Temporal trend of hepatitis B surface mutations in the post-immunization period: 9 years of surveillance (2005–2013) in eastern China

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Limited information is available about the temporal trend in the prevalence and evolution of hepatitis B virus (HBV) S-gene mutations in the post-immunization era in China. From 2005 to 2013, 1077 hepatitis B cases under 15 years of age reported through Chinese National Notifiable Disease Reporting System (NNDRS) were successfully sequenced of S-gene in Shandong province, China. A total of 97 (9.01%) cases had amino acid (aa) substitution in the “α” determinant of HBsAg. The yearly prevalence from 2005 to 2013 maintained at a relatively stable level, and showed no significant change (P > 0.05).

Multivariate logistic regression analysis demonstrated that the prevalence of “α” mutations was independently associated with the maternal HBsAg status (P < 0.05), and not with surveillance year and hepatitis B vaccination (P > 0.05). The hottest mutation position was aa126 (I126S/N and T126A, 29.63%), and aa 145 (G145R/A, 25.93%). Mutated residue 126 tended to occur less frequent, while that of residue 145 was more frequent with increasing year. Our data showed that there was no increase in the frequency of HBV “α” mutations over time during the post-immunization period. However, long-term vaccination might enhance the change of HBV mutational pattern, and G145 mutation was becoming dominant.

Hepatitis B virus (HBV) infection and HBV-related complications remains a major global public health problem. An estimated 350 million population were chronically HBV infected, with 600,000 deaths annually related to HBV worldwide. In most HBV endemic areas, maternal-infant vertical transmission is a major route for the HBV chronic infection (approaching 90%). Interruption of early HBV transmission through effective immunization is the most cost-effective strategy for the prevention of primary infection.

The surface (S) region of HBV surface antigen (HBsAg), utilized in current recombinant hepatitis B vaccine (HepB), contains a highly conserved antibody-neutralizing epitope cluster which spans amino acids (aa) 124–147 within the major hydrophilic region (MHR) of HBsAg, and is referred to as “α” determinant. The “α” determinant is considered to be in the form of two major loops with cysteine-disulfide bonds. It is well documented that neutralizing antibodies produced during natural infection, or following active or passive immunization against HBV are targeted to the conformational epitopes of the “α” determinant. Hence, single or multiple mutations occurring within this region can lead to conformational changes with altered antigenicity and can allow replication of mutated HBV in vaccinated population, posing a potential threat to the long-term success of massive vaccination. Such mutations are generally behaved as vaccine escape mutants (VEMs). Early investigations have revealed that VEMs were mainly occurred in the “α” determinant. G145R mutation was the first identified VEMs to be described in 1990, and later several other notable VEMs (I/T126S, Q129H, G130N, D144A, G145A) at different positions associated with HBV breakthrough infections have also been repeatedly documented worldwide. Apart from causing immune failure, VEMs are found in association with HBV occult infection, and

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also have been proven to reduce the binding of antibodies raised against wild-type small envelop protein under experimental conditions. In China, HepB was recommended to infants since 1992 and was introduced into the national children immunization program in 2002, with the prevalence of HBsAg among the children under 5 years of age decreasing from 9.67% in 1992 to 0.96% in 2006. With the successful control of HBV achieved by massive hepatitis B vaccination, one of the remaining concerns might focus on the issue of VEMs, which may potentially challenge the global prevention program and the eventual eradication of HBV infection.

Shandong province is located in the eastern part of China and covers an area of 156,700 km², which is inhabited by a population of 95.79 million (2010 census data). With the implementation of national immunization program, HBsAg prevalence among children aged 1–14 years has dramatically decreased from 8% in 1992 to 1.36% in 2006. Since 2005, surveillance of hepatitis B case under 15 years of age has been implemented in the province. We used the blood samples collected in this surveillance system during 2005–2013 to analyze the trend of the prevalence and pattern of HBV S-gene mutations in the era of massive hepatitis B vaccination, evaluating the influence of the current universal vaccination immunization program on HBV surface mutations.

**Methods**

**Study population and sample collection.** The surveillance on hepatitis B cases was initiated in all 140 counties of Shandong province since 2005. The definition of hepatitis B case was based on clinical and serological criteria in the hospital. After being reported through Chinese National Notifiable Disease Reporting System (NNDRS), all hepatitis B cases aged 0–14 years, including acute and chronic cases, were investigated using a standard questionnaire through interviewing with their parents by the staffs of the Center for Disease Control and Prevention (CDC) at county level. All investigators were trained at provincial CDC before the investigation. The cases’ demographic information (age, gender and ethnicity), hepatitis B vaccination history, clinical characteristics and epidemiological risk factors (maternal HBsAg status, history of blood transfusion and operation, and so on) were collected. Hepatitis B vaccination history was obtained according to immunization certificate, if not, by parents’ description. Blood samples were collected for each case in the hospitals and then transferred to the provincial CDC and stored at −70 °C until use.

The ethical approval was given by Ethics Review Committee of the Shandong CDC, and the study was conducted in accordance with the ethical standards of the Declaration of Helsinki. Written informed consents for the use of their clinical samples were obtained from the children's legal guardians.

**HBV DNA extraction and nested polymerase chain reaction.** HBV DNA was extracted from 200 ul serum with QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions. DNA was amplified by two primers, HBVSF1 (sense, nucleotides [nt] 203–221: 5′-CCTGTATTTTCCGTGCTGGTGGCTCC-3′) and HBVSR1 (antisense, nt 1002–1022, 5′-GCAGCAAGCCCAAAGAGCC-3′) in a 50-UL reaction. Amplification conditions were as follows: 94 °C for 5 min, then 35 cycles at 94 °C for 35 s, 58 °C for 30 s, 72 °C for 35 s, and finally 72 °C for 10 min. Amplified products were visualized by 1.5% agarose gel electrophoresis stained with GelRed™ and evaluated under UV light. Samples that were found to be negative in the one-step PCR were amplified by two-step PCR using HBVSF2 (sense, nt 196–213: 5′-GGTACAGCCGGGTTTTC-3′) and HBVSR2 (antisense, nt 860–881, 5′-GCCATGAAGTTAAGGGAGTAGC-3′) inner primers in second round. The amplification conditions were the same as in the one-step PCR. All necessary precautions to prevent cross-contamination were strictly followed, and negative controls were included in each step of the molecular assays.

**DNA sequencing and mutation analysis.** The positive PCR products were purified and sequenced directly with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Sequences were analyzed by an ABI Prism 3130X genetic analyzer (Applied Biosystems). The HBV genotype was identified by phylogenetic analysis using neighbor-joining method after estimation of genetic distance using the Kimura two-parameter method (Mega, v4.0), including 28 HBV sequences of different genotypes (A–H) obtained from GenBank. The reliability of the phylogenetic tree was tested using the bootstrap test with 1,000 replicates. The HBV genotype was identified by translation of the nucleotide sequence according to the S open reading frame (ORF). Amino acid mutations were identified by comparison of the target sequence with HBV reference sequences of the same genotype. The sequences obtained were submitted to GenBank with the accession numbers KX067076-KX067778.

**Statistical analysis.** Comparison of categorical variables among different groups was analyzed using χ² test or Fisher's exact test, as appropriate. The χ² test for trend was used to analyze the trend of proportions with year. A logistic regression model was used to examine associations between the prevalence of “α” mutations and putative risk factors. All statistical tests were two sided. P value < 0.05 was considered statistically significant. All statistical analysis was performed by SPSS 13.0 for Windows (SPSS Inc., IL).

**Results**

**HBV DNA profile.** From 2005 to 2013, a total of 3120 cases of hepatitis B under15 years of age (average age: 9.4 ± 4.2 years, male/female ratio: 2.05/1) were reported by NNDRS in Shandong province, China. The case number decreased with year during this period. Blood samples were collected from 2194 (70.32%) of them. Due to an insufficient volume of serum, HBV-DNA extraction was completed only for 1248 (56.88%, 1248/2194) cases, with S gene successfully sequenced in 1077 cases (average age: 9.4 ± 4.5 years, male/female ratio: 2.49/1) (Fig. 1).
Genotypes and serotypes of HBV strains. Phylogenetic analysis of S gene indicated that three HBV genotypes were circulating in Shandong province. Genotype C was predominant, accounting for 93.59% (1008/1077); followed by genotype B for 6.13% (66/1077) and genotype D for 0.28% (3/1077). No other genotypes were detected. The majority of HBV strains belonged to HBsAg subtype Adra+ (86.67%, 955/1077) based on Lys122, Arg160, Val177 and Pro178; four strains (0.37%) were specified as Adra-, based on Lys122, Arg160 and Ala177; ninety-six strains (8.91%) belonged to adw2, based on Lys122, Lys160 and Pro127; three strains (0.29%) were specified as adw3, based on Lys122, Lys160 and Thr127; thirteen strains (1.21%) belonged to ayr, based on Arg122 and Arg160; six strains (0.56%) belonged to ayr1, based on Arg122, Lys160, Pro127, Phe134 and/or Ala159.

Prevalence of “α” determinant mutations. In 1077 acquired sequences, a total of 97 (9.01%, 97/1077) strains had aa substitution in the “α” determinant region of HBsAg. The yearly prevalence of “α” mutations during 2005–2013 was 6.11% (8/131), 9.90% (10/101), 9.73% (11/113), 6.62% (9/136), 9.22% (19/206), 9.33% (7/75), 12.75% (13/102), 10.26% (8/78), and 8.89% (12/135), respectively, showing no statistically significant difference.

To further investigate the profiles of “α” determinant mutations, 403 cases with missing information on hepatitis B vaccination and/or maternal HBsAg status were excluded. Table 1 listed the virological characteristics of the remaining 674 cases with the stratification by the vaccination status and maternal HBsAg. Surveillance year, cases’ age, gender, HBeAg status, hepatitis B vaccination, maternal HBsAg status, and HBV genotypes were included in the multivariate regression model (Table 2). It was found that maternal positive-HBsAg was a risk factor independently associated with the occurrence of “α” determinant mutations, while the other factors including cases’ age, gender, HBeAg status, hepatitis B vaccination and HBV genotype were not associated.

Fifty-seven out of 674 cases with complete information had “α” mutations. Documented anti-HBs titer and HBV DNA levels were available in 42 cases among them. Of these, five cases (No. 11, 24, 29, 33, 43) had a protective level of anti-HBs (138.8, 182, 120, 21.78 and 123.38 mIU/ml, respectively). Quantification of HBV DNA levels were available in 42 cases among them. Of these, five cases (No. 11, 24, 29, 33, 43) had a protective level of anti-HBs (138.8, 182, 120, 21.78 and 123.38 mIU/ml, respectively). Quantification of HBV DNA levels were available in 42 cases among them. Of these, five cases (No. 11, 24, 29, 33, 43) had a protective level of anti-HBs (138.8, 182, 120, 21.78 and 123.38 mIU/ml, respectively). Quantification of HBV DNA levels were available in 42 cases among them. Of these, five cases (No. 11, 24, 29, 33, 43) had a protective level of anti-HBs (138.8, 182, 120, 21.78 and 123.38 mIU/ml, respectively). Quantification of HBV DNA levels were available in 42 cases among them. Of these, five cases (No. 11, 24, 29, 33, 43) had a protective level of anti-HBs (138.8, 182, 120, 21.78 and 123.38 mIU/ml, respectively). Quantification of HBV DNA levels were available in 42 cases among them. Of these, five cases (No. 11, 24, 29, 33, 43) had a protective level of anti-HBs (138.8, 182, 120, 21.78 and 123.38 mIU/ml, respectively). Quantification of HBV DNA levels were available in 42 cases among them. Of these, five cases (No. 11, 24, 29, 33, 43) had a protective level of anti-HBs (138.8, 182, 120, 21.78 and 123.38 mIU/ml, respectively). Quantification of HBV DNA levels were available in 42 cases among them. Of these, five cases (No. 11, 24, 29, 33, 43) had a protective level of anti-HBs (138.8, 182, 120, 21.78 and 123.38 mIU/ml, respectively). Quantification of HBV DNA levels were available in 42 cases among them. Of these, five cases (No. 11, 24, 29, 33, 43) had a protective level of anti-HBs (138.8, 182, 120, 21.78 and 123.38 mIU/ml, respectively). Quantification of HBV DNA levels were available in 42 cases among them. Of these, five cases (No. 11, 24, 29, 33, 43) had a protective level of anti-HBs (138.8, 182, 120, 21.78 and 123.38 mIU/ml, respectively). Quantification of HBV DNA levels were available in 42 cases among them. Of these, five cases (No. 11, 24, 29, 33, 43) had a protective level of anti-HBs (138.8, 182, 120, 21.78 and 123.38 mIU/ml, respectively). Quantification of HBV DNA levels were available in 42 cases among them. Of these, five cases (No. 11, 24, 29, 33, 43) had a protective level of anti-HBs (138.8, 182, 120, 21.78 and 123.38 mIU/ml, respectively). Quantification of HBV DNA levels were available in 42 cases among them. Of these, five cases (No. 11, 24, 29, 33, 43) had a protective level of anti-HBs (138.8, 182, 120, 21.78 and 123.38 mIU/ml, respectively).
B vaccination coverage has expanded to almost all children in China. Our finding revealed that the prevalence of "a" mutations maintained at a relatively low and stable level, and showed no significant change during the consecutive 9-year of the massive vaccine period, similar to a previous report in Taiwan. Multivariate logistic regression analysis showed that maternal HBsAg positivity (OR: 1.82, 95% CI: 1.01–3.26) was significantly associated with a decreased risk of "a" determinant mutations. This finding suggests that the high coverage of B vaccination in China may have contributed to the maintenance of low prevalence of "a" mutations. Further studies are needed to explore the long-term impact of vaccination on the genetic diversity of HBV in China.

Table 1. Prevalence of "α" determinant mutations among hepatitis B cases according to selected characteristics.

Table 2. Factors associated with the occurrence of "α" determinant mutations in multivariate logistic regression among hepatitis B cases aged 0–14 years. Odds ratio (OR) and 95% confidence interval (95% CI) for OR were calculated to assess the association between the occurrence of "α" determinant mutations and relevant factors. The statistical significance level of P < 0.05 was used.

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regression analysis also revealed that the prevalence of “α” mutations was not associated with the surveillance year, indicating that the universal infant immunization did not obviously accelerate the frequency of HBV “α” mutations over time in the post-immunization era, and also suggested a low transmissibility of the mutational viruses. However, it has been reported recently that early universal immunization might accelerate the “α” mutations in vaccinated children. Due to lack of the baseline data during pre-vaccination period in Shandong province, our study could not compare the epidemiological difference of “α” mutations before and after the massive vaccination. Further, our study showed no difference between the children with and without immunization, like the findings in four Pacific Island countries and in Thailand. It is possible that the frequency of “α” mutations was not tightly related to the single vaccination, and most of these mutations might emerge spontaneously. Because of the small sample size of unvaccinated children in the study, it is essential to conduct a further investigation with increasing sample size.

The present study found a much higher prevalence of “α” mutations among children with HBsAg-positive mother compared with those whose mother was HBsAg-negative. The reason might be the fact that most children born to HBsAg-positive mother were given additional hepatitis B immunoglobulin (HBIG) besides three doses of hepatitis B vaccine to interrupt HBV vertical transmission. Due to lack of the baseline data during pre-vaccination period in Shandong province, our study could not compare the epidemiological difference of “α” mutations before and after the massive vaccination. Further, our study showed no difference between the children with and without immunization, like the findings in four Pacific Island countries and in Thailand. It is possible that the frequency of “α” mutations was not tightly related to the single vaccination, and most of these mutations might emerge spontaneously. Because of the small sample size of unvaccinated children in the study, it is essential to conduct a further investigation with increasing sample size.

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Based on an alarming number of sequences obtained in the present study, several important findings were observed concerning the mutation patterns. Of the 11 positions where mutations occurred in “α” determinant region, the most frequent mutation position was aa 126 (I126S/N and T126A, 29.63%), and 145 (G145R/A, 25.93%), all of which were known to act as VEMS, accounting for more than half of the total mutation strains with the “α” determinant. So, aa 126 and 145 might be the hottest mutation positions among HBV-infected children. Residue 126 is located in the 1st loop structure of the “α” determinant, and aa changes in this region may lead to HBV adaptive evolution, resulting in the changes of antigenicity and virulence increased. Mutations at aa 126 had been found frequently in HBV isolates from patients with chronic HBV infection with

### Table 3.
Amino acid mutations in the “α” determinant of HBsAg from genotype C among HBV-infected children. Amino acid aa mutants were presented based on their location in the archived GenBank reference AF473543. Frequency reflected the number of cases in which the mutations was detected. Unreported mutants were considered novel.

| Amino acid mutations | Frequency | Percentage (%) | Reported function | Reference |
|----------------------|-----------|----------------|-------------------|-----------|
| I126S                | 24        | 24.49          | Vaccine escape mutant | 22        |
| I126N                | 6         | 6.12           | Vaccine escape mutant | 22        |
| P127T                | 10        | 10.2           | Unclear            | 41        |
| A128V                | 1         | 1.02           | lower reactivity in HBsAg assay | 13, 42   |
| Q129H                | 3         | 3.06           | Vaccine escape mutant | 23        |
| G130E                | 1         | 1.02           | lower reactivity in HBsAg assay | 13        |
| T131P                | 7         | 7.14           | Unclear            | 14        |
| T131N                | 4         | 4.08           | Rescue of virion secretion | 15        |
| M133T                | 4         | 4.08           | Rescue of virion secretion | 15        |
| M133H                | 2         | 2.04           | Unclear            | 22        |
| S145L                | 1         | 1.02           | Vaccine escape mutant | 24        |
| D144A                | 3         | 3.06           | Vaccine escape mutant | 25        |
| D144E                | 3         | 3.06           | Vaccine escape mutant | 26        |
| D144N                | 1         | 1.02           | Unclear            | Novel     |
| G145A                | 23        | 23.47          | Vaccine escape mutant | 27        |
| G145R                | 5         | 5.1            | Vaccine escape mutant | 9         |

### Table 4.
Amino acid mutations in the “α” determinant of HBsAg from genotype B among HBV-infected children. Amino acid aa mutants were presented based on their location in the archived GenBank reference FJ562246. Frequency reflected the number of cases in which the mutations was detected.

| Amino acid mutations | Frequency | Percentage (%) | Reported function | Reference |
|----------------------|-----------|----------------|-------------------|-----------|
| T126A                | 2         | 20.00          | Vaccine escape mutant | 22        |
| P127T                | 3         | 30.00          | Unclear            | 41        |
| Q129H                | 2         | 20.00          | Vaccine escape mutant | 23        |
| M133T                | 1         | 10.00          | Rescue of virion secretion | 15        |
| F134L                | 1         | 10.00          | lower reactivity in HBsAg assay | 13        |
| D144A                | 1         | 10.00          | Vaccine escape mutant | 25        |
or without immunization, and most of these mutations were considered to be natural occurrence\(^8\). Residue 145 is located in the 2nd loop structure, known to be the best-described escape mutant position. G145 mutation is highly associated with vaccination selection, and neutralizing antibody induced by vaccination is no longer recognizes the mutated epitope\(^9,27\). The well-known G145R/A mutations were often found in vaccination failure settings\(^10\). Again, an in vitro model revealed that HBV with a G145R mutation shared the replication efficacy with equal to wild-type virus\(^36\).

Our study found mutated residue 126 tended to occur less frequent, while that of aa 145 was more frequent with increasing year. It was largely attributable to an increasing massive vaccination and favoring selective pressure on HBV, and accordingly indicating that the substitutions in the aa positions 145 were more associated with anti-HBs immune pressure than those in other epitope sites of “α” determinant region. Our results strongly suggested although the prevalence of “α” determinant mutations showed no significant change in the post-immunization era, the patterns of these mutations had been remarkably changed under the pressure of vaccination. As a result, G145 mutation was becoming more and more dominant with the vaccination selection and needed to be incorporated into future vaccine design.

Four other types of VEMs (Q129H, S143L, D144A, D144E), which were known to be associated with virus secretion and lower reactivity in HBsAg assays, were also detected\(^23–26\). Additionally, a small amount of P127T, A128V, G130E, T131P/N, M133T/I, F134L and D144N mutations was observed in our study, including the less frequent T131N/M133T double mutations. T131N/M133T can create a novel N-linked glycosylation site, and play a role in some HBV immune escape mutants\(^15\). Further biological functional analyses of these mutations are needed to determine whether they are able to escape existing vaccines. To the best our knowledge, D144N is a new identified mutation in the present study.

So far, it is still uncertain when these mutations emerged, or their duration in circulation. A previous study proved that more than half of HBV-infected children could spontaneously lose the predominance of circulatory mutant virus by reversion to wild-type\(^37\). However, Hsu et al. confirmed that G145R virus could circulate as a stable strain for 8 years of follow-up\(^38\). Such mutations could also contribute to HBV transmission in children born to HBsAg-positive mothers and vaccinated individuals\(^39,40\).

There were still several limitations in our study. The data related to the administration of HBIG backgrounds in the studied children was not been collected. No children with HBV occult infection were involved in our study, who were reported to harbor even more mutations\(^12\). The point mutations of “α” determinant were detected by direct sequencing instead of cloning and sequencing assay and quasispecies mutations might be ignored in this study, which led to the underestimation of actual prevalence of “α” determinant mutations.

In conclusion, the present study revealed that the prevalence of “α” determinant mutations remained stable in post-immunization period. However, long-term hepatitis B vaccination enhances the change of epidemic types in “α” determinant mutations. G145 mutation was becoming dominant. Given the facts that hepatitis B vaccine, HBIG and antiviral drug would more and more widely used in the future and possibility of the transmission of such “α” determinant mutation has not be complete ruled out, an ongoing surveillance with longer years is desperately needed.

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Author Contributions
A.X. and L.Z. conceived and designed the study, B.Y. drafted the paper, J.L. and J.I.L. were responsible for data collection, B.Y., Y.F. and F.J. performed the experiments, and L.Z. helped to interpret results and contributed to the writing. All authors reviewed the manuscript.
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