Hepatitis B virus genotypes and subgenotypes in the Eastern Black Sea region of Turkey

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ABSTRACT

الأهداف: تحديد الأنماط الجينية والأنماط الجينية الفرعية لفيروس التهاب الكبد ب لدى المرضى المصابين به في منطقة البحر الأسود الغربية في تركيا.

الطريقة: شملت الدراسة 137 عينة جُمعت على مدى 5 سنوات (من يناير 2005م إلى يناير 2010م) وذلك في مستشفى فارابي التابع لجامعة كارادينيز التقنية، ترابزون، تركيا. لقد كان تحليل عينات المرضى إيجابياً من حيث ظهور مستضد التهاب الكبد السطحي وظهور الحمض النووي لفيروس التهاب الكبد ب. لقد قمنا بتحليل وتحديد الأنماط الجينية لفيروس التهاب الكبد ب باستخدام التفاعل التسلسلي المبلمر لأطوال المادة الوراثية المجزأة وذلك بواسطة جزء مضخم من منطقة الفيروس pre–S.

النتائج: لقد قمنا بتحديد النمط الجيني د في 125 عينة من أصل 137 عينة مصابة بالتهاب الكبد ب (91.3%) وذلك باستخدام التفاعل التسلسلي المبلمر لأطوال المادة الوراثية المجزأة. فيما لم يتم تحديد الأنماط الجينية في 12 عينة معزولة. ولقد كان النمط الجيني الفرعي لحوالي 122 عينة من أصل 125 (97.6%) د1، فيما كان النمط الجيني الفرعي لعينتين (1.6%)، وكان كان النمط الجيني الفرعي لعينة واحدة (0.8%) د–ديل.

خاتمة: أظهرت هذه الدراسة بأن نوع النمط الجيني السائد في غالبية العينات المعزولة من فيروس التهاب الكبد ب في منطقة البحر الأسود الغربية قد كان النمط الجيني د، والنمط الجيني الفرعي د2، ونتائج الدراسة كانت مشابهة للدراسات التي أُجريت على عينات المرضى في باقي أجزاء تركيا.

Objectives: To determine the hepatitis B virus (HBV) genotypes and subgenotypes in patients with HBV infection in the Eastern Black Sea region of Turkey.

Methods: One hundred and thirty-seven patients' samples collected over 5 years (January 2005 to January 2010) at Farabi Hospital in Karadeniz Technical University, Trabzon, Turkey were included in the study. All patients were positive for hepatitis B surface antigen (HBsAg) and HBV DNA. The HBV genotypes were determined by the polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) method using an amplified segment of the pre-S region of HBV.

Results: One hundred and twenty-five of the 137 HBV samples (91.3%) were identified as genotype D using the PCR-RFLP method. Twelve isolates had undefined patterns, 122 of the 125 samples (97.6%) were determined as subgenotype D2, 2 (1.6%) were subgenotype D1, and one (0.8%) was subgenotype D-del.

Conclusions: Similar findings in the other parts of the Turkey, the predominant patterns of HBV prevailing among patients in the Eastern Black Sea region of Turkey were of genotype D and subgenotype D2.

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Hepatitis B virus (HBV) infection remains a worldwide health problem. Around one-third of the world's population has already been infected with HBV. Diseases of chronic HBV infection range from low-viremic inactive carrier state to progressive chronic hepatitis, which may evolve to cirrhosis and hepatocellular carcinoma (HCC).[1] It has been suggested that some viral factors may correlate with differences in clinical features of HBV infection such as viral load, viral mutations, and HBV genotypes.[2-4] At least 10 HBV genotypes (from A to J) have been described based on greater than 8% sequence divergence in the entire genome of HBV. Some genotypes are divided into subgenotypes with a difference ranging from 4-8% in their nucleotide sequences.[5-8] Recent data suggests that some HBV genotypes correlate with severe liver disease or better response to antiviral treatment. For example, HBV genotype C is thought to be associated with increased risk of HCC development, while HBV genotype A or B have a better response to interferon (IFN)-based treatment than those infected with HBV genotype C or D. Compared to genotype A and B, genotype C and D patients have often late and infrequent hepatitis B e antigen (HbeAg) seroconversion.[5,9]

Turkey can be classified as a region of intermediate endemicity area with a 1.7-21% HBV surface antigen (HBsAg) prevalence.[10,11] Based on data reported for regions other than the Black Sea region, it has been shown that it is the genotype D that prevails predominantly in Turkey.[12-24] There has been only one report regarding HBV epidemiology in our region, and that was merely a report of the serological screening results of blood donors indicating that the prevalence of HBsAg was 3.9% in the Black Sea region.[25] Since the region has close borders and attracts tourist from neighboring countries like Georgia and most notably from Russia, it is thought that the status of HBV genotypes and subgenotypes should be studied for any difference from the general population in Turkey. Therefore, the aim of the present study is to determine the HBV genotypes and subgenotypes in patients with HBV infection in the Eastern Black Sea region of Turkey.

Methods. *Subjects.* The study population included 137 patients with proven HBV infection (99 men and 38 women; age 7-69 years, median age 26 years) who were admitted between January 2005 and January 2010 to the Farabi Hospital of Karadeniz Technical University, Trabzon, Turkey which is a 800-bed teaching hospital serving almost the whole Eastern Black Sea region of Turkey as a reference center for hepatitis patients. All patients participating in the study were living in the Black Sea region of Turkey. The Ethics Committee at Karadeniz Technical University approved the study protocol. We conducted the study in accordance with the principles of the Helsinki Declaration. Excluding those patients co-infected with hepatitis C virus (HCV), hepatitis D virus (HDV), or HIV, all study subjects signed an informed consent and participated voluntarily. Three milliliters of venous blood were obtained from the patients and the sera were stored at -20°C until the assay was carried out.

Serological assays. Sera were tested for HBsAg, HbeAg, and anti-Hepatitis B e antibody (Anti-HBeAb) by enzyme immunoassay using commercial kits (Architect, Abbott Laboratories, Abbott Park, IL, USA).

Hepatitis B virus DNA quantitative assay. Invisorb Spin DNA extraction kit (Invitek GmbH, Berlin, Germany) were used for HBV DNA isolation. Amplification and detection steps of all samples were performed with ABI Prism 7700 Sequence Detection System (Perkin Elmer, Foster City, CA, USA).

DNA extraction for genotyping. The HBV DNA was extracted from 200 µl of serum by proteinase K digestion in 500 ml lysis buffer (20 mM Tris [pH 8.0], 150 mM NaCl, 10 mM EDTA [pH 8.0], 0.2% sodium dodecyl sulfate (SDS), 20 mg/ml proteinase K) for 2 hours at 37°C, followed by chloroform:isoamyl alcohol (24:1) extractions and ethanol precipitation after adding 3M sodium acetate (pH 4.9). The DNA pellets were dissolved in 20 ml Tris-EDTA buffer (pH 8.0) and stored at -20˚C until used.

Pre-S gene region amplification. Ten microliters of isolated DNA sample were used in a polymerase chain reaction (PCR) for amplification of the pre-S gene region of the HBV, using primers HBVp1 (nt 2823-2845; 5'-TCA CCA TAT TCT TGG GAA CAA GA-3') and HBVp2 (nt 80-61; 5'-TTC CTG AAC TGG AGC CAC CA-3'). Amplification conditions were as follows: an initial 3 minute denaturation at 94°C, 40 cycles of amplification, including 45 seconds denaturation at 94°C, 60 seconds annealing at 53°C, and 90 seconds extension at 72°C, prolonged by 3 seconds per cycle, and 7 minutes final extension at 72°C. The samples were run on 1% standard agarose gel.[26]

Hepatitis B virus genotyping. The HBV genotypes were determined by the polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) method.[26] Ten microliters of each PCR-amplified product was used for digestion with the restriction endonucleases according to the manufacturer's specifications (New England BioLabs Inc., Beverly, MA, USA). A panel of 2 restriction enzymes was used: AvaII and DpnII. The RFLP was determined

after electrophoresis of the restricted DNA on a 7.5% polyacrylamide gel electrophoresis. Samples treated and untreated with enzymes and molecular weight marker (GeneRuler™ 100 bp DNA Ladder, Fermentas, St. Leon-Rot, Germany) were run in parallel. Gels were stained with ethidium bromide and visualized with an ultraviolet transilluminator. Genotypes were determined using PCR product size and fragment sizes (bp) as suggested by Lindh et al.[26]

Results. The study included a total of 137 HBsAg and HBV DNA positive patients (72.3% males, and 27.7% females) with a median age of 26 years ranging from 7-69 years. The HBeAg seroconversion status, HBV viral loads, probable mode of transmission, alanine transaminase (ALT) levels, and clinical diagnosis of the patients are summarized in **Table 1**.

One hundred and twenty-five of the 137 HBV samples (91.3%) could be typed with PCR-RFLP and all of them classified as genotype D. Most of the typable genotype D strains (122/125, 97.6%) belonged to subgenotype D2, 2 strains (1.6%) to subgenotype D1, and one strain (0.8%) to subgenotype D-del. The remaining 12 samples' restriction patterns were different from known pre-S RFPL genotype/subgenotype patterns. Patterns and their characteristics obtained with pre-S RFLP are shown in **Table 2**.

Table 1 - Characteristics of 137 HBV patients.

Characteristics	n	(%)
HBe Ag seroconversion		
HBe Ag positive / Anti-HBe Ab negative	84	(61.3)
HBe Ag negative / Anti-HBe Ab positive	50	(36.5)
HBe Ag negative / Anti-HBe Ab negative	2	(1.5)
HBe Ag positive / Anti-HBe Ab positive	1	(0.7)
HBV viral load (copy/ml)		
$\leq 10^3$	13	(9.5)
10^3-10^5	29	(21.2)
$\geq 10^5$	95	(69.3)
Probable mode of transmission		
Horizontal	101	(73.7)
Vertical	2	(1.5)
Unknown	34	(24.8)
ALT level (U/L)		
<15	8	(5.8)
15-44	50	(36.5)
≥45	79	(57.7)
Clinical diagnosis		
Chronic HBV infection	116	(84.7)
Acute HBV infection	15	(10.9)
Cirrhosis	5	(3.6)
Hepatocellular cancer	1	(0.7)

HBe Ag - Hepatitis B e antigen, Anti-HBe Ab - Hepatitis B e antibody, HBV - Hepatitis B virus, ALT - Alanine aminotransferase

Table 2 - The pre-S RFLP patterns and their characteristics in 125 HBV patients.

Pre-S RFLP patterns	D1 (n=2)	D2 (n=122)	D-del (n=1)
Amplicon segment size (bp)	446	446	263
AvaII restriction site position (nt)	-	-	-
Fragment sizes with AvaII (bp)	446	446	263
Number of fragments with AvaII	1	1	1
DpnII restriction site position (nt)	2843, 2943, 28	2943, 28	2943, 28
Fragment sizes with DpnII (bp)	306, 67,52, 28	306, 88, 52	123, 88, 52
Number of fragments with DpnII	4	3	3

RFLP - Restriction fragment length polymorphism, bp - base pair, nt - nucleotide, AvaII - AvaII restriction enzyme, DpnII - DpnII restriction enzyme

Discussion. The genotypes of HBV show a distinct geographical distribution, and are known to influence the course of the disease and treatment.[5,9] Therefore, it is important to know which genotypes exist in all regions of countries. Differences in the distribution of the HBV genotypes have been studied in some other parts of Turkey. However, HBV genotypes and subgenotypes are unknown in the Eastern Black Sea region, which has close borders and attracts tourists from neighboring countries like Georgia, and most notably from Russia. Therefore, in this study we report the HBV genotypes in this region.

The definitive method for HBV genotyping is sequencing of the entire genome followed by phylogenetic analysis. However, this method is both time consuming and expensive.[27,28] An RFLP method based on the amplification of an pre-S gene amplicon followed by restriction digestion was developed by Lindh et al.[26] In our study, using the PCR-RFLP method, 91.3% of the HBV samples could be classified.

In the present study, HBV genotype D was identified as the most prevalent (91.3%) genotype in patients living in the Eastern Black Sea region, as in the other parts of the Turkey. Sertoz et al[15] and Sayıner et al[16] in the Aegean region; Bozdayı et al,[20] and Atalay et al,[24] in the Central Anatolia region; Yalçın et al,[22] and Aksoy et al,[18] in the Eastern Anatolia region, and Ozdemir et al[23] in the Marmara region determined genotype D existence at the rate of 100% in their studies. In a study from the Mediterranean region, strains revealed a

predominance of genotype D/E in 85.1%, followed by genotype A in 4.4%, genotype C in 4%, and genotype F in 0.7%.[17] Sunbul et al[12] also found that genotype D was the most prevalent genotype in patients from 15 hospitals throughout the country except for the Eastern Black Sea region. Subgenotypes also demonstrate significant distinct geographical distribution in the world. Eight subgenotypes (D1-D8) and a pattern showing a 183 bp deletion in a genotype D strain (D-del) have been reported for genotype D.[28] In the present study, regarding subtypes, D2 was found most prevalent, followed by subtype D1 and D-del.

It has been suggested that HBV genotype C and D patients, compared to those of genotype A and B patients, have late or absent HBeAg seroconversion that may accelerate the progression of chronic hepatitis, thereby conferring a poor clinical outcome.[5] In the present study, it was not possible to compare the various genotypes in this respect, however, the results we obtained in our study group support these findings. Overall, 122 of the 137 patients infected with HBV genotype D (89%), have chronic HBV infection (3.65% of these have cirrhosis, and 0.7% of these have HCC) with low rate of HBeAg seroconversion (37.2%), and high viral load (69.3% of these have higher than 10^5 copy/ml viral load) and high ALT levels (57.7% have higher than 45 U/l). Furthermore, horizontal transmission was determined to be the main route with a rate of 73.7% in our patients.

In conclusion, this is the first HBV genotyping study in the Eastern Black Sea region of Turkey, indicating that similar to the other parts of Turkey the genotype D and subgenotype D2 are also the predominant types among patients in our region. Lower rates of spontaneous HBeAg seroconversion, high viral load, and severe and chronic liver disease, including cirrhosis and HCC, and horizontal transmission as a main route was remarkable in our study group. Because the genotype D and subgenotype D2 were most prevalent in the Black Sea Region of Turkey, the PCR-RFLP method, which is capable of determine these genotypes and subgenotypes, should be useful for epidemiological investigations of HBV in this region.

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