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Mutational Analysis of HBs Ag-Positive Mothers and Their Infected Children despite Immunoprophylaxis

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ABSTRACT

Hepatitis B vaccination is safe and effective, although breakthrough infection occasionally occurs in those who receive the vaccine and hepatitis B immunoglobulin (HBIg) prophylaxis. Sequence variation in their antigenic regions is one of the most powerful strategies that are used by viruses to escape recognition by B and T cell-mediated immune responses. The aim of this study was to explore the mutational profile of HBV in vertical transmission.

Six HBsAg-positive mothers and their children who developed HBV infection despite immunoprophylaxis were enrolled. After extraction of HBV DNA from sera, the full HBV genome or surface gene was amplified by Gunther and hemi-nested PCR, respectively. After sequencing, the mutational analysis on paired samples between mothers and children were carried out and compared.

Different mutations were found in four children; at least, one arose in functional and/or immune epitope activities. Of 30 amino acid changes, 11 (36.6%) were located within the known HBV immune epitopes. In three children, mutations occurred within the "a" determinant region, one mutation (B2) was identical to the mother of patient, an indication of vertical transmission. The other two (B4 and B5) were considered as vaccine escape mutations. Three children harbored wild-type HBsAg, similar to their mothers. Regarding transmission in infected children, the immunoprophylaxis had no effect and failure of vaccination was observed in 2 isolates.

These findings emphasized the need for an alternative regimen, such as the administration of boosters or a more effective HBV vaccine for high-risk children who are born to HBsAg-positive mothers.

Keywords: HBV escape mutants; HBV prophylaxis; Hepatitis B immunoglobulin

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INTRODUCTION

In adolescents or adults who are infected with HBV, 1-3% will become chronically infected, whereas up to 90% infected neonates develop chronicity.[1] WHO aims to control HBV worldwide by integrating hepatitis B vaccination for all infants and some high-risk adolescents into national programs. Maternal-neonatal transmission of HBV and the subsequent development of chronic hepatitis B in infected children could be prevented when hepatitis B immunoglobulin (HBIG) is given to newborns of HBV carriers with an initial dose of hepatitis B vaccine within 24 hours after birth, a regimen that is 85% to 95% effective in preventing HBV infection and the chronic carrier state.[2-4]

The major target of antibodies that are induced by the vaccine and HBIG is against the group-specific "a" determinant of hepatitis B surface antigen (HBsAg), commonly described as a major B-cell epitope, comprising residues 124-147. Although HBsAg positivity has declined in children since the international immunization program began, HBV variants with mutations in the common "a" determinant of the S gene have been reported worldwide as a possible cause of vaccine failure due to the immune pressure of vaccine-induced anti-HBs, [5-9] which poses a substantial risk to the community. These "a" determinant variants may go undetected by conventional HBsAg screens,[10-12] and for some mutants, vertical and horizontal transmissions and infection of the vaccinated population occur.[13-15] Thus, the majority of vaccine escape mutants have been identified in studies of vertical transmission of HBV from HBV carrier mothers to their neonates.[16-18]

The prevalence of HBV in the general population in Iran ranges between 1.7% to 2.5%.[19-21] HBV vaccination has been included in the extended program of immunization (EPI) since 1993.[22] Since then, babies who have been born to HBsAg carriers have been given HBIG and vaccine at birth. A current study indicates that 98% of the target population has received the full dose of HBV vaccine,[23], and adequate immunity (anti-HBs >10 IU/mL) has been reported to range from 69% to 90.9% in children having received a full dose.[3,24,25] Although there are data regarding the immugenicity of HBV vaccine in vaccinated Iranian children who have different levels of anti-HBs,[3,24-26] no molecular data exist on subjects who are HBsAg-positive despite receiving Immunoprophylaxis. The response rates of children born to HBsAg-positive mothers to a combination of vaccine/HBIG is approximately 86% in Iranian children. [25]

The aim of this study was to analyze variations in the HBV genomic sequence that might play a role in vertical transmission of children born to HBsAg carriers who have acquired breakthrough HBV infection despite Immunoprophylaxis.

MATERIALS AND METHODS

Study Design

Six mothers, who were chronic HBsAg carriers, and their infants, who were immunized against hepatitis B postnatally with a dose of HBIG (Hyper HEP B, USA) and three 10-µg doses of vaccine (Pasteur Institute, Iran) at 0, 1 and 6 months after birth, were enrolled in the study. All children were HBsAg-positive and all parents of the enrolled children signed an informed consent and provided the child's vaccination history, based on a booklet provided by the Health Administration Authority, Baqiyatallah Research Center for Gastroenterology and Liver Disease-Tehran Hepatitis Clinic. All patients were negative for antibodies against hepatitis C, hepatitis D, and human immunodeficiency virus.

Sera Collection

Serum samples were drawn from the mothers and children 6 months post-vaccination and stored at -80°C. The serological markers for hepatitis B (HBsAg and HBeAg/anti-HBe) were measured by ELISA (BioKit, Barcelona, Spain).

DNA Extraction

HBV DNA was extracted from a 200-µL aliquot of sera using the Qiagen Mini Blood Kit (Qiagen, Hilden, Germany) according to manufacturer's instruction. In brief, 20 µL protease was added to each serum in a 1.5-mL tube. Then, 200 µL of Al buffer was added to each tube, vortexed, and incubated for 10 minutes at 56°C. For DNA precipitation, 200 µL ethanol was added to the mixture and centrifuged for 1 minute. The contents were transferred to a collection tube with a filter. Trapped DNA was washed sequentially in buffers AW1 and AW2. After centrifugation, 50 µL of elution buffer was added, and the eluted DNA was stored at -20°C.

Polymerase Chain Reaction

HBV DNA levels were determined in all samples by real time PCR (Fast-track Diagnostics, Luxembourg). Then, all positive samples, regardless of their levels of viral load, were subjected to PCR reactions using Gunther et al methodology,[27] by a single set of primers P1 and P2 (Table 1). Five microliters of the PCR product was analyzed by electrophoresis on a 1% agarose gel, stained with ethidium bromide, and visualized under UV light.

The method of amplification of the full-length genome of Gunther et al was successful for 5 samples, with HBV DNA levels $>10^4$ copies/mL. For samples that were not positive by this method, several rounds of nested and semi-nested PCR were performed. The reactions contained lx PCR buffer, 1.5 mmol/L MgCl2, 0.2 mmol/L dNTPs, 1.5 U HotStart Taq PCR (Qiagen, Hilden, Germany), 0.25 mmol/L first- and 0.5 mmol/L second-round primers (Table 1), 5 µL extracted HBV DNA for the first round, and 1 µL of the first-round amplicon for the second-round PCR as template. The thermal profiles for all reactions were similar as those described,[28, 29] with the exception of X gene single-step PCR.[30] Five µl of PCR product was analyzed by electrophoresis on a 1% agarose gel, stained with ethidium bromide, and visualized under UV light. To avoid false-positives, all PCRs were run with precautions against cross-contamination.

Sequencing

Direct sequencing of the complete genome was performed on an automated sequencer (Perkin Elmer ABI-3130XL DNA Sequencer, Foster City, CA, USA) using 0.5 µL of the appropriate internal primers (Table 1). The results were compared and analyzed using Chromas and BioEdit (version 7.0.5.3).

Table 1. Oligonucleotide primers used for PCR and sequencing. Base positions numbered from the EcoRI site.

Primer Identification	Related HBV Protein	5' to sequence 3'	Location 5' 3'	Sense/Anti-sense
P1	Whole genome/ polymerase	CCGGAAAGCTTGAGCTCTTCTTTTTCACCTCTGCCTAATCA-	1821-1841	sense
P2	Whole genome/ polymerase	CCGGAAAGCTTGAGCTCTTCAAAAAGTTGCATGGTGCTGG-	1806-1825	antisense
S1	Surface	CCTGCTGGTGGCTCCAGTTC	55-75	sense
S2	Surface	CCACAATTCKTTGACATACTTTCCA	1003-1028	antisense
S6	Surface	GCACACGGAATTCCGAGGACTGGGGACCCTG	129-160	sense
S7	Surface	GACACCAAGCTTGGTTAGGGTTTAAATGTATACC	842-873	antisense
X1	X	TGCCAAGTGTTTGCTGACGC	1176-1195	sense
X2	X	AAGGAAAGAAGTCAGAAGG	1960-1978	antisense
PS1	Pre-S	TCAGAATTCTCACCATATTCTTGGGAACAA	2817-2839	sense
PS2	Pre-S	CACTAGTAAACTGAGCCA	668-687	antisense
PS3	Pre-S	AGTAAGCTTAGAAGATGAGGCATAGCAGC	415-434	sense
C1	Core	CGGGATCCGAGGAGTTGGGGGAGGAGTT	1726-1755	sense
C3	Core	GATCTATGTATTAGGAGGCTG	1753-1774	sense
C4	Core	CCTTATGAGTCCAAGGAATA	2478-2498	antisense

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Sequence Analysis

After allocating a sequence to an HBV genotype by analysis of the S gene, amino acid/nucleotide variations in the HBV genome were compared with a reference sequence from Okamoto (1988, accession number AB033559) and HBsAg sequences from Iranian isolates in GenBank and NCBI and our registry. Compared with the former, amino acid changes were defined as "variants" (host HLA-determined). With regard to the latter (Iranian database sequences), amino acid differences were defined as "mutations."

Statistical Analysis

Data were expressed in contingency tables, and the associations between categorical variables were analyzed by chi-square tests and Fisher exact test. A *p* value less than 0.05 indicated statistical significance.

RESULTS

Analysis of Demographic, Serological, and Clinical Data

Six mothers, who were clinically chronic HBsAg carriers, with normal liver function parameters and their infected children, were enrolled in the study. Serological data, liver function tests, and HBV DNA levels are shown in Table 2. The mean age of the mothers and children were 31.5 (±SD) and 6.5 years (±SD), respectively. All mothers and their children were anti-HBc-positive and anti-HBs-negative. All mothers were HBeAg-negative and anti-HBe-positive. Three children were HBeAg-positive and 3 were HBeAg-negative (Table 2). None of the infected, immunized children developed signs or symptoms of acute hepatitis.

Serological and Virological Analysis

Table 2 shows the clinical, serological, and virological data of the patients. There were no relationships between age, gender (for children), liver function, and serological tests (or HBeAg status of children) between mothers and children. The HBV genome was sequenced, but we obtained the full-length genome for only 5 isolates. The remaining isolates contained at least one protein (surface) that was available for sequencing (Table 2 and Figure 1).

Table 2. Demographic, serologic and virologic data of patients.

Sample	S	X	C/PreCore	PreS	Complete Genome	HBV DNA (Copy/mL)	ALT (Unit/mL)	AST (Unit/mL)	HBeAg	Anti-HBe	Mutation
M1	+	-	+	+	-	33140	40	45	Neg	Pos	S:I4T
M2	+	+	+	+	+	294560	36	42	Neg	Pos	S: S143L; PC: Q2L, 28stop; C: T67S; P: H359N, Y400H, R841K
M3	+	-	-	-	-	200<	22	30	Neg	Pos	-
M4	+	-	-	-	-	7900	16	15	Neg	Pos	-
M5	+	-	-	-	-	5800	25	23	Neg	Pos	-
M6	+	-	-	-	-	ND	28	29	Neg	pos	-
B1	+	+	+	+	+	33o32000	87	66	Pos	Neg	-
B2	+	+	+	+	+	361362000	76	60	Neg	Pos	S: S143L; PC:Q2P; P: Y400H,R841K
B3	+	+	+	-	-	ND	85	60	Pos	Neg	PC: Q2P,
B4	+	+	+	+	+	27800	34	46	Neg	Pos	S:I4N,G145A;PS1:T79A; P:I243V,A251S,N270S, H358Q,D818A,
B5	+	+	+	+	+	1690000	60	55	Pos	Neg	S: I4T, Q129R; PS2:L162R; PC:Q2L,28stop; X: R26C,F30V; H359N
B6	+	-	-	-	-	1400	22	28	Neg	Pos	-

Note: PS1, Pre-S1; PS2, Pre-S2; PC, Pre-core; S, Surface; C, Core; P, Polymerase. M, mothers; B, children.

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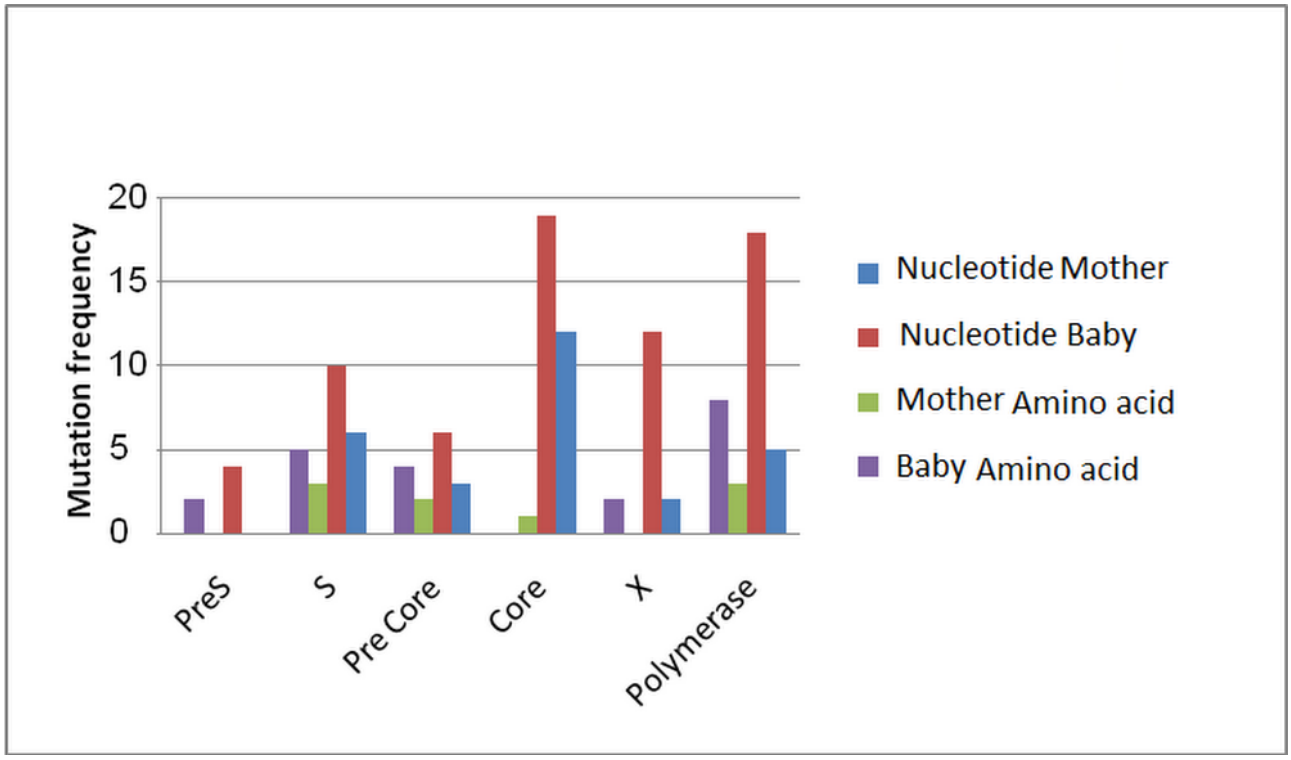

Figure 1. A diagram showing the percentages of the mutational frequencies between mothers and their children.

Genotyping

All 12 isolates belonged to genotype D, subtype ayw2, and subgenotype D1 (results not shown).

Pairwise Mutational Analysis

Table 2 shows the pairwise mutational analysis between mothers and their children.

M1 had the I4T surface protein. B1 did not contain any mutations in its full genomic sequencing at all.

M2 harbored: Surface S143L; Pre-core, Q2L and 28 stop codon; Core, T67S, and Polymerase, H359N, Y400H, R841K. B2 had Surface, S143L; Pre-core, Q2P and Polymerase, Y400H, R841K.

M3 did not contain any mutations in the surface protein. In three sequenced proteins of B3, only Q2P in the Pre-core region was detected.

M4 did not contain any mutations in the surface protein. The following mutations were found in B4: Surface, I4N and G145A; Pre-S1, T79A and Polymerase, H358Q, D818AI, I243V, A251S, N270S.

M5 did not contain any mutations in the surface protein. B5 had I4T and Q129R in the surface; L162R in Pre-S2, Q2L and 28stop codon in Pre-core, and R26C, F30V, H359N in Polymerase.

M6 and B6 did not have any mutations in their surface proteins.

Mutational Analysis of Individual Proteins

Two mutations in the Pre-S region (T79A (B4) and L162R (B5)) were observed in two children. In each sample, B2, B4 and B5 within the surface protein, one mutation was found in the "a" determinant region. In mothers, only one isolate (M2) contained an S143L mutation in the same region. Also, within the small HBsAg (however, outside the a determinant, I4T was noted in M1, B4, and B5. isolates from 4 mothers (M3, M4, M5, and M6) and 3 children (B1, B3, and B6) did not contain any mutations in the surface protein.

By polymerase protein analysis, only B4 contained 3 mutations (I243V, A251S, and N270S) in the spacer region. Within the RT region, 4 isolates (M2, B2, B4, and B5) contained 5 mutations at positions 358, 359, and 400. Mutations at positions 841 and 818 of the RNAse H domain were observed in M2, B2, and B4, respectively.

G1896A, which introduces a stop signal in codon 28 of the precore, was observed in M2 and B5. Mutations at Q2L were noted in the M2 and B5

isolates, and mutation at Q2P in B2 and B3 isolates were noted. Within the core region, despite harboring 31 nucleotide mutations (results not shown), only one amino acid substitution (T67S in M2) was observed.

Within the X region, the only mutations were R26 and F30V in sequence B5.

Table 3. The transmission status of infected children based on the pair wise mutational analysis between mothers and their babies.

Sample Code	Transmission Status
B1	Intrauterine Transmission
B2	Intrauterine Transmission
B3	Intrauterine Transmission
B4	Vaccine Escape Mutation
B5	Vaccine Escape Mutation
B6	Intrauterine Transmission

Evolutionary Analysis

Out of the 12 patients, 6 had mutations. There were 97 nucleotide substitutions, of which 67 (69%) and 30 (31%) were silent and missense, respectively (results not shown). The ratio between synonymous and nonsynonymous (dS/dN) mutations was, 2.2 indicating strong negative selection at the protein level. There were 30 amino acid substitutions: 2, 8, 6, 1, 2, and 11 substitutions were observed in the pre-S, surface, pre-core, core, X, and polymerase proteins, respectively (Fig1). Eleven of 30 mutations (36.6%) were related to the immune surveillance of the hosts: 7 (23.3%) and 4 (13.3%) mutations occurred in T helper and B cell epitopes, respectively, throughout the core, surface, and polymerase proteins (Table 2).

Transmission Status of Children

Based on the pairwise mutational analysis, the transmission status of infected children indicated that Immunoprophylaxis had no effect in the B1, B2, B3, and B6 isolates. Vaccine escape phenomenon was observed in isolates B4 and B5 (Table 3).

DISCUSSION

Little is known about the natural history of HBsAg variants. Newborns may not have a fully functional T-cell response and are susceptible to infections with mutant HBV, despite combined active and passive vaccination against HBV. Direct escape from protective anti-HBs, as observed with the failure of vaccine and HBIG prophylaxis, is caused by mutations in B cell epitopes that impede or abolish recognition by anti-HBs antibodies. Sequence variation in antigenic regions is one of the most powerful viral strategies for escaping recognition by B and T cell-mediated immunity, facilitating viral persistence. It is well documented that amino acid substitutions and insertions in the “a” determinant result in antigenic and immunogenic changes in HBsAg,[2,5-9] and as a result, HBsAg is not detected in some assays[10-12] although this was not the case in our cohort.

In this study, 6 children born to HBsAg positive mother carriers who were immunized with HBV vaccine and HBIG became carriers, and all but one had detectable levels of HBV DNA. The full-length genome was sequenced in 4 of 6 children. In the pairwise mutational analysis, 3 children harbored wild-type HBsAg, similar to their mothers (B1, B3, and B6); hence, they might have obtained the infection through transuterine transmission; the vaccine plus HBIG had no effect on prevention of the infection.

On the other hand, the S gene in the remaining 3 children contained mutations within the “a” determinant (B2, B4, and B5); one child (B2) inherited the mutation from her mother. Because none of the mothers received any vaccine, it appears that this mutation (in M2) developed by natural selection and was subsequently transmitted to the baby (B2). Other studies have shown similar results with G145R and M133L, indicating vertical transmission from mother to babies.[31,32] However, G145R in one study[33] and I126N, P120E, F134Y, and D144A in another study[34] did not present in mothers and thus, assigned as being vaccine escape phenomenon. Similarly, in the present study, B4 and B5 might have acquired their infection due to vaccine escape (Table 3).

Further, multiple mutations with known functional or immune epitope reactivity outside the surface protein were observed in the pre-core, core, pre-S, and polymerase proteins.[35-38] Of 30 amino acid changes, 11 (36.6%) were related to immune surveillance, including the T helper (Th) epitope of the core[35] and poly[36] proteins. Mutations in T cell epitopes influence the anti-HBs antibody profile through interactions between CD4+ helper T cells and B cells. Hepatitis B viruses that harbor mutated epitopes that can not be recognized by specific T-cells in a vaccinated individual will not

enhance anti-HBs production. Based on the complete genomic sequence in children, at least one amino acid mutation was involved in functional or immune epitope activity.

The failure of children to generate an adequate anti-HBs response after vaccination might be due to at least 3 reasons. First, individuals who were protected by HBV vaccine had impaired immune responses and could not develop anti-HBs antibodies. The negative status of anti-HBs in these children who were positive for HBsAg and negative for anti-HBs could have been caused by insufficient amount of antibodies to neutralize the circulating HBsAg.[39] Another reason is an *in utero* infection, wherein the blood of newborn babies might have already been infected with maternal HBV prior to delivery, rendering the HBV vaccine and HBIG useless.

Alternatively, the children might have been infected with HBV mutants or simultaneously with wild-type and mutant HBV. Because serum anti-HBs targets and neutralizes wild-type HBV, the appearance and persistence of HBsAg mutants might occur, regardless of anti-HBs positivity. Mutations in the "a" epitope were observed only in B2, B4, and B5. It is possible that small amounts of wild-type HBV in a population pool coexisted with the variant as a quasispecies but could not be detected in the sequence analysis. However, because we did not perform molecular cloning, this situation cannot be excluded. Therefore, the true proportion of patients who carry HBsAg variants should be higher than what we observed. In this scenario, the HBV population that is defective in one gene may coexist with a wild-type population, facilitating rescue of the virus.[40] These alterations may be part of a dynamic process in which existing mutants die gradually and are replaced by novel mutants that originate from the wild-type. The emergence of quasispecies under these circumstances ensures the persistence of the virus.[41]

The absence of mutations in mothers and their children was not surprising, as it has been shown that viruses that alternate between different hosts have an additional restriction on genetic variation.[42] Similarly, Hsu et al.[43] examined maternal serum and serial samples from their infants who developed an HBV infection despite Immunoprophylaxis and found that the HBV inoculums that were transmitted from mother to infant were generally (87%) the wild-type strain. Again, the co-existence of wild-type and mutant strains is another possibility for the absence of mutations.

The subjects in this study appeared to be asymptomatic. In spite of the combination of passive and active immunization, the children became HBsAg carriers. All children were anti-HBc-positive with mild increases in ALT levels. They must be followed up carefully, as chronically infected children with or without vaccine escape mutants are prone to various liver diseases, including cirrhosis and hepatocellular carcinoma later in life.[16,44]

In conclusion, these findings highlight the need for an alternative regimen, such as the administration of boosters or a more effective HBV vaccine (a third-generation or HBV DNA vaccine), for high-risk children who are born to HBsAg-positive mothers.

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