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• VIRAL HEPATITIS •

A novel stop codon mutation in HBsAg gene identified in a hepatitis B virus strain associated with cryptogenic cirrhosis

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Abstract

AIM: HBsAg is the most important serological marker for acute or chronic hepatitis B. Nevertheless, there were reports of HBsAg-negative infection caused by hepatitis B virus in recent years. We had a patient with crytogenic cirrhosis who was negative for HBsAg, positive for anti-HBs and HBeAg. This paper was to explore the pathogenic and molecular basis of the unusual serological pattern.

METHODS: HBV serologic markers were qualitatively and quantitatively determined. HBV DNA in serum was qualitatively tested using routine Polymerase chain reaction (PCR), and the viral level was determined with real-time fluorescence quantitative PCR. HBsAg gene was amplified and cloned. Four clones were sequenced. The new genomic sequences were compared with GenBank on the DNA level as well as the protein level.

RESULTS: The qualitative results of serological markers were HBsAg(-), anti-HBs(+), HBeAg(+), anti-HBe(-) and anti-HBc(+). The quantitative results of serological marker were HBsAg (S/N): 0.77 (cut off of S/N: $\geqslant$2.00), HBeAg (S/N): 56.43 (cut off S/N: $\geqslant$2.10), anti-HBc (S/C_O): 2.03 (cut off of S/C_O: $\leqslant$1.00). The viral level was as high as 1.54×10^9 copies/ml. Sequencing of the HBsAg gene clones revealed a unique point mutation at nucleotide 336 (C to A), which resulted in a novel stop codon at aa 61. The novel HBsAg gene stop mutation had not been described.

CONCLUSION: The lack of detection of HBsAg in the presence of high viral levels of replication may be caused by the existence of viral genomes harboring point mutations which resulted in stop codon upstream of the "a" determinant in HBsAg gene.

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INTRODUCTION

Hepatitis B virus (HBV) is a small DNA-containing virus with 4 overlapping open reading frames. The four genes are core, surface, X and polymerase. The surface antigen open reading frame is divided into three regions, pre-S1, pre-S2 and S, which encode three envelope proteins respectively termed large, middle and major protein. All the three envelope proteins contain the major protein, HBsAg, which consists of 226 amino acids and is the predominant protein of the 20 nm small spherical particles representing circulation excess surface protein[1,2].

Serological evidence for acute or chronic hepatitis B is provided most commonly by assays detecting the HBsAg. Its detection is believed to prove the presence of hepatitis B virus in the liver and the peripheral blood. Both the clearance of HBsAg from serum and the appearance of antibodies to HBsAg (anti-HBs) are associated with a resolution of hepatitis in acute or chronic hepatitis B infection[3]. However, the development of polymerase chain reaction (PCR) technique has permitted the detection of very low levels of HBV in patients. There are a number of reports of HBsAg-negative virus carriers[4,5]. Moreover, even cases of anti-HBs-positive carriers have been described although antibodies against the viral envelope usually neutralize the virus and confer protection from infection[6]. China is a highly endemic area for HBV infection, some studies suggested that 30 % to 40 % of HBsAg-negative patients with cryptogenic cirrhosis, chronic active hepatitis, or chronic persistent hepatitis had HBV-DNA in serum or liver tissue[7]. A well-characterized explanation for the latter pattern is surface mutation. Over the past decade many kinds of surface mutations have been described. We present here a patient with cirrhosis and active viral replication in the presence of anti-HBs. Sequencing of the HBV DNA from the patient revealed a point mutation at nucleotide 336 (C to A) in HBs-gene. This mutation led to a stop codon at 61 amino acids of HBsAg and a premature translation stop, which has not been described elsewhere up to now.

MATERIALS AND METHODS

Case report

The patient was a 56-year-old man. He was positive for HBsAg but asymptomatic in 1988. In 1990 his alanine aminotransferase (ALT) level was slightly elevated. In 1992 he developed a very severe disease which was diagnosed as severe type hepatitis B. He recovered 3 months later. He felt well from 1992 to 1998, no data about HBV serology and liver function were available during that period. However, he began to feel fatigue, weakness, abdominal distension from the beginning of 1999. His WBC and platelet were markedly decreased, but negative for HBsAg, and positive for anti-HBs. Abdominal ultrasound examination showed splenomegaly. Cryptogenic cirrhosis was diagnosed. Cirrhosis caused by HCV, alcohol, drugs, Wilson' s diease and schistosomiasis was excluded. In order to find the cause of the cirrhosis, he came to our hospital in August 2000. Serum was collected at the time and stored at -70 ℃ until analysis.

HBV serological markers detection

HBsAg, anti-HBs, HBeAg, anti-Hbe, anti-HBc-IgG and anti-HBc-IgM were tested using commercially available standard enzyme immunoassay kit (Kehua Bio-Engineering Co. LTD, Shanghai, China). HBsAg, HbeAg and anti-HBc-IgG were

quantitatively determined using Abbott reagent with IMX automatic immunoassay analyzer (Abbott Laboratories, North Chicago, IL), according to the manufacturer' s instructions.

Serum HBV DNA detection

HBV DNA detection was carried out using commercially available PCR kit (Liver Research Institute, Beijing Medical University, Beijing, China), according to the manufacturer' s instructions. Serum samples (200 μl) were digested with 1 g/L proteinase K and 0.5 % sodium dodecyl sulfate (at 37 ℃ **for** 2 hours), followed by phenol-chloroform extractions and ethanol precipitation. After centrifugation, the pellet was dissolved in 10 μl distilled water. Five μl elute was used for PCR. Thermal cycling conditions were as follows: 35 cycles of amplification were performed at 94 ℃ for 30 s and at 60℃ for 45 s. The PCR products were investigated by staining with ethidium bromide on ultraviolet transilluminatior after electrophoresis in 1.5 % agarose gel.

HBV DNA quantification

HBV DNA in serum was quantified using a commercially available real-time fluorescence quantitative PCR (FQ-PCR) kit (Da An Gene Diagnostic Center, Sun Yet-Sen Medical University, Guangzhou, China), in accordance with the manufacturer' s instructions. Briefly, 40 μl of serum was mixed with 40 μl of DNA-extracting solution (provided by the kit). The mixture was vortexed and placed in a 100 ℃ heating block for 10 min, then overnight at 4 ℃. The mixture was centrifuged for 5 min at 10 000 rpm. Two μl of supernatant was added to the tube containing FQ-PCR core reagent (provided by the kit). FQ-PCR was performed using a GeneAmp 5700 sequence detection system (Perkin Elmer, Foster City, CA). Thermal cycling conditions were as follows: at 93 ℃ for 2 min for initial denaturation, followed by 40 cycles of at 93 ℃ for 30 s, at 55 ℃ for 60 s. Analysis of raw data was done with the GeneAmp 5700 SDS Software (PE Biosystems). Data were collected at the annealing step of each cycle, and the threshold cycle (C_T) for each sample was calculated by determining the point at which the fluorescence exceeded the threshold limit. The standard curve was calculated automatically by plotting the C_T value against each standard of known concentration and calculation of the linear regression line of this curve. Calculation of the correlation coefficient was done for each run, and the minimal value was 0. 98. Sample copy numbers were calculated by interpolation of the experimentally determined standard curve.

Amplification of HBsAg gene

The primers were designed by ourselves according to the sequences published[8,10], which could amplify whole HBsAg gene (from nt 155 to 833). The procedures for HBV DNA extraction were the same as routine PCR described above. The reaction conditions were: the total volume was 30 μl, containing 50 mmol/L KCl , 10 mmol/L tris-HCl (pH9.0), 0.1 % triton 100, 0.2 mmol/L dNT, 1.5 mmol/L $MgCl_2$, 15 pmol primer 1 and primer 2, TaqDNA polymerase 2.5U, a drip of paraffin oil was added on the top of the solution. PCR conditions were as follows: at 94 ℃ for 5 min for initial denaturation, followed by 30 cycles at 94 ℃ for 1 min, at 56 ℃ for 50 s, at 72 ℃ for 10 s, at 72 ℃ for 10 min for extension. The PCR products were investigated by staining with ethidium bromide on ultraviolet transilluminatior after electrophoresis in 1.5 % agarose gel or used for HBsAg gene cloning.

Forward primer: 5' GGGAAGCTTATGGAGAACATCACATCAGGATTC3'

Reverse primer: 5' CGCGGATCCTTAAATGTATACCCAGAGACAAAA3' .

Cloning and sequencing of HBsAg gene

The amplified products of HBsAg gene were cloned using pGEM-T easy vectors system kit (Promega Co., Madison, WI.), according to the manufacture' s instructions. To detect the vectors containing the PCR products, white/blue colony selection was used. The inserted products were analyzed by electrophoresis in 1 % agarose gel after EcoR 1 digestion and PCR (as amplification of HBsAg gene described above). Four clones were sequenced. The sequence of the complete HBsAg gene was obtained by forward and reverse reading of overlapping fragments using ABI PRISM BigDye terminator cycle sequencing ready reaction kit (Perkin Elemer, Foster City, CA). To identify mutations, the new genomic sequences were compared with GenBank on the DNA level as well as the protein level.

RESULTS

All HBV serologic markers were tested repeatedly. HBsAg, HBeAg and anti HBc were tested again using Abbott reagent. The results confirmed that the patient was HBsAg-negative, anti-HBs-positive and HBeAg-positive. It was estimated that the patient had cirrhosis caused by HBV. To confirm the diagnosis, the presence of HBV DNA in serum of the patient was tested using routine PCR and HBV DNA was detected. The quantity of HBV DNA in serum was determined using real-time fluorescent quantitative PCR, which was unexpectedly as high as 1.54×109/ml (Tables 1 and 2).

Table 1 Viral and clinical examination results of the patient

Item	Jan. 1999	Apr. 1999	Oct. 1999	Aug. 2000	Nov. 2000	Mar. 2001
HBsAg	-	-	-	-	-	-
Anti-HBs	+	+	+	+	+	+
HbeAg	+	+	+	+	+	+
Anti-Hbe	-	-	-	-	-	-
Anti-HBc-IgG	+	+	+	+	+	+
Anti-HBc-IgM	+	-	-	-	-	-
HBV DNA	Nd	Nd	Nd	Nd	+	+
HBV DNA copies/ml	Nd	Nd	Nd	Nd	1.02×10^8	1.54×10^9
ALT (U/L)	49.0	56.9	67.1	74.4	69.0	68.6
Albumin (g/L)	42.3	40.4	38.5	40.3	41.5	40.8
Globulin (g/L)	28.6	30.7	28.5	34.4	31.9	32.6
Bilirubin (μmol/L)	18.9	23.2	28.6	25.8	27.7	28.4
Hemoglobin (g/L)	Nd	Nd	Nd	111	115	110
WBC (10^9/L)	Nd	Nd	Nd	2.1	2.8	2.6
Platelet (10^9/L)	Nd	Nd	Nd	26	58	40

Nd, not determiined; +, positive; -, negative. ALT, alanine aminotransferase; WBC, white blood cell.

Table 2 HBsAg, HBeAg and anti-HBc level determined using Abbott reagent

Item	Results	Cut off
HBsAg	0.77	≥2.00(S/N)
HbeAg	56.433	≥2.10(S/N)
Anti-HBc	2.033	≤1.0(S/Co)

Due to the massive production of viral particles in the absence of HBsAg and presence of anti-HBs, HBsAg gene mutation, possibly in the "a" determinant, was suspected. Therefore, HBV DNA was extracted from the serum of the patient, HBsAg gene was amplified. After electrophoresis of the PCR products corresponding to the complete HBsAg gene amplified from serum and ethidium bromide staining of the gel, a PCR fragment about 700 bp was detected (the figure was not shown). Then the PCR products of HBsAg gene were cloned, 4 clones were sequenced. The complete sequence of

the HBsAg gene was obtained by forward and reverse reading of overlapping fragments (Table 3). All of the 4 clones had the same sequence. The nucleotide and amino acid sequences of the HBsAg gene were compared with GenBank. There were no nucleotide insertions or deletions in the HBsAg gene. Surprisingly, sequencing of the HBsAg gene clones revealed a unique point mutation at nucleotide 336 (C to A), which resulted in a novel stop codon at aa 61. Thus, only a truncated version of HBsAg containing 21 amino acids could be synthesized from this gene, which lacked the entire "a" determinant. The novel HBsAg gene stop codon caused by a point substitution mutation upstream of the "a" determinant of HBsAg gene has not been described up to now.

The isolate belonged to subtype adw2 according to the amino acid sequence deduced from the nucleotide sequence of the HBsAg gene. The patient's nucleotide sequence and amino acid sequence were compared with a published sequence of the same subtype reported by Ono *et al*[9] and a Chinese consensus sequence of the same subtype (China J Microbiol Immunol, 1999; 19: 197-200). A two by two analysis of the three nucleotide and amino acid sequences demonstrated a relatively high degree of homogeneity. The nucleotide and amino acid difference was 5.28 % and 8.37 % between the patient's and Ono's sequences, and was 4.84 % and 7.92 % between the patient's and the Chinese consensus sequences, respectively. The "a" determinant of the patient's sequence differed from the Ono's sequence by only 2 amino acids and differed from the Chinese consensus sequence by another 2 amino acids, which might reflect the genetic heterogeneity of the same subtype and could not be the mutation (Table 4).

Table 3 The complete nucleotide sequence of the HBsAg gene from the patient

nt															Codon
155	ATG	GAG	AAC	ATC	ACA	TCA	GGA	TTC	CCA	GGA	CCC	CTG	CTC	GTA	TTA	15
200	CAG	GCG	GGG	TTT	TTC	TTG	TTG	ACA	AAA	ATC	CTC	ACA	ATA	CCA	CAG	30
245	AGT	CTA	GAC	TCG	TGG	TGG	ACT	TCT	CTC	AAT	TTT	CTA	GGG	GGA	ACA	45
290	CCC	GTG	TGT	CTT	GGC	CAA	AAT	TCG	CAG	TCC	CAA	ATC	TCC	AGT	CAC	60
335	**TAA**	CCA	ACC	TGC	TGT	CCT	CCA	ATT	TGT	CCT	GGT	TAT	CGC	TGG	ATG	75
380	TGT	CTG	CGG	CGT	TTT	ATC	ATC	TGC	CTC	TGC	ATC	CTG	CTG	CTA	TGC	90
425	CTC	ATC	TTC	TTG	TTG	GTT	CTT	CTG	GAC	TAT	CAA	GGT	ATG	TTG	CCC	105
470	GTT	TGT	CCT	CTA	CTT	CCA	GGA	TCA	ACA	ACA	ACC	AGC	ACC	GGA	CCA	120
515	TGC	AAA	ACC	TGC	ACG	ACT	CCT	GCT	CAA	GGC	AAC	TCT	AAG	TTT	CCC	135
560	TCT	TGT	TGC	TGT	ACA	AAA	CCT	ACG	GAC	GGA	AAC	TGC	ACC	TGT	ATT	150
605	CCC	ATC	CCA	TCA	TCT	TGG	GCT	TTC	GCA	AAA	TAC	CTA	TGG	GAG	TGG	165
650	GCC	TCA	GTC	CGT	TTC	TCT	TGG	CTC	AGT	TTA	CTA	GTG	CCA	TTT	GTT	180
695	CAG	TGG	TTC	GTA	GGG	CTT	TCC	CCC	ACT	GTC	TGG	CTT	TCA	GTT	ATA	195
740	TGG	ATG	ATG	TGG	TTT	TGG	GGG	CCA	AGT	CTG	TAC	AAC	ATC	GTG	AGT	210
785	CCC	TTT	ATG	CCG	CTG	TTA	CCA	ATT	TTC	TTT	TGT	CTC	TGG	GTA	TAC	225
830	ATT	TAA														227

Table 4 Comparison of nucleotide sequences and amino acid sequences of HBsAg gene among the 3 adw subtypes

Codon	4	9	14	24	29	45	47
Sequence(1)	ATC Ile	CTA Leu	GTG Val	AGA Arg	CCG pro	TCA Ser	CTA Leu
Sequence(2)	ATC Ile	CCA Pro	GTA Val	A**A**A Lys	CCA Pro	TCA Ser	GTA Val
Sequence(3)	ACA Thr	CTA Leu	GTG Val	AGA Arg	CCA Pro	GCT Ala	GTA Val
Codon	49	56	57	59	61	64	71
Sequence(1)	CCT pro	CCA His	ACC Thr	AAT Asn	TCA Ser	TCC Ser	GGT Gly
Sequence(2)	CTT Leu	C**A**A Glu	ATC Ile	A**G**T Ser	**TAA** stop	T**G**T Cys	GGT Gly
Sequence(3)	CTT Leu	CCA His	ACC Thr	AAT Asn	TCA Ser	TCT Ser	GGC Gly
Codon	82	83	85	94	99	100	110
Sequence(1)	ATA Ile	TTC Phe	TTC Phe	TTA Leu	GAT Asp	TAT Tyr	ATT Ile
Sequence(2)	AT**C** Ile	T**G**C Cys	T**G**C Cys	TTG Leu	GAC Asp	TAT tyr	CTT Leu
Sequence(3)	ATA Ile	TTC Phe	TTC Phe	TTG Leu	GAC Asp	TAC Tyr	CTT Leu
Codon	113	114	115	117	118	122	126
Sequence(1)	TCA Ser	ACA Thr	ACA Thr	AGT Ser	ACG Thr	AAA Lys	ACT Thr
Sequence(2)	TCA ser	TCA Ser	ACA Thr	AGC Ser	AC**C** Thr	AAC Lys	ACT Thr
Sequence(3)	ACA Thr	TCA Ser	ACT Thr	AGC Ser	ACG Thr	AAG Lys	ATT Ile
Codon	130	131	132	136	143	144	146
Sequence(1)	GGC Gly	AAC Asn	AAG Lys	TCA Ser	ACG Thr	GAT Asp	AAT Asn
Sequence(2)	GGA gly	ACC Thr	ATG Met	TCA Ser	ACG Thr	GAC Asp	AAC Asn
Sequence(3)	GGA Gly	ACC Thr	ATG Met	TCT Ser	TCG Ser	GAC Asp	AAC Asn
Codon	148	154	155	160	161	171	190
Sequence(1)	ACC Thr	TCG Ser	TCC Ser	AAA Lys	TAC Thr	TCT Ser	GTT Val
Sequence(2)	ACC Thr	TCA Ser	TCT Ser	AAA Lys	TAC Thr	TCT Ser	GT**C** Val
Sequence(3)	ACT Thr	TCA Ser	TCT Ser	AGA Agr	TTC Phe	TCC ser	GTT val
Codon	194	200	207	209	213	214	215
Sequence(1)	GCT Ala	TAT Thr	AGC Ser	GTG Val	ATA Ile	CCG Pro	CTC Leu
Sequence(2)	GTT Val	T**T**T Phe	AAC Asn	TTG Leu	ATG met	CCG Pro	CTG Leu
Sequence(3)	GTT val	TAT Thr	AAC Asn	TTG Leu	TTA Leu	CCT Pro	CTA Leu
Codon	222						
Sequence(1)	CTC Leu						
Sequence(2)	CTC Leu						
Sequence(3)	CTT Leu						

Sequence (1): adw subtype reported by Ono *et al*. Sequence (2): The patient's sequence. Sequence (3): The Chinese consensus sequence of adw subtype.

DISCUSSION

Hepatitis B virus replicates via an RNA intermediate, using a reverse transcriptase that appears to lack a proofreading function. Therefore, HBV exhibits a mutation rate more than 10-fold higher than other DNA virus[10-12]. Mutations in all 4 genes have been described. Surface gene mutation were initially noted as vaccine escape mutants, detected in 2-3 % of children in HBV endemic regions receiving HBV immunoprophylaxis at birth, and also observed in liver transplanted HBV carriers who received hepatitis B immunoglobulin to prevent re-infection of the graft[13-17]. Similar mutations could also arise in the natural course of HBV infection. The prevalence and clinical significance of naturally occurring mutations in full-length surface and overlapping polymerase genes of hepatitis B virus were analyzed in 42 patients with chronic hepatitis, mutations were observed in 10 patients (24 %) in the "a" determinant region[18,19].

The surface gene of HBV contains a dominant neutralizing epitope termed "a" determinant located between aa 121-149 of HBsAg, The production of antibodies to the "a" determinant after vaccination usually protects against HBV infection. The surface protein variants noted in most studies were clustered within the "a" determinant, especially the substitution of glycine for arginine at aa 145, which makes this epitope unlikely to bind to antibodies generated to wild-type HBsAg. However, other kinds of mutation outside of the "a" determinant have been described in recent years, including deleting and inserting mutations in the surface gene of HBV[20-22].

In contrast to the mutations mentioned above, an uncommon point mutation at nucleotide 336 (C to A) of HBsAg-gene occurred in our isolates, which resulted in a novel stop codon at aa 61. This finding could not be a laboratory error, because all sequences of four clones were the same. Because of this new stop codon introduction, only truncated molecules of surface antigen could be expressed, which contained only 60 amino acid residues and was lack of the "a" determinant. This unique mutation could well explain the patient' s unusual serologic pattern: HBsAg-negative, but HBeAg-positive, anti-HBs-positive and HBV DNA-positive. The novel HBsAg gene stop codon caused by a point substitution mutation upstream of the "a" determinant has not been described up to now. Our finding is very similar to the deletion mutation of HBsAg gene described by Weinberger *et al*. The deletion mutation located at the nucleotide 31 of the HBsAg gene, which led to a frame-shift and introduced a stop-codon after 21amino acids of HBsAg[23].

To initiate infection, a virus must attach to a host cell receptor via one of its surface proteins. Hepatitis B virus has three related surface proteins, small S, middle S, and large S. It is not clear which of these three proteins serves as the HBV attachment protein. It has been thought that the pre-S region or S region determines viral binding[24,25]. However, due to the lack of a susceptible cell line that could be used to test specific blocking reagents, which protein is involved in the initial stage of HBV infection is difficult to determine. The HBV DNA level in our patient was as high as 10^9/ml in serum, indicating that isolates that bear such truncated molecules on their surface (the mutant HBsAg was only equal to one-fourth of HBsAg from wild type) can well finish their life cycle including viral binding and entry. Our finding presented here provides the evidence that sequences in the pre-S region determine viral binding.

However, our finding raises a theoretical question: Cells infected with hepatitis B virus produce both virions and 20nm subviral (surface antigen) particles. Although hepatitis B virus encodes three envelope proteins, all of the information required to produce 20 nm HBsAg particles resides within the S protein[26]. The nucleotide sequence of the HBsAg gene predicts the existence of three hydrophobic domains, located at residues 4 to 28 (signal I), 80 to 100 (signal II) and 164 to 221 (signal III). Studies on certain artificial deletion mutants suggested that deletion of signal II completely destabilized the chain, and deletion of the signal III resulted in a nonsecreted chain[27]. How such a drastically shortened HBsAg which is lack of signal IIand signal III, can be able to form morphologically correct viral and subviral particles? Because when co-expressed with wild type S protein, the mutant polypeptide can be incorporated into particles and secreted, therefore, it is assumed that the presence of a minor population of intact genomes helps in replication and formation of intact virions. All virus isolates consist of a mixture of viral strains. Multiple variants have been found in a single host. Advances in molecular biology technique have revealed significant diversities in sequence of of HBV isolates. Sequencing results suggest that there were HBV quasispecies groups in chronically infected patients[28-31]. Actually, electron microscopy of serum samples containing mutated DNA from the patient reported by Weiinberger *et al*. revealed typical subviral particles with an average diameter of 17-20 nm, but did not reveal a single filamentous particle[23]. Our results show that lack of detection of HBsAg in the presence of high viral levels of replication may be caused by the existence of viral genomes harboring point mutation which results in stop codon upstream of the "a" determinant of HBsAg gene.

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