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ORIGINAL ARTICLE

Analysis of clinical characteristics and *S* gene sequences in chronic asymptomatic HBV carriers with low-level HBsAg

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HBV S gene;
Mutation site

Summary
Background: During the natural hepatitis B virus (HBV) infection process, some infected subjects are characterized by a sustained low serum HBV surface antigen (HBsAg) expression level. Most members in this population are chronic asymptomatic HBV carriers (ASCs). To elucidate the mechanism underlying low-level HBsAg expression in ASCs, we sequenced the *HBV S* gene in these patients to reveal specific sequence characteristics.
Methods: Overall, 1308 cases of chronic ASCs were grouped according to their HBsAg serum expression levels (10 IU/mL). The clinical characteristics of the population were analysed in detail. The *HBV S* gene was sequenced from 276 ASC cases with low-level HBsAg expression. Additionally, 100 of 1032 ASC cases with high-level HBsAg expression were randomly selected for *HBV S* gene sequencing based on age matching according to the low-level HBsAg group. A comparative analysis was conducted with the *HBV S* gene sequences from ASCs with low HBsAg expression and the HBV reference *S* gene sequences from ASCs with high HBsAg expression.
Results: The population with low-level HBsAg expression displayed the following primary clinical characteristics: mostly chronic asymptomatic HBV carriers, older age (mean age 55.09 years), HBsAg/anti-HBe/anti-HBc (core) positivity as the main serological pattern (97.1%), low HBV DNA replication (1.32 ± 1.60 $\log_{10}$ IU/mL), a low HBV-DNA positive rate (45.65%) and

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primarily genotype B (82.54%) and serotype adw (84.13%). The comparative analysis of the *HBV S* gene sequences from ASCs with low-level HBsAg showed significant mutations (including co-mutations) on both sides of the main hydrophilic region (MHR).
Conclusion: Significant mutations in multiple regions and at multiple sites (including co-mutations) on both sides of the MHR may be one cause of the low HBsAg expression level in this population.

Introduction

Since the discovery of the Australia antigen in 1963 [1], hepatitis B surface antigen (HBsAg), which was the first identified protein of the hepatitis B virus (HBV), has become one of the most important biological and serological indicators for the diagnosis of HBV infection [2]. With continuous advances and improvement in detection methods and technology, the sensitivity and accuracy of quantitative HBsAg detection has significantly improved, and the application of the results of HBsAg detection has become more extensive. Quantitative HBsAg measurement can be used to clinically diagnose the disease, evaluate the disease stage [3—8], examine the history of HBV infection [9—11], perform risk assessment in cases of chronic hepatitis B virus (CHBV) infection progression and liver cirrhosis, diagnose liver cancer and liver cancer recurrence [12—14] and evaluate the efficacy of treatment for HBV infection [15,16].

HBsAg is the main protein generated in hepatocytes by HBV and is secreted into the blood. HBsAg consists of three structural proteins [large hepatitis B (LHBs), intermediate hepatitis B (MHBs) and small hepatitis B surface (SHBs) antigen], which are all encoded in the HBV pre-S/S open reading frame. SHBs consists of 226 amino acids, MHBs is identical to SHBs but contains 55 additional amino acids at the N-terminus, and LHBs is an extension of MHBs that contains 108—109 additional amino acids [17,18]. The domain-flanking amino acid residues 99—169 in SHBs constitute the main hydrophilic region (MHR) of HBsAg. Within this region, the fragment corresponding to amino acid residues 124—147 is located in loop2 and forms the ''a'' antigenic determinant of HBsAg, which is the major antigenic determinant of the vaccine; this determinant elicits the formation of protective antibodies and is important for antibody identification and binding using commercial kits [19—22].

The concentration of HBsAg in the serum of HBV-infected patients depends both on the expression of the corresponding encoded mRNA and on the establishment of a complex equilibrium involving the interaction between HBV and the host immune system, not simply on the viral replication process [6,23—25]. During the natural process of HBV infection, a population with HBV infection characterized by a sustained low level of HBsAg in serum is formed. Studies have confirmed the presence of a population with low-level HBsAg in serum and low HBV DNA replication; these subjects are mostly chronic asymptomatic HBV carriers (ASCs) [13,17,18,26—29]. This poses a new challenge for the prevention and treatment of hepatitis B and has received widespread attention from clinicians, laboratory diagnostic specialists, epidemiologists and molecular biologists [26,27]. To date, few studies have investigated the mechanism underlying the sustained low-level HBsAg expression in ASCs. Therefore, we sequenced the *S* gene of chronic ASCs with sustained low-level HBsAg expression and compared these sequences with the established *S* gene sequences of chronic ASCs with sustained high-level HBsAg expression to reveal the characteristics of the *S* gene sequences and explore the mechanism underlying the sustained low-level HBsAg expression in these patients.

Materials and methods

Specimen collection

Serum samples were obtained from 1308 chronic ASCs from the specimen banks of the State Key Laboratory for the Diagnosis and Treatment of Infectious Diseases, the First Affiliated Hospital, College of Medicine, Zhejiang University, the Clinical Experimental Centre of Hangzhou Hospital of Infectious Diseases and the Clinical Experimental Centre of People's Liberation Army 117 Hospital. Most of the samples were obtained from eastern regions of China (Zhejiang, Anhui, Shanghai, Jiangsu, Jiangxi, Fujian and Shandong). The serum samples were collected from February 2014 to December 2015 and were stored at −70 °C. Based on the laboratory test results, more than one year of follow-up with the patients, clinical data and history of infection or natural history [9—11], the exclusion criteria were as follows:

- cases complicated with infection by human immunodeficiency virus (HIV), hepatitis C virus (HCV) or hepatitis D virus (HDV);
- cases with low-level HBsAg in the presence of acute HBV infection (acute hepatitis) or early infection;
- cases with low-level HBsAg in the conversion phase of HBsAg/anti-HBs recovery;
- cases with the long-term coexistence of positive HBsAg/anti-HBs;
- cases in which drug treatment for liver protection or enzyme reduction, immunomodulators or antiviral drugs had been administered during the six months prior to sample collection.

Research objects definition

Definition of chronic ASC: a chronic ASC is characterized by the presence of positive serum HBsAg for more than six months, normal serum amino transferase levels and no evidence of liver cirrhosis (LC) or hepatocellular carcinoma (HCC) based on the clinical criteria and ultrasound examination [11,30,31].

Definition of HBV infection cases with a low HBsAg expression level: low HBsAg expression refers to chronic ASCs who show HBV infection and serum HBsAg levels below 10 IU/mL in at least three tests during more than one year of follow-up with an interval of at least three months between samplings [24,32,33]. Based on the disease type, these cases are classified as ASCs; based on the HBV infection history, they are classified as chronic HBV infection; and based on the natural history of HBV infection, they are classified as inactive stage (inactive or low/non-replication stage) [9—11].

Grouping

The 1308 cases of ASC were grouped according to serum HBsAg levels [24,32,33]; there were 276 cases in the low-level HBsAg group ($\leq$ 10 IU/mL) and 1032 cases in the high-level HBsAg group (> 10 IU/mL). Although the mean age was higher in the low-level HBsAg group than in the high-level HBsAg group ($P < 0.05$), no significant difference was found in gender or the alanine amino transferase (ALT) levels ($P > 0.05$) between the two groups. The clinical data of the two groups are shown in Table 1.

Determination of biochemical and serological markers in chronic asymptomatic HBV carriers

The ALT and HBV markers (HBsAg, anti-HBs, HBeAg, anti-HBe, and anti-HBc) were measured using a C1600 biochemical analyser and an i2000 immunoassay instrument (Abbott Laboratories, Abbott Park, IL, USA), respectively. The ALT and HBV marker detection kits were purchased from Beijing Lidman Biochemical Technology Co., Ltd. and Abbott Laboratories Ltd., respectively. The HBsAg assay had a linear range from 0.05 to 250 IU/mL. If the HBsAg level was higher than 250.0 IU/mL, the samples were serially diluted 1:100 according to the manufacturer's instructions to obtain values that fell within the linear range.

To verify the accuracy and consistency of our quantitative measurement of HBsAg levels, another chemiluminescence immunoassay (CMIA) was performed on HBsAg-positive specimens using a Maglumi 4000 analyser and the supporting HBsAg kits. The neutralization test was used to confirm the results and indicated low HBsAg levels. Confirmation for HBsAg using the neutralization test was conducted as follows. First, 100 μL of the collected HBsAg-positive serum sample was added to the two sample cups. Then, either 100 μL of anti-HBs (1000 IU/mL, ACON, China) (for measurement) or 100 μL of normal saline (as a control) was added; the samples were mixed well and incubated in a 37 °C water bath for 30 minutes followed by HBsAg determination using the CMIA method. If [(control value − measurement value)/control value] $\geq$ 50%, the original serum was considered HBsAg-positive; otherwise, the original serum was considered a false positive for HBsAg.

HBV DNA extraction and amplification

Viral DNA was extracted from 500 μL of serum and eluted into 100 μL of H_2O (DNAse- and RNAse-free) using the NP968 Nucleic Acid Extraction System (TianLong, Suzhou, China) according to the manufacturer's instructions. The HBV DNA level was determined in all samples using a fluorescence quantitative PCR detection kit (ACON, Hangzhou, China), the lowest limit of detection (LLOD) and linear range of which were 20 IU/mL and 50-5.0 $\times$ 10^8 IU/mL, respectively, and an ABI StepOnePlus Real-Time PCR System (ABI Applied Biosystems, Foster City, CA, USA).

HBV genome sequencing

The target fragments of the *HBV S* genomes in the 276 serum samples in the low-level HBsAg group, and 100 randomly selected serum samples in the high-level HBsAg group that were chosen based on age matching according to the low-level HBsAg group were amplified by nested PCR [28]. No significant difference was found in the compositions of the two groups or in the cases in the same group from different regions ($P > 0.05$).The primers used for amplification and sequencing are shown in Table 2. The PCR amplification system included 5 μL of 5 $\times$ KAPA2G buffer A, 5 μL of 5 $\times$ KAPA enhancer, 0.1 μL of KAPA2G Robust HotStart DNA polymerase, 0.5 μL of 10 μM dNTP mix (TaKaRa, Japan), 1 μL of 10 μM of each primer, 3 μL of DNA template in each 25-μL reaction tube and water to obtain a final volume of 25 μL. The first round of amplification included pre-denaturation at 95 °C for 3 minutes, followed by 5 cycles of denaturation at 95 °C for 30 seconds, annealing at 57 °C to 53 °C for 30 seconds (1 °C decrease each cycle) and extension at 72 °C for 30 seconds; then 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 53 °C for 30 seconds and extension at 72 °C for 30 seconds, with a final extension at 72 °C for 2 minutes. The PCR products from the first round of amplification were used as the DNA templates in the second round of amplification, with the same amplification conditions and reaction system as the first round. PCR was performed using a Bio-Rad S1000 thermal cycler (Bio-Rad, Hercules, CA, USA), and the positive products were purified, recovered and submitted to Sangon Biotech Co. (Shanghai, China) for sequencing using an ABI PRISM BigDye Sequencing Kit and the ABI 3730 Genetic Analyzer (ABI Applied Biosystems).

The sequencing results were aligned and spliced using the SeqMan program of Lasergene software (DNAstar Inc., Madison, WI, USA) and compared using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Genotyping and serotyping

The *S* gene assembled with multiple target fragments was compared using the MEGA v6.0 software [34], and a phylogenetic tree was constructed using the ortho-abutting method for the obtained sequence to perform genotyping with the representative genotypes [A (AF090842 and X02763), B (AB033554, AF100309, and D00329), C (AB014381, AY123041 and X04615), D (M32138, X65259, and X85254), E (AB032431 and X75657), F (X69798, AB036910 and AF223965), G (AB064310, AF160501 and AF405706) and H (AY090454, AY090457 and AY090460)] recommended by NCBI [35]. Cases with *S* gene homology $\geq$ 96% were considered the same genotype [36]. The bootstrap was set to 1000 times. The serotype was determined according to amino acid expression at specific sites in the *S* gene sequence [37].

Table 1 Clinical data for low-level and high-level HBsAg chronic ASC groups.

Parameters	Low-level HBsAg group ($n = 276$)	High-level HBsAg group ($n = 1032$)	P
Sex			
Male	169	671	0.224
Female	107	361	
Age (y)	55.09 ± 16.45	43.63 ± 10.95	0.000
Laboratory results			
ALT (U/L)	23.73 ± 9.60	25.32 ± 10.54	0.058
HBsAg (IU/mL)	2.95 ± 3.19	$5162.32 \pm 12{,}592.25$	0.000
HBV serological marker pattern			
HBsAg/HBeAg/anti-HBc (+)	0	247	0.000
HBsAg/anti-HBe/Anti-HBc (+)	268	733	
HBsAg/anti-HBc (+)	8	20	
HBV DNA ($\log_{10}$ IU/mL)	1.32 ± 1.60	3.28 ± 2.95	0.000
HBV-DNA positive rate (%)	45.65 (126/276)	91.96 (949/1032)	0.000

Table 2 Primers used for amplification of the *S* gene in HBV.

Primer set and name	Sequence(5′—3′)	Position (nt)	Fragment size (bp)[a]	Inner or outer	Sequencing primer
I F	ACCWTATWCYTGGGAACAA	2819—2837	1554	Outer	No
I R	TCAGCAAAYACTYGGCA	1190—1174		Outer	No
I aF	ACCWTATWCYTGGGAACAA	2819—2837	981	Inner	Yes
I aR	GAYGAYGGGATGGGAATACA	617—598		Inner	Yes
I b F	GACTYGTGGTGGACTTCTC	251—269	939	Inner	Yes
I b R	TCAGCAAAYACTYGGCA	1190—1174		Inner	Yes

[a] With reference to NC_003977 from NCBI.

Establishment and verification of the reference sequence for the main genotypes of the *HBV S* gene in ASC

Based on the results of the successful *HBV S* gene sequencing of 100 cases in the high-level HBsAg group, the reference sequences of genotype B (ASC-R-B: MF458549) and genotype C (ASC-R-C: MF458550) (wild type) of the ASCs in Eastern China were established. The base that occurred with the highest frequency at each site in the main genotypes (B and C) was used as the reference base.

To verify the correctness and applicability of the ASC-R-B and ASC-R-C reference sequences established in this study, the genotype B and C sequences of ASCs in the NCBI database (defined as the NCBI-ASC-B and NCBI-ASC-C groups, respectively) and the reference sequences for genotypes B and C recommended by the NCBI [34] (NCBI-R-B: AB033554, AF100309, and D00329; NCBI-R-C: AB014381, AY123041, and X04615) were used in the homology analysis.

Comparative analysis of the established reference gene sequences for the *HBV S* gene in the high- and low-level HBsAg groups (ASC-R-B, ASC-R-C)

The *HBV S* gene sequences of ASCs in the high- and low-level HBsAg groups were compared with the established ASC-R-B and ASC-R-C reference sequences. Each case in which a base at a given site was replaced by another base and led to a change in the amino acid of the expressed protein was defined as a mutation (non-synonymous mutation) [38]. Cases in which mutations were present in more than two codons in the same *S* gene sequence were defined as multi-site co-mutations [39]. Mutation frequencies that differed significantly between the high- and low-level HBsAg groups were defined as significant mutations. Mutations with a frequency $\geq 10\%$ were defined as hotspot mutations, and mutations with a frequency $< 10\%$ were defined as non-hot spot mutations [40]. Finally, a statistical analysis of the different mutation types and their classifications was performed.

Statistical analysis

SPSS version 12.01 was used to conduct the statistical analysis. The scatter plots were produced with GraphPad Prism 5 software. The results are presented as the mean $\pm$ standard error of the mean (SEM); the HBV DNA level (IU/mL) was logarithmically transformed for analysis. Continuous and categorical variables were compared between groups using the *t*-test and the Chi^2 test or Fisher's exact test. The frequency of amino acid mutation sites was analysed using the Cochran—Mantel—Haenszel test (CMH). All *P*-values were two-tailed. Differences with $P < 0.05$ were considered significant.

Table 3 Virologic characteristics of the low-level HBsAg and high-level HBsAg chronic ASC groups.

Parameter	Low-level HBsAg group ($n = 126$)[a]	High-level HBsAg group ($n = 94$)[b]	P
Sex			
Male	75	62	0.330
Female	51	32	
Age (y)	53.64 ± 16.37	53.77 ± 11.34	0.933
Laboratory results			
ALT (U/L)	23.87 ± 9.19	24.98 ± 10.89	0.410
HBsAg (IU/mL)	3.96 ± 2.76	3738.76 ± 13203.04	0.007
HBV serological marker pattern			
HBsAg/HBeAg/anti-HBc (+)	0	16	0.000
HBsAg/anti-HBe/anti-HBc (+)	123	75	
HBsAg/anti-HBc (+)	3	3	
HBV DNA ($\log_{10}$ IU/mL)	2.77 ± 0.85	3.66 ± 1.18	0.000
HBV genotype/subgenotype			
Genotype B (%)	82.54 (104/126)	52.08 (48/94)	0.000
HBV serotype			
adw (%)	100 (104/104)	95.83 (46/48)	0.098
ayw (%)	0 (0/104)	4.17 (2/48)	
Genotype C (%)	17.46 (22/126)	47.92 (46/94)	0.000
HBV serotype			
adw (%)	9.10 (2/22)	4.35 (2/46)	0.603
ayr (%)	90.90 (20/22)	95.65 (44/46)	

[a] Calculated based on the successful S gene sequencing of 126 cases.
[b] Calculated based on the successful S gene sequencing of 94 of the 100 randomly selected cases.

Results

The basic characteristics of chronic asymptomatic HBV carriers

Successful *HBV S* gene sequencing followed by genotyping and serotyping were performed for 126 of the 276 patients, which composed the low-level HBsAg group, and 94 of 100 patients who were randomly selected from the high-level HBsAg group based on age matching according to the low-level HBsAg group. The results showed statistically significant differences ($P < 0.05$) in HBV serotype marker patterns, mean HBV DNA levels, distribution of HBV genotype, and HBV serotypes between the high- ($n = 94$) and low-level HBsAg groups ($n = 126$); however, no significant differences in sex, and alanine amino transferase (ALT) levels between the two groups were found ($P > 0.05$) (Tables 3 and 4). The genotyping results are shown in Fig. 1A and B.

Establishment and verification of the reference sequence of the main genotypes of the *HBV S* gene in ASCs

Genotyping was performed for 94 cases in the high-level HBsAg group after successful sequencing. The main genotypes were B and C. Reference sequences of genotype B (ASC-R-B) and genotype C (ASC-R-C) in ASCs were established, with the base that occurred at the highest frequency at the same site of the genotype B and C sequences selected as the reference base; the results are shown in Fig. 2A and B.

The sequences of genotypes B and C of ASCs in the NCBI (NCBI-ASC-B and NCBI-ASC-C) and the NCBI recommended reference sequences for genotypes B and C (NCBI-R-B and NCBI-R-C) were used in the nucleotide and amino acid homology analysis. Our established ASC-R-B and ASC-R-C displayed very high homology (99.6—100%) to the reported genotype B and C sequences found in ASCs in most areas of China and displayed high homology (98.2—99.6%) to most of the NCBI genotype B and C reference sequences from Asia; by contrast, they displayed much lower homologies to cases from some areas far from China (98.2% and 98.7% for Canada and Indonesia, respectively) (Table 4). Thus, ASC-R-B and ASC-R-C can serve as the *HBV S* gene reference sequences for ASCs in China.

Comparative analysis of the established *HBV S* gene reference sequences (ASC-R-B, ASC-R-C) in the high- and low-level HBsAg groups

The ASC-R-B and ASC-R-C reference sequences established in this study were compared with the *S* gene sequences of genotypes B and C, respectively, in the high- and low-level HBsAg groups. With the exception of displacement mutations, no deletion or insertion mutations were found when the *HBV S* gene of ASC was sequenced. The number of amino acid mutations and the number of hot spot mutations in the SHBs protein of genotype B in the low-level HBsAg group were higher than the numbers of such mutations in the high-level HBsAg group (Fig. 3, $P < 0.05$). The amino acid mutation sites of the low-level HBsAg group were mainly distributed on both sides of the MHR (amino acid residues 40—49 and 198—220). The number of amino acid mutations and the

Table 4 Homology analysis of the *S* gene sequences for ASC-R-B and ASC-R-C (%).

Reference sequence	Sequence for comparison	Sequence	Region	Homology (%)	
				Nucleotide	Amino acid
ASC-R-B	NCBI-ASC-B	JX661484	Shanghai	99.6	100
		JX661473	Shanghai	99.6	99.6
		GU145102	Chengdu	99.6	100
		JN406371	Wuhan	99.8	99.6
		KY470957	Yunnan	99.7	99.6
		KY470963	Yunnan	99.7	99.6
		DQ463800	Canada	98.0	98.7
		DQ463801	Canada	98.2	98.7
	NCBI-R-B	D00329	Japan	99.2	99.6
		AF100309	Shanghai	99.8	99.6
		AB033554	Indonesia	98.6	98.2
ASC-R-C	NCBI-ASC-C	EU306721	Yunnan	99.2	98.7
		EU439007	Yunnan	99.2	98.7
		FJ560989	Beijing	99.5	100
		FJ560990	Beijing	99.5	99.1
		GU145103	Chengdu	99.8	100
		JX661496	Shanghai	99.4	99.6
		JX661498	Shanghai	99.7	99.6
		AY641558	Korea	99.5	99.6
		AY641560	Korea	99.5	99.1
		AB033556	Japan	99.2	98.7
		AB222715	Japan	99.4	99.1
	NCBI-R-C	AB014381	Japan	99.5	100
		AY123041	Japan	99.5	99.1
		X04615	Japan	99.2	98.2

ASC-R-B and ASC-R-C are the reference sequences of genotypes B and C in ASCs established in this study; NCBI-ASC-B and NCBI-ASC-C are the sequences of genotypes B and C in ASCs uploaded to NCBI; NCBI-R-B and NCBI-R-C are the reference sequences of genotypes B and C in ASCs recommended by NCBI B.

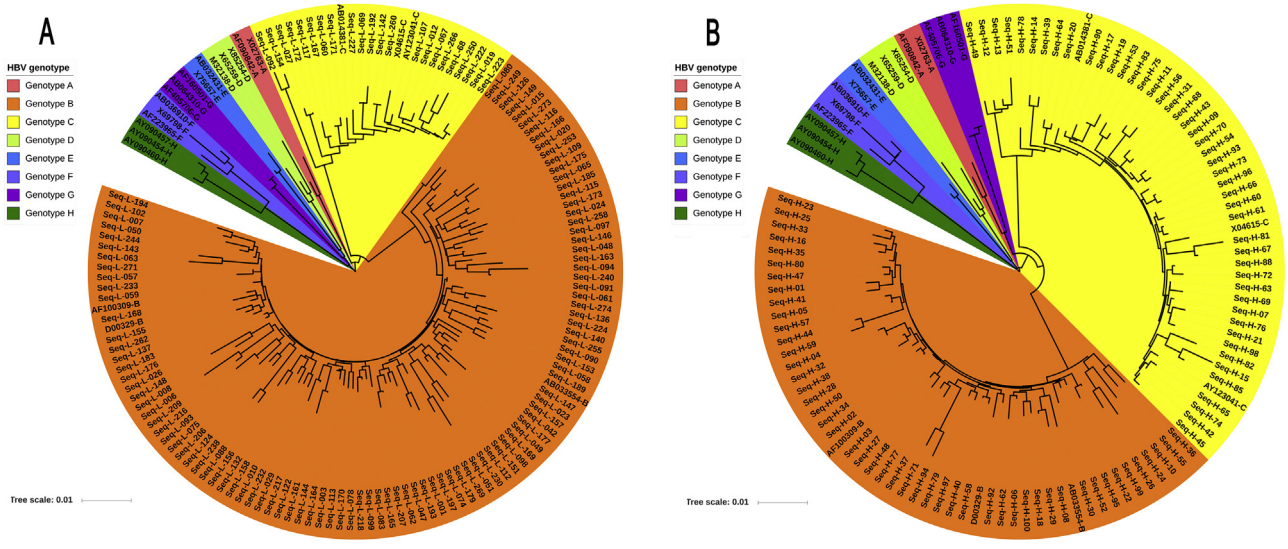

Figure 1 Comparison of *HBV S* gene sequencing and representative genotypes. A. Genotyping results of 126 cases in the low-level HBsAg group (104 cases of genotype B and 22 cases of genotype C) after successful sequencing. B. Genotyping results of 94 cases in the high-level HBsAg group (48 cases of genotype B and 46 cases of genotype C) after successful sequencing.

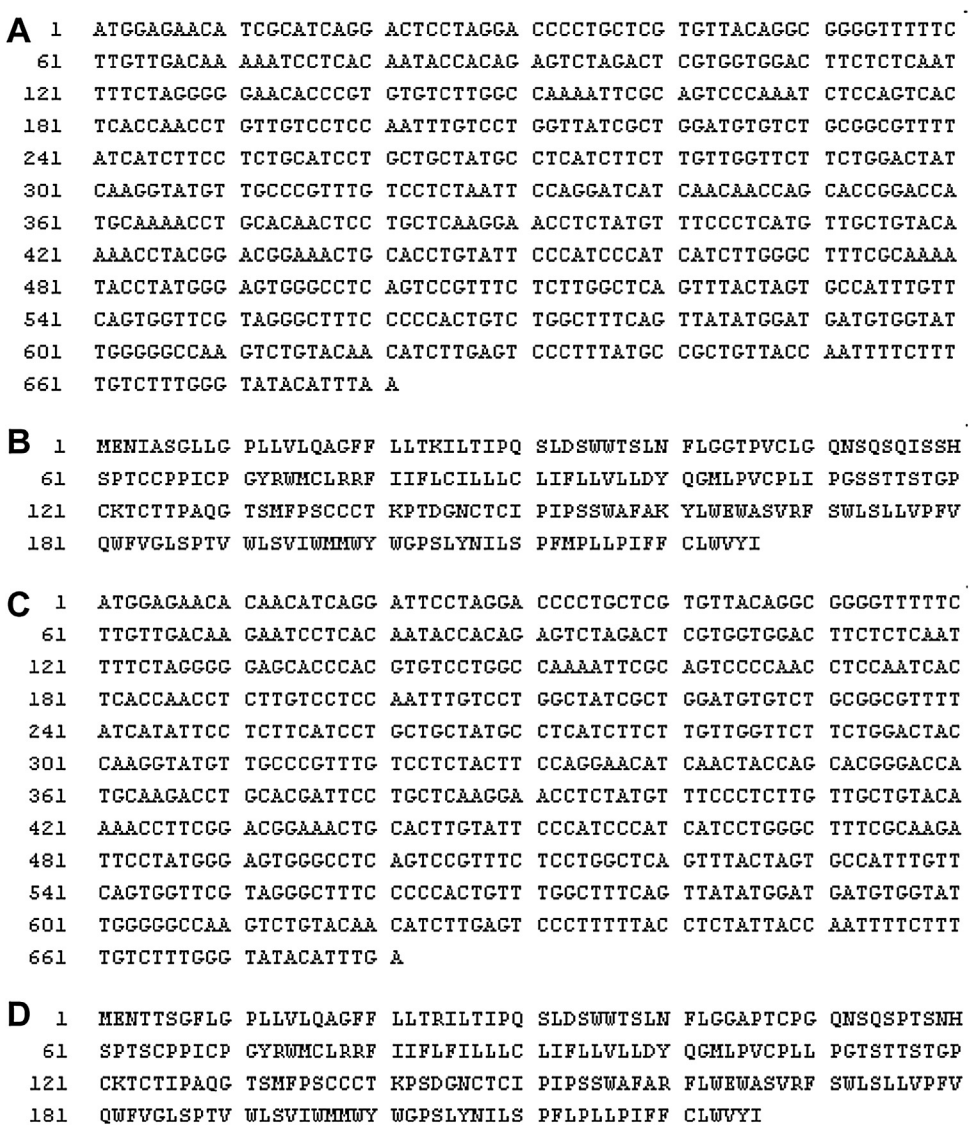

Figure 2 Reference sequences of genotypes B and C in ASC. A. Reference nucleic acid sequence of genotype B in ASC (ASC-R-B). B. Reference amino acid sequence of genotype B in ASC (ASC-R-B). C. Reference sequence of genotype C in ASC (ASC-R-C). D. Reference amino acid sequence of genotype C in ASC (ASC-R-C).

number of hot spot mutations in genotype C of the SHBs protein showed no statistically significant difference between the two groups ($P > 0.05$).

The distribution of the characteristic amino acid mutations found in the different genotypes in the two groups is shown in Table 5. In genotype B, 12 single-site mutations, 4 two-site co-mutations and 2 single-site mutations were found in the low-level HBsAg group, and 2 two-site co-mutations were found in the high-level HBsAg group; the frequency of these mutations differed significantly between the two groups (significant site mutation, $P < 0.05$). Of these mutations, 1 single-site mutation (S210R) and 3 two-site co-mutations (G44E/V + T45P/I, G44E/V + L49P/R, N40S + I208T) were not hotspot mutations. In genotype C, 5 single-site (T5A, A45T, T47A/K, Q101R, I126S/T) and 1 single-site (N3S) mutations of the low- and high-level HBsAg groups were significant site mutations ($P < 0.05$), respectively, and these sites were hotspot mutations.

Discussion

Serum HBsAg is not only an important serological marker for the screening and diagnosis of HBV infection but also an important indicator for the treatment of hepatitis B [15]. Jaroszewicz et al. [4] and Nguyen et al. [8] reported that the HBsAg levels of patients with HBV infection in Asia and Europe were different at different stages of the disease and that the patient's serum HBsAg level played a role in auxiliary diagnosis with respect to understanding the natural history of HBV infection [7]. Previously, some individuals in the HBV-infected population were reported to be characterized by a sustained low level of HBsAg expression (< 10 IU/mL), suggesting that the effective detection of low-level serum HBsAg had important clinical and epidemiological significance [24,32,33]. In this study, the clinical characteristics of 276 patients with HBV infection and low-level HBsAg were investigated, and the clinical

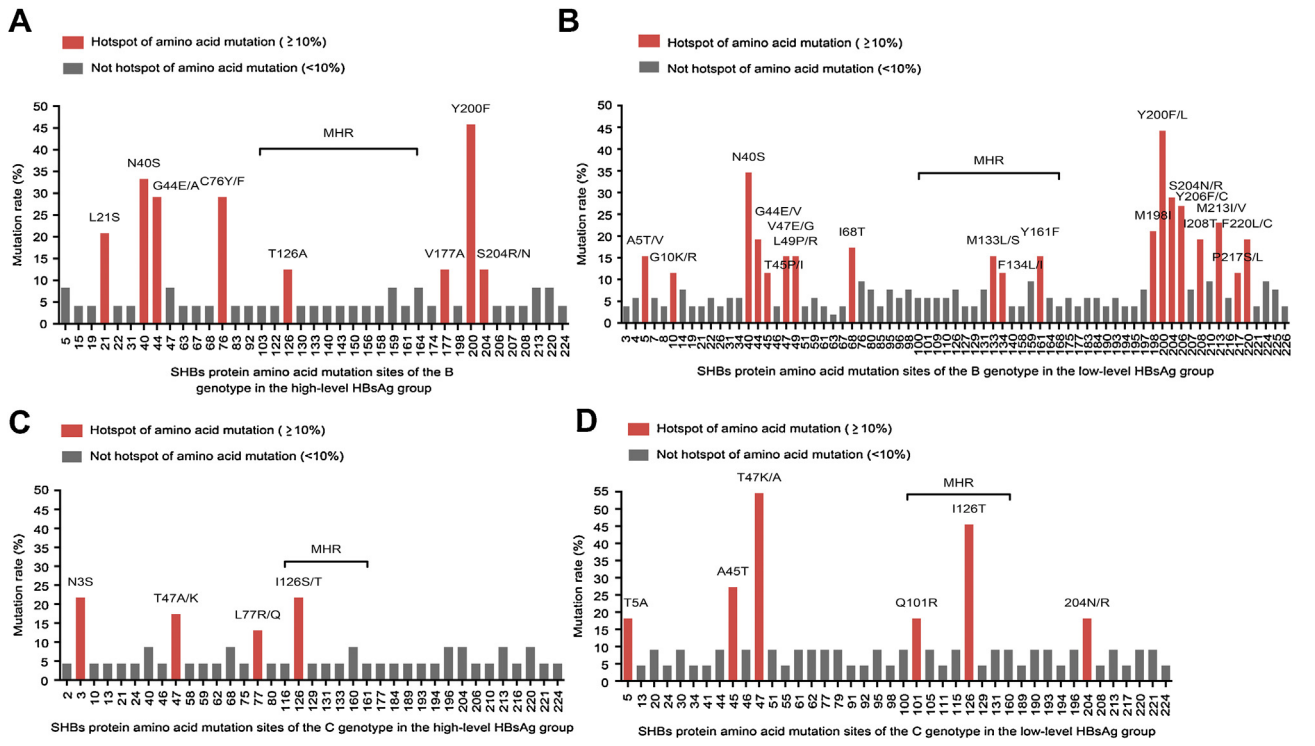

Figure 3 The distribution of SHB protein amino acid mutation sites in the two groups. A. SHB protein amino acid mutation sites of the B genotype in the high-level HBsAg group. B. SHB protein amino acid mutation sites of the B genotype in the low-level HBsAg group. C. SHB protein amino acid mutation sites of the C genotype in the high-level HBsAg group. D. SHB protein amino acid mutation sites of the C genotype in the low-level HBsAg group.

Table 5 Significant mutations of SHBs protein amino acids in the high- and low-level HBsAg groups.

Genotype	Mutation type	Low-level HBsAg group	High-level HBsAg group
B	Hotspot	G10K/R, T45P/I, L49P/R, I68T, F134L/T/I, M198I, S204R/N, Y206F/C, I208T, M213I, P217S/L, S204R/N + M213I	L21S, C76Y/F, L21S + 44E/V, N40S + 76Y/F
	Not hotspot	G44E/V + T45P/I, G44E/V + L49P/R, N40S + I208T, S210R	
C[a]	Hotspot	T5A, A45T, T47A/K, Q101R, I126S/T	N3S

[a] Because the number of C gene collected samples was low, significant mutation sites were not found in not hotspot mutations which was not listed in the table.

data from these patients were analysed. The results showed that the population with low-level HBsAg consisted primarily of patients who were chronic ASCs. A comparison of these patients with 1032 cases of chronic ASCs with high-level HBsAg expression revealed no significant differences in gender composition or ALT levels (Table 1, $P > 0.05$), whereas age, HBV serological marker patterns, HBV DNA load ($\log_{10}$ IU/mL), HBV-DNA positive rate (%), distribution of HBV genotype, and HBV serotype showed significant differences between the two groups ($P < 0.05$) (Tables 1 and 3). The patients were older in the low-level HBsAg group (55.09 ± 16.45 years) than in the high-level HBsAg group (43.63 ± 10.95 years), suggesting that the formation of low-level HBsAg was closely related to viral clearance, which was consistent with the changing trend in the natural history of the HBsAg concentration reported by Jaroszewicz et al. [4] and Nguyen et al. [8]. The HBsAg/anti-HBe/anti-HBc serological pattern was observed in 97.1% of the cases in the low-level HBsAg group, and the low replication and low positive rate of HBV DNA in this group were similar to the values reported in the literature for these parameters [32,33]. Thus, chronic asymptomatic HBV infection, older age distribution (mean age of 55.09 years), HBsAg/anti-HBe/anti-HBc positive rate (97.1%), low replication of HBV DNA (1.32 ± 1.60 $\log_{10}$ IU/mL), low HBV-DNA positive rate (45.65%), genotype B (82.54%), and serotype adw (84.13%) were the main characteristics of the ASC population with low-level HBsAg.

There are differences in the content and proportion of subviral particles at different stages of HBV infection. Pfefferkorn et al. [41] found that the content of L-HBsAg and M-HBsAg and the proportion of the total HBsAg in ASCs were lower than those of other stages of HBV infection (acute infections, HBeAg-negative CHB, HbeAg-positive phase). LHBs is more abundant on virions and filamentous SVPs than on spherical SVPs. Thus, a decline in the ratio

of virions versus spherical SVPs could contribute to the observed relative decrease of LHBs in low viraemic phases of infection (i.e., the IC phase), but the effect is likely very small because even in highly viraemic patients, the amount of small SVP exceeds that of virions by at least 1:1000. Additionally, Chai et al. [42] reported that when the expression of HBsAg was low, a small amount of HBsAg protein is preferentially used to form virus particles. This opinion has been confirmed in a study by Garcia et al. [43]. A possible reason for these two viewpoints was that the HBV infection stages of the two studies were different. Until now, there have been no reports of the distribution of L-HBSAg, M-HBSAg, S-HBSAg and the proportion of subviral particles versus viral particles in the serum of HBV ASCs with low-level HBsAg. Research in this area has been listed as the next research plan of our research team. In the alignment analysis of HBV gene sequences, we encountered difficulty in comparing results from different references, which led to issues in the assessment of results from individual studies. Overall, choosing appropriate HBV reference sequences is critical for such analyses [34,44]. The reference sequences established in this paper considered not only the nature of the research object itself (ASCs) but also regional differences (Table 4) to ensure the establishment of reference sequences that are representative and comparable to the study population. Thus, the results based on these reference sequences are reliable and authoritative.

There are five main research findings regarding possible mechanisms of low-level HBsAg expression:

- mutations in the *S* gene, particularly mutations in the region of the gene encoding the ''a'' antigenic determinant, cause changes in the 3-D conformation and the antigenic determinant of HBsAg, thereby affecting the detection of the antigen or leading to decreased secretion of HBsAg. In particular, mutations within the S protein may impair virion secretion (sE2G, sL95W and sL98V), change antigenicity (sL21R, sL95W and sL98V), impact HBV replication (sC69*) [17]. The early appearance of the stop codon in the *S* gene caused by the rtA181T/sW172*, rtV191I/sW182* and rtM204I/sW196* mutations makes it impossible for the virus to successfully synthesize and secrete HBsAg [17,26,27,45,46];
- mutation of the pre-*S*/*S* gene results in divergence between the HBV DNA level and the HBsAg concentration [18,47,48];
- either an unexplained immune tolerance exists between HBV and the host [24] or the administration of immunosuppressants enhances the body's immune tolerance so that HBsAg is not completely removed, resulting in a low level of circulating HBsAg [49];
- methylation of the *S* gene regulates HBsAg secretion [25];
- mutations within the pre-core (PC), basal core promoter (BCP) and pre-S result in low HBsAg levels in patients due to diminished HBsAg secretion [50].

Most of these findings are related to the use of antiviral therapy, which results in pre-*S*/*S* gene mutations, the development of occult hepatitis, the use of immunosuppressants or *S* gene methylation, but few studies have investigated the sequence characteristics of the *HBV S* gene in the low-level HBsAg ASC population. Therefore, in this study, we compared the *S* gene sequences of HBV infection cases of ASCs with low-level HBsAg expression with the established genotype B and C reference sequences and HBV-infected cases with high-level HBsAg. The results showed that the amino acid mutation rate and the number of mutation sites in the SHBs protein in the low-level HBsAg group, which primarily consisted of genotype B, were higher than those in the high-level HBsAg group ($P < 0.05$). The mutation sites were mostly distributed on both sides of the MHR (amino acid residues 40—49 and 198—220) (Fig. 3B). The distribution of these mutation sites was significantly different from the distribution of the mutation sites in the occult hepatitis, CHB and HBV infection with the coexistence of HBsAg and anti-HBs; in that case, most of the mutation sites are found inside the MHR [51—53].

We further analysed the amino acid mutation sites of SHBs in the high- and low-level HBsAg groups of ASCs from the perspectives of single-site mutations and multi-site co-mutations. The distribution of significant amino acid mutations found in the different genotypes in the two groups is shown in Table 5. In genotype B, hotspot (including 8 single-site mutations and 1 two-site co-mutation) and non-hotspot mutations (including 1 single-site mutation and 3 two-site co-mutations) were found in the low-level group, and 2 single-site mutations and 2 two-site co-mutations found in the high-level HBsAg group were hotspot mutations. In genotype C, hotspot mutations including 5 single-site mutations (T5A, A45T, T47A/K, Q101R, I126S/T) and 1 single-site mutation (N3S) were found in the low- and high-level HBsAg groups, respectively. In genotype B, the mutation frequencies of 2 single-site mutations, 2 two-site co-mutations in the high -level group were higher than in the low-level group, and the others in the low-level group were higher than those in the high-level group. In genotype C, the mutation frequency of 1 single-site mutation in the high-level group was higher than that in the low-level group, and the others in the low-level group were higher than those in the high-level group. HBsAg expression in HBV may be a bi-directional regulation of gene mutation. In other words, it can increase the expression level of HBsAg by the gene mutation and conversely reduce the expression level. However, whether these mutations determine the key sites of low expression of HBsAg requires further proof by the establishment of the model for expression in vitro.

Conclusion

In summary, a large-scale study of 1308 ASC cases grouped based on HBsAg level (10 IU/mL) was performed. Based on previous research results, the clinical features of cases with low-level HBsAg were analysed in detail. After establishing an *HBV S* gene reference sequence for ASC cases with a high expression of HBsAg in Eastern China, a comparative analysis of the *HBV S* gene sequence in patients with low-level HBsAg expression was performed. The results of this analysis suggest a possible mechanism for low-level HBsAg expression at the molecular level; the process of HBV immune clearance by the host appears to trigger significant mutations (including co-mutations) on both sides of the MHR, which may be one cause of low HBsAg expression.

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Disclosure of interest

The authors declare that they have no competing interest.

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