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Original Article

Distribution of hepatitis B virus genotype and cancer predicting precore and basal core promoter mutations

Surg Lt Cdr A.K. Agarwal^a, Col Sourav Sen^{b,*}, Col Debabrata Banerjee^c,
Rakesh Srivastava^d, Surg Cmde A.K. Praharaj, (Retd)^e

^a Graded Specialist (Microbiology), INHS Sanjivani, Cochin, India

^b Senior Adviser (Microbiology & Pathology) and Trained in AIDS & Virology, Army Hospital (R & R), New Delhi, India

^c Senior Adviser (Medicine & Gastroenterology), Command Hospital (Northern Command), C/O 56 APO, India

^d Assistant Professor (Microbiology), R D Gardi Medical College, Ujjain, India

^e Professor and Head (Microbiology), All India Institute of Medical Science, Bhubaneswar, India

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ABSTRACT

Background: Chronic hepatitis B (CHB) infection which is associated with an increased risk of developing liver disease including cirrhosis and hepatocellular carcinoma. Viral factors that may increase the risk for HCC development include HBV DNA level, genotypes, and naturally occurring mutations such as hepatitis B virus precore (PC) (G1896A) and basal core promoter (BCP) A1762T/G1764A double mutations. HBV genotypes and subgenotypes can significantly influence HBeAg seroconversion rates, viremia levels, mutational patterns that could significantly influence the heterogeneity in clinical manifestations and even response to antiviral therapy.

Method: 94 CHB infected individuals with detectable serum HBV DNA levels were studied. HBsAg, HBeAg, anti-HBc IgM antibody estimations were done by ELISA. HBV DNA estimation was done. The HBV genotypes were determined by TSP-PCR and 10 samples randomly selected for DNA sequencing. PC and BCP mutations were determined by DNA sequence analysis of core region.

Result: Of 94 study participant samples with detectable serum HBV DNA levels, 75 were successfully genotyped and sequenced for BCP/PC region. 30/75 (40%) harbored PC and BCP mutations. The total Double mutations of BCP at A1762T/G1764A nucleotide positions, and PC mutation at G1896A nucleotide position were seen in 29.3% and 21.3%, respectively. All 75 isolates were subtype D using TSP-PCR. However, by sequencing 2/10 were subtype A, while 8 were subtype D.

* Corresponding author. Tel.: +91 8130757368 (mobile).

E-mail address: sensourav@hotmail.com (S. Sen).

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Conclusion: Our study reinforces that D is the predominant genotype in Indian population. It reveals that Indian CHB subjects have increased prevalence of BCP & PC mutations, which possibly may lead to development of HCC.

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Introduction

Hepatitis B virus (HBV) infection is a global health problem. More than two billion people in the world have been infected by Hepatitis B virus (HBV) at some time or other in their lives. Of these, about 350 million remain infected chronically and become carriers of the virus.¹ In India, the average carrier rate is 5.0 per cent with an estimated carrier population of 50.0 million.² The clinical manifestations of HBV infection range from acute self-limiting infection or fulminant hepatic failure, inactive carrier state, chronic hepatitis with progression to cirrhosis and hepatocellular carcinoma (HCC).

There are primarily 10 genotypes (A–J) and several subtypes of HBV, based on the divergence in the entire HBV genomic sequences, this being >8% for genotypes and 4–8% for subtypes, respectively. The geographic distribution of HBV genotypes/subtypes are distinct. While genotype A is pandemic, and has mostly been reported from North America, Northern Europe, and Central Africa, genotypes B and C are found in the Far East and Southeast Asia. In India, Mediterranean region, and Middle East genotype D is prevalent, whereas genotypes E and F are seen in West Africa and in the Amerindian population. Genotype G has been seen in the USA, Germany and France, genotype H in Mexico and South America, genotype I in Vietnam and Laos and genotype J in the Ryukyu islands in Japan. HBV genotypes/subgenotypes have been observed to significantly influence HBeAg seroconversion rates, viremia levels, mutational patterns which in turn leads to heterogeneity in clinical manifestations and response to antiviral therapy.^{3–5}

HBV related HCC is the fifth most frequent cancer worldwide.⁵ HBV is responsible for contributing to more than 50% of HCC cases worldwide. Among people with chronic HBV infection, the incidence of HCC has been seen to range from 400 to 800 in males, and from 120 to 180 in females (per 100 000 person/year).^{6,7} Natural history of chronic hepatitis B (CHB) is affected by several factors that could influence the rate of HCC resulting from HBV infection, including gender with higher rates of HCC found in men, geographic region with higher rates reported in eastern and Southeast Asia and sub-Saharan Africa, environmental factors such as the presence of aflatoxin in food, age at infection with perinatal infection appearing to have a higher risk, ethnicity, heavy use of alcohol, and smoking.^{8,9} Several viral factors that are strongly linked with the outcome in HBV infection include HBV genotype, DNA levels, and HBV mutations such as hepatitis B virus precore (PC) G1896A, and basal core promoter (BCP) A1762T/G1764A double mutations.^{3,4} BCP A1762T/G1764A and Precore G1896A lead to active exacerbation of

chronic hepatitis B, liver cirrhosis, and acute fulminant hepatitis.⁹

Detection of hepatitis B virus genotypes and mutations are becoming increasingly important in planning of clinical management of CHB. The present study was a pilot study conducted to estimate the distribution of hepatitis B virus genotype and presence of naturally occurring precore and basal core promoter mutations in CHB infected patients in tertiary care center.

Material and methods

Patients study population

Between August 2010 and Dec 2011, a total of 94 HBV infected individuals already diagnosed as chronic hepatitis comprised of chronic asymptomatic carriers, CHB, cirrhosis, and HCC were enrolled.

Specimen collection and storage

All the patients were interviewed, and history of present and past illness was taken. After obtaining informed consent, collection of samples from the study participants were done by venipuncture into sterile tubes as well tubes containing anti-coagulant (EDTA). Serum & plasma samples were stored at –70 °C until assayed.

Serology

Presence of HBsAg, HBeAg, anti-HBe antibodies, and IgM HBe antibodies were determined in serum samples with commercial assay kits of ELISA as per manufacturer's instructions.

Identification of basal core promoter (BCP) & precore (PC) mutations

This was carried out using direct DNA sequence analysis of core/precore region (658bp; nucleotide positions 1730–2388) by following steps, namely:

- HBV DNA extraction from 200 µl plasma samples by using commercial kit (Qiagen) as per the manufacturer's instructions.
- HBV DNA PCR using following primers: Forward - 5'-CTGGGAGGATTGGGGGAGGAGATT-3' and Reverse - 5'-GGCGAGGGAGTTCTTCTTCTAGGGG-3'. Thermal cycling conditions consisted of an initial denaturation for 03 min at 95 °C, followed by 30 cycles of 94 °C for 45 s, 58 °C for 30 s,

Fig. 1 – 1.5% agarose gel electrophoresis picture demonstrating 658 base pairs amplicon of hepatitis B virus-deoxyribonucleic acid (nucleotide positions 1730–2388). Lane 1 shows 100 base pair ladder, lanes 2 and 3 show positive patient samples, lane 4 & 5 shows negative patient sample, and lanes 6 and 7 shows positive and negative controls, respectively.

and 72 °C for 1 min 30 s followed by final extension at 72 °C for 5 min.

- c) Agarose gel electrophoresis using migration pattern of a 100 bp DNA Ladder. Amplified product showed a band corresponding to 658bp for a positive result (Fig. 1).
- d) Post amplification amplicon purification was carried out by QIA quick PCR purification kit as per manufacturer's instruction.
- e) Sequencing of PCR amplified product of purified PCR amplicons were directly sequenced bi-directionally on ABI PRISM 3100 genetic analyzer system (Applied Biosystems, Foster City, California, U.S.A) using the PCR primers.

- f) Nucleotide alignment and mutations interpretation was done with the precore/core nucleotide sequences generated in FASTA format using CLUSTALX software 2.1 version, and aligned HBV reference strain sequence (NC_003977.1) from gene bank (Figs. 2 and 3).

HBV genotyping by Type Specific PCR (TSP-PCR) was carried out in HBV DNA positive samples by amplifying the pre-S1 region through S genes by nested PCR using type specific primers and genotype-specific DNA bands (Table 1 & Fig. 4), as described by Naito et al previously.¹⁰ P1 (sense) and S1-2 (antisense) were universal outer primers (1063 bases) for first round PCR. This was followed by 2nd round PCR where B2 was used as the inner primer (sense) with a combination called mix A for genotypes A, B, and C, and B2R (antisense) with mix B for genotypes D, E, and F. The first round PCR was carried out using initial denaturation of for 10 min at 95 °C, followed by 40 cycles consisting of 94 °C for 20 s, 55 °C for 20 s, and 72 °C for 1 min. The first round PCR product was added to two tubes containing the second sets of each of the inner primer pairs, and were amplified for 40 cycles with the following parameters: initial denaturation at 95 °C for 10 min, followed by 20 cycles of 94 °C for 20 s, 58 °C for 20 s, and 72 °C for 30 s, and an additional 20 cycles of 94 °C for 20 s, 60 °C for 20 s, and 72 °C for 30 s. Final extension was done at 72 °C for 5 min for both the tubes. The amplicon band sizes vary on agarose gel electrophoresis for the different genotypes, namely, A (68bp), B (281bp), C (122bp), D (119bp), E (167), and F (97bp).

HBV genotyping by sequencing

HBV genotypes were also determined by sequencing and phylogenetic analysis of pre-S1 through S genes in 10 study samples (Fig. 5). Amplified products of first round PCR of S-

Fig. 2 – Nucleotide alignment of the Hepatitis B virus (HBV) study sequences precore (PC) region after alignment with HBV reference strain sequence (NC_003977.1) from gene bank showing G to A at nt. 1896 with T at nt. 1858 generated in FASTA format using CLUSTALX software 2.1 version.

Fig. 3 – Nucleotide alignment of the Hepatitis B virus (HBV) study sequences basal core promoter (BCP) region after alignment with HBV reference strain sequence (NC_003977.1) from gene bank showing A1762T/G1764A double mutations generated in FASTA format using CLUSTALX software 2.1 version.

Table 1 – Primer sequences used for HBV genotyping by nested PCR.

Primer	Sequences (position, specificity, and polarity)
First round PCR	
P1b	5'-TCA CCA TAT TCT TGG GAA CAA GA-3' (nt. 2823–2845, universal, sense)
S1-2	5'-CGA ACC ACT GAA CAA ATG GC-3' (Nt. 685–704, universal, antisense)
Second round PCR	
Mix A	
B2	5'-GGC TCM AGT TCM GGA ACA GT-3' (Nt. 67–86, types A to E specific, sense)
BA1R	5'-CTC GCG GAG ATT GAC GAG ATG T-3' (Nt. 113–134, type A specific, antisense)
BB1R	5'-CAG GTT GGT GAG TGA CTG GAG A-3' (Nt. 324–345, type B specific, antisense)
BC1R	5'-GGT CCT AGG AAT CCT GAT GTT G-3' (Nt. 165–186, type C specific, antisense)
Mix B	
BD1	5'-GCC AAC AAG GTA GGA GCT-3 (Nt. 2979–2996, type D specific, sense)
BE1	5'-CAC CAG AAA TCC AGA TTG GGA CCA-3' (Nt. 2955–2978, type E specific, sense)
BF1	5'-GYT ACG GTC CAG GGT TAC CA-3' (Nt. 3032–3051, type F specific, sense)
B2R	5'-GGA GGC GGA TYT GCT GGC AA-3' (Nt. 3078–3097, types D to F specific, antisense)

gene (1063 bases) were purified by using QIA quick PCR purification kit. Then purified PCR products were directly sequenced bi-directionally on ABI PRISM 3100 genetic analyzer system (Applied Biosystems, Foster City, California, U.S.A) by using outer, first round primers (Table 1). The sequence generated were submitted online using National Center for Biotechnology Information/Basic Local Alignment Search Tool (NCBI-BLAST) [available at http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn%20&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS%20=on&LINK_LOC=blasthome] to determine the genotype which was based on nucleotide similarity of our study sequence with the available HBV sequences in gene bank.

Results

The study population consisted of 94 HBV infected individuals where in the mean age was 38.35 ± 13.32 years with a male predominance (M:F:80:14).

The detection of HBV serological markers (HBsAg, HBeAg, Anti HBeAb and IgM anti HBc) were carried out by ELISA method in all 94 HBV infected individuals serum samples. HBsAg was positive in all 94 HBV infected individuals. Of 94 serum samples, 55 (58.5%) were HBeAg positive while 39 (41.5%) were HBeAg negative and anti-HBe positive. Of 94 serum samples, 92 (97.9%) were IgM anti HBc negative and 02 (2.1%) were IgM anti HBc positive.

Fig. 4 – 1.5% agarose gel electrophoresis picture HBV genotyping by TSP-PCR demonstrating 119 base pairs amplicon of Genotype D. Lane 1 - 100 base pair ladder, lane 2 - negative control, lanes 3 - 12 HBV Genotype D amplicons.

HBV DNA detections by PCR and agarose gel electrophoresis corresponding to band of 658bp as shown in Fig. 1 and quantification by real time PCR was carried out in all 94 HBsAg positive individuals. Out of 94 HBsAg positive individuals, HBV DNA was detected in all 94 individuals.

Out of 94 HBsAg positive samples with detectable serum HBV DNA, 75 were successfully sequenced for BCP/PC

mutations and genotyped by TSP-PCR. Of 75, fifty were diagnosed as chronic hepatitis B, twenty one as chronic asymptomatic carrier and two as cirrhosis and HCC respectively.

Distribution of HBV genotypes

75 individuals were of genotype D with a 119bp by TSP-PCR (Fig. 4) but on phylogenetic analysis based on sequencing of S gene region of 10 HBV study strains found that 2 were subtype A (AFMCHBV 39 and 42) and the remaining 8 were subtype D (AFMCHBV 02, 10, 19, 40, 47, 48, 49, 55). The phylogenetic tree was constructed with s gene sequences of 10 strains along with reference HBV genotype sequences from gene bank (Fig. 5).

Distribution of BCP and PC mutations

The distribution of BCP and PC mutations among the sequences of various clinical groups of the study participant samples is shown in Table 2. Sequence analysis for BCP/PC region in 75 individuals showed that 40% study sequences harbored PC and BCP mutations while the remaining 60% had similarity with wild-type HBV sequences. Overall, double BCP mutations at A1762T/G1764A nt. positions and PC mutation at G1896A nt. position were seen in 29.3% and 21.3% study sequences, respectively. Isolated PC mutation at G1896A nt. position was seen in 10.7%, while double BCP mutations at A1762T/G1764A nt. positions alone were seen in 18.6% (Figs. 2 and 3). Lastly, 10.7% sequences harbored dual - PC and BCP, mutations. All the 30 isolates that had PC mutation at position 1896 displayed T at nt. 1858 (Fig. 2).

Fig. 5 – Phylogenetic tree analysis of pre-S1 through S gene (1063 bases) of 10 Hepatitis B virus (HBV) study sequences aligned along with reference HBV genotype sequences from gene bank using.

Table 2 – Distribution of BCP and PC mutations in study population.

Type of mutations	Total (n = 75)	Chronic asymptomatic (n = 21)	Chronic hepatitis (n = 50)	Cirrhosis liver (n = 2)	HCC (n = 2)
BCP only (A1762T/G1764A)	14	8	5	1	0
Only PC (G1896A)	08	1	7	0	0
Both BCP and PC	08	0	5	1	2
Total BCP	22	8	10	2	2
Total PC	16	1	12	1	2

Association of the BCP and PC mutations with HBeAg status, and genotypes

Of the 75 participants, 54.7% were HBeAg-positive, while 45.3% HBeAg-negative. Twenty four (70.6%) patients with HBeAg negative and anti HBe positive serology harbored mutants (G1896A/A1762T, G1764A/Both), while this 17.6% in those with HBeAg positive serology (Table 3).

Double mutations of BCP at A1762T/G1764A were seen in 9.7% of 41 HBe Ag positive but in 29.4% of 34 HBeAg negative patients. The PC stop codon mutation at G1896A was seen in 20.6% out of 34 HBeAg-negative patients, while only 01 (2.4%) HBeAg-positive patient. Seven (20.6%) HBeAg negative patients had combined BCP double mutations and PC stop codon mutation, whereas 01 (2.4%) of the HBeAg positive patient had this. Overall, total double mutations of BCP and PC were seen in 12.2% and 4.9%, respectively, of 41 HBeAg positive, but in 50% and 41.2%, respectively, of 34 HBeAg negative patients (Table 3).

All 30 patients harboring the BCP & PC mutations belonged to genotype D.

Discussion

HBV infection is an important global health problem that places a continuously increasing burden on developing countries like India. About 400 million people worldwide are chronic carriers of the virus.^{1,2}

Chronic infection with hepatitis B virus is associated with an increased risk of developing liver disease including cirrhosis and hepatocellular carcinoma. The clinical presentation and natural history of HBV infection is mediated through complex interactions between the virus and the host immune response. Viral mutations in the core or precore gene affect replication and may enhance liver injury. Recently genotypes have been identified that are linked to

clinical outcomes, drug responses, and mutations.³ Therefore, Identification of these viral factors associated with the development of severe liver disease including HCC may have important clinical implications in the management of patients with HBV infection and continues to be of great interest.

HBV genotype

In India, genotype D has been reported to be most prevalent, followed by genotype A, except in Eastern parts where genotype C has been found.^{11–15} In our study, genotype D (93%) is the predominant, followed by genotype A (7%), which is in accordance with earlier reports.

In our study, all 75 HBV DNA isolates showed characteristic banding pattern (119bp) corresponding to genotypes D by TSP-PCR. However, phylogenetic analysis of S gene sequence of 10 HBV DNA isolates showed 02 and 08 isolates respectively as genotype A and D. Previously, study conducted by Chattopadhyay et al (2006) had demonstrated 100% concordance between TSP-PCR and sequencing for HBV genotyping.¹² TSP-PCR is a simple, rapid and cost-effective method but it cannot identify genotypes G and H, and may also miss out atypical, non-specific and recombinant forms.¹⁰ There is need to verify and validate the specificity of TSP-PCR by molecular methods such as real time PCR and sequencing of HBV isolate.

HBV genotype plays a role in spontaneous HBsAg and HBeAg sero-conversions, mutations pattern in BCP and PC regions, clinical outcome, and response to interferon and antiviral therapy. Compared to those infected with HBV genotypes A and B, patients with genotypes C and D infections have lower rates of or delayed spontaneous HBeAg seroconversion. Those harboring genotypes A and B infection show higher rate of HBsAg clearance as compared to genotype C and D. The latter have late or absent HBeAg seroconversion with accelerated progression of chronic hepatitis, and poor clinical outcome.^{3,6,8,16}

Table 3 – Association of BCP, PC mutations with HBeAg status, and genotype.

Type of mutations		HBeAg +ve (n = 41)	HBeAg –ve (n = 34)	Genotype D (n = 73)	Genotype A (n = 02)
Precore	G1896G (Wild)	40	27	57	02
	G1896A (Mutant)	01	07	16	None
Basal core promoter	A1762A,G1764G (Wild)	37	24	51	02
	A1762T, G1764A (Mutant)	04	10	22	None
Precore + Basal core promoter	G1896G + A1762A + G176G (Wild)	40	27	51	02
	G1896A + A1762T + G176A (Mutant)	01	07	22	None

HBV PC mutations are observed most frequently in genotype D, most prevalent in India, followed by genotypes C and B, and are seen least frequently in genotype A. The PC mutation is usually not found in genotype A. Various studies including from India reported that genotype D was associated with more severe liver disease and HCC in younger patients than genotype A. HBV genotypes D and C are also associated with a lower rate of favorable response to interferon alpha therapy than genotypes A and B, respectively.^{3,6,8,12}

Mutations in the precore/core region

In our study, the predominant precore variation observed was a G-to-A change at nucleotide (nt.) 1896 (A1896), while the most common core promoter mutations involve a two-nucleotide substitution: A-T at nt. 1762 and G-A at nt. 1764 (T1762 and A1764). This is in accordance with studies from India and rest of the world.^{15,17,18} PC (A1896) and BCP (T1762 and A1764) are the most common and clinically significant mutations in the precore/core region. Prevalence of basal core promoter and precore mutants in precore/core region ranging from 33 to 37% has been reported in India amongst chronic HBV patients.^{5,17}

Precore mutation (G1896A)

The predominant mutation in the pre-core region is a G to A change at nucleotide 1896, creating a stop codon at codon 28, which leads to premature termination of the precore/core protein, and thus, preventing HBeAg production. In our study, PC mutation was detected in 21.3% isolates at nt. position 1896. This mutation was detected in 41.2% HBeAg-negative study participant isolates. Two out of 41 patients with HBeAg (4.9%) also displayed the PC stop codon mutation. Thus, the distribution of PC mutation in present study is in accordance with other studies from India, except from eastern part where it was found to be lower especially in HBeAg negative patients.¹⁷

Cytosine at position 1858 prohibits G-to-A mutation at nt. 1896, as this destabilizes the stem-loop structure of the RNA encapsidation signal. Interactions of this encapsidation signal with the viral DNA polymerase is an essential step in the viral replication cycle. Presence of thymine at position 1858 is common in HBV genotype D sequences. Genotype A usually shows a cytosine at this position.^{19,20} Genotype A sequences in our study revealed presence of cytosine nucleotide 1858 which is in agreement with previous data that also have found the a low frequency of precore mutants in genotypes A.²⁰ The prevalence of PC stop codon mutation has been found to be markedly varied in different countries. It ranges from 20% in China, Malaysia and Hong Kong to up to 100% in Taiwan, Korea and Japan.²¹ This variability in the prevalence of the said mutant depends of the predominant circulating HBV genotype in a given geographic region, and has been reported to be restricted to mainly genotypes B, D and.^{19–22} Our study also demonstrates this association of the G1896A mutation with HBV genotype D.

Although PC A1896 mutant was initially reported in patients with chronic active hepatitis or fulminant hepatitis, it may not be responsible for the fulminant course, since the PC

A1896 mutant has also been detected in HBeAg-negative patients with inactive liver disease. Thus, the PC mutation may only represent a mechanism utilized by the virus to escape the immune clearance of the host. Additionally, PC mutant has been reported with decreased risk of HCC.^{8,19,21–23}

BCP mutations (A1762T, G1764A)

The core promoter controls the transcription of the 2 core gene products: core and precore RNA. These mutations have been described in patients with chronic HBV, fulminant hepatitis, or who are immunosuppressed. A common hallmark of core promoter mutations is biological phenotype of enhanced viral replication.¹⁹

In our study, double mutations in the BCP at nt. 1762 and nt. 1764 were detected in 29.3% isolates. BCP mutations were detected in 50% HBeAg-negative study participant sequences, and 12.2% of HBeAg positive patients. This distribution of BCP mutation in present study is in accordance with other studies from India.^{5,17} Double BCP mutations are associated with more severe hepatic injury and potentially fulminant hepatitis by more vigorous cellular immune response, which is possibly due to increase in HBV replication. Double BCP mutations emerge most frequently with activation of hepatitis and prior to sero-conversion to anti-HBe, which may be the reason for presence of double BCP mutations in HBeAg-negative patients. The reason for higher frequency of e-antigen negativity and increased DNA levels is possibly due to differential regulation of this region by selectively down regulating the transcription of the precore mRNA but without affecting the core or pregenomic RNA. Therefore, it also serves as an alternative of the HBV to lose HBeAg and escape the host immune clearance.^{8,19,21}

In Asia-Pacific regions, the prevalence of BCP mutations in patients with CHB has been reported to be higher as compared to the PC stop codon mutation.²¹ Basal core promoter mutations have been reported to be associated with higher risk of HCC development in both black Africans and Asians.¹⁸ Liu et al demonstrated that the presence of BCP mutations is associated with an odds ratio of 3.79 for the development of HCC. It was concluded that the 1762/64 mutation can be used as an independent biomarker to predict HCC development.⁹ In our study, isolate sequences from two HCC patients displayed BCP mutations.

Lastly, we found that dual BCP mutations coexisted with PC stop codon mutation in 10% of study patient isolates, of which 7(87.5%) were HBeAg negative, and 1(12.5%) was HBeAg positive. It has been proposed that co-occurrence of BCP and PC mutations may moderately increase the HBV replication.¹⁷

Conclusion

HBV genotype influences the severity of liver disease that patients experience as well as their response to interferon based therapy. It is also thought to influence the emergence of resistant strains. Therefore, patients infected with certain genotypes of HBV that are known to be resistant to common treatment regimens can be counseled to seek alternative therapeutic options to spare them the cost and burden of treatment. The knowledge of the prevalence of HBV genotypes

in a particular region is thus of immense importance to allow the proper and effective management of HBV patients in that region. Our study once again demonstrated the predominance of HBV genotype D in Indian scenario, and that genotype D was more commonly associated with BCP & PC mutations than genotype A.

This study, in agreement with previous studies, reveals that Indian subjects have increased prevalence of BCP & PC mutations. This is of paramount clinical importance since they are known to moderate the HBV disease progression towards development of HCC. These genetic features of HBV, in addition to age, sex, HBV viral load and serum ALT levels may help identify those who are at an increased risk for liver disease progression and would therefore potentially benefit from early interventions, such as regular screening to detect disease progression and treatment. Since we are unable to follow up the study participants, the study being cross-sectional, further long term, prospective studies will be required to assess the association of HBV PC and BCP mutations with liver disease progression and complications in the Indian scenario.

Conflicts of interest

All authors have none to declare.

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