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Virologic characteristics of hepatitis B virus in patients infected *via* maternal-fetal transmission

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Abstract

AIM: To determine whether HBV with the same characteristics causes dissimilar mutations in different hosts.

METHODS: Full-length HBV genome was amplified and linked with pMD T18 vector. Positive clones were selected by double-restriction endonuclease digestion (*Eco*R Ⅰ and *Hin*dⅢ) and PCR. Twenty seven clones were randomly selected from an asymptomatic mother [at two time points: 602 (1 d) and 6022 (6 mo)] and her son [602 (S)], and the phylogenetic and mutational analysis was performed using BioEditor, Clustal X and MEGA software. Potential immune epitopes were determined by the Stabilized Matrix Method (SMM), SMM-Align Method and Emini Surface Accessibility Prediction.

RESULTS: All of the 27 sequences were genotype C, the divergence between the mother and son was 0%-0.8%. Compared with another 50 complete sequences of genotype C, the mother and her son each had 13 specific nucleotides that differed from the other genotype C isolates. AA 1-11 deletion in preS1 was the dominant mutation in the mother (14/18). The 1762T/1764A double mutation existed in all clones of the mother, 3 of them were also coupled with G1896A mutation, but none were found in the son. 17 bp deletion starting at nucleotide 2330 was the major mutation (5/9) in the son, which caused seven potential HLA class Ⅰ epitopes and one B cell epitope deletion, and produced a presumptive new start codon, downstream from the original one of the P gene.

CONCLUSION: The HBV strain in the son came from his mother, and discrepant mutation occurred in the mother and her son during infection.

Key words: Hepatic B virus; Vertical transmission; Full genome; Mutation; Phylogenetic; Deletion

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INTRODUCTION

Chronic hepatitis B virus (HBV) infection is a significant global public health problem. Approximately 3-4 billion people worldwide have been infected with HBV, and 350-400 million persons have chronic HBV infection. Vertical transmission, especially mother-to-infant transmission is the primary pathway of hepatitis B virus infection, in approximately 90% of the infants born to HBeAg positive HBsAg carrier mothers, if no immunoprophylaxis is given. HBV with the same characteristics in the mother and child interacts with the immune system and may result in different mutations to escape immune pressure.

In the present study, we isolated the full genomic sequence of HBV in an asymptomatic mother and her son by direct sequencing. Phylogenetic and mutational analysis were performed to obtain evidence of mother to infant HBV transmission, and to elucidate distinctive HBV mutations in the different hosts.

MATERIALS AND METHODS

Patient

A 31-year-old female who was first seen at the First People's Hospital of Yunnan Province, Kunming, Yunnan, China in 2005 because of allergic purpura was reevaluated. She was HBsAg and HBeAg positive since at least 1999. Her sera were obtained from two time points: 602 (1 d) and 6022 (6 mo later). When seen again, 6 mo after her initial visit, her 7-year old son's serum [602 (S)] was collected. The alanine aminotransferase (ALT) level and abdominal ultrasound examination of the mother and son were normal. There was no history of alcohol abuse, parenteral drug use and hepatotoxin exposure. Neither the mother nor her son had received immunoprophylaxis or immunotherapy.

Serological markers and HBV DNA

HBV serological markers were evaluated using commercially available enzyme-linked immunosorbent assay (ELISA) kit (Kehua Bio-Engineering Co. LTD, Shanghai, China). HBV DNA in the sera was determined using a commercially available real-time fluorescence quantitative PCR (FQ-PCR) kit (Da An Gene Diagnostic Center, Shenzheng, China). Both subjects were also tested for hepatitis C virus (HCV), hepatitis A virus (HAV) and hepatitis E virus (HEV) infection.

Amplification of full-length HBV genome

HBV DNA was extracted from the sera using proteinase K digestion followed by phenol/chloroform extraction. The complete HBV genome was amplified by polymerase chain reaction (PCR) as described previously[1]. Amplification was performed in a 96-well cycler (Bio-RAD, USA), and 25 μL PCR mixture containing 2.5 mmol $MgCl_2$, 200 μmol/L dNTP, 400 nmol/L of each primer, and 2 U of Ex Taq polymerase (Takara BioTech). The PCR reaction was performed using the following cycles: 94℃ pre-denature for 5 min, 30 cycles of 94℃ for 1 min, 56℃ for 1 min, 72℃ for 2 min; and 72℃ for 10 min as a final extension step. The full-length amplicon were purified using a gel extraction kit (HuaShun Bio-Engineering).

Cloning and sequencing of the full-length HBV genome

Purified full-length HBV-DNA was directly linked with pMD18-T vector (TaKaRa Bio-Tech), using the standard cloning techniques. White colonies were picked and the genomic length of insertions was confirmed with PCR and double-restriction endonuclease digestion with *Eco*R I and *Hin*dⅢ. DNA sequencing analysis of the correct recombinants was performed with BigDye Terminator v3.1 and 3130 Genetic Analyzer (Applied Biosystems). For each patient and time point, 9 recombinants were randomly selected and sequenced for the full-length genome of HBV.

Sequence analysis

Thirty three HBV complete genome sequences (genotypes A to H) were used in as reference[2]. Additionally, 40 complete HBV genome sequences of genotype C from GenBank were used for phylogenetic analysis. The complete genome sequences were assembled from the sequencing data, using the BioEdit sequence alignment editor software, version 7.0.5.2[3]. Alignment was performed using Clustal X software, version 1.83[4]. Phylogenetic and molecular evolutionary analysis and nucleotide differences between the isolate sequences were carried out by the MEGA program, version 3.1[5]. The genetic distance was estimated using the Kimura two-parameter algorithm[6]. The phylogenetic trees were constructed using the neighbor-joining method[7]. Bootstrap re-sampling and reconstruction was carried out 1000 times to confirm the reliability of the phylogenetic trees[8]. The HBV genotype was assigned according to the classification system reported previously.

Potential immune epitopes prediction

For a new deletion fragment (17 bp deletion in Core gene region from nt 2330), Stabilized Matrix Method (SMM) and SMM-Align method were used to predict the potential major histocompatibility classes Ⅰ/Ⅱ[9,10] (Predicted IC50 < 200), and the Emini Surface Accessibility Prediction was used to predict the potential B cell epitope[11].

RESULTS

Serological markers and HBV DNA quantification

Both the mother and son were positive for HBsAg, HBeAg and anti-HBc. They were seronegative for HAV, HCV and HEV. The HBV viral load was 2.4×10^8 copy/mL in the mother (6022) and 4.2×10^8 copy/mL in the son [602(S)]. The 602 sera were not quantified due to insufficient material.

Genotypic and serotypic relatedness

The 27 sequenced clones (Gene Bank accession No: DQ377159-377165, EU306713- EU306729, EU439005-439007) ranged in length from 3036bp to 3254 bp. The divergence of the complete genome of 18 clones for the mother was 0%-0.8%, while the divergence of the 9 clones for the son was 0.1%-0.6 %, and the divergence of the 27 clones for the mother and child was 0%-0.8%. For the 27 clones, the homogenous of S gene was 98.5%-100%. The 27 clones were 51H, 54E, 62A, 73G, 125T and 227S in the large S gene, 136A, 142K, 233R, 236S, 248P, 252R, 304H, and 354H in the P gene, and 122K,160R and 159A in the small S gene. Compared with the 33 global complete genome sequences of HBV from GenBank, phylogenetic analysis showed that with bootstrap values of 100%, the clones were classified into genotype C and C2 subgroup (Figure 1)[12]. The serotype was classified as $adrq^+$[13].

Characterization of the nucleotides and the deduced amino acid sequence

Compared with the other 50 complete sequences of

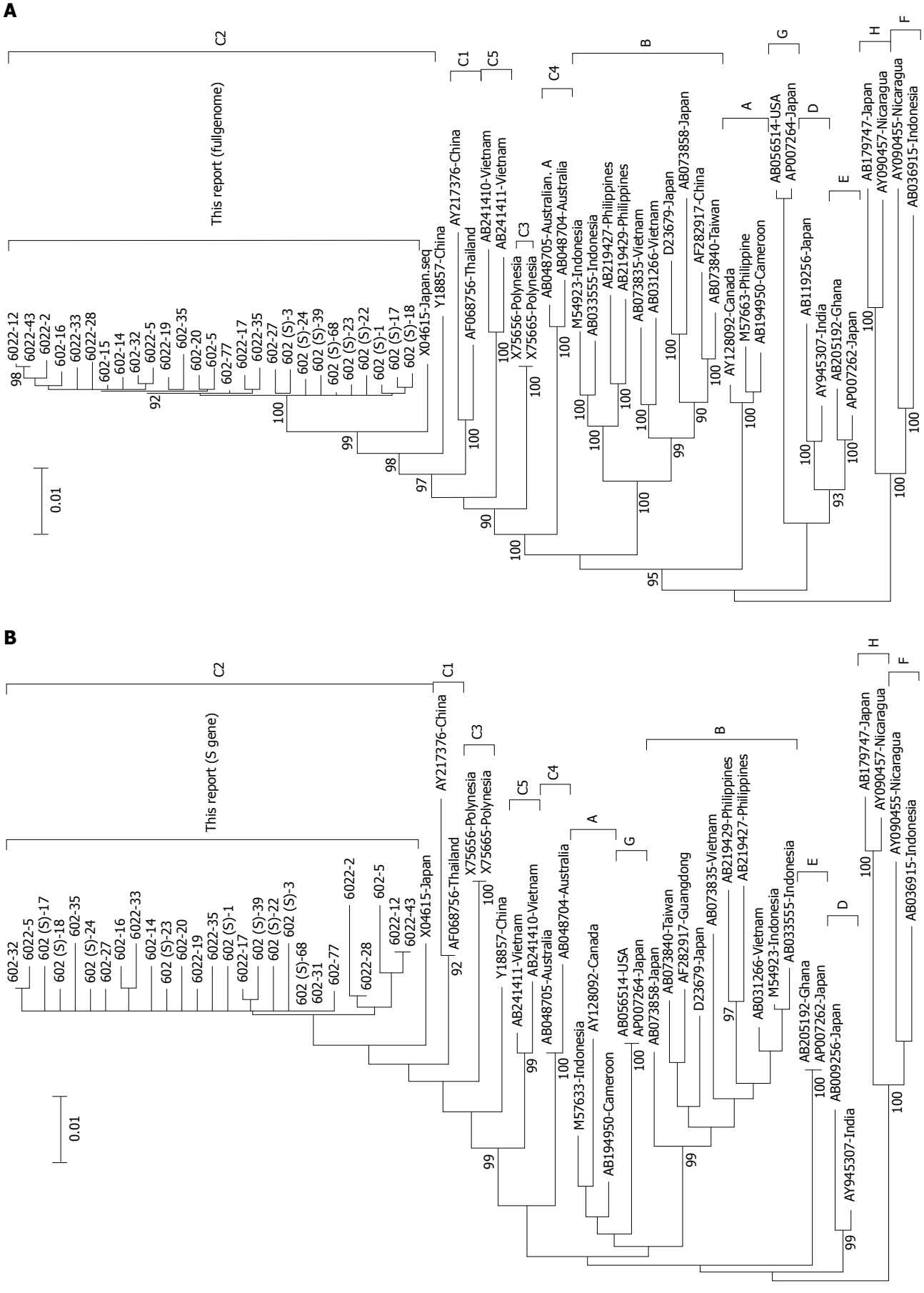

Figure 1 Phylogenetic tree constructed on the complete genome (**A**) and S gene (**B**) [Mother: 602 (1 d), 6022 (6 mo); Son: 602 (S)]. Bootstrap values are shown at the beginning of each main node. The length of the horizontal bar indicates the number of nucleotide substitutions per site. The origin of each strain has also been shown.

Table 1 MHC-I binding predictions of 144-183 residue of HbcAg, processed by the SMM method

Allele	Position (aa)	Pep length	Sequence	IC_{50} (nm)[1]
HLA A*3101	148-157	10	VVRRRGRSPR	30.9
HLA A*3101	150-159	10	RRRGRSPRRR	13.7
HLA A*3101	157-166	10	RRRTPSPRRR	19.2
HLA A*3101	159-167	9	RTPSPRRRR	38.4
HLA A*3101	165-174	10	RRRSQSPRRR	19.2
HLA A*3101	166-175	10	RRSQSPRRRR	35.4
HLA A*3101	167-175	9	RSQSPRRRR	13.7
HLA A*3201	159-167	9	RTPSPRRRR	48.6
HLA A*6801	145-154	9	ETTVVRRRGR	23
HLA A*6801	146-154	9	TTVVRRRGR	22.3
HLA B*0702	162-170	9	SPRRRRSQS	21.3
HLA B*0702	170-178	9	SPRRRRSQS	21.3
HLA B*0702	155-163	9	SPRRRTPSP	40.4
HLA B*0801	162-170	9	SPRRRRSQS	39.5
HLA B*0801	170-178	9	SPRRRRSQS	39.5
HLA B*2705	150-159	10	RRRGRSPRRR	49.5
HLA B*2705	164-173	10	RRRRSQSPRR	14.7
HLA B*2705	166-175	10	RRSQSPRRRR	33.9

[1]IC_{50} (nM) < 50 was considered as high affinity. Only IC_{50} (nM) has been shown in the Table.

genotype C, there were 13 specific point mutations in the 27 clones, the nucleotide positions were at 105T, 346C, 855C, 861T, 930G, 951T, 1110T, 1341C, 1972C, 2215C, 2246C, 2480T and 3102A. Except for C105T and G3102A which caused mutation of amino acid A158V in the large S gene and D266N in the P gene, respectively, the other substitutions resulted in the same sense mutation. Within the pre-S1 region, 14 clones of the mother had 18bp deletion from the start codon, which resulted in deletion at codons 1-11. The C-terminal truncated S protein was found in both the mother and son, which caused random point mutation in or near the hydrophilic region of S gene (nt 200, nt361, nt455, and nt565, respectively). Within the X region, 1762T/1764A double mutation was observed in all clones in the mother, but none was found in the son. The hypervariable region (HVR) sequence at positions 1751 to 1755, and the AT-rich region sequence at 1789 to 1795 in the X-ORF was conserved in all of the clones. In the precore and core regions, 3 clones of the mother had G1896A mutation and one clone had 19bp deletion, resulting in a novel stop codon of the C gene. In the P region, the YMDD motif was conserved in all the clones, 5 clones of the son had a 17 bp deletion from nt2330, and produced a 43 aa residue in the N-terminal of polymerase (Figure 2). Other random mutations occurred throughout the sequenced genomes, like a "G" insertion at nt 761 [602 (S)-39] was observed in one clone of the son and a "TT" deletion at nt2012 in one clone of the mother.

Potential immune epitopes caused by 17 bp deletion from nt2330

The 17bp deletion mutant from nt2330 in 5 clones of the son led to the deletion of aa144-183 of the HBcAg, as reported in this study after performing Blastn. By the immune epitope prediction method, potential clustal immune epitopes were located in the deletion region of Core gene, including 7 HLA class I epitopes and one B cell epitope (PRRRRSQ; Table 1, Figures 3 and 4).

DISCUSSION

Using phylogenetic and mutational analysis, clones of the mother obtained at two time points were conserved. The dominant mutants in the mother obtained at two time points were the same (18 bp deletion in preS1 region and BCP double mutation), except for the scattering mutation. Although, the son was infected from the mother, the divergence of the mother and the son was 0%-0.8%, and the dominant mutants in the son were different from the mother.

Universal infant immunization has reduced HBV infection rate in highly endemic countries with a relatively homogenous population. However, HBV transmission continues to occur *via* vertical (mother-to-child) and horizontal (sexual, parenteral and household) routes. In Asia, where genotypes B and C predominate, vertical transmission is common. In the present study, we isolated HBV strains from an asymptomatic mother and her son and analyzed the sequences phylogenetically. All the 27 clones were of the C2 subtype and adrq$^+$ serotype. Compared with 50 other complete sequences of genotype C, there were 13 specific nucleotides belonging to the mother and her son. It should be noted that there was no history of alcohol abuse, parenteral drug use or hepatotoxin exposure, and no immunoprophylaxis or immunotherapy was given to the mother and her son. The son's father was seronegative for HBV markers. Based on these findings combined with the data of divergence in the complete viral genome and homogeneity in the S gene, it can be concluded that the son became infected *via* a mother-to-infant transmission.

The pre-S1 domain is the essential binding site for hepatocyte receptors, and mutations at this region may directly influence HBV infection, and progression of liver disease. In the present study, 14 clones of the mother had aa 1-11 deletion in preS1 caused by a 18bp deletion from the original start codon of preS1. This deletion mutant has previously been reported in different clinical conditions, especially hepatocellular carcinoma (HCC) and chronic hepatitis B (CHB) infection with genotypes C and D, and fulminant hepatitis B (FHB) with genotype A in Africa. It has also been observed in isolates obtained from nonhuman primates (Table 2)[14-32]. Interestingly, there are no reports of this deletion mutation in genotypes B, E, F and G. In a heart transplant recipient who died from fulminate hepatitis B transmitted by the donor, the 18bp deletion was detected in the recipient, but not in the donor[20]. In our case, the 18bp deletion mutants in preS1 were present in 14 clones of the mother (78%), but in none of the sequenced clones in the son (0%). This phenomenon may imply that host immune pressure was the primary cause of aa 1-11 deletion in preS1. In a subsequent study (2 years later), we observed that the

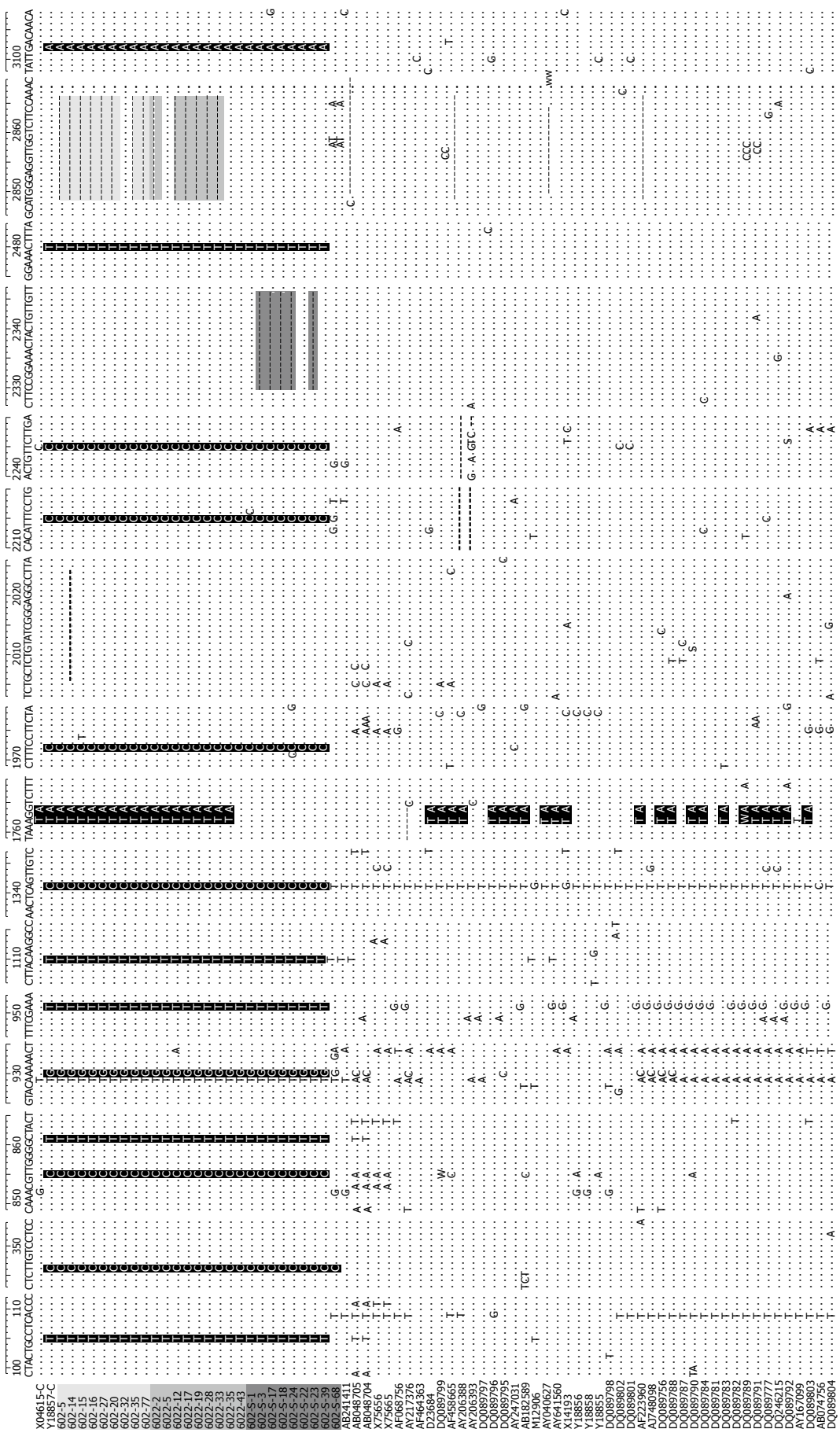

Figure 2 Nucleotide sequences of the 27 HBV/C isolates and 50 other complete genome sequences of genotype C [light gray, Mother: 602 (1 d); Gray, 6022 (6 mo); Dark gray, Son: 602-S; Sequences: Gray (deletion), black (substitution of nucleotides)]. Compared with the 50 complete genome sequences of genotype C, nucleotide positions at 105T, 346C, 855C,861T, 930G, 951T, 1110T, 1341C, 1972C, 2215C, 2246C,2480T and 3102A were specific for the 27 samples. 14 clones of the mother had an 18bp deletion from start codon; 5 clones of the son had a 17nt deletion from nt2330; 1762T/1764A double mutations were found in all clones of the mother, but none in the sequenced clones of the son. Nucleotides A1762A and G1764A double mutation comprised 54.5% of the total genotype C (42/77).

18bp deletion in preS1 had a tendency for substitution by large fragment deletions in preS1, and laboratory tests in the mother showed abnormal values (ALT > 2 times of reference values) (detailed discussion reported in a

Table 2 Deletion of aa 1-11 in preS1 in the different HBV genotypes

Genotype	GenBank No.	Clinic status	Countries/district	Ref.
Human				
Genotype C	AF223959	CHB	Vietnam	Erik *et al*, 2001
	AY217372	CHB	China	Luo *et al*, 2004
	AB195945	CHB	Japan	Horiike *et al*, 2007
	EF533714	HCC	China	[1]Gao *et al*, 2007
	AY206389	HCC	China	[1]Lin *et al*, 2002
	AB014395	HCC	Japan	Takahashik *et al*, 1998
	AY641563	HCC	South Korea	Song *et al*, 2005
	D1666	AHB	Japan	Uchida *et al*, 1995
	X98076	FHB	Switzerland	Pult *et al*, 1997
Genotype D	AB033559	CHB	Papua New Guinea	Okamoto *et al*, 1988
	AB119256	CHB	Japan	Michitaka *et al*, 2006
	M32138	Anti-HBe	France	Tong *et al*, 1990
	AY902777	FHB	Montana	Garfein *et al*, 2004
Genotype A	AF297621	FHB	Africa	Owiredu *et al*, 2001
	U87743	AHB	South Africa	Bowyer *et al*, 1997
	X69458	AHB	Zimbabwe	Chirara *et al*, 1994
Ape				
Gibbon	AB037928		East Asia	Aiba *et al*, 2003
Chimpanzee	D00220	HBsAg (+)	London	Vaudin *et al*, 1988
Gorila	AJ131567	HBsAg (+)	Japan	Grethe *et al*, 2000
Orangutan	AF193864	CHB	Bornean	Verschoor *et al*, 2001
Recombination				
Human-Gibbon	AB048705	HBsAg (+)	Australian Aborigines	Sugauchi *et al*, 2001

CHB: Chronic hepatitis B; AHB: Acute hepatitis B; FHB: Fulminant hepatitis B. [1]Sequences were submitted directly to the GenBank.

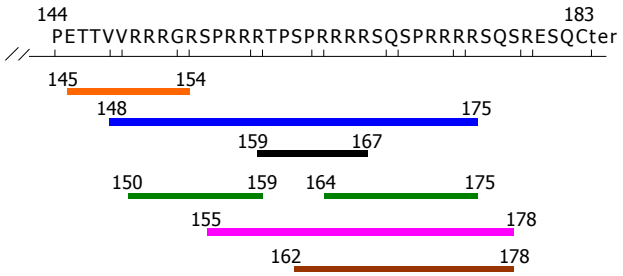

Figure 3 Potential HLA class Ⅰ epitopes of the deletion fragment in HBcAg (aa residue144-183), determined by the SMM method [only IC_{50} (nM) < 50]. Blue line: HLA A*3101 (aa 148-175); Black line: HLA A*3201(aa 159-167); Orange line: HLA A*6801 (aa 145-154); Pink line: HLA B*0702 (aa 155-178); Brown line: HLA B*0801 (aa 162-178); Green line: HLA B*2705 (aa 150-159, 164-175).

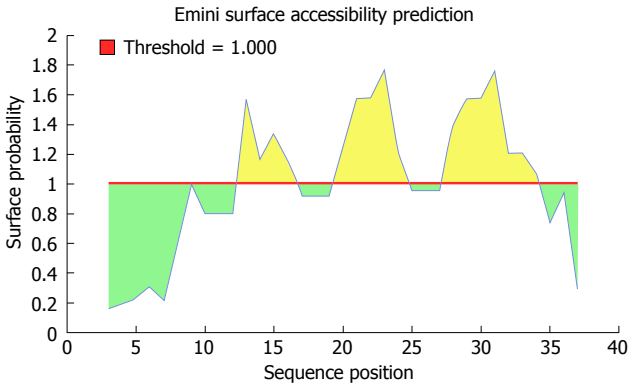

Figure 4 Predicted peptides of the potential B cell epitope of the deleted fragment in HBcAg (aa residue 144-183). A potential sequence of B cell epotide in aa residue 144-183 of HBcAg was PRRRRSQ, start position at aa 171, end position at aa 177.

separate publication). However, whether the deletion of aa 1-11 mutation in preS1 was a precursor of large fragment deletion mutation or was an isolated event under immune pressure remains to be determined. To our knowledge, no previous study has reported such deletion mutants. This deletion, which disrupts the preS1 start codon, may play an important role in enhancing the progression of chronic liver disease.

The 18 bp deletion mutants in preS1, located in the overlapping region of S/P gene region, caused six amino acid deletions, from 183 to 187 of the HBV polymerase. In the present study, all of these deletion mutants coexisted with the wild-type full-length genome. Transfection experiments in human hepatocells (HuH-7) demonstrated that the polymerase gene function was not affected by the large pre-S1 deletions, and mutant viral genomes were capable of replication[33]. This finding may explain the above observation, although deletion mutation of pre S1 was the major mutation in the mother, and the viral load in the serum remained high.

Cloning and sequence analysis showed that 3 clones of the mother and one clone of the son had premature S protein, caused by random points mutation. These mutations could affect not only the spatial structure, but also the immune reactivity. Some reports have found that C-terminal truncated S proteins exist in patients with chronic hepatitis B infection and hepatocellular carcinoma[34]. The shorted S proteins can stimulate gene expression from the endogenous HBV enhancer I and can also deregulate the expression of oncogenes such as c-myc[35], which may help clarify the tumor development mechanism of HBV. Whether the truncated S proteins

transactivate the oncogenes and/or worsen the clinical status of the subjects remains to be established.

The double mutation of nucleotide A1762T/G1764A in basal core promoter (BCP), and G1986A in the precore region are frequently observed in HBV sequences isolated from patients with chronic HBV infection, fulminant hepatitis, HCC, and in reactivation of HBV with a fulminant course[36-38], which results in mutations at two codons in the carboxyl functional region of X protein (K130M and V131I), and a stop codon at aa 28 of HBeAg, respectively. At present, there are conflicting opinions regarding 1762T/1764A hotspot mutations. Some studies suggest that these mutations decrease HBeAg expression and slightly increase viral DNA replication, and are mostly found in patients who seroconvert to anti-HBe[39,40]. By contrast, other studies indicate that this mutation is not associated with HBeAg/anti-HBe status or HBV DNA[41,42]. The lack of influence of BCP mutation on HBeAg expression was mainly indicated by its higher prevalence in genotype C than genotype B patients. In the present study, all clones of the mother from two time points had these double mutations (Figure 2); genotype C has a tendency for higher prevalence of 1762T/1764A double mutation (42 *vs* 35, 54.5%). Based on our data from two time points, we did not observe any HBeAg/anti-HBeAg seroconversion, and we did not detect the impact of BCP mutation on HBV DNA load, due to insufficient quantity of 602 sera. Besides, the 1762T/1764A double mutation was present in all clones of the mother, 3 of whom were also coupled with G1896A. The G1896A mutation is supposed to increase the stability of the stem-loop structure. Recent reports suggest that HBV subgenotypes Ba, C1, and C2 have an intermediate frequency of 1896A mutation[43]. Interestingly, although the son was infected *via* the mother, none of the BCP and 1896A mutants were found in the son (0/9). This finding is consistent with previous studies in which mutants with 1896A were seldom transmitted to the infant *via* the mother[44]. Whether the BCP and precore mutations in the mother were related to infection with genotype C, or whether she was seroconverting to anti-HBeAg, or may develop severe disease exacerbations remains to be determined.

A heterogeneous population of core antigen internal deletions (CID) has been found to be highly prevalent in chronic HBV carriers[45,46], HCC patients[47] and immune-suppressed patients[48]. Normally, CID coincides with a potent T/B-cell epitope, and is almost always found in the presence of HBV with an apparent full-length core gene[49-51]. HBcAg has been shown to be a major target of T-cell immunity[52]. However, deletion type HBc did not show any antibody response[53].

In the present study, the wild type precore/core protein and deletion type precore/core protein co-existed in the sera of both the mother and son. Out of the 27 clones, one clone in the mother had 19nt deletion at position nt2006, and 5 clones in the son had 17bp deletion at position nt2330, which caused in-frame shift and resulted in a C-terminal truncated preCore/Core protein. To the best of our knowledge, this is the first report on 17bp deletion mutant, resulting in immune epitope deletion based on the immune epitope prediction tools. Interestingly, none of these mutants were found in the mother. Therefore, we concluded that under host immune pressure, due to the loss of HLA class and B cell I epitopes, this deletion variant may evade the immune system and became dominant in the host.

The 17 bp deletion mutation occurred in the overlapping region of P/C gene, and produced 43 aa peptides of HBV polymerase, possibly resulting in a new start codon, downstream of the original one. P-ORF encodes the terminal protein (TP), polymerase (pol) and RNase H. Previous studies have identified that in a protein-primed reaction, the minimal domain of TP ranged from amino acid 20 to 175[54]. In the present report, these 5 clones had incomplete TP, based on the minimal function domain of TP. These mutants have little or no activity. Interestingly, the viral load in the son was high (4.2×10^8 copy/mL), and no similar deletion mutants were found in his mother. Whether this phenomenon coexisted with the wild-type genome, or was the function domain of TP remains to be examined.

In addition, there were other point mutations and deletion/insertion mutations scattered in the complete genome, many of these were located in the T and B lymphocytes epitope, such as P46L, C69R and F73L in X gene[55], and P54T and M132T in C gene[56], while others resulted in truncated surface or preCore/Core proteins (T361A, TT deletion at nt2012, *etc.*). Because these mutants were dissimilar in different clones, they may have been caused by spontaneous mutation or immune pressure during evolution.

In summary, the HBV strain in the son was transmitted *via* maternal-fetal transmission, based on the presence of 13 specific and common nucleotides in both the mother and son. The dominant HBV mutants were different in the mother and son, because of different immune-environments. These mutants were capable of escaping immune-pressure and were present in HBV carriers. These newly discovered variants were located within or close to the functional domains of viral proteins. Whether they can exist "silently" in the hosts, or will cause liver disease remains to be determined.

COMMENTS

Background

Mother-to-infant transmission is the main pathway of hepatitis B virus (HBV) infection. Approximately 90% infants of HBeAg positive HBsAg carrier mothers become carriers, if no immunoprophylaxis is given. HBV with the same characteristics in the child and the mother interacts with the immune system, resulting in the development of different mutations designed to escape the immune pressure.

Research frontiers

Biosoftwares are powerful tools designed to perform virologic and phylogenetic analysis, and to predict immune epitopes. Combining this knowledge with clinical data on HBV-infected patients, may provide new information on the prevention and treatment of HBV infection.

Innovations and breakthroughs

The results of our study suggest that the preS1 deletion of HBV may be

associated with disease progression. Using immune epitope prediction tools, the immune epitope deletion was found to occur in the C gene region.

Applications

Data obtained by virologic and phylogenetic analysis may provide clues to the prevention and treatment of chronic HBV infection.

Peer review

Virologic and phylogenetic analysis provides evidence of mother to infant transmission, and indicates that HBV with the same characteristics has dissimilar mutations in different hosts. Follow-up studies should greatly enhance the significance of the present report.

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