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ORIGINAL ARTICLE

Distribution of *Hepatitis B Virus* Genotypes and Its Association with Severity of Liver Disease in Patients with Chronic Hepatitis B in Uttar Pradesh, India

Ajay Kumar · Manisha Dwivedi · Sri Prakash Misra · Vatsala Misra · Sushil Narang · Renu Pandey · Balesh Kumar Tiwari

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Abstract The present study was designed to investigate the distribution of genotypes and its association with severity of liver disease in patients with Chronic Hepatitis B (CHB) in Uttar Pradesh, India. One hundred five HBsAg positive patients were selected for the study. The DNA was extracted by using pure viral DNA extraction kit. The genotype of *Hepatitis B virus* (HBV) was identified by using polymerase chain reaction- based restriction fragment length polymorphism (PCR-RFLP) method, by using *AvaII* and *DpnII* restriction enzyme to see the different patterns of cleavage that would occur at this specific site. Among 105 HBsAg positive chronic liver disease patients 58 patients were positive for HBeAg and 47 samples were HBeAg negative. Genotyping was done successfully in 91 samples. Genotype A was identified in 22% and genotype D in 78% CHB patients. Genotype A showed elevated liver enzymes much more than genotype D. The Child Pugh classification of 20 patients with genotype A was class A ($n = 2$), B ($n = 9$), C ($n = 9$) and of genotype D was class A ($n = 13$), B ($n = 50$) and C ($n = 8$). In conclusion our results showed that Genotype D was the commonest genotype in Uttar Pradesh, whereas genotype A had significantly more elevated ALT/AST levels than genotype D. ($P < 0.05$). Child Pugh Grade B was significantly more in patients with genotype D, whereas Child Pugh Grade C was more in genotype A.

Keywords HBV · CHB · Genotyping · PCR-RFLP

Introduction

Hepatitis B virus (HBV) infection is a well recognized health problem leading to significant morbidity and mortality world wide especially in the developing countries. An estimated 375 million subjects are suffering from chronic hepatitis B (CHB) infection [6]. HBV is an etiological agent of acute and chronic liver disease, including fatal fulminant hepatitis, cirrhosis and hepatocellular carcinoma, which is one of the most common human cancers and causes of death worldwide [15]. Based on genomic sequence data hepatitis B virus is classified into eight genotypes A–H [11], and four major serotypes ayw, ayr, adw and adr on the basis of complete genome and S gene sequence analysis [2, 25]. Genotypes and serotypes are useful tools in understanding the epidemiology of HBV infection. HBV genotypes have distinct geographical distributions. Genotype A and D occur frequently in Africa and Europe [5, 16], while genotype B and C are prevalent in Asia [21], genotype E is almost entirely restricted to Africa and F is found preferentially in Central and South America [20], genotype G was reported in France and in the United States [25]. The eighth genotype H has been described in Central America [2]. In India, prevalence of HBV in chronic hepatitis is very high [8]. The HBV

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A. Kumar · R. Pandey · B. K. Tiwari
Centre for Biotechnology, University of Allahabad, Allahabad, Uttar Pradesh, India

A. Kumar · M. Dwivedi (✉) · S. P. Misra · S. Narang
Department of Gastroenterology, M.L.N. Medical College, Allahabad, India
e-mail: mani_1952@rediffmail.com

V. Misra
Department of Pathology, M.L.N. Medical College, Allahabad, India

genotypes A and D have been reported from different parts of India [10, 13, 28], while C in addition to A and D has been reported and characterized recently from an eastern Indian population only [3]. Genotypes and serotypes are useful tools for understanding the epidemiology of HBV infection. Although both genotypes and serotypes segregate geographically, the same serotype can be represented by several genotypes [1]. Recently, genotypes of HBV have been reported to be an influential factor in the clinical manifestations of chronic liver disease in the host. Genotype A is associated with chronic liver disease more frequently than genotype D in Europe [18, 28]. India is a vast country, comprising of multiracial communities with wide variations in ethnicity and cultural patterns, which is attributable to its geographical location due to invasion or anthropological migrations in the past. A recent study carried out by Datta S (2008) [9] found the prevalence of HBsAg to be 2–8%, which is approximately similar to that observed from other parts of India [4, 14]. Data on the distribution of genotypes for patients with CHB is very limited in North India, thus the present study aims to investigate the distribution of genotypes and its association with liver disease in patients with CHB in Uttar Pradesh, India.

Materials and Methods

Patients

One hundred five consecutive adult patients with CHB infection, who were admitted in four medical centers of Allahabad, UP between February 2008 and April 2010, were prospectively included in the study. The medical centers were representative of all the geographic regions of the state. Those with age ranging from 20 to 75 years, who were HBsAg positive at least for a period of 6 months, were enrolled. In one hundred five patients with HBV infection 78 patients had attended the indoor/out patients, of Department of Gastroenterology and Hepatology of M.L.N. Medical College, Allahabad. The remaining 27 patients were from other prestigious nursing homes. Clinical data and clinical history of the patients was recorded. Serum was initially tested for liver function test (ALT, AST, alkaline phosphatase, serum bilirubin and serum albumin) and serological test for HBsAg. All HBsAg positive samples were stored at −70°C and used for further analysis like HBeAg, anti HBeAg and HBV genotyping.

Biochemical Analysis

The alanine amino-transferase (ALT), alanine aspartate transferase (AST), alkaline phosphatase (ALP), serum albumin and serum bilirubin of the targeted patients was assessed with the help of Kinetic Assay kit (AUTOSPAN Diagnostics Ltd., India.) following the instruction of the manufacturer. The normal value ranged between 10–40 U/l, 10–40 U/l, 60–300 mg/dl, 3.6–5.0 and <0.8 U/l at 37°C, respectively.

Serological Analysis

Viral markers of HBV, viz, HBsAg, HBeAg, anti-HbeAg, and anti-HCV were tested. HBsAg was tested by using ERBA LISA Hepatitis B kit (Transasia Bio-Medicals Ltd.), HBeAg and anti HBeAg were tested by using Micro screen HBeAg ELISA test kit (AUTOSPAN Diagnostics Ltd., India.), anti HCV were tested by using rapid card (AUTOSPAN Diagnostics Ltd., India) according to the instruction of the manufacturer.

Identification 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′-Antisence) described by Lindh et al. [17] were obtained commercially. PCR reaction mixture contained 200 µmol/l each nucleotide triphosphate, 0.4 µmol/l each primer, 1.5 mmol/l $MgCl_2$, 75 mmol/l Tris–HCl, 20 mmol/l $(NH_4)2SO_4$, 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. [17].

Statistical Analysis

Data in the text and tables are expressed as mean ± SD by using Microsoft Excel work sheet. The p value was

calculated by using ANOVA and was consider significance if $P < 0.05$.

Results

Demographic Characteristics

The study included 105 consecutive patients with CHB. 79 (75.23%) males and 26 (24.76%) females. The mean age of the patients was 43.22 ± 13.57 years. Of all the patients, 58(55.23%) were HBeAg positive compared to 47 patients (44.76%) being HBeAg negative. Of 58 HBeAg positive patients, 41 were males and 17 were females, while 38 of 47 HBeAg negative patients were males and 09 were females.

The mean value of ALT, AST, serum bilirubin and serum albumin, in patients who were HBeAg positive was 131.96 ± 84, 71.92 ± 47.22, and 4.39 ± 4.7 and 3.6 ± 0.81, respectively. The mean value of ALT, AST, serum bilirubin and serum albumin, in patients who were HBeAg negative was 79.33 ± 50.09, 54.91 ± 38.10, 2.69 ± 2.51 and 3.78 ± 0.81, respectively.

The demographic characteristics and laboratory features of HBeAg positive and HBeAg negative patients are shown in Table 1.

Distribution of HBV Genotypes and Subtypes

Genotype A was detected in 20 (22%) patients out of 91. In genotype A, 13 (65%) were having subgenotype A-1 and 7 (35%) were A-2. Genotype D was observed in 71 (78%) out of 91 patients. In genotype D, subgenotype D-2 and D-3 were 20 (28.7%) and 51 (71.3%), respectively. While genotyping failed in 3 (2.8%) and no PCR and HBV-DNA product was obtained in 11(10.4%) patients. RFLP patterns suggestive of BCEFGH were not observed in any patients. The detailed distribution of genotype among male and female patients is shown in Fig. 1. The RFLP pattern of the HBV genotype observed as in Fig. 2.

Table 1 Demographic and biochemical information of HBsAg +ve patients

Characteristic	HBeAg Positive	HBeAg negative	Total
Sex			
Male	41	38	79
Female	17	09	26
Age (Yr)	42.84 ± 13.73	43.72 ± 13.5	43.22 ± 13.57
ALT	131.96 ± 84	79.33 ± 50.9	108.42 ± 76.09
AST	71.92 ± 47.22	54.91 ± 38.10	64.30 ± 44.01
Serum Bilirubin	4.39 ± 4.7	2.69 ± 2.51	3.60 ± 3.99
Serum Albumin	3.6 ± 0.81	3.78 ± 0.81	3.68 ± 0.81

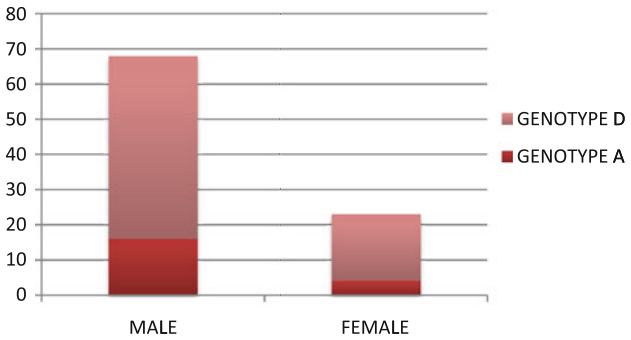

Fig. 1 Distribution of HBV genotype among male and female

Severity of Disease Associated with Genotype

Out of 91 patients where genotyping was done successfully, 20 had genotype A whereas 71 had genotype D. The Child Pugh grading in genotype A was, A $n = 2$(10%), B $n = 9$ (45%) and C $n = 9$ (45%), whereas in genotype D it was, A $n = 13$ (18.3%), B $n = 50$ (70.4%) and C $n = 8$ (11.3%). Child Pugh Grade B was significantly more in patients with genotype D whereas Child Pugh Grade C was more in genotype A.

Discussion

In our study it was observed that in Uttar Pradesh, North India the most prevalent genotype among patients with HBV related chronic liver disease was genotype D (78%) the remaining 22% had genotype A. HBV genotypes A and D have been well documented from different parts of

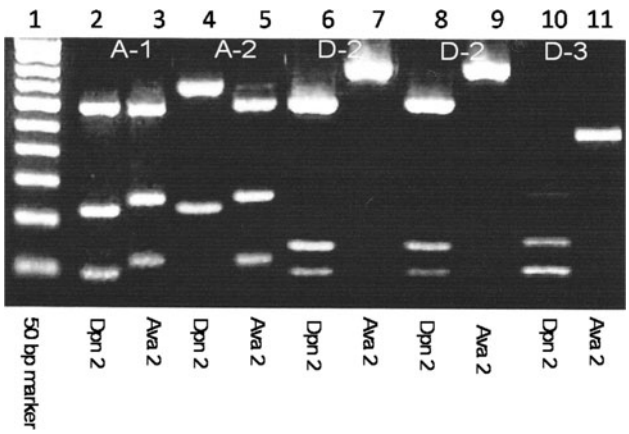

Fig. 2 The electrophoresis pattern of RFLP on digestion with restriction enzymes Dpn2 and Ava2. *Lane 1* shows 50 bp ladder. *Lane 2*, *4*, *6*, *8*, *10* shows restriction digestion band with Dpn2. *Lane 3*, *5*, *7*, *9*, *11* shows restriction digestion band with Ava2

mainland India [3, 10, 13, 27]. There is not much information regarding the prevalence of HBV genotypes from North India. Till date few studies have been done on the frequency of various HBV genotypes in India and show much variation. A study published by Thakur et al. found an equal proportion of genotype A and D [27]. A study conducted by Kumar A et al. found that the genotype D was dominant over genotype A and found genotype C in one patient; this genotype has not previously been reported from India [13]. The other studies found genotype D to be the predominant genotype on genotype A [3, 10]. Our results are in accordance with the latter studies which showed the dominancy of genotype D on A.

HBV genotypes have been shown to have a distinct geographic distribution. Genotypes B and C are prevalent in the East and South-East Asia [26]. In contrast, genotypes A and D are common in Western Europe and North America [19, 24]. In Mediterranean region [29], the Middle East and Central Asia, genotype D is dominant [12, 23]. Detection of a minor proportion of other genotypes like A, B and E is also similar to mainland India [10].

Predominance of genotypes A and B in the Indian population may indicate that HBV strains in India originated from those in Europe or vice versa. In many countries where well known waves of migration have occurred over time, the prevalence of different HBV genotypes reflects the origin of immigrants and other patterns of migration. This is exemplified by South Africa, where the most prevalent genotypes, A and D [7], correlate with migration from Northwestern Europe (UK and Netherlands), Southern Europe and India. The same explanation holds for the predominance of genotypes A and D in our population.

The variation in relative frequency of HBV genotypes in studies from India may show geographical variations. The study by Thakur et al. 2002 and Kumar A et al. 2005, both from northern India, have found high and equal proportion of genotype A and D [13, 27], but in our study we found that the genotype D was predominant over genotype A, similar results were reported in studies from Western India and the Andaman and Nicobar Islands. This raises the possibility that the Indian population originally had HBV genotype D, which has been partially replaced by genotype A, particularly in northern India, due to evidence that a few million years ago, a group of people with eastern European genetic affinities migrated into the Indian subcontinent from the northwest. In our study we found genotype A to have subgenotype A1 and A2, and genotype D to have subgenotype D2 and D3, while a study carried out by Thippavazzula Rekha et al. reported that D1 and D2 were most common and were found in 82% of patient samples of genotype D, D3 was found in one patient [22].

In our study we also analyzed that the ALT level was high in genotype A having CTP Grade B and C, while genotype D was found to be associated with less ALT elevation and had CTP Grade A and C, none of genotype A have CTP Grade A. In accordance with genotype and its association with Child Pugh Grade we stated that genotype A has severe liver disease than genotype D. This type of study was also done by Kumar A et al. 2005 and found that genotype A was more often associated with ALT elevation, HBeAg positivity, absence of anti-HBe i.e. severe liver disease [13].

This study has some limitations. This includes small sample size and we used a method based on only a part of HBV genome i.e. pre-S region, not the entire HBV genome. However, this RFLP method has previously been shown to be reliable for HBV genotyping [16]. This study proves that RFLP technique serves the purpose. There is also increasing evidence that the clinical picture, response to therapy may differ depending on which genotype has infected the patient. However, the population which has been studied may not fully represent the entire region of India and further studies are needed to study the prevalence of HBV genotype in our population. We concluded that Genotype D was the most frequent genotype in Uttar Pradesh, North Indian population. Genotype A had significantly more elevated ALT/AST levels than genotype D and genotype A had more Childs 'C' patients whereas genotype D had more of Childs 'B' patients.

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