Hepatitis B virus genotype A is more often associated with severe liver disease in northern India than is genotype D

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Background: The clinical outcome of chronic hepatitis B may depend on hepatitis B virus (HBV) genotype. Data from India on this aspect are limited and contradictory. We studied the frequency of HBV genotypes and their clinical significance. **Methods:** Stored sera from patients with chronic HBV infection were tested for HBV genotype using PCR-RFLP. Clinical data, and biochemical and serological parameters were retrieved from medical records; patients were classified as having chronic hepatitis or cirrhosis. **Results:** Of 70 patients studied (mean age [SD] 38.4 [17.0] years; 63 men; ALT 140 [177] U/L), 32 had chronic hepatitis and 38 had cirrhosis. HBeAg was positive in 50/67 (75%), and anti-HBe in 12/66 (18%). Genotype A was the commonest (37; 53%), followed by D (32; 46%) and C (1; 1%). Patients with genotype A more often had ALT elevation exceeding 1.5 times normal (30/37 [81%]) than those with genotype D (18/31 [58%]; $p<0.05$). They also more often had positive HBeAg (32/37; 86%) and negative anti-HBe (33/36; 92%) than those with genotype D (18/29 [62%] and 21/29 [72%], respectively; $p<0.05$ each). Of 37 patients with genotype A, 23 (62%) had cirrhosis and 14 (38%) had chronic hepatitis; of 32 patients with genotype D, 15 (47%) had cirrhosis and 17 (53%) had chronic hepatitis (p=ns). In the subgroup aged $\geq$25 years, genotype A patients more often had cirrhosis than those with genotype D (23/28 [82%] vs 13/23 [57%]; $p<0.05$). **Conclusion:** HBV genotypes A and D were the commonest in our population. Genotype A was more often associated with ALT elevation, HBeAg positivity, absence of anti-HBe and, among those aged 25 years and above, cirrhosis of liver, than was genotype D. [*Indian J Gastroenterol* 2005;24:19-22]

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Based on genomic sequence data, hepatitis B virus (HBV) is classified into eight genotypes (A-H).[1] Preliminary data suggest that clinical outcome of HBV infection may depend on the genotype of the infecting virus. Most studies from Asia report preponderance of genotypes B and C;[2,3,4] of these, genotype C was found to be associated with a more severe liver disease than genotype B. In contrast, in studies from Europe and North America, where genotypes A and D are common,[5,6,7] the relationship of genotype with clinical outcome remains unclear. Limited data are available from India on HBV genotypes.[8,9,10] These suggest that genotypes A and D are the most prevalent, but their relationship to severity of disease is not known.

We determined the HBV genotypes in patients with chronic liver disease in northern India and assessed their clinical significance, if any.

Methods

Patients with HBV-related chronic liver disease attending the outpatient and inpatient services of Department of Gastroenterology at our institution during the years 2000 to 2003, and testing positive for HBV DNA were studied retrospectively. Clinical data were retrieved from medical records, as were laboratory test results including hemogram, liver function tests, coagulation profile, and findings at abdominal ultrasonography, upper gastrointestinal endoscopy and liver biopsy. Liver cirrhosis was diagnosed either on histology, or on a combination of radiological, endoscopic and laboratory data. Serum specimens from the study patients that had been stored at –80°C since the time of initial evaluation were used for genotype analysis.

HBV genotyping

HBV genotyping was performed using amplification of a segment of the HBV surface gene by polymerase chain reaction (PCR), followed by restriction fragment length polymorphism (RFLP).[11] In brief, DNA was extracted from 200 µL of serum using the *QIAamp* blood kit (Qiagen; Valencia, CA, USA) according to the manufacturer's instructions. A 541-basepair DNA fragment located between nucleotides 256 and 796 of the HBV genome was amplified by PCR. The amplified product was digested using restriction enzymes *Tsp509I* or *HifI* (New England Biolabs, Beverly, MA, USA). The resulting DNA fragments were separated using 8% polyacrylamide (SRL, Mumbai) gel electrophoresis, and the restriction patterns were read under ultraviolet light after staining the gel with ethidium bromide.

In 14 selected patients, the results of RFLP were confirmed by direct sequencing of the PCR products using an automated DNA sequencer. The sequences

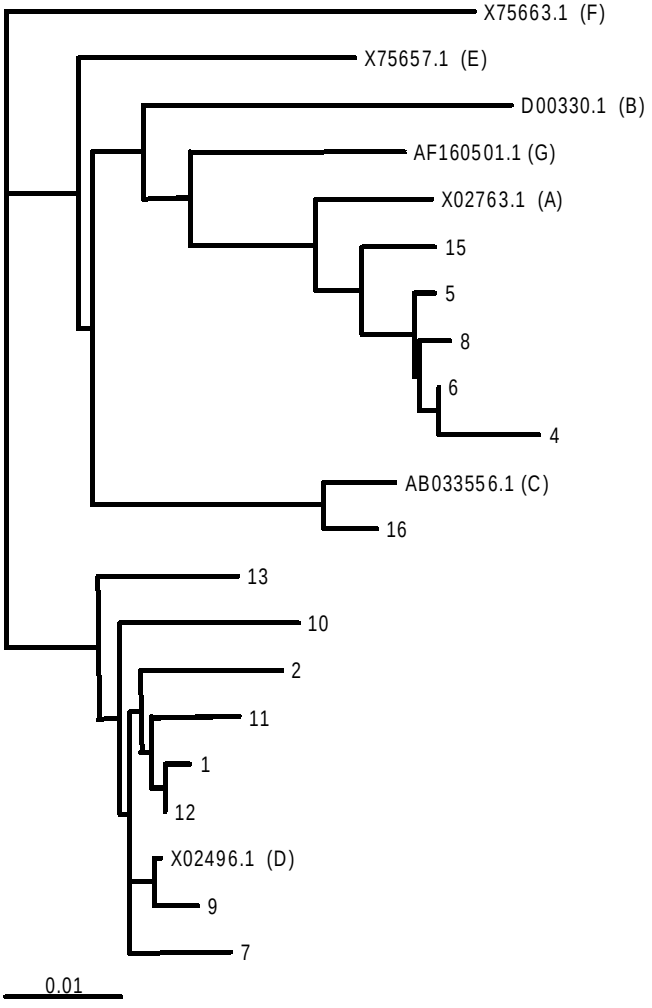

Fig: Phylogenetic tree showing genotype assignment of 14 isolates based on DNA sequencing of the PCR product. The sequences were compared with 7 prototype sequences of HBV genotypes A-G obtained from GenBank; these sequences are shown with their GenBank accession numbers, followed by their genotype in parenthesis. The phylogenetic analysis confirms the genotype assignments made using RFLP analysis (genotype A: isolates 4, 5, 6, 8 and 15; genotype D: 1, 2, 7, 9, 10, 11, 12, 13; and, genotype C: 16)

obtained were aligned with known sequences of different HBV genotypes obtained from GenBank (accession numbers X02763, D00330, ABO33556, X02496, X75657, X75663, and AF160501 for genotypes A-G, respectively) using a biological sequence comparison software *BioEdit* (version 5.0.6).

Statistical analysis

Intergroup comparisons were done using chi-squared test, Fisher's exact probability test and Mann-Whitney U test. Two-tailed p values below 0.05 were considered significant.

Results

Seventy patients were included in the study. Their baseline characteristics are shown in the Table. Most had HBV genotype A (37/70 [53%]) or D (32/70 [46%]); one patient had HBV genotype C. RFLP patterns suggestive of genotypes B, E, F, G and H were not observed in any patient.

Table: Baseline characteristics of patients studied

Total patients	70
Mean age (years)	38.4 (SD 17)
Gender	M 63, F 7 (9:1)
Median ALT (IU/L) (range)	92 (10-1168)
Median AST (IU/L) (range)	59 (12-769)
Median bilirubin (mg/dL) (range)	0.8 (0.2-20.9)
Mean albumin (g/dL) (n=69)	3.4 (SD 0.9)
Mean prothrombin time (s) (n=60)	15.8 (SD 4.2); control 11.8
Mean platelet ($x10^3/\mu L$) (n=61)	121 (SD 66)
HBeAg (n=67)	
Positive	50 (71%)
Negative	17 (24%)
Anti-HBe (n=66)	
Positive	12 (17%)
Negative	54 (77%)
Clinical manifestations	
Chronic hepatitis	32 (46%)
Cirrhosis	38 (54%)
Child-Pugh class* (n=37)	
A	12 (32%)
B	12 (32%)
C	13 (35%)

*Only patients with cirrhosis; in one patient, inadequate data precluded classification

Assignment of genotypes based on genomic sequencing in all the 14 isolates for which PCR products were sequenced was identical to that using RFLP analysis (genotype A in 5 patients, D in 8 patients, and genotype C in one patient) (Fig).

Mean ALT level in our patients was 140 (SD 177) U/L. Patients were divided into two groups based on ALT levels below and above 1.5 times the upper limit of normal (normal <40 U/L). Patients with genotype A more often had ALT elevation exceeding 1.5 times normal (30/37 [81%]) than did those with genotype D (18/31 [58%]; $p<0.05$). Genotype A patients also more often had positive HBeAg (32/37; 86%) and negative anti-HBe (33/36; 92%) than did those with genotype D (18/29 [62%] and 21/29 [72%], respectively; $p<0.05$ each).

Of the 37 patients with genotype A, 23 (62%) had cirrhosis and 14 (38%) had chronic hepatitis; in comparison, of the 32 patients with genotype D, 15 (47%) had cirrhosis and 17 (53%) had chronic hepatitis (p=ns). A subgroup analysis for patients aged 25 years and older showed that genotype A was more often associated with cirrhosis than was genotype D (23/28 [82%] vs 13/23 [57%]; $p<0.05$).

Serum bilirubin, AST, albumin and prothrombin values were similar in patients with genotypes A and D. Similarly, the distribution of Child-Pugh classes A, B and C

was similar in patients with cirrhosis infected with these two genotypes.

Discussion

Our patients with chronic HBV infection had predominantly genotypes A and D, in almost equal proportions. Till date, only three studies have been published from India on the frequency of various HBV genotypes.[8,9,10] Thakur *et al*[8] found nearly equal proportion of genotypes A and D, similar to our results. The other two studies[9,10] found genotype D to be the predominant genotype, with genotype A being responsible for only 5%-8% of patients. Further, we found HBV genotype C in one of our patients; this genotype has not previously been reported from India.

HBV genotypes have distinct geographical distribution. Genotypes B and C are prevalent in the Far East and in South-East Asia,[2,3,4] where HBV infection is highly endemic and perinatal or vertical transmission is the predominant mode of transmission. In contrast, genotypes A and D are commoner in Western Europe and North America.[5,6,7] In the Mediterranean region,[12] the Middle East[13] and Central Asia,[14] genotype D is dominant. HBV strains in India may therefore have originated from those in Europe or vice versa. Genetic studies of mitochondrial DNA and Y chromosome in the Indian population attest to the presence of significant European admixture, which is more marked in northern than in southern India.[15]

The variation in relative frequency of HBV genotypes in studies from India may reflect geographical variations. The study by Thakur *et al*[8] and our current study, both of which are from northern India, have found high proportions of genotypes A and D. In comparison, the other two studies, one from western India and the other from the Andaman and Nicobar Islands, have reported predominance of genotype D. 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 human migration from Europe over time.

In hepatitis C virus infection, viral genotypes have been related to clinical illness and response to therapy. However, in the case of HBV infection, data on relationship of clinical illness with viral genotype are limited. Most of these have compared genotypes B and C in South-East Asian countries where genotypes A and D are rare.

We found an association between genotype A and higher ALT levels, indicating greater necro-inflammation, as compared to genotype D. This inflammation may be a result of immune injury secondary to higher rate of viral replication. This is supported by our observation that genotype A was associated with positive HBeAg status, a marker of active replication. No previous large comparison of HBeAg status between genotypes A and D has been reported. In a study of 54 patients from Uzbekistan,[14] no difference in HBeAg status or ALT levels was found between genotypes A and D. In two large studies from Japan[16] and USA,[17] that included 1077 and 694 patients, respectively, HBeAg positivity was more often associated with genotype A than with other genotypes. In another study of 413 patients from Japan that compared genotypes D and C, Duong *et al*[18] showed that HBeAg/anti-HBe ratio was lower in genotype D as compared to genotype C. There are several studies comparing HBeAg status between genotypes B and C; most of these found higher HBeAg positivity rate with genotype C.[4,19,20,21] This suggests that HBV genotypes may have clinical significance; however, pathogenetic mechanisms contributing to the differences in disease between HBV genotypes remain to be clarified.

Seroconversion from HBeAg positive state to anti-HBe positivity early in the course of chronic HBV infection is associated with cessation of virus replication and reduction in the rate of progression of liver disease. In contrast, late HBeAg seroconversion after multiple episodes of reactivation and remission may lead to progression of liver injury to cirrhosis and decompensation. The association in our patients of HBV genotype A with higher ALT levels, a higher HBeAg positivity rate and presence of cirrhosis suggests that this genotype may be associated with a higher risk of progression of disease to cirrhosis of the liver. This progression may be mediated by greater necro-inflammation associated with this genotype.

Our study has some limitations. These include its retrospective nature and small sample size. Also, we used a method based on only a part of, and not the entire, HBV genome. However, this RFLP method has previously been shown to be reliable for HBV genotyping;[11] further, the results of our sequencing and phylogenetic analysis confirmed the validity of genotype assignment done using this RFLP method.

In summary, our results indicate that HBV genotype A is associated with more marked ALT elevation, a higher rate of HBeAg positivity and presence of liver cirrhosis in our population.

References

1. Kao JH, Chen PJ, Lai MY, Chen DS. Hepatitis B virus genotypes and spontaneous hepatitis B e antigen seroconversion in Taiwanese hepatitis B carriers. *J Med Virol* 2004;72:363-9.
2. Nakayoshi T, Maeshiro T, Nakayoshi T, Nakasone H, Sakugawa H, Kinjo F, *et al*. Difference in prognosis between patients infected with hepatitis B virus with genotype B and those with genotype C in the Okinawa Islands: a prospective study. *J Med Virol* 2003;70:350-4.

3. Chan HL, Wong ML, Hui AY, Hung LC, Chan FK, Sung JJ. Hepatitis B virus genotype C takes a more aggressive disease course than hepatitis B virus genotype B in hepatitis B e antigen-positive patients. *J Clin Microbiol* 2003;41:1277-9.
4. Sumi H, Yokosuka O, Seki N, Arai M, Imazeki F, Kurihara T, *et al*. Influence of hepatitis B virus genotypes on the progression of chronic type B liver disease. *Hepatology* 2003;37:19-26.
5. Sanchez-Tapias JM, Costa J, Mas A, Bruguera M, Rodes J. Influence of hepatitis B virus genotype on the long-term outcome of chronic hepatitis B in Western patients. *Gastroenterology* 2002;123:1848-56.
6. Mayerat C, Mantegani A, Frei PC. Does hepatitis B virus (HBV) genotype influence the clinical outcome of HBV infection? *J Viral Hepat* 1999;6:299-304.
7. Moriya T, Kuramoto IK, Yoshizawa H, Holland PV. Distribution of hepatitis B virus genotypes among American blood donors determined with a preS2 epitope enzyme-linked immunosorbent assay kit. *J Clin Microbiol* 2002;40:877-80.
8. Thakur V, Guptan RC, Kazim SN, Malhotra V, Sarin SK. Profile, spectrum and significance of HBV genotypes in chronic liver disease patients in the Indian subcontinent. *J Gastroenterol Hepatol* 2002;17:165-70.
9. Gandhe SS, Chadha MS, Arankalle VA. Hepatitis B virus genotypes and serotypes in western India: lack of clinical significance. *J Med Virol* 2003;69:324-30.
10. Arankalle VA, Murhekar KM, Gandhe SS, Murhekar MV, Ramdasi AY, Padbidri VS, *et al*. Hepatitis B virus: predominance of genotype D in primitive tribes of the Andaman and Nicobar islands, India (1989-1999). *J Gen Virol* 2003;84:1915-20.
11. Lindh M, Andersson AS, Gusdal A. Genotypes, nt 1858 variants, and geographic origin of hepatitis B virus — large-scale analysis using a new genotyping method. *J Infect Dis* 1997;175:1285-93.
12. Yalcin K, Degertekin H, Bahcecioglu IH, Demir A, Aladag M, Yildirim B, *et al*. Hepatitis B virus genotype D prevails in patients with persistently elevated or normal ALT levels in Turkey. *Infection* 2004;32:24-9.
13. Sallam TA, William Tong CY. African links and hepatitis B virus genotypes in the Republic of Yemen. *J Med Virol* 2004;73:23-8.
14. Kato H, Ruzibakiev R, Yuldasheva N, Hegay T, Kurbanov F, Achundjanov B, *et al*. Hepatitis B virus genotypes in Uzbekistan and validity of two different systems for genotyping. *J Med Virol* 2002;67:477-83.
15. Bamshad M, Kivisild T, Watkins WS, Dixon ME, Ricker CE, Rao BB, *et al*. Genetic evidence on the origins of Indian caste populations. *Genome Res* 2001;11:994-1004.
16. Kobayashi M, Arase Y, Ikeda K, Tsubota A, Suzuki Y, Saitoh S, *et al*. Clinical characteristics of patients infected with hepatitis B virus genotypes A, B, and C. *J Gastroenterol* 2002;37:35-9.
17. Chu CJ, Keeffe EB, Han SH, Perrillo RP, Min AD, Soldevila-Pico C, *et al*. Hepatitis B virus genotypes in the United States: results of a nationwide study. *Gastroenterology* 2003;125:444-51.
18. Duong TN, Horiike N, Michitaka K, Yan C, Mizokami M, Tanaka Y, *et al*. Comparison of genotypes C and D of the hepatitis B virus in Japan: a clinical and molecular biological study. *J Med Virol* 2004;72:551-7.
19. Yuen MF, Sablon E, Yuan HJ, Wong DK, Hui CK, Wong BC, *et al*. Significance of hepatitis B genotype in acute exacerbation, HBeAg seroconversion, cirrhosis-related complications, and hepatocellular carcinoma. *Hepatology* 2003;37:562-7.
20. Chu CJ, Hussain M, Lok AS. Hepatitis B virus genotype B is associated with earlier HBeAg seroconversion compared with hepatitis B virus genotype C. *Gastroenterology* 2002;122:1756-62.
21. Kao JH, Chen PJ, Lai MY, Chen DS. Genotypes and clinical phenotypes of hepatitis B virus in patients with chronic hepatitis B virus infection. *J Clin Microbiol* 2002;40:1207-9.

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