Turk J Gastroenterol 2005; 16 (4): 183-187

Determination of hepatitis B genotypes in patients with chronic hepatitis B virus infection in Turkey

Türkiye'deki kronik hepatit B virüs enfeksiyonlu hastalarda hepatit B virüs genotiplerinin belirlenmesi

Filiz Türe ÖZDEMİR[1], Deniz DUMAN[2], Deniz ERTEM[3], Erol AVŞAR[1-2], Fatih EREN[1], Osman ÖZDOĞAN[2], Cem KALAYCI[2], Nuray ASLAN[4], Ali Mithat BOZDAYI[4], Nurdan TÖZÜN[1-2]

Institute of [1]Gastroenterology, Department of [2]Gastroenterology and [3]Pediatric Gastroenterology, Marmara University, İstanbul

Institute of [4]Hepatoloji, Ankara University, Ankara

Background/aims: *There are significant variations in the geographic distribution of hepatitis B virus genotypes throughout the world, and some genotypes are associated with different clinical outcomes. Eight genotypes of human hepatitis B virus (designated A-H) have been described to date. To determine the hepatitis B virus genotypes in Turkish patients with chronic liver disease and compare the results with clinical characteristics of the patients.* ***Methods:*** *Fifty-four (pediatric: n=25 and adult: n=29) patients with chronic hepatitis B virus infection and with an hepatitis B virus DNA level above 5 pg/ml were entered into the trial. Restriction fragment length polymorphism method was used to determine hepatitis B virus genotype and their restriction fragment length polymorphism patterns. Hepatitis B virus DNA samples of 13 patients were sequenced automatically for further confirmation of restriction fragment length polymorphism results.* ***Results:*** *Genotype D was the dominant genotype in all of our cases. Among six restriction fragment length polymorphism patterns of genotype D reported in the literature, three (D1, D2, D6) were present in our series and D2 was the most frequent restriction fragment length polymorphism pattern (81.5%). No significant differences were observed among different genotype D restriction fragment length polymorphism patterns with respect to patients' serum ALT, AST, and hepatitis B virus DNA titer, but D2 restriction fragment length polymorphism pattern was significantly more common in younger adults compared to D1 restriction fragment length polymorphism pattern.* ***Conclusion:*** *Genotype D with D2 restriction fragment length polymorphism pattern is the dominant hepatitis B virus genotype in all age groups in Turkey.*

Key words: Hepatitis B virus, genotype, chronic hepatitis, PCR-RFLP, RFLP pattern

Amaç: *Dünya üzerinde hepatit B virüs genotiplerinin coğrafi dağılımı belirgin farklılıklar gösterir ve bu genotiplerin bazılarına eşlik eden özel klinik tablolar izlenebilir. Bugüne kadar insan hepatit B virüslerinde 8 farklı genotip (A'dan H'a kadar) tanımlanmıştır. Kronik karaciğer hastalığı olan Türk hastalarda hepatit B virüs genotiplerini belirlemek ve sonuçları hastaların klinik özellikleri ile karşılaştırmaktır.* ***Yöntem:*** *Kronik hepatit B virüs enfeksiyonlu ve hepatit B virüs DNA değerleri 5 pg/ml üzerinde olan 54 (25 çocuk ve 29 erişkin) hasta çalışmaya alındı. Hepatit B virüs genotiplerini ve «Restriction Fragment Length Polymorphism» paternlerini saptamak için «Restriction Fragment Length Polymorphism» yöntemi uygulandı. Hepatit B virüs DNA örneklerinin «Restriction Fragment Length Polymorphism» sonuçlarını teyit etmek amacıyla otomatik dizi analizleri yapıldı.* ***Bulgular:*** *Hastaların tümünde genotip D mevcudiyeti saptandı. Serimizde, genotip D'ye ait daha önce literatürde belirlenmiş olan 6 farklı «Restriction Fragment Length Polymorphism» paternlerinden sadece 3 tanesi gösterildi ve bunlardan en yaygın olanı «D2 Restriction Fragment Length Polymorphism» paterni idi (%81.5). Değişik genotip D «Restriction Fragment Length Polymorphism» paternlerine ait hastaların serum ALT, AST ve hepatit B virüs DNA düzeyleri karşılaştırıldığında aralarında anlamlı farklılık bulunmadı ancak «D2 Restriction Fragment Length Polymorphism» paterni «D1 Restriction Fragment Length Polymorphism» paternine kıyasla genç olgularda anlamlı olarak daha sık izlendi.* ***Sonuç:*** *Türkiye'de hepatit B virüs genotiplerinin dağılımı incelendiğinde, tüm yaş gruplarında D genotipinin ve bu genotipe ait «Restriction Fragment Length Polymorphism» paternlerden «D2 Restriction Fragment Length Polymorphism» paterninin baskın olduğu bulunmuştur.*

Anahtar kelimeler: Hepatit B virüsü, genotip, kronik hepatit, PCR-RFLP, RFLP paterni

INTRODUCTION

Chronic hepatitis B virus (HBV) infection is a widespread disease affecting more than 5% of the world's population. HBV is well known to have a wide genetic heterogeneity (1). In 1988, Okamoto et al. first defined four genotypes (A-D) based on a divergence of 8% or more of the complete HBV

Address for correspondence: Filiz TÜRE ÖZDEMİR
Institute of Gastroenterology, Marmara University, PK: 53 Maltepe, İstanbul, Turkey
Phone: +90 216 305 32 03 • Fax: +90 216 399 99 12
E-mail: filizture07@hotmail.com

Manuscript received: 21.03.2005 **Accepted:** 14.06.2005

genome (2). In 1992, Norder et al. compared S-gene sequences of the HBV genomes formerly classified by Okamoto and they showed that the smallest differences of S gene sequences between genomes were 4.1%. Thereafter, two new genotypes designated as E and F were defined using at least 4% differences at the level of S-gene (3), followed by G and H recently reported from Belgium and Central America (4,5). Thus, eight genotypes of HBV (A-H) in total have been defined to date.

Genotype A prevails mainly in North America, northern Europe, India, and Africa; genotype B and C in Asia; genotype D in southern Europe, the Middle East, and India; genotype E in West and South Africa; genotype F in South and Central America; genotype G in the United States and Europe; and genotype H in Central America and California (6).

HBV genotypes may have different pathogenesis according to their geographic distribution and they seem to play a role in disease behavior and outcome. Various studies have found an association between genotypes and disease severity as well as response to antiviral treatment (7-9).

The well-known variations in HBV genotype in different geographic areas of the world prompted us to investigate the situation in the Turkish population, especially in groups of children, which has been under-reported when compared to adults, and the correlation of the genotype with disease characteristics.

MATERIALS AND METHODS

Fifty-four patients with HBV DNA levels greater than 5 pg/ml as determined using a commercially available liquid-hybridization assay (Digene, USA) were studied. The study protocol was approved by the local ethics committee and informed consent was obtained from each adult patient and parents of the pediatric patients. There were 36 males and 18 females, aged 2 to 65. All patients were seronegative for hepatitis C virus.

HBV DNA was extracted from the serum samples by the standard methods and genotypes were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Serum samples were stored at -80°C until studied.

The segment of the HBV genome between nt 256 and 796 was amplified for genotyping. Sera were prepared with NaOH as previously described (10) or with proteinase K, phenol/chloroform method.

Ten microliters of the mixture from either preparation was used for PCR in a reaction volume of 50 µl (11). Primers were sense: 5'GTGGTGGACTTCTCTCAATTTTC, and antisense: 5'CGGTA(A/T)AAAGGGACTCA(A/C)AT. After an initial 3 min denaturation at 94°C, 40 cycles of amplification, including denaturation for 45 s at 94°C, annealing for 60 s at 53°C and extension for 90 s at 72°C were done and followed by a final 7 min extension at 72°C. Samples of DNA were run on 1% standard agarose gel.

PCR product (10 µl) was mixed with 1 µl (5U) of Tsp EI (MBI Fermentas), 1.5 µl of 10X buffer and 2.5 µl of water and then incubated at 65°C for 3 h. In a separate reaction, PCR product (10 µl) was mixed with 0.5 µl (5U) of HinfI (MBI Fermentas), 1.5 µl of 10X buffer and 3 µl of water and incubated at 37°C for 3 h. After incubation, the samples were run on a mixed gel containing 2% NuSieve agarose (MBI Fermentas) and 1% standard agarose. DNA was visualized by ethidium bromide staining. The restriction patterns were read visually (11) (Table 1).

Differences of S gene nucleotides (nt 256-296) between patterns were determined by automatic

Table 1. Restriction points of enzymes

Ptn.	Restriction points with HinfI*					Length of bp				Restriction points with Tsp*					Length of bp					
	379	481	529	614	633					354	480	589	633	686						
D1						526	15				X	X	X		173	164	109	43	36	16
											X	X	X							
D2			X			274	252	15				X	X		173	164	109	43	36	16
D6		X	X			252	226	48	15						282	164	43	36	16	

*Number of nucleotides, Ptn: Pattern, bp: Base pairs

DNA sequencer (ABI 310 system, Perken Elmer, USA). The nucleotide sequences of each pattern were obtained from the literature and confirmed by comparing the specific sequences in gene bank. Phylogenetic comparison was done by distance matrix/UPGMA analysis using Kimura 2-parameter by MEGA2 software package program (Figure 1) (12).

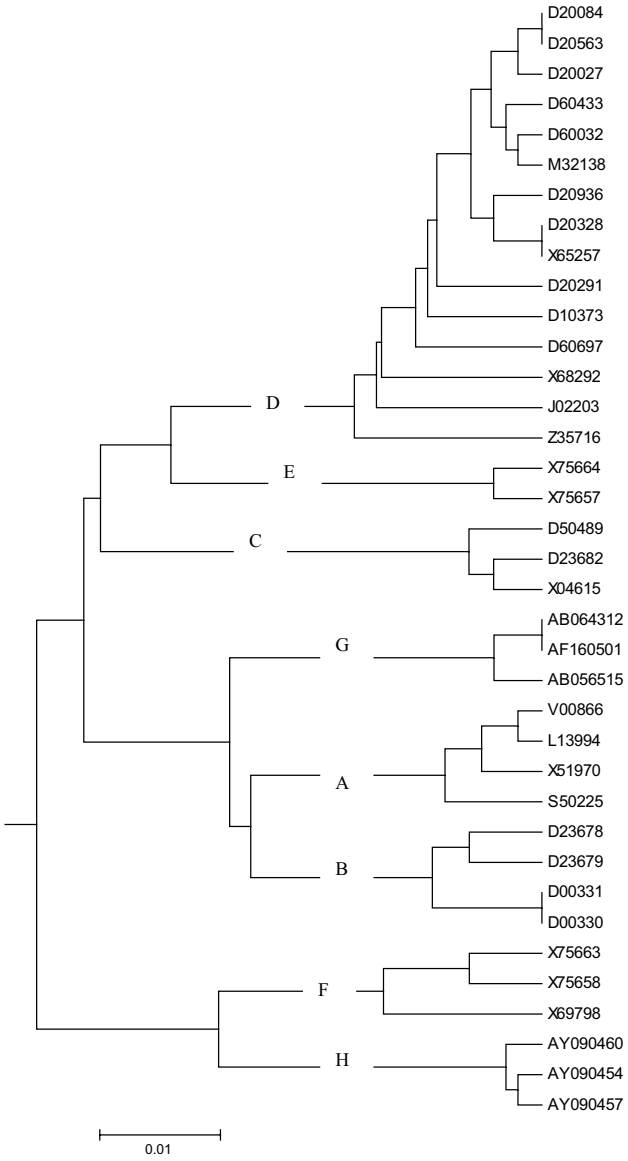

Figure 1. Phylogenetic tree obtained by distance matrix/UPGMA comparison (with Kimura-2 correction) after bootstrapping 1000 replicates of sequence segment from the S region of HBV (nt 468-718), (D20084, D20563, D20027, D60433, D60032, D20936, D20328, D20291, D10373, D60697 are our patients, the others are from the gene bank). RFLP: Restriction fragment length polymorphism

Statistical Analysis

Student's *t*-test was used for the comparison of means within groups. Correlation between continuous variables was analyzed using Spearman's test. P values less than .05 were considered significant.

RESULTS

Overall 25 pediatric (16 male, 9 female) and 29 adult (20 male, 9 female) patients were enrolled into the study. The mean ages of children and adults were 8.6 ± 3.46 (2-17) years and 39.2 ± 13.83 (20-65) years, respectively. Demographic and clinical data are summarized in (Table 2).

Table 2. Demographic and biochemical data of chronic hepatitis B patients

Characteristic	Children	Adults
Age (year)	8.88±3.7 (2-18)	40±14.12 (20-65)
Sex (F/M)	9/16	9/20
ALT (U/L)	77.8±51.57 (15-224)	121.75±116.46 (35-549)
AST (U/L)	104.56±63.66 (15-256)	95.21±95.4 (21-475)
ALP (U/L)	369.83±209.49 (119-1000)	167.19±80.43 (44-375)
GGT (U/L)	24.75±35.3 (7-171)	41.75±16.85 (12-72)
Total protein (g/dl)	7.13±0.53 (6.1-8.2)	7.6±1.19 (3.7-8.6)
Albumin (g/dl)	4.06±0.31 (3.4-4.5)	4.13±0.77 (1.8-5)
HBV DNA (pg/ml)	3026.44±1463.53 (79-5008)	2421.7±1884.6 (7-7972)

Data presented as mean±SD (range) or number of patients

All patients were HBsAg positive. HBeAg positivity was more prevalent in children than adults (24/25 vs 10/29, respectively) ($p<0.001$). Furthermore, children had significantly higher HBV DNA values than adults [3026.44±1463.53 (79-5880) vs. 2421.7±1884.6 (7-7972) pg/ml; $p=0.03$]. Thirty-four of 54 patients had a liver biopsy and 30 had chronic hepatitis and two had cirrhosis. Sixteen out of 34 cases were children. In 24 of 54 patients (44.44%), there was a family history of HBV infection.

Regarding HBV genotypes, genotype D was predominant in all of our patients. Among six different RFLP patterns of genotype D described in the literature, only D1, D2 and D6 were observed in our study, and D2 was the most frequent RFLP pattern. There was no significant dissociation among RFLP patterns in adults and children apart from

D6, which was only found in adults (Table 3). D2 RFLP pattern was more common in younger patients compared with D1 (D2, 36.7±14.2 years vs. D1, 53.2±9.6 years, p=0.028), but no significant differences were observed among different genotype D RFLP patterns with respect to patients' serum ALT, AST, HBV DNA titer, HBeAg status and histologic data.

Table 3. Dissociation of patterns

RFLP Patterns of Genotype D				
Group	D1 (%)	D2 (%)	D6 (%)	Total
Adult	5	21	3	29
Children	2	23	-	25
Total	7 (13)	44 (81.5)	3 (5.5)	54

When the past medical histories of the patients were reviewed, six of 14 children who received interferon (IFN)-α had no response to treatment and seven of 18 adults who received IFN-α experienced a relapse and were switched to lamivudine therapy by the time the current study was conducted.

DISCUSSION

We found that a single genotype, namely genotype D, was prevalent in the entire study population. Most common RFLP patterns were D1, D2 and D6. This is the first study evidencing HBV genotypes in children and also demonstrating those three RFLP patterns of genotype D in Turkey.

Sequencing of viral genomes has become a major goal of descriptive virology, and the data is used to trace routes of infection, to reconstruct the phylogenetic history of viruses and to delineate genetic subtypes. HBV is a typical example of a virus that attracts attention with its different genotypes, showing special geographic distribution around the world. A genetic classification based on the comparison of complete genomes has defined eight genotypes of HBV, which were designated from A through H (6).

Genotype D appears to predominate in the Mediterranean basin and the Middle East, and this is consistent with Turkey's geographical location in the world as a bridge between Southeast Europe and Asia. Our findings are consistent with the previous data from Turkey (13-16).

In our study, there was a family history of HBV infection in 44.4% of all patients. Recent data supported that the transmission was more likely to be via horizontal route in patients infected with genotype D in France, where genotypes A and D were more prevalent (17). In contrast, in the United States, genotype D was related to all modes of infection (18).

In the current study, as might be expected, almost all of our pediatric patients were positive for HBeAg. However, the majority of our adult patients were anti-HBe positive. Similarly, in French patients, genotype D was predominant in anti-HBe-positive patients with chronic hepatitis (16).

Genotype D was reported to be associated with a poor response to antiviral therapy (9). In our series, it was not possible to comment about the effect of genotype on the response to antiviral treatment since this was not a longitudinal study.

Lindh et al. were able to show different RFLP patterns of genotypes in their series using computer analysis, but they could not explain the correlations between these patterns and clinical outcomes (11). In our study, no significant correlation was found between genotype D RFLP patterns and liver function tests, the presence of cirrhosis, clinical course, HBeAg and anti-HBe status and the viral load; however, the number of patients for each pattern was insufficient to reach firm conclusions.

In conclusion, genotype D with D2 RFLP pattern is the dominant HBV genotype in Turkey in all age groups. Further studies in patients chronically infected with HBV are warranted to delineate the influences of different genotype D RFLP patterns on the disease behavior and response to antiviral therapy in our country.

ACKNOWLEDGEMENTS

This study was supported by grants from the Marmara University Research Fund.

REFERENCES

1. Blumberg BS, Alter HJ, Visnich S. A new antigen in leukemia sera. JAMA 1965; 191: 541.
2. Okamoto H, Tsuda F, Sakugawa H, et al. Typing hepatitis B virus by homology in nucleotide sequence: Comparison of surface antigen subtypes. J Gen Virol 1988; 69: 2575-83.
3. Norder H, Hammas B, Löfdahl S, et al. Comparison of the amino acid sequences of nine different serotypes of hepatitis B surface antigen and genomic classification of the corresponding hepatitis B virus strains. J Gen Virol 1992; 73: 1201-8.

4. Stuyver L, De Gendt S, Van Geyt C, et al. A new genotype of hepatitis B virus: Complete genome and phylogenetic relatedness. J Gen Virol 2000; 81: 67-74.
5. Arauz-Ruiz P, Norder H, Robertson BH, et al. Genotypes H: a new Amerindian genotype of hepatitis B virus revealed in Central America. J Gen Virol 2002; 83: 2059-73.
6. Keeffe EB, Dieterich DT, Han SHB, et al. A treatment algorithm for the management of chronic hepatitis B virus infection in the United States. Clin Gastroenterol Hepatol 2004; 2(2): 87-106.
7. Kao JH, Chen PJ, Lai MY, et al. Hepatitis B genotypes correlate with clinical outcomes in patients with chronic hepatitis B. Gastroenterology 2000; 118: 554-9.
8. Chu CJ, Lok AS. Clinical significance of hepatitis B virus genotypes. Hepatology 2002; 35: 1274-6.
9. Conjeevaram HS, Lok ASF. Management of chronic hepatitis B. J Hepatology 2003; 38: 90-103.
10. Lok ASF, Akarca U, Greene S. Mutations in the precore region of hepatitis B virus serve to enhance the stability of the secondary structure of the pre-genome encapsidation signal. Proc Natl Acad Sci 1994; 91: 4077-81.
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 Inf Dis 1997; 175: 1285-93.
12. Kumar S, Tamura K, Jakobsen IB, et al. MEGA2: molecular evolutionary genetics analysis software. Bioinformatics 2001; 17: 1244-5.
13. Bozdayı AM, Bozkaya H, Uzunalimoglu O, et al. Nucleotide divergences in the core promoter and precore region of genotype D hepatitis B virus in patients with persistently elevated or normal ALT levels. J Clin Virol 2001; 21: 91-101.
14. Bozdayı AM, Aslan N, Bozdayı G, et al. Molecular epidemiology of hepatitis B, C and D viruses in Turkish patients. Arch Virol 2004; 149(11): 2115-29.
15. Yalcın K, Degertekin H, Bahcecioglu IH, et al. Hepatitis B virus genotype D prevails in patients with persistently elevated or normal ALT levels in Turkey. Infection 2004; 32: 24-9.
16. Leblebicioglu H, Eroglu C, Members of the Hepatitis Study Group. Acute hepatitis B virus infection in Turkey: epidemiology and genotype distribution. Clin Microbiol Infect 2004; 10(6): 537-41.
17. Grandjacques C, Pradat P, Stuyver L, et al. Rapid detection of genotypes and mutations in the pre-core promoter and the pre-core region of hepatitis B virus genome: Correlation with viral persistence and disease severity. J Hepatology 2000; 33: 430-9.
18. Chu CJ, Kneeffe EB, Han SH, et al. Hepatitis B virus genotypes in the United States: Results of a Nationwide Study. Gastroenterology 2003; 125: 444-51.