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*Original Article*

# Amino acid substitutions in the S region of hepatitis B virus in sera from patients with acute hepatitis

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*Background:* An increase in the number of acute hepatitis patients with hepatitis B virus (HBV) of non-indigenous genotypes may reduce the efficacy of vaccination against HBV.

*Methods:* We have determined the amino acid (aa) sequences in the major hydrophilic region (MHR) in the S region of HBV in patients with acute hepatitis B and compared those with the ones from HBV strains used for the production of HBV vaccines commercially available in Japan.

*Results:* Of 48 patients studied, 11 were infected with genotype A, 11 with genotype B and 26 with genotype C HBV. The aa sequences of the nine genotype A isolates were the same as the aa sequence of J02205 which is used for the production of one of the commercially available recombinant vaccines. The aa sequences of the 11 genotype B isolates differed from the aa sequence of J02205 in two or three amino acids. Of the

26 genotype C isolates, 22 had the same aa sequence as X01587 which is used for the production of another recombinant vaccine. The remaining genotype C isolates had aa substitutions at aa131, which have a potential to alter the hydropathy and the three-dimensional structure of the MHR. The differences among the three current HBV vaccines in aa sequences in the MHR theoretically alter the hydropathy and three-dimensional structure.

*Conclusion:* Our results suggest that the transmission of HBV isolates with different genotypes or with aa substitutions in the MHR might reduce the efficacy of currently available HBV vaccines in the protection of HBV infections.

**Key words:** genotype, hepatitis B virus, major hydrophilic region, vaccine

## INTRODUCTION

ABOUT 300 MILLION people in the world are chronically infected with hepatitis B virus (HBV). Chronic infection may eventually lead to liver cirrhosis or hepatocellular carcinoma.[1-4] To prevent the transmission of this virus, vaccination has been introduced in many countries. Indeed, universal vaccination has not only reduced the number of infected individuals, but also the number of deaths related to HBV.[5,6]

In Japan, in 1985, a national project was started to vaccinate children born to HBV-infected mothers. The chances of vertical transmission from HBV-carrying mothers have decreased. Recently, the prevalence of HBV in Japan has decreased to approximately 0.6%.[7]

Because the number of individuals infected with HBV has decreased, the number of patients with acute hepatitis B, mainly caused by horizontal transmission from HBV carriers, should also have decreased. However, in Japan, the number of patients with acute hepatitis B has recently increased (Yatsuhashi H. *et al.*, 2004, unpubl. data).

The increase in the number of patients with acute hepatitis B may, in part, be the result of patients carrying novel HBV genotypes imported from abroad. For

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example, in recent years, genotype A HBV has often been detected in patients with acute hepatitis B.[8,9]

Genotype A HBV is transmitted from individuals who live in or have immigrated from other countries to Japan. Its infection is characterized by a high viral load and a long hepatitis B surface antigen (HBsAg) positivity period. The transition of acute hepatitis B with genotype A HBV infection to the chronic state has been reported recently.[8,10] Decreasing the transmission rate of genotype A HBV is therefore important for the control of the disease. Introducing universal vaccination for adolescents or adults is a measure to be considered.

The effectiveness of universal vaccination depends on the reactivity of vaccines against HBV. HBsAg binds antibody to hepatitis B surface antigen (anti-HBs) produced against HBV vaccines mainly via the 'a' determinant region (aa124–aa149). This region contains common antigenic epitopes of all subtypes (adw, adr, ayw, ayr) of HBsAg and lies in the major hydrophilic region (MHR) between aa99 and aa169. Amino acid (aa) substitutions in the MHR, particularly in the 'a' determinant region, can alter B cell epitopes of HBsAg, leading to immunological escape from the host immunity induced by either vaccination or previous infection.[11] Therefore, if HBV prevalent in Japan has aa substitutions in the MHR, the effect of universal vaccination may be reduced.

In Japan, three types of HBV vaccine (Bimmugen, The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan; Heptavax, Merck & Co., Whitehouse Station, NJ, USA; and Meinyu, Meiji Dairies, Tokyo, Japan) are now available. Efficacy and immunogenicity of vaccines are not always comparable or identical.[12,13] Whether giving a single vaccine effectively prevents the transmission of all genotypes of HBV is an important but still unsolved problem. Elucidating the aa substitutions in the MHR may give a clue to this problem.

The purpose of the present study is to determine the difference of the aa sequences in the MHR of HBV among isolates from patients with acute hepatitis and also the difference of the aa sequences among viral strains used for the production of anti-HBV vaccines, and to find ways to use currently available vaccines as effective prophylaxes.

## METHODS

### Patients

FROM 1992 TO 2001, serum samples were collected from 48 patients diagnosed with acute hepatitis B in our institutions. Only patients whose serum samples were stored at the onset of hepatitis were included in this study. All the 48 patients ran a self-limited clinical course. No patients subsequently developed fulminant hepatic failure or chronic sequelae.

The criteria for the diagnosis of acute hepatitis B were the following: (i) an acute onset of liver injury without a history of liver dysfunction and positivity for HBsAg in serum; and (ii) immunoglobulin M (IgM) antibody to HBV core antigen (anti-HBc) at a titer of more than 2.5 of cut-off index. Coinfection with a hepatitis A virus or a hepatitis C virus was excluded by serological tests. None of the patients had previously received any vaccination against HBV.

Serum samples from the 48 patients with acute hepatitis B were examined virologically, and the results were examined for correlations with clinical characteristics. Informed consent was obtained from each patient. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethics committees of our institutions.

### Determination of HBV-DNA

Hepatitis B virus DNA level was determined using transcription-mediated amplification (TMA) and a hybridization protection assay (Chugai Diagnostics Science, Tokyo, Japan) using the protocol of Kamisango *et al.*[14] The range of detection using TMA was from 3.7 log genome equivalents (LGE)/mL (i.e. $10^{3.7}$ copies/mL corresponding to 5000 copies/mL) to 8.7 LGE/mL ($10^{8.7}$ copies/mL). In seven of 34 studied serum samples, the level of HBV-DNA was lower than 3.7 LGE/mL and these were categorized as 3.7 LGE/mL.

### Genotyping HBV

Hepatitis B virus genotypes were determined using commercial enzyme immunoassay kits (Smitest HBV Genotyping kit; Genome Science, Fukushima, Japan). In brief, DNA extracted from serum was amplified by polymerase chain reaction (PCR) using three sense primers (i.e. s1: 5'-ACCAACCCTCTGGGATTCTTTCC-3', s2: 5'-ACCAATCCTCTGGGATTCTTCCC-3', and s3: 5'-AGCAATCCTCTAGGATTCCTTCC-3' [nt 2902–2924]) and an antisense primer (i.e. as1: 5'-GAGCCTGA GGGCTCCACCC-3' [nt 3091–3073]) biotinylated at the 5' end; their sequences were deduced from conserved sequences in the pre-S1 region of HBV. The biotin-labeled and amplified HBV-DNA was denatured in an alkaline solution, and tested for hybridization to probes specific for one of the seven HBV genotypes (A-G) immobilized on wells of a 96-well

microplate. Thereafter, hybridization was detected by staining with the streptavidin-horseradish peroxidase (HRP) conjugate.[15]

## Amplifying and sequencing the S region of HBV-DNA

The entire aa sequence of MHR in the S region was amplified by two-stage PCR using genotype-specific primers. The outer primers for the amplification of the first fragment were 5'-TTTCCACCAAGCTCTGCAA-3' (sense: nt 9–28) and 5'-TTCAGGGAATAACCCCATCT-3' (antisense: nt 872–853) for genotype A, 5'-CTCCA CCACTTTCCA GACT-3' (sense: nt 1–22) and 5'-CAACTCCCAATTACATATCCC-3' (antisense: nt 899–879) for genotype B and 5'-TTACAGGCGGGG TTTTTCTT-3' (sense: nt 70–89) and 5'-TACAGACTT GGCCCCCAATA-3' (antisense: nt 771–752) for genotype C. The inner primers were 5'-AGAGTCAGGGGCC TGTATTT-3' (sense: nt 35–55) and 5'-AGGGAATAA CCCCATCACTTT-3' (antisense: nt 869–849) for genotype A, 5'-TTCAAGATCCCAGAGTCAGG-3' (sense: nt 24–43) and 5'-AGGGAATATCCCCACCTTTTT-3' (antisense: nt 869–849) for genotype B and 5'-CGGGGTT TTCTTGTTGACA-3' (sense: nt 77–97) and 5'-CCCAAT ACCACATCATCCATA-3' (antisense: nt 758–738) for genotype C.

The first stage of amplification was carried out in a thermal cycler for 40 cycles (94°C, 1 min; 55°C, 1 min; 72°C, 1 min) in 100 μL reaction mixture containing 200 mM dNTPs, 1.0 mM each of primers and PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂ and 0.001% (wt/vol) gelatin) and 2 U Ampli-Taq polymerase (Perkin Elmer Cetus, Norwalk, CT, USA). PCR products (2 μL) were subjected to the second stage of amplification under the same conditions as those in the first stage. Standard precautions to avoid contamination were taken during PCR, with a negative control serum sample included in each run.

Amplification products were purified on Wizard PCR preps DNA purification resin (Promega, Madison, WI, USA), and sequenced bidirectionally with a Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Foster City, CA, USA) using the above-mentioned PCR primers. Sequencing was performed in an automated DNA sequencer (ABI 377; PE Applied Biosystems).

The nucleotide sequences of HBV isolates from the patients were compared with those of three reference HBV strains which are used for vaccine production.[16–18]

Phylogenetic trees were constructed with the Mega Program version 2.1 (Center for Evolutionary Functional Genomics, The Biodesign Institute, Tempe, AZ, USA) using the Kimura two-parameter matrix and the neighbor-joining method.[19] To confirm the reliability of phylogenetic tree analysis, boot-strap resampling, and reconstruction were carried out 500 times.

## Hydrophobicity and secondary structure analysis

The hydrophobicity profile of the MHR of the S region was predicted by computer-assisted Kyte-Doolittle analysis (an estimate of hydrophobicity based on the bulk phase partitioning of side chain hydrophobicity alone)[20] with GENETYX-MAC software (version 10.1; Software Development, Tokyo, Japan).

The secondary structures of the amino acids in the same region were predicted by computer-assisted Robson[21] and Chou-Fasman analyses[22] with the GENETYX-MAC software.

## Statistical analyses

Data were analyzed by the chi-squared test for categorical data and Student's $t$-test or the Mann–Whitney $U$-test for continuous variables. $P$-values less than 0.05 were regarded as statistically significant.

## RESULTS

### Distribution and clinical characteristics of HBV genotypes

HEPATITIS B VIRUS genotype was determined in the 48 patients with acute hepatitis B. Genotype A was detected in 11 (23%) patients, genotype B in 11 (23%) and genotype C in 26 (54%).

The clinical and demographic backgrounds of the patients with acute hepatitis B who were infected with HBV of different genotypes are shown in Table 1. The mean ages of all the groups were similar. The proportion of male to female patients was higher in genotype A infection than in genotypes B or C infection (100%, 73% and 64%, respectively: A $vs$ B, $P = 0.22$; A $vs$ C, $P = 0.01$; B $vs$ C, $P = 0.16$). The maximum alanine aminotransferase (ALT) levels were lower in patients with genotype A infection than in patients with genotypes B or C infection (1646 ± 1123, 3085 ± 1119 and 2545 ± 981 IU/L, respectively: A $vs$ B, $P = 0.01$; A $vs$ C, $P = 0.03$; B $vs$ C, $P = 0.89$). The maximum HBV-DNA levels were not significantly different between the

Table 1 Demographic and clinical differences among patients with acute hepatitis infected with HBV of distinct genotypes

| Features | Genotypes of HBV | | | Differences (*P*-value) | | |
|---|---|---|---|---|---|---|
| | A (*n* = 11) | B (*n* = 11) | C (*n* = 26) | A *vs* B | A *vs* C | B *vs* C |
| Age (years) | 30.6 ± 7.5 | 28.1 ± 5.1 | 31.1 ± 9.1 | 0.41 | 0.87 | 0.33 |
| Gender (M:F) | 11:0 | 8:3 | 15:11 | 0.22 | 0.01 | 0.16 |
| ALT (IU/L) | 1646 ± 1123 | 3085 ± 1119 | 2545 ± 981 | 0.01 | 0.03 | 0.89 |
| HBV-DNA (LGE/mL) | 6.8 ± 1.7 | 6.6 ± 2.1 | 5.2 ± 1.2 | 0.60 | 0.23 | 0.06 |

ALT, alanine aminotransferase; HBV, hepatitis B virus.

genotypes (6.8 ± 1.7, 6.6 ± 2.1 and 5.2 ± 1.2 LGE/mL, respectively: A *vs* B, *P* = 0.60; A *vs* C, *P* = 0.23; B *vs* C, *P* = 0.06).

## Amino acid sequence of the S region

The aa sequence of the S region between aa27 and aa203 was determined in the 48 sequences. Figure 1 shows a phylogenetic tree constructed using the 48 sequences and 15 published sequences (four for genotype A, three for genotype B, three for genotype C, one for genotypes D, E, F, G and H). Among the 48 sequences we studied, 11 were classified into genotype A, 11 into genotype B and 26 into genotype C.

The aa sequence of the region between aa101 and aa163 including MHR (aa111-aa156) was compared among 48 sequences and three HBV sequences (X01587, J02205 and huGK-14) currently used for anti-HBV vaccine production. As shown in Figure 2, the aa sequences of X01587 (used for Bimmugen) and J02205 (used for Heptavax) differed in eight amino acids (i.e. aa110, aa113, aa114, aa126, aa131, aa143, aa160 and aa161). The aa sequence of huGK-14, which is used for the HBV-vaccine Meinyu, differed from that of X01587 in six amino acids and from that of J02205 in two amino acids.

Nine of the 11 isolates classified into genotype A had the same aa sequence as J02205. The remaining two isolates (AB289727 and AB289728) differed from J02205 at aa161 (Fig. 2).

Ten of the 11 isolates classified into genotype B had the same aa sequence as J02205 except for two amino acids (aa114 and aa131). The remaining isolate had another aa substitution at aa112 (Fig. 2).

As shown in Figure 2, 22 of the 26 isolates classified into genotype C had the same sequence as X01587. The remaining four isolates (from patients 10, 24, 30 and 48) had the same sequence as X01587 except for one aa substitution at aa131; the threonine (aa131) of X01587 was substituted with proline for three isolates

(AB289714, AB289720 and AB289736) and with alanine for one isolate (AB289701).

## Hydrophobicity and secondary structure analysis

As mentioned above, the aa sequences of the MHR from four isolates differed from that of X01587 only at aa131. Furthermore, the aa sequence of the MHR differed between X01587 and J2205 in eight amino acids. We compared the hydropathy and secondary structure of the MHR among J02205, X01587 and two isolates with genotype C (one isolate with proline at aa131 and one with alanine at aa131). The results of Kyte-Doolittle hydropathy analysis based on the hydropathy index are shown in Figure 3. The substitution with alanine-131 was found to alter the patterns on the hydropathy plot, whereas the substitution with proline-131 was found to have little effect. A substitution with alanine-131 could increase the hydrophobicity of the first loop of the MHR, which may affect the antigenicity of HBV.

The secondary structure of our isolate with alanine-131 by Chou-Fasman analysis predicted an α-helix configuration for the region from aa126 to aa135 instead of the β-configuration predicted for the same region of X01587. The predicted secondary structure of our isolate with proline-131 coincided with that of X01587. In contrast, by Robson prediction, the secondary structure of our isolate with alanine-131 coincided with that of X01587; however, that of our isolate with proline-131 was found to have lost a turn structure between aa131 and aa134, which was predicted for X01587.

## DISCUSSION

VACCINATION IS THE key to controlling HBV infection. In countries with a high prevalence of HBV infection, universal vaccination is effective not only for controlling viral infections but also for decreasing the incidence of hepatocellular carcinoma.[5,23] Even in
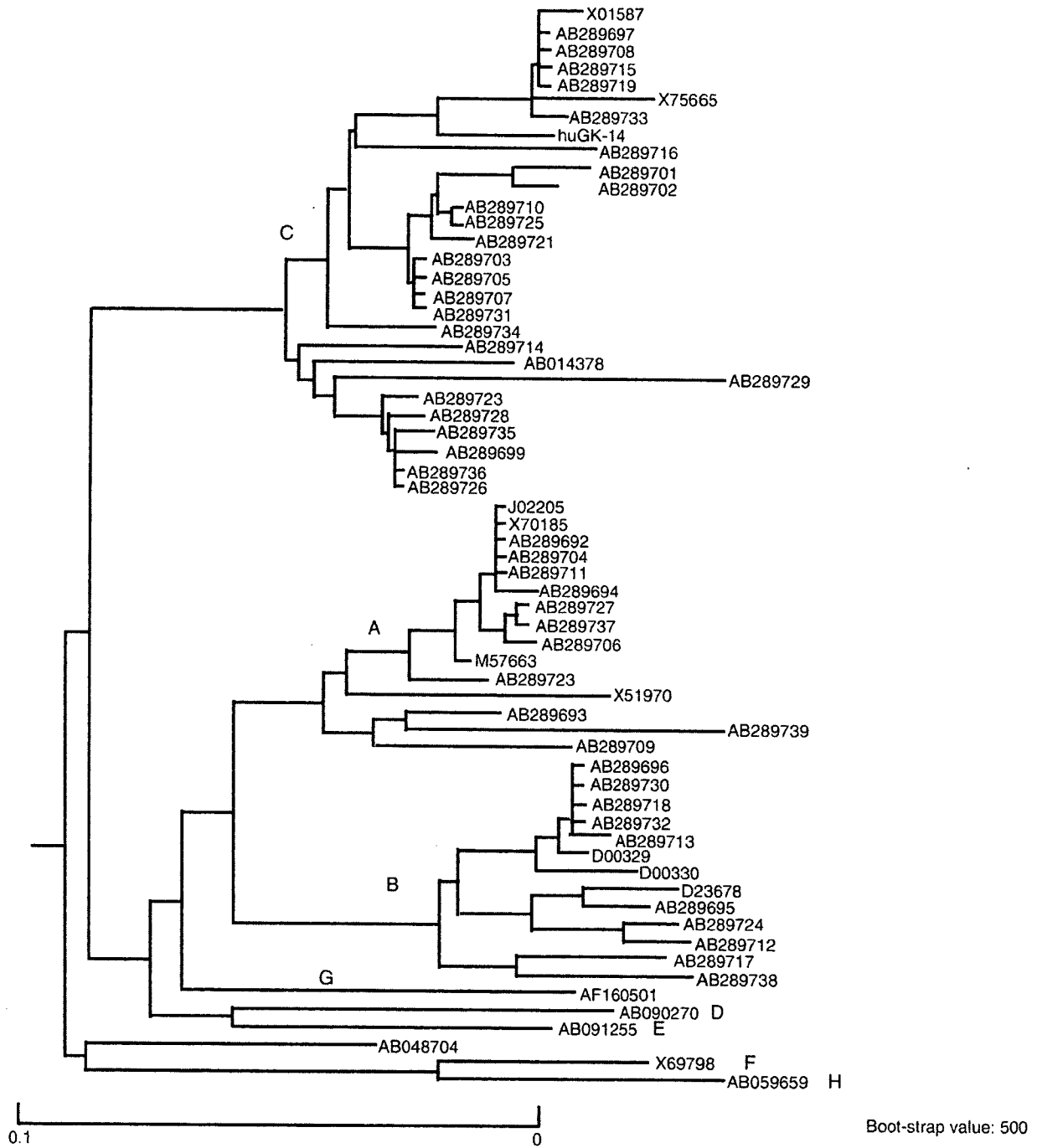
Boot-strap value: 500

0.1           0

Figure 1 Phylogenetic tree constructed using hepatitis B virus (HBV)-DNA sequences of the *S* gene. The sequences include four with genotype A, four with genotype B, three with genotype C, and those recovered from the serum of 48 patients with acute hepatitis B. J02205 (genotype A) is used for the production of Heptavax and X01587 (genotype C) is used for the production of Bimmugen. The horizontal bar indicates the number of nucleotide substitutions per site. Accession numbers are shown for the isolates that have been deposited in the DDBJ/EMBL/GenBank databases. The accession numbers for the HBV sequences from the 48 patients are also shown.
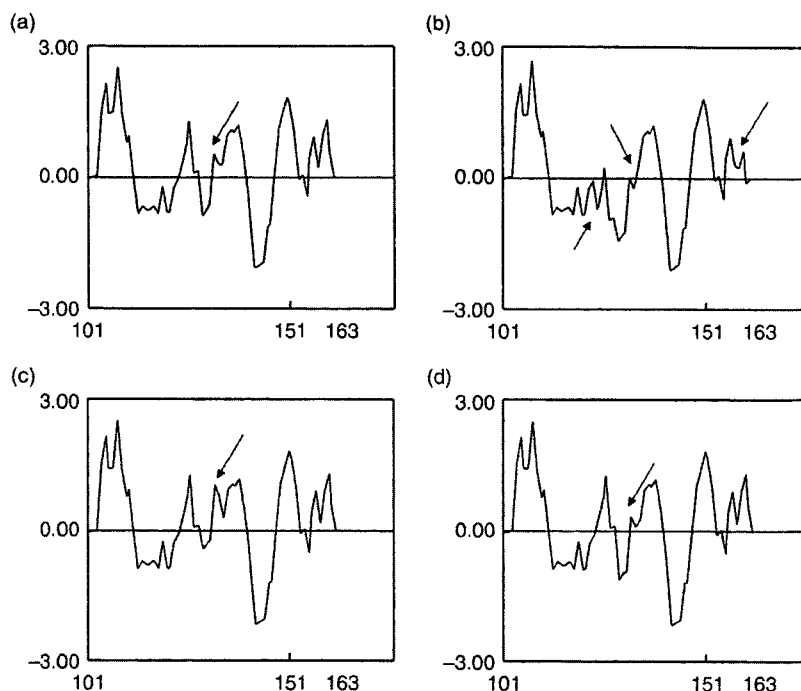
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**Figure 2** Comparison of amino acid sequences of the major hydrophilic region (MHR) of the S gene.

countries with a low prevalence of HBV infection, vaccination is very important for preventing mother-to-child transmission as well as patient-to-staff transmission.

HBV is classified into several genotypes that differ from one another in nucleotide sequence by more than 8% of the entire genome. The aa sequences of their phenotypes also differ among genotypes. The difference in the aa sequence of the 'a' determinant region may

alter the three-dimensional structure and antigenicity, and may reduce the protectivity of HBV vaccines.

As mentioned above, the aa sequences of currently available recombinant vaccines differ from each other. J02205 and X01578 differ in eight amino acids (i.e. aa110, aa113, aa114, aa126, aa131, aa143, aa160 and aa161) between aa101 and aa163. Three (i.e. aa126, aa131 and aa143) of them are included in the MHR and may alter the hydropathy and three-dimensional

Figure 3 Hydropathy profile of the major hydrophilic region (MHR) of the *S* gene elaborated using the Kyte-Doolittle hydropathy index. Arrows show the positions of amino acids which are different among X01587, J02205, AB289701 (alanine-131) and AB289720 (proline-131). (a) X01587, (b) J02205, (c) AB289701 (alanine-131), (d) AB289720 (proline-131).

structure of the region. Therefore, the antibody produced against J02205 vaccines may not completely neutralize X01578 and vice versa. Indeed, previous studies showed that antibody profiles induced by recombinant vaccines produced from different genotypes are not identical with each other,[12] which suggests that antibodies produced by recombinant vaccines might not protect viral infection with different genotypes.

As shown in Figure 2, the aa sequences of our isolates classified into genotype A are very close to the aa sequence of J02205. Therefore, the transmission of genotype A HBV is prevented by Heptavax which is made from J02205.

The aa sequences of our isolates classified into genotype B are the same as the aa sequence of J02205 except for one substitution at aa131. This aa, which is asparagine and is located in the first stem loop structure of the MHR, was substituted with threonine in our genotype B isolates. Because asparagine and threonine have an uncharged side chain and similar polarity, genotype B HBV infection may be prevented effectively by Heptavax.

The aa sequences of our isolates classified into genotype C were the same as that of X01587 except for four isolates having a substitution at aa131. Bimmugen, which is produced from X01587, may be effective for

preventing genotype C HBV infections caused by those four isolates. However, Heptavax may not be effective for preventing genotype C HBV infection because of the difference in eight amino acids as described above.

The four isolates have proline or alanine instead of threonine-131, which has never been reported before. The polarities of threonine and proline/alanine are quite different. The Kyte-Doolittle hydropathy analysis suggests that substituting threonine at aa131 with alanine or proline would increase hydrophobicity, which may then lead to a change in antigenicity. Hou *et al.* reported that some blood donors who were tested negative for serum HBsAg had a substitution of isoleucine for threonine at aa131 in the S region.[24] They suggested that the structure and antigenicity of HBV may be altered by this substitution.

The secondary structure of our isolate with alanine-131 predicted by Chou-Fasman analysis suggested an α-helix configuration instead of a β-configuration in the region from aa126 to aa135. The secondary structure of our isolate with proline-131 predicted by Robson analysis suggested that this change causes the loss of a turn structure between aa131 and aa134. Some changes in the secondary structure can affect the three-dimensional structure of the protein and thus affect antigenicity. These results suggest that the transmission of the four

isolates with an aa substitution at aa131 may not be prevented by either Heptavax or Bimmugen.

However, the protective immunity elicited by HBV vaccines, which is usually polyclonal in nature, may not be totally lost or severely affected *in vivo* by the alteration of only a single amino acid in the 'a' determinant region.[25] Also, antibodies against regions outside the 'a' determinant region may be protective.[26] The protectivity of current vaccines may be elucidated by *in vitro* binding studies using polyclonal antibodies.

It was reported that some individuals immunized with recombinant vaccines are infected with HBV with or without mutations in the 'a' determinant region.[11,27,28] HBV isolates with amino acid substitutions at aa144[29-31] or 145[11,27,28] are known to be transmitted despite vaccination. Indeed, some chronic HBV carriers are reported to have HBV with such amino acid substitutions.[32,33] We were unable to find patients who had these substitutions in the present study. However, large-scale studies are necessary to elucidate the prevalence of 'vaccine-escape mutants' in patients with acute hepatitis B.

In conclusion, we have shown that the aa sequence of the MHR in the S gene of HBV is different among isolates from patients with acute HBV infection. Current vaccination may prevent the transmission of these HBV isolates, which should be further investigated.

## ACKNOWLEDGMENT

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# The High Incidence of the Emergence of Entecavir-Resistant Mutants among Patients Infected with Lamivudine-Resistant Hepatitis B Virus

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Nagasaki, F., Niitsuma, H., Ueno, Y., Inoue, J., Kogure, T., Fukushima, K. and Shimosegawa, T. *The High Incidence of the Emergence of Entecavir-Resistant Mutants among Patients Infected with Lamivudine-Resistant Hepatitis B Virus.* Tohoku J. Exp. Med., 2007, 213 (2), 181-186 —— Hepatitis B virus (HBV) infection remains to be one of the most prevailing infection in the world, causing chronic liver diseases. Although lamivudine has been effective to suppress HBV replication, longer durations of administration can lead to the emergence of drug-resistant mutant viruses, followed by reactivation of hepatic inflammation (breakthrough hepatitis). Moreover, the optimal period of administration as well as the effects of anti-viral nucleot(s)ide such as lamivudine, adefovir, and entecavir, has not been established. To evaluate the efficacy of the anti-viral effects of entecavir for lamivudine-resistant HBV, we administered entecavir sequentially in four patients with chronic HBV infection, who demonstrated the emergence of lamivudine-resistant HBV and histological active hepatitis. The antiviral effects were evaluated by the serum viral loads and biochemical laboratory data. After follow-up periods of more than 36 months, we found high incidence in the emergence of entecavir resistant mutants (3/4, i.e., 75%). An additional mutation at the 184th amino acid, different from the previously reported lamivudine-resistant mutations (80th, 180th, and 204th), seemed to have a close relationship with the induction of entecavir-resistant mutants at least for Japanese HBV genotype C. Our observation draws attention to the possibility that the usage of entecavir for lamivudine-resistant HBV could promptly induce entecavir-resistant mutations in addition to lamivudine-resistance. —————— HBV; lamivudine; entecavir; antiviral resistant virus

Chronic hepatitis B virus (HBV) infection remains one of the major serious health problems in the world (Lee 1997). Its clinical manifestation is affected by various factors, particularly the genotype (Maddrey 2000). Eight different genotypes of HBV, designated A to H, have been already determined (Okamoto et al. 1987, 1988; Norder et al. 1992; Stuyver et al. 2000; Arauz-Ruiz et al. 2002). Furthermore, it has been reported that a genotype is composed of various subgenotypes (Norder et al. 2004). For example, genotype B causes seroconversion more frequent-

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ly than genotype C (HBV/C), and those infected with HBV/B appear to have a better prognosis (Kikuchi 2000; Orito et al. 2001). At present, HBV genotype B (HBV/B) has been clustered into five subgenotypes (Nagasaki et al. 2006a).

Lamivudine, the first approved orally administered nucleotide analogue, is well known for its safety and effectiveness in the treatment of HBV infections, including HBV cirrhosis, regardless of the HBV genotype (Yao and Bass 2000; Yao et al. 2001; Yuen et al. 2003). On the other hand, long durations of lamivudine therapy could lead to the evolution of drug-resistant mutants, which can emerge in 15% in one year, and in 50% in three years after the initiation of therapy (Lee 1997; Dienstag et al. 1999; Kapoor et al. 2000; Yao et al. 2001), and could be followed by hepatic failure, i.e., breakthrough hepatitis (Liaw et al. 2000; Santantonio et al. 2000; Lok and McMahon 2001). As we previously reported, some patients could discontinue lamivudine administration successfully (Nagasaki et al. 2006b), but it is usually considered to be very exceptional (Wang et al. 2002).

Recently, other antiviral agents, such as adefovir, entecavir, and tenofovir have revealed usefulness in suppressing the serum HBV-DNA levels. In Japan, the use of adefovir was approved in 2004 and that of entecavir in 2006. However, their long term efficacies have not been established. Especially, the usage of entecavir for lamivudine resistant HBV has been considered questionable since both lamivudine and entecavir show similar mechanisms in the development of resistant mutations.

In this study, we administered entecavir to 4 patients with chronic HBV infection, who had been previously administered lamivudine in whom the emergence of a resistant mutant had been confirmed.

## MATERIALS AND METHODS

We administered entecavir monotherapy treatment for more than 36 months in 4 patients with chronic HBV infection, who had been previously treated with lamivudine 100 mg daily and developed breakthrough hepatitis due to the emergence of lamivudine-resistant mutant virus after the cessation of lamivudine. The inclusion criteria of these patients strictly followed those of the phase III clinical trial by Bristol-Myers Japan Inc. (Tokyo); briefly, both i) the presence of active HBV viremia (> $10^5$ copy/mL), and ii) the presence of pathologi-

TABLE 1. The clinical backgrounds of the four patients.

| Patients | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Sex | M | M | F | M |
| Age | 51 | 43 | 51 | 27 |
| Duration of prior lamivudine administration | 31 | 13 | 20 | 16 |
| T. Bil (mg/dL) | 0.9 | 0.5 | 0.7 | 0.4 |
| AST (IU/L) | 61 | 34 | 39 | 37 |
| ALT (IU/L) | 74 | 62 | 61 | 102 |
| HBeAg (RIA) | + | + | + | + |
| Anti-HBe (RIA) | – | – | – | – |
| HBV-DNA (Log copy/ml) | 7.5 | 5.4 | > 7.6 | > 7.6 |
| Genotype (RFLP) | C | C | C | C |
| YMDD motif | YIDD | YMDD/YIDD | YIDD/YVDD | YIDD |

T-Bil, total bilirubin; AST, asparate aminotransferase; ALT, alanine aminotransferase; HBeAg, hepatitis B envelope antigen; Anti-HBe, hepatitis B envelope antibody; RIA, radioimmunoassay; RFLP, restriction fragment length polymorphism; Y, tyrosine; M, methionine; D, aspartic acid; I, isoleucine; V, valine; M, male; F, female.

cally proven chronic hepatitis. The study protocol was approved by institutional review board (IRB) in Tohoku University Hospital. For this clinical trial, 4 patients were initially screened and all of these patients were considered as eligible patients for this study. All of these four patients had been administered entecavir 0.5 or 1.0 mg daily for 52 weeks in a coded manner, and thereafter 1.0 mg daily by the protocol of our present study. Their clinical data are shown on Table 1. The genotype of HBV detected in all 4 patients was C, and the serum HBV-DNA level and alanine aminotransferase (ALT) were 7.5 Log copy/mL and 70.8 IU/L on average, respec-

tively. The quantitative range of HBV-DNA by polymerase chain reaction (PCR)-assay (Roche Ampricor HBV, Roche Japan Inc., Tokyo) was 2.6-7.7 Log copy/ mL.

We defined antiviral resistance as a more than 1 log10 increase in the HBV-DNA level in the serum from a patient who had an initial virologic response (Lok and McMahon 2007). We conducted HBV-DNA direct sequence analyses in the polymerase region before the administration of entecavir, and at the time antiviral resistance emerged.
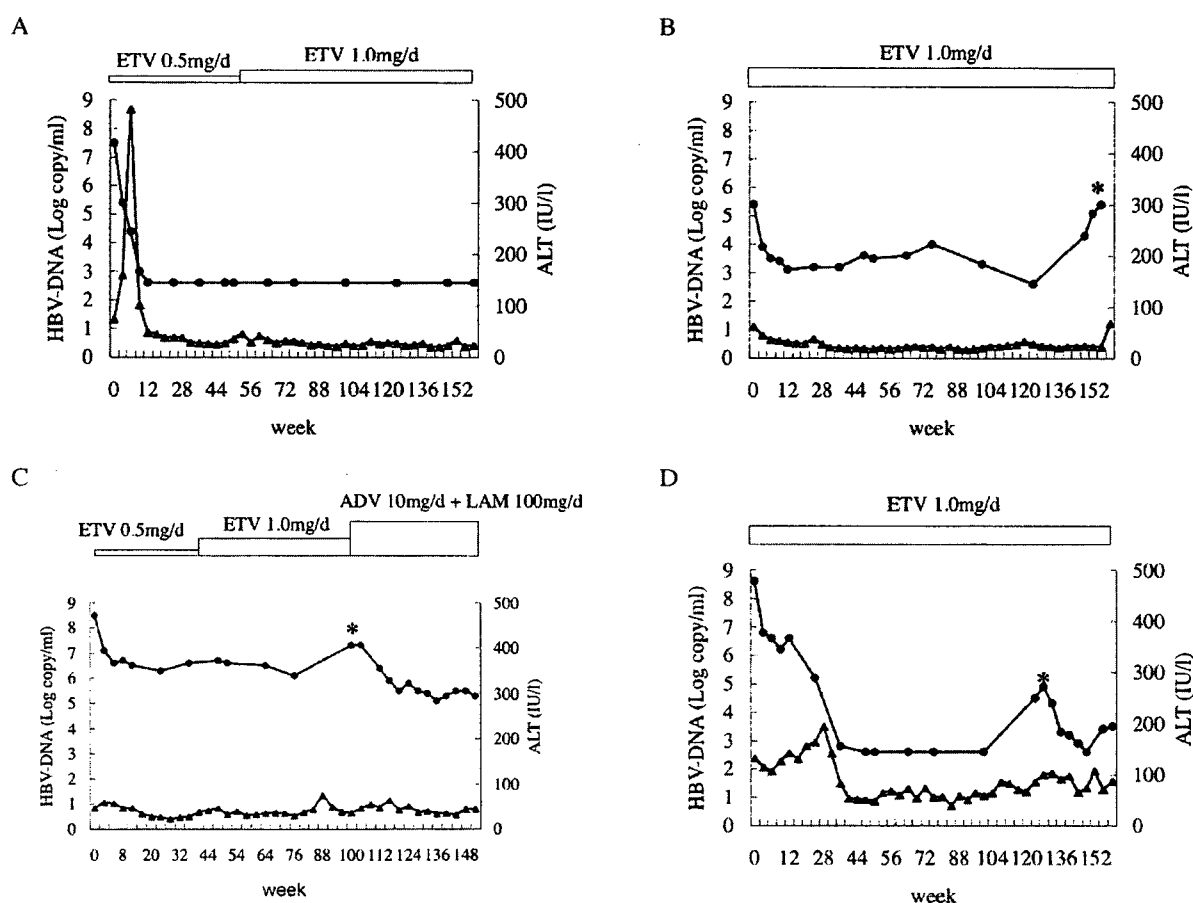


Fig. 1. Clinical course of the four patients treated with entecavir.
A: Clinical course of Patient No. 1. An entecavir-resistant mutant was not found in this patient.
B: Clinical course of Patient No. 2. An entecavir-resistant mutant was detected at the 152nd week (*).
C: Clinical course of Patient No. 3. Entecavir was administered 0.5 mg/day for 52 weeks, thereafter 1.0 mg/day. An entecavir-resistant mutant was detected at the 104th week (*), and the treatment was then changed to adefovir and lamivudine combination therapy.
D: Clinical course of Patient No. 4. An entecavir-resistant mutant was detected at the 130th week (*).
ETV, entecavir; ADV, adefovir; LAM, lamivudine; ALT, alanine aminotransferase.
—●—, HBV-DNA; —▲—, ALT.

## RESULTS

### Clinical evaluation

Patient No. 1 had a good response to entecavir without the emergence of a resistant mutant virus (Fig. 1A). On the other hand, the other three patients (75%) experienced the emergence of entecavir mutant viruses during the therapy. Although patient No. 2 at first showed continuously suppressed serum HBV-DNA levels of 2.6 to 4.0 Log copy/mL, at the 52nd week the patient showed an increase of the serum HBV-DNA levels followed by an elevation of ALT and was found to have entecavir-resistant mutant viruses (Fig. 1B). Patient No. 3 was administered entecavir 0.5 mg daily and the serum HBV-DNA level decreased from 8.5 Log copy/mL but did not decrease further below 6.0 Log copy/mL even after an increase of the entecavir dosage as shown in Fig. 1C. We changed the entecavir monotherapy to lamivudine and adefovir combination therapy at the 104th week. Thereafter, this patient showed decreases in both the serum HBV-DNA level and ALT. Patient No. 4 had been administered entecavir 1.0 mg daily which resulted in the suppression of HBV-DNA to below a detectable level by PCR assay. At the 130th week, the serum HBV-DNA level increased to 4.9 Log copy/mL (ALT 100 IU/L) and entecavir-resistant mutant viruses were revealed. Later, the serum HBV-

DNA level decreased without any change in treatment (Fig. 1D).

### The analysis of HBV sequences regarding mutations after entecavir administration

HBV amino acids (aa) in polymerase region analyses from the three patients with entecavir-resistant mutants are shown in Table 2. The HBV nucleotide sequences in the polymerase regions obtained at the initiation of the therapy and during the week entecavir-resistant mutants had emerged were determined and amino acids were analyzed by them. The drug resistant mutations as shown in the table were confirmed as follows. In the three patients the 80th aa in the A domain was substituted to leucine, and the 180th aa in the B domain was methionine. Thus, the lamivudine resistant 80th aa mutation found at the initiation of entecavir treatment had been returned to be wild type. In two patients the 184th aa was leucine, and in one patient there was a mixed pattern of threonine, isoleucine, proline, and leucine. The 204th aa in the C domain of the three patients was valine. Other domains, F, D and E showed no nucleotide and amino acid mutations.

### DISCUSSION

We administered entecavir monotherapy for more than 3 years in 4 patients with chronic HBV infection who had previously showed break-

TABLE 2. The analysis of amino acids in the HBV polymerase region by the nucleotide sequences of HBV detected from three patients with entecavir resistance at the start of the therapy and at the time resistant viruses emerged. Patient No. 1 did not have entecavir resistance and thus sequencing was not performed.

| Patient | Weeks when resistant mutation confirmed | HBV-DNA Polymerase region | | | | | | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | | A Domain | | B Domain | | | | C Domain | |
| | | 80 | | 180 | | 184 | | 204 | |
| (No.) | | 0w | Mutant | 0w | Mutant | 0w | Mutant | 0w | Mutant |
| 2 | 152 | I/L | L | L | M | T | L | I/M | V |
| 3 | 104 | I/L | L | L | M | T | T/I/P/L | M/I/V | V |
| 4 | 130 | I | L | L | M | T | L | I | V |

I, isoleucine; L, leucine; M, methionine; P, proline; T, threonine; V, valine.

through hepatitis induced by prior lamivudine administration. The administration of entecavir for these patients caused the emergence of entecavir-resistant viruses in three of the four patients, although severe liver dysfunction did not yet occur. Breakthrough hepatitis can often be fatal, especially for patients with progressed liver diseases, so we administered entecavir when virologic breakthrough was confirmed.

It was reported that lamivudine-resistant mutant HBV nucleotide sequences showed changes of methionine to isoleucine or valine at the 204th aa, leucine to methionine at the 180th aa, leucine to isoleucine at the 80th nucleotide and valine to leucine at the 173rd aa (Stuyver et al. 2001).

In this study, we determined the HBV nucleotide sequences obtained from three patients, and analyzed the amino acids in the polymerase region both before the administration of entecavir and at the 152nd, 104th and 130th week, respectively. As a result, we found at first that in all of them the resistance to lamivudine was related to the change from leucine to isoleucine at the 80th aa. They then showed the lamivudine resistant mutations after the administration of entecavir monotherapy (180th and 204th aa). It has been reported that susceptibility to entecavir decreases when mutations at both the 180 and the 204th aa emerge (Tenney et al. 2004), which is in accord with our present results.

At the 184th aa, as previously reported concerning its relation to entecavir resistance (Tenney et al. 2004), all of the patients showed the mutation from threonine to leucine, although patient No. 4 showed a mixed pattern.

Additionally, we found a new change at the 80th aa, which showed an inverse pattern of the lamivudine-resistant mutant (Yim et al. 2006). Actually, 80th mutation found at the initiation of entecavir treatment return to be wild type when entecavir-resistant mutants developed (Table 2). The results might suggest that some of the lamivudine-related mutations could disappear with the cessation of lamivudine.

We suppose that the emergence of resistance to antiviral agents in patients with sequential administration of entecavir monotherapy for chronic HBV after the emergence of a lamivudine-resistant mutant virus might have the closest relationship with the 184th aa and, additionally, the 80th aa, at least in HBV genotype C, the most common genotype in Japan.

In addition to lamivudine, adefovir has also been reported to develop resistant mutants (Fung et al. 2005). Sequential therapy for patients with chronic HBV infection could result in the emergence of multi-drug resistant mutants (Yim et al. 2006).

The discontinuation of lamivudine before the emergence of a mutant virus might be useful to prevent breakthrough hepatitis; nevertheless, the reactivation of hepatitis is sometimes fatal, especially for patients with progressed liver diseases, such as liver cirrhosis (Honkoop et al. 2000). These findings underscore the difficulties in determining the best therapy for patients with chronic HBV infection.

The results of our study suggested the possibility that entecavir monotherapy should not be the first choice for the treatment of patients with chronic HBV infection who have developed a lamivudine-resistant virus.

Further studies regarding optimal patient selection, treatment duration, and the dosages of antiviral agents including entecavir are warranted.

## Acknowledgments

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CASE REPORT

# Fulminant hepatic failure in a case of autoimmune hepatitis in hepatitis C during peg-interferon-alpha 2b plus ribavirin treatment

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## Abstract

A 27-year-old Caucasian female with hepatitis C virus (HCV) infection treated with interferon (IFN) who developed severe autoimmune hepatitis (AIH) is described. The infecting viral strain was of genotype Ib and the pre-treatment HCV viral load was at a high level. The patient was treated with pegylated IFN-alpha 2b and ribavirin, and her HCV-RNA became negative at wk 12, but after that she developed fulminant hepatic failure. The patient recovered after steroid pulse therapy consisting of methylprednisolone 1000 mg/d for three days which was administered twice. A needle liver biopsy revealed the typical pathological findings of AIH.

Key words: Autoimmune hepatitis; Interferon; Ribavirin; Hepatitis C virus; Anti-viral therapyA; Acute liver failure

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## INTRODUCTION

The most effective treatment for hepatitis C virus (HCV) infection is pegylated interferon (peg-IFN) in combination with ribavirin (RBV)[1]. This treatment exerts a variety of immuno-modulatory effects and may unmask the underlying autoimmune diseases such as autoimmune thyroiditis and rheumatoid arthritis[2,3]. In a similar fashion, interferon (IFN) was reported to induce or exacerbate autoimmune hepatitis (AIH)[4-7]. We describe a patient who developed fulminant hepatic failure with AIH during the treatment with peg-IFN and RBV. The patient had no prior history of autoimmune diseases and the severe hepatitis occurred after the HCV RNA level decreased to below the detection limit.

## CASE REPORT

### Patient

The patient was a 27-year-old Caucasian woman from South Caucasian Country. She had never consumed alcohol. She had not received blood transfusions or undergone surgery. Her sister and mother were receiving treatment for Basedow's disease, but she had no manifestation of Basedow's disease or other autoimmune diseases.

### Present illness

She was found to be infected with hepatitis C virus during her pregnancy and was referred to our department in October 2005. She hoped to receive IFN therapy to reduce the risk of transmission to her second baby. The HCV genotype was Ib and the HCV RNA viral load was 4600 kIU/mL (high range) (Table 1). Alanine aminotransferase (ALT) was 47 IU/L (normal < 35 IU/L). The markers of hepatic functional reserve were within normal ranges and ultrasonography of the liver indicated no abnormal findings. After delivery, interferon therapy was commenced with peg-IFN-alpha 2b (Peg-Intron®, Schering-Plough Corp., NJ, USA) and RBV (Rebetol®, Schering-Plough) on January 30, 2006. The clinical course of this anti-viral therapy is illustrated in Figure 1. The initial doses consisted of 100 μg peg-IFN per week and 600 mg RBV per day. This therapy was well tolerated with minimal adverse effects and HCV RNA became negative at wk 12. Around the same time, a rise of transaminase was noted: ALT, 83 IU/L (normal < 35 IU/L); and asparate aminotransferase (AST), 56 IU/L (normal < 30 IU/L). The dosage of peg-

**Table 1 Laboratory findings before interferon treatment**

| | | | | | |
|---|---|---|---|---|---|
| WBC | 5300 /μL | Total bilirubin | 0.6 mg/dL | HCV-Ab | Positive |
| Hb | 11.0 g/dL | Direct bilirubin | 0.1 mg/dL | HCV-RNA | 4600 kIU/L |
| Plt | 452 × 10³ /μL | Total protein | 7.7 g/dL | HCV genotype | Ib |
| PT | 101.8% | Albumin | 4.2 g/dL | HBs-Ag | Negative |
| | | BUN | 16 mg/dL | HBs-Ab | Negative |
| | | Creatinine | 0.8 mg/dL | ANA | × 80 |
| AST | 39 IU/L | Uric acid | 3.4 mg/dL | ASMA | Negative |
| ALT | 47 IU/L | Na | 140 mEq/L | AMA | Negative |
| LDH | 176 IU/L | K | 4.5 mEq/L | IgG | 1635 mg/dL |
| ALP | 278 IU/L | Cl | 104 mEq/L | IgA | 108 mg/dL |
| γ-GTP | 11 IU/L | Total cholesterol | 133 mg/dL | IgM | 283 mg/dL |
| ChE | 386 IU/L | Glucose | 77 mg/dL | HLA-DR | DR17/DR13 |
| | | | | allele | DRB1*0301/*1302 |

WBC: white blood cell; Hb: hemoglobin; Plt: platelet; PT: prothrombin time; AST: asparate aminotransferase; ALT: alanine aminotransferase; LDH: lactate dehidrogenase; ALP: alkaline phosphatase; γ-GTP: gamma glutamil transpeptidase; ChE: choline esterase; BUN: blood urea nitrogen; HCV: hepatitis C virus; HBs-Ag: hepatitis B virus surface antigen; HBs-Ab: hepatitis B virus surface antibody; ANA: antinuclear antibody; ASMA: anti-smooth muscle antibody; AMA: anti-mitochondrial antibody; Ig: immunoglobulin; HLA: human leukocyte antigen.

**Table 2 Laboratory findings at onset of acute liver failure**

| | | | | | |
|---|---|---|---|---|---|
| WBC | 5800/μL | BUN | 5.0 mg/dL | ANA | × 160 |
| Hb | 12.2 g/dL | Creatinine | 0.7 mg/dL | ASMA | Negative |
| Plt | 290 × 10³ /μL | Uric acid | 2.8 mg/dL | LKM-1 | Positive |
| PT | 41.4% | Na | 138 mEq/L | AMA | Negative |
| APTT | 44.4 sec | K | 3.9 mEq/L | IgG | 2436mg/dL |
| | | Cl | 105 mEq/L | IgA | 175 mg/dL |
| | | Total cholesterol | 175 mg/dL | IgM | 280 mg/dL |
| AST | 311 IU/L | Triglyceride | 67 mg/dL | | |
| ALT | 280 IU/L | Glucose | 127 mg/dL | | |
| LDH | 245 IU/L | CRP | 0.2 mg/dL | HCV-RNA | 13 kIU/l |
| ALP | 571 IU/L | | | HBs-Ag | Negative |
| γ-GTP | 108 IU/L | Tyrosin | 262 μmol/L | HBs-Ab | Negative |
| ChE | 124 IU/L | BCAA | 313 μmol/L | HBc-Ab IgM | Negative |
| Total bilirubin | 5.5 mg/dL | | | HAV | Negative |
| Direct bilirubin | 3.3 mg/dL | TSH | 0.499 μIU/mL | EBV | Negative |
| Total protein | 7.4 g/dL | T3 | 1.66 pg/mL | HSV | Negative |
| Albumin | 3.1 g/dL | T4 | 0.55 ng/dL | CMV | Negative |

APTT: activated partial thromboplastin time; CRP: C-reactive protein; BCAA: branched-chain amino acid; TSH: thyroid-stimulating hormone; LKM-1: antibody to microsomes type 1; HBc-Ab: hepatitis B virus core antibody; HAV: hepatitis A virus; EBV: Epstein-Barr virus; HSV: herpes simplex virus; CMV: cytomegalovirus.
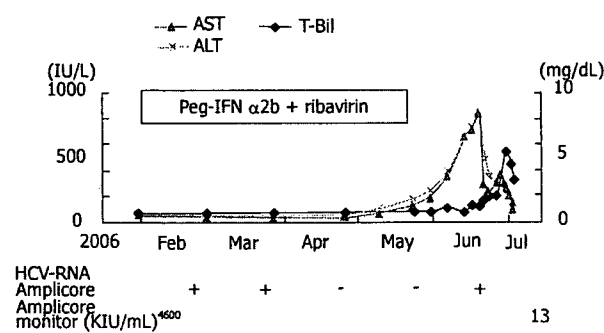


**Figure 1** Clinical course of peg-interferon (peg-IFN) plus ribavirin therapy for hepatitis C virus infection. ALT: Alanine aminotransferase; AST: asparate aminotransferase; T-Bil: total bilirubin.

(Stronger neo-minophagen C®, Minophagen Pharmaceutical Co., Ltd., Tokyo, Japan) was administrated intravenously, and ALT indicated a decreasing tendency (peak ALT was 815 IU/L). After that, she showed jaundice and a prolongation of the prothrombin time (PT) was noted. Her human leukocyte antigen (HLA)-DR serotypes were DR17 and DR13. She was admitted to our hospital on June 30, 2006.

## Physical findings on admission

Physical examination revealed: height 167 cm, weight 56 kg, blood pressure 118/64 mmHg, body temperature 37.3°C, and clear consciousness. The bulber conjunctiva was slightly icteric. No peripheral edema, vascular spiders, and flapping tremor were observed.

## Clinical course

On admission, elevations of transaminases (ALT 280 IU/L, AST 311 IU/L) (Table 2) and total bilirubin (5.5

IFN was reduced to 80 μg at wk 17, and 60 μg at wk 18 because of the persistent increase of ALT, and finally, the treatment was discontinued at wk 19. Glycyrrhizinate
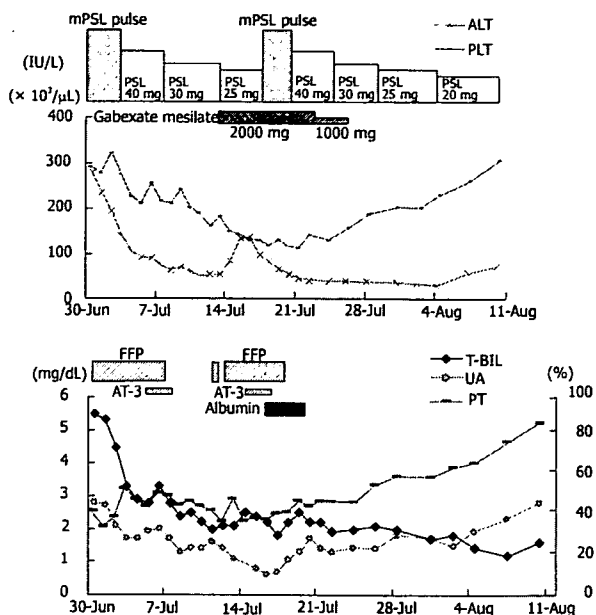
**Figure 2** Clinical course of fulminant hepatic failure. PSL: prednisolone; mPSL: methylprednisolone; UA: uric acid; PT: prothrombin time; FFP: fresh frozen plasma; AT-3: antithrombin Ⅲ.
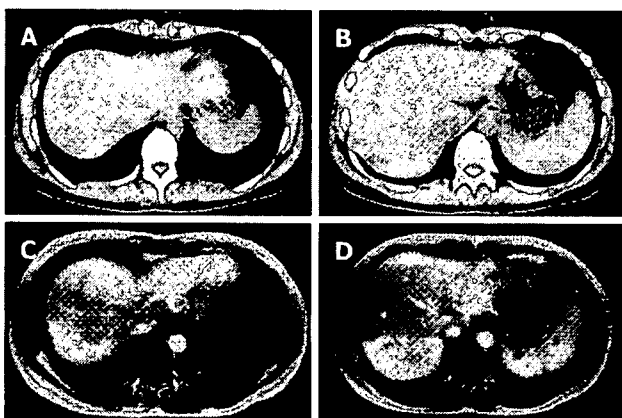


**Figure 3** A, B: Abdominal CT at the onset of hepatic encephalopathy; C, D: Enhanced abdominal MRI.

mg/mL) and decrease of blood urea nitrogen (BUN), uric acid (UA), and PT were detected. Hepatitis A, hepatitis B, cytomegalovirus, herpes simplex, and Epstein-Barr virusesl were negative. Anti-nuclear antibody (ANA) was × 160 (normal < × 80) and immunoglobulin G (IgG) 2436 mg/mL (normal < 1695 mg/mL), which were × 80 and 1635 mg/mL before the IFN treatment. Antibody to liver/kidney microsomes type 1 (LKM-1) was positive. Steroid pulse therapy (methylprednisolone 1000 mg/d for three days) was performed (Figure 2). Nevertheless, hepatic encephalopathy appeared on July 3. Abdominal computed tomography indicated massive necrosis of hepatocytes (Figure 3). Brain magnetic resonance imaging showed no abnormal findings. The findings of her electric encephalogram were typical of metabolic encephalopathy. Total parenteral nutrition was started. The protein load was restricted and lactulose enemas were performed. After
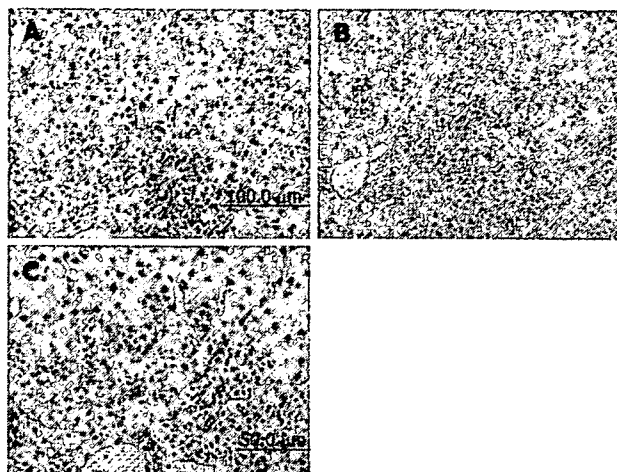


**Figure 4** Liver biopsy showing interface hepatitis with infiltration of plasma cells and rosette formations. A, C: Hepatoxyline-Eosin staining; B: Elastica-Masson staining.

that, the hepatic encephalopathy improved gradually. The ALT level rose again on July 14, and steroid pulse therapy was administered again. The ALT showed a decreasing tendency again, after which she started oral intake. PT, BUN, and UA had improved gradually. She was discharged from our hospital on August 10.

A needle liver biopsy was performed on October 16 and it showed the typical pathological features of autoimmune hepatitis. Interface hepatitis with infiltration of inflammatory cells including plasma cells and rosette formation, were found but no biliary change was noted (Figure 4). The serum ALT levels were normalized over the following six months by 10 mg of prednisolone per day.

## DISCUSSION

IFN therapy for patients with HCV infection has been reported to induce or exacerbate AIH[4-7]. In the present case, severe hepatitis occurred after HCV RNA had decreased to below the detection limit after treatment with peg-IFN with RBV for 12 wk. The patient presented the typical clinical features of serum aminotransferase elevation, positive ANA and LKM-1, and hypergammaglobulinemia, and responsiveness to glucocorticoid therapy. The pathological findings showed the typical features of AIH, which included interface hepatitis with infiltration of plasma cells, and rossete formation.

One of the explanations for the occurrence of autoimmunity in HCV patients is the loss of self-tolerance due to molecular mimicry between viral proteins and self-antigen[8]. HCV infection is known to be related to autoimmune disease, and chronic hepatitis C patients show autoantibodies such as ANA and LKM-1. HCV core protein has been reported to show homology with cytochrome P450IID6, which could be recognized by LKM-1 antibodies as seen in type 2 AIH[9,10]. Recently, AIH has been found to occur in patients during IFN therapy after liver transplantation for HCV liver cirrhosis[11,12].

In the present case, AIH occurred during IFN therapy and the patient developed fulminant hepatic failure when HCV RNA had decreased to below the detection limit. To our knowledge, there is no report of an HCV patient who developed fulminant hepatic failure after peg-IFN with RBV therapy. Peg-IFN with RBV is an established therapy for chronic hepatitis C patients but AIH should be considered as a potential complication of therapy leading to severe hepatitis. Especially, longer treatment duration (48 wk) and prolonged elevation of serum IFN levels in the pegylated-IFN could contribute to the development of autoimmune phenomenon. Liver biopsy in the early phase of acute liver injury to determine whether immunnosuppression therapy may be required for such patients. In Asians, the development of AIH during antiviral therapy is believed to be rare, although the potential risk should be taken into consideration if female young Caucasian case is treated like this report. However, the treatment of recurrence of HCV after cessation of antiviral therapy due to the emergence of autoimmune hepatitis like our case is a very difficult clinical decision to make. Probably, the interferon based antiviral therapy, the most standard anti-viral agent, could remain as the mainstream regimen for next decade. The balance between antiviral effects and possible autoimmune phenomena could be key factors as described previously[13,14]. Small molecules such as HCV protease inhibitor, either as monotherapy or combined with other small molecules, could be the first choice for the treatment of the current case in future.

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