SHORT COMMUNICATIONS

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Detection of HBV Genotype C in Hepatocellular Carcinoma Patients from North East India: a Brief Report

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Abstract

Background and Objectives: Newer genotypes of HBV have been reported from India. This study was aimed to determine the circulating genotypes of HBV in hepatocellular carcinoma patients from three different geographical locations of India. **Methods:** 141 HBV related HCC cases were included from three different hospitals across the country. Genotyping of HBV was performed by PCR using type specific primers specially designed in 70 cases. Samples of interest were confirmed by direct sequencing of the precore/core region of HBV genome. **Results:** Genotypes could be detected in 40 (57.14%) out of the 70 HBV DNA positive HCC cases by type specific primers. HBV genotype D was documented in 20 (50%), genotype A in 10 (25.0%) and genotype C in 10 (25.0%) of these HCC cases. Genotype C of HBV was detected only in the samples from North East India. No significant difference was observed for the biochemical profile. **Conclusion:** Although Genotype D is the major HBV genotype in India followed by A, detection of HBV genotypes C in HCC patients indicates a changing epidemiology of the virus in India that may require region based management of the virus.

Keywords: HBV- HCC- genotype C- North East India- South India and North India

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Introduction

Hepatitis B virus (HBV) is a major public health problem with over 360 million chronically infected people worldwide and accounting for about 600,000 deaths from HBV- related liver disease or hepatocellular carcinoma annually (Shepard et al., 2006).

On the basis of genetic differences, HBV have been classified into eight genomic groups (A-H). Entire genome sequences within each group diverge from other groups by more than 8%. These eight groups have distinct geographical distributions (Norder et al., 1993; Norder et al., 2004). Genotype A is distributed globally and is the main genotype found in Europe, North America, Africa and India. Genotypes B and C are predominant in East Asia, Southeast Asia and Australia. Genotype D is mainly found in the Middle East and Mediterranean countries but has been reported globally, whereas genotype E seems to be predominant in West Africa. Genotype G has been characterized in samples from USA, Mexico and France and genotypes F and H are found exclusively in Central and South America (Campos et al., 2005; Weber, 2006).

Different genotypes have shown different responses to interferon-alpha (IFN-α) treatment. Compared to genotype C, genotype B has a higher rate of response to IFN-α treatment in HBeAg (+) CHB, and genotypes E, F, and H appear to be more sensitive to IFN-α than genotype G (Wai et al., 2002; Erhardt et al., 2009).

The study was aimed to determine the circulating genotypes of HBV in hepatocellular carcinoma patients from three different geographical regions of India with special reference to north east India using type specific primer sets. The findings are the initial reports of a study on the whole genome analysis of HBV genome from three sites across India.

Materials and Methods

Patient enrolment

HCC patients were included as per EASL 2001 guidelines. Consecutive cases of HBV related HCC cases as; 40 from North India, 50 from South India and 51 from North East India were recruited in the study. Ethical guidelines of 1975, Helsinki were followed. HBV DNA was extracted using phenol chloroform method. Serological studies were done by available 3rd generation ELISA kits.

HBV Genotyping by PCR

Genotyping of HBV was identified by PCR using type specific primers designed for the precore region of HBV with slight modification to the published sets of primers

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(Datta et al., 2008). The details of the primers used and PCR conditions are tabulated in Table 2. Six genotypes (A–F) of HBV could be identified by specific bands of the second PCR. To avoid false-positive results, instructions to prevent cross-contaminations were followed, and the results were considered valid only when they were obtained in duplicate.

Primer sequences used and PCR condition

A band of size 787 bp indicated the presence of HBV genotype C. Again a band size of 370 bp indicated the presence of genotype D while a PCR product size of 147 bp confirmed the presence of HBV genotype A (Figure 1).

Amplification of Precore/Core Gene

For sequence analysis, nested PCR for amplification of Precore/core was performed. For the first-stage PCR, 25 µl of reaction mixture, containing 2 µl of the DNA sample, 1X PCR buffer (10mM Tris–HCl, pH 9.0, 50mM KCl, 1.5mM $MgCl_2$, 0.01% gelatin, and 0.1% triton X-100), 10mM of each dNTP, 100 ng of each outer primer, and 1U of taq DNA polymerase, was amplified in a thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) for 35 cycles. Each cycle entailed denaturation at 95°C for 60 sec, primer annealing at 55°C for 30 sec, and extension at 72°C for 60 sec with a final extension step at 720C for 10 min. After the first amplification, 1 µl of the PCR product was re-amplified for another 35 cycles with 100 ng of each inner primer. For the Precore/core region, the outer sense primer was 5'-CTGGGAGGAGTTGGGGGA-30, nucleotide positions 1770–1787; the outer antisense primer was 50- CAATGCTCAGGAGAC TCTAA-3', nucleotide positions 2476–2495; the inner sense primer was 5'-GGTCTTTG TACTCGGAGGCT-30, nucleotide positions 1788–1808;and the inner antisense primer was 50-GTCAGAAGGCAAAAAAGAGA- 3', nucleotide positions 2467–2486.

Nucleotide Sequencing and Phylogenetic analysis

Sequences were aligned using the Clustal W program (Thakur et al., 2002) and the phylogenetic tree analysis was performed using the neighbour-joining method (bootstrap re sampling test with 1,000 replicates) in MEGA version 3.0 software (Nakai et al., 2001).All the cases were sequenced for the confirmation of genotype and mutational analysis and submitted to the Gene Bank database (JQ038375-JQ038414).

Other tests

HBeAg status was confirmed with enzyme immunoassay (EIA; Abbott Laboratories, Abbott Park, IL). HBV viral load was also done using RT PCR. Serum AFP levels were determined using an Immulite-100 automated immunoassay system (Diagnostic Products, Los Angeles, CA, USA). Staging of the HCC cases were done based on the radiological findings.

Results

Demographic characteristics

The mean age was 50.1±7.07, 50.4± 6.36 and 49.04762

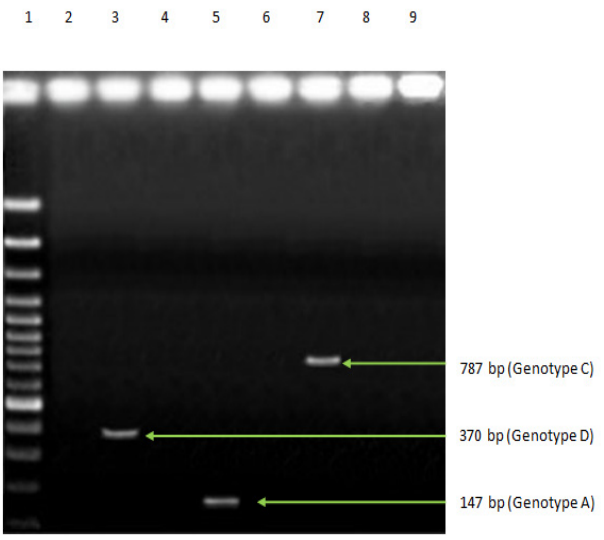

Figure 1. Gel Photographs Showing Different Bands for Three Different Genotypes of HBV: Lane 1,100 bp ladder; Lane 2, Blank; Lane 3, 370 bp (genotype D); Lane 5, 147 bp (Genotype A); Lane 7, 787 bp (Genotype C), Lanes 8, 9, Blank.

Table 1. Region Wise Break up of HCC Cases with Respect to Direct Sequencing for HBV Genotyping

Conditions	North India	South India	North East India
HBV related HCC cases included (141)	40	50	51
HBV DNA positive cases with high viral load (70)	17	21	32
Genotypes confirmed by type specific primers and then confirmed by direct sequencing	10	10	20

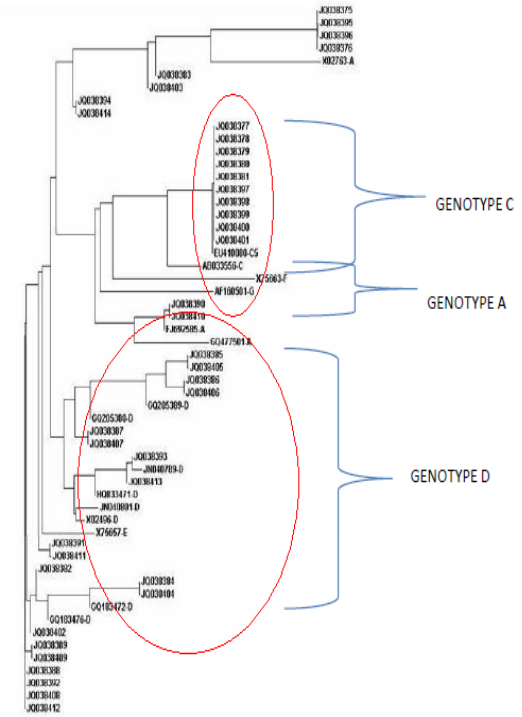

Figure 2. Phylogenetic Tree Confirming Genotype C in HCC Cases

Table 2. List of Primers and PCR Conditions (35 cycles of PCR).

Primer Name	Primer Sequence	PCR Condition
GTA-F	5'-CGGAAACTACTGTTGTTAGACGACGGGAC-3'	
GTA-R	5'-AATTCCTTTGTCTAAGGGCAAATATTTAGTGTGGG-3'	
GTD –F	5'-ACAGCATGGGGCAGAATCTTTCCACCAG-3'	95- 10 minutes
GTD-R	5'-CCTACCTTGTTGGCGTCTGGCCAGG-3'	
GTC -F	5'-CCTGAACATGCAGTTAATCATTACTTCAAAACTAGG-3'	
GTC -R	5'-AGCAGGGGTCCTAGGAATCCTGATGTTG-3'	95-60 seconds
GTB-F	5'-CCGCTTGGGGCTCTACCGCCCG-3'	60.1-45 seconds
GTB-R	5'CTCTTATGCAAGACCTTGGGCAGGTTCC-3'	72-45 seconds
GTE-F	5'-CTAATGACTCTAGCTACCTGGGTGGGTGTA-3'	
GTE-R	5'-CCATTCGAGAGGGACCGTCCAAGAAAGC-3'	72- 7 minutes
GTF-F	5'-ACAGCATGGGAGCACCTCTCTCAACGACA-3'	
GTF-R	5'-AGAGGCAATAGTCGGAGCAGGGTTCTG-3'	

± 14.14 years for North, South and North East India respectively that were more or less comparable. The male female ratio of 9:1(9 male and 1 female), 4:1(8 male and 2 female) and 4:1(16 male and 4 female) from North, South and North East India respectively indicates a clear male predominance in accordance to many previous studies (Table 3).

Biochemical characteristics

The ALT levels of 113.89 ± 26.87, 68.2 ± 12.5 and

Table 3. Clinical and Virological Characteristics of Hepatitis B virus Infected HCC Patients from Three Different Geographical Locations of India. (ALT, alanine transaminase; AST, aspartate transaminase; ALP, alkaline phosphatase; ALB T.BIL, Total Bilirubin).

Characteristics	North India (n=10)	South India (n=10)	North east India (n=20)
Male: female	9:01	4:01	4:01
Mean age	50.1±7.07	50.4± 6.36	49.04762 ± 14.14
		Age	
<40 years	3	1	4
> 40 years	7	9	16
ALT (U/L)	113.8889 ± 26.87	68.2 ± 12.5	66.10201± 14.14
AST (U/L)	59.56 ± 6.36	42.7± 39.27	49.19724± 31.63
ALP (U/L)	933.89 ± 88.38	196.5 ± 113	195.5 ± 182.37
ALB (g/L)	3.54 ± 0.1	3.64 ± 0.52	3.72 ± 0.87
		T.BIL (mg/dl)	
	0.84 ± 0.21	1.06 ± 0.53	1.37 ± 0.87
Mean platelet count (104/mm^3)	2.21 lakhs ± 0.52	2.063 ± 0.47	2.186 ± 1.19
a-Fetoprotein (ng/ml)	4234.731 ± 13.44	115,000 ± 12.73	25,809 ± 11.31
		Family history of HCC	
	1 (10%)	1 (10%)	3 (15%)
		Child-Pugh class	
A	7 (70%)	5 (50%)	11 (55%)
B	3 (30%)	5 (50%)	9 (45%)
HBeAg (positive)	6 (60%)	6 (60%)	13 (65%)
HBV DNA (positive)	8 (80%)	7 (70%)	16 (80%)
Log10 HBV DNA (copies/ml)			
Low (< 5)	3 (30.0)	2 (20.0)	4 (20.0)
High (≥ 5)	7 (70.0)	8 (80.0)	16 (80.0)
		Genotype	
A	2 (20%)	2 (20%)	6 (30%)
D	8 (80%)	8 (80%)	4 (20%)
C	-	-	10 (50%)

Table 4. Comparison among the Three Genotypes

Serial Number	Parameters	Genotypes		
		A (10)	C (10)	D (20)
1	HBeAg status			
	HBeAg +ve	6 (60%)	7 (70%)	12 (60%)
	HBeAg -ve	4 (40%)	3 (30%)	8 (40%)
2	Log 10 HBV DNA (copies/ml)			
	Low (<5)	3 (30%)	2 (20%)	3 (15%)
	High (≥5)	7 (70%)	8 (80%)	17 (85%)
3	AFP (ng/mL)			
	<20 ng/ml (%)	1 (10%)	1 (10%)	4 (20%)
	20-400 ng/ml (%)	2 (20%)	1 (10%)	2 (10%)
	>400 ng/ml (%)	8 (80%)	9 (90%)	14 (30%)
4	Child-Pugh class			
	A	5 (50%)	4 (40%)	12 (60%)
	B	5 (50%)	6 (60%)	8 (40%)

66.10± 14.14 U/L and the AST levels of 59.56 ± 6.36, 42.7± 39.27 and 49.20 ± 31.63 U/L were observed in cases from North, South and North East India respectively. The SGOT and SGPT values were raised in cases from Northern India. There were no major differences for other biochemical parameters such as albumin and bilirubin among the patients from the three groups. Alfa Feto protein levels were raised in majority of the cases from all groups (Table 2).

Virological Status

Majority of the cases that were genotyped were with HBsAg positivity from all the three regions. On the other hand HBeAg was positive in 60% cases from North and South India and in 65% cases from North East India. High HBV viral load was documented in majority of the cases from all the three regions (Table 2).

A total of 380 cases of HCC with different aetiologies were screened. Out of the 380 total HCC cases screened from the three regions, 257 were HBV related HCC cases while 89 were HCV related and another 34 were cryptogenic cases of HCC. Among the 257 cases of HBV related HCC cases, 141 cases were positive for HBV DNA and were with high HBV viral load. Again 70 of these 141 cases were subjected to genotyping by type specific primer. The region wise break up of HCC cases with respect to direct sequencing for HBV genotyping has been shown in Table 1.

HBV Genotypes

HBV genotype D was observed in 20 (out of 40) of the total cases while genotype A was found in 10 out of the 40 (25.0%) cases. On the other hand a HBV (HBV genotype C) was documented in the remaining 10 out of 40 (25.0%) hepatocellular carcinoma cases. The genotypes of the same cases were confirmed by direct sequencing. Genotype C of HBV was observed only in North east India and was the predominant genotype associated with hepatocellular carcinoma cases from the region (Figure 2).

Type specific primer based PCR and its specificity in detecting the HBV genotype

A total of 70 patients were found with HBV DNA positivity (50%) by the qualitative PCR performed. Again genotypes could be detected in 40 (57.14%) out of these 70 cases by the type specific primers designed. Although the PCR based on type specific primer is an easy and cost effective method but is not highly specific.

Correlation of HBV genotypes with other parameters

When the HBV genotypes were compared with respect to HBeAg status, no significance difference was observed. It was found that 70% of the HBV genotype C were having HBeAg =ve indicating the replicative form of the virus. Most of the cases of genotype C and D were found with higher viral load.AFP levels were significantly elevated in HBV genotype C cases of North East India. It was a non significant findings with respect to the child plug scores among the three genotypes studied (Table 4).

Discussion

Detection of genotype A and D of HBV in most of the cases of HCC is in support of the circulating genotypes of HBV in this country which have been well documented in studies from different parts of India (Thakur et al., 2002; Banerjee et al., 2006; Chattopadhyay et al., 2006a; Chattopadhyay et al., 2006b, Erhardt et al., 2009; Wai et al., 2010). Our study findings are in support of many other North Indian studies which reported genotype D to be predominant with a low frequency of genotype A and also with HBV genotype distribution documented from western and southern parts of India (Thakur et al., 2002; Banerjee et al., 2006; Chattopadhyay et al., 2006a; Chattopadhyay et al., 2006b, Erhardt et al., 2009; Wai et al., 2010).On the other hand emergence of genotype C in eastern part of India has been reported by only few studies (Datta et al., 2006; Datta et al., 2007; Datta et al., 2008) as described by the an article of Dutta et al., (2008) where trade, use of illicit drugs, trafficking and visits to and from different countries have been attributed for the drastic

change in HBV epidemiology of India and specially of eastern and north eastern parts of India.Also, in the eastern part of India, sub genotypes Aa/A1, Cs/C1, D1, D2 and D3 are prevalent as documented by two separate studies of Banerjee et al., (2008a) and Banerjee et al., (2008b). However, data on HBV sub genotype distribution is scanty from India (Nakai et al., 2001). The only sub genotype. High nucleotide sequence similarity with viral genome isolated from south East Asian sub genotype Cs/C1 strain, genotype C strains from eastern Indian patients was suggested to be a recent introduction to eastern Indian population (Kumar et al., 2005; Vivekanandan et al., 2004). This report of detection of genotype C may be considered as result of gene influx and may be attributed to human travelling. The possible root of influx of this genotype can be achieved by whole genome analysis of these sequences. Further the association of HBV genotype C with HCC needs to be keenly observed as in the current study, 50% of the HCC cases from North East India were infected with genotype C which is known to have a aggressive course of action.

Detection of HBV genotype C in the current study in 50% of the HCC cases from North East India is in accordance with high prevalence of HBV genotype C in neighbouring countries like Myanmar (Nakai et al., 2001) where it was found as high as 77% among the migrant travellers, around 49% in Bangladesh (Wang et al., 2014), pretty high incidence in Thailand (Louisirirotchanakul et al., 2012), Northern Provinces of China with exceedingly high percentage of genotype C cases (Zeng et al., 2005) and Yunnan (Wang et al., 2014). Also, Genotype was reported to be the second highest circulating genotype of HBV in many surrounding parts such as in Arunachal Pradesh which is one of the north eastern state sharing boarder to China and the study site (Kumar et al., 2011), southern provinces of China (Zeng et al., 2005) and few parts of Yunnan (Shen et al., 2015).

There is also a need to study the course of the disease associated with genotype C infected patients and the response to the antiviral drugs available in Indian context. There are reports indicating that newer genotypes of HBV such as genotype C from eastern part of the country are a serious matter of concern in management of HBV in India. Association of genotype C as a risk factor has been studied in details and was reported to increase the risk of HCC and was found to be associated with poor surgical outcome for hepatocellular carcinoma patients. The influx of newer genotype to north east India may be due to the long porous international border it shares with countries such as china, Bhutan, Bangladesh and Burma where genotype C of HBV is the predominant genotype. Human travelling and intra venous drug abusers may be the source of its transportation to north east India. Also, the screening of immigrants from places with high endemicity of HBV and particularly of genotype C needs to be screened for reducing the influx to the native population. HBV genotyping by phylogenetic analysis based on nucleotide sequences is the most reliable genotyping method. However, this is not an appropriate method for large scale genotyping. Several groups have reported the genotyping of HBV by the restriction fragment length polymorphism method. However, these methods were not so sensitive and specific apart from being expensive. However the current method of genotyping is a cheap and rapid method. The specificity of the technique is however not cent percent and need further modification in the protocol.

Though type specific primer based genotyping is a rapid and cost effective method for HBV genotyping as reported from other studies and it may be used for rapid diagnosis but can't be considered as the best methods available for genotyping. The detection of newer genotypes of HBV is an indication to the changing epidemiology of the virus in this country. Frequent reports of HBV genotype C and emergences of new mutations have immense importance in determining the clinical outcome, HCC development, transplantation outcome, efficacy of vaccination and response to anti viral drug. A region based approach for HBV related HCC management is of extreme importance. Preventive measures to reduce the spread of newly introduced genotype to other parts of the country are also essential.

Conflict of Interest

All authors declare that they have no conflict of interest.

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