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• *BRIEF REPORTS* •

# Distribution of hepatitis B virus genotypes in patients with chronic hepatitis B in Turkey

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## Abstract

**AIM:** Hepatitis B virus (HBV) strains isolated worldwide has been classified into eight genomic groups deduced from genome comparisons and designated as genotypes A to H. We aimed to investigate prevalence of HBV genotypes and subtypes in Turkey.

**METHODS:** A total of 88 chronic hepatitis B (CHB) patients from 15 hospitals throughout the country were included. Patients who were HBsAg positive in serum at least for 6 mo, who had HBV-DNA in serum and elevation of ALT levels more than two times upper limit of normal, and who had percutaneous liver biopsy within 6 mo were included. Genotyping of HBV was done by restriction fragment length polymorphism (RFLP). The patients received subcutaneous 9 MU interferon-α 2a thrice a week for a period of 6 mo.

**RESULTS:** Genotype D was detected in 78 of 88 (88.7%) patients, however, genotyping failed in two patients (2.3%), while no product was obtained in eight (9.0%) patients. Regarding subtypes, D2 was more prevalent (67 patients between 78% and 85.9%) followed by subtype D2+deletion (seven patients of 78 or 8.9%), subtype D1 (three patients of 78% or 3.9%) and subtype D3 (one patient of 78% or 1.3%). Thirty-three patients (37.5%) were HBeAg positive compared to 55 (62.5%) anti-HBe positive patients. The endpoint for the viral response of HBeAg positive patients was 27.2%, while it was found 52.7% in HBeAg negative patients ($P$<0.05). Long-term persistent viral response was 29.5% for all patients.

**CONCLUSION:** This multi-center study indicates that the predominant genotype with CHB patients in Turkey like in other Mediterranean countries is genotype D.



**Key words:** Chronic hepatitis B; Genotype; Interferon-α 2a

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## INTRODUCTION

Hepatitis B virus (HBV) is associated with acute and chronic viral hepatitis. Approximately 5% of the world population is infected with HBV and those with chronic hepatitis B (CHB) infection have a risk to develop cirrhosis and hepatocellular carcinoma[1]. The antigenic determinants of hepatitis B surface antigen (HBsAg) are classified into four sub-types, or on the basis of serotypes, where those subtypes are also divided into sub-divisions within nine serotypes[2]. Eight genotypes of HBV ranging from A to H have been identified[3,4]. Epidemiological studies suggest that these serotypes are common in different parts of the world with various frequencies as follows: genotype A in Western Europe and North America, genotype B in North and Southeastern Asia, genotype C in Asia and Pacific, genotype D in Southern Europe (Mediterranean countries, Middle East), genotype E in Africa, and genotype F in South America and Alaska, genotype G in some European countries and North America, and genotype H has been recently reported from Central America[5,6].

Data on the distribution of genotypes for patients with CHB are very limited in our country. The present study aims to investigate the prevalence of distribution of genotypes and its relation with the treatment in patients with CHB in Turkey.

## MATERIALS AND METHODS

### *Patient selection*

Eighty-eight consecutive adult patients with chronic HBV infection who were admitted in 15 medical centers of Turkey, between March 2001 and October 2002, were prospectively included into the study. The medical centers were representative of all the geographic regions of the country. Those with ages ranging from 16 to 65 years, who are HBsAg positive at least for a period of 6 mo, who are HBeAg or anti-HBe and HBV-DNA positive (qualitative) in serum, whose serum alanine aminotransferase (ALT) levels were at least 1.5 times or higher than the upper limits of normal range for at least a period of 6 mo, and those with chronic active hepatitis in percutaneous liver biopsy, but had no previous antiviral therapy, were included in the study. A percutaneous liver biopsy was performed in

all patients except the ones with a previous liver biopsy within 6 mo. Those with malignancy, depression, anti-HIV positivity, cirrhosis, history of antiviral therapy and thyroid dysfunction, and pregnant women were excluded. Also patients who were positive for anti-HCV and anti-HDV were excluded.

### Biochemistry and serologic markers

Liver biochemical tests, ALT (0-46 IU/L), aspartate aminotransferase (AST) (8-46 IU/L), and gamma-glutamyl-transpeptidase (GGT) (7-49 IU/L) were measured in all patients at initial examination, third, sixth months and during the follow-up at each institution. Serological markers HBsAg, hepatitis B e antigen (HBeAg), anti-HBe, anti-HBc IgM, anti-HBc IgG, anti-HBs, anti-HAV (IgM and IgG), anti-HCV and anti-HDV total (IgM+IgG) were tested during first evaluation by commercially available kits (Axsym, Abbott Laboratories, North Chicago, IL, USA) at each institution.

### Determination of HBV genotypes

Serum samples from each subject were taken at first examination and stored at -70 ℃ until they were studied. Patients' sera from other samples were transferred at approximately -15 ℃ into dry ice boxes. HBV-DNA was isolated with high pure viral nucleic acid kit (Roche Diagnostics, Mannheim, Germany) according to the instructions of the manufacturer. Briefly 200 μL sera were used for DNA extraction and 50 μL elution buffer was used for elution of DNA. Five microliters of HBV-DNA were added to a 45 μL reaction mixture for amplification. HBV primers (HBV-1 and HBV-2) described by Lindh *et al*[7], were obtained commercially. The sequences of PCR primers used in this study are shown in Table 1. 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 (MIR-D40 Sanyo) for 40 cycles (1 min at 94 ℃, 1 min at 55 ℃, 2 min at 72 ℃). PCR products were separated in 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. Standard procedures for reducing contamination were strictly followed. 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*[7]. As described previously, most genotype D strains produce either of two pre-S RFLP patterns (arbitrarily designated D1 and D2) due to a single nucleotide difference (at nt 2 847). Same nomenclature or terms (D1 and D2) were used for subtyping of genotype D in this paper.

**Table 1 Primer sequences used for HBV genotyping by PCR-RFLP**

| | |
|---|---|
| HBV-1 | 5′- TCACCATATTCTTgggAACAAgA -3′ (nt 2 823-2 845, sense) |
| HBV-2 | 5′- TTCCTgAACTggAgCCACCA -3′ (nt 80–61, antisense) |

### Treatment schedule

The interferon-α 2a (Roferon, Roche, Basel, Switzerland) 9 MU was subcutaneously given thrice a week for a period of 6 mo. Patients were assessed before the treatment, at 3 mo, at the end of the treatment and 6 mo after the discontinuation of therapy, and they all underwent a thorough physical examination, whole blood counts, urinary analysis as well as assessing their biochemical parameters and HBV-DNA. At 3 mo assays, the treatment was discontinued for patients with PCR HBV-DNA positivity. For those whose response was detected, treatment lasted to achieve the period of 6 mo.

### Criteria for response to therapy

Response to interferon (IFN) therapy was evaluated in accordance with HBV-DNA clearance from serum (viral response), normalization of serum ALT levels (biochemical response) and HBeAg/anti-HBe seroconversion (serological response). After 6 mo of treatment, PCR HBV-DNA negative patients were defined as persistent long-term viral response. HBsAg clearance from serum was called complete response[5,8].

### Statistical analysis

Data in the text and tables are expressed as mean±SD. Differences between groups were examined by $\chi^2$ test, Fisher's exact test, Bonferroni's correction and Wilcoxon sign assay. A *P* value of less than 0.05 was considered to be significant.

## RESULTS

### Demographic characteristics

The study included 88 patients with CHB, 59 (67%) male and 29 (33%) female. The mean age was 35±11 years. Of all the patients, 33 (37.5%) were HBeAg positive compared to 55 patients (62.5%) being anti-HBe positive. Of 33 HBeAg positive patients, 20 were males and 13 were females, while 39 of 55 anti-HBe positive patients were males and 16 were females. The demographic characteristics and laboratory features of HBeAg positive and anti-HBe positive patients are shown in Table 2.

### Genotype outcomes

Genotype D was detected in 78 of 88 (85.9%) patients, however, genotyping failed in two patients (2.3%), while no PCR and HBV-DNA product was obtained in eight (9.0%) patients. Regarding subtypes, D2 was more prevalent (67

**Table 2 Baseline characteristics of HBeAg positive and anti-HBe positive patients (mean±SD)**

| Characteristics | HBeAg positive (*n* = 33) | Anti-HBe positive (*n* = 55) | Total (*n* = 88) |
|---|---|---|---|
| Sex | | | |
| Male | 20 | 39 | 59 |
| Female | 13 | 16 | 29 |
| Age (yr) | 28±10 | 39±9.9 | 35±11 |
| ALT | 113±66 | 148±107 | 135±96 |
| AST | 69±32 | 98±90 | 87±75 |
| GGT | 33.6±21.5 | 49.8±40.6 | 43.6±35.5 |
| Knodell score | 7.5±3.4 | 7.6±3.2 | 8.2±3.8 |

patients among 78 or 85.9%) followed by subtype D2+deletion (seven patients of 78 or 8.9%), subtype D1 (three patients of 78 or 3.9%) and subtype D3 (one patient of 78 or 1.3%).

### *Assessment of response to therapy*

ALT and AST values in patients before therapy and at months 3 and 6 during therapy and at month 6 after the treatment are given in Table 3.

Bonferroni's correction and Wilcoxon sign assay were also performed for patients, whose ALT and AST values between pre-treatment, at third month, at sixth month and 6 mo after the therapy were found to be significantly different from Friedman variance analysis. It was found that there was a significant difference between the initial ALT value and values of 3, 6 and 12 mo. At third month, a viral response occurred in 53.4% of the patients. A total of 41 patients discontinued the treatment, 35 HBV-DNA positive patients along with six who failed to continue due to several reasons (two hyperthyroidism, two severe depression, one activated lung tuberculosis, and one interstitial lung disease development). Forty-seven patients who showed viral response at 3 mo were continued until 6 mo. Thirty-eight (43.1%) out of those patients who accomplished the treatment at 6 mo were PCR HBV-DNA negative compared to 9 (10.2%) positive. During the sixth month, 26 patients (29.5%) continued long-term persistent response, while eight patients (9.1%) developed relapse. Four patients did not come up for control. The viral, biochemical and serological responses of HBeAg and anti-HBe positive patients at 3, 6 and 12 mo (6 mo after the treatment) are listed in Table 4.

When viral response rates of months 3 and 6 were compared, the response rate in HBeAg negative patients group was higher than that in HBeAg positive patients (at 3 mo $P<0.01$, at 6 mo $P<0.05$). Long-term persistent response (viral response+biochemical response) was found to be 18.1% in HBeAg positive patients compared to 30.9% in HBeAg negative patients. Although the viral response rate was higher in HBeAg negative, the difference was not statistically significant ($P>0.5$). The long-term persistent response rate was 26.1% in all patients. During the course of the treatment, 24.2% of the HBeAg positive patients developed seroconversion. HBsAg was lost only in two (2.3%) patients, one developed anti-HBs and the other had no occurrence of anti-HBs during follow-up.

When the response rates were evaluated in accordance with the subtypes of genotype D, it was found that 85.9% of the patients had D2 subtype at the beginning of the treatment, while the genotype detected in 91.2% of the patients with persistent response is D2. No statistically significant difference was found ($P>0.5$). The genotype distribution before the treatment and for patients with long-term persistent response is shown in Table 5.

**Table 3** ALT and AST values pre-treatment, during and post-treatment (mean±SD)

| | ALT | AST |
|---|---|---|
| Before treatment | 135.0±96.0 | 87.0±75.0 |
| 3rd mo | 56.8±20.1 | 40.6±11.9 |
| 6th mo | 62.3±38.7 | 53.3±17.4 |
| 6th mo after therapy | 43.3±23.0 | 35.2±19.4 |

**Table 4** Main outcome measurements for patients with CHB treated with interferon-α 2a

| Response outcome | | Total | HBeAg positive | Anti-HBe positive | *P* |
|---|---|---|---|---|---|
| Viral response | *n* | % | % | % | |
| 3rd mo | 47 | 53.4 | 36.3 | 63.6 | <0.01 |
| 6th mo | 38 | 43.1 | 27.2 | 52.7 | <0.05 |
| 12th mo | 26 | 29.5 | 18.1 | 36.3 | >0.1 |
| Biochemical response | | | | | |
| 3rd mo | 30 | 34.1 | 21.2 | 41.8 | >0.5 |
| 6th mo | 26 | 29.5 | 24.2 | 32.7 | >0.5 |
| 12th mo | 25 | 28.4 | 24.2 | 30.9 | >0.5 |
| Serological response | | | | | |
| 3rd mo | 4 | 12.1 | 12.1 | – | – |
| 6th mo | 7 | 21.2 | 21.2 | – | – |
| 12th mo | 8 | 24.2 | 24.2 | – | – |

## DISCUSSION

The success of CHB treatment depends on some parameters. It is higher in females as a gender who have lower HBV-DNA levels, higher ALT levels, moderate and severe hepatitis damages, whereas the response to treatment rate is lower in males with mild liver damage, higher HBV-DNA levels and lower ALT levels[9]. Recently, the relation between HBV genotype and treatment success has also been examined. Studies suggest that genotype C is associated with more severe liver damages compared to genotype B[10]. Kao *et al*[11], reported that the most prevalent genotypes in Thailand are B and C, and genotype C is associated with more severe liver damages, while genotype is mostly associated with HCC. In another study, 73 patients received IFN therapy, and 34 patients were registered as control group. Genotype B was determined in 38% of the patients compared to genotype C in 60%. The response rate in genotype B was significantly higher than that in genotype C[12]. In the present study patients from 15 medical centers throughout Turkey were included. Since all of the genotypes determined are genotype D, the relation between the severity of liver disease and genotype could not be evaluated. No statistically significant difference was found in subtypes between the pre-treatment patients and those with a persistent response ($P>0.5$). There are few reports in the literature on genotype study for patients with CHB in Turkey. Multi-center national study including the cases of acute viral hepatitis B, genotype D was determined in 147 of 158 patients, and genotyping failed in 11 cases[13]. Yalcin *et al*[14], also found genotype D in the 32 CHB patients and 12 inactive HBsAg carrier. These findings show that the prevalent genotype in patients with viral hepatitis B in

**Table 5** Subtype of genotype distribution in patients

| Subtype of genotype | Pre-treatment (*n* = 78) | | Patients who completed the treatment (*n* = 31) | | Non-responder (*n* = 47) | |
|---|---|---|---|---|---|---|
| | *n* | Percentage | *n* | Percentage | *n* | Percentage |
| - D2 | 67 | 85.9 | 29 | 93.5 | 38 | 80.9 |
| - D2+deletion | 7 | 8.9 | 2 | 6.5 | 5 | 10.6 |
| - D1 | 3 | 3.9 | – | – | 3 | 6.4 |
| - D3 | 1 | 1.3 | – | – | 1 | 2.1 |

our country is genotype D. Interferons have antiviral, immunomodulatory and antiproliferative effects[15]. Interferon-α is the primary antiviral agent used in the treatment of CHB patients. In a meta-analysis of 24 studies by Craxi *et al*[16], the effect of interferon was evaluated on HBeAg positive CHB patients. Six to twelve months after treatment with interferon, persistent ALT normalization was 26.2%, HBeAg deletion was 24.3%, persistent HBV-DNA negativity was 23.4% and HBsAg negativity was 5.6%. The persistent viral response ranged from 15% to 40%[17]. The recommended dosage for IFN is 9-10 million s.c. thrice a week for a period of 4-6 mo[18].

In our study the response to IFN was 53.4% for all patients at 3rd mo, 43.1% at 6th mo and 29.5% at 12th mo (6 mo after the end of therapy). It has been reported that, with IFN monotherapy, the development rate for HBeAg seroconversion is approximately 20-30%[19]. HBeAg seroconversion was 12.1% at 3rd mo, 9.1% at 6th mo and 3.0% at 12th mo and 24.2% in total. HBsAg deletion was around 8% in average as a result of treatment in CHB patients. Such cases are called complete response[20]. We had complete response in 2.3% of our patients.

Mutation is possible in various sections of HBV genome. The most common is the mutation in precore region (G1896A), and HBeAg cannot be synthesized in such mutants. The persistent response to interferon is lower in precore mutant CHB patients compared to "wild type" HBV patients. One of the reasons is spontaneous seroconversion of HBeAg in some patients, which is facilitated by IFN. Some studies demonstrated that HBV genotype may influence HBeAg seroconversion. Patients with genotype C have lower rates of spontaneous HBeAg seroconversion and high cirrhosis than genotype B patients[2,21,22]. It has been reported that the response rate is higher in genotype A and B patients treated with interferon compared to genotype D and C patients[11,23]. These data will oblige the organization of clinical trials for antiviral therapies to be based on genotypes in the future.

HBeAg negative disease accounts for up to 30% of CHB globally[24]. Prevalence of precore mutants vary depending on the geographical regions; it is common in the Mediterranean countries and Asia, whereas very rare in North America and Western Europe. This variation may be associated with prevalent HBV genotype because these mutants are only present in patients infected with HBV genotypes B-D. Recently many investigators in Europe have assumed that CHB patients with HBeAg negativity are more than HBeAg positive patients[25]. Prevalence rates in the Mediterranean region (Italy, Greece and Israel) are approximately 50-80%, with 40-55% in East Asia (Hong Kong, Taiwan and China) and at least 15% in South Asia (India)[26]. Seroprevalance data show that from 1975 to 1985, 58% of 584 chronically infected Italian patients studied were HBeAg positive, while 42% were HBeAg negative. However, from 1992 to 1997, HBeAg positive disease made up only 10% of the 834 CHB patients studied. The remaining 90% were HBeAg negative and/or anti-HBe antibody positive[5]. In the present study, 62.5% of our patients were precore mutant. In a previous multi-center study, it was found to be 54.2% and response to IFN therapy was significantly higher in precore mutants compared to HBeAg positive patients[27]. These findings also suggest that CHB patients in Turkey are mostly precore mutants. It has been reported that HBeAg positive cases and precore mutant cases provide distinct responses to IFN therapy[28-30]. In our study, the long-term persistent viral response was 18.1% in HBeAg positive cases, while it was found to be 36.3% in precore mutants. The persistent viral response was 29.5% in all patients. Although long-term persistent response was higher in precore mutants compared to HBeAg positive patients, the difference between them was not statistically significant ($P$>0.5).

In conclusion, this multi-center study indicates that the predominant genotype with CHB patients in Turkey like in other Mediterranean countries is genotype D. Precore mutants are prevalent among patients with CHB in Turkey as well. A higher response was obtained although the persistent viral response to IFN therapy was not statistically significant in terms of HBeAg positive CHB.

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