Prevalence of Mutations in Basal Core Promoter and Precore Region of Hepatitis B Virus in Vaccinated and Nonvaccinated Individuals of the Aboriginal Nicobarese Tribe of Car Nicobar Island, India

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Key Words

Hepatitis B virus · Basal core protein · Precore gene · Mutation · Nicobarese · Aboriginal · Hepatocellular carcinoma · Cirrhosis

Abstract

The aim of this study was to explore the prevalence of basal core promoter (BCP) and precore gene (PC) mutations in hepatitis B virus (HBV) isolates among the Nicobarese tribe and their relationship with genotypes and HBeAg status. A total of 726 blood samples were collected from two villages of the Car Nicobar Island where mass vaccination was performed in the year 2000. HBV DNA was isolated and the BCP and PC regions were amplified and sequenced directly. The samples positive for HBV DNA were tested for HBsAg, HBeAg and anti-HBe. Among the 211 and 515 samples collected from vaccinated and nonvaccinated persons, 16 and 82 were positive for HBV DNA, respectively. Among the vaccinated individuals, only 1 had a mutation in both the BCP and PC gene. Among the nonvaccinated subjects, 3 (4.5%) had an A1762T mutation, 8 (12.1%) had a G1764A mutation, 11 (16.7%) had a G1896A mutation and 4 (6.1%) had a G1899A

mutation. The HBeAg-negative subjects had a significantly higher frequency of BCP and PC mutations than the HBeAg-positive subjects. The prevalence of a PC mutation was higher than that of a BCP mutation. The present study stresses the need for the continuous surveillance of subjects with BCP and PC mutations, as the mutations may contribute to the progression of liver disease.

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Introduction

Over 350 million people worldwide are infected chronically with hepatitis B virus (HBV), of whom 250 million reside in Asia [1, 2]. HBV infection is associated with different clinical outcomes and leads to a chronic carrier state in 5–10% of people infected in adult life and 85–90% of those infected in infancy [3]. HBV replicates by reverse transcriptase that lacks proofreading function. As a result, HBV exhibits a higher rate of mutation than most DNA

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viruses. Although development of mutation is constrained due to overlapping reading frames, mutations have been described in all genes. Mutations in the precore (PC)/core gene and basal core promoter (BCP) regions are associated most frequently with viral persistence and clinical course of chronic hepatitis [4]. The PC open reading frame of HBV codes for the protein HBeAg. The presence of HBeAg in the serum of an individual is associated with active viral replication and is a highly infective stage. Subsequently, there is a loss of HBeAg from the serum, and the subject undergoes seroconversion to anti-HBe. Usually these parameters indicate a decrease of viral replication and remission of liver disease. However, HBV variants have been described that do not produce HBeAg despite continuing replication of infectious virions. The most common variant has a G-to-A transition at nucleotide (nt) 1896 of the PC region, converting codon 28 from that coding for tryptophan to a stop codon (TGG to TAG) [5]. HBV strains with this mutation do not produce HBeAg at all, and such strains might get selected over nonmutant strains. The core promoter region regulates the transcription of the PC region. A double mutation at nt 1762–1764 changing AGG to TGA in the BCP region (BCP mutation) has been suggested to downregulate HBeAg production. This mutation is thought to be associated with enhanced viral replication and has been found during chronic infection [6]. In a cross-sectional study, both the BCP mutations and the PC A1896 mutations were found to be highly and equally significant for the HBeAg-negative phenotype displayed [7]. Due to the requirement of base pairing at the pregenomic RNA level, the presence of an A1896 mutation is thought to be restricted to genotypes that have a T1858, as in the case of genotypes B, C, D and E. The absence of this mutation in genotypes A and F has also been attributed to the presence of C at nt 1858 [8, 9]. In view of these clinical implications of the PC and BCP regions of HBV, it was considered necessary to know the prevalence of these mutations among HBeAg-negative chronic HBV carriers among the Nicobarese, a tribe with a very high HBV carrier rate. The Andaman and Nicobar Islands, a Union Territory of India, is the home of six heritage/traditional tribes, constituting about 10% of the total population of these islands. Hepatitis B infection is highly endemic among these tribes [10]. The epidemiology of HBV infection among Nicobarese (a Mongoloid tribe that constitute more than 98% of the tribal population of the islands) appears to be unique with a high prevalence of the chronic carrier state (22.2%) and a comparable proportion of the population (26.3%) positive for anti-HBs [11]. Considering the high endemicity of

HBV infection among these aboriginal tribes, a pilot project of mass hepatitis B vaccination was initiated in the year 2000 in two villages of the Car Nicobar Island inhabited exclusively by Nicobarese [12]. More than 95% of the vaccinated people developed an anti-HBs antibody titer of more than 10 mIU/ml, indicating seroprotection after the third dose of vaccination, but the proportion of seroprotected people dropped to 85.5% after 3 years and stayed at the same level 5 years after the vaccination [13, 14]. The present study is a part of the follow-up study on the efficacy of the vaccination 10 years later. This is the first study of its kind done on this tribal community of India, which has a very high endemicity of hepatitis B.

Subjects and Methods

Study Subjects

A total of 726 blood samples were collected from two villages of the Car Nicobar Island. These 726 samples included 211 samples from individuals who belonged to the cohort that participated in the vaccination study in 2000 as part of the pilot project of mass vaccination and 515 samples were from individuals belonging to the nonvaccinated cohort. Informed consent was obtained from the subjects/guardians prior to collection of samples. Serum was separated from the blood samples and brought to the laboratory of RMRC, Port Blair, maintaining cold chain conditions and stored at -86° until processed. An attempt to isolate HBV DNA was made for all of the samples. The samples positive for HBV DNA by PCR were tested for HBsAg, anti-HBs, anti-HBc, HBeAg and anti-HBe using commercially available enzyme-linked immunosorbent assay kits (General Biologicals Corporation, Hsin Chu, Taiwan).

Isolation of HBV DNA and Amplification of the Surface Gene, BCP and PC Regions

DNA was isolated from 200- μ l serum samples by phenol/isoamyl alcohol/chloroform extraction after incubation with proteinase K. Extracted DNA was subjected to HBV detection by nested PCR of the surface (S) gene following a published protocol [15, 16]. Extracted DNA was subjected to nested PCR using two sets of primers as described by Kumar et al. [17]. Polymerase chain reaction (PCR) was performed on 5 μ l of DNA extract in a 50- μ l reaction mix containing a final concentration of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.25 μ M each of the 4 dNTPs, 2.5 U Taq DNA polymerase (Bangalore Genei, Bangalore, India) and 0.6 mM each of the primers. First-round PCR with the help of outer primers P1 (5'-GTT GCA TGG AGA CCA CCG TGA AC-3') and P2 (5'-CTT CTG CGA CGC GGC GAT GGA GA-3') was carried out with initial denaturation at 94° for 3 min, followed by 30 cycles of 94° for 1 min, 60° for 1 min and 72° for 1 min 30 s, followed by primer extension at 72° for 7 min. Second-round PCR using 5 μ l of the first PCR product with the help of inner primers P3 (5'-CAT AAG AGG ACT CTT GGA CT-3') and P4 (5'-GGC GAG GGA GTT CTT CTT C-3') was performed with initial denaturation at 94° for 3 min, followed by 30 cycles of 94° for 1 min, 55° for 1 min and 72° for 1 min 30 s. A single cycle for primer extension was done at 72° for 7 min.

Table 1. Details of the samples with BCP and PC mutations in HBV isolates

Sample No.	Age, years/sex	Status	HBsAg	Anti-HBs, mIU/ml	Anti-HBc	HBeAg	Anti-HBe	Genotype	BCP		PC	
									A1762	G1764	G1896	G1899
1	37/M	V	P	2.4	P	N	P	D	T	A	A	A
2	50/M	NV	P	1	P	N	P	D	T	A	A	A
3	27/M	NV	P	2	P	N	P	D	–	A	–	–
4	26/M	NV	P	2	P	N	P	D	–	–	A	–
5	22/F	NV	N	8	P	N	N	D	T	A	–	A
6	26/F	NV	N	3	P	N	N	D	–	–	–	A
7	22/F	NV	P	4	P	N	P	D	–	A	A	–
8	37/M	NV	P	1.9	P	N	P	D	–	–	A	A
9	65/M	NV	P	0.1	P	P	–	D	–	A	A	–
10	28/M	NV	P	0.2	P	N	P	D	–	A	A	–
11	63/F	NV	N	0.2	P	N	N	D	–	–	A	–
12	32/F	NV	P	0.3	P	N	P	D	T	A	A	–
13	32/M	NV	P	0.8	P	N	P	D	–	–	A	A
14	29/F	NV	P	1.1	P	N	P	A	–	–	A	–
15	27/M	NV	P	1	P	N	P	D	–	A	A	A

V = Vaccinated; NV = nonvaccinated; P = positive; N = negative.

DNA Sequencing

PCR primers were used for direct sequencing of the amplified DNA from both directions using the Big Dye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, Calif., USA) in a 3130 Automated DNA Sequencer (Applied Biosystems). Mutations were recorded only when detected in both the forward and reverse sequences.

Genotyping, BCP and PC mutant analysis were carried out by sequence comparison with other known sequences from different HBV genotypes as performed using MEGA5 [18] and SeqScape software (Applied Biosystems).

HBV Genotyping

Genotyping of HBV was performed on the basis of phylogenetic relationship taking 345 base pair sequences of part of the S gene along with representative reference sequences from different genotypes as described earlier for HBV [19, 20].

Statistical Analysis

Data was compared and statistical significance was tested using Epi Info 7 software (www.cdc.gov/epiinfo/). $p < 0.05$ was considered statistically significant.

Results

Among the 211 samples from vaccinated persons, 16 (7.6%, 95% CI: 4.4, 12.0) were positive for HBV DNA, while among the 515 samples from nonvaccinated persons, 82 (15.9%, 95% CI: 12.9, 19.4) were positive. Thus, among the total number of 726 subjects, 98 (13.5%, 95%

CI: 11.1, 16.2) were positive for HBV DNA. Bidirectional sequencing of BCP and PC genes was successfully achieved for 82 (82/98) samples positive for HBV DNA, which included 16 from vaccinated subjects and 66 from nonvaccinated subjects. Further analysis presented here is confined to these 82 subjects in whom sequencing of the BCP and PC gene was successful. The few mutations that were found in the BCP and PC region were clustered at nt positions 1762, 1764, 1896 and 1899.

Out of these 82 HBV DNA-positive subjects, 71 (86.6%) were negative for HBeAg, of which 42 (out of 71; 59.2%) were positive for anti-HBe. Among the 82 samples, 35 (42.7%) were positive for HBsAg. All the samples detected with a mutation in either of BCP, PC or both, except 3, were found to be HBsAg positive (table 1). Out of the 71 subjects negative for HBeAg, 27 (38%) were positive for HBsAg.

Mutations among Vaccinated Individuals

All of the 16 isolates from vaccinated individuals whose BCP/PC gene was sequenced had T at nt 1858. Only 1 (6.3%) sample, obtained from a 37-year-old male, had mutations in both the BCP and PC region. He was positive for HBsAg and anti-HBe, but negative for HBeAg. The anti-HBs titer of the sample was 2.4 mIU/ml (subject 1 in table 1). The HBV isolate was classified into genotype D (fig. 1).

Fig. 1. Phylogenetic analysis of the partial S gene showing the genotype of HBV with BCP and PC mutations.

Table 2. Demographic data in with respect to the PC and BCP mutations in HBV among the nonvaccinated subjects

Variables	Total	HBeAg (+)	HBeAg (–)	Anti-HBe ¹ (+)	Anti-HBe ¹ (–)
Age group					
0–9 years	1 (1.5)	0 (0)	1 (100)	0 (0)	1 (100)
10–19 years	4 (6.1)	1 (25.0)	3 (75.0)	0 (0)	3 (75.0)
20–29 years	17 (25.7)	2 (11.8)	15 (88.2)	11 (64.7)	4 (23.5)
30–39 years	12 (20.4)	2 (16.6)	10 (83.4)	9 (75.0)	1 (8.3)
40–49 years	10 (15.2)	1 (10.0)	9 (90.0)	5 (50.0)	4 (40.0)
50–59 years	13 (19.7)	3 (23.1)	10 (76.9)	8 (61.5)	2 (15.4)
60 years and above	9 (13.6)	2 (22.2)	7 (77.8)	2 (22.2)	5 (55.6)
Gender					
Male	30 (39.8)	8 (26.7)	22 (73.3)	15 (50.0)	15 (50.0)
Female	36 (60.2)	2 (5.5)	34 (94.5)	20 (55.6)	16 (44.4)
HBV DNA positive by sequencing	66	11 (16.7)	55 (83.3)	35 (53.0)	20 (30.3)
Mutation					
In BCP					
A1762T	3 (4.5)	0 (0.0)	3 (5.5)	2 (5.7)	1 (5.0)
G1764A	8 (12.1)	1 (9.1)	7 (12.7)	6 (17.1)	1 (5.0)
In PC					
1858T	65 (98.5)	11 (100)	54 (98.2)	34 (51.5)	20 (100)
1858C	1 (1.5)	0 (0.0)	1 (1.8)	1 (2.8)	0 (0.0)
G1896A	11 (16.7)	1 (9.1)	10 (18.2)	9 (25.7)	1 (5.0)
G1899A	4 (6.1)	0 (0.0)	4 (7.3)	2 (5.7)	2 (10.0)
In BCP+PC	7 (10.6)	1 (9.1)	6 (11.0)	5 (14.3)	1 (5.0)

Data in parentheses indicate percentages. ¹ Samples positive for HBeAg were not tested for anti-HBe, i.e. only samples negative for HBeAg were subjected to anti-HBe testing.

Mutations among Nonvaccinated Individuals BCP Mutation

Among the 66 nonvaccinated subjects whose BCP/PC gene was sequenced, 3 (4.5%) had an A1762T mutation and 8 (12.1%) had a G1764A mutation. A total of 3 (4.5%) had both A1762T and G1764A mutations. None of the HBeAg-positive samples had an A1762T mutation. Both A1762T (5.5% vs. 0%; $p = 0.4278$) and G1764A (12.7% vs. 9.1%; $p = 0.7358$) mutations were observed to be not significantly different in subjects with an HBeAg-negative status that in those who were HBeAg positive (table 2). These mutations were also found to be higher in anti-HBe-positive cases than in anti-HBe-negative cases.

PC Mutation

Among the 66 nonvaccinated subjects, 11 (16.7%) had a G1896A mutation and 4 (6.1%) had a G1899A mutation. A total of 4 (6.1%) HBV isolates had both G1896A and G1899A mutations. One of the HBeAg-positive samples had a G1896A mutation, but none of the HBeAg-positive samples had a G1899A mutation. Both G1896A

(18.2 vs. 9.1%; $p = 0.4601$) and G1899A (7.3 vs. 0%; $p = 0.3560$) mutations were observed to be not significantly different in HBeAg-negative subjects compared to HBeAg-positive individuals (table 2). These mutations were found to be more frequent in anti-HBe-positive cases than in anti-HBe-negative cases. Two HBV isolates with a single mutation in the PC region were negative for HBsAg (table 2).

Both BCP and PC Mutations

The presence of both BCP and PC mutations were observed in 7 (10.6%) of the cases and was found to be not significantly different in HBeAg-negative cases compared to HBeAg-positive cases (11 vs. 9.1%; $p = 0.8581$). One HBV isolate with both BCP and PC mutations was negative for HBsAg.

C1858T Mutation

In the present study, 65 isolates had T at nt 1858 and 1 had C. Among these 65 isolates with T at nt 1858, 64 belonged to the D genotype and 1 to the A genotype. The

isolate with C at nt 1858 was of the D genotype. Demographic data in respect to the BCP and PC mutations in HBV among the nonvaccinated subjects is provided in table 2. No mutation was detected at nt 1814 and 1862 in any of the samples. All of the individuals harboring a mutation either in BCP/PC region or both were nonseroprotected (anti-HBs titer ≤ 10 mIU/ml) and anti-HBc positive.

Discussion

The BCP (nt 1744–1804), residing in the overlapping X open reading frame region, controls transcription of both PC and core regions. PC mutations frequently occur temporally related to core gene mutations/deletions. A variety of PC/core mutants have been described in various parts of the world and are known to be associated with increased risk of hepatocellular carcinoma (HCC) as well as the progression of liver diseases [21–23]. In the present study, BCP mutations A1762T and G1764A were observed in 5.5 and 12.7% of the HBeAg-negative subjects. PC mutations G1896A and G1899A were observed in 18.2 and 7.3% of the HBeAg-negative subjects, which is slightly lower than observed in earlier studies [21, 22, 24]. A double-mutation in the BCP region was observed in 4 HBeAg-negative individuals, which includes 1 vaccinated individual. The anti-HBs titer of the vaccinated individual with BCP and PC mutations was ≤ 10 mIU/ml, so there is a probability of having been reinfected with HBV. The HBeAg-negative subjects had a significantly higher frequency of mutation in both BCP and PC nucleotides than HBeAg-positive subjects. Earlier studies have also shown a high prevalence of BCP and PC mutations in HBeAg-negative individuals [21, 24, 25]. However, 1 HBeAg-positive individual had a G1896A mutation. A study conducted in Germany showed the presence of G1896A mutations in 2.2% of the HBeAg-positive subjects [26].

Changes at positions 1762 and 1764 have been described to be especially common in subjects with C1858 [8, 27], but in the present study the presence of such mutations was observed in subjects with T1858. The majority of the HBV isolates in the present study was genotype D with T at nt 1858. The same finding was observed in a study conducted in Brazil [28]. T1858 associated with genotypes A, C and D had been found as well in a survey in the United Arab Emirates [29]. In the study conducted in Brazil, PC mutations were observed to be more frequently associated with genotype D of HBV [28].

Among the 82 individuals positive for HBV DNA, 35 were HBsAg positive. The rest were positive for either an-

ti-HBs or anti-HBc indicating occult HBV infection. Occult HBV infection is a condition which may be defined as serologically undetectable hepatitis B surface antigen (HBsAg-ve), despite the presence of circulating HBV DNA with or without serological markers of a previous infection (anti-HBc and/or anti-HBs positive) [17, 30]. Core gene mutations are epidemiologically associated with disease activity, whereas the PC stop mutation may be an innocent bystander. In contrast, the double mutations in the BCP downregulate HBeAg expression and are likely to increase viral replication with enhanced disease activity [4]. Mutation G1896A in the PC region leading to a G-to-A shift could induce a stop codon and subsequently suppress the expression of HBeAg [5]. Though this would stop the production of HBeAg, the HBV DNA would still be synthesized and eventually contribute to the progression of liver disease to a more advanced stage [16]. It was once reported that G1896A correlated with severe forms of liver diseases [31]. There is strong epidemiological evidence that a PC A1896 mutation as well as T1762 and A1764 of BCP are associated with fulminant hepatitis [32]. It was suggested that the fulminant course was due to either a vigorous immune response of the host or to high levels of HBV DNA or to high transcription efficiency of the virus [27]. In our HBV isolates, all of the BCP and PC mutations were observed in genotype D and only G1896A was observed in genotype A. Genotype D was earlier found to be the predominant genotype of HBV circulating among these tribal populations of India [15]. There are therapeutic implications of this finding as subjects with genotype D have more severe disease [33]. Earlier reports also indicated a significant correlation between BCP mutations of HBV and HCC [34].

The present study is the first of its kind to report the prevalence of these mutations among the Nicobarese tribe living in the islands where hepatitis B is highly endemic. In contrast to an earlier study carried out in India [24], the majority of the HBV isolates in our study were of genotype D with T at nt 1858, in which the prevalence of a PC stop mutation is higher than that of a BCP mutation. The detection of BCP and PC mutations in vaccinated individuals is of grave concern as it is likely to increase viral replication and enhance disease activity [4].

Conclusions

Although there is no conclusive evidence for association of these mutations with severity of complications, the present study stresses the need for the continuous

surveillance of these subjects with BCP and PC mutations as they may contribute to the progression of liver disease to a more advanced stage. Frequent examination of individuals with chronic HBV infections for the presence of these mutations may be useful for identifying individuals who require preventive antiviral treatment and for the prediction of development of HCC. However, the wide range of chronic liver disease, from inactive carrier to liver cirrhosis and HCC, cannot be explained completely by the presence of PC and core variants. There may be an interactive mechanism between host genetic factors and virus variation within the core protein or other proteins. The exact clinical relevance of these variants needs further investigation. Future studies should focus on interactions of these HBV mutations with host factors, as well as the development of high throughput methods for the detection of HBV mutations for the prediction of HCC.

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Ethics Approval

The study was cleared by the institutional Ethics Committee.

Disclosure Statement

The authors do not have any commercial or other associations that may pose a conflict of interest.

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