High Prevalence of Hepatitis B Virus Pre-S Mutant in Countries Where It Is Endemic and Its Relationship with Genotype and Chronicity

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It has been reported that hepatitis B virus (HBV) mutants carrying mutations in the pre-S region can be found in infected patients. In this study, we investigated the prevalence of the HBV variant with the pre-S mutant in different geographic regions, including countries with low and high levels of endemic HBV infection, and analyzed the correlation with clinical findings. We examined 387 HBV DNA-positive serum samples from individuals among 12 countries, consisting of Vietnam, Myanmar, Thailand, China, Korea, Nepal, Japan, Russia, Spain, United States, Bolivia, and Ghana. HBV pre-S mutants were detected in 71 (18.3%) of 387 serum samples tested. This mutant was the most prevalent in Vietnam (36%), followed by Nepal (27.3%), Myanmar (23.3%), China (22.4%), Korea (14.3%), Thailand (10.5%), Japan (7.7%), and Ghana (4.3%). In contrast, no case with this mutation was found in Russia, Spain, United States, and Bolivia. Among the HBV deletion mutations, 15.5% (11 of 71) occurred in the pre-S1 and 46.5% (33 of 71) in the pre-S2 regions. Eight (11.3%) cases had a mutation in both the pre-S1 and pre-S2 regions. In addition, a point mutation at the pre-S2 starting codon was observed in 19 (26.7%) cases. The detection rate of the HBV mutant in patients with hepatocellular carcinoma was significantly higher than in other patients (P < 0.05). Furthermore, these mutants were found more frequently in genotype B (25%) and genotype C (24.5%) than in other genotypes (P < 0.05). Our results indicated that there was a high prevalence of HBV pre-S mutation in regions of endemic HBV infection in Asia. Furthermore, the pre-S mutation appeared to be correlated with hepatocellular carcinoma and HBV genotypes.

Hepatitis B virus (HBV) is a small, enveloped 3.2-kb DNA virus with four open reading frames (ORFs). HBV envelope proteins are encoded by three overlapping envelope genes contained within a single ORF: pre-S1, pre-S2, and S. Depending on the translated initiation site among S, pre-S2, or pre-S1, three different-size proteins are produced; a small hepatitis B surface protein (small HBs), containing 108 or 119 additional amino acid residues; and a large hepatitis B surface protein (large HBs), containing 55 additional amino acid residues; and a large hepatitis B surface protein (large HBs), containing 108 or 119 additional amino acid residues, depending on the serotype. The pre-S region has been proved to mediate hepatocyte attachment of the virus (amino acids 21 to 47 in pre-S1) (1, 20, 21, 26), to contain B-cell and T-cell epitopes (5, 16, 17) and a binding site for neutralizing anti pre-S2 antibody (amino acids 120 to 145) (1, 20, 26); and an S promotor for controlling the production of middle HBs and small HBs.

Naturally occurring HBV with the pre-S mutation has been reported in patients with chronic infections, fulminant hepatitis (32), post-lamivudine treatment (14), and post-liver transplantation (29). It is known that the rate of occurrence has varied in the literature (4, 7–9, 27, 31). However, the clinical significance of this mutation is still unclear. We therefore investigated the correlation of the HBV pre-S mutation with clinical manifestation of liver diseases as well as the association with HBV genotypes in various geographic regions, including areas of high and low endemicity of HBV infection.

MATERIALS AND METHODS

Patients. Sera were obtained from 387 HBV surface antigen (HBsAg)-seropositive patients in 12 countries: Vietnam (114, ranging in age from 6 years to 78 years; mean = 42 ± 13 years), Nepal (11, ranging in age from 38 years to 69 years; mean = 50 ± 10 years), Myanmar (30, ranging in age from 17 years to 71 years; mean = 45 ± 15 years), China (58, ranging in age from 7 years to 70 years; mean = 41 ± 15 years), Korea (7, ranging in age from 25 years to 74 years; mean = 50 ± 16 years), Thailand (19, ranging in age from 19 years to 44 years; mean = 33 ± 9 years), Japan (39, ranging in age from 18 years to 74 years; mean = 45 ± 15 years), Ghana (24, age is not available), Russia (39, ranging in age from 5 years to 15 years; mean = 10 ± 3.4 years), Spain (28, ranging in age from 23 years to 67 years; mean = 47 ± 11 years), United States (12, ranging in age from 32 years to 73 years; mean = 45 ± 16 years), and Bolivia (6, ranging in age from 40 years to 67 years; mean = 20 ± 15 years).
years to 55 years; mean $= 44 \pm 5$ years). Among these patients, we obtained the clinical diagnosis in 355 cases, consisting of 39 acute hepatitis, 80 asymptomatic carrier, 3 fulminant hepatitis, 90 liver cirrhosis, and 49 hepatocellular carcinoma (HCC) (Table 1). Clinical diagnosis was based on liver function tests, hepatitis virus markers, autoantibodies, tumor markers, and ultrasonography. Some patients, mainly those with chronic hepatitis for interferon therapy, were diagnosed by histopathology of the liver. Informed consent for participation in this study was obtained from each individual. All sera were collected from 1998 through 2002 and stored at $-40^\circ$C or below until used. During this period, the sera were thawed one or two times for another assays.

### Serologic test for HBV markers
Antigen and antibody related to HBV were tested by using a commercially available enzyme-linked immunosorbent assay kit (Abbott Laboratory Co., North Chicago, Ill.).

### Detection of HBV DNA by PCR for screening
Nucleic acids were extracted from 100 ml of serum samples with the SepaGene RV-R kit (Sanko Junyaku Co., Ltd., Tokyo, Japan). HBV DNA was detected by nested reverse transcription-PCR with AmpliTaq Gold DNA polymerase (Perkin-Elmer, Norwalk, Conn.). The sequences of the PCR primers were 5'-TGC CAA CTG GAT CCT TCG (S2-2, antisense, nucleotides 1625 to 1607) for the outer primer pair (233 bases) and 5'-GTC CCC TTC TTC (P1, sense, nucleotides 2817 to 2839) and 5'-AGA AGA TGA GGC ATA GCA (S1, antisense, nucleotides 668 to 687) for the inner primer pair (118 bases). Both the first- and second-round PCRs were done with 40 cycles of amplification at 94°C for 20 s, 55°C for 20 s, and extension at 72°C for 30 s, followed by extension at 72°C for 7 min. The sensitivity of this PCR assay allowed detection of as few as 10 copies of HBV DNA. To avoid false-positive results, instructions to prevent cross contamination were strictly followed (11), and results were considered valid only when they were obtained in duplicate.

### HBV genotyping by PCR
Genotyping of HBV was identified by PCR with type-specific primers designed from the pre-S1 through S genes of HBV that were originally reported by Naito et al. (19). Briefly, HBV DNA was amplified by nested PCR with AmpliTaq Gold DNA polymerase (Perkin-Elmer). The first-round PCR was done with the universal primers, and the second-round PCR with type-specific primers. The specificity of this assay system was confirmed by nucleotide sequencing and phylogenetic analysis as reported previously (19).

### Amplification of HBV pre-S region and sequencing analysis
The pre-S1 and pre-S2 regions were amplified by heminested PCR with 5'-TCA CCA TAT TCT TGG GAA CAA GA-3' (P1, sense, nucleotides 2817 to 2839) and 5'-GGC ACT AGT AAA CTG GTC CTT-3' (MD24, sense primer, nucleotides 1392 to 1421) and 5'-GTT CAC GGT GGT CTC CAT G-3' (S1, antisense, nucleotides 668 to 687) for the outer primer pair (1,085 bases) and 5'-AGA AGA TGA GGC ATA GCA-3' (S2-2, antisense, nucleotides 1625 to 1607) and 5'-GTT CAC GGT GGT CTC CAT G-3' (P1, sense, nucleotides 2817 to 2839) and 5'-AGA AGA TGA GGC ATA GCA-3' (S1, antisense, nucleotides 668 to 687) for the inner primer pair (545 bases). Both the first- and second-round PCRs were done with 40 cycles of amplification at 94°C for 20 s, annealing at 55°C for 20 s, and extension at 72°C for 3 min, and elongation for 7 min in the last cycle. The second-round PCR was performed in the same conditions for 40 cycles except the annealing temperature was at 60°C.

The pre-S amplicons were isolated by 1% agarose gel electrophoresis and purified with the QIAquick gel extraction kit (Qiagen Inc., Chatsworth, Calif.). Recovered PCR products were then subjected to direct sequencing with primers of P1 and S4R, with an ABI Prism Big Dye terminator cycle sequencing ready reaction kit (Perkin Elmer). Sequences of amplified DNA were determined with a sequencer (ABI model 373 and 310; Applied Biosystems, Foster City, Calif.). Nucleotide and deduced amino acid sequences were edited and analyzed with the Genetyx version 5.2 for Windows (Genetyx, Tokyo, Japan).

### Quantiﬁcation of viral load
Quantitation of HBV DNA was done by real-time PCR as reported by Chen et al. (3). The detection limit of this assay was 3.73 x 10^3 genome equivalents per ml. The sequences of primers and probes were HBc1, 5'-AGT GTG GAT TCG CAC TCC T-3' (sense, nucleotides 2290 to 2287); HBc1R, 5'-GAC TCC TTC TTC TTC TAG GAC ACC TG-3' (antisense, nucleotides 2387 to 2365); and HBcP1, 5'-CCA AAT GCC CCT ATC TTA TCA ACG TGC AGA GGT GAC AAG-3' (TaqMan probe, nucleotides 2303 to 2331).

### Statistical analysis
Statistical analysis was performed with Student's t test for comparison of means and $\chi^2$ analysis for categorical variables. A $P$ value (two-tailed) of less than 0.05 was considered statistically significant.

## Results

### Prevalence of the HBV pre-S mutation and sequence analysis
HBV pre-S deletion mutations were detected in 71 of 387 cases (18.3%), with the highest rate in Vietnam (36%), followed by Nepal (27.3%), Myanmar (23.3%), China (22.4%), and Korea (11%).

### Statistical analysis
Statistical analysis was performed with Student's $t$ test for comparison of means and $\chi^2$ analysis for categorical variables. A $P$ value (two-tailed) of less than 0.05 was considered statistically significant.

## Table 1. Patients

| Country | No. of cases | Age, yr (mean ± SD) | M/F ratio | Clinical diagnosis* (no. of cases) |
|---------|--------------|---------------------|-----------|-----------------------------------|
| Vietnam| 114          | 47 ± 14             | 3.3       | ASC 4  | 25  | 3  | 6  | 41  | 27  |
| Nepal  | 11           | 50 ± 10             | 8         | AH 7   | 3   | 1  | 10 | 7   |
| Myanmar| 30           | 45 ± 15             | 3.6       | CH 18  | 4   | 9  | 8  |
| China  | 58           | 41 ± 15             | 1.4       | LC 4   | 19  | 9  |
| Korea  | 7            | 50 ± 16             | 6         | HCC 4  |
| Thailand| 19           | 33 ± 9              | 19        | ED 15  |
| Japan  | 39           | 45 ± 15             | 2.5       | NA 15  |
| Russia | 24           | NA                  | NA        | NA 9   |
| Spain  | 28           | 41 ± 11             | 6         | HCC 21 |
| United States| 12 | 50 ± 12 | 2 | HCC 12 |
| Bolivia| 4            | 44 ± 5              | 5         | HCC 6  |
| Total  | 387          | 40 ± 18             | 2.8       | HCC 80 |

* ASC, asymptomatic carrier; AH, acute hepatitis; FH, fulminant hepatitis; CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma; NA, not available.

## Table 2. HBV genotypes

| Country | No. of cases with HBV genotype: |
|---------|---------------------------------|
| Vietnam| 67                              |
| Nepal  | 1                               |
| Myanmar| 30                              |
| China  | 3                               |
| Korea  | 2                               |
| Thailand| 7                               |
| Japan  | 1                               |
| Russia | 2                               |
| Spain  | 17                              |
| United States| 5   |
| Bolivia| 5                               |
| Total  | 84                              |

## Table 3. HBV genotypes

| Country | No. of cases with HBV genotype: |
|---------|---------------------------------|
| Vietnam| 67                              |
| Nepal  | 1                               |
| Myanmar| 30                              |
| China  | 3                               |
| Korea  | 2                               |
| Thailand| 7                               |
| Japan  | 1                               |
| Russia | 2                               |
| Spain  | 17                              |
| United States| 5   |
| Bolivia| 5                               |
| Total  | 84                              |
Korea (14.3%), Thailand (10.5%), Japan (7.7%), and Ghana (4.2%). However, they were not detected in Russia, Spain, United States, and Bolivia (Table 3). Among 71 mutations detected, 15.5% appeared in the pre-S1 and 46.5% in the pre-S2 region. These mutations were identified frequently in the 3′ terminus of the pre-S1 and/or the 5′ terminus of the pre-S2 region (Fig. 1A and 1B). In pre-S1 deletion mutations, the hepatocyte binding site of HBV located in amino acids 21 to 47 (1, 20, 26) and/or the S-promoter region (2) were partially or totally truncated in 15 cases (Fig. 1A). In addition, a point mutation with an amino acid substitution at the starting codon of the pre-S2 region (from methionine to valine, isoleucine, or threonine) was observed in 19 of 71 (26.7%) mutants tested. The number of deleted amino acids varied from 1 to 127, but no frameshift was found.

**Correlation among pre-S mutation, clinical findings, and genotypes of HBV.** The highest prevalence of the mutation was observed in China (4.2%). However, they were not detected in Russia, Spain, United States, and Bolivia. In particular, a high prevalence (over 20%) of the HBV pre-S mutant was found in four countries, including Vietnam, Myanmar, Nepal, and China, which are regions where HBV is highly endemic. In particular, 36% patients found in Vietnam were infected with the HBV pre-S mutant. On the other hand, no cases with such a mutant were seen in Russia, Spain, United States, and Bolivia. In Russia, we tested serum samples from children only. This may be one reason why we could not detect such a mutant in this country even if HBV is highly endemic in Russia. In fact, no case with the pre-S mutant was found among patients less than 20 years of age, although the prevalence increased with age in this study.

**Relationship between viral load and pre-S mutation of HBV.**
We compared the viral load of HBV in serum samples between the wild-type (66 cases) and pre-S mutation types (34 cases) of HBV. The results showed that the HBV DNA level in pre-S mutation cases with chronic infection was lower (4.8 ± 1.35 log10 copies/ml) than that of the wild type (5.19 ± 1.37 log10 copies/ml), but this difference was not statistically significant.

**DISCUSSION**

The pre-S1 and pre-S2 regions, together with the major hydrophilic region (amino acids 100 to 160 in the S gene), exposed at the surface of HBV particles, are highly immunogenic and potentially under selective pressure by the immune system (18). The pre-S1 region contains the binding site for HepG2 cells in the liver membrane within residue pre-S1 (amino acids 21 to 47) (21). The pre-S2 region has been found to bear the binding site for polymerized human serum albumin, which is believed to be involved in the attachment of HBV to the human hepatocyte membrane (25). Chimpanzees immunized with peptides derived from the 5′-terminal part of the pre-S2 sequence (amino acids 120 to 140) were protected against HBV infection (20). Therefore, mutations of the pre-S gene might significantly modify the course of HBV infection (8).

Recently, although the occurrence of HBV with the pre-S deletion mutation in liver disease patients has been reported by several groups, its clinical significance has remained obscure (4, 24, 28, 30). In the present study, we found a significant difference in the detection rate of the HBV pre-S deletion mutation among 12 countries tested. Importantly, a high prevalence (over 20%) of the HBV pre-S mutant was found in four countries, including Vietnam, Myanmar, Nepal, and China, which are regions where HBV is highly endemic. In particular, 36% patients found in Vietnam were infected with the HBV pre-S mutant. On the other hand, no cases with such a mutant were seen in Russia, Spain, United States, and Bolivia. In Russia, we tested serum samples from children only. This may be one reason why we could not detect such a mutant in this country even if HBV is highly endemic in Russia. In fact, no case with the pre-S mutant was found among patients less than 20 years of age, although the prevalence increased with age in this study.

We found four patterns of the pre-S mutant: a pre-S1 in-frame deletion, a pre-S2 in-frame deletion, both pre-S1 and pre-S2 deletions, and a point mutation in the start codon of pre-S2. The 3′ terminus of the pre-S1 and 5′ terminus of pre-S2 were the favored sites for the deletion mutations. Interestingly, the pre-S1 mutant had a truncated hepatocyte binding site and/or S-promoter region. In vitro studies showed that pre-S1 mutants involving the binding sites for transcription factors of the S promoter, such as NF1 and SP1, might lead to a decrease in HBsAg secretion, retention of HBsAg,
| Isolate (Country) | Pre-S1 | Hepatocyte binding site (aa 21-47) | S promoter (nt 3045-3160) |
|------------------|--------|-----------------------------------|--------------------------|
| D50517 (Wild type) | MGQWSSKPRG | PLGFFPDHQL DPAFGANSMN PDQWDPNPKD | HAPEANVQGA GAFPGQFTPP HQLGLQWSPQ AQGLITTVPA APPASTNRQ SGRQGTP1SP PRD5HPQA 119 |
| VT63 (Vietnam)   |        |                                   |                          |
| VT72             |        |                                   |                          |
| VT88             |        |                                   |                          |
| VT105            |        |                                   |                          |
| VT128            |        |                                   |                          |
| VT140            |        |                                   |                          |
| VT162            |        |                                   |                          |
| VT190            |        |                                   |                          |
| VT233            |        |                                   |                          |
| VT266            |        |                                   |                          |
| VT272            |        |                                   |                          |
| VT303            |        |                                   |                          |
| VT333            |        |                                   |                          |
| VT444            |        |                                   |                          |
| VT552            |        |                                   |                          |
| VT717            |        |                                   |                          |
| VT746            |        |                                   |                          |
| VT787            |        |                                   |                          |
| VT87             |        |                                   |                          |
| (Vietnam)*       |        |                                   |                          |
| VH153            |        |                                   |                          |
| VH178            |        |                                   |                          |
| VH183            |        |                                   |                          |
| VH274            |        |                                   |                          |
| VH381            |        |                                   |                          |
| (Nepal)          |        |                                   |                          |
| NE559            |        |                                   |                          |
| NE560            |        |                                   |                          |
| MY7260           |        |                                   |                          |
| MY19             |        |                                   |                          |
| MY505            |        |                                   |                          |
| MY626            |        |                                   |                          |
| MY644            |        |                                   |                          |
| MY863            |        |                                   |                          |
| CN415            |        |                                   |                          |
| CN427            |        |                                   |                          |
| CN430            |        |                                   |                          |
| CN444            |        |                                   |                          |
| CN458            |        |                                   |                          |
| CN460            |        |                                   |                          |
| CN493            |        |                                   |                          |
| TH72             |        |                                   |                          |
| TH12             |        |                                   |                          |
| IM565            |        |                                   |                          |
| IM569            |        |                                   |                          |
| IM570            |        |                                   |                          |
| CA283            |        |                                   |                          |
| (China)          |        |                                   |                          |
| (Thailand)       |        |                                   |                          |
| (Japan)          |        |                                   |                          |
| (Ghana)          |        |                                   |                          |
FIG. 1. Multialignment of the amino acid sequences in the pre-S1 (A) and pre-S2 (B) genes of HBV isolated in this study. D5017, wild type of HBV derived from GenBank databases. Dots represent amino acids that are identical to those of the HBV wild type (top line), and dashes represent deletions.

| Isolate (Country) | Pre-S2 |
|-------------------|--------|
| D5017 (Wild)      |        |
| VT53 (Vietnam)*   |        |
| VT57               |        |
| VT87               |        |
| VT98               |        |
| VT108              |        |
| VT128              |        |
| VT140              |        |
| VT162              |        |
| VH190              |        |
| VT21               |        |
| VT23               |        |
| VT26               |        |
| VT260              |        |
| VT68               |        |
| VT78               |        |
| VT88               |        |
| VT88 (Vietnam)*    |        |
| VH15               |        |
| VH153              |        |
| VH168              |        |
| VH194              |        |
| VH274              |        |
| VH290              |        |
| NE5590 (Nepal)     |        |
| NE5590 (Nepal)     |        |
| NE3516 (Myanmar)   |        |
| MY180              |        |
| MY186              |        |
| MY253              |        |
| MY53               |        |
| MY628              |        |
| MY644              |        |
| MY683              |        |
| CN415 (China)†     |        |
| CN425              |        |
| CN427              |        |
| CN430              |        |
| CN433              |        |
| CN468              |        |
| CN469              |        |
| CN453              |        |
| TH72 (Thailand)    |        |
| TH142 (Japan)      |        |
| IM559              |        |
| IM556              |        |
| IM569              |        |
| QA283 (Ghana)      |        |

* VT: Ho Chi Minh City of Vietnam, VH: Hanoi of Vietnam
and a large S protein in the cytoplasm, resulting in the ground-glass-like hepatocyte phenomenon (13, 15). Thus, the overproduction and accumulation of the large S protein could give rise to severe, prolonged hepatocellular injury.

The pre-S2 gene is known as the hypervariable region of the nucleotide sequence. The pre-S2 mutant deleted the site containing several epitopes for T and B cells, i.e., HLA-3 restricted T-cell epitopes (amino acids 109 to 123), B-cell epitopes for neutralizing (amino acids 109 to 129), and the binding site for putative neutralizing anti-pre-S2 antibody (amino acids 120 to 145) (7). In our study, nearly 35% of HBV pre-S mutants were seen in HCC patients. Regarding the relationship between HBV mutants and HCC occurrence, Ohno et al. (22) showed that the pre-S1 sequences carried a transcriptional transactivation domain that can stimulate the transcription of transforming growth factor alpha gene in vitro. Furthermore, Jacobczak et al. (10) revealed that acceleration of hepatocellular carcinogenesis could result from a synergistic effect of transforming growth factor alpha and HBsAg coexpression on hepatocyte proliferation.

Although both pre-S1 and pre-S2 mutants could be detected at different replicative stages, the pre-S mutant prevails at stages which have lower HBV DNA level and might be associated with HCC development. Fan et al. (6) suggested that this mutant may potentially play a role in the pathogenesis of HBV-related HCC by expressing the pre-S2 deletion mutant in vitro. Furthermore, one study from Taiwan reported that 50% of HCC samples had a pre-S1 or pre-S2 deletion mutant, but no mutant was found among chronic carriers without cirrhosis or HCC (26). Taken together, the specific sites of pre-S truncation, the higher rate of the pre-S2 mutant, and its predilection for HCC patients suggested that the pre-S mutant might play an important role in chronicity and hepatocellular carcinogenesis in the course of HBV infection.

We also investigated the correlation between the pre-S mutant and genotypes of HBV. Our data showed a high prevalence of this mutant in patients infected with genotypes B and C. These two genotypes are major genotypes of HBV distributed in Asian countries, and genotype C was found to be correlated with severe liver diseases, together with a higher rate of HBeAg positivity and a high HBV DNA level (12). Furthermore, it has also been suggested that genotype C has a higher rate of mutation in the core promoter regions (23). However, the question of whether or not the pre-S mutant is linked to the distribution of HBV genotypes and endemicity requires further study.

In conclusion, we found that the HBV pre-S mutant prevailed in various countries where HBV is highly endemic. This mutant might play a role in the persistence of HBV and in the process leading to HCC in these countries.

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