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• VIRAL HEPATITIS •

A novel hepatitis B virus mutant with A-to-G at nt551 in the surface antigen gene

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Abstract

AIM: Hepatitis B surface antigen (HBsAg) mutant of hepatitis B virus (HBV) is one of the important factors that result in immune escape and cause failure of immunization. In this study we reported and characterized a novel HBV mutant with A-to-G at nt551 and intended to provide theoretical data for prevention of HBV infection in China.

METHODS: A methodology comprising polymerase chain reaction (PCR) amplifying, M13 bacteriophage cloning and nucleotide sequencing was used to analyze the sera of the pediatric patient who was hepatitis B (HB) immune failure. Expression plasmids containing the mutant S gene and a wild-type (*adr*) S gene were constructed respectively and the recombinant HBsAg were expressed in COS-7 cells under the regulation of SV40 early promoter. The recombinant proteins were investigated for their immunological reactivity with different monoclonal antibodies (mAb) against 'a' determinant and vaccine-raised human neutralizing antibodies.

RESULTS: It was found that there was a new point mutation at nt551 of the HBV (*adr*) genome from A to G, leading to a substitution of methionine (Met) to valine (Val) at position 133 in the 'a' determinant of HBsAg. Compared to the wild-type HBsAg, the binding activity of the muant HBsAg to mAbs (A6, A11 and S17) and to vaccine-raised human anti-hepatitis B surface antibody (anti-HBs) decreased significantly.

CONCLUSION: According to the facts that the patient has been immunized with HB vaccine and that the serum is anti-HBs positive and HBsAg negative, and based on the nucleotide sequence analysis of the mutant HBV S gene and its alteration of antigenicity, the HBV is considered to be a new vaccine-induced immune escape mutant different from the known ones.

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INTRODUCTION

From late 1980's, there has been increasing number of reports on hepatitis B (HB) patients with atypical HBV serological markers. Analysis of HBV in those patients demonstrated mutants[1-6]. Mutations could be found within the C gene, S gene, P gene and X gene[7,8]. HBsAg encoded by the S gene carries the common determinant 'a', as well as 'd' or 'y' and 'w' or 'r' subtype determinant, and is classified into four major subtypes, i.e. *adw*, *adr*, *ayw*, *ayr*. Two amino acid (aa) residues encoded by S gene at codon positions 122 and 160 have been postulated to determine the different antigenic subtypes[9]. The most important S gene mutations are those affecting the antigenicity of HBsAg 'a' determinant (aa 124 to 147) to which the major immune-target of neutralizing polyclonal antibodies reacted. In this way, the HBsAg mutants can escape detection by current methods and influence the effect of HBV vaccines.

There have been several reports on HBV S gene mutants affecting amino acid positions 126, 129, 131, 141, 144 and 145 of HBsAg[10-12]. In this report, we described a rare variant of HBV isolated from a pediatric patient whose serum was negative for HBsAg and positive for anti-HBs. Sequence analysis revealed the substitution at position 133 (Met to Val) in the 'a' determinant within S gene. Using a panel of three mAbs (A6, A11 and S17) against 'a' determinant and vaccine-raised anti-HBs, the recombinant mutant HBsAg showed less binding activity than the wild-type HBsAg. Taken together, the data we presented clearly demonstrated that the substitution results in antigenic alteration and may allow the mutant virus to escape the detection by standard HBsAg assays.

MATERIALS AND METHODS

Patient

The patient, male, 4 years old, was born to a HBV carrier mother. He had been immunized with HBV vaccine on a conventional 0-1-6 schedule, i.e., 3 injections of HBV vaccine were given at 0, 1 and 6 month (s) old respectively. The virus markers of his serum were anti-HBs positive, HBsAg negative and HBeAg positive, as well as positive for HBV DNA by PCR and high level of alanine transaminase (ALT) 200 IU· L^{-1}.

Extraction and amplification of HBs DNA

The viral DNA was extracted from the serum sample using the standard method. Briefly, 100 μl serum was treated with proteinase K, phenol and chloroform, and then DNA was precipitated with ethanol. The resulted DNA was resuspended in 20 μl didistilled water for later use. A nested PCR method[13,14] was used to amplify HBV S gene fragment. The external primers were 5'-ACATACTCTGTGGAAGGC-3' (nt2 756 to nt2 773, forward) and 5'-TATCCCATGAAGTTAAGG-3' (nt884 to nt867, reverse). The internal primers were 5'-CGGGATCCATATTCTTGGGAACAAG-3' (nt2 826 to nt2 844, forward, underlined is a BamHI site) and 5'-CACTGCAGGGTTTAAATGTATACCCA-3' (nt839 to nt821, reverse, underlined is a PstI site). The PCR was carried out for 30 cycles, each cycle including 94 ℃ denaturation for 1 minute, 50 ℃ annealing for 1 minute and 72 ℃ chain elongation for 2 minutes.

Nucleotide and amino acid sequence analysis

The PCR-amplified HBV S gene fragments (about 1.2 kb) were

cleaned with a QIAquick spin column (Qiagen). The DNA was digested with both BamHI and PstI, and then ligated by T4 DNA ligase with M13mp18 RF DNA cut with the same restriction enzymes. The ligated DNA was used in the transformation of *E.coli* JM105 cells and the recombinant phages were recognized by loss of β-galactosidase activity in the culture plate containing X-gal and IPTG. The single-stranded recombinant DNA was prepared according to the standard protocol[15]. The S gene sequence was determined on an ABI PRISM™ 377XL sequencer (PE Applied Biosystems, USA) and sequence analysis was performed using Release 6.70 of the PCGENE package (IntelliGenetics Co.). The HBsAg ‘a’ determinant coding regions of 48 defined HBV genotypes downloaded from National Center for Biotechnology Information (NCBI) were analyzed comparatively.

Construction of expression plasmids and transient protein expression

The process followed the reference[16-18]. Briefly, the construction of recombinant wild-type and mutant HBsAg expression plasmids started with a plasmid pS300 which was constructed from pSP65 carrying the SV40 early promoter sequence, the preS/S gene and the poly (A) signal sequence of HBV. For construction of the major HBsAg expression plasmid, the preS1 and preS2 regions were deleted by restriction enzyme digestion. COS-7 cells were cultured in DMEM/HG medium supplemented with 5 % fetal calf serum, two million units/ml of ampicillin and one million units/ml of kanamycin under the condition of 5 % CO_2, 37 ℃. The cells in 60-mm dishes were transfected with 10 μg of the expression plasmids using calcium phosphate precipitation method. 72 hours later, the transfected cells were collected into 1.5-ml Eppendorf tubes, washed with 10 mM PBS (pH7.4) and resuspended with 500 μl (for each dish) of 10 mM PBS (pH7.4). The cells were disrupted by freezing and thawing for 5 times, and then centrifuged at 8 000 rpm for 5 minutes. The supernatants contained the recombinant HBsAg proteins.

Recombinant HBsAg antigen immunoassay and epitope analysis

A solid phase radioimmunoassay (RIA) method was applied for detecting the binding activity of the expressed HBsAgs with mAbs. In brief, polystyrene beads were coated with mAbs against different epitopes of HBsAg respectively and then incubated with the expressed HBsAgs overnight at room temperature. The beads were washed thoroughly and incubated with ^{125}I-labeled anti-HBs (Beijing Atomic Energy Institute). Bound antibodies were detected as counts per minute (cpm) in LKB 1 272 gamma counter. To evaluate the reactivity of vaccine-raised human anti-HBs to recombinant wild-type HBsAg and mutant HBsAg, an enzyme-linked immunosorbent assay (ELISA) was established as follows[19-21]: plates were coated with size filtrated and volume concentrated antigen from expression cell culture; Plasma from five HBV vaccinated individuals were pooled and serially diluted human anti-HBs was incubated in the plates at 37 ℃ for 90 minutes. Bound human IgG was detected by a second incubation with horseradish peroxidase (HRP) conjugated murine monoclonal anti-human IgG; The reactivity was determined by enzyme catalyzed OPD colour development and the results were expressed as absorbance units at 490 nm.

RESULTS

HBs variant nucleotide and amino acid sequence analysis

The HBs DNA sequence of the novel mutant was shown in Figure 1. The adenosine (A) at nt519 and the guanosine (G) at nt633 indicated that the mutant belonged to *adr* subtype[22]. Sequence comparison between the mutant and 48 defined HBs genotypes revealed a new nucleotide mutation at nt551 from A to G, leading to the amino acid alteration at position 133 from Met to Val in the ‘a’ determinant. The mutant was first reported and its sequence data have been deposited with GenBank DNA databases under the accession number AF052576. The comparative analysis of HBsAg ‘a’ determinant coding regions of different HBV genotypes was shown in Figure 2.

DNA SEQUENCE 1203 BP; 254 A; 354 C; 265 G; 330 T; 0 OTHER

ATGGGAGGTT GGTCTTCCAA ACCTCGACAA GGCATGGGGA CGAATCTTTC TGTTCCCAAT 2907
CCTCTGGGAT TCTTTCCCGA TCACCAGTTG GACCCTGCGT TCGGAGCCAA CTCAAACAAT 2967
CCAGATTGGG ACTTCAACCC CAACAAGGAT CACTGGCCAG AGGCAAATCA GGTAGGAGCG 3027
GGAGCATTCG GGCCAGGGTT CACCCCACCA CACGGCGGTC TTTTGGGGTG GAGCCCTCAG 3087
GCTCAGGGCA TTTTGACAAC AGTGCCAGTA GCACCTCCTC CTGCCTCCAC CAATCGGCAG 3147
TCAGGAAGAC AGCCTACTCC CATCTCTCCA CCTCTAAGAG ACAGTCATCC TCAGGCCATG 3207
CAGTGGAACTC CACAACATT CCACCAAGCT CTGCCAGACC CCAGAGTGAG GGGCCTATAC 052
TTTCCTGCTG GTGGCTCCAG TTCCGGAACA GTAAACCCTG TTCCGACTAC TGCCTCACCC 112
ATATCGTCAA TCTTCTCGAG GACTGGGGAC CCTGCACCGA ACATGGAGAG CACAACATCA 172
GGATTCCTAG GACCCCTGCT CGTGTTACAG GCGGGGTTTT TCTTGTTGAC AAGAATCCTC 232
ACAATACCAC AGAGTCTAGA CTCGTGGTGG ACTTCTCTCA ATTTTCTAGG GGGAGCACCC 292
ACGTGTCCTG GCCAAAATTC GCAGTCCCCA ACCTCCAATC ACTCACCAAC CTCTTGTCCT 352
CCAATTTGTC CTGGTTATCG TTGGATGTGT CTGCGGCGTT TTATCATATT CCTCTTCATC 412
CTGCTGCTAT GCCTCATCTT CTTGTTGGTT CTTCTGGACT ACCAAGGTAT GTTGCCCGTT 472
TGTCCTCTAC TTCCAGGAAC ATCAACTACC AGCACGGGAC CATGCAAGAC CTGCACGATT 532
CCTGCTCAAG GAACCTCT**G**TG TTTCCCTCT TGTTGCTGTA CAAAACCTTC GGACGGAAAC 592
TGCACTTGTA TTCCCATCCC ATCATCCTGG GCTTTCGCAA GATTCCTATG GGAGTGGGCC 652
TCAGTCCGTT TCTCCTGGCT CAGTTTACTA GTGCCATTTG TTCAGTGGTT CGTAGGGCTT 712
TCCCCCACTG TTTGGCTTTC AGTTATATGG ATGATGTGGT ATTGGGGGCC AAGTCTGTAC 772
AACATCTTGA GTCCCTTTTT ACCTCTATTA CCAATTTTCT TTTGTCTTTG GGTATACATT 832
TAA 835

Figure 1 The complete nucleotide sequence of the mutant S gene. The A-to-G mutation site at nt551 of HBV genome is in bold letter. The underlined are the EcoRI-like site, the initiation codon of HBsAg and the amino acid codon (ATG to GTG) respectively. The first C of the EcoRI-like site (GAACTC) is counted as nt1. The GenBank accession number of the sequence is AF052576.

The amino acid position on the 'a' determinant of HBsAg

```
        Gene names      124                             133             137     139
147
        (subtypes)      Cys                             Met             Cys     Cys                     Cys
※HPBA11A  (adr)     TGC ACGATTCCTGCTCAAGGAACCTCT ATG TTTCCCTCT TGT TGC TGT
ACAAAACCTTCGGACGGAAAC TGC
※HPBADRM  (adr)     --- ---------- --- --------- --- --- --- ---------- ---
§HPBCGADR (adr)     --- ---------- --- --------- --- --- --- ---------- ---
#HBVADR   (adr)     --- ---------- --- --------- --- --- --- ---------- ---
§HBVADR4  (adr)     --- ---------- --- --------- --- --- --- ---------- ---
※HPBB4HST1(adr)     --- ---------- --- --------- --- --- --- ---------- ---
§HPBADR1CG(adr)     --- ---------- --- --------- --- --- --- ---------- ---
§HPBADRA  (adr)     --- ---------- --- --------- --- --- --- ---------- ---
§HEHBVAYR (ayr)     --- ---------- --- --------- --- --- --- ---------- ---
※HPBC5HK02(ayw)     --- ---------- --- --------- --- --- --- ---------- ---
※HPBB5HK01(ayw)     --- ---------- --- --------- --- --- --- ---------- ---
※HPBC4HST2(adr)     --- ---------- --- C-------- --- --- --- ---------- ---
※HPBADRC  (adr)     --- ---------- --- --------C --- --- --- ---------- ---
※HPBCG    (adr)     --- ---------- --- --------- --- --G --- ---------- ---
※HPBC6T588(adr)     --- ----------C------ --- --------- --- --- --- ----------T------ ---
§HPBADWZ  (adw)     --- ----C------------------- --- --------A --- --- --- ---------A----------- ---
※HPBADW3  (adw)     --- ----C------------------- --- --------A --- --- --- ---------A----------- ---
#HBVP6CSX (adw)     --- --A-C------------------- --- --------A --- --- --- ---------A----------- ---
※HPBA2HYS2(adw)     --- --A-C------------------- --- --------A --- --- --- ---------A----------- ---
#HBVP4CSX (adw)     --- --A-C------------------- --- --------A --- --- --- ---------A----------- ---
#HBVCGWITY(adw)     --- --AGC------------------- --- --------A --- --- --- ---------A----------- ---
※HPBA3HMS2(adw)     --- --A-C------------------- --- --------A --- --- --- ---------A----T------ ---
※HPBA1HKK2(adw)     --- --A-C------------------- --- --------A --- --- --- ---------A----T------ ---
※HPBADW2  (adw)     --- --A-C------------------- --- --------A --- --- --- ---------A----T------ ---
※HPBADW1  (adw)     --- --A-C------------------- --- --------A --- --- --- ---------AT---T------ ---
#HVHEPB   (ayw)     --- ----C------------C-A---- --- --------A --- --- --- ---------A----T-----T ---
§HPBADWZCG(ayw)     --- ----C------------C-A---- --- --------A --- --- --- ---------A----T-----T ---
#HBVP3CSX (ayw)     --- ----C-A----------------- --- -A------C --- --- --- --C----------------T ---
#HBVP6PCXX(ayw)     --- ----C-A----------------- --- -A------C --- --- --- --C----------------T ---
#HBVP2CSX (ayw)     --- ----C------------------- --- -A------C --- --- --- --C----------------T ---
#HBVAYWGEN(ayw)     --- ----C------------------- --- -A------C --- --- --- --C----------------T ---
#HBVP5PCXX(ayw)     --- ----C------------------- --- -A------C --- --- --- --C----------------T ---
#HBVP4PCXX(adr)     --- ----C------------------- --- -A------C --- --- --- --C----------------T ---
§HBU46935 (adr)     --- -----CA----------------- T-- -A------A --- --T --- --------------------T ---
§HPBMUT   (ayw)     --- -T--C------------------- --- -A------C --- --- --- --------------------T ---
§XXHEPAV  (ayw)     --- ----C------------------- --- -A------C --- --- --- --C----------------T ---
§HBVAYWE  (ayw)     --- ----C------------------- --- -A------C --- --- --- --C----------------T ---
§HPBAYW   (ayw)     --- -T--C-A----------------- --- -A------C --- --- --- --C----------------T ---
§HBVAYWC  (ayw)     --- ----C------------------- --- -A------C --- --- --- --C----------------T ---
#HBVAYWMCG(ayw)     --- ----C------------C-A---- --- -A------C --- --- --- --C----------------T ---
§HPBADW2  (adw)     --- ----C------------------- --- -A------C --- --- --- --C----------------T ---
§HBVDNA   (adw)     --- ----C------------------- --- -A------C --- --- --- --C----------------T ---
§HPBVCG   (adw)     --- ----C------------------- T-- A-------A --- --- --- --------------------T ---
#HBVADW   (adw)     --- ----C------------C-A---- -A- --------A --- --- --- ---------A----T-----T ---
§HBVADW2  (adw)     --- ----C------------C-A---- --- ---------A --- --- --- ---------A----T-----T ---
#HBVXCPS  (adw)     --- ----C------------C-A---- --- ---------A --- --- --- ---------A----T-----T ---
§HBVAYWCI (adw)     --- ----C------------C-A---- --- ---------A --- --- --- ------A-------T-----T ---
§HBVADW4A (adw)     --- --A-C---T---A----------- --- --------C --- --- --- TCC-----C------------ ---
```

Figure 2 Comparative analysis of the HBsAg 'a' determinant coding regions of different HBV genomes. HBsAg 'a' determinant is a conformational epitope which has a special two-loop construction kept by the disulfide bonds between Cys124 and Cys137, Cys139 and Cys147, respectively. 48 HBV genome sequences were downloaded from National Center for Biotechnology Information (NCBI), USA (*http://www.ncbi.nlm.nih.gov*). "Gene names" are their names in the original gene databases. Here the ※ labeled ones are from DDBJ, the # labeled are from EMBL, and the § labeled are from GenBank.

Recombinant HBsAg transient expression in COS-7 cell

The recombinant wild-type HBsAg and mutant HBsAg were expressed under the regulation of SV40 early promoter in COS-7 cells in a transient fashion. Only secreted HBsAg in culture supernatant was examined for expression. There was no obvious expression yield difference between the wild-type and mutant recombinant HBsAg based on protein silver staining on SDS-PAGE.

Immunoreactivity analysis

To compare the reactivity of recombinant wild-type HBsAg and mutant HBsAg to antibodies, the quantity of the antigens must be the same. Because both the wild-type and mutant HBV were known to be adr subtypes, an anti-'d' determinant mAb, S4 (Shanghai Institute of Biological Products), was used for the standardization of the HBsAgs. After series of dilution and detection, both HBsAg preparations were adjusted to a protein concentration of 2.1 ng/ml. Three different anti-'a' determinant mAbs, A6, A11 and S17, were selected to characterize the binding activity of the expressed HBsAgs. In the condition of the same concentration of HBsAg proteins determined by anti-'d' mAb, the reactivity of the mutant HBsAg to three anti-'a' mAbs were unexceptionally weaker than that of the wild-type HBsAg, as shown in Table 1. The recombinant wild-type and mutant HBsAgs were also tested for their relative reactivity to vaccine-raised human anti-HBs. Clearly, pooled human HBV vaccinated antisera decreased its binding strength to the mutant HBsAg by about 5-fold by ELISA assay, as shown in Table 2. As control, the cell culture supernatant without recombinant plasmid transfection did not bind to human antisera in this assay. The results demonstrated that the Met-to-Val substitution at amino acid position 133 of HBsAg led to the antigenic alteration.

Table 1 Detection of immunoreactivity of the expressed HBsAgs to anti-'a' monoclonal antibodies by radioimmunoassay[a]

Anti-'a' monoclonal antibodies	Wild-type HBs (133Met)	Mutant HBs (133Val)
A6	1118 (5.82)	774 (3.93)
A11	932 (4.80)	744 (3.76)
S17	945 (4.87)	630 (3.14)

[a]Counter per minute (cpm), the number in the parentheses is P/N value. According to the solid RIA kit producer's recommendation, P/N=(sample cpm-background)/(negative control cpm-background). Untransfected cells were used as negative control, average cpm was 240. Blank polystyrene beads were used as background, average cpm was 58. P/N$\geqslant$ 2.10 is considered to be positive reactivity. The more the P/N value, the stronger the reactivity.

Table 2 Immunoreactivity of vaccine-raised human anti-HBs to ricombinant wild-type HBsAg and to recombinant mutant HBsAg in an ELISA assay

Plate coated with	Wild-type rHBsAg				Mutant rHBsAg				Control
Vaccine-raised anti-HBs	1:1	1:2	1:4	1:8	1:1	1:2	1:4	1:8	1:1
Absorbance at 490 nm	2.45	1.32	0.71	0.28	0.53	0.28	0.15	0.06	0.04

DISCUSSION

The hepatitis B virus has three envelope proteins, and the major envelope protein is S protein, consisting of 226 amino acids. A hydrophilic region in S protein (aa 124 to 147) is designated as 'a' determinant, an antigenic determinant common to all HBV subtypes. 'a' determinant is a conformational epitope which is made up of a special two-loop structure kept by the disulfide bonds between Cys124 and Cys137, Cys139 and Cys147, respectively. This structure projects out from the surface of the HBV particle[23]. The HBV DNA template is transcribed by cellular RNA polymerase to pregenomic RNA, which in turn is reverse transcribed to DNA by virus polymerase. This unique way of HBV replication means a significant tendency to mutation[8,19]. On the other hand, the pressure of immunoprophylaxis with HB immunoglobulin and/or vaccines is another important cause to result in escape mutants[23-28]. Up to date, in the reports about HBV vaccine-induced escape mutants, the most popular one is that with glycine to arginine at aa145 of HBsAg. The mutation decreased the binding activity of HBsAg to mAbs[23,29]. There was another vaccine-induced escape mutant of HBV from an immunized child with anti-HBs positive. The aspartic acid at aa144 was substituted by an alanine in HBsAg, and anti-'a' monoclonal antibody assay showed the mutant HBsAg had weak reactivity[12]. We also reported a different mutant of HBV with isoleucine at aa126 replaced by serine[30]. It seemed that the Ser126 mutant was not an antibody-induced escape mutant since anti-HBs was not detected in the patient. Besides, the mutations, situated closely adjacent to the 'a' determinant, could also change the entire immunodominant region structure and therefore weaken the antigenicity even though no mutations were found within this '124-147' region[31]. Hence, the classical definition of the 'a' immunodominant region may need to be extended to require adjacent amino acids to support its comformation[32-34].

In this report, we characterize a novel HBV mutant with A-to-G at nt551. The substitution of Met to Val at position 133 in the 'a' determinant of HBsAg results in the decrease of reactivity of the recombinant HBsAg to anti-'a' mAbs and vaccine-raised human anti-HBs. Since the major B-cell antigenic epitope resides in the group specific 'a' determinant region, which is reported to be conformational[35], the data we presented clearly demonstrated that the mutation affects the conformation of the 'a' determinant and alters the antigenicity of HBsAg, leading to HBsAg escape from the detection by standard HBsAg assays. Our observations further indicate that the mutation in the HBV surface gene may lead to a considerable decrease of properly folded surface antigens which may render the virus particle less immunogenic in producing an effective neutralizing anti-HBs to clear the virus. According to the fact that the patient has been immunized with HBV vaccine and that the serum is anti-HBs positive and HBsAg negative, the HBV variant we report here is considered to be a new vaccine-induced immune escape mutant.

This antigenically divergent HBV mutant is important for both clinical and diagnostic reasons[36,37]. Therefore, further studies using expressed mutant HBs proteins and accumulation of additional cases will be required for elucidation of the mechanism of the loss of antigenicity.

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