Table 4 Multivariate analysis of predictors of survival after initial diagnosis of extrahepatic metastases among 151 patients

Variable	Hazard ratio	95% CI	P
PS (0 *vs* 1-4)	5.576	2.431-12.152	< 0.001
Vp (0-2 *vs* 3, 4)	4.792	2.137-10.712	< 0.001
Treatment (performed *vs* not performed)	4.134	1.539-11.011	0.003
Child pugh stage (A *vs* B, C)	2.372	1.247-4.914	0.008

Causes of death

Twenty-five patients were still alive at the end of this study while 126 patients died. Of the latter group, intrahepatic tumor stages at the first diagnosis of extrahepatic metastases were T0-2 in 17 patients and T3-4 in 109 patients. One hundred and twelve (89%) patients died of intrahepatic HCC or liver failure. Fourteen (11%) patients died of extrahepatic HCC (Table 5). Eight patients died of respiratory failure due to lung metastases. Four patients died of bone metastases-related disease. Two patients died of obstructive jaundice due to portohepatic node metastasis.

Of the 4 patients who died of bone metastases-related disease, 3 died of intracranial hypertension due to skull metastasis. Another patient died of vertebra metastasis-related disease. He was 69-year old at first diagnosis of bone metastases. He suffered from complete spinal cord injury due to vertebral metastasis with gradual worsening of PS. Finally, PS changed to 4 and the patient died of aspiration-related pneumonia. The survival period after first diagnosis of extrahepatic metastases was 11.5 mo.

Among the 14 patients who died of extrahepatic HCC, 3 had chronic hepatitis, 7 had cirrhosis of Child-Pugh grade A, 3 had cirrhosis of Child-Pugh grade B, and 1 had cirrhosis of Child-Pugh grade C. All patients who died of extrahepatic HCC with the exception of that with Child-Pugh grade C had some hepatic reserve until death. Intrahepatic tumor stage at first diagnosis of extrahepatic metastases was T0 (3 patients), T1 (4 patients), T2 (1 patient), T3 (5 patients), and T4 (1 patient). All 8 patients with intrahepatic tumor stage T0-T2 were treated previously for intrahepatic HCC. Eight of 17 (47%) patients with intrahepatic tumor stage T0-T2 died of extrahepatic metastases. On the other hand, 6 of 109 (6%) patients with intrahepatic tumor stages T3 and T4 died of extrahepatic metastases. The mortality rate of patients with intrahepatic tumor stage T0-T2 was significantly higher than that of patients with intrahepatic tumor stages T3 and T4 ($P = 0.001$) (Table 6).

Table 5 Clinical profile of 14 patients who died of extrahepatic metastases during the follow-up period

Case	Presentation	Site	Intrahepatic HCC stage	Sex	Age (yr)	Child-Pugh stage	Etiology
1	R	Lung	T3	M	65	A	HCV
2	R	Lung	T4	M	35	CH	HBV
3	R	Lung	T3	M	56	A	HBV
4	R	Lung, vertebra	T0	M	40	CH	HBV
5	R	Lung, vertebra	T1	M	69	A	HBV
6	R	Lung, LN	T0	M	63	B	HBV
7	R	Lung, vertebra, nasal	T0	M	50	A	HBV
8	R	Lung	T3	M	73	A	NBNC
9	I	Skull	T1	M	57	A	HCV
10	I	Skull	T2	F	72	C	HCV
11	I	Skull	T3	M	56	B	HCV
12	A	Vertebra	T3	M	69	A	HCV
13	O	Lung, rib, LN	T1	M	74	A	HCV
14	O	Vertebra, LN	T1	M	70	B	HCV

All patients with intrahepatic tumor stage T0-T2 were treated previously for intrahepatic HCC. R: respiratory failure; CH: chronic hepatitis; LN: lymph node; NBNC: no hepatitis B virus or hepatitis C virus; I: intracranial hypertension symptom; A: aspiration-related pneumonia; O: obstructive jaundice.

Table 6 Causes of death of 126 HCC patients with extrahepatic metastases

Intrahepatic tumor stage	Intrahepatic HCC or liver failure	Extrahepatic HCC
T0-2 ($n = 17$)	53% (9/17)	47% (8/17)
T3-4 ($n = 109$)	94% (103/109)	6% (6/109)

DISCUSSION

The prognosis of HCC patients with extrahepatic metastases is unsatisfactory[16,17] and often not well known[18]. In the present study, we assessed the clinical features and prognosis of 151 consecutive HCC patients with extrahepatic metastases. The incidence of extrahepatic metastases from HCC was 15.2%. The most frequent metastatic sites were the lung, lymph nodes, bone, and adrenal gland. The cumulative survival rates of the 151 patients after the initial diagnosis of extrahepatic metastases at 6, 12, 24, and 36 mo were 44.1%, 21.7%, 14.2%, 7.1%, respectively. The median survival period was 4.9 mo (range, 1-37 mo). The mortality rate due to extrahepatic metastases from HCC was 11% (14/126).

Extrahepatic metastases have been reported to occur in 13.5%-42% of HCC patients[22-24]. In this study, the prevalence of extrahepatic metastases was 15.2%. Though we screened all HCC patients at regular intervals for intra/extra hepatic metastases, not all patients received a full metastatic follow up based on the use of several diagnostic techniques. Since the majority of HCC patients with extrahepatic metastases were asymptomatic, it is possible to miss asymptomatic metastases such as those in the lungs, distant lymph nodes, muscles and rectum.

Based on the initial diagnosis of intrahepatic HCC, Natsuizaka *et al*[16] reported that patients with advanced HCC develop extrahepatic metastases significantly more frequently than those with less advanced HCC. At the initial diagnosis of extrahepatic metastases, many HCC patients with extrahepatic metastases have been reported

to have advanced intrahepatic stage[16,22]. In our study, 123 (81%) patients with extrahepatic metastases had intrahepatic tumor stages T3 (28%) and T4 (53%), at the initial diagnosis of extrahepatic metastases, suggesting that HCC patients with advanced intrahepatic tumor stage (T3, T4) are at risk of developong extrahepatic metastases, and that such patients should be followed up carefully.

On the other hand, our study identified 28 (19%) patients with early intrahepatic tumor stage (T0-T2) at the initial diagnosis of extrahepatic HCC. Eight of the 17 (47%) patients later died of extrahepatic metastases. With regard to previous treatment, 27 of 28 patients with early intrahepatic tumor stage were treated previously for intrahepatic HCC. Considering the possibility of extrahepatic metastases, HCC patients with early intrahepatic tumor stage should be followed up carefully, particurally those who have been treated previously for intrahepatic HCC. This also includes HCC patients who have received complete resection or ablation.

In this study, the most frequent metastatic sites were the lungs, lymph nodes, bones, and adrenal glands. Other studies have reported similar findings[16,22]. HCC is thought to spread mainly *via* the hematogenous route, thus causing intra/extra hepatic metastases. Most of HCCs are hypervascular tumors. Moreover, HCC tends to invade vessels, such as portal and hepatic veins. Therefore, HCC could spread through the lung and systemic circulation via the hepatic or portal vein. This could explain why the lung is the most frequent site of metastases in HCC. Most of HCC patients with lung metastases are asymptomatic. To detect lung metastases from HCC, chest CT should be performed at regular intervals during routine metastasis follow-up.

Though there is no standard treatment for extrahepatic metastases of primary HCC, several authors have reported the use of various treatment modalities for extrahepatic m etastases[7,15,23,25-29]. Some reports have described successful treatment of extrahepatic metastases with no or few intrahepatic HCC[7,25-27]. However, only few HCC patients can undergo surgical resection of extrahepatic metastases because of hepatic reserve or intraheatic tumor stage. In this study, the prognosis of 3 patients after surgical resection of extrahepatic metastases seemed good. These 3 patients had good hepatic reserve, no intrahepatic HCC (PS = 0) and no intra/extra hepatic HCC and are expected to have good prognosis. The clinical features of HCC patients with extrahepatic metastases varied widely. All patients were not symptomatic and thus not necessary to receive treatment of extrahepatic metastases. Thus, treatment of extrahepatic metastases from primary HCC must be performed carefully taking into consideration the clinical features.

Multivariate analysis in our study identified PS, portal venous invasion, treatment for extrahepatic metastases, and Child-Pugh grade as important determinants of survival after the initial diagnosis of extrahepatic metastases. Ishii *et al*[17] reported that brain metastases, number of metastatic tumors and primary tumor status are important factors for survival. In our study, only two patients had brain metastases. With regard to the number of metastatic tumors, we might miss asymptomatic metastases. Thus,

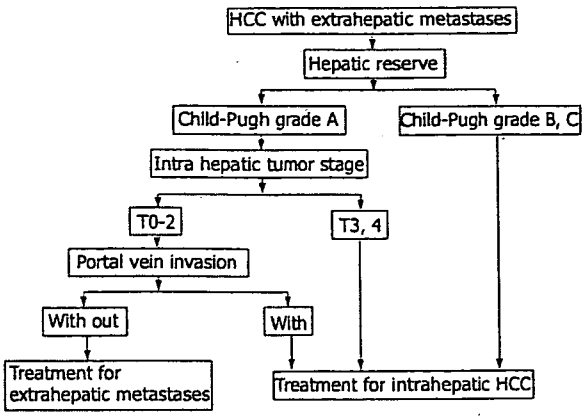

Figure 2 Initial sites to be treated.

we did not include brain metastasis and number of metastatic tumors in this multivariate analysis. Treatment of extrahepatic metastases was an important determinant of survival in our study. There might be selection bias of patients treated for extrahepatic metastases because many of them had good hepatic reserve. HCC patients with poor hepatic reserve did not receive treatment for extrahepatic metastases in this study. Regardless of such bias, treatment of extrahepatic metastases might be important for improvement of prognosis.

With regard to the cause of death, many HCC patients with extrahepatic metastases died of intrahepatic HCC or liver failure and few (11%) died of extrahepatic HCC. Of the 14 patients who died of extrahepatic metastases, 10 had good hepatic reserve and 8 had early intrahepatic tumor stage, at the initial diagnosis of extrahepatic metastases. Usually, HCC patients with good hepatic reserve, no or few intrahepatic HCCs, and those without portal venous invasion show relatively good prognosis. According to the univariate analysis of HCC patients with extrahepatic metastases, patients with early intrahepatic tumor stage have a significantly better prognosis than those with advanced intrahepatic tumor stage. In our study, the mortality rate due to extrahepatic metastases with early intrahepatic tumor stage was significantly higher than that due to those with advanced intrahepatic tumor stage. This might be explained by the differences in survival periods between these intrahepatic tumor stage groups. Extrahepatic metastases with early intrahepatic tumor stage can spread during the relatively long survival period, and few patients die of extrahepatic metastases. Extrahepatic metastasis with early intrahepatic tumor stage is a very important cause of death of HCC patients. Successful treatment of extrahepatic metastases in HCC patients with early intrahepatic tumor stage might improve the prognosis.

In conclusion, the majority of HCC patients with extrahepatic metastases should undergo treatment for intrahepatic HCC. Selected HCC patients with critical extrahepatic metastases could undergo treatment for extrahepatic metastases. However, these selected patients must have good hepatic reserve, intrahepatic tumor stage: T0-T2, and are free of portal venous invasion (Figure 2). The important sites of critical metastases from primary

HCC are the lungs, bones and the portohepatic node. Further studies are needed for the improvement of the prognosis of HCC patients with extrahepatic metastases.

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Feasibility of Freeze-Dried Sera for Serological and Molecular Biological Detection of Hepatitis B and C Viruses[▽]

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We compared hepatitis B virus (HBV) surface antigen, anti-hepatitis C virus (HCV) antibody, and HCV RNA quantification in frozen and freeze-dried serum samples to assess the usefulness of freeze-dried sera for detection of HBV and HCV. The results indicated that freeze-dried sera as well as frozen sera can be useful for serological and molecular biological analyses of HBV and HCV.

Freeze-dried sera are generally used as standards for hematobiochemical tests. In frozen and freeze-dried sera, protein, lipid, and electrolyte levels remain relatively stable, but enzyme levels show a greater degree of variation in freeze-dried sera than frozen sera (6). Nevertheless, freeze-dried sera can be stored at room temperature for a long time and are therefore easier to handle than frozen sera.

For the study of hepatitis B virus (HBV), dried blood spot (DBS) samples have been used for detecting hepatitis B virus surface antigen (HBsAg) and antibody to hepatitis B core antigen (2, 8). Recently, DBS samples allowed the development of a simple, sensitive, and appropriate test for quantifying HBV DNA and studying HBV genetic variants (5). As for hepatitis C virus (HCV), dried sera are used for the test of anti-HCV antibody (Ab) (2), and DBS samples allowed the development of a simple, sensitive, and reliable test for detection and genotyping of HCV RNA (1, 7). However, there is no report on their usefulness in HCV RNA quantification. We conducted serological and molecular biological tests to detect HBV and HCV using frozen and freeze-dried serum samples to determine the feasibility of freeze-dried sera.

The Atomic Bomb Casualty Commission established the Adult Health Study (AHS) longitudinal cohort in 1958; since then, the Atomic Bomb Casualty Commission and its successor, the Radiation Effects Research Foundation (RERF), have examined about 20,000 atomic-bomb survivors and controls biennially in outpatient clinics in Hiroshima and Nagasaki. We selected at random 12 consecutive HBsAg-positive and 25 consecutive anti-HCV Ab-positive individuals among 6,121 AHS longitudinal cohort subjects who underwent hepatitis screening from 1993 through 1995. Their serum samples were stored by both freezing and freeze-drying methods.

First, the procedure used for the preparation of frozen serum samples was as follows: Blood obtained from the AHS subjects was kept at room temperature for 20 min. Serum was then divided into four equal parts and stored in 1.5-ml polypropylene tubes at −80°C until use. These samples were thawed by leaving them at room temperature for 30 min and mixed well by inversion before use. Second, the procedure used for the preparation for freeze-dried serum samples was as follows. A 0.4-ml portion of the serum was separated as mentioned above and stored in a glass tube at −80°C. After 1 week of storage, the samples were freeze-dried using a freeze-dryer, sealed, and stored at room temperature (20 to 25°C) until use. These samples were reconstituted by the volumetric method using diethyl pyrocarbonate-treated Milli-Q water and mixed well before use.

The tests for HBsAg and anti-HCV Ab using fresh serum samples in hepatitis screening from 1993 through 1995 were described previously (3, 4). In screening tests, an anti-HCV Ab titer of $\geq 2^{12}$ was defined as a high titer. In the present study, HBsAg and anti-HCV Ab were measured by enzyme immunoassay (EIA) (International Reagents Corporation, Kobe, Japan) and second-generation EIA (International Reagents Corporation), respectively. Measured values of ≥1.0 for HBsAg and anti-HCV Ab were defined as positive. An anti-HCV Ab titer of ≥50 was defined as a high titer.

Serum RNA was extracted from 100 μl of frozen or reconstituted freeze-dried serum samples using SepaGene RV-R (SankoJunyaku Co., Tokyo, Japan). The prepared RNA was reverse transcribed with random primers (6-mer) and reverse transcriptase (ReverTra Ace; Toyobo Co., Tokyo, Japan). HCV RNA was quantitated by real-time PCR using fluorescence resonance energy transfer probes. Primers and probes were designed within a highly conserved 5′ untranslated region (UTR) and also targeted homologous regions of genotypes 1a, 1b, 2a, and 2b. The oligonucleotide sequences of the primers were as follows: HCVNC2, 5′-CCTGTGAGGAACTACTGTC-3′, and HCVNC1, 5′-CAACACTACTCGGCTAGCAGTC-3′. The hybridization probes were as follows. Probe NCJ-LC (5′-GAACCGGTGAGTACACCGGAAT) was labeled at the 5′ end with the fluorophore Red 640 and phosphorylated at the 3′ end. Another probe, NCJ-FL (5′-GGGAGAGCCATAGTGGTCTGC), was labeled with fluorescein isothiocyanate at the 3′ end. PCR was performed in a total volume of 20 μl,

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containing 5 mM $MgCl_2$, 6 pmol of NCJ-LC, 4 pmol of NCJ-FL, 10 pmol of the two PCR primers, 2 μl of LightCycler-FastStart DNA Master hybridization probe mix (Roche Diagnostics Co.), and 1 μl of synthesized cDNA solution. The PCR cycling program consisted of an initial denaturing step at 95°C for 10 min and 50 amplification cycles of 95°C for 15 s, 55°C for 6 s, and 72°C for 10 s. Once the threshold was chosen, the point at which the amplification plot crossed the threshold was defined as the threshold cycle (C_T). The calculated C_T value is predictive of the quantity of target RNA copies. The standard curve was calculated using serially diluted plasmids containing nucleotide sequences of the HCV 5′ UTR, to obtain control fragments for determination of HCV copy numbers. All assays were conducted in duplicate.

The positive-negative results of HBsAg in frozen and freeze-dried serum samples were consistent with results using fresh serum samples. The concordance in measurement of anti-HCV Ab among fresh, frozen, and freeze-dried serum samples was not complete but was satisfactory. Both frozen and freeze-dried serum samples of one case tested negative for anti-HCV Ab, despite testing positive in the 1993-1995 hepatitis screening. One freeze-dried serum sample of another case tested positive for anti-HCV Ab, despite testing negative in the 1993-1995 screening (Table 1). For these two patients with discrepant results, the specimen yielding a positive result contained only low titers of anti-HCV Ab; subsequent testing for HCV RNA by quantitative or qualitative PCR was negative in both cases (data not shown). Furthermore, 86% (18/21) of the fresh serum samples yielding high anti-HCV Ab titers by passive hemagglutination also yielded high anti-HCV Ab titers on subsequent testing of both frozen and freeze-dried serum samples by EIA; frozen and freeze-dried samples from the remaining three cases produced low-positive results. These results might be due to decay of the antibody during storage or differences in criteria for high antibody titer between the previous and present kits. However, the results for high anti-HCV Ab titers were completely consistent in frozen and freeze-dried serum samples.

TABLE 1. Comparison of detection of HBsAg and anti-HCV Ab in frozen, freeze-dried, and fresh serum samples

Substance tested, sample type, and EIA result[a]	No. of fresh serum samples with PHA[b] result		Concordance (%)
	Positive	Negative	
HBsAg			
Frozen			
Positive	12	0	100
Negative	0	25	
Freeze-dried			
Positive	12	0	100
Negative	0	25	
Anti-HCV Ab			
Frozen			
Positive	24	0	97
Negative	1[c]	12	
Freeze-dried			
Positive	24	1[c]	95
Negative	1[c]	11	

[a] HBsAg was measured by EIA; anti-HCV Ab was measured by second-generation EIA.
[b] HBsAg was measured with a reverse passive hemagglutination (PHA) test kit; anti-HCV Ab was measured with a second-generation PHA test kit.
[c] HCV infection status was negative with quantitative or highly sensitive qualitative PCR.

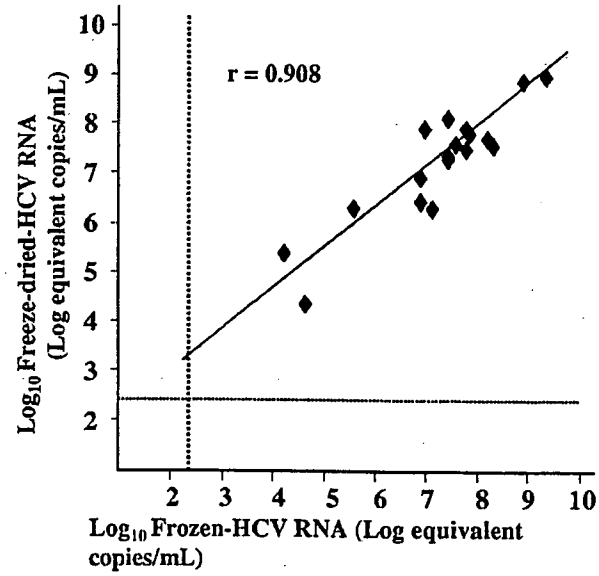

FIG. 1. Correlation of HCV RNA levels in frozen and freeze-dried serum samples. The correlation between $\log_{10}$-transformed HCV RNA quantities for pairs of frozen and freeze-dried serum samples from 18 cases analyzed by real-time PCR was significant ($r = 0.908$, $P < 0.0001$).

A linear relationship in the range of 2.0×10^2 to 2.0×10^9 equivalent copies/ml was observed between C_T values and quantity of RNA copies ($r > 0.99$) (data not shown). Real-time PCR detected HCV RNA in 18 of 25 frozen and freeze-dried serum samples from anti-HCV-positive cases in the 1993-1995 hepatitis screening. The correlation between HCV RNA concentration in frozen and freeze-dried serum samples was significant ($r = 0.908$, $P < 0.0001$) (Fig. 1).

The intra-assay variability was determined by assaying two frozen serum samples containing HCV RNA of genotype 1b and 2a (respectively, 9×10^5 and 1.3×10^6 copies/ml) 10 times in a single day, and the respective coefficients of variation (CVs) were 6.2% and 2.9%. The respective interassay CVs calculated by assaying each of these serum samples once a day for 10 days were 3.6% and 4.3%.

On the whole, results for fresh, frozen, and freeze-dried serum samples for HBsAg and anti-HCV Ab demonstrated very good agreement, indicating that these methods and storage conditions are appropriate for serological assays of HBV and HCV. Furthermore, results of a newly developed highly sensitive and high-range HCV RNA quantitative assay for frozen and freeze-dried serum samples showed good correlation. We expected that the PCR products of HCV RNA would vary depending on storage method and conditions; however, the results showed no marked differences during 10 years of storage. The use of sera of AHS subjects stored from 1969 can further advance the study of the evolution of HBV/HCV as well as the natural history of viral liver diseases.

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Emergence of a Novel Lamivudine-Resistant Hepatitis B Virus Variant with a Substitution Outside the YMDD Motif[▽]

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Lamivudine is a major drug approved for treatment of chronic hepatitis B virus (HBV) infection. Emergence of drug-resistant mutants with amino acid substitutions in the YMDD motif is a well-documented problem during long-term lamivudine therapy. Here we report a novel lamivudine-resistant strain of HBV with an intact YMDD motif, which included an amino acid substitution, rtA181T, in the reverse transcriptase (RT) domain of HBV polymerase. The substitution also induced a unique amino acid substitution (W172L) in the overlapping hepatitis B surface (HBs) protein. The YMDD mutant strains were not detected even by using the sensitive peptide nucleic acid-mediated PCR clamping method. The detected nucleotide substitution was accompanied by the emergence of an additional nucleotide substitution that induced amino acid change (S331C) in the spacer domain. The rtA181T mutant strain displayed a threefold decrease in susceptibility to lamivudine in in vitro experiments in comparison with the wild type. In vivo analysis using human hepatocyte-chimeric mice confirmed the resistance of this mutant strain to lamivudine. We developed a method to detect this novel rtA181T mutation and a previously reported rtA181T mutation with the HBs stop codon using restriction fragment length polymorphism PCR and identified one patient with the latter pattern among 40 patients with lamivudine resistance. In conclusion, although the incidence is not high, we have to be careful regarding the emergence of lamivudine-resistant mutant strains with intact YMDD motif.

Hepatitis B virus (HBV) is a small, enveloped DNA virus that causes chronic hepatitis and often leads to cirrhosis and hepatocellular carcinoma (4, 12, 33). To date, interferon and three nucleoside and nucleotide analogs (lamivudine, adefovir dipivoxil, and entecavir) have been approved by the United States Food and Drug Administration for the treatment of chronic HBV infection. Lamivudine, an oral cytosine nucleoside analogue, potently inhibits HBV replication by interfering with RNA-dependent DNA polymerase (10, 16, 22). Lamivudine therapy suppresses HBV replication in most patients and improves transaminase levels and liver histology (16, 22, 25, 30). However, prolonged therapy results in the emergence of drug-resistant mutants in 24% and 70% of patients after 1 and 4 years of therapy, respectively, followed by increases in viral load and re-elevation of transaminase levels (18).

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Most lamivudine-resistant strains show amino acid substitutions in the YMDD (tyrosine-methionine-aspartate-aspartate) motif in the C domain of HBV polymerase. In addition to the emergence of the YMDD mutation, rtL180M and rtV173L mutations in the B domain of HBV polymerase are frequently observed (1, 9). In vitro analyses have confirmed that the rtL180M mutation augments the level of lamivudine resistance and enhances viral replication, while the rtV173L mutation enhances only viral replication (9, 23). On the other hand, only a few uncommon mutations associated with lamivudine resistance have been reported so far (3, 7, 24, 34). In the C domain of HBV polymerase, rtM204S and rtD205N were detected in patients with lamivudine resistance (3, 7). In the B domain, rtL180C and rtA181T were associated with lamivudine resistance (7, 24, 34). Yeh et al. (34) reported the emergence of rtA181T mutants in 4 of 23 patients who received long-term lamivudine therapy. The mutant appeared concomitantly with or after emergence of YMDD motif mutants and persisted thereafter. The nucleotide substitution in the FLLA motif resulted in early termination of the overlapping HBs gene transcription by creating a stop codon (TGG to TGA). Yeh et al. (34) demonstrated that the mutation reduced the

susceptibility to lamivudine in vitro. They also detected such mutations in virus from a patient with leukemia and speculated that truncated HBs gene might be related to the development of leukemia (7).

Analyzing nucleotide and amino acid sequences of HBV in patients who developed a breakthrough, we identified a novel mutant that showed nucleotide substitutions in the B domain of the reverse transcriptase. The G residues of nucleotides 669 and 670 were mutated to T and A, respectively, and associated with the amino acid substitution rtA181T. The substitutions also induced the amino acid substitution W172L in the overlapping HBs protein. Since the nucleotide substitution was associated with nucleotide and amino acid substitutions in the putative spacer region of the polymerase, we checked the importance of these substitutions for resistance to lamivudine in vitro. We also analyzed the resistance of this new strain in vivo using a human hepatocyte-chimeric mouse (27, 31). Furthermore, we analyzed the susceptibility of the mutant strain to adefovir and entecavir. When used alone or in combination with lamivudine, these drugs are known to be effective against wild-type as well as lamivudine-resistant HBV (2, 5, 14, 17, 32). Infrequent emergence of resistance compared with lamivudine resistance has been reported for both of these two drugs (2, 5). We also developed a detection system to identify the novel and previously reported (7, 34) nucleotide substitutions to study the incidence of such mutations.

MATERIALS AND METHODS

Antiviral compounds. Lamivudine [(−)-β-L-2′,3′-dideoxy-3′-thiacytidine] was provided by GlaxoSmithKline (Stevenage, Herts, United Kingdom). Adefovir {9-[2-(phosphonomethoxy)ethyl]-adenine} was provided by Gilead Sciences (Foster City, CA), and entecavir {2-amino-1,9-dihydro-9-[(1S,3R,4S)-4-hydroxy-3-(hydroxymethyl)-2-methylenecyclopentyl]-6H-purin-6-one, monohydrate} was provided by Bristol-Myers Squibb Pharmaceutical Research Institute (Wallingford, CT).

Analysis of virological markers. Hepatitis B surface antigen (HBsAg), hepatitis B envelope antigen (HBeAg), and antibody against HBeAg (anti-HBe) were quantified by enzyme immunoassay kits (Abbot Diagnostics, Chicago, IL). HBV-DNA was measured by real-time PCR using a Light Cycler (Roche, Mannheim, Germany). The primers used for amplification were 5′-TTTGGGCATGGACA TTGAC-3′ and 5′-GGTGAACAATGTTCCGGAGAC-3′. The amplification condition included initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 5 s, and extension at 72°C for 6 s. The lower detection limit of this assay was 300 copies.

Cloning of HBV DNA and plasmid construction. HBV DNA was extracted from 100 μl of each serum sample by SMITEST (Genome Science Laboratories, Tokyo, Japan) and was dissolved in 20 μl H_2O. Full-length HBV DNA was amplified using the above HBV DNA samples by the method of Gunther et al. (13). Nucleotide sequence positions were numbered from the unique EcoRI site. The 1.4-genome-length HBV DNA amplified from the serum of a patient who showed lamivudine resistance was cloned into plasmid vector pTRE (Takara Bio, Tokyo, Japan) (patient strain). In brief, the PCR product amplified using serum from the patient was cleaved with BamHI and ApaI (HBV positions 1400 to 2600) and cloned into pcDNA3 (Invitrogen, San Diego, CA), and the resulting construct was named pcDNA3-1. Similarly, the PCR product was cleaved with ApaI and BamHI (HBV positions 2600 to 3215 and 1 to 1400) and cloned into pBlueScript SK+ (Stratagene, La Jolla, CA), and the resulting construct was named pB-1. The KpnI-BamHI fragment from pB-1 and the KpnI-ApaI fragment from pcDNA3-1 were cloned into pcDNA3-1. Finally, the plasmids were cleaved with HindIII and NotI within the multicloning site and cloned into plasmid vector pTRE. As a laboratory strain, we employed a plasmid containing a 1.4-genome-length wild-type genotype C HBV (wild-type strain; GenBank accession number AB206816) (31). To introduce the nucleotide substitutions into the S331C/rtA181T patient and wild-type strains, site-directed mutagenesis was performed with a QuikChange site-directed mutagenesis kit (Stratagene).

TABLE 1. In vitro susceptibility of the S331/rtA181 mutant to lamivudine[a]

Strain		S331/rtA181 mutation	Lamivudine IC_{50} (μM)	Resistance (fold)
Source	Type			
Patient	WT	−/−	0.19 ± 0.01	1
	S331C	C/−	0.23 ± 0.01	1.2*
	rtA181T	−/T	0.58 ± 0.08	3**
	S331C/rtA181T	C/T	0.57 ± 0.06	3**
Laboratory	WT	−/−	0.23 ± 0.04	1
	S331C	C/−	0.3 ± 0.05	1.3*
	rtA181T	−/T	0.88 ± 0.2	3.9**
	S331C/rtA181T	C/T	0.98 ± 0.12	4.3**

[a] Experiments were performed in triplicate. Values are expressed as means ± SD. WT, wild type. *, not significant; ** $P < 0.001$ compared to the wild type.

The eight plasmids with and without amino acid substitutions in the spacer and reverse transcriptase domain are listed in Table 1.

Cell culture, transfection, and determination of IC_{50}. HepG2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal bovine serum at 37°C in 5% CO_2. Cells were seeded to semiconfluence in six-well tissue culture plates. Transient transfection of the plasmids into HepG2 cells was performed using TransIT-LT1 (Mirus, Madison, WI) according to the instructions provided by the supplier. To determine 50% inhibitory concentrations (IC_{50}s) for each antiviral drug, various concentrations of lamivudine, adefovir, and entecavir were added after 24 h to the culture plate containing the cells, and cells were harvested after 5 days. The medium containing the drugs was changed on days 1, 3, and 4. A plasmid encoding β-galactosidase (β-Gal) was cotransfected to adjust the transfection efficiency. The β-Gal enzyme assay was performed with a β-Gal enzyme assay system (Promega, Madison, WI). All experiments were performed in triplicate. GraphPad Prism software (GraphPad Software, Inc.) was used to determine the best-fit values for individual dose-response equations.

Analysis of replicative intermediate of HBV by Southern blot hybridization and quantitation. The cells were harvested at 3 or 5 days after transfection and lysed with 250 μl of lysis buffer (10 mM Tris-HCl [pH 7.4], 140 mM NaCl, and 0.5% [vol/vol] NP-40) followed by centrifugation for 2 min at 15,000 × *g*. The core-associated HBV genome was immunoprecipitated by mouse anticore monoclonal antibody 2A21 (Institute of Immunology, Tokyo, Japan) and subjected to Southern blot analysis after sodium dodecyl sulfate–proteinase K digestion followed by phenol extraction and ethanol precipitation. The DNA was detected with a full-length HBV-DNA probe labeled by the DIG DNA labeling and detection kit (Roche Diagnostics, Basel, Switzerland) according to the instructions provided by the manufacturer. Quantitative analysis was performed by real-time PCR with SYBR green using a Light Cycler. The HBV-specific primers used for amplification were 5′-TTTGGGCATGGACATTGAC-3′ and 5′-GGT GAACAATGTTCCGGAGAC-3′. The amplification conditions included initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 5 s and extension at 72°C for 6 s. The lower detection limit of this assay was 300 copies.

Evaluation of effects of antiviral drugs on mutant strains using human hepatocyte-chimeric mice. Human hepatocyte-chimeric mice were generated and used in the drug evaluation studies as described previously (27, 31). Briefly, human hepatocytes were transplanted into urokinase-type plasminogen activator-transgenic SCID mice, which are immunodeficient and develop liver failure. The transplanted cells were characterized in terms of in vivo growth potential and function. The human hepatocytes progressively repopulated the murine host liver and were susceptible to cultured-cell-line-produced HBV. All animal protocols were performed in accordance with the guidelines of the local committee for animal experimentation. The mice were inoculated with 50 μl of serum samples containing wild-type and newly identified drug-resistant strains. Serum samples obtained from mice were stored at −80°C before further analyses. After stable high-level HBV viremia was confirmed, the mice were administered food containing 30 mg of lamivudine/kg of body weight/day. The nucleotide sequences of wild-type and mutant strains were confirmed by sequencing analysis.

Detection of rtA181T mutants by PCR with restriction fragment length polymorphism (RFLP). HBV DNA extracted from serum samples were amplified by

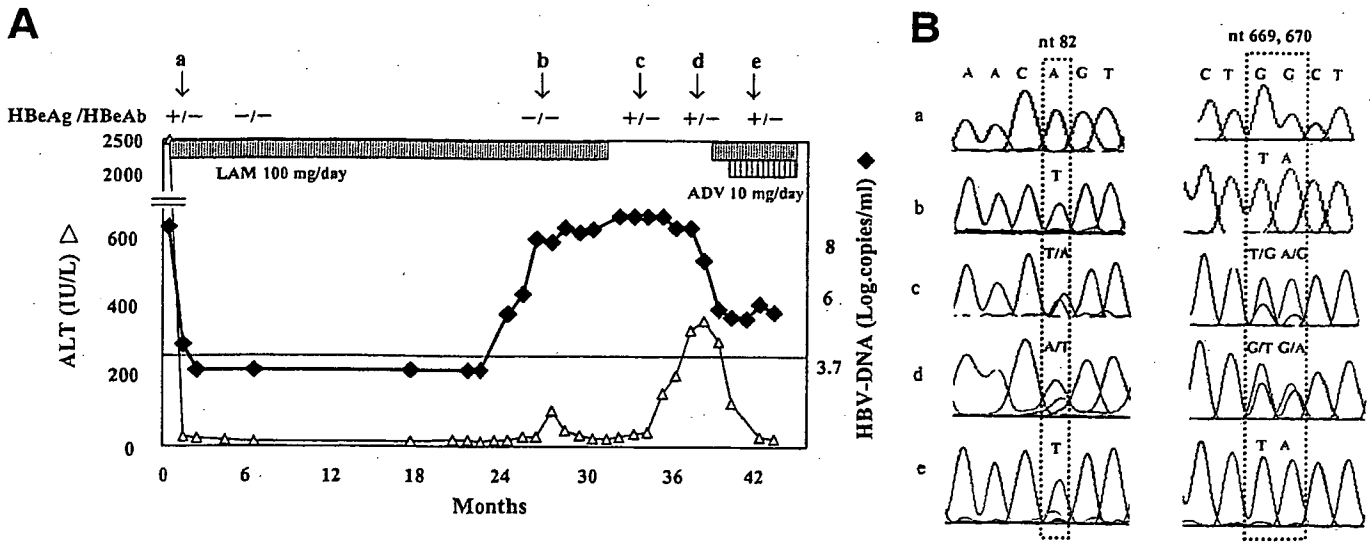

FIG. 1. (A) Clinical course of a patient who developed breakthrough without emergence of YMDD mutants during lamivudine therapy. Arrows a to e indicate time points of serum sampling for direct sequencing and RFLP PCR. (B) Nucleotide sequence analysis of the reverse transcriptase/polymerase gene of hepatitis B virus by direct sequencing. Time points of serum sampling (see panel A) were as follows: (a) just before lamivudine treatment, (b) after breakthrough, (c) after cessation of lamivudine treatment, (d) just before readministration of lamivudine, and (e) during adefovir and lamivudine therapy. Note that the wild type reappeared during the cessation of therapy (c and d), but it disappeared after readministration of the drug (e).

PCR using the primers 5′-GCCCGTTTGTCCTCTACTTCCA-3′ and 5′-ACCA CTGAACAAATGGCACTAGTAAGCTGA-3′. The reverse primer was designed to introduce an EspI site (GCTCAGC) into only wild-type sequences. The PCR was performed in a total volume of 25 μl, consisting of a reaction buffer (100 mmol/liter Tris-HCl [pH 8.3], 50 mmol/liter KCl, and 15 mmol/liter $MgCl_2$), 0.2 mmol/liter of each deoxynucleoside triphosphate, 1 μl of the DNA solution, 10 pmol of each primer and 1 U of *Taq* DNA polymerase (Gene Taq; Wako Pure Chemicals, Tokyo, Japan) with 0.2 μg of anti-*Taq* high (Toyobo Co., Osaka, Japan). The amplification conditions included an initial denaturation at 94°C for 2 min, 35 cycles of amplification (denaturation at 94°C for 1 min, annealing of primer at 58°C for 1 min, extension at 72°C for 2 min), and final extension at 72°C for 7 min. Two μl of PCR products was digested with 5 U of EspI and subjected to electrophoresis in a 3.5% agarose gel.

Statistical analysis. Data are expressed as means ± standard deviations (SD). Group comparisons were performed using the Student *t* test. A *P* value of less than 0.05 was considered statistically significant.

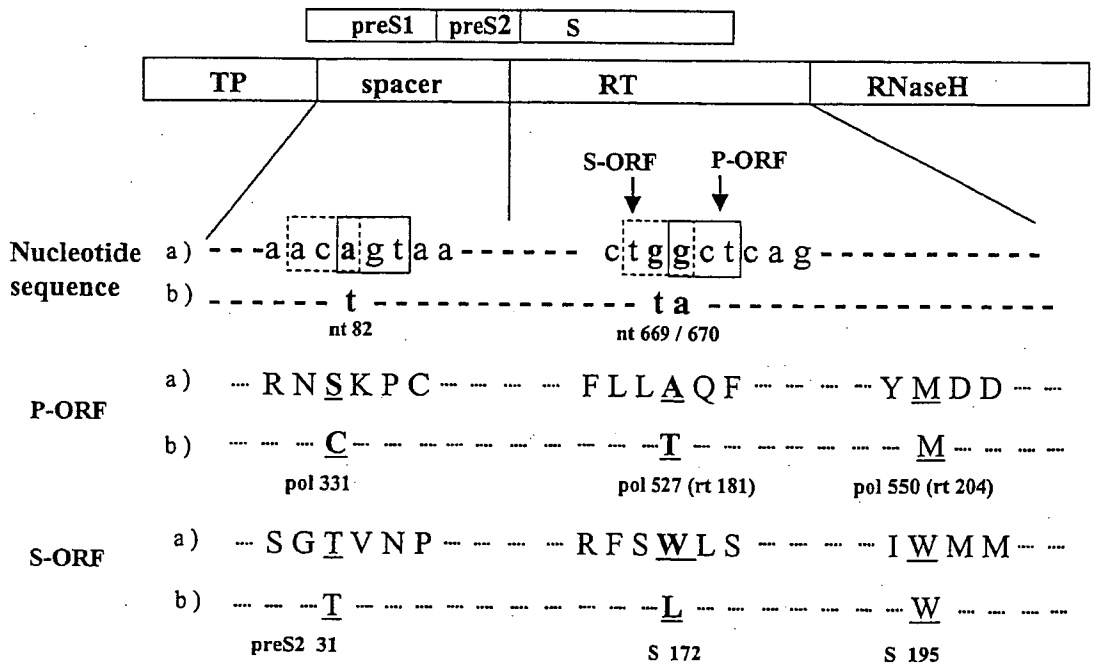

FIG. 2. Comparison of nucleotide sequences and amino acid sequences of two overlapping open reading frames, reverse transcriptase/polymerase and the HBs gene of the hepatitis B virus, before and after viral breakthrough. Sequences obtained from serum samples before (a) and after (b) breakthrough were compared. See Fig. 1A for time points of serum sampling. Nucleotide sequence numbers are those of typical HBV (e.g., accession no. AB206816 [31]), which starts from a unique EcoRI site.

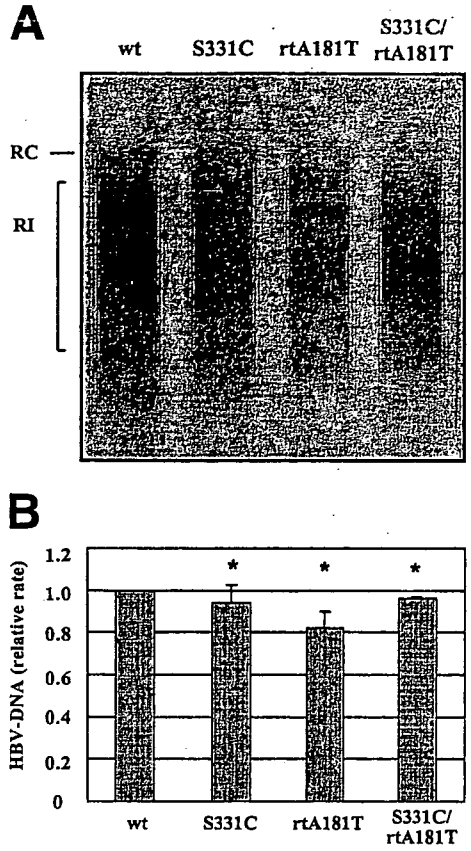

FIG. 3. Replication ability of wild-type HBV and three mutants (S331C, rtA181T, and S331C/rtA181T). Plasmids containing 1.4-genome-length HBV were transiently transfected into HepG2 cells. (A) The replicative intermediates were analyzed by Southern blot hybridization. Core-associated replicative intermediates of HBV DNA were isolated from HepG2 cells at 3 days after transfection. The positions of relaxed circular DNA (RC) and replication intermediates (RI) are indicated. (B) Quantitative analyses of core-associated intermediates of HBV. Experiments were performed in triplicate. Values are relative to those of the wild type and are expressed as means ± SD. *, not significant compared to the wild type.

RESULTS

Isolation of a novel lamivudine-resistant strain with an intact YMDD motif. The novel lamivudine-resistant strain of HBV was isolated from a 44-year-old Japanese man with chronic HBV infection (Fig. 1A). In this patient, lamivudine successfully reduced the HBV level at the initial stage of treatment, but viral breakthrough was observed at 24 months after the beginning of therapy. The patient was very punctual and confirmed that he took lamivudine with perfect compliance. The HBV viral load reached up to 8.5 log copies/ml, but nucleotide sequence analysis showed no YMDD mutation. The YIDD and YVDD mutants were not detected even with a peptide nucleic acid-mediated PCR clamping method sensitive for detection of YMDD mutants (6). The analysis also showed that this isolate belonged to genotype C of HBV. Comparison by the direct sequence method of nucleotide sequences obtained before and after the viral breakthrough showed three nucleotide substitutions that induced two amino acid substitutions in both spacer (polS331C) and reverse transcriptase (polA527T or rtA181T) domains of the polymerase (Fig. 1B and 2). The latter nucleotide substitutions induced an amino acid change in the overlapping HBs protein (W172L) (Fig. 2). Twelve HBV genomes were cloned from the serum of this patient after viral breakthrough, and eleven of them showed the above amino acid substitutions. Only one clone showed the wild-type sequence. The new strain of HBV became undetectable when lamivudine therapy was discontinued, and this strain outcompeted the wild-type strain upon administration of the drug (Fig. 1B). These results prompted us to study the significance of each of these mutations.

Effect of substitutions on HBV replication. To assess the effect of nucleotide substitutions on HBV replication, four plasmids containing 1.4-genome-length patient-specific HBV genome (Table 1) were generated and transfected into HepG2 cells. In comparison with the patient's wild-type strain, the replication capacities of the S331C, rtA181T, and S331C/rtA181T mutants were not different (94%, 82%, and 96%, respectively), suggesting that these mutants can replicate at almost the same rate as the wild-type strain (Fig. 3).

Susceptibility of mutants to lamivudine in vitro. To analyze the role of the polS331C and rtA181T mutations in lamivudine resistance, four patient-specific strains and four laboratory strains were transfected into HepG2 cells (Fig. 4; Table 1). A single amino acid substitution in the spacer region did not contribute to resistance in either patient or laboratory strains. In contrast, an amino acid substitution in the polymerase (rtA181T) induced resistance that was 3.0 and 3.9 times greater than that of patient and laboratory strains ($P < 0.001$), respectively. The presence of both of these amino acid changes induced 3.0 and 4.3 times greater resistance in each of the above strains. Thus, the spacer mutation had little effect on the susceptibility to lamivudine (Table 1).

We also compared the rtA181T mutant identified in this study with the rtA181T mutant reported previously, which had premature termination in the HBs protein (7, 34), for replication ability and susceptibility to lamivudine. Although the HBs antigen produced to culture supernatant was different between the two strains (52.5 ± 8.2 and 4.4 ± 0.6 IU/ml, respectively), there was no noticeable difference in replication ability and lamivudine sensitivity between the two mutants (data not shown).

Assessment of drug resistance of novel mutations in vivo using human hepatocyte-chimeric mice. To confirm the lamivudine resistance of the novel mutant strain, two human hepatocyte-chimeric mice were each inoculated with a serum sample obtained from the patient who developed breakthrough without mutations in the YMDD motif (Fig. 1A). The serum was obtained during breakthrough while the patient was still taking the drug. Twelve weeks after the inoculation of the serum samples, both mice developed high-level viremia (7.8 and 6.6 log copies/ml, respectively). Direct sequence analysis showed that the nucleotide sequence of the virus that replicated in the chimeric mice was in accordance with the mutant strain. Cloning and sequencing analysis showed that only 1 of 12 clones obtained from the inoculum was wild type, while the remaining 11 clones were rtA181T mutants with an intact YMDD motif. We also analyzed the serum of the two infected mice before and after lamivudine therapy. All 11 and 15 clones before and all 11 and 12 clones during therapy had the

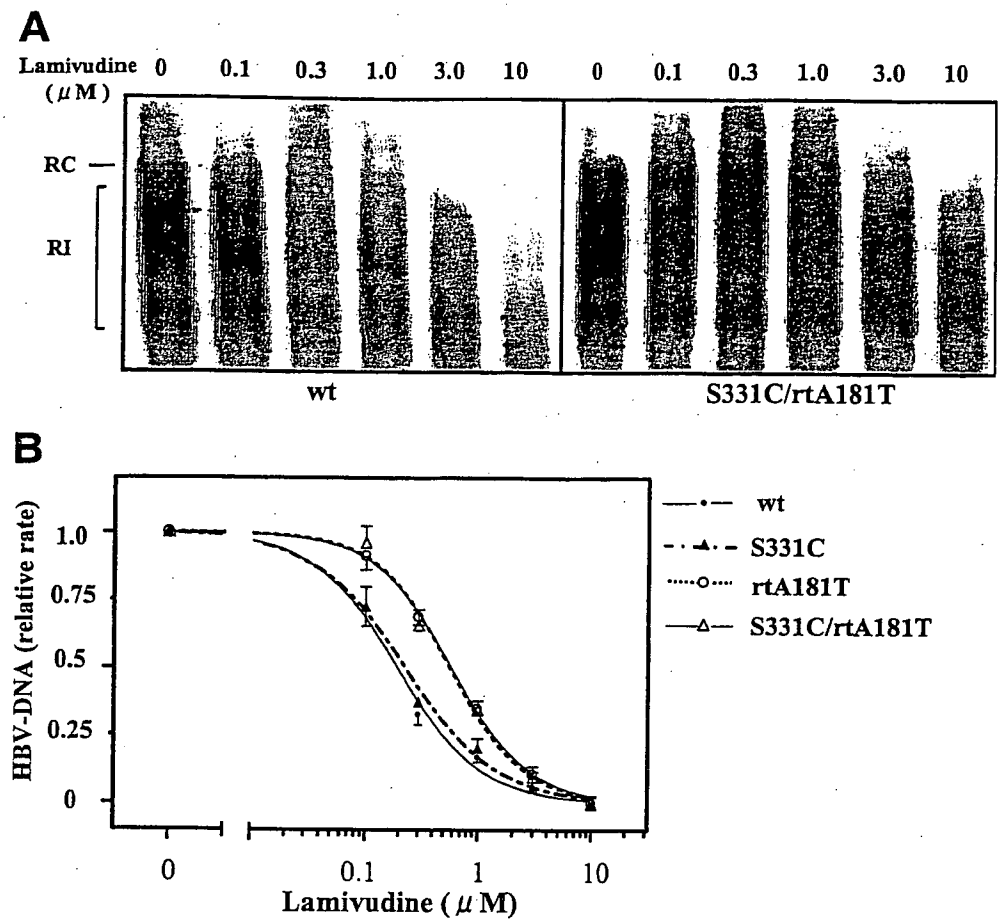

FIG. 4. In vitro analyses of susceptibility of wild-type HBV and three mutants (S331C, rtA181T, S331C/rtA181T) to lamivudine after transient transfection into HepG2 cells. Cells were transiently transfected with plasmids containing 1.4-genome-length HBV and treated with the indicated amount of lamivudine. (A) Southern blot analysis of replicative intermediate. Representative results for the wild type (wt) and the S331C/rtA181T mutant are shown. The positions of relaxed circular (RC) and replication intermediate (RI) forms of HBV DNA are indicated. (B) Dose-response curves of the four HBV strains against lamivudine. The curves were used to estimate the lamivudine IC_{50}s for each HBV strains. Values are relative to no-lamivudine controls for each strain. Experiments were performed in triplicate. Values are expressed as means ± SD.

rtA181T mutation (data not shown). Two other mice were inoculated with wild-type HBV obtained from a patient not treated with lamivudine as a control, and both mice also developed high-level viremia (8.3 and 9.3 log copies/ml, respectively). Thirteen weeks later, the viremia reached plateau and the mice were fed food containing lamivudine. After 6 weeks of treatment, the mean viral load decreased by 2.8 log copies/ml in the wild type, whereas it decreased by only 0.39 log copy/ml in the mutant ($P < 0.001$) (Fig. 5).

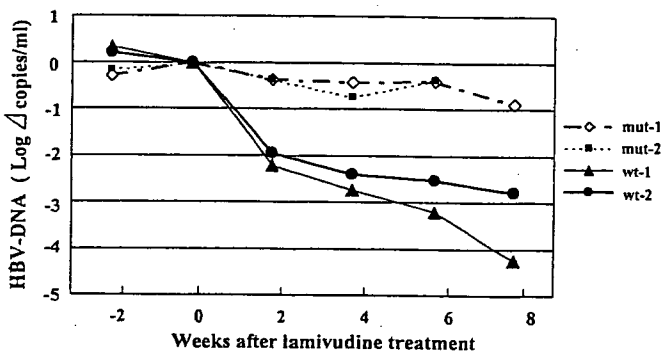

FIG. 5. In vivo analyses of the effect of lamivudine on wild-type and S331C/rtA181T mutant HBV. Four human hepatocyte-chimeric mice were inoculated with serum samples containing wild-type or mutant HBV. One of the animals fed with lamivudine died 6 weeks after the beginning of therapy.

Susceptibility of mutants to adefovir and entecavir in vitro. We also analyzed the effects of adefovir and entecavir against the S331C/rtA181T mutant using a transient-transfection assay with HepG2 cells. The IC_{50}s of these drugs for the mutant strain and wild type were almost the same (Table 2).

Detection of rtA181T mutant in patients treated with lamivudine. In this study, we developed a RFLP PCR method to detect the rtA181T mutants, by which we were able to detect mutant strains even when they were mixed with the wild type (Fig. 6). The system also detected the rtA181T (HBs stop) mutant reported by Chien et al. (7) and Yeh et al. (34). Using this method, we analyzed 40 patients who showed viral breakthrough (increase in viral load equal to or more than 1 log) during lamivudine therapy. We found that only one of these patients was positive (Fig. 6A). Nucleotide sequence analysis of serum samples obtained from this patient showed that the

TABLE 2. In vitro susceptibility of the S331/rtA181 mutant to lamivudine, adefovir, and entecavir[a]

Patient strain	S331/rtA181	Lamivudine		Adefovir		Entecavir	
		IC_{50} (μM)	Resistance (fold)	IC_{50} (μM)	Resistance (fold)	IC_{50} (nM)	Resistance (fold)
WT	−/−	0.19 ± 0.01	1	0.37 ± 0.1	1	0.19 ± 0.02	1
S331C/rtA181T	C/T	0.57 ± 0.06	3**	0.36 ± 0.08	0.98*	0.23 ± 0.05	1.2*

[a] Experiments were performed in triplicate. Values are expressed as means ± SD. WT, wild type. *, not significant; ** $P < 0.001$ compared to the wild type.

mutant strain had the rtA181T mutation with a truncated HBs antigen, as reported previously (7, 34). The YMDD motif of HBV detected in this patient was of the wild type. All 39 remaining patients with viral breakthrough were positive for YIDD and/or YVDD mutants. The RFLP PCR analysis of these 39 samples showed that four contained a small amount of rtA181T mutants (Fig. 6B). Nucleotide sequence analyses of these samples showed that they contained only a small amount of rtA181T mutants with a truncated HBs antigen (Fig. 6C).

Finally, we examined the presence of YMDD or rtA181T mutants in eight patients who showed a poor response with lamivudine treatment (HBV viral load above 6.0 log copies/ml after 6 months of treatment). None of these patients tested positive for both of these mutations (data not shown).

DISCUSSION

In this study, we identified a novel lamivudine-resistant strain of HBV with an intact YMDD motif in a patient who received long-term lamivudine therapy. YMDD mutants were not detected even by a sensitivity-enhanced detection method, which was reported previously by our group (6). The double nucleotide substitutions (GG to TA) induced amino acid substitutions in both polymerase (rtA181T) and HBs antigen (HBs W172L). One might assume that the compliance of the patient was poor. However, the patient was very punctual and confirmed that he took lamivudine with perfect compliance.

Our study demonstrated that the rtA181T mutation reduced the susceptibility to lamivudine 3.0- to 3.9-fold in vitro (Table 1). Furthermore, we also confirmed lamivudine resistance of this mutant strain in vivo using human hepatocyte-chimeric mice. The amino acid substitution in the reverse transcriptase (RT) domain is similar to that reported previously (7, 34). However, in contrast to our results, the mutant strains in the latter reports emerged with or after those with the mutation in the YMDD motif (YIDD or YVDD) and took over them (34). There are two additional differences between the substitutions we identified and those described by Yeh et al. (34), as detailed below.

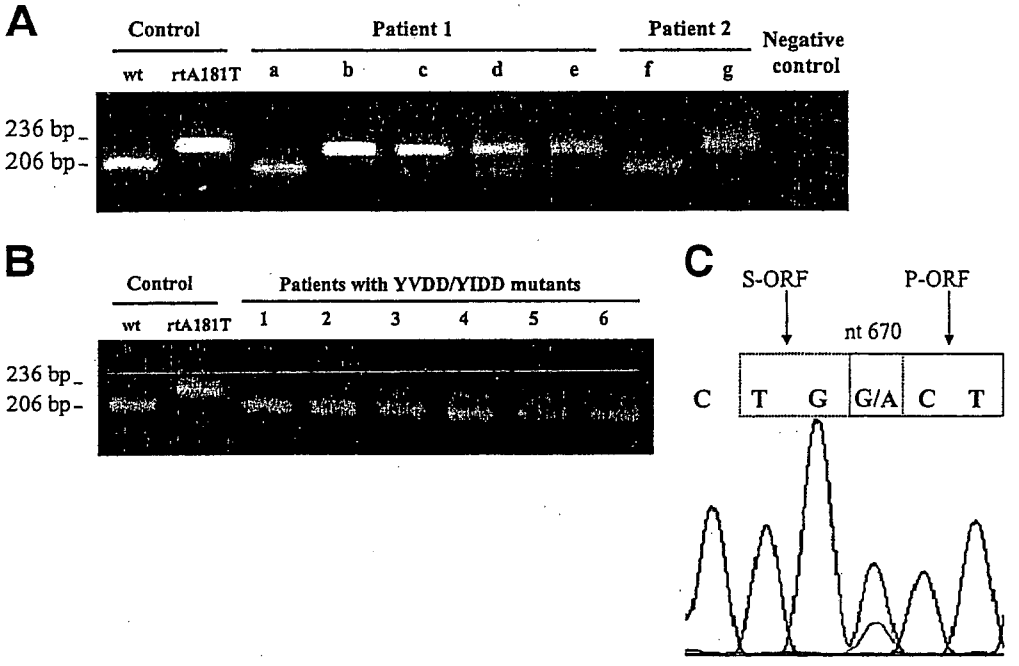

FIG. 6. Detection of the rtA181T mutant by RFLP PCR assay. PCR-amplified DNA fragments were treated with EspI, which digests only wild-type sequences, and separated in a 3.5% agarose gel. (A) Agarose gel electrophoresis of RFLP PCR products. Wild-type and rtA181T mutant plasmids were used as controls. See Fig. 1A for the time points of serum sampling (a to e) for patient 1 and see Fig. 1B for a comparison with nucleotide sequence analyses. f and g indicate the time points before and after viral breakthrough for patient 2. (B) Agarose gel electrophoresis of RFLP PCR products using HBV DNA samples obtained from 39 patients who showed lamivudine breakthrough. Of the 39 samples, 35 were wild type (lanes 1 and 2). The remaining four samples (lanes 3 to 7) showed partial digestion, suggesting a mixture of wild-type and mutant strains. (C) Nucleotide sequence analysis of a sample by RFLP PCR suggested the presence of a wild-type–mutant mixture (lane 5 of panel B).

Firstly, the HBs antigen was prematurely terminated in the mutant strain reported by Yeh et al. (34). In this regard, a similar amino acid substitution of the B domain of the polymerase FLLA motif in woodchuck hepatitis virus (WHV) treated with lamivudine was reported (15, 28). The HBs antigen in these WHV mutant strains also had premature stop codons. These findings suggest that the mutant strains of HBV and WHV cannot replicate and spread by themselves because of the lack of HBs antigen. Such strains are thought to replicate by using in vivo-supplied HBs antigen from wild-type strains as helper antigens. In contrast, the novel strain identified in this study had no premature termination of the HBs gene. The in vitro study suggested that the strain had a replication ability similar to that of the wild type. Furthermore, we also showed that the strain infected and reached a high viral load in human hepatocyte-chimeric mice. Although the inoculum contained only a small amount of wild-type strain (one of 12 clones), all clones obtained from mouse serum were mutant strains (rtA181T). Considering these results and the fact that the index patient showed high viral titers after breakthrough (more than 7.6 log copies/ml), this mutant strain can spread and replicate by itself and has strong replicative ability.

Secondly, the substitutions identified in this study appeared with nucleotide and amino acid substitutions in the spacer region of the polymerase (S331C). There are only a few studies that reported the function of the spacer domain (19–21, 28), leaving the biological significance of this region unknown. The substitution in the spacer region reappeared with the A181T mutation in the RT domain in the index patient after the patient restarted lamivudine therapy. Although our study showed no significant contribution of this mutation to drug resistance (Fig. 3 and 4; Table 1), the significance of the mutation in this region (fingers in the HBV polymerase homology model [8]) should further be investigated.

Recently, the amino acid substitutions rtA181T and rtA181V were reported to emerge with resistance against adefovir (11, 32). Tillmann et al. (29) reported one case in which the virus developed the rtA181T mutation during famciclovir breakthrough. The A556T mutation of WHV, analogous to the rtA181T mutation of HBV, has been reported to be associated with lamivudine resistance (15, 28). These results indicate that the amino acid substitutions at position 181 may associate with resistance against many nucleoside analogues, including lamivudine, famciclovir, and adefovir. Although our in vitro study indicated that the rtA181T mutant had no resistance against adefovir and the animal study showed that combination therapy with lamivudine and adefovir effectively reduced the virus load in woodchucks (15), such combination therapy did not produce sufficient suppression of HBV in the index patient (Fig. 1A). The amino acid substitution at position 181 has to be further analyzed with regard to resistance to anti-HBV drugs.

The rtA181T mutation detection system using RFLP PCR developed in this study is a useful tool, as we were able to distinguish the wild type from all mutants with nucleotide substitutions in a given region. The system also enabled us to monitor the fluctuation of the wild-type/mutant ratio during therapy against HBV (Fig. 1 and 6). The incidence of rtA181T mutants with an intact YMDD motif is rare in Japanese patients with chronic HBV infection treated with lamivudine. Interestingly, 4 of the 39 (10%) patients who developed lamivudine breakthrough and were positive for YMDD mutants were found to have small amounts of rtA181T mutant strains. Different from the previous report (34), the mutants did not take over another strain and were not preceded by exacerbation. We have to monitor these patients carefully for further population change of mutants and for exacerbation of hepatitis.

A recent study reported that the prevalence of genotype A HBV infection is increasing in Japan and that the incidence of disease chronicity is higher than for other genotypes (26). It is thus expected that an increasing number of the sexually active population will receive nucleoside analogue therapy against HBV and multiple mutant strains can potentially emerge and spread along with long-term treatment. There is an increasing possibility of emergence of novel mutants resistant to multiple anti-HBV drugs. The importance and significance of the rtA181 mutations, including the novel mutant strain identified in this study, should be investigated further to develop more useful treatment strategies.

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Prolonged Negative HCV-RNA Status Led to a Good Outcome in Chronic Hepatitis C Patients with Genotype 1b and Super-High Viral Load

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Key Words
IFN-α_{2b} + ribavirin, combination therapy · Hepatitis C virus genotype 1b · High viral load · IFN-β, induction therapy

Abstract
Objective: We examined whether a sustained negative HCV-RNA status for 48 weeks affects the outcome in patients with genotype 1b and super-high viral load, and also investigated whether the outcome is affected by the induction therapy of twice-daily pre-administrated interferon (IFN)-β. ***Methods:*** 78 eligible patients were divided into four groups. 40 were patients assigned to the short treatment protocol. 13 patients received 3 MU IFN-β twice daily for 2 weeks followed by IFN-α_{2b} + ribavirin for 22 weeks (β-induction group: group 1). 27 patients received IFN-α_{2b} + ribavirin for 24 weeks (standard combination group: group 2). 38 patients were assigned to the maintenance treatment protocol. All of the 13 in the β-induction group (group 3) and 21 of 25 patients in the standard combination group (group 4) who were negative HCV-RNA PCR at week 24 had IFN monotherapy to maintain a negative HCV-RNA result for 48 weeks. ***Results:*** An HCV-RNA-negative status at week 24 was observed in 96% (25/26) of groups 1 and 3 versus in 79% (41/52) of groups 2 and 4 ($p < 0.01$). The sustained virological response (SVR) was 38% (5/13) in group 1 and 11% (3/27) in group 2 ($p < 0.05$). In the maintenance treatment, SVR was observed in 46% (6/13) of group 3 and 32% (8/25) of group 4 (NS). ***Conclusions:*** A sustained negative HCV-RNA status for 48 weeks might be associated with viral elimination in patients with genotype 1 and super-high viral load.

Introduction

Hepatitis C virus (HCV) infection is estimated to affect 170 million individuals worldwide [1], including 2 million people in Japan [2]. Chronic HCV infection often progresses into liver cirrhosis including the development of associated complications such as gastroesophageal varices and hepatocellular carcinoma over the course of 20–50 years [3–6]. Interferon (IFN) is the only effective treatment for HCV infection, and is widely used. The beneficial effects of IFN in patients with chronic HCV infection have been clearly defined and include decreases in serum transaminase concentration, eradication of the virus, and improvement of liver histology [7–10]. However, a sustained virological response (SVR) is rarely obtained by

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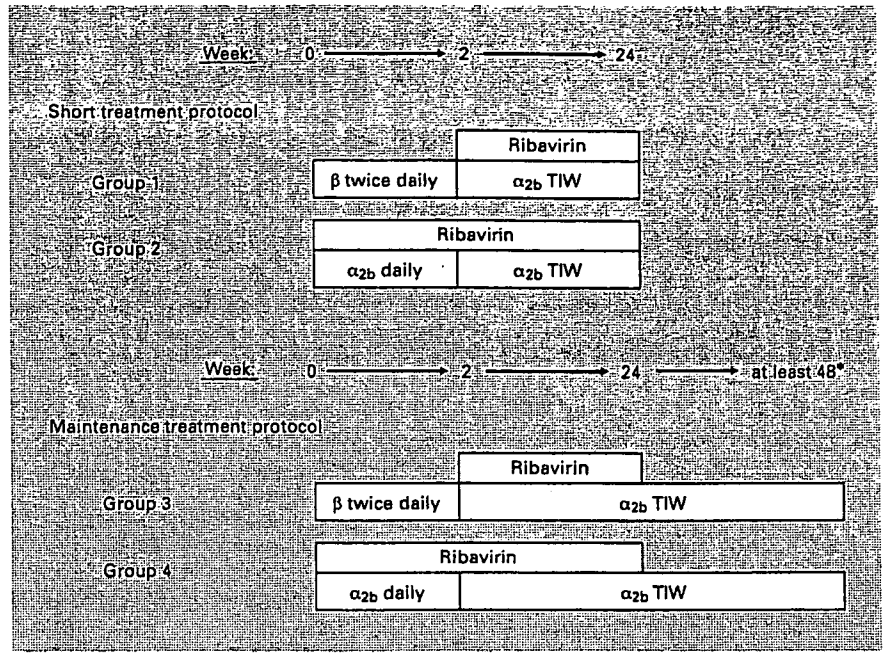

Fig. 1. Study design showing the two different protocols of IFN therapy. * Maintenance treatment was designed to sustain a negative HCV-RNA PCR result for 48 weeks.

IFN monotherapy in poor response categories (cirrhosis, high viral load, genotype 1/4) [11–16]. Recent advances of various IFN treatments such as consensus IFN, ribavirin combination, and pegylated IFN can achieve a relatively high SVR in those patients [17–25].

In Japan, the patients with genotype 1 and high viral load is most prevalent [26]. The oral administration of ribavirin has been permitted for only 24 weeks by medical insurance until December 2004 [27]. Because the relapse rate is higher in combination therapy only for 24 weeks [20, 22], we conducted prolonged IFN monotherapy after ribavirin combination. Recently, it was reported that not only the treatment duration but also the duration of therapy with an undetectable HCV-RNA load are associated with the probability of a long-term antiviral response during pegylated IFN/ribavirin combination therapy, and that patients infected with genotype 1 would require a continuous non-detectable viral load in serum for 36 weeks to attain 90% probabilities of SVR [28]. In this study, we designed a clinical trial consisting of combination therapy followed by prolonged IFN monotherapy, which was continued for 48 weeks from the time of the first negative HCV-RNA PCR result for HCV genotype 1 patients with high viral load. We also investigated whether the outcome of IFN therapy is affected by the induction therapy of twice-daily pre-administered IFN-β.

Materials and Methods

Patients

A total of 78 adult patients were recruited for this study. All patients were infected with HCV genotype 1b and had super-high viral load (>500 KIU/ml) as determined by Amplicor HCV monitor assay (Roche Molecular Diagnostics Co., Tokyo, Japan). The detection range of the assay was between 0.5 and 500 KIU/ml (a standard sample containing 10^5 copies/ml of HCV was assigned a titer of 10^5 IU/ml). Patients eligible for study participation were required to satisfy the following criteria: (1) aged from 20 to 65 years; (2) a recent liver biopsy within 3 months of the start of therapy; (3) diagnosis of chronic hepatitis by the conventional classification; (4) positive for HCV-RNA of genotype 1b in serum within 3 months in titers of >500 KIU/ml by the Amplicor HCV monitor assay; (5) abnormal serum alanine aminotransferase levels for >6 months; (6) leukocyte count >3,000/mm^3, platelets >100,000/mm^3; (7) serum bilirubin <2.0 mg/dl; (8) lack of liver cirrhosis, hepatocellular carcinoma, autoimmune hepatitis, alcoholic liver disease and any other chronic liver diseases (positive for serological markers of hepatitis B virus); (9) lack of psychiatric illnesses, including depression, or conditions affecting the bone marrow, alimentary, cardiovascular or pulmonary systems, and (10) no immunosuppressive or antiviral therapy within 6 months prior to entry.

IFN Protocol

Patients were treated with the combination therapy of IFN and ribavirin: 6–10 million units (MU) of IFN-α_{2b} subcutaneously administered three times weekly; oral ribavirin administered twice daily at a total dose of 600 or 800 mg for patients whose weight was less or more than 60 kg, respectively. The IFN therapy protocol is described in figure 1. At the start of the therapy, the physicians in

charge explained the purpose and method of the clinical trial as well as potential adverse events during the twice-daily IFN-β induction. The physicians also explained the information including the result of clinical trials of combination therapy for 48 weeks in other countries, such as the SVR rate, HCV-RNA relapse rate and adverse events. After giving sufficient informed consent, the patients themselves decided whether or not to be treated by twice-daily IFN-β induction and also decided whether or not to be treated by additional IFN monotherapy to sustain a negative HCV-RNA result for 48 weeks. According to the patients' decision, four therapeutic groups were divided as follows:

Short Treatment Protocol: 40 patients were treated by this protocol for 24 weeks. 13 patients were treated by 3 MU of IFN-β twice-daily administered for 2 weeks followed by the combination therapy for 22 weeks (group 1). 27 patients were treated by the standard combination therapy for 24 weeks (group 2).

Maintenance Treatment Protocol: 38 patients were treated by this protocol. 13 patients were treated by 3 MU of IFN-β twice-daily administered for 2 weeks followed by the combination therapy (group 3). 25 patients were treated by the standard combination therapy (group 4). For consistency with current guidelines, patients who were HCV-RNA-positive by PCR at month 6 were removed from the study and considered as non-responders. The patients who had an undetectable HCV-RNA load in serum at month 6 had an additional minimum of 24 weeks' IFN monotherapy as maintenance treatment. The maintenance treatment was designed to sustain a negative HCV-RNA PCR result for 48 weeks.

The study was approved by the Institutional Review Boards of the participating clinical sites before study initiation, and the study was conducted according to the Declaration of Helsinki. Written informed consent was obtained from all patients.

Virological Response to IFN

The virological response to IFN was determined by measuring serum HCV-RNA levels with the Amplicor HCV monitor assay at days 2, 3, 8, 15, 29 and every 28 days thereafter. Negative samples on the Amplicor HCV monitor assay were re-examined by the Amplicor qualitative assay, which has a detection limit of HCV-RNA of 0.2 KIU/ml. SVR was defined as a negative serum HCV-RNA during the 6 months following completion of IFN administration. All patients other than those with SVR were considered to be non-responders.

Histological Analysis

All patients underwent liver needle biopsy under sonographic guidance in the 3 months prior to the start of IFN administration. Baseline liver histology of chronic hepatitis was classified, based on the extent of fibrosis, into five stages (F0 (no fibrosis), F1 (mild fibrosis), F2 (moderate fibrosis), F3 (severe fibrosis), or F4 (cirrhosis)), and based on activity into four grades (A0 (no activity), A1 (mild activity), A2 (moderate activity), or A3 (severe activity)), according to the method of Desmet et al. [29].

Statistical Analysis

Baseline clinical characteristics were compared between the treatment groups using Fisher's exact test or the Mann-Whitney U-test. Treatment efficacy was analyzed by Fisher's exact test. p values <0.05 were considered statistically significant.

Results

Characteristics of the Patients

There were no significant differences in the general characteristics of the patients in demographic, biochemical, virological and histological features between the β-induction group (group 1) and standard combination group (group 2) in the short treatment protocol. There were no significant differences in the background characteristics between the β-induction group (group 3) and standard combination group (group 4) in the maintenance treatment protocol. Among the four therapeutic groups, background characteristics were also not significant, except the history of previous IFN monotherapies: the rate of previous IFN monotherapies in the short standard combination group (group 2) was significantly lower compared with other therapeutic groups ($p < 0.05$) (table 1).

HCV-RNA Clearance

HCV-RNA negativity and the week after starting therapy are shown in table 2. 96% (25/26) of the β-induction group (groups 1 and 3) had undetectable HCV-RNA load in serum 24 weeks after starting therapy. In comparison, 79% (41/52) of the standard combination group (groups 2 and 4) had undetectable HCV-RNA load in serum 24 weeks after starting therapy. There was a significant difference in the HCV-RNA status at 24 weeks between the β-induction group (groups 1 and 3) and the standard combination group (groups 2 and 4) ($p < 0.05$). Of the patients who received maintenance IFN monotherapy, 39% (5/13) in the β-induction group (group 3) and 43% (9/21) in the standard combination group (group 4) had detectable HCV-RNA during IFN monotherapy (breakthrough). The residual patients completed IFN monotherapy to sustain a negative HCV-RNA PCR profile for 48 weeks. In the patients with a negative HCV-RNA status for 48 weeks, 25% (2/8) in the β-induction group (group 3) and 33% (4/12) in the standard induction group (group 4) had re-appearance of HCV-RNA after IFN monotherapy. The periods of IFN maintenance monotherapy were 32.4 ± 6.2 weeks in the β-induction group (group 3) and 38.5 ± 6.9 weeks in the standard combination group (group 4) ($p < 0.05$).

HCV-RNA Dynamics and the Time of HCV-RNA Negativity

The first and second phase of HCV-RNA dynamics are shown in figure 2. An early significant decline in HCV-RNA was observed in the β-induction group (groups 1

Table 1. Baseline characteristics of the patients according to four therapeutic groups

	Short treatment protocol (n = 40)		Maintenance treatment protocol (n = 38)		p value
	β-induction group (group 1, n = 13)	standard combination group (group 2, n = 27)	β-induction group (group 3, n = 13)	standard combination group (group 4, n = 25)	
Mean age, years[a]	55.8 ± 5.6	54.6 ± 10.3	54.0 ± 9.2	56.7 ± 10.4	n.s.
Male:female	7:6	13:14	10:3	18:7	n.s.
Basal WBC, $\times 10^3/mm^3$	4.7 ± 1.4	4.5 ± 1.5	4.7 ± 1.3	4.9 ± 1.6	n.s.
Basal Hb, g/dl	14.4 ± 1.3	14.6 ± 1.0	15.2 ± 1.0	14.8 ± 1.1	n.s.
Basal ALT, IU/l[a]	72.4 ± 36.1	68.9 ± 31.7	73.8 ± 40.1	62.7 ± 26.2	n.s.
Platelets, $\times 10^4/mm^3$ [a]	16.4 ± 4.7	14.4 ± 4.4	16.7 ± 5.8	15.2 ± 3.7	n.s.
Serum HCV-RNA, KIU/ml	>500	>500	>500	>500	n.s.
Histological findings[b]					
Staging 0	0	0	0	0	n.s.
Staging 1	5	10	7	13	n.s.
Staging 2	4	7	3	8	n.s.
Staging 3	4	10	3	4	n.s.
Staging 4	0	0	0	0	n.s.
Grade 0	0	0	0	0	n.s.
Grade 1	4	9	4	10	n.s.
Grade 2	8	17	8	13	n.s.
Grade 3	1	1	1	2	n.s.
History of previous IFN monotherapies	6	5*	7	13	n.s.

[a] Data are mean ± SD. [b] Classified by the method of Desmet et al. [29]. n.s. = Not significant.
* The rate of previous IFN monotherapies in short standard combination group was significantly lower compared with other therapeutic groups ($p < 0.05$).

Table 2. HCV-RNA disappearance and the week after starting therapy

Weeks	β-Induction group (groups 1 and 3, n = 26)	Standard combination group (groups 2 and 4, n = 52)	p value
2	12% (3/26)	4% (2/52)	0.191
4	35% (9/26)	10% (5/52)	<0.01
8	62% (16/26)	29% (15/52)	<0.01
12	73% (19/26)	52% (27/52)	0.059
16	96% (25/26)	69% (36/52)	<0.01
20	96% (25/26)	77% (40/52)	<0.05
24	96% (25/26)	79% (41/52)	<0.05

and 3) on days 7 and 14 in the standard combination group (groups 2 and 4). Twice-daily administration of IFN-β accelerated HCV-RNA decline in the second phase against IFN/ribavirin combination therapy. As a result of early viral decline, HCV-RNA disappearance was attained in a shorter period in the β-induction group (groups 1 and 3) (table 2). That was significant with the standard combination group (groups 2 and 4) at weeks 4, 8, 16, 20 and 24. The mean time to the first negative HCV-RNA PCR result was 8.4 ± 6.2 weeks in the β-induction group (groups 1 and 3) and 14.5 ± 6.9 weeks in the standard combination group (groups 2 and 4) ($p < 0.01$).

Virological Response

Table 3 shows SVR rates. In patients who received the short treatment protocol, SVR was observed in 5 of 13 patients (38%) in the β-induction group (group 1) and in 3 of 27 patients (11%) in the standard combination group (group 2) ($p < 0.05$). In the patients who received the maintenance treatment protocol, SVR was observed in 6 of 13 patients (46%) in the β-induction group (group 3) and in 8 of 25 patients (32%) in the standard combination group (group 4) (NS).

Adverse Events

Table 4 summarizes the laboratory abnormalities and adverse events recorded for 24 weeks after initiation of IFN therapy. There were no patients with leukocyte counts <1,000/mm^3, hemoglobin concentrations

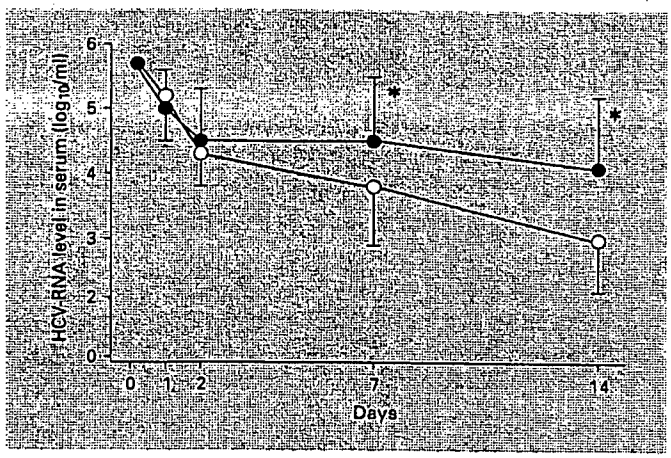

Fig. 2. Mean (±SD) of viral load in the serum during the first 14 days of treatment for patients chronically infected with HCV genotype 1b and super-high viral load (>500 KIU). The two treatment groups included: (1) twice-daily administration of IFN-β (groups 1 and 3) (○) and (2) combination therapy with ribavirin and daily IFN-α_{2b} (groups 2 and 4) (●). * $p < 0.05$.

Table 3. Sustained virological response rate to two different antiviral regimens with or without β-induction

	β-Induction group (groups 1 and 3; n = 26)	Standard combination group (groups 2 and 4; n = 52)	p value
Short treatment protocol	38% (5/13)	11% (3/27)	<0.05
Maintenance treatment protocol	46% (6/13)	32% (8/25)	n.s.

n.s. = Not significant.

Table 4. Number of patients who had laboratory abnormalities or adverse events

	β-Induction group (groups 1 and 3; n = 26)	Standard combination group (groups 2 and 4; n = 52)
Leukocytes <1,000/mm^3	0	0
Hb <10 g/dl	6	19
Hb <8.5 g/dl	0	0
Platelets <50,000 /mm^3	2	2
Albumin <3.5 g/dl	14	0
Albumin <3.0 g/dl	0	0
Proteinuria/day		
<1 g	7	0
1–3.5 g	10	0
>3.5 g	8	0
ALT elevation[a]	9	3

[a] ALT elevation was considered positive when ALT of anytime during IFN therapy increased more than twofold of the baseline ALT.

<8.5 g/dl and serum albumin level <3.0 g/dl. Incidences of hypoalbuminemia (<3.5 g/dl) and proteinuria were observed only in patients treated with the β-induction. A total of 8 patients had severe proteinuria (>3.5 g/day). ALT elevation (twofold against the baseline) was significantly higher in the β-induction group (groups 1 and 3) (9/26 vs. 3/52; $p < 0.01$). However, 2 weeks of β-induction therapy was completed in all patients and these adverse events recovered after the completion of β-induction. During maintenance IFN monotherapy (groups 3 and 4), the laboratory abnormalities and adverse events were not observed. No patients discontinued because of these adverse events during the therapeutic periods.

Discussion

Genotype 1 is the most prevalent genotype of HCV in most geographical areas, including Japan. Recent studies have revealed important information about viral dynamics following initiation of IFN therapy [30–32]. For genotype 1 patients, the antiviral effectiveness of IFN (blocking virus production, free virion clearance rate, and HCV-infected cell death rate) has been shown to be significantly lower than that for non-genotype 1 patients [32]. Failure to clear the virus can be observed at three different phases: during the initial treatment period (nonresponse), during maintenance treatment after an initial response (breakthrough), and after treatment discontinuation (relapse) [20, 22]. In IFN-resistant patients, a high prevalence of those three reactions was observed. Thus to obtain a high SVR rate, high prevalence of undetectable HCV-RNA and low rates of breakthrough and relapse would be desirable.

In the present study, an HCV-RNA-negative status at 24 weeks was significantly high and early in the β-induction group, while an HCV-RNA-negative status at 24 weeks was obtained in 79% of the patients in the standard combination group. While ribavirin combination achieved similar results even for 48 weeks [20, 22], the early disappearance and high rate of an HCV-RNA-nega-

tive status was obtained in the genotype-1-infected patients of super-high viral load (>500 KIU) by the induction of twice-daily administration of IFN-β. Twice-daily administration of IFN-β is associated with early virus elimination [33–37]. However, adverse events during administration of IFN-β can include marked elevation of serum alanine aminotransferase, decreased platelet count, and proteinuria especially in the patients treated with twice-daily administration [33–38]. To take advantage of the antiviral efficacy, the upper limit of duration of twice-daily administration of IFN-β needs to be established. Some reports demonstrated that about 70–85% of the patients treated with twice-daily administration of IFN-β could tolerate continuing treatment for 4 weeks [33, 34]. In our study, all patients treated with the twice-daily IFN-β induction protocol could tolerate continuing induction treatment for 2 weeks. The patients treated with β-induction had a relatively high SVR rate with or without IFN monotherapy. Although the significance of induction therapy remains unclear, our results suggest that induction therapy might be beneficial for genotype-1-infected patients of super-high viral load (>500 KIU).

A relatively high rate of breakthrough (approx. 40%) might be caused by the short duration of ribavirin usage or background of super-high viral load (>500 KIU). In Japan, the oral administration of ribavirin has been permitted for only 24 weeks by medical insurance until December 2004 [27]. Thus, it was our design for this study that prolonged IFN monotherapy would be continued for 48 weeks from the time of first negative HCV-RNA PCR result. As a result, we obtained a relatively low prevalence of relapse. The relapse rates were 33% in patients treated with standard combination therapy and 25% in those treated with IFN-β induction therapy. These low rates of relapse were similar to the result of pegylated IFN/ribavirin combination therapy for 48 weeks [23–25]. We obtained a relatively high SVR rate for the patients with genotype 1 and super-high viral load (>500 KIU) by the limited treatment with a 6-month course of ribavirin. In addition, the SVR rates were higher in the β-induction group than in the standard combination group with or without IFN monotherapy. In particular, patients treated with IFN monotherapy followed by combination therapy with twice-daily pre-administration of IFN-β had a SVR rate of 46%. Moreover, no patients discontinued because of adverse events during the treatment protocol.

Generally, the beneficial effect of induction therapy remains controversial. In non-1b patients, high rates of SVR are obtained without induction [32]. In patients with genotype 1b and a high viral load, various studies including induction-dosing trials showed greater rates of early viral clearance. However, there were a few reports suggesting that early viral clearance was associated with a high prevalence of SVR [37, 39, 40]. Vrolijk et al. [41] demonstrated that daily induction therapy might be beneficial for IFN-resistant patients, but only when combined with adequate maintenance therapy of long duration. Drusano and Preston [28] demonstrated that not only the treatment duration but also the duration of therapy with an undetectable HCV-RNA load are associated with the probability of a long-term antiviral response during pegylated IFN + ribavirin combination therapy, and that patients infected with genotype 1 require a continuous non-detectable viral load in serum at least for 32 weeks. Indeed, some reports described that a sustained negative status of HCV-RNA for 2 or more years by long-term IFN therapy correlated with SVR in patients with genotype 1b and high viral load. However, a limitation was found in the patients with viral load over 3 Meq/ml or 500 KIU who were treated with IFN monotherapy [42, 43]. Long-term IFN therapy can be associated with an increased risk of development of adverse effects. In the present study targeted for the patients with genotype 1b and super-high viral load (>500 KIU), relatively high rates of SVR were obtained by combination therapy for 24 weeks followed by prolonged IFN monotherapy for an average of totally 56 weeks with twice-daily pre-administration of IFN-β as induction. The SVR rate of prolonged group was not inferior to 48 weeks of pegylated IFN/ribavirin combination therapy [23–25]. However, the significance of induction therapy was diluted in the maintenance protocol, because a prolonged negative HCV-RNA status led to a decrease in the relapse rate in the standard combination group.

The reason for the importance of a sustained long-term negative HCV-RNA status is unclear. One line of speculation suggests that after the disappearance of HCV-RNA in serum, HCV persists in hepatocytes. In the presence of IFN, which blocks viral production, newly infected hepatocytes would not be observed. Although the considerable variation in infected cell half-life could reflect individual differences in cellular immunity against HCV, immune control through faster killing of infected cells may have an important role in successful IFN treatment [30]. If the killing of infected cells by cytotoxic T lymphocytes functions adequately, removal of infected cells would be completed and SVR would be observed. Even if cytotoxic T lymphocytes do not function due to a quasi-species diversity and high viral load [14, 15], we could attain SVR to be sustained for 48 weeks from the time of the first