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• *BRIEF REPORTS* •

High level of hepatitis B virus DNA after HBeAg-to-anti-HBe seroconversion is related to coexistence of mutations in its precore and basal core promoter

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Abstract

AIM: G1896A mutation in precore or A1762T/G1764A mutations in basal core promoter are suspected to be responsible for patients with detectable level of HBV DNA in serum after seroconversion from HBeAg to anti-HBe. However, G1896A variant has impaired, while A1762T/G1764A variant may have intact replication ability. They themselves or their coexistence status may play different roles in such meaningless seroconversion. For these reasons, the significances of these two types of mutations were comparatively investigated in this study.

METHODS: One hundred and sixty-five sera with positive anti-HBe and HBV DNA were collected from different patients. Mutations of G1896A and A1762T/G1764A among these serum samples were detected using competitively differentiated PCR. HBV DNA was demonstrated using real-time quantitative PCR.

RESULTS: G1896A and/or A1762T/G1764A mutations were detected in 89.1% (147/165) out of patients with detectable HBV DNA in serum after HBeAg-to-anti-HBe seroconversion. The positive rate of G1896A variants was significantly higher than that of A1762T/G1764A mutations (77.6% *vs* 50.3%, $\chi^2 = 26.61$, $P<0.01$). The coexistence positive rate of these two types of mutations was 38.8% (64/165). Coexistence mutations were found in 77.1% (64/83) out of sera with A1762T/G1764A mutations, and in 50.0% (64/128) out of sera with G1896A mutation. Compared with variants with G1896A mutation only, the coexistence mutations were predominant in patients with high level of serum HBV DNA, and related to higher total bilirubin, lower serum albumin and progressive liver diseases.

CONCLUSION: The coexistence of G1896A mutation and A1762T/G1764A mutations is very common, and responsible for the major cases with high level of HBV DNA in serum and progressive liver diseases after HBeAg-to-anti-HBe seroconversion. This coexistence mutation variant may have higher pathogenicity and replication ability.

Key words: Hepatitis B; Hepatitis B virus; Viral load; Mutant

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INTRODUCTION

In the natural history or during the antiviral therapy of chronic HBV infection, seroconversion from HBeAg to anti-HBe is usually accompanied by a decrease in viral replication and remission of liver disease[1-4]. However, viral replication and liver damage persist in about 10% of patients after seroconversion[5-8]. The characteristic laboratory findings of these patients are that there are detectable levels of HBV DNA, and HBV variants with mutations in precore, core or basal core promoter (BCP) regions[5,6,9,10]. Among these mutations, HBV variants with a G-to-A mutation at nucleotide 1 896 (G1896A) or double-point mutation in BCP, A-to-T mutation at nucleotide 1 762 and G-to-A mutation at nucleotide 1 764 (A1762T/G1764A) are the commonest[9-14], and may be responsible for these meaningless seroconversions. However, G1896A variant has impaired, while A1762T/G1764A variant may have intact replication ability[15-17]. They themselves or their coexistence status may play different roles in such meaningless seroconversions. For these reasons, G1896A mutation and A1762T/G1764A mutations were detected using competitively differentiated polymerase chain reaction (CD-PCR) in this study, and the serum viral loads of patients with infections of G1896A variant, A1762T/G1764A variant or coexistence mutation variant of these two types of mutations were comparatively studied.

MATERIALS AND METHODS

Samples

One hundred and sixty-five serum samples with positive

anti-HBe were selected out from 300 continuous serum samples with positive HBsAg and HBV DNA from different patients in the Department of Infectious Diseases, the Third Affiliated Hospital, Sun Yat-Sen University. The serum markers of HBV were demonstrated by ELISA. The HBV DNA level was quantified using fluorescein quantitative PCR (Taqmen, Roche). These samples were divided into three groups according to the level of HBV DNA in serum, low-level group ($\geqslant 10^3$ to $<10^5$ copies/mL), median level group ($\geqslant 10^5$ to $<10^7$ copies/mL) and high-level group ($\geqslant 10^7$ copies/mL). There were 61 samples in the low-level group, 58 samples in median level group and 46 samples in the high-level group.

Reagents

Mutant-type control for CD-PCR, recombinant plasmids pG1896A and pHB-BCP2 were constructed before. Wild-type control for CD-PCR, pTZ19U-HBV that contained double copies of HBV DNA (adw) were presented by Professor Huang Zhimin, Sun Yat-Sen University. T4 DNA ligase and pfu DNA polymerase were purchased form Promega Company (USA). Anti-digoxigenin (anti-DIG) and anti-fluorescein (anti-FITC) labeled with horseradish peroxidase were purchased from Roche Company (USA). Primers shown in Table 1 were designed with the Omega 2 software and synthesized in Bioasia Biological Engineering Company (Shanghai, China).

Table 1 Primers and probes for detections of G1896A or A1762T/G1764A mutations

Denomination	Sequences (5′→3′)
PCP	BIO-GAGAC TCTAA GGCTT CTCGA TACAG AGCTG AGG
PCMd	DIG-CTCAC GCTAC ATTGT GTGCC TTGGG TGGCT TCA
PCWd	FITC-GTCCG TAGTC TCGTT GTGCC TTGGG TGGCT TGG
BCP1-M	DIG-GCTGA CGATG CGATG GGGAG GAGAT TAGGT TAATG A-3′
BCP1-W	FITC-CGTCC GTAGT GCCGA GGGAG GAGAT TAGGT TAAAG G-3′
PCA	CCCAG CAGAG AATGG CTTGC CTGAG TGCAG TATG
PCSc	CCCGA ATTCC ACCGT GAACG CCCAT CAG
PCAc	CCCAA GCTTG CAGTA TGGTG AGGTG AGCAA TG

BIO: the abbreviation of biotin; DIG: the abbreviation of digoxigenin; FITC: the abbreviation of fluorescein isothiocyanate. The underlined nucleotides had no relationship with HBV.

G1896A mutation detection

The method of CD-PCR was described in detail in previous paper. G1896A mutation was detected using CD-PCR with a few modifications. Briefly here, a 30-μL PCR reaction was performed. The reaction mixture contained 10 mmol/L Tris-HCl, pH 8.5, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 20 μmol/L dNTPs, 2 U pfu DNA polymerase, 20 pmoL FLU-PCWd, 20 pmoL DIG-PCMd, 10 pmoL PCA and 5 μL plasmid or extracted DNA. The cycling conditions were as follows: 2 cycles (first set) of 94 ℃ for 60 s, 53 ℃ for 120 s and 72 ℃ for 120 s, followed by 35 cycles (second set) of 94 ℃ for 30 s, 65 ℃ for 30 s, 94 ℃ for 40 s and 72 ℃ for 60 s. The PCR products were then hybridized with solidified biotin-labeled probe PCP in two different holes of microtiter plate. The color reaction was obtained after the captured PCR products reacted with horseradish peroxidase-labeled anti-DIG or anti-FITC respectively. Wild-type and G1896A mutant-type plasmids were used as positive control and negative control.

A1762T/G1764A mutation detection

A1762T/G1764A mutation was detected using CD-PCR just like G1896A mutation. The main differences were that BCP1-M and BCP1-W were used as competitive primers and recombinant plasmid pHB-BCP2 was used as positive control.

DNA sequencing

To confirm the results of CD-PCR, three samples of each group, the G1896A mutation group, A1762T/G1764A mutation group and the groups with positive or negative for both types of mutation were selected for DNA sequence analysis. Fragments of HBV BCP, precore and core regions were analyzed using DNA sequencing after they were amplified using primer PCSc and PCAc, and cloned into plasmid pUC19.

Statistical analysis

For statistical analysis, *t* tests, χ^2 examination or Fisher exact probability analysis was used. SPSS 10.0 for Windows was used for all statistical analysis. $P<0.05$ was considered statistically significant.

RESULTS

Detections of G1896A and A1762T/G1764A mutations

G1896A and A1762T/G1764A mutations in patients with positive HBV DNA in serum after seroconversion were very common. HBV strains with G1986A and/or A1762T/G1764A mutations were predominant (89.1%, 147/165). Compared with A1762T/G1764A mutations, the positive rates of G1896A variants were significantly higher (77.6% *vs* 50.3%, $\chi^2 = 26.61$, $P<0.01$) in these patients. The coexistence positive rate of these two types of mutations was 38.8% (64/165). Coexistence mutations were found in 77.1% (64/83) out of sera with A1762T/G1764A mutations, and in 50.0% (64/128) out of sera with G1896A mutation.

Confirmation analysis of G1896A and A1762T/G1764A mutations

The CD-PCR results of 12 selected samples were confirmed as expected by DNA sequence analysis. It suggests that the results of CD-PCR are believable.

Relationship of mutations with serum HBV DNA level

The relationship of G1896A and A1762T/G1764A mutations to serum HBV DNA level in these patients is shown in Table 2. From low, median to high level of HBV DNA, the total positive rates of G1896A mutation decreased in turn, while the total positive rates of A1762T/G1764A mutations increased. Since coexistence of G1896A and A1762T/G1764A mutations were very common in these patients, the mutations of G1896A only, A1762T/G1764A only and their coexistence were separately considered (Table 2). The status of mutations of G1896A only was the same as that of total G1896A mutation. The status of

Table 2 Relationship between CD-PCR results and serum HBV DNA load in 165 serum samples with detectable HBV DNA in serum after HBeAg-to-anti-HBe seroconversion

	No. (%) of CD-PCR results				
	Total G1896A	Total A1762T/G1764A	G1896A only	A1762T/G1764A only	Coexistence
Low-level group (n = 61)	53 (86.9)[b]	20 (32.8)	35 (57.4)[d]	2 (3.3)	18 (29.5)
Median level group (n = 58)	45 (77.6)	32 (55.2)[e]	23 (39.6)	10 (17.3)	22 (37.9)
High-level group (n = 46)	30 (65.2)	31 (67.4)[f]	6 (13.0)	7 (15.2)	24 (52.2)[g]

[1]χ^2 = 7.08, [b]P<0.01, [2]χ^2 = 21.81, [d]P<0.01, compared with high-level group. [3]χ^2 = 6.06, [e]P<0.05, compared with low-level group. [4]χ^2 = 8.20, [f]P<0.01, compared with median level group. [5]χ^2 = 5.65, [g]P<0.05, compared with low-level group.

Table 3 Main clinical data of 147 serum samples with G1896A and/or A1762T/G1764A mutations

	G1896A only (n = 64)	A1762T/G1764A only (n = 19)	Coexistence (n = 64)
Age (yr)	40.5±12.8	43.2±14.2	40.7±12.8
Sex (no. male/no. female)	55/9	18/1	59/5
Alanine aminotransferase (IU/L)	101.9±158.3	121.8±183.1	116.7±137.3
Total bilirubin (μmol/L)	25.2±39.2	24.8±40.6	32.5±55.2[b]
Serum albumin (g/L)	38.4±5.3	39.1±5.8	37.7±5.3[d]
Clinical diagnosis			
Chronic hepatitis Mild (n)	23	3	13
Median (n)	20	10	14
Gravies (n)	12	4	22[f]
Liver cirrhosis	9	2	15[g]

[1]t = –2.85, [b]P<0.01, [2]t = 6.37, [d]P<0.01, compared with group of G1896A only. [3]χ^2 = 18.3, [f]P<0.01, [4]χ^2 = 4.03, [g]P<0.05, compared with group of mild type of chronic hepatitis B.

mutations of A1762T/G1764A only could not be analyzed because of the limited case numbers. The status of coexistence was the same as that of total A1762T/G1764A mutations. In high-level group, HBV variants with coexistence of G1896A mutation and A1762T/G1764A mutations were predominant.

Relationship of mutations with main clinical data

The main clinical data of 147 serum samples with G1896A mutation and/or A1762T/G1764A are shown in Table 3. The patients with coexistence mutation variant infections were related with higher total bilirubin and lower serum albumin as compared with patients who were infected by HBV variants of G1896A mutation only. For clinical diagnosis, coexistence mutations were more often to be found in progressive liver diseases (gravies type of chronic hepatitis B and liver cirrhosis), while G1896A mutation only is found more often in benign liver diseases (mild or median type of chronic hepatitis B).

DISCUSSION

CD-PCR is a rapid method for point mutation screening, and can detect mutations with high specificity, efficiency and rapidity. Using this technique in this study, G1896A and/or A1762T/G1764A mutations were detected in 89.1% out of patients with detectable HBV DNA in serum after HBeAg-to-anti-HBe seroconversion. It suggests that G1896A and/or A1762T/G1764A mutations are major causes of this meaningless seroconversion. G1896A mutation was detected in up to 77.6% of such patients. However, the variant with G1896A mutation is usually accompanied by a decrease in HBV replication and remission of liver disease[9,15,18-22], and can be considered as favorable factor of response to interferon treatment[23]. That means G1896A mutation may not be responsible for the meaningless seroconversion, especially for patients with progressive liver diseases. This view is further supported by those variants with G1896A mutation only which were closely related to low level of HBV DNA and benign liver diseases, when HBV DNA level and clinical data were taken into account in this study.

The A1762T/G1764A variant is usually accompanied by increase in HBV replication and decrease in HBeAg secretion, and may be related to liver deterioration[9,24-28], or at least not to affect HBV DNA level[18]. A1762T/G1764A mutations were detected in half of the patients, and coexisted with G1896A mutation in 77.1% out of all A1762T/G1764A mutations in this study. The coexistence mutation variant was also found to be related to high level of HBV DNA in serum. These results suggest that A1762T/G1764A mutations, especially the coexistence mutations, may play more important roles in the meaningless seroconversion than G1896A mutation. Other data show that A1762T/G1764A mutations take place years early than G1896A before seroconversion[13]. Thus, these results suggest that, when it subsequently occurs in the genome, G1896A mutation can decrease the replication of wild-type HBV but A1762T/G1764A variant. The reason may be that the transcription of A1762T/G1764A variant is regulated in a different manner and by different transcriptional factors[16,17,29].

The coexistence of G1896A mutation and A1762T/G1764A mutations are very common[25], have a clear link with chronic active hepatitis[26], are associated with the degree of histological injury[30] and are found to be related to high level of HBV DNA, higher total bilirubin, lower serum albumin and progressive liver diseases in this study. Thus, this coexistence mutation variant might have higher pathogenicity and replication ability. However, some research demonstrated that the dual mutations occurred less frequently in patients with high level of serum HBV DNA[5]. For these reasons, the coexistence of G1896A

mutation and A1762T/G1764A mutations is worth some further extensive investigations.

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