J. Microbiol. Biotechnol. (2008), **18**(10), 1722–1728

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Low Frequency of Precore Mutants in Anti-Hepatitis B e Antigen Positive Subjects with Chronic Hepatitis B Virus Infection in Chennai, Southern India

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Received: January 24, 2008 / Accepted: May 28, 2008

The natural course of chronic hepatitis B (CH-B) virus infection is reportedly variable, and the long-term outcomes in hepatitis B e antigen (HBeAg)-negative chronic hepatitis B infection are distinct from HBeAg-positive chronic hepatitis. However, the molecular virological factors that contribute to the progression of liver disease in the south Indian setting remain largely unclear. We prospectively studied 679 consecutive patients for HBsAg, HBeAg, anti-HBe, and HBV DNA by qualitative PCR. Randomly selected samples were subjected to bidirectional sequencing to reveal core/precore variants. Of the total 679 chronic HBV cases investigated, 23% (154/679) were replicative HBV carriers. Furthermore, amongst the 560 HBV DNA samples analyzed, 26% (146/560) were viremic. Among the 154 HBeAg positive cases, HBV DNA was positive in 118 cases (77%), significantly (p<0.001) higher than the anti-HBe positive (7%) (28/406) cases. Significant increase in liver disease (p<0.01) with ALT enzyme elevation (p<0.001) was observed in both HBe and anti-HBe viremic cases. Interestingly, low frequencies of mutations were seen in the precore region of the HBV strains studied. HBV precore and core promoter variants were less often detected in subjects with "e" negative chronic HBV infection and, therefore, may not have a prognostic role in determining liver disease sequelae in this part of tropical India.

Keywords: Hepatitis B virus, HBV, India, precore/core mutants

Of the estimated 50 million new cases of hepatitis B virus (HBV) infection diagnosed annually, 5–10% of adults and up to 90% of infants become chronically infected. Asia constitutes 75% of the global pool of HBV infection, where it is reportedly the leading cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) [21, 24], and India has intermediate HBV endemicity. The prevalence of hepatitis B surface antigen (HBsAg) in India varies from 1–13%, with an average of 4.7% [18, 22, 25, 29]. A high prevalence rate of HBsAg has been reported among a few Indian tribes [23, 26–28]. HBV is considered to be the etiology of chronic hepatitis and cirrhosis in 40–60% of subjects [17, 28], and ~80–90% of primary liver cancers have been reported to be associated with HBV infection in India [17, 28], where chronic HBV infection is reportedly acquired in childhood, presumably before 5 years of age. Chronic hepatitis B is not a static disease and the natural history of the disease is affected both by viral and host factors [20]. Prevention of HBV infection through vaccination, therefore, is presumed to be the main strategy for decreasing the incidence in India.

India, with a carrier rate of 3%, contributes nearly 10% of the HBV carriers in the world. Without any organized HBV prevention programme, and with 25 million live births each year, nearly 1 million HBV infections are added to the HBV pool yearly, contributing to its rapid expansion [2]. In this situation, HBV epidemiology is presumed to be an important determinant of the global HBV burden in the future. Molecular epidemiological data regarding HBV infection provides information about the emerging worldwide epidemiology of HBV, which is likely to shift its focus to South Asia in general, and India in particular, in the years to come, in view of the growing HBV burden therein, in the absence of interventions.

Genetic alterations have been observed in the precore and core nucleotide sequences of HBV strains from anti-HBe Indian patients. In a recent Indian study, a substantial proportion of anti-HBe positive chronically infected individuals continued to circulate preC wild-type HBV [14], and also documented a HBeAg positive CHB patient with a pre-C

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mutant. Further more a substantial proportion of anti-HBe positive subjects carry the precore wild-type strain, which suggests that predominance of pre-C mutants can never be solely responsible for the absence of HBeAg [16]. The HBV profile of Indian patients with chronic HBV, a zone of intermediate prevalence, is believed to be an important determinant of the global HBV burden in the near future. Hence, in a prospective, cross-sectional study, we investigated the biochemical serological, histological, and molecular aspects of HBV among subjects positive for chronic HBV infection at a tertiary referral center in Chennai, southern India.

MATERIALS AND METHODS

Patients and Serological Studies

Between 2002 and 2004, 679 patients with long-term HBV infection, referred from various gastroenterology departments to the National Reference Centre for Viral Hepatitis, Department of Microbiology, Dr. ALM PG IBMS, University of Madras, Chennai, India, were studied. The long-term chronic hepatitis B was defined as those with steady positivity for HBsAg in their serum for >6 months, persistently increased levels of alanine transaminase (ALT) for >6 months, and either USG/CT scan or histologically identified liver disease. Patients were excluded for one or more of the following reasons: HBV-infected transient HBe seroconverted carriers (defined as persistent HBV infection with both HBe and anti-HBe positive sera by ELISA); superinfected and co-infected with HIV; liver disease with evidence of other noninfectious etiologies (autoimmune, metabolic, and toxic); and alcohol abuse (more than 5 alcoholic drinks a week). The study was approved by the University of Madras's institutional ethics committee.

Eligible patients included males and females aged between 20 and 75 years, with detectable serum HBsAg, HBeAg/anti-HBe at the time of screening and for at least 6 months before the study entry, as assessed by ELISA. The available biochemical, clinical, and liver histopathological data were used to correlate with the virological profile. Accordingly, 679 HBV-infected cases (568 men, 111 women) with >6 months of HBsAg positivity were analyzed with the available clinical, biochemical, and histological assessment information from the patient's clinical case history records. All subjects were interviewed on the basis of a predesigned questionnaire regarding occupation, contact with HBV-related liver disease patients, past history of jaundice (its nature, etiology, and outcome), blood transfusion, surgery/dental/gynecological procedures, radiological intervention, exposure to syringes and needles, ear pricks, sharing of razors and blades, mother being HBsAg positive, information on high-risk behaviors such as intravenous drug abuse, sexual promiscuity, tattooing, *etc.* None had a history of hemodialysis or organ transplantation.

The patients were classified into 4 groups, according to HBeAg positivity and presence of HBV DNA. All anti-HBe positive patients were also HBeAg negative. The groups were as follows: group I, 118 HBeAg positive patients with HBV DNA positive; group II, 36 HBeAg positive without DNA positive; group III, 28 patients with DNA in anti-HBe positive group; and group IV, 378 anti-HBe positive patients without HBV DNA. All four groups were assessed with available data of age, sex, ALT enzyme, liver disease assessed by either USG/CT scan, or disease proven histologically.

DNA Extraction and PCR Assays

DNA was extracted from serum samples by standard Proteinase K digestion, phenol chloroform, and ethanol precipitation methods. HBV DNA precore/core sequence was amplified by PCR using primers spanning the precore/core region (MDD2: 5'-GCG AAG CTT GAG GAA TAA AGC CCC GTA AA-3' and HDM3: 5'-GCG CTG CAG GAG TTG GGG AGG AGA TTA-3') [12]. A 100 µl amplification reaction was performed in first round and then 50-µl of the amplified product was subjected to nested-PCR using a set of internal primers (MDN5: 5'-GCG AAG CTT AGA TCT CTG GAT GCT GGA-3' and HDB2: 5'-GCG CTG CAG GAG GCT CTA GGC ATA AAT-3') and using the same thermocycling profile used for the first round reaction.

Nucleotide Sequencing and Computer-Assisted Sequence Analysis

PCR products were separated by 2% agarose gel electrophoresis and subsequently purified using a commercial kit (Qiaquick gel extraction kit; QIAGEN, U.S.A.) as per the manufacturer's instructions. The purified PCR products (0.1 to 0.5 µg) were sequenced with the Big-Dye Terminator cycle sequencing ready reaction kit (Perkin Elmer, Foster City, CA, U.S.A.) and subjected to bidirectional direct sequencing. The data were collected and analyzed with the sequence analysis software (ABI Prism 377). The nucleotide sequences were aligned along with a consensus sequence derived out of EMBL database sequences of the precore region of HBV, using the multiple sequence alignment programs Clustal X version 1.8 and Genedoc software.

Statistical Methods

The median values of ALT levels according to age, HBeAg/anti-HBe, with or without DNA, and liver status were analyzed by nonparametric tests such as the Mann-Whitney U test. Whenever variables were continuous, the results were analyzed by the *t*-test. The evaluation was performed using the Statistical Package for Social Sciences (SPSS Version 13.0; IL, U.S.A.).

RESULTS

The mean age of the patients was 46±12 years (SD); 568 patients (84%) were male and 111 (16%) were female. The age group and the overall risk factors analyzed are given in Table 1. Out of 679 chronic HBV patients analyzed, 154 (23.4%) were replicative carriers. HBV DNA was analyzed in 560 cases, of which 146 (26%) were viremic. The breakup of HBV DNA positivity in different groups of chronic HBV infection cases are shown in Table 2. Among the 154 HBeAg positive cases, HBV DNA was positive in 118 cases (77%), which was significantly ($p<0.001$) higher than anti-HBe positive cases found to be DNA positive, which was 28 (7%). Although HBV DNA positive patients in both groups had significant elevation of ALT ($p<0.001$), a significant level of liver diseased patients were observed in the anti-HBe positive patients with DNA positivity compared with HBe DNA positive groups ($p<0.05$).

The overall liver status of 251 patients is shown in Tables 2 and 3. Forty-one percent of liver abnormality was seen among the HBe positive patients compared with 39% in anti-HBe positive patients. The liver cirrhosis (29%) and

Table 1. Demographic profile of 679 patients with chronic hepatitis B virus (HBV) infection.

Characteristics	Numbers (%)
Age, Year. Mean (and SD)	46 (12)
Age groups	
<30	82 (12%)
31–40	143 (21%)
41–50	221 (33%)
>50	233 (34%)
Sex	
Male	568 (84%)
Female	111 (16%)
Risk factors (n=608)	
Sexual promiscuity	48 (8%)
BT/hospitalization/surgery	33 (5%)
IVDU	10 (2%)
Tattoo	49 (8%)
Exposure to unsterilized instruments	126 (21%)
H/o Jaundice	43 (7%)
Unknown	299 (49%)
Liver function test	
ALT, IU/l	
Median (range)	55 (45–65)
Less than 50	173 (37)
Greater than 50	300 (63)
HBV profile	
HBeAg (%)	154 (23%)
Anti-HBe (%)	525 (77%)
DNA positivity (%)	146 (23%)
Liver status	
Liver normal by USG (%)	151(60%)
Liver abnormal by USG (%)	55 (22%)
CHB with cirrhosis	10 (4%)
CHB without cirrhosis	26 (10%)
HCC	9 (4%)

chronic hepatitis with cirrhosis (11%) was significantly higher (p<0.01) in the DNA positive anti-HBe positive groups (Group III). In both USG/CT scan and histopathological level, liver abnormality (p<0.01) was significantly higher in DNA positive HBe seroconverted cases (Group III *vs* Groups I, II, and IV) compared with the HBe group (Tables 2 and 3). Liver abnormality was significantly (p<0.01) higher in chronic HBV-infected patients with the age of above 50 years.

We analyzed the core/precore mutant in eight randomly selected DNA positive HBe seroconverted (anti-HBe positive) patients (4 chronic hepatitis, 2 cirrhosis, and 2 HCC) along with one HBe positive sample as a control. The 1,896 stop codon mutation was not seen in our patient's sample, and low frequencies of mutations were observed in the precore region. All the nine cases were positive for HBV DNA by PCR and their nucleotide sequences were determined

Table 2. Characteristics of 679 patients with chronic hepatitis B virus (HBV) infection according to hepatitis B e antigen (HBeAg) positivity.

Characteristics	HBeAg+ve (n=154)	Anti-HBeAg+ve (n=525)	p Value
Age (years)			**0.582**
Mean (SD)	46 (12)	46 (12)	
Age group			
< 30	20 (13%)	62 (12%)	
31–40	35 (23%)	108 (21%)	
41–50	43 (28%)	178 (34%)	
>50	56 (36%)	177 (34%)	
Sex *N* (%)			**0.204**
Male	125 (81)	443 (84)	
Female	29 (19)	82 (16)	
ALT (IU/l)[a]			**<0.001**
Less than 50 (n=173)	49 (34)	124 (38)	
Greater than 50 (n=300)	97 (66)	203 (62)	
HBV DNA (n=146)			**<0.001**
DNA positivity (%)	118 (77%)	28 (7%)[b]	
Liver status (n=251)[c]			**0.024**
Liver normal	60 (58)	91 (61)	
Liver abnormal by USG (%)	42 (41)	58 (39)	
CHB with cirrhosis (%)	6 (6)	4 (3)	
CHB without cirrhosis (%)	8 (8)	18 (12)	
HCC(%)	0 (0)	9 (6)	

[a]ALT values not available for 206 cases.
[b]HBV DNA PCR was done for 406 anti-HBe positive cases.
[c]Liver status not known for 52 HBeAg and 376 anti-HBe positive cases.

and analyzed (GenBank accession numbers for HBV core/precore nucleotide sequences: DQ222407, DQ222408, DQ222409, DQ222410, DQ222411, DQ222412, DQ222413, DQ222414, and DQ222415).

Variations in the Precore Region

The precore stop codon mutation at nt-1896 was not observed in any of the HBV isolates analyzed in this study. Mutation at nt-1899 was observed in two (P1 and P5) patients with chronic hepatitis B of the eight anti-HBe positive patients. A1899 mutation was also seen in the HBe positive case analyzed (P7). In another patient (P11), a mixed population of both 1899 variant and wild type was observed. The HBV precore sequences of two of the anti-HBe patients (one diagnosed with cirrhosis and another with chronic hepatitis B) had a mutation at nt-1862 (G→T) leading to a change of valine to phenyl alanine at codon 17. It was also identified that these HBV isolates had "C" at nt-1858. Other polymorphisms in the precore region included mutations at nt-1846 (A→T; Ser→Ser), nt-1850 (T→A; Ser→Thr), and

Table 3. Characteristics of 679 patients with chronic hepatitis B viral (HBV) infection according to HBV DNA positivity.

Characteristics	HBeAg Positive (*n*=154)		anti-HBeAg Positive (*n*=406)[a]		*p* Value
	Group I (*n*=118)	Group II (*n*=36)	Group III (*n*=28)	Group IV (*n*=378)	
Age, Year					**0.157**
Mean (and SD)	45 (12)	47 (12)	50 (11)	44 (12)	
Age group					
<30	16 (14%)	4 (11%)	2 (7%)	52 (14%)	
31–40	28 (24%)	7 (19%)	3 (11%)	81 (21%)	
41–50	35 (30%)	8 (22%)	8 (29%)	135 (36%)	
>50	39 (33%)	17 (47%)	15 (54%)	110 (29%)	
Sex					**0.408**
Male (%)	97 (82%)	28/ (78%)	26 (93%)	318 (84%)	
Female (%)	21 (18%)	8 (22%)	2 (7%)	60 (16%)	
ALT, IU/L[b]					**<0.001**
Median (range)	42 (35–230)	51 (35–260)	160 (38–467)	55 (35–160)	
Less than 50	35 (30)	14 (50)	2 (7)	72 (37)	
Greater than 50	83 (70)	14 (50)	26 (93)	136 (63)	
Liver status[c]					**0.002**
Liver normal (%)	55 (60)	5 (50)	8 (29)	62 (63)	
Liver abnormal by USG (%)	25 (27)	3 (30)	8 (29)	17 (17)	
CHB with cirrhosis (%)	5 (5)	1 (10)	3 (11)	1 (1)	
CHB without cirrhosis (%)	7 (8)	1 (10)	8 (29)	10 (10)	
HCC (%)	0 (0)	0 (0)	1 (4)	8 (8)	

[a]A total of 525 DNA PCRs were done an 406 anti-HBe positive cases.
[b]ALT values not available for 206 cases.
[c]Liver status not known for 52 HBeAg and 376 anti-HBe positive cases Groups I and III, Positive for HBV DNA; Groups II and IV, Negative for HBV DNA by PCR.

nt-1888 (G→A; Gly→Gly). No deletion or insertion mutations were observed in any HBV precore sequences analyzed.

Variations in the Core Region

Multiple sequence variations have been observed in the core region of all the HBV strains from the e-CHB analyzed. Several point missense mutations were observed in the core region of the HBV strains at positions nt-1934 (T→A; Ser→Thr), nt-1938 (T→C; Val→Ala), nt-1979 (A→G; Ile→Val), and nt-2020 (G→T; Glu →Asp). Sequence variations in the deduced amino acid sequence precore/ core region are summarized in Table 6. Notably, in all the eight HBeAg negative anti-HBe positive cases, there were mutations at nt-1934 (T→A; Ser→Thr) and nt-1979 (A→G; Ile→Val) at proximal core codons 12 and 27 respectively. It was observed that these two mutations were not seen in the HBV core sequence of HBe positive patients. Besides these missense mutations, there were many silent mutations in the core nucleotide sequences that included nucleotide substitutions at nt-1915 (G→T), nt-1951 (T→G), nt-1975 (T→G), nt-2013 (G→T), nt-2029 (G→A), nt 2035 (G→T), nt- 2038 (A→G), nt-2045 (T→C), nt 2053 (C→T), nt-2059 (A→T), nt-2074 (T→A), nt-2074 (T→C), nt-2077 (T→C), nt-2080 (G→T), nt-2083 (T→C), and nt-2089 (T→G) in the core region of the HBV isolates.

Table 4 Age-specific correlation of liver status in patients with chronic HBV infection.

Groups Age, Yr	Liver abnormality by CT,USG & histopathology[a]	*p* Value
<30 (*n*=74)	7/26 (27)	
31–40 (*n*=119)	13/44 (30)	**0.017**
41–50 (*n*=186)	30/74 (41)	
>50 (*n*=181)	48/84 (57)	

[a]Numerator, Numbers positive; Demoninator, Total and numbers in parentheses indicate percentage.

Discussion

In our report on HBV infection in chronically infected patients in the southern part of India, a fairly large chronically infected patient's sample was systematically obtained, by using a multistage sampling methodology, among the various hospital referred cases. Majority of men were positive for both anti-HBe and HBV DNA compared with women ($p<0.001$). Most of the subjects aged 40 years and above were HBe seroconverted. The low prevalence of PC and BCP mutants, despite a very high prevalence of HBeAg negative infection, moderate to severe USG/CT findings, characterize the chronic HBV-infected cohort in the present study. However, a high level of viremia in anti-HBe positive

Table 5. Clinical and virological profile of the randomly selected chronic HBV infected patients for core/precore analysis.

Sl no.	Patient ID	Clinical status	ALT (IU/l)	HBeAg	anti-HBe	HBV DNA
1	P1	Chronic hepatitis without cirrhosis	234	–	+	+
2	P8	Chronic hepatitis without cirrhosis	267	–	+	+
3	P9	HCC	38	–	+	+
4	P7	Chronic HBV infection	79	+	–	+
5	P6	HCC	94	–	+	+
6	P5	Chronic hepatitis without cirrhosis	65	–	+	+
7	P11	Chronic hepatitis without cirrhosis	467	–	+	+
8	P3	Cirrhosis	421	–	+	+
9	P12	Cirrhosis	327	–	+	+

patients is associated with a rapid progression of liver disease, and several investigators have demonstrated that progressive liver damage, characterized by active replication, occurs in 10% of anti-HBe positive HBV carriers [16, 21].

The HBV DNA from HBeAg seroconverted patients is low (7%) in the present study in comparison with the usual >50% positivity rate reported among HBV-infected subjects in diverse clinical states in India [8, 14]. Elevated aminotransferase levels with HBV DNA positivity is significantly higher (p<0.05) in aged chronically HBV-infected patients. Patients aged >50 years were spontaneously seroconverted from HBeAg to anti-HBe and often accompanied a significant flare in aminotransferase levels. This is intriguing, but is possibly due to the study subjects, which included a majority that were characterized clinically and not histopathologically, and who were referred by gastroenterologists from various hospitals to our referral centre for analyzing the HBV virological profile.

In the present study, the G to A mutation at nt-1896 was not observed in any of the HBV strains analyzed. This was in contrast to the observations from our earlier study [30]. Globally, the lower prevalence of PC mutants has been reported [6, 11] and also from Western India, where 62% of e-negative chronic hepatitis B (e-CHB) infection had wild-type sequences in the PC region. There are a number studies reporting a higher prevalence [4, 6, 9, 15, 19, 32] of HBV mutants existing as quasispecies in a dynamic equilibrium (mix) with the wild-type viral sequences. Although many studies have shown that the genetic diversity of HBV is highest during periods of high viral replication and a mounting immune response, we observed low frequency of mutation in our patients, which opens many questions of the e-CHB in this region.

Table 6. The mutation site resulting in amino acid changes in the core/precore ORF of HBV in the sequenced samples.

Study cases	Precore (codon position/ amino acid)			Core (codon position/ amino acid)			
	13 S	17 V	29 G	12 S	13 V	27 I	40 E
P11		F		T		V	
P7			D				D
P9				T		V	
P5			D	T		V	D
P6				T		V	
P3	T	F		T		V	
P1			D	T		V	D
P8				T		V	
P12					A	V	

Our results are consistent with those of studies from Japan [10], that mutations in the core region can be frequently detected in patients with chronic HBV infections and that these mutations were more often found in HBeAg negative patients. Absence of HBeAg in some patients despite viral replication, although there is no mutation preventing the HBe Ag production, suggests that additional mechanisms might exist outside the precore region. Codon 12 is a helper T-cell epitope, and codon 27 is a part of the CTL epitope. It is possible that mutations in these codons of immune recognition sites might influence immune modulation, which could contribute to the development of viral persistence [5]. Our earlier study [30] and others also confirmed that precore/core variants of HBV were defective in HBeAg synthesis in Indian patients with chronic HBV infection. It is suggested that interferon therapy is ineffective in completely eliminating mutant HBV infection and that relapse rate is high in chronic HBV infection with precore mutant forms [7]. Therefore, the emergence of precore/core HBV mutants may have therapeutic implications in view of their potential to develop persistence and liver damage in the infected patients.

HBeAg negative patients with HBV DNA had significantly more severe liver disease than in other groups, suggesting that the accumulation of mutations due to host immune pressure in the due course of viral persistence could lead to the progression of liver damage. It is unlikely that the severity of illness is dependent solely on the prevailing precore/core sequence; it probably relates to such other factors like viral load, host immune response, *etc.* Some studies have shown an association of mutations in the preC/C region with the severity of liver disease [1, 16]. A recent study from India has shown that chronic or fulminant hepatitis B was

not associated with precore or core HBV mutants [14]. Research data have suggested a direct correlation between precore stop codon mutation and mutations in the core gene [3, 20]. Even though we did not observe precore stop codon mutation, it was notable that the two patients who had HBV with T1862 precore mutation also had a high frequency of mutations (>10 mutations) in the core sequence compared with the other HBV isolates studied.

Our study has some limitations; these include that it is a single-point assessment, and the number of samples that were used for analyzing the core/precore mutation in this study without genotyping was too small to produce sufficient reliability coefficients, which in turn could cause imprecise estimates to generalize our findings with true-score confidence intervals. The absence of 1896 and low prevalence core/precore mutation in our study contrasts with its reportedly high prevalence (37–85%) among HBeAg negative individuals with diverse outcomes of HBV infection [6, 9, 13, 15, 19]. Moreover, we used a method based on only a part of, and not the entire, HBV genome. However, randomly selected patients subjected to core/precore sequencing by a population-based bidirectional method have previously been shown to be reliable for HBV mutant analysis [30].

In summary, although the most common precore stop codon mutation was not detected, there was a low frequency of precore defects in the HBV isolates analyzed in this study. The study indicates that core mutations can be frequently detected in patients with chronic HBV infection. Prospective studies on the sequence variations of the preC/C region of the HBV genome and about the molecular mechanisms in relation to progression of liver disease would provide better understanding of the biological significance of these viremic HBeAg minus strains in India.

Acknowledgments

We sincerely thank the study participants, and highly appreciate the cooperation and assistance rendered by the Gastroenterologists. This work was supported by a grant-in-aid for "Referral Center for Chronic Hepatitis Serology and Molecular Virology" from the Indian Council of Medical Research (ICMR), Government of India.

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