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ORIGINAL ARTICLE

Characteristics of amino acid substitutions within the ''a'' determinant region of hepatitis B virus in chronically infected patients with coexisting HBsAg and anti-HBs

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KEYWORDS
Chronic hepatitis B;
HBsAg;
Anti-HBs;
''a'' determinant region;
Amino acid substitution

Summary
Objectives: Simultaneous positivity for both hepatitis B surface antigen (HBsAg) and antibodies to HBsAg (anti-HBs) is an atypical serological profile in chronic hepatitis B (CHB) patients. The exact mechanisms underlying the uncommon profile remains unclear. The aim of this study was to analyze the characteristics of amino acid substitutions within the ''a'' determinant region in a large cohort of CHB patients with coexistence of HBsAg and anti-HBs.
Methods: In total 8687 CHB patients, of which 505 had coexisting HBsAg and anti-HBs, were enrolled in this study. Mutations within the ''a'' determinant region in 131 HBsAg+/anti-HBs+ patients and 150 age and gender matched HBsAg+/anti-HBs− patients were determined by direct sequencing and the characteristics of amino acid substitutions were analyzed.
Results: The prevalence of coexistence of HBsAg and anti-HBs in the CHB patients was 5.81%. Compared to the control subjects, there were more amino acid substitutions in HBsAg+/anti-HBs+ patients (30.5% vs. 12.7%, $P < 0.001$), especially within the first loop of the ''a'' determinant region. The most frequent amino acid substitution was located at position s126 and the predominant substitution was sI126T in HBsAg+/anti-HBs+ patients with genotype C. The frequency of additional N-glycosylation sites in HBsAg+/anti-HBs+ patients and the control subjects was 3.8% and 0.6%, respectively.

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Conclusions: The accumulation and diversity of amino acid variations within ''a'' determinant region might contribute to the coexistence of HBsAg and anti-HBs. These findings extend understanding of the genetic mechanism of this atypical serological profile in CHB patients.

Introduction

Chronic hepatitis B (CHB) virus infection is a global public health problem, which plays a critical role in the development of cirrhosis and liver cancer [1]. In theory, hepatitis B surface antigen (HBsAg) is recognized by the antibody to HBsAg (anti-HBs) which contributes to neutralization of HBsAg and clearance of virus particles [2]. There should not be simultaneous positivity for both HBsAg and anti-HBs in the serological profile from the same patient. However, in routine clinical practice, patients with coexisting HBsAg and anti-HBs have been reported in several previous studies [3—24].

The mechanism underlying the simultaneous detection of HBsAg and anti-HBs is still largely unknown [25—29], and clinical outcomes of this atypical serological profile are also controversial. The mutations within the ''a'' determinant region (aa124-147) of HBV Surface (*S*) gene, which altered the antigenic conformation and antigenicity of HBsAg, might be one of the possible mechanisms underlying this atypical serological profile [25—28]. Therefore, we conducted this study to analyze the characteristics of amino acid substitutions within the ''a'' determinant region in CHB patients with coexistence of HBsAg and anti-HBs.

Methods

Patients

From June 2014 to May 2016, 8687 patients with chronic HBV infection from Tianjin Second People's Hospital and Tianjin institute of Hepatology were enrolled in this study, of which 505 carrying both HBsAg and anti-HBs. Among the patients with positive anti-HBs, the *S* gene was successfully sequenced and analyzed in 131 patients. One hundred and fifty age and gender matched patients with HBsAg+/anti-HBs— were selected as control group in the present study. Seropositive patients for hepatitis C virus (HCV) or human immunodeficiency virus (HIV) were excluded from this study. All subjects gave their consent and the ethical approval for this study was obtained from the Faculty of Health Science Ethics Committee of Tianjin Second People's Hospital.

Laboratory assessment

The serum concentrations of HBsAg and anti-HBs were assayed by the electrochemical luminescence analysis using the Roche Cobas E601 analyzer (Roche Cobas E601, Germany). The quantitative determination of HBsAg concentration > 0.05 IU/mL was defined as positive, and the threshold level of anti-HBs > 10 IU/L was defined to be positive. HBV DNA was extracted from sera and the viral *S* gene was amplified by nested PCR with the primers listed in Supplementary Table 1. The PCR products were purified and the genomic sequences (Supplementary Table 2) were obtained subsequently by using an ABI 3730xl DNA Analyzer. HBV genotyping were performed by phylogenetic analysis (Fig. 1) comparing with the reference sequences of genotypes A-J (A: X02763, X51970, AF090842; B: D00329, AB073846, AB602818; C: X04615, AY123041, AB014381; D: X65259, M32138, X85254; E: X75657, AB032431; F: X69798, AB036910, AF223965; G: AF160501, AB064310, AF405706; H: AY090454, AY090457, AY090460; I: AB562462, FJ023671; J: AB486012).

Statistical analyses

All analyses were performed using the SPSS 25.0 software (SPSS Inc., Chicago, IL, USA). A *P*-value less than 0.05 was considered to indicate a significant difference.

Results

Prevalence of the coexistence of HBsAg and anti-HBs

The prevalence of coexistence of HBsAg and anti-HBs among CHB patients has been reported to vary between 2.43% and 8.9% in recent years [3,5—8,10—16,18—21,24]. In our study, among the 8687 CHB patients, 505 were identified carrying both HBsAg and anti-HBs. The prevalence of HBsAg+/anti-HBs+ patients in this cohort was 5.81% (Supplementary Fig. 1).

The baseline characteristics of patients

The *S* gene was successfully sequenced and analyzed in 131 HBsAg+/anti-HBs+ patients in the present study, 150 age and gender matched patients with HBsAg+/anti-HBs— were selected as control group. No significant differences were found between HBsAg+/anti-HBs+ patients and control subjects in terms of the distribution of age and gender ratio ($P > 0.05$, all) (Supplementary Fig. 2).

Phylogenetic analysis showed that two HBV genotypes (B and C) were identified in the 281 CHB patients. Among them, 43 (15.3%) patients were infected with genotype B, 238 (84.7%) with genotype C (Fig. 1A). There were no distinct subgroups observed within each genotype regarding the case and the control group (Fig. 1B). Subgenotype and serotype analyses were shown in Supplementary Fig. 3—6 and Supplementary Table 2. In our study, most the HBV-B patients are subgenotype B2 and serotype *adw2*, while most the HBV-C patients are subgenotype C1 and serotype *adrq+*.

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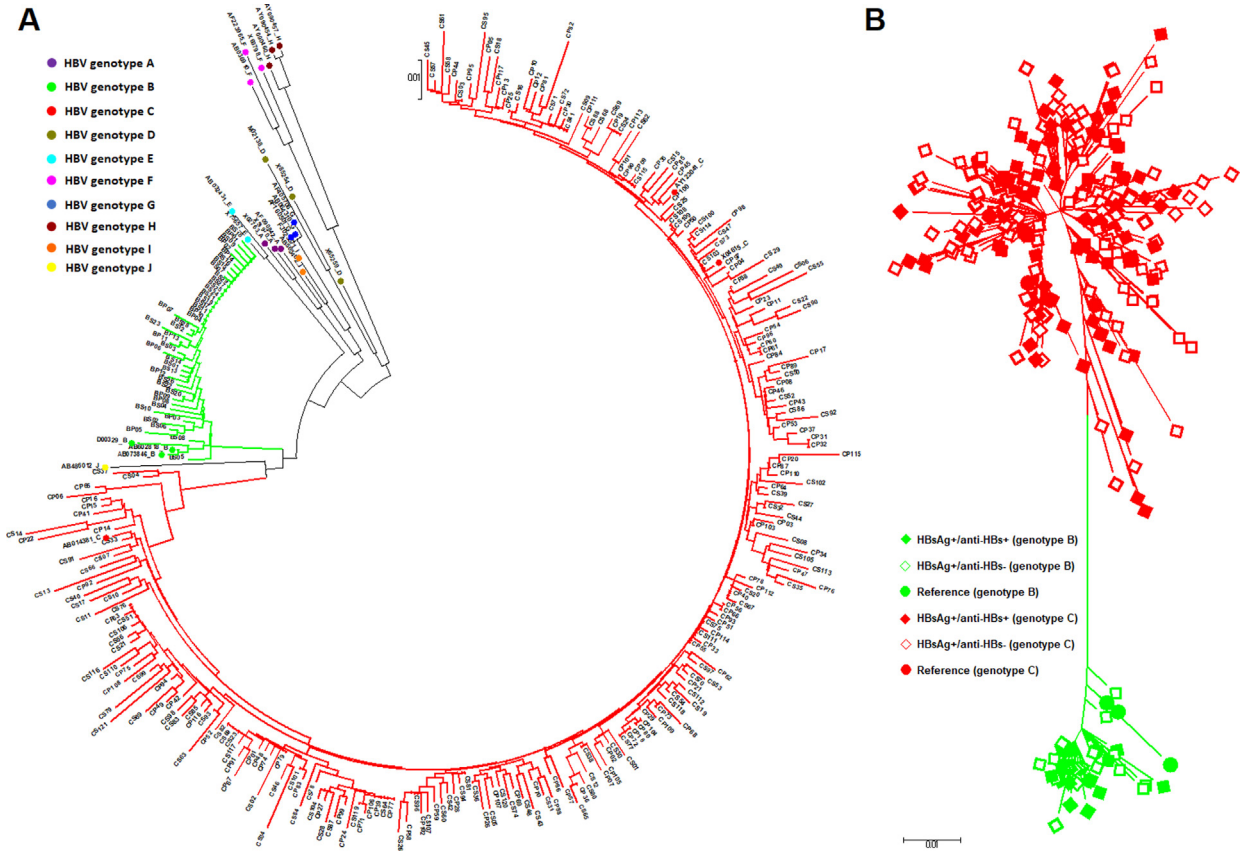

Figure 1 A. Phylogenetic tree is constructed by the neighbor joining method using MEGA6, based on the sequences of HBV *S* gene in 281 CHB patients including 131 HBsAg+/anti-HBs+ patients and 150 HBsAg+/anti-HBs− patients. Genotype and Genbank accession number, indicated with different colored dots, are shown in each branch of the references. Subgroup B and subgroup C are indicated in green and red, respectively. B. The distribution of the patients with different genotypes from the case and the control group is indicated in a maximum likelihood phylogenetic tree.

Analysis of amino acid substitutions within the ''a'' determinant region

The sequences of the ''a'' determinant region were translated into amino acid sequences and compared with the reference sequences of the corresponding genotypes, as shown in Supplementary Fig. 7. Forty HBsAg+/anti-HBs+ patients and 19 HBsAg+/anti-HBs− patients had amino acid substitutions within the ''a'' determinant region, respectively. Among those HBsAg+/anti-HBs+ patients, 36 patients carried single substitution and 4 patients carried multiple substitutions. While among those HBsAg+/anti-HBs− patients, 16 patients carried single substitution and 3 patients carried multiple substitutions. There were not significant effects of age, gender as well as genotype on the substitution prevalence in HBsAg+/anti-HBs+ patients and HBsAg+/anti-HBs− patients (Fig. 2A−C). No significant differences were found regarding the mean age between the patients with substitution and those without substitution (Fig. 2D−M).

The proportions of amino acid substitutions within ''a'' determinant were then compared between the HBsAg+/anti-HBs+ patients and HBsAg+/anti-HBs− patients. There were more amino acid substitutions in the HBsAg+/anti-HBs+ patients (30.5% vs. 12.7%, $P < 0.001$) (Fig. 3A). When stratified by genotype, there were more amino acids variability within the ''a'' determinant region in HBsAg+/anti-HBs+ patients with genotype C (29.1% vs. 12.4%, $P = 0.001$, Fig. 3C), but not genotype B (42.9% vs. 13.8%, $P = 0.084$, Fig. 3B). Higher proportion of substitutions in the HBsAg+/anti-HBs+ patients were observed regardless of gender and age (Fig. 3D−G). When stratified by the loop of ''a'' determinant region (aa 124-147), more amino acid substitutions were observed within the first loop (aa124-137) (Fig. 3A−G), but not in the second loop (aa139-147) (Fig. 3A−G), which was consistent with the results derived from the previous studies [3,6,9,10,15,18,21] (Supplementary Fig. 8).

Analysis of substitution sites within the ''a'' determinant region

In the current study, amino acid substitutions within ''a'' determinant were detected in HBsAg+/anti-HBs+ patients at positions s126, s127, s129, s130, s131, s133, s137, s140, s143, s144 and s145, as shown in Fig. 4 A and B. When stratified by genotype (Fig. 4C), substitutions in s127, s140 and s143 only occurred in patients with genotype B, while substitutions in s130, s137, s144 and s145 only occurred in

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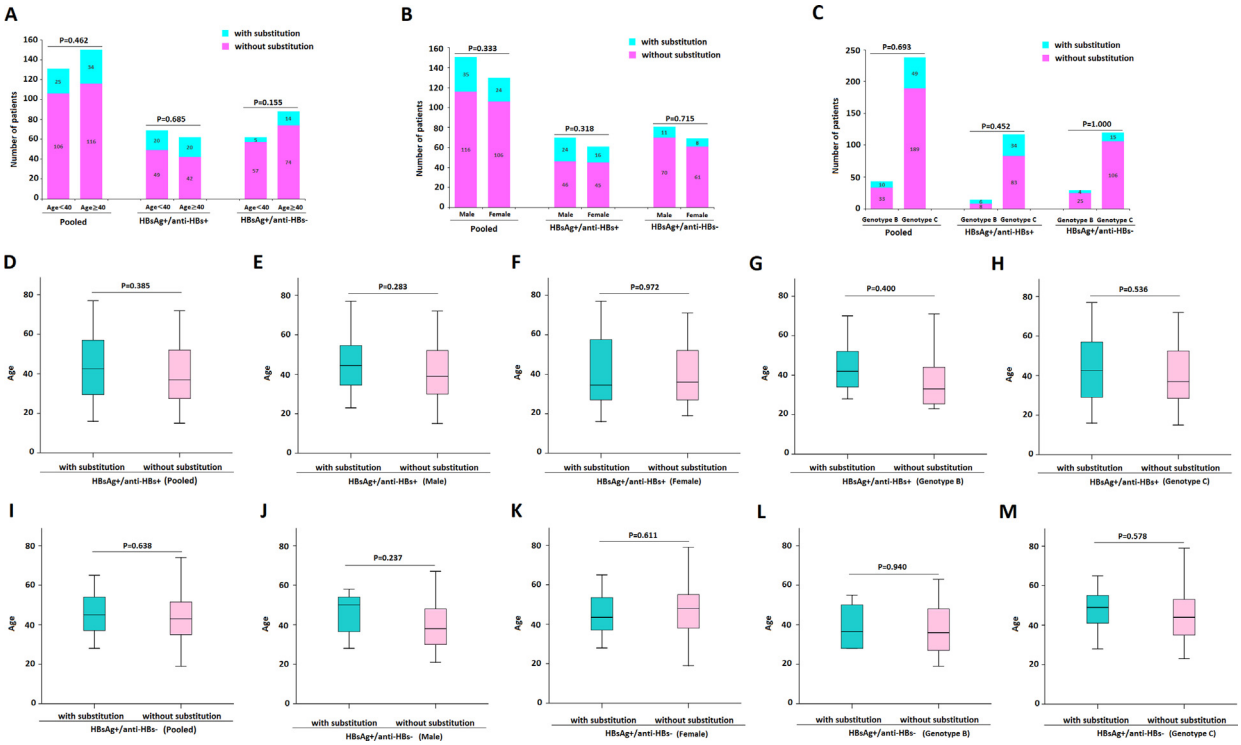

Figure 2 The effects of (A) age, (B) gender and (C) genotype on the substitution prevalence. D—H. Comparison of the mean age between the HBsAg+/anti-HBs+ patients with substitution and those without substitution. I—M. Comparison of the mean age between the HBsAg+/anti-HBs— patients with substitution and those without substitution.

genotype C patients. However, substitutions in s126, s129, s131 and s133 were observed both in genotype B and genotype C patients.

Compared with the control subjects, the most frequent substitution was located at position s126 in HBsAg+/anti-HBs+ patients ($P = 0.003$), especially in the patients with genotype C ($P = 0.011$) (Fig. 4D). Regarding position s126, the predominant substitution was sI126T (16, 64.0%), followed by sI126S (3, 12.0%), sI126N (2, 8.0%) and sI126V (2, 8.0%) (Fig. 4E and F). Most of the sI126T substitutions (14, 87.5%) belong to single amino acid substitution, only two (12.5%) in combination with other substitutions (sI126T/sG130N, sI126T/sM133T, respectively) (Fig. 4E and G).

Analysis of additional N-linked glycosylation sites

Five HBsAg+/anti-HBs+ patients were found harboring additional N-linked glycosylation sites (Fig. 5): sT126A/sT131N/sM133T/sT140I (Patient No. BP-01, Fig. 5A); sI126T/sG130N (Patient No. CP-15, Fig. 5B); sI126S/sT131N/sM133T (Patient No. CP-17, Fig. 5C); sQ129N (Patient No. CP-24, Fig. 5D); sG130N (Patient No. CP-26, Fig. 5E), while sQ129R/sG130N was only found in one HBsAg+/anti-HBs— patient (Patient No. CS-11, Fig. 5F). The frequency of additional N-glycosylation sites in HBsAg+/anti-HBs+ patients and the control subjects was 3.8% and 0.6%, respectively.

Discussion

In the present study, we attempted to investigate the characteristics of amino acid substitutions within the ''a'' determinant region in CHB patients with coexisting HBsAg and anti-HBs. As a result, we found there were more amino acid substitutions in HBsAg+/anti-HBs+ patients compared to the control subjects, especially within the first loop of the ''a'' determinant region. The most frequent amino acid substitution was located at position s126 and the predominant substitution was sI126T. Moreover, 3.8% HBsAg+/anti-HBs+ patients were found harboring additional N-linked glycosylation sites. Our findings are consistent with the previous observations [9,10,15,18,19,21,24] and provide new insights into the characteristics of amino acid substitutions within the ''a'' determinant region in patients with coexistence of HBsAg and anti-HBs in a large cohort of Chinese population. These results also have important implications for future work.

Firstly, Our results together with the data derived from previously reported studies (Supplementary Table.3) indicate that position s126 is a hotspot with higher frequency of amino acid substitutions among all the 24 positions in the ''a'' determinant region, while amino acid substitutions in position s139 has not been reported yet (Supplementary Table.3). Moreover, substitution sC137 is uncommon, which had been ever discovered as sC137S in one patient with genotype D [16] (Supplementary Table.3). To the best of our knowledge, the present study showed for the first time that

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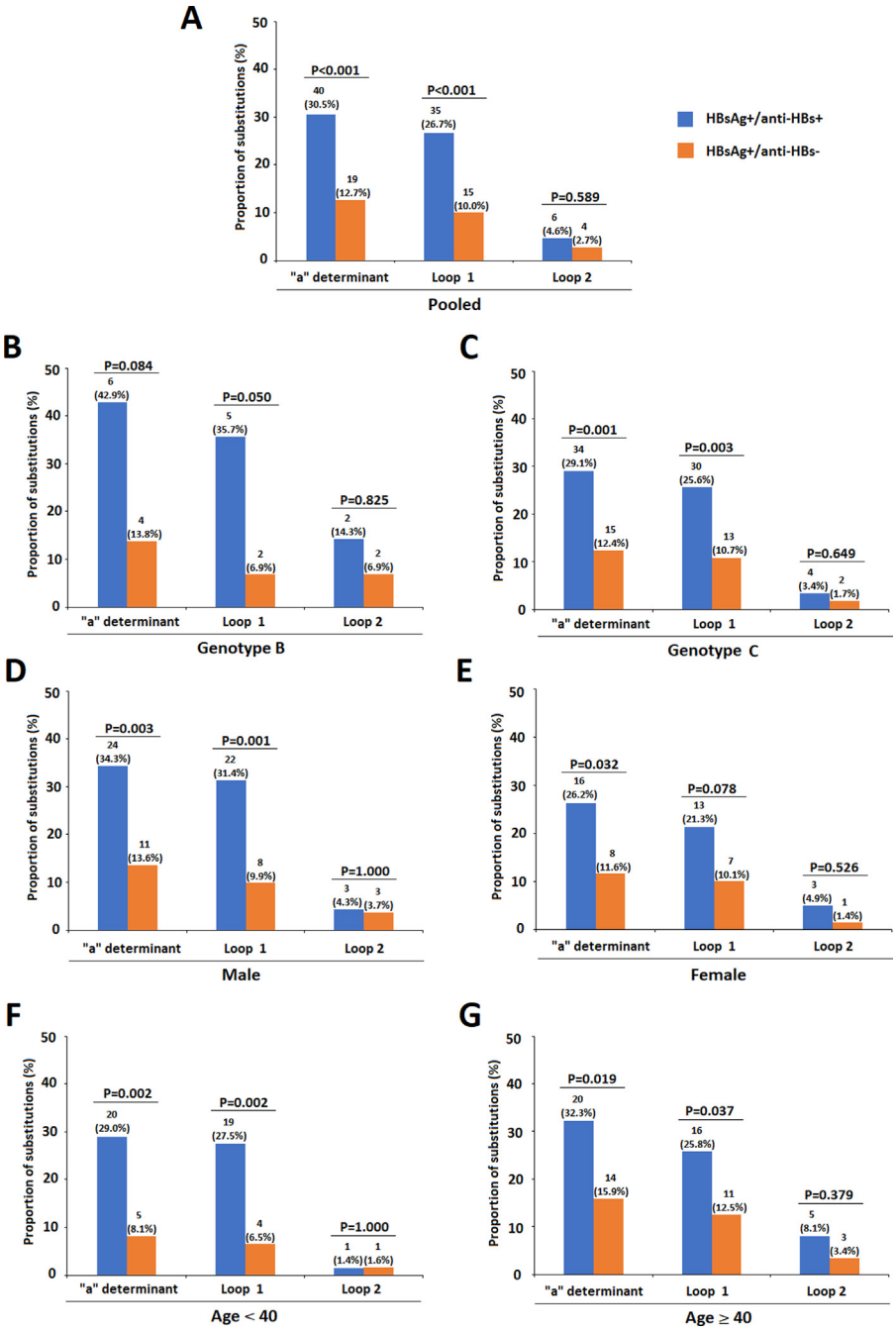

Figure 3 Comparison of the proportions of amino acid substitutions between HBsAg+/anti-HBs+ patients and HBsAg+/anti-HBs− patients. (A) Pooled, (B) genotype B, (C) genotype C, (D) Male, (E) Female, (F) Age<40, (G)Age≥40. Loop1: aa124-aa137; Loop2: aa 139- aa147.

sC137R occurred in HBsAg+/anti-HBs+ patients with genotype C. The exact mechanisms of different inclinations to develop amino acid substitutions in certain positions of the ''a'' determinant region warrants further investigation.

Secondly, biased amino acid substitution patterns in certain positions were identified (Fig. 4E and F). Regarding sI126, substitution sI126T predominated in HBsAg+/anti-HBs+ patients with genotype C, compared with other substitutions such as sI126S, sI126N and sI126V (Fig. 4E and F), which was consistent with most of the previous reports [6,9,10,15,17−19,21,22,24] (Supplementary Fig. 9). It would be of great interests to explore the exact roles of this substitution bias in clinical outcomes in future studies.

Thirdly, the preferred amino acid substitutions with genotypic heterogeneity was revealed herein as well as others [8,9,11,15,18,19]. The distribution of HBV genotypes varies geographically and ten genotypes of HBV have been identified and labelled A-J. In addition, natural history of HBV infection largely depends on the mode of transmission. It might be difficult to compare studies reporting different genotypes as they are directly linked to the mode of transmission, except in Asia where there is a large

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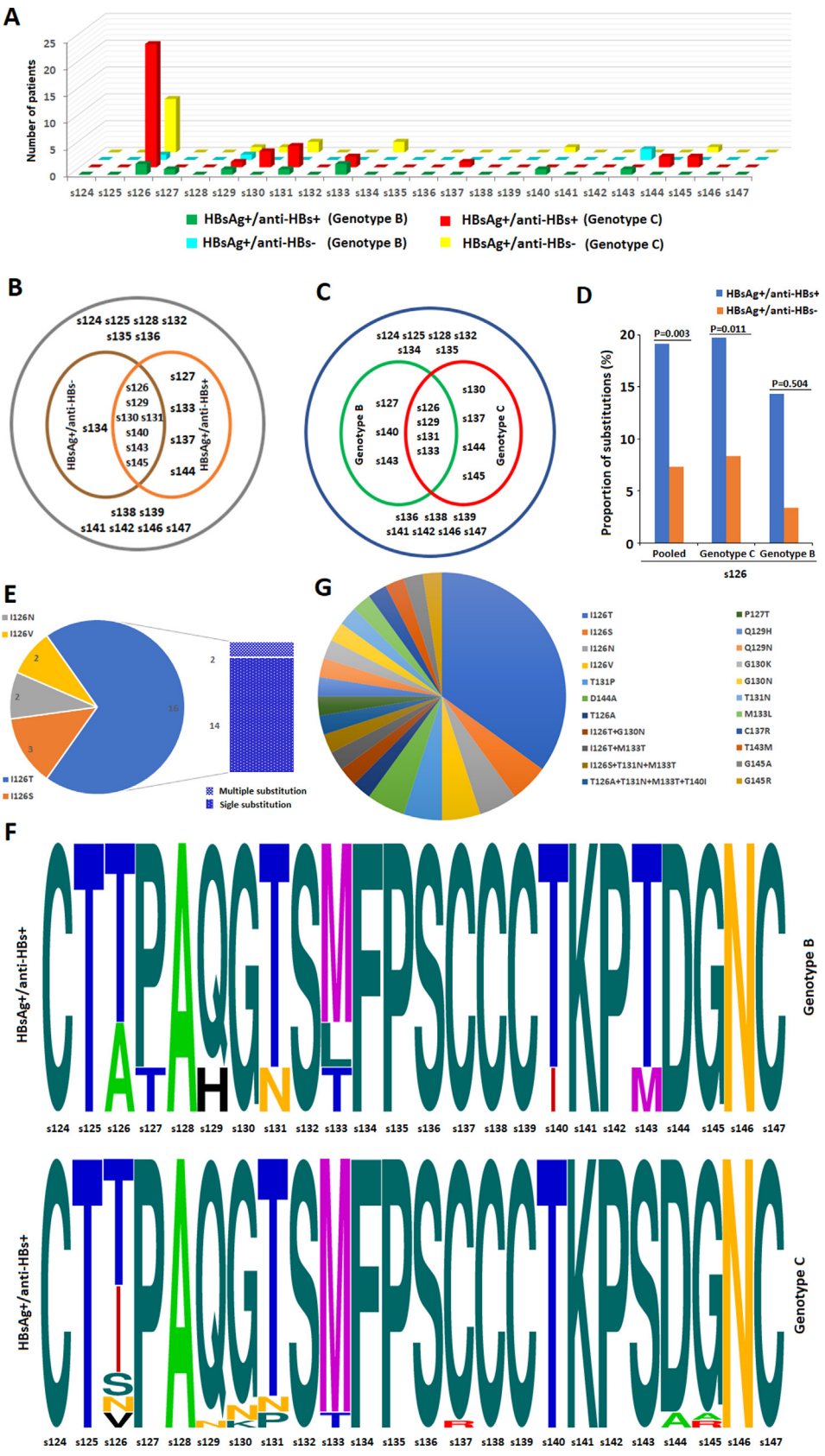

Figure 4 Analysis of substitution sites within the ''a'' determinant region. A. Comparison of the substitution positions in patients with genotype B and genotype C. B. Distribution of the substitution positions in HBsAg+/anti-HBs+ patients and HBsAg+/anti-HBs− patients. C. Distribution of the substitution positions in HBsAg+/anti-HBs+ patients with genotype B and genotype C. D. Comparison of the proportions of amino acid substitutions between HBsAg+/anti-HBs+ patients and HBsAg+/anti-HBs− patients regarding position

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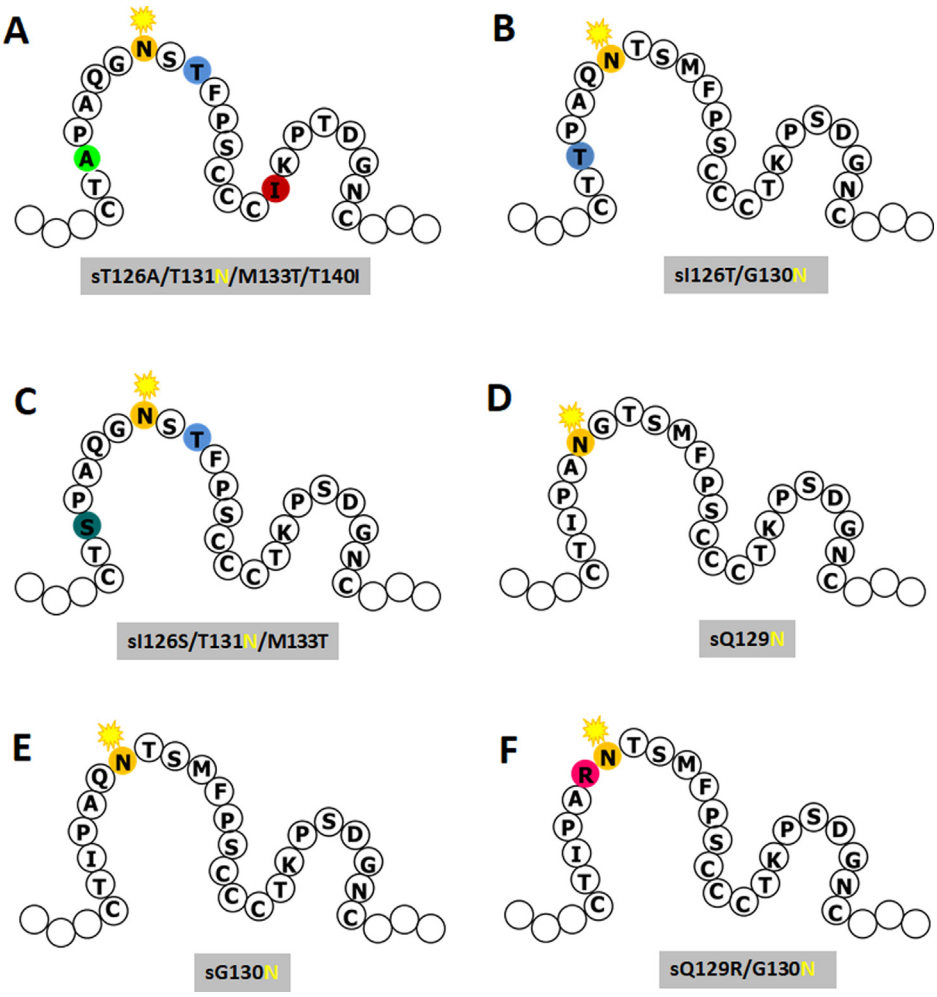

Figure 5 Analysis of additional N-linked glycosylation sites. Five HBsAg+/anti-HBs+ patients (A—E) and one HBsAg+/anti-HBs— patient (F) harbor additional N-linked glycosylation sites.

dominance of genotype C and B mostly acquired from mother to child infection. Different viral genotypes and subtypes which influencing the immunological status and selection of certain amino acid substitutions deserves further attention.

Fourthly, Our results demonstrated that additional N-linked glycosylation sites were formatted in HBsAg+/anti-HBs+ patients. Amino acid substitutions in position s129, s130 and s131 within the ''a'' determinant region might introduce an additional ''N-X-T/S'' motif for viral envelope N-linked glycosylation [14,23,24,30]. Whether these additional N-linked glycosylation sites could play a specific role in viral fitness and evolution warrants further investigation.

Last but not the least, among 131 HBsAg+/anti-HBs+ patients with sequencing data analyzed in this study, only 40 patients were found to have amino acid substitutions (Fig. 2). However, there were still 91 HBsAg+/anti-HBs+ patients without any amino acid substitutions within the ''a'' determinant region. So, there might be some other factors involved in this atypical serological pattern in chronic HBV infection [25—29]. In addition, most chronic HBV patients in China are infected at or shortly after birth, there should be no significant difference in age between patients HBsAg+/anti-HBs— and HBsAg+/anti-HBs+ since both types of patients are infected at the same age, as shown in Supplementary Fig. 2. It is not a case of HBsAg+/anti-HBs— patients suddenly starting to make anti-HBs after several years. With age the anti-HBs levels in HBsAg+/anti-HBs+ patients may diminish since the body is not being stimulated by the original neutralizing epitope. However, other minor non-neutralizing epitopes may maintain a certain level of anti-HBs. Though there was no significant correlation of anti-HBs levels and the age of the HBsAg+/anti-HBs+ in current

s126. E. Amino acid substitution patterns in position s126 in HBsAg+/anti-HBs+ patients. F. Graphical representation of amino acid substitution patterns within each position of the ''a'' determinant region in HBsAg+/anti-HBs+ patients using Weblogo. G. Distribution of substitution patterns in HBsAg+/anti-HBs+ patients.

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study (Supplementary Fig. 10), the longer period of follow-up study with more samples would be helpful to clarified this phenomenon. The exact mechanisms underlying the concurrent presence of HBsAg and anti-HBs need to be well elucidated in future work.

There are also some potential limitations in the study with descriptive character, principally limited to the same geographic and racial cases. In addition, clone-based sequencing and ultra-deep pyrosequencing might be more reliable tools to identify amino acid substitutions with relatively low frequency. Furthermore, the full disease picture of the enrolled patients and long-term follow-up are not available, which should be considered when interpreting the results.

In conclusion, our data indicate that the accumulation and diversity of amino acid variations within ''a'' determinant region might contribute to the coexistence of HBsAg and anti-HBs. These findings extend understanding of the genetic mechanism of this atypical serological profile in CHB patients.

Ethical approval

Ethical approval for this study was obtained from the Ethics Committee of Tianjin Second People's Hospital.

Disclosure of interest

The authors declare that they have no competing interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at: https://doi.org/10.1016/j.clinre.2019.08.005.

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