Cloning of HBV S Gene with Artificial Mutation Sites in 'a' Determinant and Analyzing the Protein Antigenicity Expressed

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[ABSTRACT] HBV (hepatitis B virus) vaccine is an effective way to control HBV infection, so viruses must be in variance to escape the clearance of host's immunity. The mutations induced by HBV vaccine majored in 'a' determination. In order to study mutations those how to influence the biologic characteristic of S gene, especially in antigenicity and immunogenicity of HBsAg, We constructed series variant clones of HBV S gene 'a' determination according to data of epidemiology. After verified by sequence, we constructed a series variant clone of HBV S gene with "site-mutation method of PCR (gene SOEing PCR, gene splice of overlap extending PCR)". After cellular expression, the antigen expressed by different mutant clones were all recognized by corresponding monoclonal antibody and not done by common HBsAb with immunofluorescence assay. In a conclusion, the mutations in "a "determination can alter the biologic characteristic of S gene, especially in the antigenicity of HBsAg, in a extend. The series mutant clones of "a" determination, which we constructed, may be provide the basic theory for how to develop new HBsAg detected kit, vaccine or HBV immunoglobulin(HBIG).

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1 Introduction

Hepatitis B virus (HBV) is a major human pathogen causing acute and chronic liver inflammation. It bears the clearance from host, and must avoid it through gene variance or other ways. Hereinto, variance of surface gene is the most common form. Surface antigen mutations of HBV may lead to immune escape and cause failure of immunization. In Europe and North America, HBV with mutations in the portion of the S gene coding the "a" determinant of the hepatitis B surface antigen (HBsAg) have been documented in small numbers of infants born to HBV-infected mothers following post-natal HBV vaccine and hepatitis B immune globulin (HBIG) prophylaxis and in many liver transplant recipients who develop HBV re-infection despite HBIG prophylaxis[1]. In order to study mutations in 'a' determination of HBV S gene those how to influence the biologic characteristic of S gene, especially in antigenicity and immunogenicity of HBsAg, we used gene SOEing PCR (gene splice of overlap extending PCR), and constructed expression plasmids of HBV S gene containing mutation sites (T126S, M133L, D144A) in 'a' determinant. According to investigates of epidemiology, these mutations are frequently encountered in patients with HBV infection [2,3]. Data of cellular expression presented below confirmed the importance of the mutation in 'a' determinant.

2 Materials and Methods

2.1 Plasmids and cells

pcDNA3 were obtained from Invitrogen company(USA)，pCR3.1/SHBV(contain wild HBV S gene）and BHK-21 cells are of our lab.

2.2 Chemicals and enzymes

Pfu enzyme，EcoR I，Smal I and Xhol-I endonuclease were obtained from Kejian company,Wuhan; CIAP,123bp mark，goat anti mice fluorescence antibody from Gibico BRL (USA).

2.3 Methods

2..3.1 Site-directed mutation in 126,133 and 144 positions of HBV S gene

2.3.2 PCR3.1/SHBV and pcDNA$_3$

PCR3.1/SHBV and pcDNA$_3$ were both digested by EcoR I, 37℃,5 hours（hereinto, the linear pcDNA3 after digestion were prepared for next clone. Complex from the former after digestion were dephosphored by CIAP, connected with the linear pcDNA3 and provided the templets of the next gene SOEing PCR.

2.4 Gene SOEing PCR：

2.4.1 Primers

Left primer, ZL：5'-GCGCTGAACATGGAGAACATCAC-3 (nt157 to nt180);

Right primer, ZR: 5'-CCATCTTTTTGTTTTGTTAQGG-3' (nt860 to nt838);

Primer SP6R: 5'-AGCATTTAQGTGACACTATAQAATAQG-3 (located at SP6 promoter of pcDNA3). Sited-directed mutation primers at 126, 133 and 144 positions in HBV S gene:126 position, 126L (left primer): 5'-CTGCATGACTAQTGCTCAAGGAAC-3' (nt439 to nt415), 126R (right primer):

5‘-TTCTTGAGCACTAGTCATGCAGGTCC-3‘ (nt460 to nt434）; 133 position, 133L (left primer): 5‘-CCAGGAACCTCTCTGTATCCCTCC-3‘ (nt 455 to nt 431), 133R (right primer): 5-AGGGATACAGAGAGG TTCCTTGAGCA-3‘ (nt476 to nt 450); 144 position (left primer): 5‘-AACCTTCGGCCGGAAATTGC-3‘ (nt 492 to nt 472), 144R (right primer): 5‘GTGCAATTTCCGGCCGAAGGTTTG-3‘ (nt 513 to nt 489).

2.4.2. Gene SOEing PCR

Methods were executed as described by Servant, et al[4].In precise, the first PCR: the upstream -part of S gene with different position mutations were amplified by ZL and 126R or 133R or 144R; the downstream–part of S gene with different mutations were amplified by SP6R and 126L or 133L or 144L.The second PCR were all amplified by ZL and SP6R.We extracted the second PCR productions of HBV S gene with T126S，M133L or D144A mutation。

The fragments for amplified by PCR were rounded to be 92℃ 30 s, 50℃ 45s and 72℃ 60 s for 30 cycles, and the final extension step being 10 min at 72°C, Amplification products were resolved by agarose gel electrophoresis, stained with ethidium bromide, and quantified for the next PCR ligations or cloned.

2.5 Clone and determination of variant HBV S gene

2.5.1 Clone of HBV S gene with sited-mutation

After digesting pcDNA3 by Xhol and EcoR V, we purified the linear vector. All second PCR productions were digested by Xhol, connected with the above vector, and at last transferred into *E.coli*. Ligation products were transferred into DH5 α with routine principle, sifted and maintained at 37℃, poke out single clone of DH5 α and cultured, Both of the newly-constructed vectors were confirmed by restriction endonuclease digestion, PCR with specific primers and finally by DNA sequencing (Baosheng Co., Dalian, China).

2.5.2 Analysis by sequencing

After sifting and determination by digestion with EcoR I and Xhol, mutation clones were sequenced and analyzed by ALIGN soft.

2.6 Cells and transfection

To study the biological property of mutant clone, BHK cells were divided into 5 groups (wild strain group, negative control, T126S, M133L and D144A group) and seeded in 8-well dishes at the density of $1\sim2 \times 10^4$ cells/cm^2 in DMEM containing 10% fetal calf serum. cells were cultured for 3 days continued with DMEM. BHK-21 cells were transfected with the appropriate plasmids constructs(20 μ g of purified DNA per 10cm dish)by lipofectamine （Boehringer Mannheim Biochemicals Co.USA. At the end of incubation, cells were fixed and detected by immunofluorescence assay 3 days post-transfection with monoclonal antibody of HBsAg or common HBsAb.

3 Results

3.1 Results of Gene SOEing PCR（the first and second PCR）

The site-mutation PCR were executed by SOEing PCR, the products of the first PCR and second PCR were showed. The ladder-liked fragments with different length were amplified in the first PCR（Fig 1.1, 1.2）and the same length of products were emerged in the second PCR (Fig 1.3).

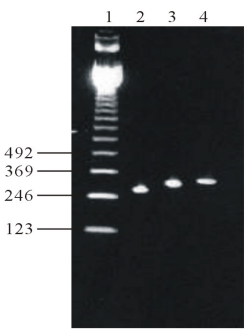

Fig 1.1

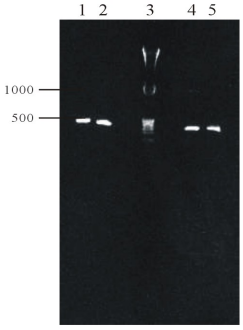

Fig 1.2

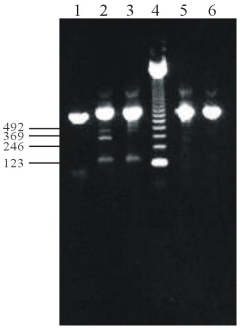

Fig 1.3

The upstream-part of S gene by first PCR（figure.1.1） the downstream-part of S gene by first PCR（figure 1.2）the second PCR （figure. 1.3）. Note Fig. 1.1:lane 2,3 and 4 were the upstream-part of S gene by first PCR with mutations at 126,133 and 144 position and the weight were 378 bp, 399bp and 432bp separately; lane 1 is 1kb Mark. Fig.1.2: lane 1,2,4 and 5 were the downstream-part of S gene by first PCR with mutations at 126, 133,144 and 144 position, and weight is 380bp, 359bp and 326bp separately（all containing muticlone sites and full-length SP6 promoter of pcDNA$_3$）; lane 3 is 1kb Mark. Fig3. 1.3: lane 1 is the product by ZL and ZR, 703bp; lane 2,3,5 and 6 are products through ZL and SP6R for 126,133 and 144 position separately, 758bp（all containing muticlone sites, full-length SP6 promoter and mutation sites）; lane 4 is 123bp Mark.

3.2 Align of postulated translation after sequencing

The data were testified after sequencing. After analyzing by ALIGN soft, the homologies of amino acids was almost in 100% among the strains of Wshbvaa, T126Saa, M133Laa, and M144aa, except for the mutant sites we constructed (Wshbvaa amino acid sequence which was translated in theory is simplify for the wild strain sequence of HBV s antigen in "a" determination; T126Saa for the mutant strain sequence at 126 site of HBV s antigen in a determination; M133Laa for mutant strain at 133 site and D144Aaa for mutant strain at 144 site). (Figure 2)

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                0         10        20        30        40        50
                *         *         *         *         *         *
Wshbvaa         MENITSGFLGPLLVLQAGFFLLTRILTIPQSLDSWWTSLNFLGGTTVCLGQNSQSPTSN
T126Saa         ...........................................................
M133Laa         ...........................................................
D144Aaa         ...........................................................

                60        70        80        90        100       110
                *         *         *         *         *         *
Wshbvaa         HSPTSCPPTCPGYRWMCLRRFIIFLFILLLCLIFLLVLLDYQGMLPVCPLIPGSSTTSTGP
T126Saa         ...........................................................
M133Laa         ...........................................................
D144Aaa         ...........................................................

                120       130       140       150       160       170       180
                *         *         *         *         *         *         *
Wshbvaa         CRTCMTTAQGTSMYPSCCCTKPSDGNCTCIPIPSSWAFGKFLWEWASARFSWLSLLVPFVQ
T126Saa         ......S....................................................
M133Laa         ..........L................................................
D144Aaa         ..................A.........................................

Wshbvaa         WFVG
T126Saa         ...G
M133Laa         ...G
D144Aaa         ...G
```

Figure 2. Align of postulated translation after sequencing

3.4 Detection of immunofluorescence assay

The cells were transfected by the differenced mutant clones of HBV S gene and 3 days later, the cells were recognized by monoclonal antibody of HBsAg or HBsAb. The fluorescence were found in the cells of different group by monoclonal antibody, but there were no sign in common HBsAb reorganization (The data not shown) (10×40).(Figure 3)

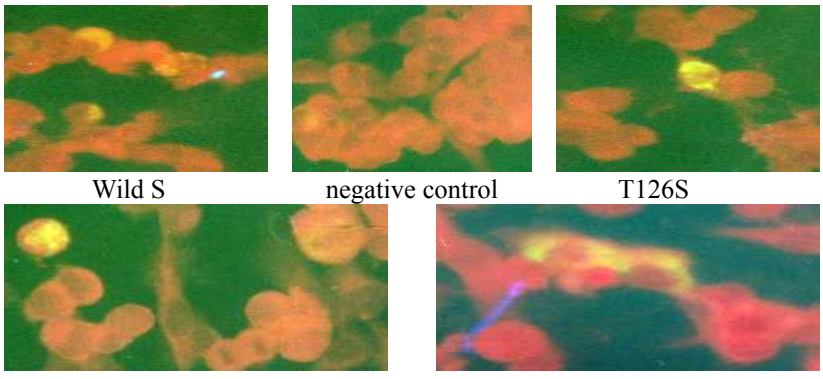

Figure 3. Detection of immunofluorescence assay

5. Discussion

HBV vaccine has been proven to be effective in prevention HBV infection. During the natural infection and vaccination, 'a' determination in HBsAg is thought as the most important motif to elicited the protective antibody. After vaccination, about 90-95% of people can induce the protective antibody against 'a' determinant. But there are still some people can be infected by HBV, though in these patient HBsAb can be detected. Data showed that the variances of HBV S gene 'a'determinant may lead to the above phenomenon. Further more, the variances may escape detection by certain commercial HBsAg kit a, have a selective advantage in carriers treated with passive immunazition(HBIG)and become a dominant clone[4,~6].

Carman et al found G145R strain in a carrier of HBV and persisted for 5 years. Synchronously, the G145R variant sera from patients could infect chimpanzee, auto-replicate completely, and have pathogenic effect[7, 8].

Recently, Sayiner AA, et al (2007) found that different commercial kits recognized variant HBsAg depressively, even could not, expressed by G145R, M133A or other mutation positions plasmids, and those variant positions are most common emergence in patients [9]. In the chronic, variance of HBV S gene is also very common; positions of mutation are focus on loop I of 'a' determinant. In addition, in patients with unsuccessful vaccination or protection for reinfection after HBIG therapy(such as organ graft), variances are also found in 'a' determinant，but those often mustered on loop II [10].

All sites, single or with others, in HBV 'a' determinant can be elicited to be variance and those variances can cause antigenic alteration of HBsAg more or less in different patients. So, it is crux to definitude which variant strains may alter antigenicity and immunogenicity of HBsAg in vivo (especially high frequent mutation positions), how escape the immune supervision and lead to persistence of HBV infection [11]. Therefore, the hinge to solve above problem is to analyze biological characteristics of protein HBV S gene expressed in vivo and in vitro.

According to data of epidemiology, we used gene SOEing PCR and constructed the expressed plasmids of HBV S gene containing mutation sites (T126S, M133L and D144A) in 'a' determinant. Sequence analysis confirmed that the homologies of nucleotides or amino acids between the wild HBV and mutants were 100% except for the target mutation sites. Meanwhile, variant clones with different mutation sites were transfected into BHK cells and showed the variant clones constructed can be expressed in eukaryotic cells after detection by immunofluorescence assay. The results showed that proteins expressed could precisely fold, maintain natural second construct, and take on good antigenicity. So, the series variant clones we constructed can be used in developing new blended multivalence vaccine, HBIG or HBsAg detected kits, otherwise, also provide the basis for analyzing biological charateristic of S gene variant in vivo, especially in antigenicity and immunogenicity in the future.

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