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Hepatitis B virus genotype G is an extremely rare genotype in Japan

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Abstract

Background: Hepatitis B virus (HBV) has been classified into seven genotypes (A–G). HBV genotypes have a geographically characteristic distribution. Since HBV genotype G (HBV/G) was identified recently, little is known about the distribution of HBV/G in Japan. The aim of this study was to clarify this issue.
Patients and methods: Seven hundred and twenty-one serum samples obtained from patients with HBV in Japan were investigated. The patients included 149 asymptomatic carriers, 325 with chronic hepatitis, 129 with liver cirrhosis, and 118 with hepatocellular carcinoma. Six HBV genotypes (A–F) were determined by restriction fragment length polymorphim targeting to the S region of the HBV genome. Furthermore, HBV/G was investigated by polymerase chain reaction with hemi-nested primers derived from an HBV/G-specific nucleotide sequence.
Results: Of the 721 serum samples investigated, 12 subjects were classified as having HBV/A, 88 HBV/B, 610 HBV/C, 3 HBV/D, and 1 HBV/F. Seven subjects had a mixed infection with distinct genotypes, two with HBV/A and HBV/D, and five with HBV/B and HBV/C. HBV/G was not identified among the 721 samples.
Conclusion: HBV/G was not identified in a large cohort of patients with HBV, either single or dual infection. HBV/G seems to be an extremely rare genotype in Japan.

Keywords: Distribution; Genotypes; Hepatitis B virus; Japan; Polymerase chain reaction; Restriction fragment length polymorphism

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1. Introduction

Hepatitis B virus (HBV) infects approximately 350 million individuals worldwide and can cause a wide spectrum of liver disease [1]. HBV has been classified into seven genotypes based on an entire genome difference of more than 8% [2–4]. HBV genotypes have a geographically characteristic distribution [5]. HBV genotype A (HBV/A) and HBV/D are the most common genotypes worldwide, and account for the majority of cases in Europe and Africa. HBV/B and HBV/C are found in East Asia. HBV/E is confined to Africa, and HBV/F has been identified in indigenous populations of Central and South America. In 2000, a unique strain harboring a 36-base pair (bp) insertion into the core region was identified in France and was phylogenetically classified into the seventh genotype, G [4]. Thereafter, HBV/G was revealed to be distributed in San Francisco [6,7], Germany [8], Mexico [9], and Canada [10], and accounted for 1–5% in these areas. Although little is known about the virological and clinical characteristics of HBV/G, one of its unique characteristics is frequent coinfection with the other genotypes. In San Francisco, eight of the eight HBV/G patients were coinfected with HBV/A [6,7], and all of the HBV/G isolates from Canada were also coinfected with HBV/A, or HBV/A and HBV/C [10].

In Japan, HBV/C is the most common genotype, accounting for approximately 85% of all genotypes, and HBV/B follows with 12% [11–13]. However, little is known about the distribution of HBV/G in Japan. We have formerly investigated the 540 sera from patients with hepatitis B collected in and around Nagoya, and found that there were no HBV/G among them [14]. However, the serum samples in the study was obtained from a restricted area, a central part of Japan, therefore, further studies including serum samples collected from the other part of Japan had been required to conclude how often HBV/G distributed in Japan. Moreover, since HBV/G is frequently coinfected with the other genotypes, there is a possibility that HBV/G might exist as a minor population in the sera classified into the other six genotypes (A–F). At this time, to elucidate this issue, we conducted nationwide study of the distribution of HBV/G by analyzing sera obtained from patients with hepatitis B, including those whose genotypes were already known, using hemi-nested polymerase chain reaction (PCR) with HBV/G-specific primers. We also discussed the issues of HBV/G to date.

2. Materials and methods

2.1. Patients

Seven hundred and twenty-one serum samples were collected from patients with HBV in Japan. The patients resided in Hokkaido, Iwate, Yamagata, Niigata, Tokyo, Kanagawa, Nagano, Nagoya, Kyoto, Fukuoka, and Okinawa. The patients in this study were overlapped with some of the previous report [11]. They included 470 (65.1%) males and 251 (34.8%) females. The mean ± S.D. age was 43.6 ± 14.9 years (Table 1).

Table 1
Demographics of the 721 patients in this study

Sample	721
Gender (M:F)	470:251
Age (year)	43.6 ± 14.9
ALT (IU)	78.8 ± 115.8
ALP (IU)	240.8 ± 155.2
γ-GTP (IU)	52.2 ± 96.2
T. bil (mg/dl)	0.99 ± 1.60
HBeAg (%)	45.2
HBV DNA[a] (LGE/ml)	5.69 ± 1.84
Diagnosis	
Asymptomatic carrier	149
Chronic hepatitis	325
Liver cirrhosis	129
Hepatocellular carcinoma	118

Abbreviations: ALT, alanine aminotransferase; ALP, alkaline phosphatase; γ-GTP, gamma-glutamyl transpeptidase; LGE, log genome equivalents; T. bil, total bilirubin; TMA, transcription-mediated amplification.

[a] Value was calculated using available data of transcription-mediated amplification of 255 subjects.

2.2. Detection of hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg) and HBV DNA level

HBsAg was detected by a particle-agglutination test using a commercial kit (Serodia: Fujirebio, Tokyo, Japan), and HBeAg was detected by ELISA using a commercial kit (Serodia: Kokusai-shiyaku, Tokyo, Japan), following the manufacturer's recommendations. Levels of HBV DNA were determined by the transcription-mediated amplification (TMA) method (Chugai Industry, Tokyo, Japan), and the results were expressed as log genome equivalents (LGE) per millilitre.

2.3. Determination of six HBV genotypes (A–F) by restriction fragment length polymorphism (RFLP)

DNA was extracted from 100 μl of serum samples using commercial kits (Smitest EX R&D: Genome Science, Fukushima, Japan) under manufacturer's recommendation. The extracted DNA was amplified in a 50-μl reaction mixture containing 0.5 μM of a sense primer MF1 (5′-YCC TGC TGG TGG CTC CAG TTC-3′: nt. 55–75), 0.5 μM of an antisense primer MR2 (5′-AAG CCA NAC ART GGG GGA AAG C-3′: nt. 730–709), 2.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems Japan Co. Ltd., Tokyo, Japan), 0.2 mM each dNTPs, 3 mM $MgCl_2$, and 1× AmpliTaq Gold Buffer. The reactions were performed in a GeneAmp PCR system 9600 thermocycler. The sample was denatured at 96 °C for 9 min, and subjected to 40 cycles of PCR (95 °C for 1 min; 60 °C for 1 min; 72 °C for 1 min) followed by 72 °C for 5 min at final extension in a 96-well cycler (GeneAmp 9600; Perkin-Elmer, Norwalk, CT, USA). The amplified product

was subjected to the second round PCR with a sense primer MF2 (5′-GTC TAG ACT CGT GGT GGA CTT CTC TC-3′: nt. 246–271) and MR2 under the same condition as the first round PCR. The second round PCR product with the length of 485 bp was subjected to the digestion with five kinds of restriction enzymes. Genotype B could be distinguished by digestion with *Ear*I because of no recognition site of it was existed. Similarly, genotype C also could be distinguished by digestion with *Alw*I, as no recognition site of it was found within the amplified product. Only genotype E had a recognition site of *Nci*I, and only genotype F had no recognition site of *Hph*I. Finally, the distinction between genotypes A and D were done by digestion with *Nla*IV. Genotype A has a recognition site of *Nla*IV, result in the generation of fragments of 220 and 265 bp. While genotype D had two recognition site of *Nla*IV, result in generation of fragments of 34, 186, and 265 bp. Therefore, genotypes A and D were distinguished by if each of 220 and 186 bp were observed, respectively. The digested amplicon were run on 3% agarose gel stained with ethidium bromide and observed under UV light [15].

2.4. Identification of HBV/G

Nucleic acids extracted from serum were subjected to PCR with hemi-nested primers designed on the 36-bp insertion in the C gene of HBV/G genomes. In brief, the DNA was amplified by the first round of PCR for 40 cycles with HBHKF1 (sense: 5′-ACG GGG CGC ACC TCT CTT TAC-3′ [nt. 1519–1539]) and HBHKR2 that involved the 36-bp insertion characteristic of HBV/G (antisense: 5′-AGC CAA AAA GGC CAT ATG GCA-3′ [nt. 17–37 in the core gene of HBV/G]) in the presence of AmpliTaq Gold (Applied Biosystems, Foster City, CA). The second round of PCR was performed for 40 cycles on the product of the first-round PCR with HBHKF2 (sense: 5′-GCA CTT CGT TTC ACC TCT GCA-3′ [nt. 1581–1601]) and HBHKR2. Then, the products were examined for fragments of 357 bp [15].

3. Results

3.1. Demographics, laboratory findings, and diagnosis of the patients

The mean value of alanine aminotransferase (ALT), alkaline phosphatase, gamma-glutamyl transpeptidase, and total bilirubin in the sera was 78.8 ± 115.8 IU, 240.8 ± 155.2 IU, 52.2 ± 96.2 IU, 0.99 ± 1.60 mg/dl, respectively (Table 1). Three hundred and twenty-six patients (45.2%) were positive for HBeAg. The mean value of HBV DNA measured by TMA was 5.69 ± 1.84 LGE per millilitre. One hundred and forty-nine patients (20.1%) were diagnosed as asymptomatic carriers, 325 (45.1%) with chronic hepatitis, 129 (17.9%) with liver cirrhosis, and 118 (16.4%) with hepatocellular carcinoma.

Table 2
Six genotypes (A–F) and HBV genotype G in 721 subjects from Japan

Genotype	No.	No. of HBV genotype G
A	12	0
A+D	2	0
B	88	0
B+C	5	0
C	610	0
D	3	0
F	1	0

3.2. HBV/G among 721 serum samples

Of the 721 serum samples investigated, 12 subjects were classified as having HBV/A, 88 HBV/B, 610 HBV/C, 3 HBV/D, and 1 HBV/F (Table 2). Seven subjects had a mixed infection with distinct genotypes, two with HBV/A and HBV/D, and five with HBV/B and HBV/C. HBV/G was not identified among the 721 samples.

4. Discussion

Several lines of evidence about the clinical significance of HBV genotypes have been accumulated in recent years. HBV/C causes more severe liver diseases than HBV/B by prolonging active hepatitis accompanying HBeAg production [16,17]. In a Western study, the rate of sustained remission after seroconversion was higher in genotype A than in genotype D hepatitis in patients who seroconverted to anti-HBe, and mortality related to liver disease was more frequent in genotype F than in genotype A or genotype D hepatitis [18]. Clinical data concerning HBV/G are very limited. One previous study analyzed 165 patients living in San Francisco and showed that the ALT level was higher in HBV/G than in HBV/C, and HBeAg was more prevalent in HBV/G than in HBV/C or HBV/D [7]. Further studies with a large sample size are warranted to confirm these findings.

Coinfection with distinct genotypes was seen also in other than HBV/G. In this study, coinfections with HBV/A and HBV/D as well as HBV/B and HBV/C were observed. In the previous study, analyzed 256 sera from the USA, Japan, Uzbekistan, Bangladesh, South Africa, and Cameroon, coinfection with distinct genotypes was identified in 28 subjects (10.9%) [19]. The occurrence of coinfection with distinct genotypes is important in virological aspects. It is reported that genomic recombination between distinct genotypes resulted in hybrid HBV strains, which causes distinct degree of liver diseases [20,21]. In such cases, genomic recombination never occurs without coinfection with distinct genotypes. However, clinical implication of coinfection with distinct genotypes per se still remains unanswered.

Ten years before the classification of HBV/G by Stuyver et al. [4], a unique strain with a 36-nucleotide insertion into the core region, which is known to a characteristic of HBV/G nowadays [22], was isolated from a homosexual man with hu-

man immunodeficiency virus infection [23]. Laboratory findings of his serum showed a few curious values. One was that HBeAg was detected in his serum in spite of a stop codon existing in the precore region of its genome, generally aborting the production of HBeAg at the stage of translation. Stuyver et al. also observed the same phenomenon, detection of HBeAg despite the stop codon in the precore region, and speculated that HBV/G might harbor another mechanism for producing HBeAg. Two years later, the mystery was solved by demonstration of coinfection with HBV/A in four of four sera with HBV/G [6]. It was explained that the HBeAg in the sera was produced by the coinfected precore wild type HBV/A. Furthermore, it was revealed that eight of the eight HBV/G patients from San Francisco were coinfected with HBV/A [7], and three of the three HBV/G patients were coinfected with HBV/A, or HBV/A and HBV/C in Canada [10]. These findings of the high frequency of coinfection of HBV/G with other genotypes give rise to another question, of whether HBV/G is competent to replicate by itself. An inoculation experiment in chimpanzees or an expression study in cultured cells would be required to answer this question.

The entire genome sequence of HBV/G has been reported from France [4,24], the USA [22], and Germany [8] so far. Interestingly, the sequence homology of these strains was surprisingly high. In one study in the USA, 10 HBV/G isolates, including 8 from San Francisco as well as 2 from France (FR1 [4] and B1-89 [24]), had a sequence homology of 99.3–99.8% among themselves [22]. Furthermore, another report from Germany showed that the HBV/G isolate (235/01) was nearly identical (sequence homology of the entire length was 99.7%) to both B1-89 and FR1 [8]. There are a few possible explanations for this finding. One possibility is that there are epidemiological links among French, German, and American HBV/G. A patient with HBV/G from Germany [8] and a homosexual male patient with HBV/G from San Francisco [23] were both positive for human immunodeficiency virus type-1. Thus, HBV/G might spread among a specific population, such as homosexual men or intravenous drug users. This would be also associated with the fact that HBV/G was not found among the patients in the current study, in which homosexual and intravenous drug were not included. The other possibilities are that HBV/G has a high genetic stability or was introduced into humans very recently. The mutation rate of HBV has been estimated to be 4.57×10^{-5} per site per year [25]. Thus, HBV/G might have an exceptionally low mutation rate under specific conditions, or the time since its introduction into humans might not have been long enough to gain a genetic diversity like that of the other six genotypes. To elucidate this issue, more HBV/G isolates from a wide variety of areas should be investigated.

In conclusion, HBV/G was investigated in a large cohort of patients with HBV from various areas in Japan, but no HBV/G isolate was identified, in either single or dual infection. The finding of the current nationwide study, the same as that of the previous study investigated the patients in a restricted area, indicates that HBV/G is extremely rare in Japan. Further studies with a large sample size from various areas in the world are required to further reveal the virological and clinical characteristics of HBV/G.

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Long-term follow-up of chronic hepatitis B after the emergence of mutations in the hepatitis B virus polymerase region

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SUMMARY. Treatment of chronic hepatitis B has been greatly improved by the use of lamivudine, but mutations occur in the polymerase region of hepatitis B virus (HBV) and lamivudine-resistant mutants frequently develop. The emergence of lamivudine-resistant strains of HBV is a problem for treating chronic hepatitis B using lamivudine. We observed biochemical and virological changes in 15 patients with chronic hepatitis B for a median period of 29 months (range: 4–42 months) after the emergence of lamivudine-resistant mutants of HBV. Patterns of mutation of the polymerase gene were examined by sequencing the LLAQ motif in domain B and the YMDD motif in domain C. Exacerbation of liver dysfunction occurred in 14 (93.3%) of the 15 patients at a median of 4 months after the emergence of mutations. However, exacerbation of liver dysfunction was observed only in four patients (26.7%) at the time of appearance of the first mutations and in 80.0% of the patients at the time of appearance of the second mutations. Increase in serum alanine aminotransferase (ALT) levels was significantly greater at the time of appearance of second mutations ($P = 0.0096$). In most cases, wild-type HBV was mutated with the substitution of only rtM204I at first, and rtL180M/M204I mutations and then rtL180M/M204V mutations subsequently appeared. Further mutations of the polymerase region caused clinical deterioration. Thus as mutations emerge in the polymerase region, the clinical outcome deteriorates. Thus, monitoring the patterns of mutation of the polymerase gene is useful when using lamivudine for treating HBV.

Keywords: breakthrough, hepatitis B virus, lamivudine, LLAQ, mutation, YMDD.

INTRODUCTION

Lamivudine is a nucleoside analogue that suppresses the replication of hepatitis B virus (HBV) by inhibiting the viral RNA-dependent DNA polymerase. Treatment of chronic hepatitis B has been greatly improved by the use of lamivudine, and the rates of seroconversion (loss of HBe antigen and appearance of anti-HBe) in HBe antigen-positive patients have been reported to be 16–22% after 1 year and 35–40% after 3 years of lamivudine therapy [1–4]. Moreover, in HBe antigen-negative patients, normalization of alanine aminotransferase (ALT) and suppression of serum HBV-DNA to undetectable levels have been achieved [5]. However, it has been reported that lamivudine-resistant HBV mutations of the polymerase region develop in 30% of patients after 1 year and in 49–57% of patients after 3 years of lamivudine therapy [3,6]. Breakthrough hepatitis induced by lamivudine-resistant mutations is sometimes difficult to treat and can be fatal, and is one of the biggest problems in lamivudine treatment of chronic hepatitis B. Although new nucleoside analogues such as adefovir dipivoxil and entecavir used in the United States, Europe, Australia and some Asian countries have demonstrated clinical activity against lamivudine-resistant strains of HBV [7–9], lamivudine is still a key drug in the treatment of chronic hepatitis B. Elucidation of the clinical course of hepatitis B after the emergence of lamivudine-resistant mutations is important. In this paper, we report the long-term biochemical and virological changes in HBV after the emergence of mutations in chronic hepatitis B patients.

Abbreviations: ALT, alanine aminotransferase; HBV, hepatitis C virus; PCR, polymerase chain reaction; IFN, interferon.

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PATIENTS AND METHODS

Patients

During the period from March 1999 to February 2002, 40 patients with chronic hepatitis B were treated with lamivudine (100 mg/day) at the Hokkaido University Hospital, and HBV mutations emerged in 15 (37.5%) patients.

Table 1 Patients' characteristics at the start of lamivudine treatment

Total	$n = 15$
Age median (range)	36 (23–59)
Sex	
Male	11 (73.3%)
Female	4 (26.7%)
Background liver disease	
Chronic hepatitis	13 (86.7%)
Cirrhosis	2 (13.3%)
Knodell's Histologic Activity Index ($n = 10$)	
Necroinflammatory score	6.9 ± 3.1
Fibrosis score	1.9 ± 1.3
HBeAg positive	14 (93.3%)
ALT	407 ± 439 (IU/L)
HBV-DNA	7.7 ± 1.3 (LGE/mL)

Age is expressed as median (range) and values of Knodell's Histologic Activity Index and ALT and HBV-DNA are expressed as mean ± SD.

Laboratory testing

Serum ALT levels and HBe antigen, anti-HBe and serum HBV-DNA levels were checked biweekly or at least once a month. HBe antigen and anti-HBe levels were determined using radioimmunoassay kits (Abbot, North Chicago, IL, USA), and serum HBV-DNA levels were measured using transcription-mediated amplification (TMA) assay kits (Chugai Diagnostic Science Co., Ltd, Tokyo, Japan).

Sequencing of the polymerase region

DNA was extracted from 200 μL of serum of each patient by using a QIAamp DNA blood kit (Qiagen, Chatswoth, CA, USA). Five microlitres of DNA template was mixed with 12.5 μL of PCR Master Mix (Promega, Madison, WI, USA), 0.4 μM of sense and antisense primers, and 5.5 μM nuclease-free water for amplification by PCR. The sense primer for PCR was 5′-TGGCTATCGCTGGATGTGTCT-3′ and the antisense primer was 5′-TTGTTCAGTGGTTCG-TAGGGC-3′. The conditions of polymerase chain reaction (PCR) were as follows: 94 °C for 2 min for the initial incubation, 94 °C for 30 s for denaturing, 57 °C for 30 s for annealing, 72 °C for 1 min for extension for 35 cycles, and a final extension step of 72 °C for 5 min. The DNA product was purified by using a QIAquick PCR Purification Kit (Qiagen). The PCR products were reacted by using an ABI PRISM BigDye Terminator v3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with the sequence primer of 5′-CCCTCATGTTGCTGTA-CAAAACCT-3′. Then sequence reaction products were purified by using a DyeEx Spin Kit (Qiagen) and sequenced by using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). We checked two motifs of the HBV polymerase region, the leucine–leucine–alanine–glutamine (LLAQ) motif from codon rt179 to codon rt182 in domain B of the polymerase region, and the tyrosine–methionine–aspartate–aspartate (YMDD) motif from codon rt203 to codon rt206 in domain C. Emergence of lamivudine-resistant mutations was defined as detection of LLAQ and/or YMDD motif mutations. Sequencing of the polymerase region was performed at intervals of 2 weeks to 3 months partly retrospectively, and serial changes in mutation patterns of the HBV polymerase region were observed. In this study, the first mutation was defined as any mutation in the polymerase region detected for the first time and the second or further mutations were defined when new patterns of mutations emerged after previous mutations.

Statistical analysis

Mann–Whitney's *U*-test was used to compare the periods to the emergence of mutations, and Student's *t*-test test was used to compare serum ALT and HBV-DNA levels. All *P*-values were two-sided, and $P < 0.05$ was considered to be statistically significant. All serum HBV-DNA levels less than the limit of detection (<3.7 LGE/mL) were analysed as being 3.7 LGE/mL, and levels above the upper limit of detection (more than 8.8 LGE/mL) were analysed as being 8.8 LGE/mL.

RESULTS

Patients

We observed biochemical and virological changes, including patterns of mutations in the polymerase region, in those 15 lamivudine-resistant patients. Baseline characteristics of the patients at the start of lamivudine treatment are shown in Table 1. The 15 patients included 11 males and four females with a median age of 36 years (range: 23–59). Liver biopsies were performed in 10 patients. In those 10 patients, the necroinflammatory score of hepatitis was 6.9 ± 3.1 (mean ± SD) and the fibrosis score was 1.9 ± 1.3 in the Knodell's Histologic Activity Index [10]. HBe antigen was positive in 14 (93.3%) of the 15 patients. The mean serum ALT level and mean serum HBV-DNA level in the 15 patients were 407 ± 439 IU/L and 7.7 ± 1.3 log genome equivalent (LGE)/mL, respectively. The median follow-up periods were 35 months (range: 27–50 months) from the start of lamivudine treatment and 29 months (range: 4–42 months) after the emergence of lamivudine-resistant mutations.

Period to the emergence of mutations

The median period from the start of lamivudine therapy to emergence of mutations was 16 months (range: 5–34). The

median period was 22 months (range: 12–30 months) in patients with HBV-DNA levels <7.7 LGE/mL (mean serum HBV-DNA level of 15 patients at the start of lamivudine therapy), and it was 15 months (range: 7–18) in patients with higher levels. Patients with higher serum HBV-DNA levels developed mutations more rapidly ($P = 0.0056$).

Patterns of mutation in the HBV polymerase region

Mutation patterns of the polymerase region changed serially (Table 2). The median number of changes in mutation pattern during the follow-up period was 2 (range: 1–4). In nine (60.0%) of the 15 patients, the first mutation pattern was wild type in domain B and YIDD (rtM204I) in domain C. The first mutation patterns of the LLAQ motif in domain B were LMAQ (rtL180M) in five patients (83.3%) and LLTQ (rtA181T) in one patient (16.7%), and those of the YMDD motif in domain C were YIDD (rtM204I) in 10 patients (76.9%) and YVDD (rtM204V) in three patients (23.1%). Only four patients (26.7%) showed liver dysfunction at the time of the appearance of the first mutations. In 10 patients, further mutations appeared in the polymerase region 7 months (range: 1–27 months) after the appearance of the first mutations, and exacerbation of liver dysfunction occurred in eight (80.0%) of the 10 patients. Second or further mutations occurred in domain B and/or domain C, and each mutation caused exacerbation of liver dysfunction. In most cases, mutation with substitution of only rtM204I appeared at first, and mutation with rtL180M/M204I and then mutation with rtL180M/M204V subsequently emerged. Further mutations of the polymerase region caused worse clinical outcomes (Table 3).

Serum ALT and HBV-DNA levels

Exacerbation of liver dysfunction was observed frequently. Serum ALT levels remained within the normal range in only one patient (6.7%), and they were lower than 100 IU/L in five patients (33.3%), 100–500 IU/L in four patients (26.7%), and exceeded 500 IU/L in five patients (33.3%). The median period from the emergence of mutations to the start of exacerbation of liver dysfunction was 4 months (range: 2–10), and the peak serum ALT level was 367.8 ± 385.8 IU/L (mean ± SD). The peak serum ALT level after the appearance of the first mutations was 191.8 ± 281.6 IU/L and that after the appearance of the second mutations was 570.6 ± 390.3 IU/L, exacerbation of liver dysfunction being significantly more

Table 3 Patterns of mutations in the polymerase region and exacerbation of liver dysfunction

Domain C: wild Domain B: wild	→	YIDD wild	→	YIDD LMAQ	→	YVDD LMAQ
n		12		3		10
Extraction of liver dysfunction (ALT >200 IU/L)		4 (33.3%)		2 (66.7%)		7 (70.0%)

Table 2 Mutation patterns of the HBV polymerase region

case	First mutation	Second mutation	Third mutation	Fourth mutation	Follow-up period (months)
1	LLAQ YIDD*	LMAQ YVDD*			42
2	LLTQ YMDD	LMTQ YVDD	LMAQ YVDD		36
3	LLAQ YIDD				35
4	LLAQ YIDD	LMAQ YIDD*	LLAQ YIDD		33
5	LLAQ YIDD	LMAQ YVDD*	LLAQ YIDD		32
6	LLAQ YIDD*	LMAQ YIDD*	LMAQ YMDD†	LMAQ YVDD*	31
7	LMAQ YVDD				29
8	LLAQ YIDD†	LLAQ YMDD	VLAQ YMDD		26
9	LMAQ YIDD	LMAQ YVDD†			24
10	LLAQ YIDD				19
11	LLAQ YIDD				19
12	LLAQ YIDD	LMAQ YVDD*			17
13	LMAQ YVDD	LLAQ YIDD*			13
14	LMAQ YVDD†				11
15	LMAQ YMDD	LMAQ YVDD†			8

*Liver dysfunction: ALT >500 IU/L; †Exacerbation of: ALT >200 IU/L.
Values with '*' and '†' mean that the mutations were accompanied with exacerbation of liver dysfunction. Values with '†' mean ALT levels of 200–500 IU/L and '*' mean more than 500 IU/L.

severe after the appearance of second mutations ($P = 0.0096$).

The mean serum HBV-DNA level was 5.00 ± 0.39 LGE/mL (mean ± SE) at the first detection of mutations. The peak value of serum HBV-DNA levels was 6.27 ± 0.59 LGE/mL after the appearance of the first mutations, and it increased further after the appearance of the second mutations, peaking at 7.18 ± 0.54 LGE/mL (Fig. 1).

Clinical course

The clinical courses of 11 patients who did not use other antiviral drugs such as interferon (IFN) or new nucleoside analogues after the emergence of mutations were observed. The median follow-up period was 30 months (range: 8–42). Serum ALT levels remained within the normal range in only one patient and normalized in five patients after temporary worsening. In two patients, seroconversion of HBe antigen to anti-HBe occurred after temporary worsening of liver function, and serum HBV-DNA levels were decreased to undetectable levels. However, the exacerbation of liver dysfunction continued in five patients.

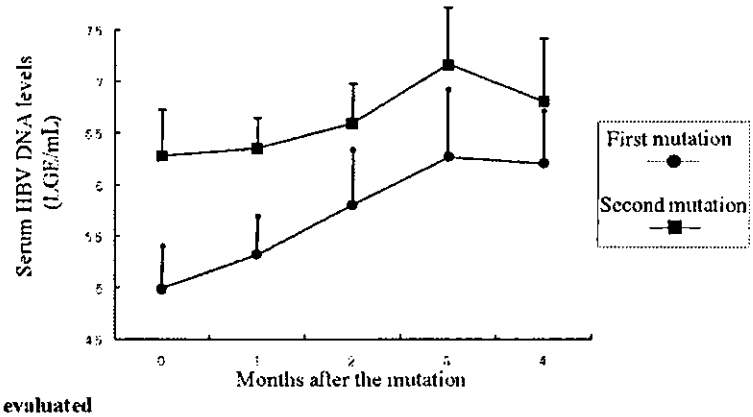
Four patients with severe breakthrough hepatitis were treated with IFN, and the median follow-up period for those patients was 6.5 months (range: 3–14). Six million units of IFN-beta was administered everyday for the first 4 weeks and thereafter three times a week for 20 weeks. Serum ALT levels were normalized in three patients, and serum HBV-DNA levels decreased to undetectable levels in two patients. One of the three patients who had HBe antigen before IFN treatment achieved seroconversion.

DISCUSSION

Lamivudine is a nucleoside analogue that suppresses replication of HBV by inhibiting the viral RNA-dependent DNA polymerase. It has been reported that resistance to lamivudine often develops after 6 months of treatment [11,12] Mutations occur in the polymerase region of HBV-DNA, and HBV becomes resistant to lamivudine. There have been many reports on mutations in the polymerase region and viral resistance, most of them focusing on the YMDD motif from codon rt203 of domain C and the LLAQ motif from codon rt179 of domain B of the HBV polymerase region [13–16]. In this study, we observed changes in serum ALT and HBV-DNA levels, HBe antigen, and anti-HBe in relation to serial changes in mutation patterns of the HBV polymerase region for a median period of 29 months after the emergence of mutations.

Serum HBV-DNA levels increased as soon as the first mutation occurred in the polymerase region, indicating that monitoring of serum HBV-DNA levels is important and that serum HBV-DNA level can be used as a predictive factor for the appearance of mutations as reported previously [17]. On the contrary, increases in serum ALT levels were delayed and ALT level peaked at a median of 4 months after the emergence of mutations. It has been reported that biochemial breakthrough phenomena are usually observed several months after the first detection of strains resistant to lamivudine [18,19]. This time lag is thought to be due to the duration until the occurrence of second or further mutations in the polymerase region.

In our series, rtL179V, rtL180M and rtA181T mutations were observed in domain B, and rtM204I and rtM204V mutations were observed in domain C. These mutations are almost the same as those reported previously [20,21]. In most cases, a mutation with only rtM204I appeared at first, and rtL180M/M204I mutations and then rtL180M/M204V mutations appeared subsequently in the polymerase region. Exacerbation of liver dysfunction at the time of appearance of the first HBV mutations occurred in only 26.7% of our patients. However, when further mutations appeared in 10 patients (66.7%) a median of 7 months after the appearance



No. evaluated					
First mutation	15	15	14	12	11
Second mutation	10	10	9	8	7

Fig. 1 Changes of serum HBV-DNA levels after the emergence of mutations. The changes of serum HBV DNA levels after the first mutations to the second were shown by closed circles and after the second mutations to the third by closed squares. Serum HBV-DNA levels were measured by TMA assay. Vertical bars mean standard errors.

of first mutations, worsening of liver impairment occurred in 80.0% of the patients. Further mutations resulted in worsening of clinical courses. Although mutations of the YMDD motif in domain C have stronger effects on resistance to lamivudine than those of the LLAQ motif in domain B, it has been reported that single C-domain mutants have remarkedly decreased abilities of replication [22–24] and that B-domain mutation rtL180M rescues the defective replication competence of domain C mutants [25,26]. In another study, the effect of the addition of rtL180M mutation was examined by using a three-dimensional homology model of the catalytic core of HBV, and it was also shown that the rtM204V mutant is more resistant to lamivudine than the rtM204I mutant and that rtL180M substitution makes each mutant more resistant to lamivudine *in vitro* [27]. These results explain our finding that exacerbation of liver dysfunction occurred more frequently in cases with further mutations because rtM204V and/or rtL180M mutations were detected more frequently in our series when further mutations occurred in the polymerase region. Although small amounts of HBV mutants could not be detected because of the detection limit of direct sequencing, our results showed the significant correlation between accumulation of mutations and exacerbation of liver dysfunction. Direct sequencing was a useful method to detect mutations in the polymerase region clinically. But it is more effective to detect especially second or further mutations by more sensitive methods such as peptide nucleic acid mediated PCR clamping [28,29] because these mutations frequently caused severe liver dysfunction.

Exacerbation of liver dysfunction occurred without increase in serum HBV-DNA level after the emergence of mutations in some of our patients, indicating that some other factors may also lead to exacerbation of liver dysfunction. The polymerase gene of HBV overlaps with the surface antigen gene, and mutations in the polymerase region result in a change in the relevant amino acid of the surface antigen. It is possible that changes in the amino acid of the surface antigen may induce exacerbation of liver dysfunction.

In 11 patients, antiviral drugs other than lamivudine were not used. Fatal cases after discontinuation of lamivudine treatment have been reported [30], and it has been reported that lamivudine still suppresses the replication of wild-type HBV-DNA after the emergence of mutations [11,31]. We therefore continued lamivudine treatment after the appearance of lamivudine-resistant mutations. Serum ALT levels were normalized in six patients, and two of those six patients achieved seroconversion after temporary worsening. Thus, good clinical courses following temporary worsening were observed in some cases, indicating that observation without using other antiviral drugs is one of choices for patients with breakthrough hepatitis. However, exacerbation of liver dysfunction continued in five of our patients, and HBe antigen reappeared in two of them who had anti-HBe before the emergence of mutations. It has been reported that breakthrough hepatitis can be fatal in patients with advanced liver disease [32]. Patients with continuous liver dysfunction after emergence of mutations and patients with advanced liver disease should therefore be treated with new antiviral drugs such as adefovir dipivoxil.

Although the follow-up period was short, a good clinical response to IFN in patients with breakthrough hepatitis was obtained. Suzuki *et al.* [33] reported that IFN therapy was effective for lamivudine-resistant HBV mutants and that it may induce virological and clinical improvement accompanied by seroconversion. Although IFN has some side-effects and it is difficult to use for cirrhotic patients, it is one of the treatment options for breakthrough hepatitis. However, it has also been reported that serum HBV-DNA levels increased again and that hepatitis reccurred after reducing the dose of IFN [33]. Indeed, hepatitis recurred after cessation of IFN treatment in one of our patients. Care should therefore be taken in reducing the dose of IFN or terminating IFN treatment.

CONCLUSIONS

Exacerbation of liver dysfunction was observed frequently after the emergence of mutations in the HBV polymerase region. In most cases, the mutation pattern was a substitution of only rtM204I at first, and rtL180M/M204I mutations and then rtL180M/M204V mutations subsequently appeared. Exacerbation of liver dysfunction became more severe as more mutations occurred in the polymerase region. These results suggest that monitoring the patterns of mutation of the polymerase gene is useful when using lamivudine for treatment of HBV.

In some cases, serum ALT levels were normalized and seroconversion was achieved after temporary worsening of liver impairment, indicating that treatment is not necessarily required for all cases after the appearance of lamivudine-resistant mutations.

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Progressive Disappearance of Anti-Hepatitis B Surface Antigen Antibody and Reverse Seroconversion after Allogeneic Hematopoietic Stem Cell Transplantation in Patients with Previous Hepatitis B Virus Infection

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Reactivation of resolved hepatitis B virus (HBV) infection, which is known as reverse seroconversion (RS), has been reported as a rare complication of allogeneic hematopoietic stem cell transplantation. We retrospectively studied HBV serologic markers in 14 recipients with pretransplant anti-hepatitis B surface antigen antibody (anti-HBs). Progressive decreases in anti-HBs titer were observed in all cases. In 12 cases, anti-HBs titer had decreased to under the protective value. RS occurred in seven cases after disappearance of anti-HBs. Although reseroconversion occurred in five cases, two cases remained in an HBV-carrier status after resolution of hepatitis. In the other five cases, RS did not occur even after disappearance of anti-HBs. The actual risks of anti-HBs disappearance and RS were estimated to be 75.0% and 39.8% at 2 years and 100.0% and 70.0% at 5 years, respectively. In conclusion, RS is a late-onset complication with high frequency that can be predicted by careful monitoring of progressive decrease in anti-HBs titer.

Keywords: Hepatitis B virus, Reverse seroconversion, Reactivation hepatitis.

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Appearance of anti-hepatitis B surface antigen antibody (anti-HBs) and clearance of hepatitis B virus (HBV) from serum usually indicate resolution of hepatitis in patients infected with HBV. However, most patients in whom HBV has been eliminated from the serum still have HBV DNA in the liver that is detectable by using polymerase chain reaction (PCR) (*1*). Reactivation of this dormant HBV in the liver has been observed in an immunocompromised status such as hematopoietic stem cell transplantation (HSCT), renal transplantation, intensive chemotherapy, or use of rituximab (*2–5*). Reactivation of hepatitis in anti–HBs-positive patients is known as reverse seroconversion (RS). There have been several case reports of RS occurring after allogeneic HSCT (allo-HSCT) as a rare complication (*6–12*). However, precise frequency of RS and results of long-term follow-up after RS have not been reported. In some cases, disappearance of anti-HBs was observed several months before RS (*4, 6, 7, 9*). In this study, we investigated the time course of immunologic status against HBV and the incidence of RS in patients with preHSCT anti-HBs.

PATIENTS AND METHODS

Patients

Fifty-six patients who had undergone allo-HSCT and had been followed for at least 1 year after the transplantation in our institute during the period from February 1990 to March 2003 were enrolled as subjects of this study. Fourteen of the 56 patients were preHSCT anti-HBs positive. Thirteen of the 14 patients were also positive for anti–hepatitis-B core antigen antibody (anti-HBc), and one patient was negative for anti-HBc. Patients' characteristics are shown in Table 1. T1
We retrospectively studied hepatitis B surface antigen (HBsAg) (Clinical Laboratory Improvement Amendments [CLIA]), anti-HBs (CLIA), hepatitis B e antigen (HBeAg) (radioimmunoassay [RIA]), anti-hepatitis e antigen antibody (anti-HBe) (RIA), and HBV-DNA (PCR) in those 14 patients using cryopreserved serum samples stored at −20°. No patients had a prior history of vaccination or HBV-specific immunoglobulin (Ig) usage. All donors were negative for HBsAg, and seven donors were confirmed to be negative for anti-HBs. Anti-HBs in the other seven donors who donated bone marrow before 1998 were not investigated in our institute or in the Japan Marrow Donor Program because RS was not commonly recognized as a complication of allo-HSCT at that time. Therefore, there was no donor who was confirmed to be anti-HBs positive in this study. The follow-up period varied from 15 to 92 months (median 48 months). Ten grams of Ig was administered intravenously on day 0 and every other week until day 100 for prophylaxis of opportunistic infection. Chronic graft-versus-host disease (cGvHD) was observed in 10 cases, and prednisolone was administered for treatment of cGvHD in 2 of those 10 cases. Only one case (case 1 in Fig. 1A) F1
had relapse of hematologic malignancy during the follow-up period.

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C型慢性肝炎　肝組織内HCV-RNAの動態

C型慢性肝炎の肝組織内RNA量の測定
—プラス鎖RNA，マイナス鎖RNA別—

Measurements of strand-specific hepatic HCV-RNA quantities of patients with hepatitis C virus infection

髭　修平

Key words：肝組織内HCV-RNA，プラス鎖HCV-RNA，マイナス鎖HCV-RNA，strand-specific RT-PCR法

はじめに

C型肝炎ウイルス(HCV)の大部分は肝細胞内で増幅し，ダイナミックに肝細胞内外を移動している．HCVを含むフラビウイルス科のRNA複製に関しては，ウイルスの非構造蛋白質や宿主蛋白質の作用も含めて次第に明らかになってきた[1,2]．HCVの複製の過程においては相補的なマイナス鎖RNAを合成し，それを鋳型としてプラス鎖(genomic)RNAを合成する．したがって，マイナス鎖RNAは増幅の中間体と考えられ，その検出はウイルス増殖の直接的な証明となり得る．肝組織内のHCV-RNA量の測定は臨床的に簡便ではないが，更に，プラス/マイナス鎖RNA量の特異的測定には方法論的にも注意が必要である．

本稿では著者の肝組織内のRNA量測定結果を含めて臨床的意義について述べる．

1. 肝組織内HCV-RNAの測定方法

生検あるいは手術による肝組織から核酸を抽出した．検体重量と核酸量には相関を認め，平均すると肝組織1mgあたりの抽出核酸量は1.6μgであった．

肝組織からの核酸抽出にはAGPC法を用いた．抽出したRNA溶液にreverse transcriptaseを用いて逆転写反応を行った．その際，HCV-RNAの検出にはrandom primerを，プラス鎖RNA，マイナス鎖RNAの検出には，各鎖に特異的なanti-sense primer，sense primerを使用した(strand-specific RT-PCR法)．作製されたcDNAにTaq polymeraseを加えてPCRを施行し増幅したが，その定量には標識ヌクレオチドを用いたRT-PCR法を用いた[3]．

2. マイナス鎖特異的HCV-RNA検出の問題点と対策

strand-specific RT-PCRを施行する際，特にマイナス鎖HCV-RNAの検出に関しては，偽陽性の出現についての注意が重要である．その理由として，①virus RNAによるself-priming，②細胞内核酸によるrandom priming，③加えたprimerのfalse primingなどが想定されている[4]．偽陽性出現への対策として，逆転写反応の際に，①ホウ素化合物でRNAの3′末端を修飾する，②tagged primerを用いる，③熱に安定で，逆転写酵素とDNAポリメラーゼ活性を併せもつrTthを使用する，④マイナス鎖が有

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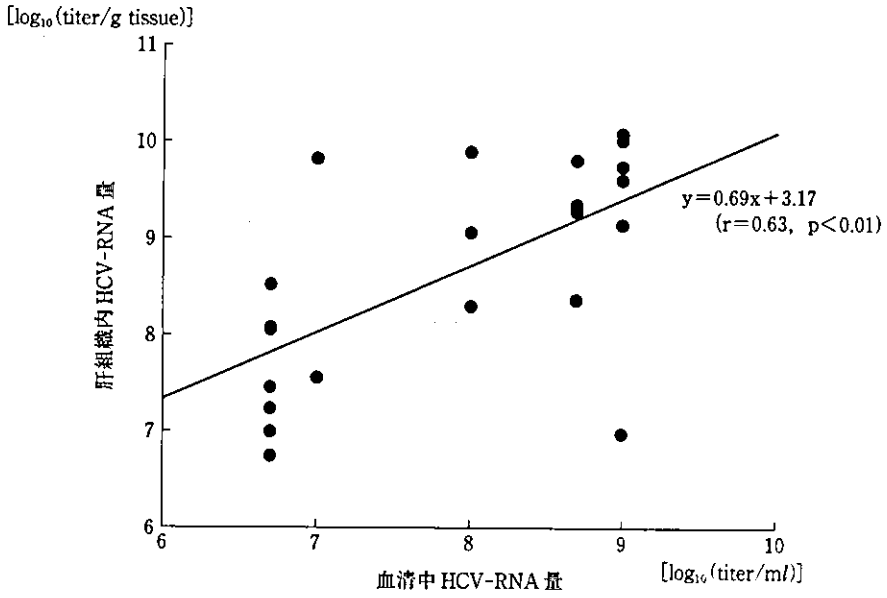

図1 C型慢性肝疾患患者の血清中および肝組織内HCV-RNA量

するpoly A^+領域を指標にRNAを分離後，逆転写を行う，などの方法により検出の特異性が上昇することが報告されている．①では核酸抽出物を$NaIO_4$および$NaBH_4$で処理後に逆転写反応を行う[5]．②ではcDNA合成時に使用するプライマーの5′末端側にHCV-RNAとは異なる塩基配列('tag')をもったものを使用する[6]．③ではrTthを，70℃の高温でMn^{2+}添加下に使用する[6]．この酵素はMn^{2+}キレートで逆転写酵素活性を失活させ，Mg^{2+}添加でDNA依存のポリメラーゼ活性を活性化することができる．通常の逆転写反応は42℃以下で行われることが多いが，反応温度の高い方が感度・特異性ともに上昇する．④は，プラス鎖RNAの3′UTR(非翻訳領域)のpoly(U)配列に対応して有するマイナス鎖5′末端のpoly A^+をビオチン標識oligo (dT) probeでハイブリダイズして分離することでマイナス鎖のみを逆転写反応に進める[7]．

本稿に示す著者の成績は，上記①のGunjiらの方法に準じて以下の化学処理を施した．すなわち，核酸抽出物に0.5M sodium acetate(pH 5.0)，20mM $NaIO_4$を加え，30℃で12時間反応後，10% ethylene glycolを加えてエタノール沈殿した．沈殿物を蒸留水で溶解し，0.1N $NaBH_4$を加えて0℃で1時間反応後に0.1N acetic acidを加えてエタノール沈殿させ，沈殿物を以後の逆転写反応に用いた．

3. 肝組織内のHCV-RNA量

肝組織内のHCV-RNA量と対応する血清中のHCV-RNA量を測定した結果，両者に相関を認めた($r=0.63$, $p<0.01$, 図1)．したがって，血清中RNA量から肝組織内のRNA量の推測が可能であった．肝組織1gと血清1ml中のHCV-RNA量を比較すると，肝内RNA量が血清中に比べて数十倍多かった．また，肝組織からはプラス鎖，マイナス鎖HCV-RNAの両方が検出された．両者には相関を認め($r=0.63$, $p<0.01$)，マイナス鎖RNA量はプラス鎖RNA量の数%程度であった(図2)．

KnodellのHAI(histological activity index)スコアにおける肝の線維化別の肝組織内HCV-RNA量(平均±標準偏差)は，プラス鎖はスコア0群で9.1±1.0，スコア1群で8.6±1.0，スコア3群で8.7±0.8，スコア4群で7.8±1.1[$\log_{10}$(titer/g tissue)]，マイナス鎖はスコア0群で

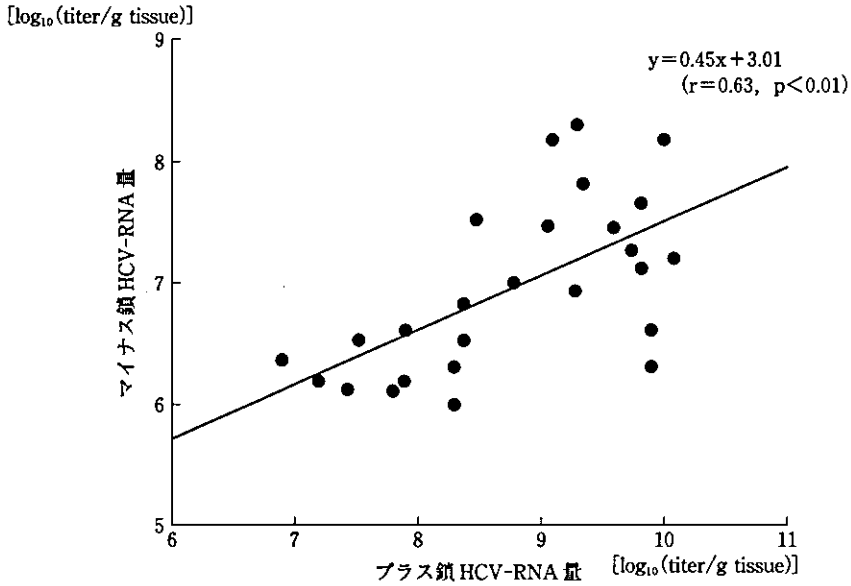

図2 肝組織内のプラス鎖HCV-RNA量とマイナス鎖HCV-RNA量

7.1±0.8，スコア1群で6.9±0.2，スコア3群で7.1±0.7，スコア4群で6.4±0.3[$\log_{10}$(titer/g tissue)]であった(**図3-B**)．プラス鎖HCV-RNA量に関しては，スコア0群とスコア4群の間に有意差($p<0.05$)を示し，肝硬変症例でウイルス量低下を認めた．

一方，肝炎の活動性をHAIのgrade Iからgrade IIIのスコアの合計で示した場合，肝組織内のHCV-RNA量と組織活動性の間に一定の関連は認めなかった(**図3-A**)．更に，血清ALT値と肝組織内HCV-RNA量との間には，プラス鎖，マイナス鎖のいずれとも相関を認めなかった(**図4**)．また，プラス鎖HCV-RNAに対するマイナス鎖HCV-RNAの比率も，肝の組織所見(HAIスコア)や血清ALT値との相関を認めず，HCVはこれらの所見とは無関係に増幅していると考えられた．

4. 肝内HCV-RNA量測定の臨床的意義

前述のとおり，肝組織内のHCV-RNAは血清中のそれに比して単位容積あたりで多量に存在しており，マイナス鎖HCV-RNAが検出される事実と合わせ，肝内でのHCV増殖の裏付けと考えられる．プラス鎖あるいはマイナス鎖RNA量が肝組織内でのHCV増殖の指標と考えた場合，これらは肝組織の活動性や血清ALT値とは相関しなかった．このことは，肝細胞障害が肝組織内HCV増殖に直接的に関連するのではなく，免疫学的反応などの他の要因による結果であることを示している．Negroらも，strand-specific RT-PCR法を用いた肝組織内HCV-RNAの半定量測定成績から，HCVの増幅の程度については血清中HCV-RNA量測定の方が臨床的に有用であると報告している[8]．

おわりに

肝組織内のHCV-RNA量の測定は，肝内HCVの増幅の程度を知るための直接的な検査法である．肝組織内HCV-RNA量は血清中HCV-RNA量とは相関するが，肝組織所見や血清ALT値とは関連せず，C型肝炎がHCVの直接的肝細胞障害以外の機序により起こることを示している．

マイナス鎖HCV-RNAはHCVが組織内で増幅していることの直接的な証明となるが，その検出系の確立には十分な注意が必要である．

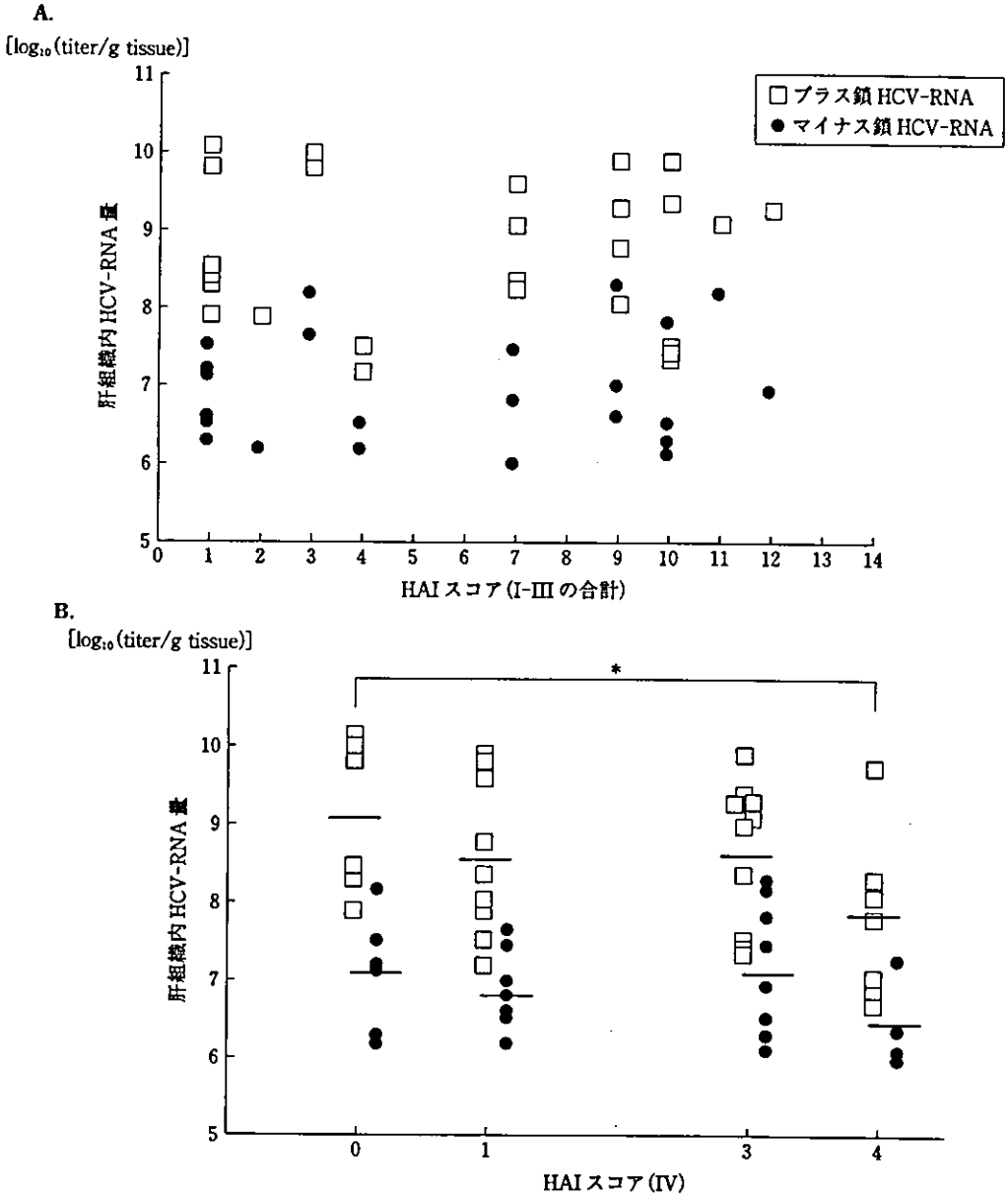

図3 C型慢性肝疾患患者の HAI スコアと肝組織内 HCV-RNA 量 (プラス鎖・マイナス鎖別)

A: HAI grade I-III のスコア合計と肝組織内 HCV-RNA 量

B: HAI grade IV のスコアと肝組織内 HCV-RNA 量

(*$p<0.05$)

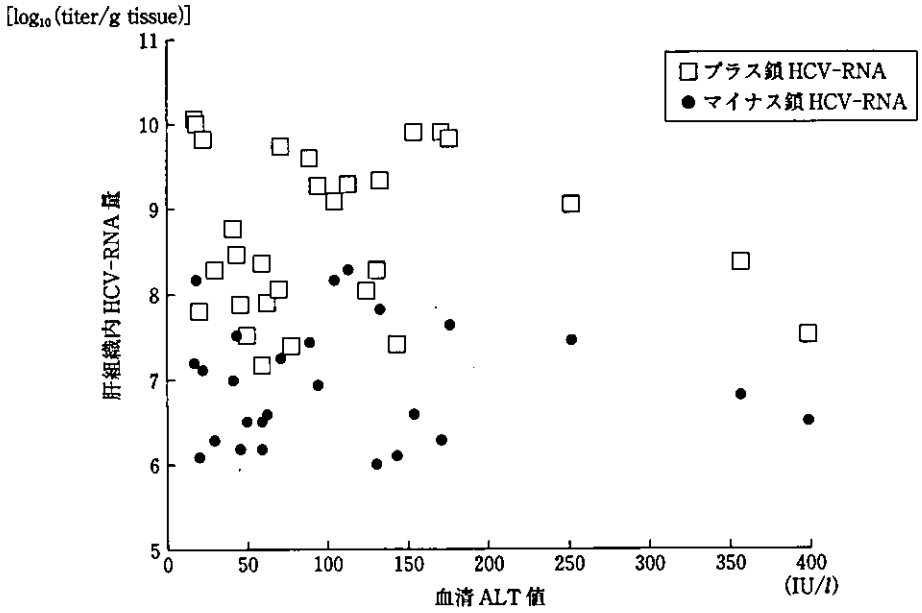

図4 C型慢性肝疾患患者の血清ALT値と肝組織内HCV-RNA量(プラス鎖・マイナス鎖別)

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B型慢性肝炎・肝硬変治療症例集

——抗ウイルス薬／ラミブジン・アデホビルピボキシル——

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症例７．ラミブジン投与中止後に肝炎の再燃を起こし，ラミブジン再投与により改善した症例

髭　修平

患者背景

30 歳　男性

診断名	B 型慢性肝炎	罹病期間	10 年	入院 or 外来	入院
既往歴	特記すべき事なし				
家族歴	父：B 型肝硬変症，母および弟：HBV キャリア				
生活歴	飲酒歴，喫煙歴なし				

前治療薬

薬剤名	用量	用法	評価
インターフェロンβ	600 万単位 / 日	静脈内投与，4 週間	無効

病　歴

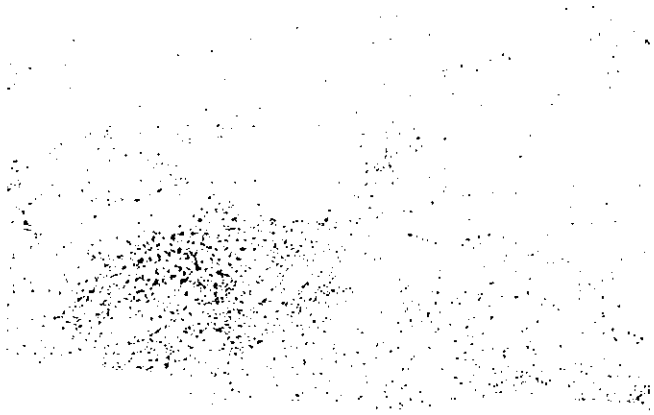
1991 年，眼科処置時に B 型肝炎を発見され，1991 年，1992 年，1997 年の 3 回，インターフェロン治療を受けたが改善せず，HBe 抗原陽性のまま肝炎の再燃を繰り返していた。2000 年 7 月 6 日に再治療を目的として当科に入院した。

経　過

治療開始前の肝生検は F2/A2 の組織像であった（図 1）。2000 年 7 月 10 日からラミブジン 100 mg/ 日を投与開始した。開始初期 4 週間は，インターフェロンを併用した（IFN β 300 万単位 / 日）。治療開始後，HBV-DNA 量の減少と HBe 抗原価の低下を認めたものの，HBe 抗体価の上昇がみられないため，2001 年 3 月から HB ワクチンを併用した。HB ワクチンは 1 回 20 μg を月 1 回の皮下投与とした。投与中に，HBe 抗原価の低下，HBe 抗体価の上昇傾向を認めたが，6 回の投与終了後には治療前のレベルに戻った。2002 年 4 月から再度 HB ワクチン治療を行ったが，同様の反応性で，セロコンバージョンに達しない状態であった。2003 年 3 月からラミブジンの内服を隔日とし，2003 年 7 月には，一旦，内服中止とした。同年 10 月には ALT 値が 100（IU/L）を越し，HBV-DNA 量も 8.22（log copies/mL）にまで上昇した。翌月，HBV-DNA 量が低下傾向になったことを確認した後にラミブジンの投与を再開したところ，2004 年 2 月にはセロコンバージョンを認めた（臨床経過図）。

症例7．ラミブジン投与中止後に肝炎の再燃を起こし，ラミブジン再投与により改善した症例

図1　治療開始前の肝生検組織像



F2/A2 の所見であった。

考　察

本症例の治療経過やポイントを整理すると，

1）20 歳代に 3 回のインターフェロン治療歴があり，さらに今回の治療でもラミブジンに追加してインターフェロンを併用しているにも拘らずセロコンバージョンが得られておらず，インターフェロン治療には抵抗性である。

2）ラミブジン投与後，速やかな抗ウイルス効果を示し，HBV-DNA 量は低値で維持されたが，HBe 抗原は抗原価の低下を認めるものの陽性のままで，HBe 抗体も上昇せず，セロコンバージョンには至らなかった。我々の治療成績では，HBe 抗原陽性例に対するラミブジン投与後のセロコンバージョンは治療後 6 ～ 7 カ月以内に得られるものが多く，このまま治療を継続して著効を得る可能性は低いと予想された。

3）HB ワクチン治療を 2 クール施行した。いずれも，一時的には HBe 抗原 /HBe 抗体価に治療の反応を認めたが，最終的にセロコンバージョンを得る程度には至らなかった。

4）ラミブジンを連日投与から隔日投与に減量したところ，HBV-DNA 量は上昇傾向を示したが，4 カ月間の中では ALT 値を上昇させるレベルには至らなかった。

5）ラミブジンを中止したところ，2 カ月後には HBV-DNA 量の著明な上昇を認め，3 カ月後には ALT 値も有意に上昇した。

6）HBV-DNA 量がピーク値を越して減少傾向に入った事を確認後にラミブジン内服を再開したところ，速やかにセロコンバージョンが得られたが，内服再開時には，既に HBe 抗原低