Incidence of HBV variants with a mutation at nt551 among hepatitis B patients in Nanjing and its neighbourhood

Chun-Ling Ma, De-Xing Fang, Kun Yao, Fa-Qing Li, Hui-Ying Jin, Su-Qin Li, Wei-Guo Tan

Chun-Ling Ma, De-Xing Fang, Fa-Qing Li, Hui-Ying Jin, Su-Qin Li, Wei-Guo Tan, Huadong Research Institute for Medical Biotechnics, Nanjing 210002, Jiangsu Province, China
Kun Yao, Microbiology and Immunology Department, Nanjing Medical University, Nanjing 210029, Jiangsu Province, China
Supported by the Natural Science Foundation of Jiangsu Province, No. BJ2000039
Correspondence to: Chun-Ling Ma, Microbiology and Immunology Department, Nanjing Medical University, Nanjing 210029, Jiangsu Province, China. mchunling@hotmail.com
Telephone: +86-25-84542419 Fax: +86-25-84541183
Received: 2004-03-19 Accepted: 2004-04-21

Abstract

AIM: To investigate the epidemiology of hepatitis B virus (HBV) strains with a mutation at nt551 in surface gene among hepatitis B patients in Nanjing and its neighbourhood.

METHODS: By using mutation-specific polymerase chain reaction (msPCR) established by our laboratory for amplifying HBV DNAs with a mutation at nt551, 117 serum samples taken from hepatitis B patients were detected.

RESULTS: The results showed that 112 samples were positive for nt551A, 4 samples were positive for nt551G. One sample was positive for nt551T. No nt551C of HBV DNA was found. The incidence of HBsAg mutants with G, C, T, A at nt551 among 117 samples was 3.42%, 0%, 0.85%, 95.73%, respectively.

CONCLUSION: In Nanjing and its neighbourhood, hepatitis B patients are mainly infected with wild genotype HBV. The incidence of mutants with a mutation at nt551 in HBV genome is significantly lower than that in wild genotype HBV DNA (P<0.01). The necessity of adding components of HBsAg mutants to HBV vaccine needs further investigation.

© 2005 The WJG Press and Elsevier Inc. All rights reserved.

Key words: Hepatitis B; Hepatitis B virus; nt551; Mutation

INTRODUCTION

Hepatitis B surface antigen (HBsAg) is considered to be one of the best markers for the diagnosis of acute and chronic HBV infection. But in some patients, this antigen cannot be detected by routine serological assays despite the presence of virus. One of the most important explanations for the lack of detectable HBsAg is that mutations which occur within the "a" determinant of HBV S gene can alter expression of HBsAg and lead to changes of antigenicity and immunogenicity of HBsAg accordingly. As a result, these mutants cannot be detected by diagnostic assays. Thus, it is essential to find specific and sensitive methods to detect the new mutants and further investigate their distribution[1,2].

HBV is a hepatotropic DNA virus. In the reverse transcription process of DNA replication, HBV DNA template is transcribed by cellular RNA polymerase to pregenomic RNA, which is reversely transcribed to DNA by viral polymerase, and as a consequence of the unique way of HBV replication it is a significant tendency of mutation[3-5]. Substitution, deletion and frame-shift by insertion or deletion of short sequences have been found in four open reading frames[6-7]. The diversity is also shown in different serological subtypes such as adr, adw, ayr and ayw, which have a common "a" determinant. It is well known that "a" determinant is the common antigenic epitope of all subtypes of HBsAg. A large antigenic area of "a" determinant is called the major hydrophilic region (MHR). Mutations within MHR of HBsAg have been considered to be associated with vaccine failure and chronic infection[1,2,8]. These mutations have been reported repeatedly since Carman et al[9] found the first case of immune escape mutants in 1990. The point mutation reported most commonly in immunized children causes a substitution from Arg to Gly at position 145 of HBsAg[10].

Other reports about substitutions in HBsAg such as 120, 121, 126, 129, 131, 133, 141, 144 are found repeatedly[11,12]. These findings of HBV immune escape mutants have caused attention of scientists all over the world in recent years. Immune escape of HBV mutants is known to be associated with HBV genome itself, but the immune pressure is considered to be one of the most important factors that result in escape mutants[12-14].

The immune escape variants could influence the effect of HBV vaccine, it is argued that the components of mutant HBsAg should be added into the HBV vaccine in the future[15,16,17]. However, in order to achieve this aim, it is necessary to confirm firstly the mutants that are the big problems among hepatitis B patients. At present, it is very important to find new immune escape mutants and further investigate their distribution. Specific and sensitive assays are essential for investigating the distribution of mutants. To detect the mutant HBV DNA, msPCR is a potential method. Our laboratory has discovered a mutation of A-to-G at nt551 of HBV genome, which can result in the alteration of Met to Val at 133aa of HBsAg[18]. To investigate the distribution of mutants with a mutation at nt551 among hepatitis B patients in Nanjing and its neighbourhood in China, we collected 326 serum samples from hepatitis B patients in different hospitals in Nanjing. One hundred and seventeen positive samples for HBV surface gene were detected by using msPCR which is sensitive and specific to the mutation at nt551 in HBV genome.

MATERIALS AND METHODS

Collection of serum samples

Three hundred and twenty-six serum samples from hepatitis B
patients were provided by Nanjing Jinling Hospital, Bayi Hospital and Nanjing Children’s Hospital. Viral markers were tested by using the enzyme immune assay (ELA). ALT levels of all the samples were abnormal. Diagnosis of hepatitis B was made according to the revised standard of hepatitis B diagnosis established at the Tenth National Symposium on Viral Hepatitis in Xi’an in 2000.

**Extraction of HBV genome DNAs**

By using the HBV DNA extraction method, all the 326 serum samples were prepared and stored at -20°C.

**Amplification of HBV S DNAs**

After nested PCR amplification, we achieved 101 HBV S DNAs. Thirty-five samples were positive for HBsAg and anti-HBs, and the other 66 samples were negative for HBsAg and positive for anti-HBs.

Primers for nested PCR to amplify HBV S DNA were designed according to the known HBV genome sequences and the main popular subtypes, adr and adw in China.

Primers for the first-round: P1’: 5’ACATCATCTGTGGAAAGGC 3’, nt2756-nt2773; the upstream primer; P6’: 5’TATCCCATGAAGTTAAGG 3’, nt884-nt867, the downstream primer.

Primers for the second-round: PEC: 5’CGGAATTCACCATATCTTGGAAAGA 3’, nt282-nt284; the upstream primer; PPS: See the above.

Amplification of HBV DNA fragments for control

To achieve HBV DNA fragments with an A at nt551, the wild genotype HBV S DNA as template was amplified by using the primer pair P551A-PPS under the condition of regular PCR. The HBsAg mutant with G at nt551 as template was amplified by using the primer pair P551G-PPS to achieve the HBV DNA fragments with a G at nt551. HBV DNA fragments with C or T at nt551 were achieved by introducing mutation in a PCR. The PCR primer sequences were as follows: P551A: 5’TCTCTGCTCAAGAACCCTCTA 3’, nt532-nt551, upstream primer; P551G: 5’TCTCTGCTCAAGGAAACCTCTG 3’, nt532-nt551, upstream primer; P551C: 5’TCTCTGCTCAAGGAAACCTCTC 3’, nt532-nt551, upstream primer; P551T: 5’TCTCTGCTCAAGGAAACCTCTT 3’, nt532-nt551, upstream primer; P551C-PPS and P551T-PPS. The electrophoresis results of them were as follows.

**Detection by using msPCR**

In order to amplify HBV DNA specifically, 101 serum samples were amplified by primer pair P551A-PPS using msPCR method. The annealing temperature of PCR was at 90°C for 90 s and the concentration of primers was at 0.8 nmol/mL in 25 μL reaction volume. Thirty cycles of amplification were performed, each at 94°C for 30 s, at 70°C for 30 s, and at 72°C for 1 min. Then the negative samples were amplified by primer pairs P551G-PPS, P551C-PPS and P551T-PPS respectively. For primer pairs P551C-PPS and P551T-PPS, the annealing temperature was at 72°C and the concentration of primers was at 0.2 nmol/mL in 25 μL reaction volume, and other conditions were similar to the amplification by P551A-PPS and P551G-PPS.

**Statistical analysis**

χ²-test was used to assess the statistical significance of differences. P<0.01 was considered statistically significant.

**RESULTS**

**HBV DNA fragments for control**

The HBV S DNA with A, G, C or T at nt551 was amplified respectively for control. The amplified DNA fragments were 314 bp in length. This result is shown in Figure 1.

![Figure 1](image)

**Detection by using msPCR**

One hundred and one serum samples positive for HBV S DNA were detected. After gel electrophoresis, 35 samples (positive for HBsAg and anti-HBs) were positive for P551A-PPS amplification, that is to say, each of these 35 samples was a positive control HBV DNA. Lane 1: DNA marker; lane 2: HBV DNA with an A at nt551; lane 3: HBV DNA with G at nt551; lane 4: HBV DNA with C at nt551; lane 5: HBV DNA with T at nt551. B: The msPCR amplification for No.127, No.210 and No.216 by using P551G-PPS. Lane 1: DNA marker; lanes 2-6: No.8 of nt551A (negative control), No.57 (positive control), No.127, No.210 and No.216 were amplified by P551G-PPS respectively. C: The msPCR amplification for No.127, No.210 and No.216 by using P551C-PPS. Lane 1: DNA marker; lanes 2-6: No.8 of nt551A (negative control), No.127, No.210 and No.216 were amplified by P551C-PPS respectively. D: The msPCR amplification for No.127, No.210 and No.216 by using P551T-PPS. Lane 1: DNA marker; lanes 2-6: No.8 of nt551A (negative control), No.2 of nt551T (positive control), No.127, No.210 and No.216 were amplified by P551T-PPS respectively.

**Investigating results**

By using msPCR, 101 serum samples positive for HBV S DNA were detected and the other 16 samples were taken from the result we detected before. The results showed that 112 samples were positive for nt551A, 4 samples were positive for nt551G (one of them was taken from the previous DNA sequence). One sample was positive for nt551T. No nt551C of HBV DNA was...
found. Thus, the incidence of HBsAg nt551G mutant, nt551C mutants, nt551T mutants, and nt551A of wild genotype HBV DNAs was 3.42%, 0%, 0.85% and 95.73% respectively among the 117 samples. The statistical analysis results are summarized in Tables I-3.

Table 1 Incidence of nt551 A and nt551 G (%)

|        | nt551 A | nt551 G |
|--------|---------|---------|
| Number of case | 112     | 4       |
| Incidence       | 112/117 (95.37) | 4/117 (3.42) |

bP<0.01 vs wild-type HBV DNA (nt551 A).

Table 2 Incidence of nt551 A and nt551 C (%)

|        | nt551 A | nt551 C |
|--------|---------|---------|
| Number of case | 112     | 0       |
| Incidence       | 112/117 (95.37) | 0/117 (0) |

bP<0.01 vs wild-type HBV DNA (nt551A).

Table 3 Incidence of nt551 A and nt551 T (%)

|        | nt551 A | nt551 T |
|--------|---------|---------|
| Number of case | 112     | 1       |
| Incidence       | 112/117 (95.37) | 1/117 (0.85) |

bP<0.01 vs wild-type HBV DNA (nt551A).

DISCUSSION

It is well known that mutants of HBsAg are able to cause infection and horizontal transmission despite the presence of anti-HBs[8-22]. It has been found that increasing use of HBV vaccine has an overwhelming positive influence on the prevention of hepatitis B viral infection, but has no effective impact on those mutants[23]. Mutations in the coding region of "a" determinant of HBsAg could not be detected in some routine assays[24-23].

In our laboratory a specific and sensitive method for monitoring the HBsAg mutant with a mutation at nt551 has been established. The method of msPCR is different from DNA specifically under different conditions.

Additionally, the concentration of primers is also an important factor for this msPCR. In short, different primers amplify HBV DNA specifically under different conditions.

In our study, all serum samples detected were taken from hepatitis B patients in Nanjing and its neighbourhood and four mutants of nt551G were found. Their detailed clinical data were as follows: No.57 taken from a man was negative for HBsAg and positive for anti-HBs. His ALT level was considered to be abnormal. No.127 taken from a 3-year-old boy was negative for HBsAg and positive for anti-HBs and his ALT level was considered to be abnormal.

The reason why all these four samples were negative for HBsAg and positive for anti-HBs might be that the mutations of A→G at nt551 could cause a change from Met to Val at 133aa of HBsAg and the structure of HBsAg was altered accordingly. The affinity between HBsAg and anti-HBs might diminish subsequently. The alteration of Met to Val at 133aa of HbsAg can reduce the combination ability between HBsAg and mAb of anti-HBs.

The detection results of these four samples have shown that the anti-HBs loses its protection against HBsAg as usual. On the contrary, they may form immune pressure by recognizing and combining with wild genotype HBV strains, but those mutants having no epitopes can survive and multiply. Therefore, a specific and sensitive method should be used to find those mutants of HBsAg and provide theoretical data for clinical diagnosis and treatment of hepatitis B.

In conclusion, most HBV infections are caused by wild genotype HBV strains. Whether it is necessary to add the component of HBsAg mutants to HBV vaccine needs further study and investigation.

REFERENCES

1. Koyanagi T, Nakamuta M, Sakai H, Sugimoto R, Enjoji M, Koto K, Iwanoto H, Kumazawa T, Mukaide M, Nawata H. Analysis of HBs antigen negative variant of hepatitis B virus: unique substitutions, Glu129 to Asp and Gly145 to Ala in the surface antigen gene. Med Sci Monit 2001; 6: 1165-1169

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

3. Kfouri Baz EM, Zheng 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. Transfus Med 2001; 11: 355-362

4. Dong J, Cheng J, Wang Q, Huangj F, Shi S, Zhang G, Hong Y, Li L, Si C. The study on heterogeneity of hepatitis B virus DNA. Zhonghua Yixue Zazhi 2002; 82: 81-85

5. Kreutz C. Molecular, immunological and clinical properties of mutated hepatitis B virus mutants. J Cell Mol Med 2002; 6: 113-143

6. Zhong S, Chan JY, Yeow W, Tam JS, Johnson PJ. Hepatitis B envelope protein mutants in human hepatocellular carcinoma tissues. J Viral Hepat 1999; 6: 195-202

7. 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

8. Yukiimasa N, Ohkushi H, Fukasawa K, Fukushima K, Takagi Y, Gomi K. Hepatitis B virus gene mutations in the sera of three patients with coexisting hepatitis B virus surface antigen and anti-surface antibody. Rinsho Byori 2000; 48: 184-188

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

10. Yang X, Lei J, Zhang Y, Luo H, Huang L, Zheng Y, Tang X, Li L. A novel stop codon mutation in S gene: the molecular basis of a patient with cryptocogenic cirrhosis. Zhonghua Yixue Zazhi 2002; 82: 400-402

11. Zhu Q, Lu Q, Xiong S, Yu H, Duan S. Hepatitis B virus S gene mutants in infants infected despite immunoprophylaxis. Chin Med J (Engl) 2001; 114: 352-354

12. Santantonio T, Gunther S, Sterneck M, Rendina M, Messner M, Launois B, Francavilla A, Pastore G, Will H. Liver graft infec-
tion by HBV S-gene mutants in transplant patients receiving long-term HBIG prophylaxis. *Hepatogastroenterology* 1999; **46**: 1848-1854

13 **Rodriguez-Frias F**, Buti M, Jardi R, Vargas V, Quer J, Cotrina M, Martell 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

14 **He C**, Nomura F, Itoga S, Isobe K, Nakai T. Prevalence of vaccine-induced escape mutants of hepatitis B virus in the adult population in China: a prospective study in 176 restaurant employees. *J Gastroenterol Hepatol* 2001; **16**: 1373-1377

15 **Cooreman 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

16 **Cooreman 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

17 **Komatsu H**, Fujisawa T, Sogo T, Isozaki A, Inui A, Sekine I, Kobata M, Ogawa Y. Acute self-limiting hepatitis B after immunoprophylaxis failure in an infant. *J Med Virol* 2002; **66**: 28-33

18 **Heijtink RA**, van Bergen P, van Roosmalen MH, Surmen CM, Paulij WP, Schalm SW, Osterhaus AD. Anti-HBs after hepatitis B immunization with plasma-derived and recombinant DNA-derived vaccines: binding to mutant HBsAg. *Vaccine* 2001; **19**: 3671-3680

19 **Banerjee K**, Guptan RC, Bisht R, Sarin SK, Khandekar P. Identification of a novel surface mutant of hepatitis B virus in a seronegative chronic liver disease patient. *Virus Res* 1999; **65**: 103-109

20 **Chen WN**, Oon CJ. Hepatitis B virus surface antigen (HBsAg) mutants in Singapore adults and vaccinated children with high anti-hepatitis B virus antibody levels but negative for HBsAg. *J Clin Microbiol* 2000; **38**: 2793-2794

21 **Oon CJ**, Chen WN, Goo KS, Goh KT. Intra-familial evidence of horizontal transmission of hepatitis B virus surface antigen mutant G145R. *J Infect* 2000; **41**: 260-264

22 **Chen WN**, Oon CJ, Koh S. Horizontal transmission of a hepatitis B virus surface antigen mutant. *J Clin Microbiol* 2000; **38**: 938-939

23 **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

24 **Weinberger KM**, Bauer T, Bohm S, Ilg W. High genetic variability of the group-specific a-determinant of hepatitis B virus surface antigen (HBsAg) and the corresponding fragment of the viral polymerase in chronic virus carriers lacking detectable HBsAg in serum. *J Gen Virol* 2000; **81**: 1165-1174

25 **Chen WN**, Oon CJ, Moh MC. Detection of hepatitis B virus surface antigen mutants in paraffin-embedded hepatocellular carcinoma tissues. *Virus Genes* 2000; **20**: 263-267

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

27 **Jolivet-Reynaud C**, Lesenechal M, O’Donnell B, Beccquart L, Foussadier A, Forge F, Battail-Poitot N, Lacoux X, Carman W, Jolivet M. Localization of hepatitis B surface antigen epitopes present on variants and specifically recognised by anti-hepatitis B surface antigen monoclonal antibodies. *J Med Virol* 2001; **65**: 241-249

28 **Wu DY**, Ugozzoli L, Pal BK, Wallace RB. Allele-specific enzymatic amplification of beta-globin genomic DNA for diagnosis of sickle cell anemia. *Proc Natl Acad Sci USA* 1989; **86**: 2757-2760

Edited by Wang XL and Zhu LH