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# Influence of Hepatitis B Virus Genotypes on the Development of PreS Deletions and Advanced Liver Disease

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Hepatitis B virus (HBV) mutants with deletions in the preS region have not been evaluated for association with viral genotypes. In a case-control study, HBV DNA samples collected from 80 each of carriers infected with HBV genotype B or C were examined for preS deletions. PreS deletion mutants were found in a total of 37 of 160 (23%) HBV carriers. Carriers with preS deletion mutants were older ($56.0 \pm 12.7$ vs $49.3 \pm 16.9$ years, $P < 0.05$), were infected more frequently with HBV genotype C (84% vs 40%, $P < 0.05$), and had more advanced disease, such as liver cirrhosis and hepatocellular carcinoma (54% vs 31%; $P < 0.05$), than did those without such mutants. In a multivariate analysis, genotype C (odds ratio [OR] = 9.3, $P < 0.001$) and advanced liver disease (OR = 3.1, $P < 0.01$) were the most significant variables in association with preS deletions. A direct repeat sequence (TCAGG) was found at the start or at the end of preS1 deletions in 6 of the 20 (30%) cases examined, and preS2 deletions in these cases were clustered over the 5′-terminal half of this region. These results indicate that the development of preS deletion mutants depends on HBV genotypes and that it may be associated with progressive liver disease. ***J. Med. Virol. 70:537–544, 2003.*** 

**KEY WORDS:** case-control study; chronic hepatitis; genotypes; hepatitis B virus; hepatocellular carcinoma; preS region

## INTRODUCTION

Hepatitis B virus (HBV) is a DNA virus with a relaxed circular, partially double-stranded genome of ~3,200 base pairs (bp) in size. HBV DNA has four partially overlapping open reading frames (ORFs) that encode viral envelope proteins, nucleocapsid as well as hepatitis B e antigen (HBeAg), DNA polymerase/reverse transcriptase, and the X protein. The envelope is composed of three forms of hepatitis B surface antigen (HBsAg): the large (coded for by the preS1/S2/S gene), middle (the preS2/S gene), and small (the S gene) envelope proteins [Tiollais et al., 1985]. The preS regions contain a regulatory element with the nucleotide (nt) sequence of CCAAT, which controls transcription of the envelope gene [Lu et al., 1995]; their products are implicated in the attachment of HBV to the surface of hepatocytes for initiating infection [Neurath et al., 1989; Ishikawa and Ganem, 1995]. In addition to their functional and structural roles, preS1 and preS2 regions are reported to have T- and B-cell recognition sites [Chisari and Ferrari, 1995].

Several lines of evidence indicate a role of preS deletion mutants in the clinical course of persistent HBV infection. Naturally occurring mutants with deletions in preS1 and preS2 regions tend to accumulate during a later course of persistent HBV infection [Gerken et al., 1991; Santantonio et al., 1992; Minami et al., 1993; Melegari et al., 1994; Nakajima et al., 1994; Kidd-Ljunggren et al., 1995; Pollicino et al., 1995; Fan et al., 2001; Tai et al., 2002], suggesting involvement

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in viral clearance. PreS deletion mutants might aggravate liver disease by means of causing cytotoxicity from the storage of large envelope proteins in the cytoplasm of hepatocytes [Bock et al., 1999b]. Furthermore, integration of the truncated preS/S gene is proposed to enhance the development of hepatocellular carcinoma by means of expressing a transactivating capacity [Caselmann et al., 1990].

Currently, seven genotypes of HBV have been identified by a sequence divergence >8% in the entire genome, named by capital letters A–G [Okamoto et al., 1988; Norder et al., 1994; Stuyver et al., 2000]. Effects of preS deletion mutants on the clinical consequence in hosts, however, have not been evaluated with respect to HBV genotypes. As the preS1/S2 region exhibits the highest genetic variability in the four ORFs, among HBV isolates of distinct genotypes [Bowyer and Sim, 2000], HBV genotypes may influence mutations in the preS region. In a case-control study, naturally occurring preS deletion mutants were compared between 80 Japanese carriers of HBV genotype B or C, with special reference to clinical outcome.

## MATERIALS AND METHODS

### Patients With Chronic Hepatitis B

Carriers of HBV who visited hospitals in Akita, Nagoya, Niigata, and Okinawa in Japan were recruited to this study. Eighty carriers infected with HBV genotype C were selected, along with 80 carriers infected with HBV genotype B; they were matched for gender, mean age, degree of liver disease, and frequency of HBeAg (Table I). These carriers were followed up for ≥12 months and were classified into four clinical stages: asymptomatic carrier state without subjective symptoms along with persistently normal levels of alanine aminotransferase (ALT) in serum; chronic hepatitis with ALT levels exceeding the upper limit of normal in the absence of cirrhosis; liver cirrhosis with clinical evidence of cirrhosis demonstrated by ultrasonography (i.e., coarse liver architecture, nodular liver surface, and blunt liver edges) and evidence of hypersplenism (i.e., splenomegaly on ultrasonography and a platelet count of $<100{,}000/mm^3$); and hepatocellular carcinoma diagnosed by ultrasonography and computed tomography (CT). A fine-needle biopsy of the liver was undertaken as required. Patients who were co-infected with hepatitis C virus (HCV) or with human immunodeficiency virus type 1 (HIV-1) were excluded from the study, and none of these patients was infected with hepatitis delta virus. None had received antiviral treatment during the follow-up period. The study protocol was approved by the ethics committees of the participating institutions, and informed consent was obtained from each HBV carrier.

### Markers of HBV Infection

HBsAg was determined by enzyme immunoassay (EIA) with commercial kits (HBsAg EIA; Dinabot, Tokyo, Japan). HBeAg and the corresponding antibody (anti-HBe) were determined by EIA with commercial kits (HBeAg/anti-HBe EIA; Dinabot). Genotypes of HBV were determined by EIA with a commercial kit (HBV GENOTYPE EIA, Institute of Immunology, Tokyo, Japan). HBV genotypes were distinguished by combinations of epitopes on preS-region products, detected by monoclonal antibodies, specific for genotypes [Usuda et al., 1999, 2000; Kato et al., 2001]. HBV DNA was determined by transcription-mediated amplification hybridization protection assay (TMA) (DNA probe Chugai-HBV; Chugai Diagnostics, Tokyo, Japan). The detection range of this assay was 3.7–8.7 log genome equivalents per milliliter (LGE/ml).

TABLE I. Baseline Characteristics and Frequency of PreS Deletion Mutants in Patients Infected With HBV Genotype B or C

| Features | Genotypes of HBV | | Differences (*P*) |
|---|---|---|---|
| | B (n = 80) | C (n = 80) | |
| Male | 57 (71%) | 56 (70%) | Matched |
| Age (mean ± SD yr) | 51.5 ± 18.0 | 50.3 ± 14.4 | Matched |
| Liver diseases | | | |
| AC : CH : LC : HCC | 22: 29: 17: 12 | 22: 29: 17: 12 | Matched |
| HBeAg positive | 13 (16%) | 18 (23%) | Matched |
| Median ALT (IU/L) (range) | 37 (10–612) | 39 (12–491) | NS |
| No. of patients with ALT | | | |
| <2 × ULN[a] | 62 (78%) | 61 (76%) | NS |
| 2–4 × ULN[a] | 11 (14%) | 10 (13%) | NS |
| >4 × ULN[a] | 7 (8%) | 9 (11%) | NS |
| Mean HBV DNA (LGE/ml) | 5.2 ± 1.7 | 5.6 ± 1.6 | NS |
| No. of patients with HBV DNA | | | |
| <3.8 (LGE/ml) | 27 (34%) | 27 (34%) | NS |
| ≥3.8 (LGE/ml) | 53 (66%) | 53 (66%) | NS |
| PreS deletion mutants | 6 (8%) | 31 (39%) | <0.05 |

HBV, hepatitis B virus; AC, asymptomatic carrier state; CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma; HBeAg, hepatitis B e antigen; ALT, alanine amino transferase; NS, not significant; LGE, log genome equivalents; ULN, upper limit of normal.
[a]Upper limit of normal-35 IU/L.

### Amplification of the PreS1/S2 Region

Serum samples were stored at −40°C until tested. Nucleic acids were extracted from 100 μl of serum, using a DNA extractor kit (Genome Science Laboratory, Fukushima, Japan). HBV DNA was amplified by polymerase chain reaction (PCR) in a 96-well cycler (GeneAMP9600, Perkin-Elmer Cetus, CA) with nested primers that produced 658-bp amplicons encompassing the entire preS1/S2 gene. The primers for the first-round PCR were HBPS1 (sense: 5′-CGC AGA AGA TCT CAA TCT CGG-3′ [nt 2417–2437]) and HBPS2 (antisense: 5′-CGA GTC TAG ACT CTG TGG TA-3′ [nt 256–237]), and those for the second-round PCR were HBPS3 (sense: 5′-GGG TCA CCA TAT TCT TGG GAA-3′ [nt 2814–2834]) and HBPS2. The first round was carried out for 35 cycles (94°C for 1 min, 55°C for 1 min, and 72°C for 1 min [7 min in the last cycle]), and the second round for 30 cycles under the same condition. PCR products were run by electrophoresis on 3.0% (wt/vol) high-resolution agarose gel (MetaPhor Agarose; FMC BioProducts, Denmark), stained with ethidium bromide and visualized in an ultraviolet (UV) transilluminator.

PreS deletion mutants were distinguished on the basis of size, which was smaller than that expected (658 bp) on electrophoresis; it was confirmed that they were not products of artifacts with PCR [Minami et al., 1993]. They are considered representative of preS deletion mutants in sera from HBV carriers [Minami et al., 1993; Fan et al., 2001; Preikschat et al., 2002]. PreS deletion mutants were considered to be present when the size of the amplicons was shorter by >2% than that expected for the wild-type HBV [Fan et al., 2001]. In some serum samples, various small amplicons were detected in the presence or absence of full-size amplicons.

### Sequencing the PreS Region

PreS amplicons in small sizes were purified through 3.0% (wt/vol) agarose gel with commercial kits (Qiagen, Hilden, Germany). Purified HBV DNA fragments were sequenced by the dideoxy method with a Big Dye Deoxy Terminator cycle sequencing kit in a fluorescent 3100 DNA sequencer (Applied Biosystems, Foster City, CA).

### Statistical Analysis

Statistical differences were evaluated using the Mann-Whitney nonparametric test, Fisher's exact probability test, and Student's *t*-test, as appropriate. Differences were considered significant for a *P*-value of $<0.05$.

## RESULTS

### Frequency of PreS Deletion Mutants

Table I compares demographic, clinical, and virological characteristics between 80 carriers of HBV genotype B and 80 carriers of HBV genotype C in a case-control study. Overall, preS deletion mutants were found in sera from 37 of 160 (23%) HBV carriers; they were predominant over the wild type in 12 (32%) of them. They were detected in 6 of the 80 (8%) carriers of HBV genotype B, significantly less often ($P < 0.05$) than in 31 of the 80 (39%) carriers of HBV genotype C.

Table II compares various features between carriers with and without preS deletion mutants. Carriers with preS deletion mutants were older ($56.0 \pm 12.7$ vs $49.3 \pm 16.9$ years, $P < 0.05$), were more frequently infected with HBV genotype C (84% vs 40%, $P < 0.05$), and had more severe liver disease (liver cirrhosis and hepatocellular carcinoma) (54% vs 31%, $P < 0.05$) than was found in patients without these factors. No significant

TABLE II. Demographic, Clinical, and Virological Characteristics of HBV Carriers With or Without PreS Deletion Mutants

| Features | PreS deletion mutants | | Differences (*P*) |
|---|---|---|---|
| | Positive (n = 37) | Negative (n = 123) | |
| Age (mean ± SD years) | 56.0 ± 12.7 | 49.3 ± 16.9 | <0.05 |
| Male | 25 (68%) | 88 (72%) | NS |
| Advanced liver disease | | | |
| LC and HCC | 20 (54%) | 38 (31%) | <0.05 |
| Median ALT (IU/L) (range) | 43 (15–201) | 37 (10–612) | NS |
| No. of patients with ALT | | | NS |
| <2 × ULN[a] | 25 (68%) | 98 (80%) | |
| 2–4 × ULN[a] | 7 (19%) | 14 (11%) | |
| >4 × ULN[a] | 5 (13%) | 11 (9%) | |
| HBeAg positive | 6 (16%) | 25 (20%) | NS |
| Mean HBV DNA (LGE/ml) | 5.1 ± 1.5 | 5.3 ± 1.7 | NS |
| No. of patients with HBV DNA | | | |
| <3.8 (LGE/ml) | 12 (32%) | 42 (34%) | NS |
| ≥3.8 (LGE/ml) | 25 (68%) | 81 (66%) | NS |
| HBV genotype | | | <0.05 |
| B | 6 (16%) | 75 (60%) | |
| C | 31 (84%) | 48 (40%) | |

HBV, hepatitis B virus; LC, liver cirrhosis; HCC, hepatocellular carcinoma; ALT, alanine aminotransferase; ULN, upper limit of normal; LGE, log genome equivalents; HBeAg, hepatitis B e antigen; NS, not significant.
[a]Upper limit of normal-35 IU/L.

differences were noted in gender, HBeAg status, and ALT, as well as HBV DNA levels, between the carriers with and without preS deletion mutants. There were no substantial differences in the prevalence of preS deletion mutants among the patients with distinct ALT levels.

The frequency of preS deletion mutants was compared among carriers in distinct clinical stages of liver disease, and between different HBV genotypes (Fig. 1). Of carriers infected with HBV genotype C, the prevalence of preS deletion mutants increased gradually with the aggravation of liver disease. Thus, preS deletion mutants occurred in 23% of asymptomatic carriers, in 38% of patients with chronic hepatitis, and in 52% of patients with advanced liver disease (i.e., liver cirrhosis and hepatocellular carcinoma combined); they occurred significantly more frequently ($P < 0.05$) than in asymptomatic carriers. Albeit to a lesser extent, a similar increase in the frequency of preS deletion mutants pari passu with the aggravation of liver disease was observed in the carriers of HBV genotype B. Thus, they were found in 5% of asymptomatic carriers, in none of the patients with chronic hepatitis, and in 17% of patients with liver cirrhosis and hepatocellular carcinoma combined. A significant difference ($P < 0.05$) was observed between patients with chronic hepatitis and those with advanced liver disease (i.e., liver cirrhosis and hepatocellular carcinoma).

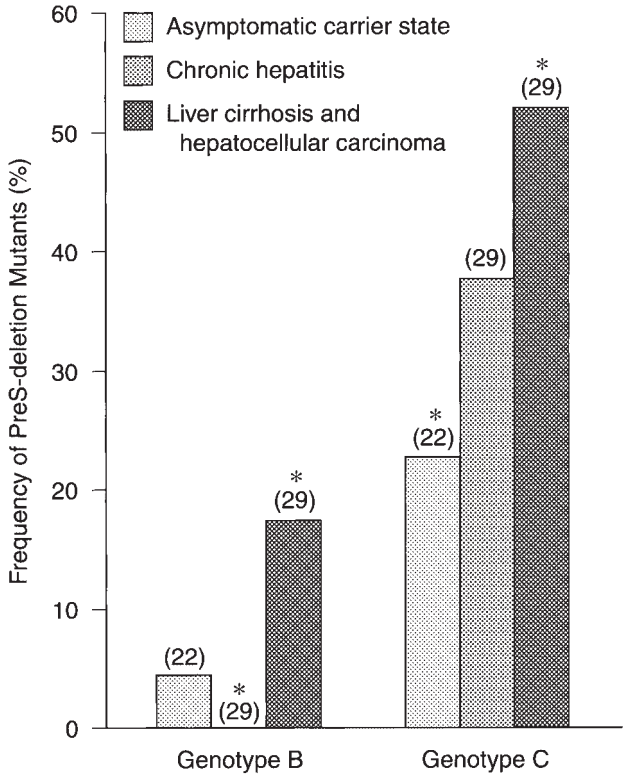


Fig. 1. Frequency of preS deletion mutants in carriers infected with hepatitis B virus (HBV) genotypes B and C who had clinical diseases of different severity. The number in parentheses on each column represents the number of patients in its category. *$P < 0.05$.

### Factors Influencing the Development of PreS1/S2 Deletions

Clinical and virological factors were evaluated for association with preS deletion mutants by multiple logistic regression analysis (Table III). Genotype C (odds ratio [OR] = 9.3, $P < 0.001$) and advanced liver disease such as liver cirrhosis and hepatocellular carcinoma (OR = 3.1, $P < 0.01$) were significant variables for the emergence of preS deletion mutants.

### Sequence Analysis of PreS Deletions

Thirty-seven short HBV DNA fragments were purified on gel electrophoresis and sequenced (Table IV and Fig. 2). The length of preS deletions in these fragments ranged from 9 to 294 bp. Deletions were in frame in 35 (95%) cases, except in cases 8 and 37. The deletions in preS1, preS2, and preS1/S2 occurred in 20 (54%), 24 (65%), and 7 (19%) cases, respectively. Remarkably, the binding site for aggregated human serum albumin, spanning amino acid positions 17–28 in the preS2-region product [Itoh et al., 1986], was conserved in 23 of the 24 (96%) preS2 deletion mutants, while the hepatocyte receptor, stretching over amino acids 21–47 in the preS1-region product [Neurath et al., 1986], was preserved in only 10 of the 20 (50%) preS1 deletion mutants. HBV isolates, in which the initiation codon of the preS2 region was either converted to a stop codon or deleted, were found in 10 of the 37 (27%) cases. The direct repeat sequence of TCAGG at the start or end of the deletion was found in 6 of the 20 (30%) preS1 deletion mutants. PreS2 deletions clustered in the 5′-terminal half of the preS2 region and occurred in 23 of 24 (96%) cases with them. Deletions of the CCAAT-box in the S-promoter region [Lu et al., 1995] were found in 10 of the 37 (27%) preS deletion mutants (one asymptomatic carrier, four patients with chronic hepatitis, one patient with liver cirrhosis, and four patients with hepatocellular carcinoma).

## DISCUSSION

HBV genotypes influence the clinical manifestation of chronic liver disease in hosts and virological conse-

TABLE III. Odds Ratios for the Emergence of PreS Deletion Mutants Estimated by Multiple Logistic Regression Analysis

| Variables | OR | 95% CI | *P* |
|---|---|---|---|
| Male | 0.6 | 0.2–1.5 | 0.488 |
| Age >50 yr | 1.4 | 0.5–3.7 | 0.280 |
| LC and HCC | 3.1 | 1.2–8.0 | <0.01 |
| ALT >2 × ULN[a] | 2.2 | 0.8–6.3 | 0.124 |
| $HBeAg^+$ | 0.6 | 0.2–1.9 | 0.387 |
| Genotype C | 9.3 | 3.4–25.5 | <0.001 |
| HBV DNA >3.7 LGE/ml | 1.0 | 0.4–2.6 | 0.928 |

OR, odds ratio; CI, confidence interval; LC, liver cirrhosis; HCC, hepatocellular carcinoma; ALT, alanine aminotransferase; ULN, upper limit of normal; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; LGE, log genome equivalents.
[a]Upper limit of normal-35 IU/L.

TABLE IV. PreS-Deletion Mutants in 37 Individuals With Chronic HBV Infection

| Case no. | Isolate name | Age/sex | HBeAg | Disease | Genotype | Deletion in | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | PreS1 (bp) | PreS2 (bp) | CCATT in S-promoter |
| 1 | MA9V | 66F | – | AC | B | 168 | (T)[b] | |
| 2 | YA32 | 40M | + | AC | C | 69[a] | (T)[b] | |
| 3 | TH13 | 34F | + | AC | C | 267 | | + |
| 4 | NA14 | 67F | – | AC | C | | 33 | |
| 5 | AKI31 | 56F | – | AC | C | | 24 | |
| 6 | NA25 | 78M | – | AC | C | | 45 | |
| 7 | AKI30 | 52F | – | CH | C | 189 | | |
| 8 | IW21 | 24F | – | CH | C | 203 | | |
| 9 | FU24 | 52M | – | CH | C | 183[a] | | |
| 10 | Ho28 | 39M | – | CH | C | 183[a] | | + |
| 11 | Ho4 | 63M | – | CH | C | | 108 | + |
| 12 | AKI10 | 41M | – | CH | C | | 48 | |
| 13 | MA23 | 54F | – | CH | C | | 48 | |
| 14 | AKI38 | 37F | – | CH | C | | 54 | |
| 15 | Ho36 | 59F | – | CH | C | | 51 | |
| 16 | AKI32 | 62M | – | CH | C | 270 | 54[b] | + |
| 17 | NA58 | 48M | + | CH | C | 115[a] | 9[b] | + |
| 18 | RY13 | 63M | – | LC | B | 195 | | |
| 19 | TH79 | 53M | – | LC | B | | 27 | |
| 20 | RY19 | 63F | – | LC | B | 246 | 45[b] | + |
| 21 | TH106 | 73M | + | LC | C | 51[a] | (I)[b] | |
| 22 | NA35 | 51M | – | LC | C | | 39 | |
| 23 | To44 | 68M | – | LC | C | | 21 | |
| 24 | FU31 | 72M | – | LC | C | | 30 | |
| 25 | NA32 | 56F | – | LC | C | | 48 | |
| 26 | M12 | 60F | + | LC | C | 195 | 30 | |
| 27 | YA74 | 72M | – | HCC | B | 171 | 63[b] | + |
| 28 | IW52 | 46M | – | HCC | B | 57 | 9 + 9 | |
| 29 | IW58 | 51M | – | HCC | C | 18 | | |
| 30 | TH109 | 76M | + | HCC | C | 294 | | + |
| 31 | TH110 | 46M | – | HCC | C | 294 | (I)[b] | + |
| 32 | KU33 | 67M | – | HCC | C | 183[a] | | + |
| 33 | TH112 | 40M | – | HCC | C | | 27 | |
| 34 | KU34 | 60M | – | HCC | C | | 48 | |
| 35 | NA10 | 50M | – | HCC | C | | 33 | |
| 36 | Ho58 | 61M | – | HCC | C | | 45 | |
| 37 | YA86 | 70M | – | HCC | C | 52 | 66[b] | |

HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; bp, base pairs; AC, asymptomatic carrier state; CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma.
[a]With direct repeat sequence (TCAGG) at the beginning and end of the deletion.
[b]With missense in the initiation codon in the preS2 region (in parentheses) or deletion thereof.

quences of the persistent HBV infection [Okamoto et al., 1990; Buckwold et al., 1997; Lindh et al., 1999; Kao et al., 2000; Orito et al., 2001a,b]. Thus, the carriers of HBV genotype C have more advanced liver disease than those of HBV genotype B, and they are different in the prevalence of the double mutation in the core promoter (T1762/A1764) or the point mutation in the precore region (A1896) that decreases or abolishes the expression of HBeAg [Lindh et al., 1999; Orito et al., 2001b]. PreS deletion mutants are frequent in the persistent HBV infection; they also seem to correlate with the progression of liver disease [Gerken et al., 1991; Santantonio et al., 1992; Minami et al., 1993; Ohno et al., 1993; Melegari et al., 1994; Nakajima et al., 1994; Kidd-Ljunggren et al., 1995; Pollicino et al., 1995; Fan et al., 2001; Tai et al., 2002]. The frequency of preS2 deletion mutants, however, varies widely in the countries in which they have been reported, ranging from 3% (1/30) in Italy [Pollicino et al., 1995], through 12% (4/34) in Thailand [Kidd-Ljunggren et al., 1995] and 27% (6/22) in Italy [Santantonio et al., 1992] to 40% (17/43) in Japan [Ohno et al., 1993].

In the present case-control study, the influence of HBV genotypes on the emergence of preS deletion mutants was examined. PreS deletion mutants were detected more frequently in the carriers of HBV genotype C than in those of HBV genotype B. These results may shed light on uneven prevalence rates of preS deletion mutants over the world, where distinct HBV genotypes prevail. They are in agreement with a recent report by Tai et al. [2002], who found preS2 deletion mutants more often in carriers of HBsAg subtype *adr* (corresponding to genotype C) than subtype *adw* (corresponding to genotype B) (8/13 or 62% and 8/23 or 35%, respectively). Furthermore, preS deletion mutants were found to be associated closely with advanced liver disease, such as liver cirrhosis and hepatocellular carcinoma. These observations are in agreement with a previous study from Japan in which HBV mutants were found with a deletion in the surface genes in none (0/12) of the asymptomatic

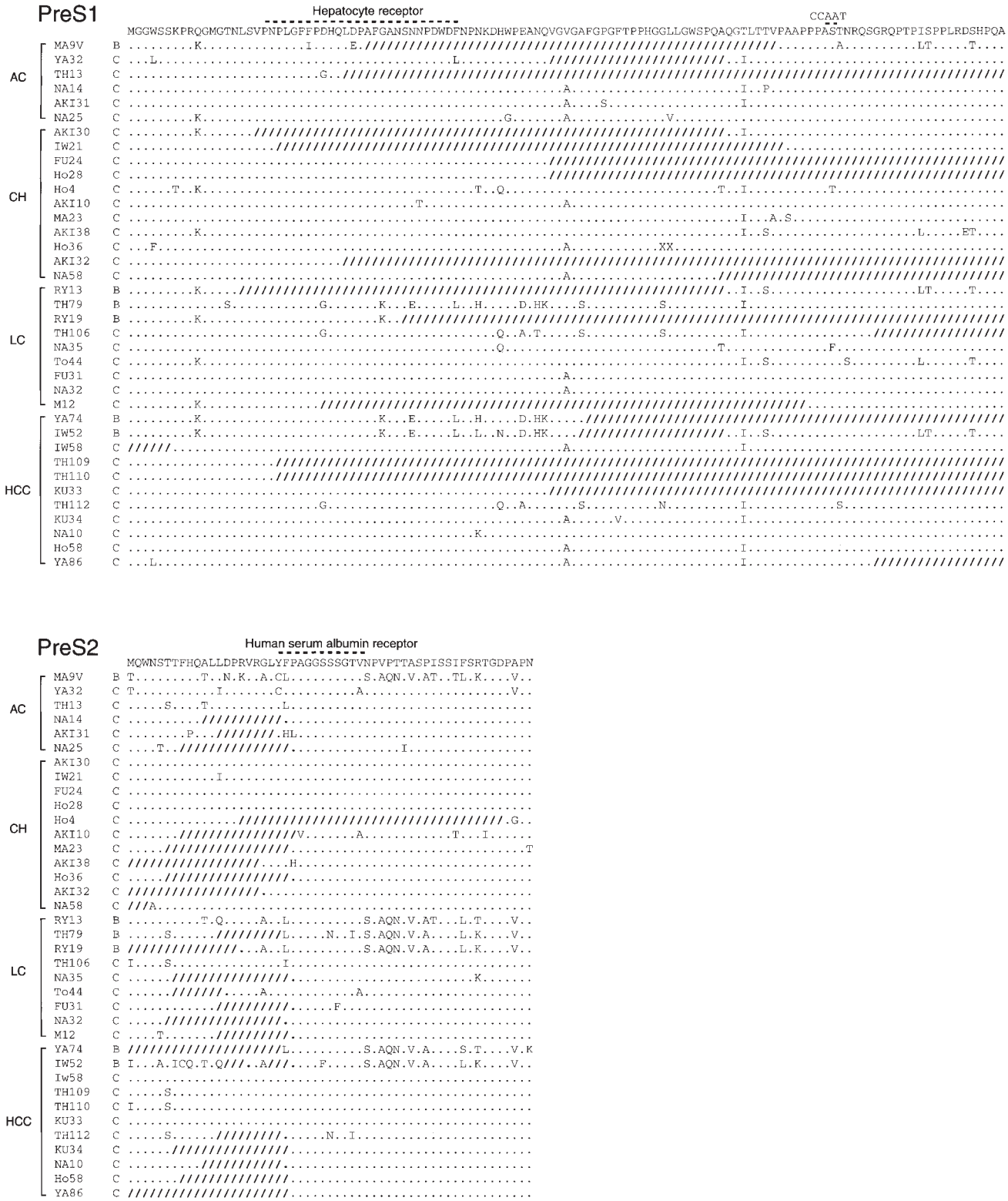


Fig. 2. Amino acid positions of preS deletions in 37 cases. The consensus sequence of hepatitis B virus (HBV) isolates of genotype C is shown at the top. Dots represent the same amino acids as in the consensus sequence, and slashes represents deletion of amino acids. HBV isolate names correspond to those in Table IV. Above the sequences, the proposed site for binding with hepatocytes (amino acids 21–47 in the preS1 region [Neurath et al., 1986]), the binding site for aggregated human serum albumin (amino acids 17–28 in the preS2 region [Itoh et al., 1986]), and the nucleotide position corresponding to a CCAAT element in the S-promoter region [Lu et al., 1995] are indicated by dotted lines. AC, aymptomatic carrier state; CH, chronic hepatitis; LC liver cirrhosis; HCC, hepatocellular carcinoma.

carriers, in 50% (8/16) of patients with chronic hepatitis, in 50% (2/4) of those with liver cirrhosis, and in 64% (7/11) of patients with hepatocellular carcinoma [Ohno et al., 1993].

PreS deletion mutants have been shown to occur preferentially in the exacerbation of chronic HBV infection [Minami et al., 1993]. Kao et al. [2001] reported that carriers of HBV genotype C have more severe acute exacerbations than do those with HBV genotype B in a long-term follow-up study, and that the immune active phase is longer in carriers of genotype C than in carriers of genotype B. The frequency and severity of acute exacerbation in HBV carriers are closely associated with the development of liver cirrhosis [Liaw et al., 1988]. Combined, these lines of evidence would point to distinct frequencies of preS deletion mutants between the carriers of genotypes B and C, making differences in the severity of liver disease. To corroborate this reasoning, preS deletion mutants in asymptomatic carriers and in patients with chronic hepatitis were detected less frequently in carriers of genotype B than in carriers of genotype C (1 of 51 [2%] vs 16 of 51 [31%], $P < 0.05$) in the present study. Taken together, preS deletion mutants would be associated with the development of more severe liver disease in carriers of HBV genotype C than in carriers of genotype B. As such, preS deletion mutations would be another molecular biological difference between genotypes B and C, giving further evidence for pathogenic differences among HBV genotypes. The present case-control study has an intrinsic limitation of a cross-sectional observation. Further studies for evaluating the influences of HBV genotypes on the emergence of preS deletion mutants are required in a longitudinal observation.

Nakajima et al. [1994] found the direct repeat, with a sequence of TCAGG, at both ends of preS1 deletions five times in HBV DNA samples from carriers of HBsAg subtype *adr* (corresponding to genotype C) and three times in those of the other subtypes. These investigators proposed the relevance of subtype *adr* to preS1 deletions. The same preS1 deletion was reported by Gerken et al. [1991]. In the present study, the direct TCAGG repeat was detected in preS1 deletions in HBV DNA samples of genotypes B and C for four and five times, respectively, and the TCAGG sequence was located at the beginning and at the end of defects in 6 of 20 (30%) cases of preS1 deletions. Hence, the TCAGG repeats within the preS1 region per se do not appear to explain the frequency of preS1 deletion mutants different between genotypes B and C, although they may be involved in the generation of deletions.

PreS deletion mutants accelerate the storage of large envelope proteins in hepatocyte cytoplasm, which in turn would induce cytotoxic effects toward the development of end-stage liver disease [Bock et al., 1999b]. Specifically, HBV mutants with a defect in the CCAAT-box in the S-promoter region may lead to the retention of S-proteins in the endoplasmic reticulum, thereby aggravating HBV-associated liver disease [Bock et al., 1999a]. In the present study, preS deletion mutants were detected in 20 of the 58 (34%) patients with advanced liver disease (liver cirrhosis and hepatocellular carcinoma combined), while a defect in the CCAAT-box in the S-promoter region was less frequent and occurred in only 9 of the 116 (8%) patients with clinical disease.

Among patients with advanced liver disease in Japan, preS deletion mutants were less frequent in those infected with HBV genotype B than in those infected with genotype C (5 of 29 or 17% vs 15 of 29 or 52%, $P < 0.05$) (Fig. 1). Even of the patients infected with HBV genotype B, however, they were more frequent in patients with advanced liver disease than in those with chronic hepatitis (5 of 29 or 17% vs 0 of 29 or 0%, $P < 0.05$). Likewise, HBV DNA samples from approximately one-half of patients with hepatocellular carcinoma in Taiwan were found to have preS1- or preS2 deletions [Tai et al., 2002]. Recently, Preikschat et al. [2002] reported that complex HBV populations with mutations in the core promoter, core gene, and preS gene are associated with the development of cirrhosis among recipients of kidney transplants in the long run.

The precise mechanism for hepatocarcinogenesis in the persistent HBV infection remains obscure. HBV genotypes may have a role in this process, and preS deletion mutants might interpret a part of the genotype difference in the development of hepatocellular carcinoma. The double mutation in the core promoter (T1762/A1764) is more frequent in HBV strains from patients with hepatocellular carcinoma than in those with inactive disease [Baptista et al., 1999; Kao et al., 2003]. To find any differences between them would require examination of the frequency of core-promoter mutation in patients with and without preS deletion mutants.

## REFERENCES

Baptista M, Kramvis A, Kew MC. 1999. High prevalence of 1762(T) 1764(A) mutations in the basic core promoter of hepatitis B virus isolated from black Africans with hepatocellular carcinoma compared with asymptomatic carriers. Hepatology 29:946–953.

Bock CT, Kubicka S, Manns MP, Trautwein C. 1999a. Two control elements in the hepatitis B virus S-promoter are important for full promoter activity mediated by CCAAT-binding factor. Hepatology 29:1236–1247.

Bock CT, Tillmann HL, Manns MP, Trautwein C. 1999b. The pre-S region determines the intracellular localization and appearance of hepatitis B virus. Hepatology 30:517–525.

Bowyer SM, Sim JG. 2000. Relationships within and between genotypes of hepatitis B virus at points across the genome: footprints of recombination in certain isolates. J Gen Virol 81:379–392.

Buckwold VE, Xu Z, Yen TS, Ou JH. 1997. Effects of a frequent double-nucleotide basal core promoter mutation and its putative single-nucleotide precursor mutations on hepatitis B virus gene expression and replication. J Gen Virol 78:2055–2065.

Caselmann WH, Meyer M, Kekule AS, Lauer U, Hofschneider PH, Koshy R. 1990. A trans-activator function is generated by integration of hepatitis B virus preS/S sequences in human hepatocellular carcinoma DNA. Proc Natl Acad Sci USA 87:2970–2974.

Chisari FV, Ferrari C. 1995. Hepatitis B virus immunopathogenesis. Annu Rev Immunol 13:29–60.

Fan YF, Lu CC, Chen WC, Yao WJ, Wang HC, Chang TT, Lei HY, Shiau AL, Su IJ. 2001. Prevalence and significance of hepatitis B virus (HBV) pre-S mutants in serum and liver at different replicative stages of chronic HBV infection. Hepatology 33:277–286.

Gerken G, Kremsdorf D, Capel F, Petit MA, Dauguet C, Manns MP, Meyer zum Buschenfelde KH, Brechot C. 1991. Hepatitis B defective virus with rearrangements in the preS gene during chronic HBV infection. Virology 183:555–565.

Ishikawa T, Ganem D. 1995. The pre-S domain of the large viral envelope protein determines host range in avian hepatitis B viruses. Proc Natl Acad Sci USA 92:6259–6263.

Itoh Y, Takai E, Ohnuma H, Kitajima K, Tsuda F, Machida A, Mishiro S, Nakamura T, Miyakawa Y, Mayumi M. 1986. A synthetic peptide vaccine involving the product of the pre-S(2) region of hepatitis B virus DNA: protective efficacy in chimpanzees. Proc Natl Acad Sci USA 83:9174–9178.

Kao JH, Chen PJ, Lai MY, Chen DS. 2000. Hepatitis B genotypes correlate with clinical outcomes in patients with chronic hepatitis B. Gastroenterology 118:554–559.

Kao JH, Chen PJ, Lai MY, Chen DS. 2001. Acute exacerbations of chronic hepatitis B are rarely associated with superinfection of hepatitis B virus. Hepatology 34:817–823.

Kao JH, Chen PJ, Lai MY, Chen DS. 2003. Basal core promoter mutations of hepatitis B virus increase the risk of hepatocellular carcinoma in hepatitis B carriers. Gastroenterology 124:327–334.

Kato H, Orito E, Sugauchi F, Ueda R, Gish RG, Usuda S, Miyakawa Y, Mizokami M. 2001. Determination of hepatitis B virus genotype G by polymerase chain reaction with hemi-nested primers. J Virol Methods 98:153–159.

Kidd-Ljunggren K, Ekdahl K, Oberg M, Kurathong S, Lolekha S. 1995. Hepatitis B virus strains in Thailand: genomic variants in chronic carriers. J Med Virol 47:454–461.

Liaw YF, Tai DI, Chu CM, Chen TJ. 1988. The development of cirrhosis in patients with chronic type B hepatitis: a prospective study. Hepatology 8:493–496.

Lindh M, Hannoun C, Dhillon AP, Norkrans G, Horal P. 1999. Core promoter mutations and genotypes in relation to viral replication and liver damage in East Asian hepatitis B virus carriers. J Infect Dis 179:775–782.

Lu CC, Chen M, Ou JH, Yen TS. 1995. Key role of a CCAAT element in regulating hepatitis B virus surface protein expression. Virology 206:1155–1158.

Melegari M, Bruno S, Wands JR. 1994. Properties of hepatitis B virus pre-S1 deletion mutants. Virology 199:292–300.

Minami M, Okanoue T, Nakajima E, Yasui K, Kagawa K, Kashima K. 1993. Significance of pre-S region-defective hepatitis B virus that emerged during exacerbation of chronic type B hepatitis. Hepatology 17:558–563.

Nakajima E, Minami M, Ochiya T, Kagawa K, Okanoue T. 1994. PreS1 deleted variants of hepatitis B virus in patients with chronic hepatitis. J Hepatol 20:329–335.

Neurath AR, Kent SB, Strick N, Parker K. 1986. Identification and chemical synthesis of a host cell receptor binding site on hepatitis B virus. Cell 46:429–436.

Neurath AR, Seto B, Strick N. 1989. Antibodies to synthetic peptides from the preS1 region of the hepatitis B virus (HBV) envelope (env) protein are virus-neutralizing and protective. Vaccine 7:234–236.

Norder H, Courouce AM, Magnius LO. 1994. Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. Virology 198:489–503.

Ohno T, Mizokami M, Wakita T, Kakumu S. 1993. Defective short variants of S region in chronic HBV carriers (in Japanese). Nippon Rinsho 51:297–303.

Okamoto H, Tsuda F, Sakugawa H, Sastrosoewignjo RI, Imai M, Miyakawa Y, Mayumi M. 1988. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. J Gen Virol 69:2575–2583.

Okamoto H, Yotsumoto S, Akahane Y, Yamanaka T, Miyazaki Y, Sugai Y, Tsuda F, Tanaka T, Miyakawa Y, Mayumi M. 1990. Hepatitis B viruses with precore region defects prevail in persistently infected hosts along with seroconversion to the antibody against e antigen. J Virol 64:1298–1303.

Orito E, Ichida T, Sakugawa H, Sata M, Horiike N, Hino K, Okita K, Okanoue T, Iino S, Tanaka E, Suzuki K, Watanabe H, Hige S, Mizokami M. 2001a. Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. Hepatology 34:590–594.

Orito E, Mizokami M, Sakugawa H, Michitaka K, Ishikawa K, Ichida T, Okanoue T, Yotsuyanagi H, Iino S. 2001b. A case-control study for clinical and molecular biological differences between hepatitis B viruses of genotypes B and C. Japan HBV Genotype Research Group. Hepatology 33:218–223.

Pollicino T, Campo S, Raimondo G. 1995. PreS and core gene heterogeneity in hepatitis B virus (HBV) genomes isolated from patients with long-lasting HBV chronic infection. Virology 208: 672–677.

Preikschat P, Gunther S, Reinhold S, Will H, Budde K, Neumayer HH, Kruger DH, Meisel H. 2002. Complex HBV populations with mutations in core promoter, C gene, and pre-S region are associated with development of cirrhosis in long-term renal transplant recipients. Hepatology 35:466–477.

Santantonio T, Jung MC, Schneider R, Fernholz D, Milella M, Monno L, Pastore G, Pape GR, Will H. 1992. Hepatitis B virus genomes that cannot synthesize pre-S2 proteins occur frequently and as dominant virus populations in chronic carriers in Italy. Virology 188: 948–952.

Stuyver L, De Gendt S, Van Geyt C, Zoulim F, Fried M, Schinazi RF, Rossau R. 2000. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. J Gen Virol 81:67–74.

Tai PC, Suk FM, Gerlich WH, Neurath AR, Shih C. 2002. Hypermodification and immune escape of an internally deleted middle-envelope (M) protein of frequent and predominant hepatitis B virus variants. Virology 292:44–58.

Tiollais P, Pourcel C, Dejean A. 1985. The hepatitis B virus. Nature 317:489–495.

Usuda S, Okamoto H, Iwanari H, Baba K, Tsuda F, Miyakawa Y, Mayumi M. 1999. Serological detection of hepatitis B virus genotypes by ELISA with monoclonal antibodies to type-specific epitopes in the preS2-region product. J Virol Methods 80:97–112.

Usuda S, Okamoto H, Tanaka T, Kidd-Ljunggren K, Holland PV, Miyakawa Y, Mayumi M. 2000. Differentiation of hepatitis B virus genotypes D and E by ELISA using monoclonal antibodies to epitopes on the preS2-region product. J Virol Methods 87: 81–89.