

RESEARCH COMMUNICATION

Identification of a Hepatitis B Virus Core Promoter Mutant by PCR- RFLP in Patients Suffering from Chronic Liver Disease, Uttar Pradesh, India

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Abstract

Objective: The present study was designed to identify a core promoter mutation in the HBV genome in patients suffering from HBV related chronic liver disease. **Materials and Methods:** 154 chronic liver disease patients were selected for study of DNA extracted using a pure viral DNA extraction kit. The core promoter mutation was detected by the polymerase chain reaction- based restriction fragment length polymorphism (PCR-RFLP) method, using the Sau 3AI restriction enzyme to see if cleavage would occur at this specific site. **Results:** Among the total of 154, 78 patients were found positive for HBsAg and 71 samples were found to be positive for HBV DNA by first round PCR. The over all prevalence of core mutant was 51(71%) in the 71 patients. 11 (68.75%) of 16 patients, excluding 1 patients with mixed type mutation was detected in inactive HBsAg carriers, 39 (81.25%), excluding 2 patients with mixed type was detected in chronic hepatitis B, and 4/7 (57%) in patients with liver cirrhosis were found. **Conclusion:** Our study concluded that the prevalence of the core promoter mutation in the BCP region was higher in the patients with chronic hepatitis B than in liver cirrhosis and HBsAg carriers. The Sau3AI assay, which is much more convenient than sequencing, was shown to be useful for the detection of the core promoter mutant in an extensive number of clinical samples. Monitoring and detection of HBV variants by PCR-RFLP in chronic infection may improve the management of these patients

Key Words: HBeAg - BCP - PCR-RFLP- core promoter mutant

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Introduction

Hepatitis B virus (HBV) is a partially double-stranded DNA virus, which is a major causative agent of chronic, acute hepatitis, liver cirrhosis and hepatocellular carcinoma in Asian, African and Southern European countries. An estimated 350 to 400 million carriers are found around the world (Wang et al., 2005) and 1 to 2 million people have died from this virus (Mahoney, 1999). In India the prevalence rate of hepatitis is reported to vary from 1 to 13 percent; with an average of 4.7 percent (Thyagarajan et al., 1996).

Hepatitis B envelope antigen (HBeAg) is considered as a marker for viral replication but seroconversion from HBeAg to anti-HBeAg positive generally indicates low level of viral production and low level of serum HBV DNA. This generally correlates with improvement of liver disease. HBV has a mutation rate 10-fold higher than other DNA viruses (Tong et al., 2005). In the natural course of chronic infection, loss of HBeAg expression and appearance of antibodies (anti-HBeAg) are usually accompanied by cessation of viral replication. The HBV variants appear during HBeAg seroconversion and they

bring mutations in the precore region (PC) that prevent HBeAg synthesis. The most common of these mutations is a G to A substitution at nucleotide 1896, which prevents the production of HBeAg by introducing a premature stop codon into the open reading frame (ORF) of the PC. This mutation converts codon 28 of the pre core sequence to a termination codon (TGG-TAG) and therefore it prevents expression of HBeAg (Lok et al., 1994).

Another common HBeAg variant is the basal core promoter mutant (BCP) characterized by point mutation in the promoter of both HBeAg mRNA and core protein mRNA. 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 et al., 2005). As opposed to precore variants, core promoter mutations can be detected in patients who are either HBeAg +ve or -ve but most frequently found in the HBsAg +ve and HBeAg -ve/ anti-HBeAg +ve. The prevalence of the core promoter variant is about 40%, evenly distributed among the major HBV genotypes. The present study aimed to identify a hepatitis B virus core promoter mutation in patients suffering from chronic liver disease.

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Materials and Methods

Subjects

One hundred and fifty four patients from the Department of Gastroenterology and Hepatology, Moti Lal Nehru Medical College, Allahabad, India were studied from July 2007 to Oct 2008, in a random manner after obtaining consent from the institutional ethical committee.

Biochemical Analysis

The alanine amino-transferase (ALT), and serum bilirubin of the targeted patients was assessed with the help of Kinetic Assay kit (Span Diagnostics Ltd.) following the instruction of the manufacturer. The normal value ranged between 10- 40 U/L and <0.8U/L at 370 C respectively.

Serological Analysis

Viral markers 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 DNA by PCR

All the HBsAg +ve samples were screened for the HBV DNA. DNA was extracted from 200µl serum by using a High Pure Viral Nucleic acid Kit (Roche, Penzberg, Germany) and stored at -80°C. 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'GCATGGAGACCACCGTGAAC3': sense) and P1-2(5' GGAAAGAAGTCAGAAGGCAA 3':antisense) and subjected to 35 cycle of 94°C for 1 min, 55°C for 1.2 min, 72°C for 2 min. The PCR product was analyzed by agarose gel (2%) electrophoresis to see the HBV DNA bands under Trans gel illuminator (BioRad. Quantity One USA).

Detection of core promoter mutation

If HBV DNA bands was seen then the nested PCR was done. 5 µl of the first round PCR product was mixed with 45 µl of a PCR reaction mixture(Roche, Penzberg, Germany) containing 400 nM of the primer P2-1(5'CATAAGAGG ACTCTTGGACT3':sense) and P2 – (5'GGCAAAAAGAGAGTA ACTC3':antisense), 30 cycles of 94°C for 1 min, 55°C for 1 min and final extension at 72°C for 1.5 min. The amplified product was analyzed by agarose gel (2%) electrophoresis to see the HBV DNA bands under Trans gel illuminator, if the band was visible then we proceed it for the next step.

A 10µl aliquot of the nested PCR products with molecular size pf 307 bp was mixed with 10 µl of buffer containing 1U of Sau 3A (Roche, Penzberg, Germany) and incubated at 37°C for 60 min. Restricted products were then loaded on an agrose gel (4% - 3 Gel agrose tinny (R.D. Applied biosciences, New Delhi): 1 agrose) 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 197 bp and 110 bp, and there is no digestion

Table 1. Core Promoter Mutations in Relation to the Clinical Status of Chronic Liver Disease

Type	Active HBsAg carrier	Chronic hepatitis B	Liver cirrhosis
Wild	4 (25.0%)	7 (14.6%)	3 (42.9%)
Mutated	11 (68.8%)	39 (81.3%)	4 (57.1%)
Mixed	1 (6.3%)	2 (4.2%)	0 (0.0%)

Table 2. Factors Affecting Mutation in the Pre-core Region

Variable	Mutated	Wild	Mixed
Sex- M/F	36/18	10/4	3/0
ALT			
Normal	43	11	0
Abnormal	11	3	3
Serum bilirubin	>0.8	>0.8	<0.8
HBeAg			
-ve	41	2	3
+ve	13	12	0

occurred in the wild type HBV DNA.

Results

HBV DNA positivity

Among 154 chronic liver disease patients 78 patients were found positive for HBsAg and 71 samples were found to be positive for HBV DNA by the first round of PCR. First round of PCR generated a 369 bp DNA fragment that was visible on 2% agarose gels.

Core promoter mutation

Overall, the A1762T/G1764A mutations in the BCP were found in 51(71%) of the 71 patients. In inactive HBsAg carriers 11 (68.75%) of 16 patients, excluding 1 patient with mixed type mutation in BCP was detected. In patients with chronic hepatitis B, the A1762T/G1764A mutation were found in 39 (81.25%), excluding 2 patients with mixed type mutation. In patients with liver cirrhosis the A1762T/G1764A mutations were found in 4 of 7 (57%). The prevalence of the A1762T/G1764A mutations in the BCP is higher in the patients with chronic hepatitis B than in liver cirrhosis and HBsAg carrier state (Table 1).

Factor affecting the mutation in the core promoter region

Besides clinical status serum levels of ALT and HBV DNA were predicting factors for A1762T/G1764A mutation analysis. A1762T/G1764A mutations were more frequent in HBeAg-negative patients than in HBeAg patients (Table 2).

Discussion

HBV has a DNA genome and its replication strategy includes RNA-dependent DNA polymerase (Summers, 1982), which may raise the mutation rate of a DNA virus close to that found with retroviruses (Chun et al., 2000). Thus, numerous mutations in the HBV genome have been reported. Many investigators have reported conflicting results about the association between the BCP and the precore mutations and the severity and progression of chronic liver disease (Chun et al., 2000; Liu et al., 2004).

The A1762T/G1764A mutation rate was high in patients with chronic hepatitis B, inactive HBsAg carriers and in liver cirrhosis respectively. There was about 13-24% difference in A1762T/G1764A mutation between the patients with chronic hepatitis B, liver cirrhosis and HBsAg carriers but Song et al (2006) reported that there were no significant differences in the A1762T/G1764A mutation between the patients with chronic hepatitis B and liver cirrhosis. This result indicated that the core promoter (A1762T/G1764A) mutation mostly occurs in early immune clearance phase regardless of clinical outcomes and these mutants are persistently selected in the natural course of chronic HBV infection. The overall prevalence of the core promoter mutation in the patients with chronic liver disease is about 80.3% in our study including in active HBsAg carriers, chronic hepatitis B, liver cirrhosis, but Xiao-Mou Peng et al reported 89.1% out of patients with detectable HBV DNA in serum after HBeAg to anti-HBeAg seroconversion (Peng et al; 2005).

In our study we found that mutations in BCP region were about 71%. The mutation rate in HBsAg carrier, chronic hepatitis B and liver cirrhosis was 68.75%, 81.25% and 52.14% respectively. We also found that out of 71, 46 (64.7%) were HBeAg -ve and 25 (35.3%) were HBeAg +ve. In all HBeAg -ve samples we identified the core promoter mutation only in 41 samples i.e. 89.1%, while Hussain et al (2005) reported that all HBeAg-negative individuals in the study were found to contain virological mutations that are associated with decreased HBeAg production. Sequencing of the 21 patient samples in this study revealed basal core promoter mutations in 55.5 and 8% of HBeAg-negative and HBeAg positive individuals, respectively. The A1762T/G1764A variant is usually accompanied by increase in HBV replication and decreases in HBeAg secretion, and may be related to liver function deterioration (Kramvis et al., 1999; Laras et al., 2002).

This study has shown that PCR-RFLP analysis of the HBV core promoter region provides a rapid approach to detect several common viral mutants in HBV-infected patients, even in the absence of DNA sequencing facilities (Barros et al., 2004). The PCR-RFLP method is particularly useful for preliminary characterization of viral variants during natural course of infection. Monitoring and detection of HBV variants by PCR-RFLP in chronic infection may improve the management of patients. Characterization of core promoter mutants may also be useful for design of vaccines based on viral epitopes that are different from those expressed by HBsAg (Pumpens and Grens, 1999). The limitation of this study is that chronic liver disease was clinically diagnosed in most patients. Therefore, unknown proportion of chronic liver disease might be misclassified into different status of chronic liver diseases.

We conclude that the prevalence of the core mutation in the BCP region is higher in patients with chronic hepatitis B than in liver cirrhosis and HBsAg carriers. The Sau3AI assay, which is much more convenient than sequencing, was shown to be useful for the detection of the T 1762 A1764 mutant in an extensive number of clinical samples. Monitoring and detection of HBV

variants by PCR-RFLP in chronic infection may improve the management of these patients.

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