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

Hua-Biao Chen, De-Xing Fang, Fa-Qing Li, Hui-Ying Jing, Wei-Guo Tan, Su-Qin Li

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 mutant 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 HBV 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.

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, 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 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 distilled water for later use. A nested PCR method[13,14] was used to amplify HBV S gene fragment. The external primers were 5’-ACATACTCTGTTGGGAAGGC-3’ (nt2 756 to nt2 773, forward) and 5’-TATCCCATGAAATTAAAGG-3’ (nt884 to nt867, reverse). The internal primers were 5’-CGGATCATATTCTGGGAAACAG-3’ (nt2 826 to nt824, forward, underlined is a BamHI site) and 5’-CAGTCGAAAGTTTAATGATATC-3’ (nt839 to nt821, reverse, underlined is a PstI site). The PCR was carried out for 30 cycles, each cycle including 94 °C denaturation for 1 minute, 50 °C annealing for 1 minute and 72 °C chain elongation for 2 minutes.

Nucleotide and amino acid sequence analysis

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

http://www.wjgnet.com/1007-9327/9/304.htm

Chen HB, Fang DX, Li FQ, Jing HY, Tan WG, Li SQ. A novel hepatitis B virus mutant with A-to-G at nt551 in the surface antigen gene. World J Gastroenterol 2003; 9(2): 304-308

Copyright © 2003 by The WJG Press ISSN 1007-9327

World Journal of Gastroenterology
cleaned with a QIAquick spin column (Qiagen). The DNA was digested with both BamHI and PstI, and then ligated with 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 ‘α’ 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 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 restriction enzyme digestion. The preS1 and preS2 regions were deleted by sequence, the HBsAg ‘α’ determinant coding regions of 48 defined HBV genotypes 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 nt551 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 ‘α’ 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 ‘α’ determinant coding regions of different HBV genotypes was shown in Figure 2.
## The amino acid position on the ‘a’ determinant of HBsAg

| Gene names | 124 | 133 | 137 | 139 |
|------------|-----|-----|-----|-----|
| (subtypes) | Cys | Met | Cys | Cys |
| HPB11A (adr) | ATT | ATG | TGG | TGT |

| | ACAAACCTTCGGACGGAAAC TGC |
|----------------|-----------------------|

| HPBA11A (adr) | ATT | ATG | TGG | TGT |
|----------------|-----------------------|

**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 *italic* 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 ad subtype, 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 unexpectedly 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, as shown in Table 2. The results demonstrated that the Met-to-Val substitution did not bind to human antisera in this assay.

**Table 1** Detection of immunoreactivity of the expressed HBsAgs to anti-‘a’ mAbs monoclonal antibodies by radioimmunossay

| 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)          |

“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 2.10 is considered to be positive activity. The more the P/N value, the stronger the reactivity.

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

| Plate coated with       | Wild-type HBsAg | Mutant HBsAg | 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[21]. 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[18,19]. On the other hand, the pressure of immunoprophylaxis with HB immunoglobulin and/or vaccines is another important cause to result in escape mutants[25-28]. 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[25,29]. 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[30]. We also reported a different mutant of HBV with isoleucine at aa126 replaced by serine[19]. 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[15]. Hence, the classical definition of the ‘a’ immunodominant region may need to be extended to require adjacent amino acids to support its conformation[27,34].

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 HBsAg proteins and accumulation of additional cases will be required for elucidation of the mechanism of the loss of antigenicity.

**REFERENCES**

1. Cabreiro M, Bartolom J, Caramelo C, Barril G, Carreno V. Molecular analysis of hepatitis B virus DNA in serum and peripheral blood mononuclear cells from hepatitis B surface antigen-negative cases. Hepatology 2000; 32: 116-123
2. Grethe S, Monazahian M, Bohme I, Thonssen R. Characterization of unusual escape variants of hepatitis B virus isolated from a hepatitis B surface antigen-negative subject. J Virol 1998; 72: 7692-7696
3. Bruce SA, Murray K. Mutations of some critical amino acid residues in the hepatitis B virus surface antigen. J Med Virol 1995; 46: 157-161
4. Weinberger KM, Zoulek G, Bauer T, Bohm S, Jilg W. A novel deletion mutant of hepatitis B virus surface antigen. J Med Virol 1999; 58: 105-110
689

Shinji T, Koide N, Hanafusa T, Hada H, Oka T, Takayama N, Shirahata H, Nakamura M, Uijke K, Yamoto Y, Tsuji T. Point mutations in the S and pre-S2 genes observed in two hepatitis B virus carriers positive for antibody to hepatitis B surface antigen. Hepatogastroenterology 1998; 45: 500-502.

Schories M, Peters T, Rasenack J. Isolation, characterization, and biological significance of hepatitis B virus mutants from serum of a patient with immunologically negative HBV infection. J Hepatol 2000; 33: 799-811.

Brunetto MR, Rodriguez UA, Bonino F. Hepatitis B virus mutants. Intervirology 1999; 42: 69-80.

Carman WF, Thomas HC. Genetic variation in hepatitis B virus. Gastroenterology 1992; 102: 711-719.

Stirk HJ, Thornton JM, Howard CR. A topological model for hepatitis B surface antigen. Intervirology 1992; 33: 148-158.

Seddigh-Tonekaboni S, Waters JA, Jeffers S, Gehrie R, Ofenloch B, Horsch A, Hess G, Thomas HC, Karayiannis P. Effect of variation in the common 'a' determinant on the antigenicity of hepatitis B surface antigen. J Med Virol 2000; 60: 113-121.

Rozynskiy L, Harrison TJ, Fang ZL, Ling R, Lochman I, Orsagova I, Pliskova L. Unusual hepatitis B surface antigen variant in a child immunized against hepatitis B. J Med Virol 2000; 61: 11-14.

Ni F, Fang DX, Gan R, Li Z, Duan S, Xu Z. A new immune escape mutant of hepatitis B virus with an Asp to Ala substitution in aa144 of the envelope major protein. Res Virol 1995; 146: 397-407.

Zhang SL, Han XB, Yue YF. Relationship between HBV viremia level of pregnant women and intrauterine infection: neatened PCR for detection of HBV DNA. World J Gastroenterol 1998; 4: 61-63.

Dong J, Cheng J, Wang Q, Wang G, Shi S, Liu Y, Xia X, Si C. Cloning and sequence analysis of truncated S gene from circulation of patients with chronic hepatitis B virus infection. Zhonghua Ganzhangbing Za zhi 2001; 39: 163-165.

Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning: A Laboratory Manual. 2nd ed. New York: Cold Spring Harbor Laboratory Press 1990: 203-233.

Fang DX, Li FQ, Tan WG, Chen HB, Jing HY, Li SQ, Lin HJ, Zhou ZX. Transient expression and antigenic characterization of HBsAg of HBV nt551 A to G mutant. World J Gastroenterol 1999; 5: 73-74.

Gu B, Ren H. Expression of HBsAg by using various eukaryotic expression vectors. Zhonghua Ganzhangbing Za zhi 1999; 7: 98-100.

Jeantet D, Chemin I, M andrand B, Zoulif M, Trep o C, Kay A. Characterization of two hepatitis B virus populations isolated from a hepatitis B surface antigen-negative patient. H epatology 2002; 35: 1215-1224.

Hasegawa K, Huang J, Rogers SE, Blum HE, Liang T. Enhanced replicability of a hepatitis B virus mutant associated with an epidemic of fulminant hepatitis. J Virol 1994; 68: 1651-1659.

Gu B, Ren H, Zhang D. Expression of recombinant inserting mutants of HBsAg in vitro and its antigenic analysis. Zhonghua Yi xue Za zhi 1999; 79: 139-142.

Cooremans MP, van Roosma MF, te Morsche R, Sunnen CM, de Ven EM, Jansen JB, Tytgat GN, de Wit PL, Paulij WP. Characterization of the reactivity pattern of murine monoclonal antibodies against wild-type hepatitis B surface antigen to G145R and other naturally occurring ‘a’ loop escape mutations. H epatology 1999; 30: 1287-1292.

Okamoto H, Imai M, Tsuda F, Tanaka T, Miyakama Y, Mayumi M. Point mutation in the S gene of hepatitis B virus for a dIy or w/ r subtypic change in two blood donors carrying a surface antigen of compound subtype ady or adw. J Virol 1987; 61: 3030-3034.

Carman WF, Zanetti AR, Karayiannis P, Waters J, Manzillo G, Tanzi E et al. Vaccine-induced escape mutant of hepatitis B virus. Lancet 1990; 336: 325-329.

Chiou HL, Lee TS, Kuo J, Mao YC, Ho MS. Altered antigenicity of ‘a’ determinant variants of hepatitis B virus. J Gen Virol 1997; 78: 2639-2645.

Karthigesu VD, Allison LM, Ferguson M, Howard CR. A hepatitis B virus variant found in the sera of immunized children induces a conformational change in the HbaAg ‘a’ determinant. J Med Virol 1999; 58: 346-352.

He JW, Lu Q, Zhu QR, Duan SC, Wen YM. Mutations in the ‘a’ determinant of hepatitis B surface antigen among Chinese infants receiving active postexposure hepatitis B immunization. Vaccine 1998; 16: 170-173.

Cooremans MP, Leroux-Roels G, Paulij WP. Vaccine- and hepatitis B immune globulin-induced escape mutations of hepatitis B virus surface antigen. J Biomed Sci 2001; 8: 237-247.

Chen WN, Oon CJ, Koh S. Horizontal transmission of a hepatitis B virus surface antigen mutant. J Clin Microbiol 2000; 38: 939-939.

Heijlink RA, van Bergen P, van Roosma MF, Sunnen CM, Paulij WP, Schalm SW, Osterhaus AD. An anti-HBs after hepatitis B immunization with plasma-derived and recombinant DNA-derived vaccine: binding to mutant HBsAg. Vaccine 2001; 19: 3671-3680.

Fang DX, Gan RB, Li ZP, Duan SC. A hepatitis B virus variant with an IletoSer mutation at aa126 of HBsAg. Sheng Wu Hu Xueyue Shengwu Wu Li Xuebao 1996; 28: 429-433.

Kfoury Baz EM, Zhong J, Mazuruk K, Van Le A, Peterson DL. Characterization of a novel hepatitis B virus mutant: demonstration of mutation-induced hepatitis B virus surface antigen group specific ‘a’ determinant conformation change and its application in diagnostic assays. Transfusion 2001; 11: 355-362.

Rozynskiy L, Harrison TJ, Fang ZL, Ling R, Lochman I, Orsagova I, Pliskova L. Unusual hepatitis B surface antigen variant in a child immunized against hepatitis B. J Med Virol 2000; 61: 11-14.

Banerjee K, Guptan RC, Bislht R, Sarin SK, Khandekar P. Identification of a novel surface variant of hepatitis B virus in a seronegative chronic liver disease patient. J Med Virol 1999; 63: 103-109.

Ijaz S, Torre F, Tedder RS, Williams R, Naoumov NV. Novel immunossays for the detection of hepatitis B surface escape mutants and its application in liver transplant recipients. J Med Virol 2001; 63: 210-216.

Koyanagi T, Nakamuta M, Sakal H, Sugimoto R, Munehika E, Koto K, Iwamoto H, Kumazawa T, Mukaide M, Nawata H. Analysis of HBsAg negative variant of hepatitis B virus: unique substitutions, Glu129 to Asp and Gly145 to Ala in the S gene. J Virol 2001; 6: 1165-1169.

Rodriguez-Frias F, Brito M, Jardi R, Vargas V, Quer J, Cotrina M, Martel M, Esteban R, Guardia J. Genetic alterations in the S gene of hepatitis B virus in patients with acute hepatitis B, chronic hepatitis B and hepatitis B liver cirrhosis before and after liver transplantation. Liver 1999; 19: 177-182.

Coleman PF, Chen YC, Mushahwar IK. Immunoassay detection of hepatitis B surface antigen mutants. J Med Virol 1999; 59: 19-24.

Edited by Zhang JZ