The possible role of S gene mutations of hepatitis B virus in intrauterine transmission

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Research

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Abstract

Background: Many hepatitis B virus (HBV) substances could inevitably enter fetuses and occurred neonatal intrauterine transmission. HBV often occurs mutation, especially S gene, and may lead to different outcomes on intrauterine transmission. We explored the associations between HBV S gene mutations of hepatitis B surface antigen positive (HBsAg-positive) mothers and intrauterine transmission.

Methods: A total of 399 HBsAg-positive mothers and neonates were recruited and their general demographic information was collected between June 2011 and July 2013. The mothers with HBV DNA levels ≥ 10^6 IU/ml were selected, 22 mothers whose neonates occurred HBV intrauterine transmission were in the HBV intrauterine transmission group (GT) and 22 mothers were randomly selected from the remaining controls were in the control group (GC). Maternal whole-genome HBV DNA was extracted, amplified, cloned, and sequenced. Obtained sequences were adjusted, genotyped, and analyzed for mutation rates. A case-control study was designed to analyze the relationship between mutations in the S gene of HBV and intrauterine transmission.

Results: Fifty-five neonates were found to have experienced intrauterine transmission (13.78%). Genotype B (4.55%), genotype C (88.64%) and inter-genotype B/C (6.81%) were found in the 44 HBsAg-positive mothers. The mutation rates of the S gene, in both genotypes B (0.58% vs 1.41%, $P = 0.040$) and C (7.56% vs 14.71%, P 0.001), were lower in group T than in group C. Missense substitutions such as L84I, P47S, K10Q, A41P, M133L, A60V, and I42T only existed in group C. The mutation rates of G73S, I126T, and I126S in group C were higher ($P < 0.001$, $P < 0.001$, $P = 0.010$). Deletions occurred in the S gene. The occurrence of intrauterine transmission with maternal mutation A90V was higher ($P < 0.001$). This may have increased the risk of neonatal HBsAg expression ($P = 0.022$).

Conclusions: The HBV S gene mutations of HBsAg-positive mothers may reduce the occurrence of HBV intrauterine transmission. It is possible for HBsAg-positive mothers infected with A90V to develop HBV chronic infection and transmit it to the fetus during pregnancy, resulting in neonatal HBV infection.

Background

Hepatitis B virus (HBV) infection is a major global obstacle to public health, with approximately 2.57 billion people infected, including cases of chronic hepatitis B (CHB) and hepatocellular carcinoma (HCC).(1) In China, intrauterine transmission is an important mode of mother-infant transmission in HBV infections because it cannot be fully blocked by hepatitis B vaccines and hepatitis B immunoglobulin (HBIG).(2, 3) The viral particles that enter the fetus during pregnancy change at different stages after birth, may disappear or make the neonates infected with HBV when the viruses continuous existence. The incidence of intrauterine transmission in China is 3.00%-43.33%,(4–6) of which 80%-90% of neonates(7, 8) develop chronic HBV infection after one year of age. In the past few decades, scholars have done much research to ascertain the complex mechanisms, but much remains unclear. Both the load and the characteristics of the virus may affect this process.(9, 10)

HBV is a small, enveloped 3.2-kb DNA virus with four open reading frames (ORFs): S gene, C gene, P gene, and X gene.(11) It is classified into eight genotypes (A to H).(12–15) The genotypes of B and C, which primarily transmit vertically, are most common in China; the genotype B is mainly found in central and southern China, whereas genotype C predominates in the northern areas.(16, 17) HBV has a higher mutation rate than other DNA viruses, owing to its lack of a proof-reading function for viral polymerase and the greatly increasing pressure of host immunities which cause sequence heterogeneity.(18, 19) So far, published data on the S gene, including the Pre-S1, the Pre-S2, and the S region,(11) which changes hepatitis B surface antigen (HBsAg) antigenicity, seems to be highly variable.(20) It is known that mutations of the S gene can directly affect the abilities of the HBV infection and its immunoresponse to hepatitis B vaccines. The “α” region (aa 124–147), one of the major mutated regions in the S region, as well as the Major Hydrophilic Region (MHR)(aa 32–76 and aa 100–160), are highly valuable in HBsAg detection and virus clearance as the most important functional epitopes.(21, 22) It seems more likely that the “mirror changes” of the genetic functions occur when overlapped with the reverse transcriptase (RT) region.(23) These mutations give rise to HBV immune escape, occult infection, resistance to drug therapy, and tolerance of immunoprophylaxis.(24–26)

Presently, little reports(27–30) showing that some S gene mutations are related to intrauterine infection. This study concentrates on the characteristics of the virus in neonates at birth, constitutes the first comprehensive analysis of the relationship between different maternal mutations of different genotypes (mutation hotspots and non-hotspots, and deletions) and intrauterine transmission before neonatal immunoprophylaxis. Further initially investigate the possible role of meaningful mutations in intrauterine transmission. There is hope that we will find virological evidence of intrauterine transmission. It will point us in the right direction for utilizing timely, effective, and targeted immunoprophylaxis to reduce the risk of HBV infection in neonates.

Methods

A total of 399 HBsAg-positive pregnant women, who were undergoing prenatal examinations and delivered babies between June 2011 and July 2013 in the Obstetrical and Gynecological department of the Third People’s Hospital of Taiyuan in Shanxi Province, China. The initial search criteria were being HBsAg positive and single neonates. The face-to-face interviews and electronic medical records were obtained. The study was reviewed and approved by the Ethics Committee of Shanxi Medical University, Taiyuan, China, and conducted in accordance with the Declaration of Helsinki. Informed written consent was obtained from all participants. Three ml maternal peripheral blood before delivery within 24 hours and three ml neonatal femoral venous blood within the 24 hours after birth (before giving the HBIG and hepatitis B vaccine) were collected.

Serological markers and serum HBV DNA assays

Serological markers were detected by electrochemiluminescence immunoassay (ECLIA) kits (Roche diagnostics GmbH, Germany) and expressed as a COI (COI: cut-off index). Serum HBV DNA levels were

tested by a real-time PCR-TaqMan kit (DAAN Gene Co. Ltd., Sun Yat-sen University, Guangdong, China) and expressed as IU/ml.

There is no exact definition of intrauterine transmission worldwide. We want to explore the characteristics of the HBV in the fetus as much as possible to achieve the purpose of studying the mechanism of intrauterine transmission. The definition we have chosen that excluded the effects of some factors after birth on the study, even if it was not possible to completely exclude it. HBsAg-positive (> 1.00 COI) and/or HBV DNA-positive (> 200 IU/ml) in neonatal femoral venous blood that within 24 hours of birth, before breastfeeding, and before giving the HBIG and hepatitis B vaccine was defined as HBV intrauterine transmission.(5)

HBV DNA extraction and amplification

We selected mothers with HBV DNA levels ≥ 10^6 IU/ml whose neonates were affected by intrauterine transmission as the HBV intrauterine transmission group (GT), and randomly select the same number of mothers in the remaining mothers with HBV DNA levels ≥ 10^6 IU/ml as the control group (GC). The maternal HBV DNA was extracted by the QIAamp DNA Mini kit (QIAGEN, Hilden, Germany).

Considering the heterogeneity of genome (quasi species), two HBV DNA fragments was amplified with the PCR amplification systems, including 3 μl of DNA template, 10 μl of 5 × *TransStart® FastPfu* buffer, 1 μl *TransStart® FastPfu* DNA polymerase, 4 μl of dNTPs (2.5 mM)(TransGen Biotech, Beijing, China), 30 μl ddH_2O, and 1 μl for each primer (10 μM) (Sangon Biotech, Shanghai, China) in a 50 μl reaction under the following conditions: initial denaturation at 94°C for five min, followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 68°C for 80 sec (DF/DR) or 110 sec (SF/SR), with a final extension of 10 min at 68°C (Table 1).

HBV DNA cloning and sequencing

PCR products were purified using the Gel Extraction Kit (OMEGA Bio-tek, Norcross, America), then cloned into the *pEASY®*-Blunt Zero vectors and transformed into Trans1-T1 Phage Resistant Chemically competent cells (TransGen Biotech, Beijing, China). Four or five positive clones of each fragment were chosen for sequencing (Sangon Biotech, Shanghai, China) (Table 1).

HBV DNA genotyping

The DNAStar software, SeqMan software (DNASTAR, Madison, WI, USA) (31) and Mega 6.0 software package were used to edit, splice, and compare with reference sequences downloaded from NCBI by constructing Neighbor-joining (NJ) phylogenetic trees, respectively. Kimura's two-parameter model calculated distances among the sequences.

(*Genotype A*: AF090842, X02763, X51970. *Genotype B*: D00329, AB073846, AB602818. *Genotype C*: AB014381, M12906, X04615. *Genotype D*: M32138, X65259, X85254. *Genotype E*: X75657, AB032431.

Genotype F: AB036910, AF223965, X69798. *Genotype G*: AF405706, AB064310, AF160501. *Genotype H*: AY090454, AY090457, AY090460).

Sequence analysis

A nucleotide substitution which means contrasted with the reference sequence. The mutation rate of the S gene was calculated by the number of mutation sites dividing the total sites, and more than ten percent were defined as mutation hotspots. Finally, a case-control study was used to analyze the association between S gene mutations and intrauterine transmission. GT was set as the case group and GC was set as the control group.

Statistical analysis

Data was analyzed with the SAS statistical package (Version 9.4, SAS Institute Inc, Cary, North Carolina, USA). Data for continuous variables is presented as mean ± standard deviation (SD) or as median and range, and differences between data were analyzed by a student's *t* test or Wilcoxon signed-rank test. The dichotomous data was compared by a Chi-square test or Fisher's exact test. Odds ratios (*OR*) and 95% confidence intervals (*CI*) were estimated using unconditional logistic regression to analyze the relationship between maternal factors and HBV intrauterine transmission. $P < 0.05$ was considered statistical significance for the test.

Results

Characteristics of subjects

A total of 399 pairs of HBsAg-positive mothers and neonates from Taiyuan (Shanxi Province, China) were recruited. They were all ethnic Han with mean ages of 27.7 ± 4.4 years (18 to 48 years). The mean ± SD (range) of maternal gestational weeks was 39.1 ± 1.1 weeks (35 to 42 weeks). The incidence of intrauterine transmission was 13.78% (55/399). There were 113 mothers with HBV DNA levels $\geq 10^6$ IU/ml, of which 22 were in GT, and 22 of the remaining 91 mothers were selected for GC. The mean ages of the 113 mothers were 25.3 ± 3.4 years (21 to 33 years) in GT and 27.8 ± 4.4 years (20 to 45 years) in GC ($t = 2.578$, $P = 0.011$, Student's *t* test). In the end, 44 HBsAg-positive mothers whose HBeAg tests were all positive were selected and the general characteristics were summarized in Table 2. The median ages of the 44 mothers were 24.0 years and 26.5 years in GT and GC, respectively (20 to 45 years)($P = 0.015$). One of the mothers in GC was 45 years old. There was difference in the mode of delivery distribution between the two groups ($P = 0.001$), but no significant statistical difference was observed in other characteristics of the mothers and neonates ($P > 0.05$).

HBV genotypes of sequences

A total of 442 HBV clones were obtained including 220 clones from GT and 222 from GC. The mean ± SD (range) number of clones per sample was 10 ± 1.3 (6 to 14). There was no difference between the two

groups (10 ± 1.7 vs 10 ± 0.4, $P = 0.814$). The genotype C accounted for the largest proportion of the clones, about 88.64% (39/44). Two mothers (4.55%) of genotype B and three mothers (6.81%) of inter-genotype B/C were found as well (Figure 1). No relationships between genotypes and intrauterine transmission were found (Fisher's exact test, $P = 0.622$).

Mutations of S gene

In genotype B, the mutation rate of GT was 0.58% (7/1203), lower than that of the GC (1.41%, 17/1203) ($\chi^2 = 4.21$, $P = 0.040$). The mutation rate in genotype C of GT was 7.56% (91/1203), lower than GC (14.71%, 177/1203)($\chi^2 = 31.06$, $P < 0.001$).

Mutation hotspots, L84I, P47S, K10Q, A41P, and M133L existed in GC; L85F, I4T, and F80S were only in GT. The mutation rate of A90V in GT was significantly higher than in GC ($P < 0.001$), but I68T was lower than in GC ($P = 0.310$) (Table 3). The mutations in genotype C below 10% were analyzed (Table 4). A60V and I42T only occurred in GC. The mutation rates of G73S, I126T, and I126S were all higher in GC ($P < 0.001$, $P < 0.001$, $P = 0.010$). The mutation rate of L98V was higher in GT, but no great statistical significance was observed ($P = 0.128$). Two mutations were found in the RT region that overlapped with the S region: C446G and T531G.

Deletions of S gene

Deletions were found in the Pre-S1 region. Eight clones in GT began at nt 2847-2850 (12 to 18 bp) and two clones began at nt 3047 (39 bp). Ten clones in GC began at nt 2847-2849 (12 to 21 bp) and ten clones began at nt 3016 (183 bp). Deletion rates in GC were not statistically higher than GT (4.5% vs 9.01%, $\chi^2 = 3.48$, $P = 0.062$).

The possible role of A90V in intrauterine transmission

We placed the S gene mutation hotspots as well as variables of the factors that were associated with intrauterine transmission into multivariate logistic regression models. Maternal HBeAg tests were equivalent between the two groups so that possible influence on intrauterine transmission could be eliminated. The study demonstrated that maternal mutation A90V could associated with a greater risk of intrauterine transmission (Table 5). It was observed that median neonatal HBsAg with maternal mutation A90V was higher than with the non-mutation (1.43 COI vs 0.65 COI, $Z = 2.30$, $P = 0.022$, Wilcoxon signed-rank test).

Discussion

Neonates born to HBsAg-positive mothers are more at risk of immunity failure and chronic infection with HBV, even after immunoprophylaxis. Many substances still inevitably enter fetuses, although the placental barrier can block some maternal HBV antigens. These substances may jeopardize neonatal immunity and cause lifelong dysfunction of the immune system.(32) These special groups have drawn

more attention from physicians and public health experts about whether they were affected by intrauterine transmission or not.

HBV has been constantly evolving and unpredictably mutating in the process of copying. HBV genotypes appear during this long-term accumulated mutation process. The major genotype among the forty-four mothers recruited from Taiyuan was C (88.63%), which was consistent with the distributions reported in northern China. Current reports(33) give different conclusions about the role of genotypes in intrauterine transmission. This study found the same possibility of causing intrauterine transmission. Further studies are needed to consider the associations between HBV genetic diversity and geographical distribution.

S protein (HBsAg), eliciting a host resistance to HBV infection, is often under high immune pressure and induces frequent mutations in the S gene.(34) Meanwhile, mutations in the S gene can alter the S protein in turn. Some mutations that occurred in HBsAg-positive mothers could change the HBsAg in neonates. Viral glycoproteins represent important targets for any antiviral immune response.(35) There are four promoters in the HBV genome (SP □, SP □, XP, CP) in which SP □ and SP □ encode HBsAg. Many mutations in the SP □ area (nt 2809 ~ 3152) were found in our study. K10Q, L84I, P47S, and A60V existed in the controls, while L85F appeared in GT. The mutation rate of G73S was higher in the controls, but A90V was the opposite. The mutation A90V could cause an increased risk of HBV intrauterine transmission associated with neonatal HBsAg. This result was different from Hai Cheng, et al,(28) whose research found that maternal A90V occurred less frequently in the HBV intrauterine infection group. However, Chen B.F. et al(36) supported that it was associated with HBV chronic infection. The study thinks that the mothers with A90V had the corresponding HBsAg structures that could not properly bind to the receptors of lymphocytes and arouse immune responses to clear the HBV virus. The long-term high replication of HBV, and changed structures of HBV which antigens are more likely to be transmitted to neonates during pregnancy, thus increases the risk of intrauterine transmission. The mothers are more likely to enter a state of chronic infection, which is also unfavorable for neonates. Mothers with the mutation A90V need to pay more attention because they may be prone to intrauterine transmission, and the same with L85F. We recommend them to take antiviral drugs during pregnancy to reduce HBV DNA, cut down the proteins expressed which could enter the fetus. Other mutations that mainly found in GC are indicate the changes in the structure of HBsAg caused by the mutation affect its assembly and secretion, making it unable to be expressed normally. Moreover, deletions in this area were found and may change HBV sequences, possibly causing interference or termination of HBsAg expression at the transcriptional level.(37) Even though no difference of deletion rate was found, it was higher in the GC still had clues.

The “α” region and the Major Hydrophilic Region (MHR), are two hydrophilic regions playing leading roles in detection of HBsAg and the clearing of viruses.(38) The mutations P47S, A41P, M133L, A60V, G73S, I42T, and I126T/S are found in GC. The mutation T531G overlapped with RT region. Some scholars have pointed out that the mutations in the RT region overlapping with the S region may cause changes in the epitope of the S protein(34) and may alter the antigenicity, immune recognition, and kinetics of viral replication, especially in “α” determinants.(10) Previous studies have held views that the mutation I126T affects the structure of HBsAg, that M133T is more inclined to appear in HBV occult infection and HCC,

(39) and that G145R correlatives with immune escape.(22, 40) These views provide some clues for our research. Maternal mutations in these areas that viral particles may not easily enter can cause neonates to lose the ability to assemble or normally secrete HBsAg, even if the viral particles enter them. Neonatal HBsAg tests in GC are still negative, which means HBsAg is lower than one COI; however, neonatal HBV DNA is still at a low level of replication and may become occult HBV infection in the future, which also requires our attention. For mothers, although the HBV DNA are at high levels, their expressed HBsAg structures have changed. Altered antigens have impaired the formation of the HBsAg-anti-HBs complex and have inhibited the immune response, resulting in immune escape. It is easier for this to turn into HBV chronic infection, which eventually leads to HCC, for it cannot be completely eliminated. Our baseline data also shows that HBV was detected earlier in the mothers.

It should be noted that although there were differences in maternal age between the two groups in Table 2, it may be due to the fact that a 45-year-old mother in GC. Meanwhile, we found that the mother had only two mutation sites in the S gene, which were A60V and I42T (non-hotspots), which indicated that the high mutation rates in GC was not because of the age. There are still some limitations: we only explored the S gene while the whole-genome mutations played their part in intrauterine transmission together. The results will be beneficial in guiding our later research to further explore the influence of mutations in other ORFs on intrauterine transmission.

Conclusions

In conclusion, this study has comprehensively explored the relationships between S gene mutations and intrauterine transmission. It was speculated that the mutation of A90V may be closely correlated with intrauterine transmission. L85F, I4T, and F80S may be useful biomarkers for predicting intrauterine transmission. L84I, A41P, M133L, P47S, A60V, and I42T were also first reported as biomarkers in this study. G73S and I126T/S may reduce the incidence of intrauterine transmission. It must be noted that this study was significantly different from the others. We mainly studied the genotype C and different types of mutations. The number of cloned sequences was somewhat larger and may have caused the different results we found. We may need to further confirm our discoveries in the genotype B.

Declarations

Ethics approval and consent to participate: The submitted work is original, not have been published elsewhere in any form or language, and not under consideration for publication elsewhere, in whole or be split up into several parts. Results were presented honestly and without fabrication, falsification or inappropriate data manipulation. All authors ensure the author group, the Corresponding Author, and the order of authors are all correct at submission. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee (the Ethics Committee of Shanxi Medical University, 2015LL073) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

Consent for publication: Informed written consent was obtained from all participants. The manuscript is approved by all authors for publication.

Availability of data and materials: The datasets generated and analyzed during the current study are not publicly available due the data will continue to do genetic and epigenetic analysis but are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests in this section.

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Authors' contributions: S.P Wang (The corresponding author) conceptualized, administrated the project and edited.

J.X Wu (The first author) curated the data, performed the software, analyzed, visualized and wrote the original draft. Y.L Feng administrated the project and edited language. Z.Q Yang curated the data, Investigated and performed the experiment. R.J Zhang performed the experiment. D.D Wang and L.Z Yi performed the experiment and visualized. S.Y Feng and B Wang investigated and supervised.

Acknowledgments: Not applicable.

Abbreviations

HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; HBsAg-positive, hepatitis B surface antigen positive; CHB, chronic hepatitis B; HCC, hepatocellular carcinoma; HBIG, hepatitis B vaccines and hepatitis B immunoglobulin; ORFs, open reading frames; aa, amino acid; MHR, Major Hydrophilic Region; RT, reverse transcriptase; ECLIA, electrochemiluminescence immunoassay; COI, cut-off index; HBeAg, hepatitis e antigen; nt, nucleotides; S protein, small protein.

References

1. Trepo C, Chan HLY, Lok A. Hepatitis B virus infection. Lancet. 2014;384(9959):2053-63.
2. Razavi-Shearer D, Gamkrelidze I, Nguyen MH, Chen DS, Van Damme P, Abbas Z, et al. Global prevalence, treatment, and prevention of hepatitis B virus infection in 2016: a modelling study. Lancet Gastroenterol. 2018;3(6):383-403.
3. Pan CQ, Duan ZP, Bhamidimarri KR, Zou HB, Liang XF, Li J, et al. An algorithm for risk assessment and intervention of mother to child transmission of hepatitis B virus. Clin Gastroenterol Hepatol. 2012;10(5):452-9.
4. Xu DZ, Yan YP, Choi BC, Xu JQ, Men K, Zhang JX, et al. Risk factors and mechanism of transplacental transmission of hepatitis B virus: a case-control study. J Med Virol. 2002;67(1):20-6.

5. Guo Z, Shi XH, Feng YL, Wang B, Feng LP, Wang SP, et al. Risk factors of HBV intrauterine transmission among HBsAg-positive pregnant women. J Viral Hepat. 2013;20(5):317-21.
6. Shao Q, Zhao X, Yao Li MD. Role of peripheral blood mononuclear cell transportation from mother to baby in HBV intrauterine infection. Arch Gynecol Obstet. 2013;288(6):1257-61.
7. Fattovich G, Bortolotti F, Donato F. Natural history of chronic hepatitis B: special emphasis on disease progression and prognostic factors. J Hepatol. 2008;48(2):335-52.
8. Liaw YF, Chu CM. Hepatitis B virus infection. The Lancet. 2009;373(9663):582-92.
9. Kazim SN, Wakil SM, Khan LA, Hasnain SE, Sarin SK. Vertical transmission of hepatitis B virus despite maternal lamivudine therapy. Lancet. 2002;359(9316):1488-9.
10. Tong S, Revill P. Overview of hepatitis B viral replication and genetic variability. J Hepatol. 2016;64(1 Suppl):S4-S16.
11. Tiollais P, Pourcel C, Dejean A. The hepatitis B virus. Nature. 1985;317(6037):489-95.
12. Okamoto H, Tsuda F, Sakugawa H, Sastrosoewignjo RI, Imai M, Miyakawa Y, et al. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. J Gen Virol. 1988;69 (Pt 10)(10):2575-83.
13. Norder H, Courouce AM, Magnius LO. Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. Virology. 1994;198(2):489-503.
14. Stuyver L, De Gendt S, Van Geyt C, Zoulim F, Fried M, Schinazi RF, et al. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. J Gen Virol. 2000;81(Pt 1):67-74.
15. Arauz-Ruiz P, Norder H, Robertson BH, Magnius LO. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. J Gen Virol. 2002;83(Pt 8):2059-73.
16. Zeng G, Wang Z, Wen S, Jiang J, Wang L, Cheng J, et al. Geographic distribution, virologic and clinical characteristics of hepatitis B virus genotypes in China. J Viral Hepat. 2005;12(6):609-17.
17. Yin J, Zhang H, He Y, Xie J, Liu S, Chang W, et al. Distribution and hepatocellular carcinoma-related viral properties of hepatitis B virus genotypes in Mainland China: a community-based study. Cancer Epidemiol Biomarkers Prev. 2010;19(3):777-86.
18. Chotiyaputta W, Lok AS. Hepatitis B virus variants. Nat Rev Gastroenterol Hepatol. 2009;6(8):453-62.
19. Sede M, Lopez-Ledesma M, Frider B, Pozzati M, Campos RH, Flichman D, et al. Hepatitis B virus depicts a high degree of conservation during the immune-tolerant phase in familiarly transmitted chronic hepatitis B infection: deep-sequencing and phylogenetic analysis. J Viral Hepat. 2014;21(9):650-61.
20. Xu H, Peng M, Qing Y, Ling N, Lan Y, Liang Z, et al. A Quasi species of the pre-S/S gene and mutations of enhancer II/core promoter/pre-C in mothers and their children infected with hepatitis B virus via mother-to-infant transmission. J Infect Dis. 2006;193(1):88-97.
21. Carman WF, Korula J, Wallace L, MacPhee R, Mimms L, Decker R. Fulminant reactivation of hepatitis B due to envelope protein mutant that escaped detection by monoclonal HBsAg ELISA. The Lancet.

1995;345(8962):1406-7.

22. Wu C, Deng W, Deng L, Cao L, Qin B, Li S, et al. Amino acid substitutions at positions 122 and 145 of hepatitis B virus surface antigen (HBsAg) determine the antigenicity and immunogenicity of HBsAg and influence in vivo HBsAg clearance. J Virol. 2012;86(8):4658-69.
23. Solmone M, Vincenti D, Prosperi MC, Bruselles A, Ippolito G, Capobianchi MR. Use of massively parallel ultradeep pyrosequencing to characterize the genetic diversity of hepatitis B virus in drug-resistant and drug-naive patients and to detect minor variants in reverse transcriptase and hepatitis B S antigen. J Virol. 2009;83(4):1718-26.
24. Carman WF, Zanetti AR, Karayiannis P, Waters J, Manzillo G, Tanzi E, et al. Vaccine-induced escape mutant of hepatitis B virus. Lancet. 1990;336(8711):325-9.
25. Raimondo G, Pollicino T, Cacciola I, Squadrito G. Occult hepatitis B virus infection. J Hepatol. 2007;46(1):160-70.
26. Zou H, Chen Y, Duan Z, Zhang H, Pan C. Virologic factors associated with failure to passive-active immunoprophylaxis in infants born to HBsAg-positive mothers. J Viral Hepat. 2012;19(2):e18-25.
27. Su HX, Xu DZ, Li D, Zhang JX, Lu J, Choi BC, et al. Heterogeneity analysis of the hepatitis B virus genome in intrauterine infection. J Med Virol. 2005;77(2):180-7.
28. Cheng H, Su HX, Wang SP, Shao ZJ, Men K, Li MZ, et al. Association between genomic heterogeneity of hepatitis B virus and intrauterine infection. Virology. 2009;387(1):168-75.
29. Su HX, Zhang YH, Zhang ZG, Li D, Zhang JX, Men K, et al. High conservation of hepatitis B virus surface genes during maternal vertical transmission despite active and passive vaccination. Intervirology. 2011;54(3):122-30.
30. Wang LJ, Xu YF, Liu XX, Chen Y. Single-nucleotide substitution of Hepatitis B virus in intrauterine infection. J Viral Hepat. 2015;22(4):433-40.
31. Swindell SR, Plasterer TN. SEQMAN. Contig assembly. Methods Mol Biol. 1997;70:75-89.
32. Zhang L, Gui XE, Teter C, Zhong H, Pang Z, Ding L, et al. Effects of hepatitis B immunization on prevention of mother-to-infant transmission of hepatitis B virus and on the immune response of infants towards hepatitis B vaccine. Vaccine. 2014;32(46):6091-7.
33. Lin CL, Kao JH. The clinical implications of hepatitis B virus genotype: Recent advances. J Gastroenterol Hepatol. 2011;26 Suppl 1(1440-1746):123-30.
34. Li H, Song XF, Hu TT, Ren H, Hu P. A strong conservative tendency in HBV transcriptase (RT): a majority of natural RT mutations derived from the S gene. Liver Int. 2016;36(7):963-70.
35. Seeger C, Mason WS. Hepatitis B virus biology. Microbiol Mol Biol Rev. 2000;64(1):51-68.
36. Chen BF. Hepatitis B virus pre-S/S variants in liver diseases. World J Gastroenterol. 2018;24(14):1507-20.
37. Pollicino T, Amaddeo G, Restuccia A, Raffa G, Alibrandi A, Cutroneo G, et al. Impact of hepatitis B virus (HBV) preS/S genomic variability on HBV surface antigen and HBV DNA serum levels. Hepatology. 2012;56(2):434-43.

38. Pollicino T, Cacciola I, Saffioti F, Raimondo G. Hepatitis B virus PreS/S gene variants: pathobiology and clinical implications. J Hepatol. 2014;61(2):408-17.
39. Hou J, Wang Z, Cheng J, Lin Y, Lau GK, Sun J, et al. Prevalence of naturally occurring surface gene variants of hepatitis B virus in nonimmunized surface antigen-negative Chinese carriers. Hepatology. 2001;34(5):1027-34.
40. Xue Y, Wang MJ, Yang ZT, Yu DM, Han Y, Huang D, et al. Clinical features and viral quasispecies characteristics associated with infection by the hepatitis B virus G145R immune escape mutant. Emerg Microbes Infect. 2017;6(3):e15.

Tables

Table 1. Primers for PCR amplification of HBV DNA

Primer	Position (nt)	Sequence (5’ - 3’)
DF	381-397	GTCTGCGGCGTTTTATC
DR	1803-1822	AAGTTGCATGGTGCTGGTGA
SF	1825-1848	TCACCTCTGCCTAATCATCTCATG
SR	997-1019	GCAAAGCCCAAAAGACCCACAAT
MF	*pEASY*-Blunt Zero 354-370	GTAAAACGACGGCCAGT
MR	*pEASY*-Blunt Zero 205-221	CAGGAAACAGCTATGAC

Table 2. General characteristics of HBsAg-positive mothers and neonates

Characteristics	GT (n=22)	GC (n=22)	*P*-value
Maternal Characteristics			
Age [years, *M (Q)*]	24.0 (3. 0)	26.5 (6.0)	0.015 [a] *
Highest educational levels [N (%)]			
High school	16 (72.7)	14 (63.6)	0.517 [b]
≥ High School	6 (27.3)	8 (36.4)	
Gestational weeks [*M (Q)*]	39.0 (2.0)	38.5 (2.0)	0.731 [a]
Mode of delivery			
Vaginal delivery	18 (81.8)	7 (31.8)	0.001 [b] *
Caesarean section	4 (18.2)	15 (68.2)	
History of abortion			
Yes	11 (50.0)	12 (54.5)	0.763 [b]
No	11 (50.0)	10 (45.5)	
Logarithmic HBV DNA (IU/ml) [*M (Q)*]	7.9 (0.8)	7.7 (0.8)	0.664 [a]
Serum HBsAg (COI*10^3)[*M (Q)*]	1.0 (1.9)	0.7 (0.6)	0.069 [a]
Serum hepatitis B e-antigen (HBeAg)(COI*10^3)[*M (Q)*]	1.1 (0.5)	1.0 (0.3)	0.972 [a]
Neonate Characteristics			
Gender [N (%)]			
Boy	9 (40.90)	12 (54.50)	0.365 [b]
Girl	13 (59.10)	10 (45.50)	
Apgar score [*Mean* ± *SD*]	9.32 ± 0.48	9.59 ± 0.50	0.072 [c]
Weight [grams, *M (Q)*]	3300.0 (600.00)	3200.0 (400.00)	0.897 [a]
Height [centimeters, *M (Q)*]	50.0 (0.0)	50.0 (2.0)	0.233 [a]
Serum HBeAg (COI)[*M (Q)*]	74.3 (165.3)	84.5 (112.2)	0.916 [a]

* $P < 0.05$; [a] Wilcoxon signed-rank test; [b] chi-square test; [c] Student's *t* test

Table 3. Occurrence of mutation hotspots in the S gene in GT and GC

Genotype	Regions	Base mutations	GT n (%)	GC n (%)	*P*-value	Amino acids
B genotype	Pre-S1	C3097A	- [b]	10 (50.00)	-	L84I
		C3100T	16 (100.00)	- [b]	-	L85F
		C2986T	- [b]	10 (50.00)	-	P47S
		A2875C	- [b]	10 (50.00)	-	K10Q
	Pre-S2	G110C	- [b]	8 (40.00)	-	A41P
	S	T165C	10 (62.50)	- [b]	-	I4T
		A551T	- [b]	10 (50.00)	-	M133L
C genotype	Pre-S1	C3116T	126 (61.76)	42 (20.79)	< 0.001 [a] *	A90V
	Pre-S2	- [b]	- [b]	- [b]	-	-
	S	T393C	51 (25.00)	- [b]	-	F80S
		T357C	40 (19.61)	48 (23.76)	0.310 [a]	I68T

[a] Chi-square test; [b] No mutations were found; * $P < 0.05$.

Table 4. Relationships between mutations in the S gene and HBV intrauterine transmission

Regions	Base mutations	GT n (%)	GC n (%)	*P*-value	Amino acids
Pre-S1	C3026T	- [b]	30 (7.39)	-	A60V
	G3064A	2 (0.98)	30 (14.85)	< 0.001 [a] *	G73S
Pre-S2	T114C	- [b]	40 (19.8)	-	I42T
S	T531C	2 (0.98)	20 (9.9)	< 0.001 [a] *	I126T
	T531G	3 (1.47)	13 (6.44)	0.010 [a] *	I126S
	C446G	10 (4.9)	9 (4.46)	0.128 [a]	L98V

[a] Fisher’s exact test; [b] No mutations were found; * $P < 0.05$.

Table 5. Multivariate analysis of intrauterine transmission

Factors	Adjusted *OR* [a] (95% *CI* [b])	*P*-value
C3116T (A90V)		
Non-mutation	1.00	0.006*
mutation	8.72 (1.89-40.31)	
Maternal age [years]		
< 30	1.00	0.181
≥ 30	0.13 (0.01-2.55)	

[a] odds ratio; [b] confidence interval; * $P < 0.05$.

Figures

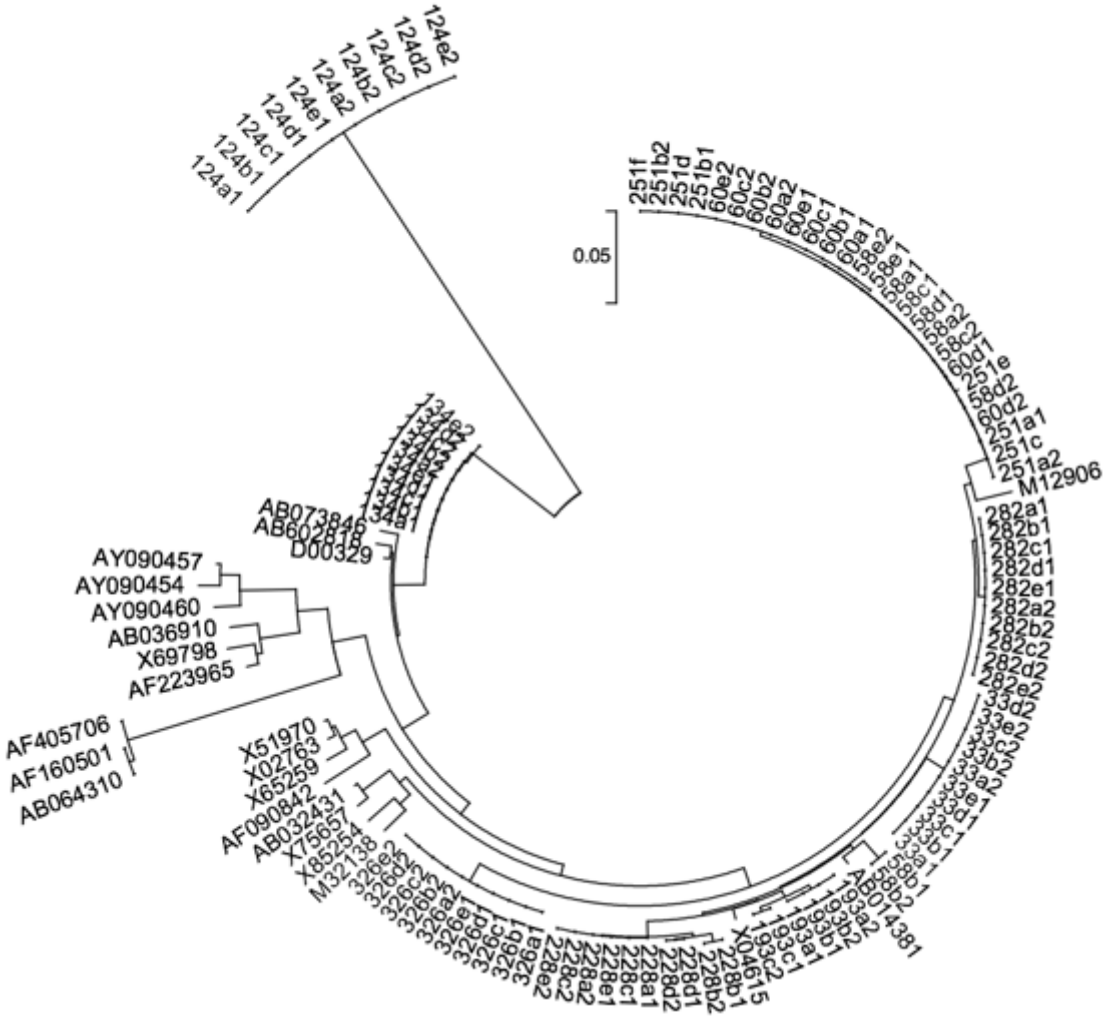

Figure 1

Short title of figure: Figure 1. Result of genotypes in HBV whole-genome sequences (partial). detailed legend: The viral sequences were clearly divided corresponding to genotype classification compared with reference sequences from NCBI. a) The combination of uppercase letters and Arabic numerals is the reference sequences of genotype; b) The combination of Arabic numerals and lowercase letters is the maternal obtained sequences, ‘a-e’ means strains (quasi species), e.g. 58a1: ‘58’ refers to the maternal ID, the letter ‘a’ refers to the one of the strains, ‘1’ refers to one of the fragments.