replicative intermediates of HBV by Southern blotting analysis. (A) A result of a representative experiment of Southern blotting analysis. + indicates the mutant nucleotides in each nucleotide and − indicates the wild-type nucleotides. RC, relaxed circular HBV DNA; ds-L, double-stranded linear HBV DNA; ss, single-stranded HBV DNA. (B) Amount of replicative intermediates measured by densitometry, in comparison with the density of 150 pg HBV marker. The bars indicate the mean value of triplicate experiments ± standard deviation. Means of HBsAg assayed by ELISA were shown as markers for transfection efficiency. *, $P<0.05$ in comparison with the all-wild construct.

Distribution of HBcAg in tissue samples

To evaluate the results of in vitro study, immunohistochemical examination of liver specimens was performed using the same primary antibody to HBcAg (Fig. 6). In the samples obtained from three of five consecutive fulminant hepatitis B patients, both the nucleus and the cytoplasm of hepatocytes were stained (Figs. 6A–C). In contrast, nuclear staining was rarely observed in acute hepatitis B patients (Figs. 6D–F). In a chronic hepatitis B patient for positive control, nuclear staining was observed (Fig. 6G). Although the cytoplasmic HBcAg was observed more strongly in the tissue samples of fulminant hepatitis than the pBFH2-transfected cells in vitro, it was shown that HBcAg was retained in the nucleus of hepatocytes of these fulminant hepatitis patients. It was considered that the findings from the tissue samples supported the results from pBFH2-transfected cells, in which the core particle-associated replicative intermediates of HBV were retained.

Discussion

A large body of evidence has demonstrated that HBV-related liver disease is immune-mediated (Chisari, 1997; Kondo et al., 1997; Perrillo, 2001). Whereas strong and multispecific T cell responses are observed during acute HBV infection, patients with chronic hepatitis B tend to have weak and narrowly focused immune responses (Chisari, 1997). However, immunocompromised hosts such as patients undergoing hemodialysis can develop fulminant hepatitis B (Igaki et al., 2003), indicating that both viral factors as well as the host immune response are involved in the pathogenesis of fulminant hepatitis. In our study, all of the five consecutive patients with fulminant hepatitis B were over 60 years old and might have had relatively weaker immune responses to HBV. Therefore, the HBV strain obtained from them is considered to be appropriate for the study of the viral factors that lead to fulminant hepatitis.

The fulminant strain of genotype B2 in this study had several mutations including A1762T/G1764A and G1896A, and additionally, G1862T was found in the precore region. It was reported that G1862T was found frequently in the HBV of fulminant hepatitis patients without A1762T/G1764A and G1896A in China, where genotype B2 HBV is prevalent (Hou et al., 2002). In that report, G1862T caused a reduction of HBeAg, which might be associated with the development of fulminant hepatitis. However, another report using a genotype D strain described that the mutation did not change the expression of HBeAg but reduced the expression of the core protein and impaired HBV replication (Guarnieri et al., 2006). These reports are controversial and further analysis of this mutation is needed.

This study demonstrated that the precore G1862T mutation caused the accumulation of the core particle-associated HBV DNA in the cells, in conjunction with the core promoter A1762T/G1764A and precore G1896A mutations. nt 1862 could have two possible functions in the replication of HBV based on the position in the precore

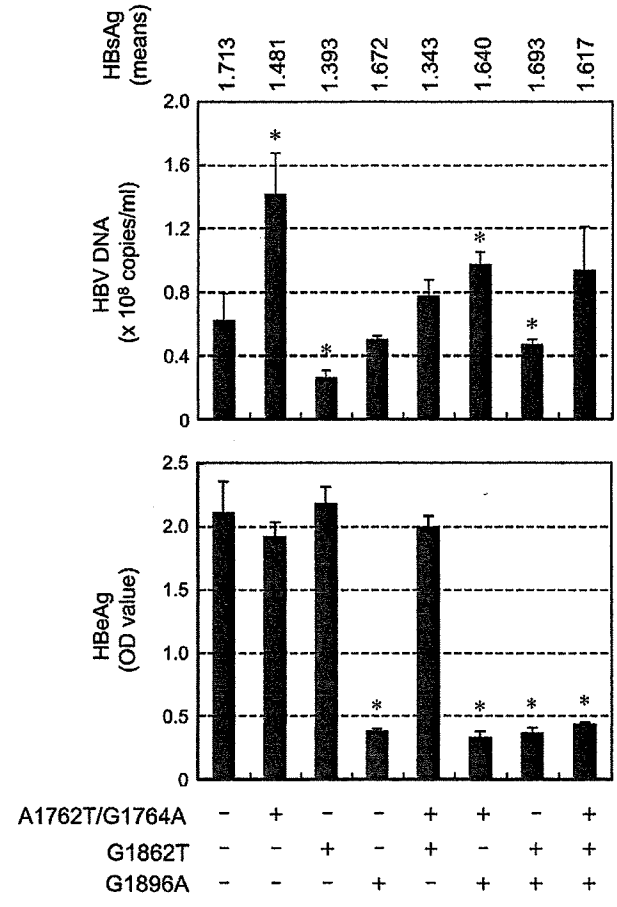


Fig. 4. Released HBV particle and HBeAg in the culture supernatant. The amount of HBV particle was measured by real-time PCR and HBeAg was assayed by ELISA. In the top, the means of HBsAg in the culture supernatant are shown. Values represent means of triplicate experiments ± standard deviation. *, $P<0.05$ in comparison with the all-wild construct.

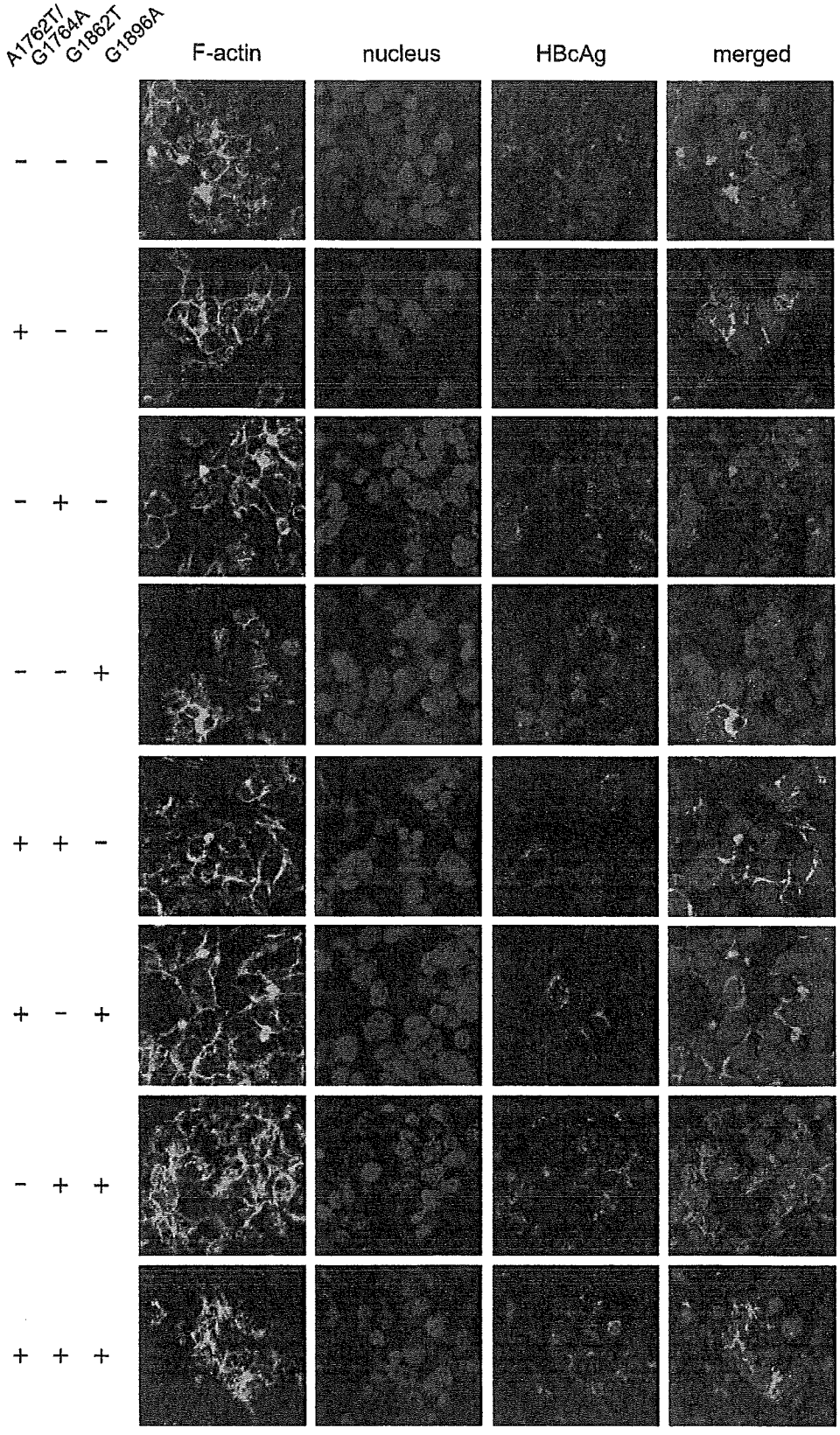


Fig. 5. Comparison of the distribution of HBcAg in the transfected HepG2 cells observed by confocal microscopy according to the HBV mutations. HBcAg was stained using a rabbit polyclonal anti-HBcAg antibody (Dako), and F-actin and nucleus were stained simultaneously.

Table 2
The summarized results of the intracellular/extracellular HBV DNA and distribution of HBcAg in the nucleus according to HBV mutations.

HBV mutations			Intracellular	Extracellular	HBcAg distribution[b]
A1762T/G1764A	G1862T	G1896A	HBV DNA[a]	HBV DNA[a]	
−	−	−	+	+	N>C
+	−	−	+	++	C>N
−	+	−	±	±	C>N
−	−	+	+	+	N>C
+	+	−	+	+	C>N
+	−	+	+	++	C>N
−	+	+	+	±	N>C
+	+	+	++	+	N>C

[a] ++ indicates that the amount of HBV DNA was significantly larger than the all-wild (A1762/G1764, G1862, and G1896) construct, and ± indicates that the amount was significantly lower than the all-wild construct. + indicates no significant change relative to the all-wild construct.

[b] N>C indicates that the cells having HBcAg in the nucleus were observed more predominantly than those having HBcAg in the cytoplasm, and C>N indicates the opposite frequency.

sequence: (i) its mutation converts aa 17 of valine to phenylalanine in the precore peptide and can affect processing of the precore/core protein into HBeAg (Chen et al., 2008; Hou et al., 2002) and (ii) it is within the bulge of the ε signal, which works as the template for the primers to synthesize the negative-strand DNA (Nassal and Rieger, 1996), and is recognized by HBV polymerase in the encapsidation of the pgRNA into the core particle (Rieger and Nassal, 1995). As for the former, aa 17 in the precore peptide is located at the −3 position of the signal peptidase cleavage site, and phenylalanine in this position is considered a "forbidden" amino acid, which may abrogate the cleavage of the precore/core protein by the signal peptidase. Although the effect of G1862T on the production of HBeAg is controversial (Chen et al., 2008; Guarnieri et al., 2006; Hou et al., 2002), it could be supposed that the single mutation of G1862T has a suppressive effect on HBV replication, at least in in vitro studies using genotype D strains (Chen et al., 2008; Guarnieri et al., 2006). A protein of 22 kDa (p22) including precore peptide, which is a product from the precore/core protein other than HBeAg, was reported to inhibit encapsidation (Kimura et al., 2005; Scaglioni et al., 1997). Hence, it was suspected that the possibly increased p22, which might not be cleaved due to G1862T, might lower the capacity of HBV encapsidation and replication. The finding in this study that the replication capacity was overcome by the precore stop mutation G1896A, which abrogates the precore/core protein, supports this speculation. In view of the function of the bulge of the ε signal, G1862T could make the extended templates for the initiation of the negative-strand DNA replication. Whereas the primer of TGAA is made using the 3′-part of the bulge as the template in wild-type HBV, the extended primer of TGAAA or TGAAAA which can anneal to the direct repeat 1 (DR1) and the continued 5′ nucleotides (5′-CTTTTTCACCTCTGCCT-3′, italic-typed nucleotides are the DR1 sequence and underlined nucleotides are complement to the extended primer) can be made in the presence of G1862T. Although it is unknown whether the extended primer can actually initiate the extension of the negative-strand DNA, the primer may have some positive effects on reverse transcription. Some of the possible effects of G1862T on the viral replication can be affected by other mutations and the phenotype may be changed.

The core promoter mutation of A1762T/G1764A, which is known to enhance the transcription of pgRNA and reduce the precore mRNA (Moriyama et al., 1996), was described as being associated with the cytoplasmic distribution of HBcAg (Kawai et al., 2003; Liu et al., 2009). In this study, the localization of HBcAg was changed to the nucleus when both G1862T and G1896A were present. Whereas HBcAg is a 21 kD protein (p21) and can be transported across the nuclear membrane by diffusion, the core particle, which consists of approximately 180 subunits of a core protein, cannot diffuse across it (Forbes, 1992; Kawai et al., 2003). It has been speculated that high density HBcAg resulting from the increased pgRNA in the presence of A1762T/G1764A might favor the formation of the core particle in the cytoplasm, and the release of the particle as an infectious virus particle after envelopment. A part of the incompletely processed precore/core protein, p22, can also assemble into the core particle, but the particle cannot encapsidate the pgRNA (Scaglioni et al., 1997). The empty core particle could be detected also in this study using the polyclonal anti-HBcAg antibody. As G1862T reduces HBcAg expression (Guarnieri et al., 2006) and G1896A abrogates the precore/core protein, the presence of these mutations may lower the density of p21 and p22 leading to inefficient assembly of the core particle in the cytoplasm. After that, unassembled p21 is transported across the nuclear membrane by the function of the putative nucleolar localization signal of HBcAg (Ning and Shih, 2004) and be assembled in the nucleus, followed by formation of the core particle. As the particles cannot be transported across the nuclear membrane, they may accumulate in the nucleus.

The core promoter mutations of A1762T/G1764A change overlapping X protein, and there is a possibility that the change of X protein may have effect on HBV replication or localization. A recent

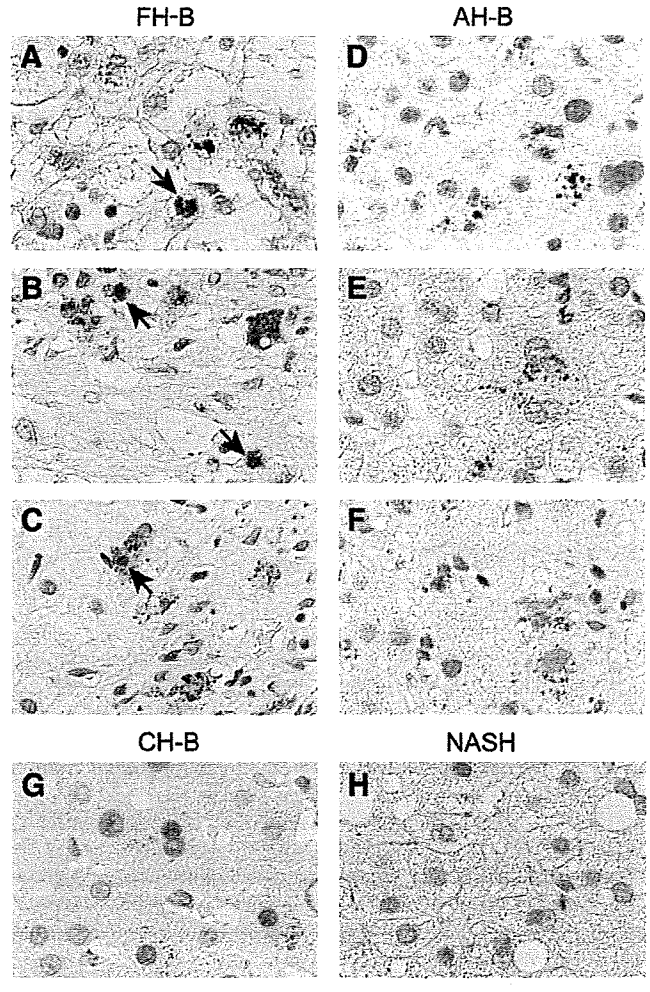


Fig. 6. HBcAg immunohistochemical examination of the liver tissue samples obtained from fulminant hepatitis B (FH-B) patients and acute hepatitis B (AH-B) patients using a rabbit polyclonal anti-HBcAg antibody (Dako). (A–C) Tissue samples of three of five consecutive fulminant hepatitis B patients from whom the FH-2 strain was isolated. The arrows indicate the hepatocytes with nuclear HBcAg staining. (D–F) Tissue samples obtained from three acute hepatitis B patients. (G) A sample obtained from a chronic hepatitis B (CH-B) patient as positive control. (H) A sample of a nonalcoholic steatohepatitis (NASH) patient as negative control.