High Conservation of Hepatitis B Virus Surface Genes during Maternal Vertical Transmission despite Active and Passive Vaccination

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Key Words

Hepatitis B virus · Vertical transmission · Heterogeneity · Mutation

Abstract

Objective: Our purpose was to explore the relationship between hepatitis B virus (HBV) gene heterogeneity and maternal vertical transmission. **Methods:** HBsAg-positive mothers and their neonates were selected and classified into a vertical infection neonate group (group N), a vertical infection mother group (group M) and a control group (group C). Serum HBsAg and HBeAg were examined. HBV gene fragments, including the pre-S1, and pre-S2 and S coding regions, were amplified and sequenced, and the genotype and serotype of the sequences were identified. Mutation sites and frequency of mutations were then compared between group N and group C. **Results:** A total of 104 HBV clone sequences were obtained. All obtained sequences belonged to genotype C and serotype adr. Upon comparing sequences between group N and group C, 4 nonsynonymous mutations were found with significant difference in mutation frequency ($p < 0.05$). When the mothers were both HBsAg and HBeAg positive, 10 nonsynonymous mutations were found. The frequencies of these mutations were significantly lower in group N than in group C ($p < 0.05$). **Conclusion:** The 10 HBV mutations were negatively associated

with vertical transmission when maternal HBeAg was positive. Furthermore, the species that were vertically transmitted to the fetus were mainly wild-type.

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Introduction

Although newborns with HBsAg-positive mothers are immunized with the hepatitis B (HB) vaccine and hepatitis B immunoglobulin (HBIG) after birth, vertical transmission of HBV could not be completely prevented by this method. After infection either before or at birth, approximately 80–90% of the neonates become chronic asymptomatic carriers and some develop hepatitis [1, 2]. In China, vertical transmission is one of most common transmission routes for hepatitis B virus (HBV) [2]. Thus, preventing vertical transmission has become a key measure to control the spread of hepatitis B in China [1]. However, the mechanism of vertical infection is unclear. Multiple factors may be involved in infection, such as the maternal serological status, molecular biological characteristics of the virus and susceptibility of the fetus.

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If the mother is positive for both HBsAg and HBeAg and her baby does not receive immunoprophylaxis, the risk of the baby developing an HBV infection by the age of 6 months is 70–90%, and about 90% of them remain chronically infected [3, 4]. If the mother is HBeAg negative, the risk of perinatal infection among infants is 10–40%, and 40–70% of these infants remain chronically infected [3–5]. The majority of these infected infants are asymptomatic carriers and mainly become infected from vertical transmission. Even with immunoprophylaxis, perinatal transmission may occur. Boot et al. [6] evaluated HBV infections in babies in the Netherlands who were born to HBsAg-positive women and received passive immunization with HB vaccinations. They found that 12 of 1,743 babies (0.7%) were HBV infected. In Korea, despite administration of the HBIG and HB vaccine to infants with HBsAg-positive mothers, the failure rate of HBV immunoprophylaxis was 4.2% in 2008 [7]. In an Australian study, Wiseman et al. [8] reported that the rate of perinatal HBV transmission from HBV DNA-positive mothers was 4/138 (3%), while the rate was 4/61 (7%) from HBeAg-positive mothers and 4/47 (9%) from mothers with very high HBV DNA levels. In China, combining HB vaccine with HBIG resulted in a protection rate of 95–97% in the neonates born to HBsAg-positive mothers, and inoculation with the HB vaccine alone led to a protection rate of 87.8% of the newborn infants [1]. Xu et al. [9] found that 3.7% of the infants born to HBsAg-positive mothers were HBsAg positive within 24 h of birth. In the women who were HBeAg positive, the intrauterine infection rate was 9.8%.

The failure of HBV immunoprophylaxis may be attributed to many causes, such as vertical transmission, viral mutation, maternal blood infectivity, low response to the vaccine and inadequate immunization. Escape mutations of HBsAg have occasionally been found in infants with failed immunoprophylaxis. For example, the prevalence of 'a' determinant mutants (amino acids 121–149 of HBsAg) in vaccinated Taiwanese carrier children was 7.8–25% from 1984 to 2004 [10, 11]. Ni and Chen [11] thought that mutations in the 'a' determinant play some roles in infecting immunized children. In most cases, 'a' determinant vaccine escape mutants were not found in infected babies despite immunoprophylaxis [12, 13], suggesting that vaccine escape variants are not an important cause of failure to prevent HBV vertical transmission.

In a previous study, we found that the main risk factors for transplacental transmission of HBV were maternal serum HBeAg positivity, history of threatened preterm labor and virus existing in the villous capillary endothelial cells of the placenta [9]. The majority of HBsAg-positive

pregnant women are asymptomatic chronic carriers, with a pool of quasi-species of HBV in their sera. The characteristics of the viral genome from all pregnant women were not fully consistent, which may be related to the outcome of vertical transmission. We analyzed the relationship between HBV gene heterogeneity and intrauterine infection in previous studies, and we found that the fetuses from mothers in whose sera HBV strains had certain mutations were not easily infected [14, 15]. However, there was possible confounding bias from the maternal serum HBeAg status because it was an important risk factor. In this study, we added some subjects, compared the HBV sequences from the vertical infection group and control group after stratification by maternal HBeAg status and analyzed the characteristics of the viral genome. The aim of this study was to identify significant virology factors to elucidate the mechanism of vertical infection.

Materials and Methods

Subjects and Specimen Collection

The subjects included HBsAg-positive mothers and their neonates. Full-term mothers were admitted for delivery to the Maternal and Child Health Hospital of Shaanxi Province, Xi'an, China, between August 1997 and 2002. HBsAg-positive mothers did not receive HB vaccine and HBIG before or during pregnancy and did not receive antiviral treatment during pregnancy. Signs of premature delivery and abortion were not found in any of the pregnancies. All subjects signed an informed consent form and were assured that personal information would be confidential.

Venous blood specimens from the pregnant women and femoral vein blood specimens from neonates were obtained within 24 h after birth and before immunoprophylaxis. Sera were separated and stored at –20° for testing of HBV markers. Neonates were immunized with high-titer HBIG and HB vaccine within 24 h after birth. Vaccination followed the 0-, 1- and 6-month schedule.

Criteria for HBV Vertical Transmission

Occurrence of HBV vertical transmission was defined as when neonate venous blood specimens were positive for both HBsAg and HBV DNA within 24 h after birth and before immunoprophylaxis, blood specimens were still positive in the first or seventh month, and the mother was an HBsAg carrier.

Six neonates who met these criteria were classified as the vertical infection neonate group (group N), and their HBsAg-positive mothers were classified as the vertical infection mother group (group M). Thirteen HBsAg-positive mothers whose neonates were negative for both HBsAg and HBV DNA at birth from the same study population were selected and classified as the control group (group C).

All mothers from groups M and C were positive for both HBsAg and HBV DNA but negative for antibodies against hepatitis A, C, D and E. The fathers of the neonates in group N were not infected by HBV.

HBV Serological Marker Assay

Serum HBsAg and HBeAg were examined by enzyme-linked immunosorbent assay (ELISA), using a commercial kit (Kehua, Shanghai, China). The results were evaluated by a Multiskan MK3 ELISA detector. Positive/negative ≥ 2.1 were considered positive.

HBV PCR Amplification and Sequencing

PCR of a 1.2-kb fragment of the HBV genome including the pre-S1, pre-S2 and S coding regions was performed on serum samples as follows. HBV DNA was extracted using a commercially available kit (QIAmp DNA blood mini-kit, Qiagen Inc., USA) and then amplified with the high-fidelity PCR System (Roche Corporation) according to the manufacturers' specifications using the following primer pairs: 5'-CGGGATCCCATA-TTCTTGGGAACAAG-3' (nt2826–2844) and 5'-CACTGCAG-GGTTTAAATGTATACCCA-3' (nt839–821) [14]. The PCR conditions were as follows: 5 min at 94° followed by 35 amplification cycles of 45 s at 94°, 1 min at 60° and 2 min at 72°. Optimal positive and negative controls were included in each extraction and amplification process.

PCR-amplified viral DNA was separated by gel electrophoresis, recovered and digested by restriction enzymes *Pst*I and *Bam*HI. HBV DNA fragments were ligated to the pUC19 plasmid vector and transformed into competent *Escherichia coli* DH5 α cells. Clones from each of the subjects were then obtained. Plasmid DNA from a single transformant colony was extracted and verified with restriction enzyme digests and sequencing. Automatic sequencing was carried out using an ABI 3100 DNA automatic sequencer (Applied Biosystems Inc., USA) by Beijing AuGCT Biotechnology Co.

Genotype and Sequence Analysis

Genotypes were identified based on 96% homology with S genes of different genotypes. Serotypes were determined based on HBsAg amino acids 122, 127, 160 and 159 [16]. To define the HBV genotype, sequences of isolates were compared with a set of GenBank reference sequences with known HBV A–H genotypes. The accession numbers of the 8 reference sequences were as follows: X02763 [17], D00329 [18], M12906 [19], X02496 [20], X75664, X75663 [16], AF160501 [21] and AY090454 [22]. The HBV genotype was confirmed by constructing phylogenetic trees with the Kimura 2-parameter model and calculating the similarity rate with reference sequences. The statistical robustness within each phylogenetic tree was confirmed with a bootstrap analysis using 1,000 replicates. Phylogenetic analysis was performed with Clustal 1.8 and MEGA 4.1. Sequence similarity and mutation positions of nucleotide and amino acid sequences were analyzed by the DNASTAR software package (DNASTAR Inc., Madison, Wisc., USA). Multiple comparisons of all clone sequences with each reference sequence (genotypes A–H) were carried out by the ClustalW program integrated in MegAlign software, and the similarity rates of each genotype were obtained and presented as means $\pm$ standard deviation.

Statistical Analysis

Mutation Analysis

A nucleotide substitution in an isolated sequence compared with the HBV reference sequence of Chinese with C genotype (Chinese HBV-C) [23, 24] was considered a point mutation. Point mutations that were found in only 1 sequence or from 1 subject

were not calculated and analyzed. The frequency of subjects with specific point mutations and frequency of clones with specific point mutations were calculated. The difference in frequencies between 2 groups was assessed by Fisher's exact test and corrected for multiple testing with the Benjamini-Hochberg method at a false discovery rate of 0.05.

Mutation Covariation

The covariation among specific point mutations was investigated by calculating the binomial correlation coefficient (ϕ) for the simultaneous presence of mutations at 2 positions in the same isolate. Statistically significant pairs of mutations were assessed by Fisher's exact test and corrected for multiple testing with the Benjamini-Hochberg method (false discovery rate = 0.05).

Cluster Analysis

Mutational clusters [25] were defined as clusters of ≥ 3 mutated positions which were significantly correlated with each other and were identified using an average linkage hierarchical agglomerative clustering. The details of this data analysis procedure have been described elsewhere [26, 27].

Briefly, the ϕ coefficients were transformed to dissimilarity values by mapping $\phi = 1$ (maximal positive association) to dissimilarity 0 and $\phi = -1$ (maximal negative association) to dissimilarity 1, with linear interpolation in between. The resulting partial dissimilarity matrix was then used as the input for average linkage hierarchical agglomerative clustering, and undefined dissimilarity values were ignored in computing average dissimilarities between clusters. In order to assess the stability of the resulting dendrogram, confidence values for all subtrees in the dendrogram were computed by 100 replicates of the clustering procedure on sequence sets bootstrapped from the original 47 clone sequences [28, 29]. Higher bootstrap values indicate that the association of mutations into a group is not due to sampling bias.

Statistical analysis was performed by software SPSS version 16 (SPSS Inc., Chicago, Ill., USA) on a computer. The difference in mean age and gender was analyzed by Student's *t* test and Fisher's exact test. Similarities of all clones isolated with each of the 8 genotypes of reference strains were analyzed by ANOVA test, and the difference in similarities between 2 genotypes was analyzed by Dunnett's *t* test. $p < 0.05$ was considered statistically significant.

Results

Subject Characteristics

A total of 457 HBsAg-positive mothers and their 461 neonates (including 4 sets of twins) joined this study and completed the survey and test for HBV markers. Among the subjects, 79 mothers were HBeAg positive and 7 babies were persistently positive for HBsAg. Twenty-six of 33 neonates who tested positive for HBsAg within 24 h of birth became negative after immunization. Six of 7 babies infected by HBV were entered into group N; the other baby was excluded because of failure to amplify the 1.2-kb fragment of the HBV genome.

Table 1. Main characteristics and HBV serological status of subjects

Groups	Subjects	Mothers				Newborns		
		HBsAg	HBeAg	age, years	clones	HBsAg	gender	clones
Vertical infection group	M1	+	+	24	6	+	male	5
	M2	+	+	26	5	+	male	6
	M3	+	–	25	5	+	female	6
	M4	+	+	24	5	+	female	5
	M5	+	+	28	5	+	male	6
	M6	+	+	24	5	+	female	4
Control group	C1	+	+	23	3	–	female	ND
	C2	+	+	25	3	–	male	ND
	C3	+	+	24	3	–	female	ND
	C4	+	+	26	2	–	male	ND
	C5	+	+	28	2	–	male	ND
	C6	+	+	26	2	–	male	ND
	C7	+	+	24	3	–	female	ND
	C8	+	+	23	3	–	female	ND
	C9	+	–	25	4	–	male	ND
	C10	+	–	25	4	–	female	ND
	C11	+	–	24	4	–	male	ND
	C12	+	–	27	4	–	male	ND
	C13	+	–	24	4	–	female	ND

+ = Positive for HBV marker; – = negative for HBV marker; ND = not done.

The mothers from group M and group C were ethnic Han women with mean ages of 25.17 ± 1.6 and 24.54 ± 1.26 , respectively ($t = 0.927$, $p = 0.367$, Student's t test). There was no difference in gender distribution between group N (3 male: 3 female) and neonates of group C (8 male: 5 female) (Fisher's exact test, $p = 1.000$). There were 5 HBeAg-positive mothers in group M and 8 in group C. The main characteristics and the HBV serological status of the different groups are listed in table 1.

Genotype and Serotype

After cloning and sequencing, 104 HBV clones, including 31 clones from group M, 32 clones from group N and 41 clones from group C, were obtained in total. These clones included 1.2-kb fragments of the HBV genome from the pre-S1 region to the S region.

The neighbor-joining phylogenetic tree with 1,000 bootstrap replications was constructed on 1.2-kb gene fragments of genotype A–H reference sequences, Chinese HBV-C [23, 24] and 104 clone sequences (fig. 1). In this neighbor-joining tree, all obtained sequences were clearly clustered into genotype C (M12906). Compared with the A–H genotypes reference sequences, all clone sequences were most similar to the reference sequence of

genotype C among the 8 genotypes. The mean similarity rate of the C genotype was $97.42 \pm 0.57\%$, which was significantly higher than that of the other genotypes, ranging from 85.00 ± 0.17 to $92.23 \pm 0.20\%$ (1-way ANOVA, $F = 11,749.36$, $p < 0.001$; Dunnett's t test, $p < 0.001$). Then all clone sequences compared with the Chinese HBV-C sequence, the mean similarity rate was $98.17 \pm 0.60\%$, indicating that the 104 HBV clones belonged to genotype C, and their serotypes were adr based on HBsAg amino acids 122, 127, 160 and 159 [16, 30]. These HBV sequences isolated from subjects were consistent with the genotype and serotype distributions of HBV in China [23, 28, 31].

The mean similarity rates of nucleotide sequences in each pair of mother and neonate in the vertical infection group were 99.55, 99.33, 99.44, 99.36, 99.55 and 98.96%. The high similarity rates indicated a remarkable consistency and homology from mother to neonate.

Mutation Sites of Nucleotide and Amino Acid Sequences

The majority of the nucleotide sequence mutations were substitutions; few insertions or deletions of single nucleotides were observed in several sequences. To ex-

Table 2. Significant HBV point mutations between 2 groups

Group	Patients	Frequency of point mutation				
		C2875A	G3212A	T434C	T462C	A802G
Group N	6	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Group C	13	7 (53.8)	7 (53.8)	7 (53.8)	7 (53.8)	8 (61.5)
p value		0.044	0.044	0.044	0.044	0.044
Amino acid substitution		Q10K (pre-S1)	W3Stop (pre-S2)	L448P (P)	M103T (S)	T571A (P)

Frequency of point mutation: the frequency of patients with a specific mutation divided by the total number of patients from 1 group (numbers of cases with percentages in parentheses). p values were assessed by Fisher's exact test and corrected with the Benjamini–Hochberg method. The amino acid site was counted from the methionine of the pre-S1, pre-S2 and S region, respectively.

Fig. 1. The neighbor-joining phylogenetic tree constructed on the pre-S1, and pre-S2 and S gene of 9 reference nucleotide sequences and 104 cloned nucleotide sequences isolated from 6 pairs of mothers and neonates in the vertical infection group (group M and group N) and 13 HBsAg-positive mothers of the control group (group C). Reference sequences of HBV genotypes are denoted according their GenBank accession number. N = Neonates of group N; M = mothers of group M; C = mothers of group C; Chinese HBV-C = HBV reference sequence of Chinese with C genotype. Bootstrap values (percentages, 1,000 replications) >70 are denoted in major branches.

plore the relationship between HBV vertical infection and viral gene heterogeneity, we analyzed and compared virus sequences between group N and group C. Five point mutations (C2875A, G3212A, T434C, T462C and A802G) were associated with vertical infection. The frequencies of mutations were significantly different between group N and group C ($p < 0.05$; table 2). The five nonsynonymous substitutions were located in the pre-S1, pre-S2, S and P regions. Particularly, G3212A was a nonsense mutation that resulted in a stop codon in the pre-S2 region and incomplete expression of large and middle HBsAg protein. This substitution was observed in 7 of 13 mothers from group C and was absent from group N.

Stratification Analysis by Maternal HBeAg Status

HBeAg positivity is an important risk factor for HBV vertical infection and perinatal transmission [9, 29]. A fetus whose mother is positive for both HBsAg and HBeAg is easily infected by HBV [9, 29, 32]. Therefore, the subjects in the 2 groups were stratified by maternal HBeAg status to exclude possible confounding bias. Because there was only 1 neonate of group N whose mother was negative for HBeAg, we did not compare his sequences with that of the HBeAg-negative mothers from group C. We obtained 26 HBV sequences from 5 neonates with HBeAg-positive mothers of group N and 21 HBV sequences from 8 HBeAg-positive mothers of group C (table 1). The mothers' ages and the neonates' genders were balanced between group N and group C. The mean divergences of nucleotide sequences and amino acid sequences were 1.87 ± 0.76 and $1.68 \pm 0.60\%$ in group N and 2.88 ± 0.67 and $2.24 \pm 0.51\%$ in group C, which were not significantly different between the two groups ($t = 3.362$,

Table 3. Significant HBV point mutations between 2 groups after stratification by maternal HBeAg positivity

Group	Patients	Frequency of point mutation									
		C2875A	C3000A	G3212A	A162G	C339T	T434C	T462C	T531G	A802G	T810C
Group N	5	0 (0)	2 (40.0)	0 (0)	2 (40.0)	2 (40.0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Group C	8	7 (87.5)	8 (100.0)	7 (87.5)	8 (100.0)	8 (100.0)	7 (87.5)	7 (87.5)	7 (87.5)	7 (87.5)	7 (87.5)
p value		0.007	0.035	0.007	0.007	0.035	0.007	0.007	0.007	0.007	0.007
Amino acid substitution		Q10K (pre-S1)	H51Q (pre-S1)	W3Stop (pre-S2)	N3S (S)	P62L (S)	L448P (P)	M103T (S)	I126S (S)	T571A (P)	F219S (S)

Frequency of point mutation: the frequency of patients with a specific mutation divided by the total number of patients from 1 group (numbers of cases with percentages in parentheses). p values were assessed by Fisher's exact test and corrected with the Benjamini-Hochberg method. The amino acid site was counted from the methionine of the pre-S1, pre-S2 and S region, respectively.

$p = 0.069$; $t = 4.787$, $p = 0.648$, respectively). Ten mutations (C2875A, C3000A, G3212A, A162G, C339T, T434C, T462C, T531G, A802G and T810C) were associated with vertical infection, all of which had significant differences in frequency of occurrence between group N and group C (p values from 0.007 to 0.035) (table 3). Five mutations (C2875A, G3212A, T434C, T462C and A802G) were also found with significant difference in unstratified results (table 2).

The positions of 10 nonsynonymous substitutions in the HBV genome are shown in table 3. Q10K and H51Q were located in the pre-S1 region, W3Stop in the pre-S2 region, N3S, P62L, M103T, I126S and F219S were in the S region, and L448P and T571A in the P region. These substitutions were mainly found in group C and occurred at lower frequencies or were absent from sequences of group N. The mutations Q10K, W3Stop, L448P, M103T, I126S, T571A and F219S were completely absent from group N and occurred with a frequency of 87.5% in group C. These 7 mutations were not found in the mothers of group M either. Mutations H51Q, N3S and P62L occurred with a lower frequency (40.0%) in group N and at a higher frequency (100%) in group C. The 3 mutations were also found in one of the mothers of group M. None of the 10 mutations were found in the 5 HBeAg-negative control mothers, with the exception of A802G, which was observed in 1 mother.

Interestingly, these mutations seemed to be divided into 2 clusters and show covariation. To identify significant covariation patterns of pairwise correlations of the 10 mutations in 47 clone sequences of group N and group C, we calculated the binomial correlation coefficient (ϕ) and its statistical significance for major mutation pairs (table 4). The Q10K mutation, along with 6 other muta-

tions (W3Stop, L448P, M103T, I126S, T571A and F219S), were positively correlated ($\phi = 0.936$). Mutations H51Q, N3S and P62L were also positively correlated (ϕ from 0.918 to 0.958). To confirm the 2 covariation clusters, we performed an average linkage hierarchical agglomerative cluster analysis. The topology of the dendrogram (fig. 2) suggested that the 10 mutations were divided into 2 clusters. Cluster I comprised Q10K, W3Stop, L448P, M103T, I126S, T571A and F219S, and cluster II comprised H51Q, N3S and P62L. All mutations of each covariation cluster were simultaneously present or absent from the same clone sequence. It may be associated with the phylogenetic relationship of sequences. The 9 clone sequences from group C (including C6-2, C5-1, C3-2, C7-2, C6-3, C7-1, C2-3, C4-2 and C8-1) with cluster I and II mutations belonged to a close phylogenetic group (fig. 1). Another 7 clone sequences from group C (including C4-3, C8-3, C2-2, C3-3, C7-3, C8-2 and C3-1) with cluster II mutations also belonged to another close phylogenetic group (fig. 1).

Discussion

In this study, when mothers were positive for HBsAg and HBeAg, the frequencies of 10 point mutations of HBV were significantly lower in neonates of the vertical infection group than in mothers of control group, and the species that vertically transmitted to fetus were predominantly wild-type. This finding suggests that the 10 HBV mutations were negatively associated with vertical transmission when maternal HBeAg was positive.

In our previous studies [14, 15], we analyzed the relationship between intrauterine HBV infection and virus gene heterogeneity by comparing HBV sequences from

Table 4. Significantly correlated pairs of mutations

Mutation A	Frequency	Correlated mutation		Covariation frequency ^b	ϕ	p value
		mutation B	frequency ^a			
Q10K	10 (21.3)	W3Stop	9 (19.1)	9 (90.0)	0.936	1.38×10^{-10}
		L448P	9 (19.1)	9 (90.0)	0.936	1.38×10^{-10}
		M103T	9 (19.1)	9 (90.0)	0.936	1.38×10^{-10}
		I126S	9 (19.1)	9 (90.0)	0.936	1.38×10^{-10}
		T571A	9 (19.1)	9 (90.0)	0.936	1.38×10^{-10}
		F219S	9 (19.1)	9 (90.0)	0.936	1.38×10^{-10}
N3S	26 (55.3)	H51Q	25 (53.2)	25 (96.2)	0.958	5.10×10^{-11}
		P62L	24 (51.1)	24 (92.3)	0.918	3.10×10^{-10}

Figures are numbers of cases with percentages in parentheses.

^aFrequencies of mutations in 47 cloned sequences of group N and group C were determined.

^bPercentages were calculated for sequences containing each A mutation.

p values for covariation were significant at a false discovery rate of 0.05 following correction for multiple comparisons.

Fig. 2. Dendrogram obtained from average linkage hierarchical agglomerative clustering, showing 2 clusters of covariation from 47 HBV nucleotide sequences of group N and group C. The length of the branches reflects the distances between mutations in the original distance matrix. Bootstrap values, indicating the significance of clusters, are reported in the boxes.

mothers (group M) and neonates (group N) of the intra-uterine HBV infection group and HBsAg-positive mothers from the noninfection group (group C). We concluded that some specific HBV mutations were associated with intrauterine infection. In this study, we compared the HBV sequences between group N and group C. The sequences from group M were not analyzed. It is difficult to clearly distinguish these species that were transmitted to the fetus from the mixed quasi-species pool of HBV in the mothers. In an HBsAg-positive mother, 1 species may have been transmitted to the fetus through the placenta, resulting in vertical infection, while other species may not have been transmitted in this way. The species obtained from group N were transmitted to the fetus by vertical transmission, while the species from group C were not transmitted due to the influence of virological character or maternal status. By comparing the nucleotide sequences between group N and group C, 5 point mutations were found with significant differences in the frequency of mutations. These mutations were confirmed after stratification by maternal HBeAg-positivity.

Maternal HBeAg-positivity is an important risk factor in vertical transmission of HBV, and it was correlated with the viral load. The viral load of HBeAg-positive carriers was often found to be higher than that of HBeAg-negative carriers [29, 33, 34]. Furthermore, the HBV DNA levels in HBeAg-positive people were very high (approximately 10^5 – 10^{10} copies/ml in sera), whereas after HBeAg seroconversion, the levels may fall below 10^4 copies/ml [29]. The risk of perinatal infection is 5–20% in infants born to

HBsAg-positive mothers and 70–90% in infants born to HBeAg-positive mothers [32]. In this study, the 13 HBeAg-positive mothers from 2 groups did not display premature delivery and abortion during pregnancy. All neonates were immunized with HBIG and HB vaccine. Why were some neonates infected by vertical transmission and some not although their mothers were all positive for HBeAg? The gene heterogeneity of the virus may contribute to vertical infection if the maternal factors were similar and the fetus was generally susceptible to HBV.

Escape mutations in the S gene encoding HBsAg are associated with passive or active immunization at a time point when the virus is already present in the host and possibly replicating, such as during failure of passive/active HB immunization in newborns of HBV-infected mothers [35]. McMahon et al. [36] investigated breakthrough of HBV after successful active vaccination in a large study and found that escape mutants were rarely detected and accompanied by wild-type infection. The authors suggested that escape mutants seemed to play a minor role in breakthrough of HBV vaccination. Basuni et al. [12] screened vaccine escape mutations in infected children and their mothers in Pacific Island countries. Although the opportunity for the emergence of vaccine escape mutants in these populations was high, they found no 'a' determinant vaccine escape mutants. This finding suggests that vaccine escape variants are not an important cause of failure to prevent HBV transmission. Liu et al. [37] observed the influence of HBV gene heterogeneity on failure of HBV vaccination in eastern China, but they found no difference in the S gene mutation rate or genotypes between the failure group and control group. However, high viral load is an important risk factor for failure of HBV vaccination.

In our study, we found 10 nonsynonymous mutations when comparing the HBV sequences of neonates from HBeAg-positive mothers of group N and HBeAg-positive mothers of group C. The frequencies of mutations in group N were significantly lower than that in group C. The cluster I mutations, which included Q10K, W3Stop, L448P, M103T, I126S, T571A and F219S, were absent from group N. The species transmitted to a fetus by vertical transmission was rarely mutated in the 10 amino acid positions, especially the 7 point mutations of cluster I. Although the results are preliminary, they may provide virologic and epidemiologic evidence for screening high-risk pregnant women for HBV vertical transmission and for developing more targeted interruption measures.

The 10 point mutations are potentially disadvantageous to the development of infection because they may

influence entry and infectivity of virus. For example, the G3212A mutation was found to result in a stop codon at the third codon of the pre-S2 region, which terminated translation of L-HBsAg and M-HBsAg. Blanchet et al. [26] suggested that activity of the pre-S domain upon viral entry only depends on the integrity of the first 75 amino acids, while large deletions overlapping the pre-S2 domain were compatible with infectivity. In our study, the stop codon in the pre-S2 region resulted in deletion of approximately 180 amino acids of L-HBsAg and almost full deletion of M-HBsAg. Such a large deletion of envelope proteins probably affects viral attachment and entry into cells and is usually associated with a lower virus load. In this situation, passive or active vaccination could have a much better chance of preventing transmission. Now the related experiments on the infectivity of virus with the stop codon substitution are carried out in hepatocytes and trophoblastic cells.

The pre-S1 infectivity determinants were confined to the N-terminal 75 amino acid residues. In our study, the Q10K and H51Q substitutions in the pre-S1 region were prevalent in group C, while few of these substitutions were observed in group N. The 2 mutations were also found in HBeAg-negative asymptomatic carriers with low viral loads [34]. However, future experiments are required to determine if 1 or several nonsynonymous mutations in pre-S1 infectivity determinants influence infectivity of virus. The 126S substitutions in the common 'a' determinant of the S region was only observed in group C, so it may be associated with immunity escape [7, 38, 39] and did not have the ability to reduce infectivity [27].

The other mutations in the S and P regions may have no obvious effect on immunoprophylaxis and infectivity. The functional significance of these mutants remains to be determined. The limitations in this study included a sample size that was small and the subject viral loads that were not measured. Now that we have begun a cohort study using HBsAg-positive pregnant women and their neonates, efforts will be made to enroll more subjects in this study, accurately detect correlative viral markers and pay greater attention to the effect of mutations on viral breakthrough after vaccination and viral infectivity to clarify the mechanism of HBV vertical infection.

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