Mutations in surface and polymerase gene of chronic hepatitis B patients with coexisting HBsAg and anti-HBs

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Abstract

AIM: To investigate the clinical significance and presence of mutations in the surface (S) and overlapping polymerase gene of hepatitis B patients with coexisting HBsAg and anti-HBs.

METHODS: Twenty-three patients with chronic hepatitis B were studied. Of the 23 patients, 11 were both positive for hepatitis B virus (HBV) surface antigen (HBsAg) and antibody to HBV surface antigen (anti-HBs), 12 were negative for anti-HBs while positive for HBsAg. DNA was extracted from 200 μL serum of the patients. Nucleotide of the surface and overlapping polymerase gene from HBV-infected patients was amplified by PCR, and the PCR products were sequenced.

RESULTS: Forty-one mutations were found within the surface gene protein of HBV in 15 patients (10 with coexisting HBsAg and anti-HBs). Six (14.6%) out of 41 mutations were located at “α” determinant region in 5 patients (4 positive for HBsAg and anti-HBs). Eleven mutations (26.8%) occurred in the downstream or upstream of “α” determinant region. Lamivudine (LVM)-selected mutations were found in three patients who developed anti-HBs, which occurred in amino acid positions (196, 198, 199) of the surface protein and in YMDD motif (M204I/V) of the polymerase protein simultaneously. Presence of these mutations did not relate to changes in ALT and HBV DNA levels.

CONCLUSION: Besides mutations in the “α” determinant region, mutations at downstream or upstream of the “α” determinant region may contribute to the development of anti-HBs. These mutations do not block the replicating competency of HBV in the presence of high titer of anti-HBs.

INTRODUCTION

Hepatitis B virus (HBV) infection leads to a wide spectrum of liver diseases, including acute self-limited infection, asymptomatic carrier state, fulminant and chronic hepatitis, which could result in life-threatening sequelae, such as liver cirrhosis and hepatocellular carcinoma[3]. In acute self-limited infection, clearance of HBV is associated with seroconversion from HBsAg to anti-HBs due to the coordination of humoral and cellular immune response[4]. However, this condition is very rare in chronic infection patients, especially in patients infected at birth, partly because of inadequate humoral and cellular immunity of the host[5]. Furthermore, the prevalence of HBV mutations that could escape from humoral and cellular immunity may result in persistent virus infection.

The S gene of HBV has three open reading frames (ORF), including preS1, preS2 and S region. The surface gene contains a neutralizing epitope named “α” determinant region located at the codon positions 124-147. Mutations in this region could alter the antigenicity of HBsAg, causing failure of anti-HBs to neutralize HBsAg and escaping from the host's immune system, resulting in active viral replication and liver disease[6]. It is reported that mutations of some epitopes located at downstream of the “α” determinant region may also affect the neutralization domain[7]. The surface gene overlaps with the catalytic domains of polymerase[8]. Thus, mutations in the surface gene have an effect on the polymerase gene while the polymerase gene mutations also impact the surface gene[9].

The present study was to analyze the prevalence of mutations in the surface and polymerase gene of HBV in patients with coexisting anti-HBs and HBsAg.
MATERIALS AND METHODS

Patients
Sera were obtained from 23 Chinese patients with chronic HBV infection. Presence of HBsAg, anti-HBs, hepatitis e antigen (HBeAg), antibody to HBeAg (anti-HBeAg) was detected by commercially available kits according to the instructions. All patients were positive for HBsAg. Of the 23 patients, 11 were positive for anti-HBs (No. 1 to 11) and 20 were positive for HBeAg. Fifteen out of the 23 patients had elevated alanine aminotransferases (ALT) levels. Virolological and biochemical parameters of 8 patients positive for anti-HBs and HBsAg at the time of HBV sequence analysis were analyzed. No patients had a history of HBV vaccination or hyper immune globulin therapy. Three patients positive for HBsAg and anti-HBs had a history of lamivudine (LMV) therapy for more than 1 year. All patients were negative for antibody to hepatitis C virus. Sera were stored at -20°C for DNA extraction.

Primer synthesis
For polymerase chain reaction (PCR), primers were synthesized according to the published sequences. Sequences of the primers for amplifying the surface gene of HBV are as follows: HBV S1: 5’-TTACAGCGGCTTTTC-3’ (nt 197, sense); HBV S2: 5’- AAGGGACTCAAGATG-3’ (nt 789, anti-sense). Primers of HBV P1, 5’-GTATTTCCATCCCCATCC-3’ (nt 599, sense) and HBV P2, 5’-CAAGGCCAGATGCCAT-3’ (nt 1033, anti-sense) were used for amplification of polymerase gene of HBV.

PCR amplification
DNA was extracted from 200 μL serum using a blood DNA kit (Omega, USA). Two microlitres of DNA template and 1 μL of each of the primers, 2 μL of 10 × dNTP and 0.5 U of Taq DNA polymerase (Promega, US) were used in a volume of 50 μL for PCR. After denaturation at 94°C for 5 min, the reaction for amplification of the surface gene with primers HBV S1 and HBV S2 was carried out at 94°C for 30 s, at 56°C for 1 min, and at 72°C for 1 min for 35 cycles, and a final extension at 72°C for 10 min. The reaction with primers HBV P1 and HBV P2 for amplifying the polymerase gene was performed at 94°C for 30 s, at 56°C for 30 min, at 72°C for 1 min for 35 cycles, and a final extension at 72°C for 10 min.

Sequencing of PCR products
The PCR products were purified by centrifugation. Direct sequence of the gene was determined using Taq Dye-Deoxy terminator sequencing kits. Sequencing reactions were analyzed on an automated DNA sequencer (model 377, ABI100, Applied Biosystem). Deduced amino acid sequences were compared with the reported consensus sequence of genotype C, subtype adw, HBV clone (PAK66, PIWK146). Mutations were determined as sequence different from the consensus sequence.

The nucleotide sequence data presented in this paper could be found in the DDBJ/EMBL/GeneBank nucleotide sequence databases with the access numbers AB014381, AB033554, AY812744, AY812743, AY800249, AY123424, AF100309.

Table 1 Clinical data of two groups of patients

| Clinical factors          | Positive anti-HBs | Negative anti-HBs | P     |
|---------------------------|-------------------|-------------------|-------|
| Age in years              | 40.3 ± 13.2       | 42.5 ± 14.7       | 0.767 |
| Sex, M/F                  | 5/6               | 7/5               | 0.6531|
| Patients with HBsAg, n(%) | 11 (100)          | 12 (100)          |       |
| Patients with HBeAg, n(%) | 9 (81.8)          | 11 (91.7)         |       |
| Patients with anti-HBe, n (%) | 1 (9.1)         | 0 (0)             |       |
| ALT in IU/L               | 65.9 ± 30.5       | 98.9 ± 42.0       | 0.6541|
| HBV-DNA (log)             | 7.0 ± 1.60        | 6.87 ± 0.9        | 0.5263|
| Number of amino acid residues | 34/41 (82.9) | 7/41 (17.1)       |       |

Fisher’s exact test was used for the categorized data; two-tailed Student’s t test was used for ALT levels, age and HBV DNA levels (log, copies mL⁻¹).

Statistical analysis
Two-tailed Student’s t test was used to assess the difference in ALT levels, age, HBV DNA levels between the two groups of patients. Fisher’s exact test was used for the analysis of difference in mutations between the two groups. P < 0.05 was considered statistically significant.

RESULTS
Comparison of the clinical features between the two groups of patients is shown in Table 1. There was no significant difference in ALT levels, age, HBV DNA levels between the two groups (P > 0.05). The relevant biochemical and virological parameters of 8 patients (No.1 to 6, No.8 and 10) are shown in Table 2.

Nucleotide and deduced amino acid sequences of surface region and polymerase gene of HBV were performed in 23 patients. Comparison with the published HBV sequence showed that 21 (91.3%) out of 23 patients were infected with genotype C, 1 with genotype B and 1 with genotype D15 (65.2%). Of the 23 patients who developed amino acid mutations in the surface gene protein, 10 were positive for anti-HBs and 5 were negative for anti-HBs. Mutations at the “α” determinant region were observed in 5 patients (5/15, 33.3%) (Figure 1). Forty-one mutations were found at 27 amino acid positions within the surface gene of HBV, and 34 mutations (82.9%, 34/41) were presented in the patients with coexisting HBsAg and anti-HBs. Six (14.6%) out of 41 mutations were located at the “α” determinant region, and 4 mutations were presented in the first loop (positions 124-137), the others were in the second loop (positions 139-147, S143T, G145R). Six mutations at amino acid residues 40 (N40S) and 47 (T47V, T47K, T47R) coincident with HLA class I-restricted (CTL) epitope were observed in 5 patients, 11 mutations (26.8%) occurred in 6 patients within the major hydrophilic regions of upstream and downstream of the “α” determinant region (amino acid positions 99-169), 6 mutations at 3 amino acid positions (196, 198 and 199) associated with LMV-selected.
Table 2  Virological and biochemical follow-up data of 8 patients

| No. | Sex | Age | ALT | HBVDNA | a-HBs | HBeAg | ALT | HBVDNA | a-HBs | HBeAg | ALT | HBVDNA | a-HBs | HBeAg | ALT | HBVDNA | a-HBs | HBeAg |
|-----|-----|-----|-----|--------|-------|-------|-----|--------|-------|-------|-----|--------|-------|-------|-----|--------|-------|-------|
| 1   | M   | 57  | 22  | 0      | -     | -     | 20  | 0      | -     | -     | 76  | 8.63   | -     | +     | -   | 87     | 6.38  | +     |
| 2   | M   | 36  | 48  | 9.38   | -     | +     | 82  | 8.53   | -     | +     | 21  | 4.04   | -     | +     | 97  | 3.78   | +     | +     |
| 3   | F   | 65  | 25  | 4.04   | +     | -     | 58  | 5.04   | +     | -     | 55  | 6.76   | +     | -     | 55  | 5.08   | +     | -     |
| 4   | M   | 45  | 45  | 6.86   | +     | +     | 80  | 6.61   | +     | +     | 59  | 5.57   | -     | +     | 93  | 6.99   | +     | +     |
| 5   | M   | 55  | 156 | 7.32   | +     | +     | 116 | 7.11   | +     | +     | 112 | 7.75   | +     | +     | 149 | 5.80   | +     | +     |
| 6   | F   | 30  | 19  | 6.91   | +     | +     | 33  | 7.70   | +     | +     | 20  | 8.86   | -     | +     | 15  | 9.08   | +     | +     |
| 8   | M   | 38  | 18  | 0      | +     | -     | 19  | 0      | +     | -     | 20  | 0      | -     | -     | 21  | 4.43   | +     | -     |
| 10  | M   | 72  | 20  | 5.18   | +     | +     | 25  | 7.04   | +     | +     | 18  | 6.26   | -     | +     | 48  | 4.15   | +     | +     |

* Positive result; negative result; ALT: alanine aminotransferase; HBV DNA: HBV DNA levels (log, copies ml⁻¹).

Table 3  Mutations of HBV in polymerase and HBsAg protein

| Mutant in patients | Position with HBsAg protein sequence change | Position with polymerase protein sequence change |
|--------------------|---------------------------------------------|-----------------------------------------------|
|                     | (n)             | 145 | 196 | 198 | 199 | 173 | 180 | 204 | 223 |
| Wild type           |                 |     |     |     |     |     |     |     |     |
| 1                  |                 |     |     |     |     |     |     |     |     |
| 3                  |                 |     |     |     |     |     |     |     |     |
| Wild type           |                 |     |     |     |     |     |     |     |     |
| 10                 |                 |     |     |     |     |     |     |     |     |
| 11                 |                 |     |     |     |     |     |     |     |     |
| 13                 |                 |     |     |     |     |     |     |     |     |

Mutation were observed in 5 patients.

Because the S gene overlaps with the major catalytic domain of the polymerase gene, the mutations near the YMDD motif of the polymerase gene were studied. Eight mutations within amino acid residues 518-569 of the polymerase gene were observed at 4 positions (V173L, L180M, M204I/V, S223A) in 5 patients. Three patients who received long term LMV therapy and developed anti-HBs at the time of sequencing, had YMDD mutations (M204I/V) in polymerase gene and the S gene mutations at amino acid positions 196, 198 and 199 (Table 3). Five out of 15 (33.3%) patients who had amino acid mutations did not develop anti-HBs, while T131N, L162Q, W196L mutations in the S gene and L180M mutation in polymerase gene were simultaneously observed in only one of these patients.

DISCUSSION

HBV is the most common etiologic agent of chronic and often fatal liver diseases world wide. HBV variants present during natural infection or anti-virus therapy, and contribute to disease persistence. The S gene of HBV is crucial for binding and infectivity, and “α” determinant of the surface gene may form a target of humoral neutralizing antibody. Mutations in the region affect the binding of anti-HBs to corresponding HBsAg[13], produce escape from the neutralizing antibody, result in persistent infection and replication of HBV, even the relatively high titer anti-HBs develops.

In the present study, 15 patients had amino acid mutations in the surface gene of HBV, and 10 of them were positive for anti-HBs. Forty-one mutations were found in 23 patients, and 34 (82.9%) mutations were presented in the patients with coexisting HBsAg and anti-HBs. only 6 mutations within the “α” determinant region were observed in 5 patients (4 for anti-HBs positive). Passive or active immune therapy may develop the escape mutation[11,12], which has a point mutation from guanosine to adenosine at nucleotide 587 (condon 145, G145R). Mutation of G145R, however, was only seen in 1 patient in our study, and it may be the reason why no patient in our cohort received active or passive hepatitis B immunization. The data suggest that mutation within the “α” determinant region may play an important role in the presence of anti-HBs. A resent study showed that mutation in the “α” determinant region contributes to the most therapy failure, but there are still some therapy failures associated with mutations in the major hydrophilic region of the surface gene located at downstream or upstream of the “α” determinant (positions 99-169)[13]. In the present study, 26.8% mutations were observed in the above mention region, suggesting that these mutations also change the antigenicity of HBsAg and contribute to the development of anti-HBs. One patient who had amino acid mutation (T131N) in the “α” determinant region did not develop anti-HBs. It may be due to the relatively lower sensitive assays because it was reported that anti-HBs complexed with HBsAg could be detected in nearly all patients with chronic hepatitis B when tested by a highly sensitive immunoassay[10].

Lamivudine-selected mutations in the S gene of HBV have been demonstrated by sequencing HBV isolated from the serum of patients treated with long-term LMV[15,16] In addition, LMV-selected mutations within the HBsAg protein downstream of the “α” determinant (I195M, W196S and M198I) lead to a decrease in the antigenicity of the protein and binding to the anti-HBs antibodies, therefore poorly inhibiting their interaction with wild-type HBsAg[17]. In our study, the change of methionine to isoleucine (rtM204I) or valine (rtM204V) was found in the YMDD motif of the polymerase gene protein in three patients who received long-term LMV therapy. These patients also had W196L/W196F or W199C mutations within the surface gene protein of HBV simultaneously.

The surface gene of HBV also includes the putative
Hepatitis B virus (HBV) immunopathogenesis. The consensus sequences of A, B and D different from those of genotype C are listed in parentheses. Dashes mean residues identical to these reference residues.

Figure 1  Amino acid mutations in the surface gene of HBV. Positions of mutation in deduced amino acid residues are indicated by vertical line below the surface protein of HBV. The consensus sequences of A, B and D different from those of genotype C are listed in parentheses. Dashes mean residues identical to these reference residues.

In fact, patients positive for anti-HBs have more amino acid mutations, especially mutations in the crucial region of the surface gene associated significantly with the presence of anti-HBs. But presence of these mutations is not related to clinical features, ALT levels, HBV DNA levels. This result is similar to the report from Taiwan, which revealed a frequent frequency of mutations at amino acid positions 40 and 47 of the surface gene in patients with chronic hepatitis B, suggesting that these mutations change CTL recognition and contribute to chronic infection in some patients.

In conclusion, the presence of mutations in the “α” determinant of surface gene is not high in patients with coexisting anti-HBs and HBsAg. The mutations at the major hydrophilic region of the surface gene contribute to the development of anti-HBs in these patients and produce escape from the neutralizing antibody, and lead to persistent infection. Long-term LMV therapy could induce YMDD mutation in the polymerase gene and surface gene of HBV.

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