Original Article

Detection of Mutations in Precore and Basal Core Region in HBeAg Negative Chronic Hepatitis B Patients from Jaipur

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ABSTRACT

Introduction: Chronic hepatitis B (CHB) has been considered a global health problem for years. Different mutations in the precore and basal core promoter (PC/BCP) region result in HBeAg negative hepatitis. G1896A, which is the most frequently observed mutation in the PC region, acts by insertion of a premature stop codon in the concerned ORF while the A1762T/G1764A double mutation in the BCP region acts by termination of HBeAg expression. Various mutants and genotypes are significantly associated with diagnostic failure, response to antiviral therapy, liver fibrosis, cirrhosis and hepatocellular carcinoma (HCC). Therefore, determination of these mutations and their correlation with clinical manifestations is of utmost importance specially in HBeAg negative CHB patients.

Methodology: 10 HBsAg positive, HBcIgM**,** and HBeAg negative CHB patients with viral load $>10^3$ IU/mL attending SMS hospital, Jaipur during year 2021 were tested for nested PCR and sanger sequencing was done for 261 bp fragment of PC/BCP region. Multiple sequence alignment and mutation analysis was done using MEGA software.

Results: Genotype D was detected in 9/10 (90%) samples and genotype A in 1/10 (10%) samples. Mutations were detected in 5/10 samples (50%) and all belonged to genotype D. Out of these 5 isolates, 3 (30%) showed the classical PC mutation G1896A, whereas in the BCP region double mutation (A1762T/G1764A) was detected in 2 (20%) cases.

Conclusion: HBV genotypes and PC/BCP mutations are useful for predicting disease prognosis and help clinicians in planning individualized treatment regimens for HBeAg negative CHB patients.

Keywords: Basal core mutations, CHB, Genotype, HBeAg, HCC, Precore mutation.

INTRODUCTION

Even after the introduction of effective vaccines more than 35 years ago, chronic hepatitis B (CHB) is still one of the most important public health problems worldwide. As per the WHO report 2020, about one third of the world population is infected with *Hepatitis B Virus* (*HBV*). 257 million chronic hepatitis and more than 880000 deaths/year have been reported worldwide where majority of the cases belonged to cirrhosis and hepatocellular carcinoma (HCC).[1] Genomically, *HBV*, member of *Hepadnaviridae* family, has partially double stranded circular DNA genome of 3.2 kb length which has over-lapping four open reading frames (ORF).[2] During viral replication, an error prone reverse transcriptase activity result in emergence of mutations that lead to severe clinical effects.[3,4]

The most common mutation in the precore (PC) region is G to A change at nucleotide (nt) 1896 which disrupts the production of hepatitis e antigen (HBeAg) resulting in HBeAg negative disease. On the other hand, the most common basal core mutation is a double mutation A1762T/G1764A. It down regulates but not completely abolishes the production of HBeAg.[5] G1896A mutation is mostly found in genotypes B and D followed by genotype C. Genotype A is believed to be incompatible with this variant because of the presence of C (cytosine) at nt1858 which makes a pair with nt1896 in the epsilon stem loop structure at pregenomic (pg) encapsidation while non A genotypes usually harbor T at nt1858.[6] In contrast to the PC G1896A, the BCP mutant has been reported to be associated with all genotypes.[7] HBeAg negativity caused by these mutations are associated with active exacerbation of CHB, severe liver diseases like liver cirrhosis, HCC and fulminant hepatitis. Apart from the above mentioned two mutations some other PC and BCP mutations like G1899A,

C1653T, T1753V, C1766T and T1768A have also been seen to be associated with HCC and other severe liver diseases.[8,9]

Detection of such mutations is becoming increasingly important in planning clinical management of HBeAg negative CHB patients. Hence, this study was done to detect various preC/C mutations in HBeAg negative CHB patients.

METHODS

The study was conducted at the state Virus Research and Diagnostic Lab (VRDL) of Department of Health Research (DHR), Department of Microbiology of Sawai Man Singh medical college, Jaipur during the year 2021. Ten HBsAg positive, HBc IgM and HBeAg negative CHB patients attending hospital, with viral load $>10^3$ IU/mL were shortlisted and patients with alcoholic liver disease, *HCV* and *HIV* infection were excluded from the study. Informed consent and institutional ethical clearance (Ethical clearance no 2273/MC/EC/2016 dated 29.03.2016) was obtained. Blood samples from all these patients were collected in EDTA.

Molecular testing: Extraction of DNA was done using EasyMag automated extraction system by bioMerieux. It works on magnetic silica-based principle. Total nucleic acid was extracted from 400µL of plasma in the final elution volume of 40µL. Nested PCR was done to amplify PC/BCP region using primers described by Kumar et al.[10] The first round PCR was done and the cycling conditions were involving initial denaturation at 95°C for 15 minutes, 40 cycles including denaturation at 94°C for 30 seconds, annealing at 52°C for 50 seconds and extension at 72°C for 50 seconds followed by final extension at 72°C for 10 minutes. The second round PCR was done using initial denaturation at 95°C for 15 minutes followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 44°C for 30 seconds and extension at 72°C for 50 seconds. Final extension was done at 72°C for 10 minutes.

Agarose gel electrophoresis: The amplified products were electrophoresed on 1.5% agarose gel. 2 µL of product was loaded and electrophoresis was done at 55V for two hours. The bands were analyzed with a gel documentation system. All the products corresponded to 261 bp when compared with 100 bp DNA ladder.

Genome sequencing and analysis: Forward strands were sequenced using BigDye Terminator cycle sequencing Ready Reaction Kit ver 3.0 (Applied Biosystems, USA) on 3500 DX Genetic analyzer (ABI, USA) by Sanger sequencing method. Genotype determination was done on the basis of the sequences showing the highest matching score when subjected to NCBI BLAST. All the sequences were analyzed using MEGA11 software for mutation analysis.

RESULTS

The study consisted of 10 CHB infected patients with mean age of 43.5 years (standard deviation ± 18.17). Males were found to be in predominance 8/10 (80%) as compared to the females 2/10 (20%). Mean viral load was 2.11 x 10^7 IU/mL. *HBV* Genotype D was detected in 9 patients (90%) while genotype A was found in 1 sample (10%). Mutations were detected in 5/10 samples (50%) while 5 samples belonged to wild type (50%). Out of these 5 isolates, 3 (30%) showed the classical PC mutation G1896A, whereas 2 (20%) cases were found to be showing BCP mutation A1762T/G1764A (Figure).

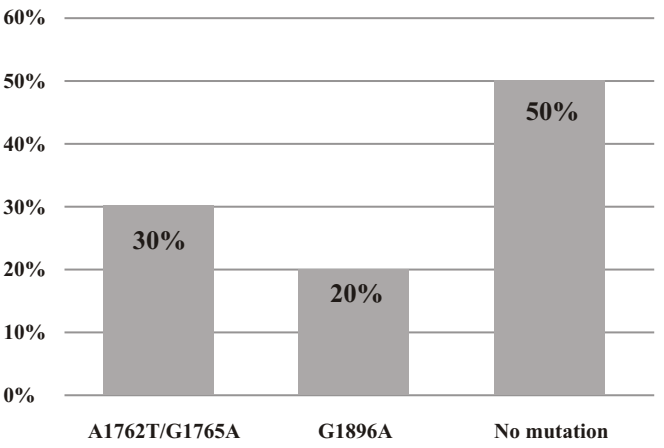

Figure : Distribution of mutations in HBeAg negative CHB patients.

In reference to the genotypes, no mutation was found in the sample with genotype A. While 2/9 (22.22%) samples were

Table : Distribution of mutations among different genotypes

Genotypes	Total patients	G1896A	A1762T/G1764A
A	1	0 (0%)	0 (0%)
D	9	3 (33.33%)	2 (22.22%)
Total	10		

found to be having double BCP mutation A1762T/G1764A and 3/9 (33.33%) samples were having PC mutation G1896A (Table).

DISCUSSION

CHB infection has continuously been considered a major health concern worldwide. India contributes 15% of total *HBV* infection reported globally where 80-90% cases belong to HCC.[11-12] Studies across the world say that different spectrum of the disease depends on viral replication, interaction with host immunological system and genetic heterogeneity including genotypes and mutations.[13]

Based on the nucleotide difference of >8% in genomic sequence, eight genotypes (A-H) and two novel genotypes, I and J have been reported so far.[14] In India, as compared to other genotypes, genotype D has been more associated with advanced liver diseases.[9] Majority of the patients in this study were found to be infected with genotype D (90%) followed by A (10%) which is similar to the previous studies from India who reported 79.5% genotype D and 20.5% genotype A 20.5% and 80% genotype D and 20% genotype A.[15-16]

There is a high rate of nucleotide substitution (10^{-4} to 10^{-6} substitutions/site/year) in HBV as a result of the error prone reverse transcription during the viral replication which leads to the mutations with significant implications for diagnosis, treatment, prevention, and prognosis of the disease.[2]

PC and BCP region control transcription of PC mRNA and mutations in this region mostly result in HBeAg negative hepatitis. The most frequent PC mutation G1896A is a nonsense mutation which cause G to A change and convert codon 28 from TGG (tryptophan) to a premature stop codon TAG subsequently leading to the termination of HBeAg expression.[17] On the other hand the most common mutation in BCP region is a double mutation A1762T/G1764A which prevent HBeAg expression by inserting premature stop codon or may enhance the pgRNA transcription leading to weak immune response causing HCC.[9] In this study, PC mutation G1896A was observed in 33.33% isolates which was higher than that of BCP double mutation A1762T/G1764A (22.22%). It is in accordance with the other previous studies. Lau et al[5] observed 29.3% PC mutation while 23.6% BCP mutation, Malik et al[15] observed 27.7% G1896A mutation, though Chouhan et al[18] observed A1762T/G1764A mutation a bit higher (36%) than PC mutation (33%).

G1896A mutation is frequently observed with genotype D but not with genotype A. The reason behind this is the presence of thymine (T) at nt1858 in genotype D. It pairs with nt1896 in the pgRNA loop, at the ε encapsidation signal. While genotype A usually has C at nt1858 which prevents G to A change at nt1896 and destabilizes the stem loop structure of the RNA encapsidation signal.[17] These findings were similar as observed in our study. All the isolates harboring G1896A mutation belonged to genotype D and all of them were having T at nt1858. This finding is in agreement to the previously reported studies. Kheirabad AK et al[19] reported 34.1% G1896A mutation and all belonged to genotype D. Pahal et al[20] mentioned in their review G1896A mutation to be observed more with genotype D. Agarwal et al[16] observed 21.3 % PC mutation in genotype D and all were having T1858. Chouhan et al[18] reported 33% G1896A mutation, all associated with genotype D.

Role of G1896A and A1762T/G1764A has been described by Kim et al[21] in the progression of advanced liver diseases in CHB. PC mutation causes resistance to interferon (IFN) alpha-2b, immunomodulators, and pegylated IFN (PEG IFN) alpha-2a thus influence response to antiviral therapy.[17] This mutation has also been considered to be the root cause of severe liver damage in HBeAg negative CHB patients. The presence of PC mutants in 94.4% of HCC cases demonstrates its high association with HCC.[15]

Limitations of the study: Small sample size is the limitation of this study hence more detailed study on a larger CHB population needs to be done. To study a better picture of different mutations, a large sample size of patients with all categories of *HBV* viral load is recommended. Clinical correlation and follow up of patients are also recommended.

CONCLUSION

Various mutants along with different *HBV* genotypes are found to be significantly associated with diagnostic failure, response to antiviral treatment, vaccine escape, liver fibrosis, cirrhosis, and HCC. Understanding the correlation between these mutations and the clinical outcomes plays an important role in therapeutic directions. Hence, it is of utmost importance to determine the genotypes and mutations specially in HBeAg negative CHB patients, before the start of any type of treatment.

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