



Original Research Article

## Identification of Hepatitis B Virus Pre-Core Mutant and Association with HBV Genotype by PCR- RFLP in Patients Suffering From Chronic Liver Disease, Uttar Pradesh: North India

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### ABSTRACT

**Objective:** Hepatitis B virus (HBV) genotype appears to show varying geographic distribution. Molecular epidemiological study of HBV in particular areas in India is still limited. This study was aimed to identify the pre-core mutation and its association with HBV genotype in patients of chronic liver disease from north India.

**Methodology:** A total of 122 hepatitis B surface antigen (HBsAg)-positive samples were analyzed. HBV genotype was identified by polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) created by restriction enzyme *Ava II* and *Dpn II*. Mutations in pre-core were identified by PCR-RFLP created by restriction enzyme *Bsu 361*.

**Results:** Genotype HBV/A and HBV/D was detected in 22 (20.8%) and 84 (79.2%) respectively out of 106 CLD patients. Over all TGG→TAG mutation in the precore region was 46 (43.8%) of 106 patients with HBsAg positivity. Mutation in precore region was detected in HBsAg carrier, chronic hepatitis B patients, and liver cirrhosis 6 (33.3%), 10 (23.8%), 26 (56.5%) respectively, excluding 1 (5.5%), 3 (7.1%), 3 (6.5%) patients with mixed type mutation also. In genotype A it was observed that the precore mutation was 50%, wild was 45% and 5% were mixed type. Whereas in genotype D it was 56% wild, 37% mutated and 7% mixed type.

**Conclusion:** In conclusion our study suggested that genotype D is the most prevalent genotype in north India followed by genotype A. The prevalence of genotype D was similar in all of the clinically defined HBV disease groups. The overall prevalence of pre-core mutation was 43.7%. Liver cirrhosis had more pre-core mutation compared with chronic hepatitis B and inactive carriers. In our study pre-core mutation was 50% in genotype A and 37% in genotype D

**Key wards-** HBV, Pre-core mutation, HBeAg

### INTRODUCTION

Hepatitis B virus (HBV) is a well known agent of acute and chronic hepatitis,

liver cirrhosis and hepatocellular carcinoma. Around 400 million people worldwide carrier of HBV of which more than 250

million reside in Asia (Wang Y et al 2005), and 1 to 2 million people have died from it (Mahoney FJ et al 1999). It has a partially double-stranded DNA, having 3.2-kb genome size and replicate via reverse transcription of RNA intermediate (Locarnini S et al 2003). In the natural history or during the antiviral therapy of chronic HBV infection, sero conversion from HBeAg to anti-HBeAg is usually accompanied by a decrease in viral replication and remission of liver disease (Ganem D et al 2004). However such a serology profile may also be seen in individuals who harbor precore (PC) and basal core promoter (BCP) mutants where replicative infection continues. Precore variants were the first major immune escape mutants of HBV to be discovered (Wai CT et al 2004) and is a most common. In this there is substitution of G to A at nucleotide 1896, which prevents the production of HBeAg by introducing a premature stop codon into the open reading frame of the precore region. This mutation converts codon 28 of the pre-core sequence to a termination codon (TGG→TAG) and therefore it prevents expression of HBeAg (Lok AS 1994, Tong S et al 2005). Because the precore region is not essential for HBV replication nor hepatitis B core antigen (HbcAg) expression, the G1896A variant is replication-competent and is infectious. The presence of precore scape mutants should be considered in individuals who exhibit HBeAg negativity, anti-HBeAg positivity, HBV DNA positivity, and elevated serum aminotransferase levels. However, the precore variants are not uniformly pathogenic and thus, co-mutations or host factors presumably explain the more virulent forms of precore mutant-associated disease.

The most frequent core promoter mutation is the double A1762T and G1764A nucleotide exchange, which results in a substantial decrease in HbeAg expression

but enhanced viral genome replication (Tong S et al 2005). It is evenly distributed among the major HBV genotypes. The present study aimed to identify the hepatitis B virus pre-core mutation in patients suffering from hepatitis B related chronic liver disease and also correlate with the genotype.

## **MATERIALS AND METHODS**

### ***Patients:***

A total of 421 patients with chronic liver disease were enrolled during Jan 2012 to May 2014 in the Jeevan Jyoti Hospital and Vanadana Women's Hospital a unit of Jeevan Jyoti Hospital, Allahabad. This study included new patients as well as returning patients attending for a follow-up visit. They were followed at every 3 months interval. At every visit, serological and biochemical tests were performed. For the analysis of genotype and core mutation, serum samples were used with written informed consent and the study protocol.

### ***Biochemical Analysis:***

The alanine amino-transferase (ALT), aspartet amino-transferase (AST), serum bilirubin and serum albumin of the targeted patients was assessed with the help of ALT (SGPT) Modified UV (IFCC), Kinetic Assay (Span Diagnostics Ltd.) AST (SGOT) Modified UV (IFCC), Kinetic Assay (Span Diagnostics Ltd.) and Bilirubin T & D manufactured by RFCL Ltd. Sidcul. Haridwar, Uttarakhand and Liquid Gold Albumin test kit manufactured by Span Dignostics Ltd respectively followed by the instruction of the manufacturer. The normal value ranged between 10- 40 U/L, 10 – 40 U/L, <0.8U/L and 3.5 mg% at 37<sup>0</sup> C respectively.

### ***Serological Analysis:***

Viral marker of HBV, viz, HBsAg and HbeAg were tested. HBsAg was tested by using ERBA LISA Hepatitis B kit (Transasia Bio-Medicals Ltd.) following

instruction of the manufacturer. HBeAg were tested by using Micro screen HBeAg ELISA test kit (Span Diagnostics Ltd.) according to the instruction of the manufacturer.

#### ***Detection of HBV Genotype:***

Serum samples from each subject were taken at first examination and stored at -70 until they were studied. HBV-DNA was isolated with high pure viral nucleic acid kit (Roche Diagnostics, Penzberg, Germany) according to the instructions of the manufacturer. Briefly 200 µL sera was used for DNA extraction and 50 µL elution buffer was used for elution of DNA. Five micro liters of HBV-DNA were added to a 45 µL reaction mixture for amplification. HBV primers (HBV-1 5'TCACCATATTCTTGGGAACAAGA 3' -Sense and HBV-2 5'TTCCTGAACTGGAGCCACCA 3'-Antisense) were obtained commercially. PCR reaction mixture contained 200 µmol/L each nucleotide triphosphate, 0.4 µmol/L each primer, 1.5 mmol/L MgCl<sub>2</sub>, 75 mmol/L Tris-HCl, 20 mmol/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, and 0.25 µL Taq polymerase (5 U/µL, MBI Fermentas) to which a 50 µL reaction volume was added. Samples were thermocycled (Bio Rad USA) for 40 cycles (1 min at 94, 1 min at 55, 2 min at 72). PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light. A result was considered positive when the band of the appropriate size (approximately 446 and 479 bp amplicon for genotype D and genotype A, respectively) was visible in the gel. The genotypes of HBV were determined by the restriction fragment length polymorphism (RFLP) created by Ava2 and Dpn2 action on an amplified segment of the pre-S region according to the method described by Lindh et al (Lindh et al 1998).

#### ***Detection of precore mutation :***

5 µl of the extracted DNA was mixed with 45 µl of a PCR reaction mixture (Roche, Penzberg, Germany) containing 400 nM of the primer P1-1 (5' CAAGCCTCCAAGCTGTGCCTTGGGTG GCCTT 3':sense) and P1-2(5' GTATGGTGAGGTGAACAATG 3': antisense) and subjected to 35 cycle of 94<sup>0</sup>C for 1 min, 55<sup>0</sup>C for 1.2 min, 72<sup>0</sup>C for 2 min. The PCR product was analyzed by agarose gel (2%) electrophoresis on to see the HBV DNA bands under Trans gel illuminator. A 10µl aliquot of the nested PCR products with molecular size pf 194 bp was mixed with 10 µl of buffer containing 1U of Bsu 361 (Roche, Penzberg, Germany) and incubated at 37<sup>0</sup>C for 60 min. Restricted products were then loaded on an agarose gel (4% - 3 Gell agarose tinny (Applied biosystem): 1 agarose) and electrophoreses together with marker with 50 bp to analyzed the cleavage at the restricted pattern. Expected sizes for bands to be generated from the mutated HBV DNA were 160 bp and 34bp, and there is no digestion occurred in the wild type HBV DNA ( Lindh M et al 1995).

## **RESULT**

### ***HBsAg/ HBeAg status:***

It was observed that out of 421 patients 122 (29%) was positive for HBsAg i.e prevalence of hepatitis B was 29% in patients with chronic liver disease. The ALT level was normal in 16 (13%) while 106(87%) showed HBsAg positivity with elevated ALT level (Normal value <40 U/mL). All HBsAg positive patients were diagnosed for HBeAg and found 32 patients were positive for it.

### ***HBV Genotype:***

DNA was isolated from all HBsAg positive patients but we found the PCR product only in 112 and there was no amplification in remaining 10 samples during the PCR which amplify the pre-S

region of HBV genome and used for characterization of HBV genotype.

Genotype A and D was detected in 22 (20.8%) and 84 (79.2%) respectively out of 106, while genotyping failed in 6 sample. RFLP patterns suggestive of BCEFGH were not observed in any patients.

**Pre-core Mutation:**

Over all TGG→TAG mutation in the precore region was 46 (43.8%) of 106 patients with HBsAg positivity. Mutation in precore region was detected in HBsAg carrier, chronic hepatitis B patients, and liver cirrhosis 6 (33.3%), 10 (23.8%), 26 (56.5%) respectively, excluding 1 (5.5%), 3 (7.1%), 3 (6.5%) patients with mixed type mutation also. (Table.1).

**Factors affecting pre-core mutation:**

Besides clinical status serum levels of ALT/AST and serum bilirubin were predicting factors for (TGG→TAG) mutation analysis. Precore (TGG→TAG) mutations were 51.4% in HBeAg-negative patients while 12.5% in HBeAg positive patients. Mixed type mutation was more (9.4%) in HBeAg negative patients than HBeAg positive patients (0.0%) (Table -2). We also see the biochemical factor including ALT/AST and serum bilirubin was significantly less in the mutated as compare with the wild and mixed type samples.

**Pre-core Mutation in HBV Genotype:**

In genotype A it was observed that the precore mutation was 50%, wild was 45% and 5% were mixed type. Whereas in genotype D it was 56% wild, 37% mutated and 7% mixed type. (Figure. 1,2)

Type	Inactive HBsAg Carrier	Chronic Hepatitis B		Liver Cirrhosis	
		HBeAg+ve	HBeAg -ve	HBeAg +ve	HBeAg -ve
Wild	11(61.1%)	14(87.5%)	15(57.7%)	14(87.5%)	3(10%)
Mutated	6(33.3%)	2(12.5%)	8(30.8%)	2(12.5%)	24(80%)
Mixed	1(5.6%)	0(0%)	3(11.5%)	0(0%)	3(10%)

Table: 1. Pre-core mutation in relation to the clinical status of chronic liver disease

Variable	Wild	Mutated	Mixed	P value
Sex- M/F	43/14	32/10	5/2	< 0.05
ALT	48.8±81.87	76.04±55.02	35.9±20.96	< 0.05
AST	69.68±50.80	60.48±38.80	32.48±13.42	< 0.05
Serum Bilirubin	2.81±2.26	2.65±1.86	2.12±1.17	< 0.05
Serum Albumin	3.59±0.82	3.81±0.89	3.67±0.44	< 0.05
HBeAg +ve	28	4	0	< 0.05
HBeAg -ve	29	38	7	< 0.05

Table: 2. Factors affecting pre-core mutation

## DISCUSSION

It has been reported that HBV genotype shows a geographic distribution (Chan HL et al 2004). In addition, it was reported that the occurrence of the mutations in the BCP and the precore regions depend on HBV genotypes (Nakayoshi T et al 2003, Chan HL et al 2004). India is a vast country, having long history of population influx from different countries resulting in different patterns of chronic disease in different parts (Sen U et al 2002). In India, prevalence of HBV in chronic hepatitis is very high (Chattopadhyay S et al 2006). Of the HBV genotypes, HBV/A, HBV/C and HBV/D, has been reported from different parts of India (Kumar A et al 2005, Chattopadhyay S et al 2006), while HBV/A and HBV/D, has been reported and characterized from northern Indian population (Thakur V et al 2002). In our study we have also found that Genotype D (76.8%) was predominant over genotype A was 20.4%.

HBV has a DNA genome and its replication strategy includes RNA-dependent DNA polymerase (Summers J et al, 1982), which may raise the mutation rate of a DNA virus close to that found with retroviruses (Chun YK et al., 2000).

The presence of pre-core mutants should be considered in individuals who exhibit HBeAg negative, HBsAg positive, Anti HBe positive, HBV DNA positivity and elevated aminotransferase levels. However, the pre-core variant is not uniformly pathogenic and thus co-mutations or host factors presumably explain the more virulent forms pre-core mutant associated disease. The outcome of HBV infection depends on the interplay between the virus, the hepatocytes and the hosts' immune response. When the balance is disrupted by the emergence of particular HBV mutants with altered phenotype, the changed virus-cell relationship might cause hepatocyte

necrosis and lead to the development of fulminant or severe hepatitis (Bartholomeusz A et al 2001)

The presence of the G1896A mutation is restricted to specific viral genotypes (B, C, D and E). These HBV genotypes are not uniformly distributed around the world. This mutation is more prevalent in geographic region where genotypes B, C and D are predominant, such as Asia and the Mediterranean area, where it can be detected in more than 50% of the individual with chronic hepatitis B. It is significantly less prevalent in North America and Europe, where genotype A is more common.

The incidence of HBeAg negative chronic hepatitis B has increased in many countries (Lok AS et al 2001). In our study HBeAg negative patients were predominated. This does not represent the true prevalence of the HBeAg negative disease. However, it does not signify its high prevalence. Information about prevalence of PC and BCP mutations is also important in terms of response to interferon, development of fulminant hepatitis, rate of lamivudine resistants and development of hepatocellular carcinoma (Locarnini S. 2004).

According to Orito et al, in the PC region, the wild type is significantly more frequent in HBeAg positive patients regardless of the HBV genotype, and the mutant type is significantly more frequent in hepatitis Be antibody (HBeAb) –ve patients. Briefly, PC mutation is associated with the proportion of HBeAg positive patients, and PC mutation may improve hepatitis via seroconversion into HBeAb (Orito E et al 2001).

In our study we found that the mutation in the PC region was about 43.8%. The mutation rate in inactive HBsAg carrier, chronic hepatitis B and liver cirrhosis were 33.3%, 23.8% and 56.5% respectively. We also estimated that the mutation rate in

HBeAg –ve patients (51.4%) was slightly higher than the HBeAg +ve patients (12.5%), while mixed type mutation was found only in HBeAg -ve patients (9.5%) not in HBeAg +ve patients (0.0%). In another study it was found that in HBeAg negative patients, 27 (46%) had precore mutation compared with 6.7% HBeAg positive patients. This trend was seen in all the chronic liver disease patients. Several observations reported that precore mutation is seen predominantly in HBeAg negative patients (Gandhe SS 2003, Tsugeno H 2002). In agreement with Niitsuma et al and Gandhe et al., a pre-core mutant cannot be the principal factor responsible for the absence of HBeAg (Gandhe SS et al 2003, Niitsuma H et al 1995)

Kao et al detected the PC mutation in the asymptomatic carriers (ASC) was 3% and in patients with hepatocellular carcinoma was 64% (Kao JH et al 2003).

Sibnarayan Datta reported over all prevalence of PC mutation from eastern India was 15.6% (Datta Sibnarayan et al 2008) and Satoru Hagiwara reported the prevalence of PC mutation in CLD patients was 34% (Hagiwara Satoru et al 2006).

In another report it was estimated that the 1896 PC mutation was 10% in HBsAg carrier, 2% in HBeAg +ve ASC, 12.5% in CHB and 30% in liver cirrhosis (Byung-Cheol Song et al 2006).

Vivekanandan Perumal reported the prevalence was about 19.6% in patients combined both the group with normal and abnormal ALT (Vivekanandan Perumal et al 2008).

Abbas Zaigham reported the prevalence of PC mutation was 31% in HBsAg –ve patients and 15.8% in HBsAg +ve patients in Pakistan (Abbas Zaigham et al 2006). A study done by Vutien P in Asian American Patients show 17% had precore mutation. (Vutien P et al 2013)

The PC mutant was detected more often in subject with genotype D than those with genotype A (Vivekanandan Perumal et al 2008, Akarca US et al 1994), while in our study it was in genotype A (50%) and in genotype D (37%). The genotype HBV/A has cytosine at nucleotide position 1858, which is located opposite the nucleotide 1896 in the stem loop structure of pregenomic RNA. A 1896 PC mutation in genotype A subjects thus results in an unstable C-A base pairing with consequent disruption of the stem loop structure (Pollack JR et al 1993). The low frequency of 1896 PC mutation in genotype D in our study is not keeping with the previous report.

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