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SCIENCE DIRECT

Diagnostic Microbiology and Infectious Disease 53 (2005) 57–60

DIAGNOSTIC MICROBIOLOGY AND INFECTIOUS DISEASE

www.elsevier.com/locate/diagmicrobio

Genotyping of hepatitis B virus isolated from chronic hepatitis B patients in the south of Turkey by DNA cycle-sequencing method

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Received 5 February 2005; accepted 16 April 2005

Abstract

The 8 genotypes of hepatitis B virus (HBV A–H) show a distinct geographic distribution and influence the course of disease and the prognosis of treatment. In this study, we have genotyped 50 HBV isolates circulating in the south of Turkey by DNA cycle sequencing, based on their compatibility with reference sequences of a part of S gene. In our cases, all 50 (100%) HBV sequences from the patients demonstrated full compatibility with the sequences of ayw subtype viruses in genotype D. However, we have found some nucleotide sequence variations within genotype D, 47 (94%) of which were related to HBVGEN1 (Z35716 genotype D) and 3 (6%) were related to HBVDNA (X68292, genotype D).

Keywords: HBV; Genotypes; PCR; Cycle sequencing

1. Introduction

Hepatitis B virus (HBV) belongs to the family of hepadnaviruses. The virus associated with acute hepatitis, chronic hepatitis, and development of hepatocellular carcinoma (Maynard, 1990). HBV sequences have been classified based on their phylogenetic relationships into 8 genetic groups, termed A–H, based on an intergroup divergence of 8% or greater of complete nucleotide sequence (Okamoto et al., 1988; Norder et al., 1994; Arauz-Ruiz et al., 2002). Heterogeneity of HBV DNA may cause the production of heterogeneous viral proteins including the proteins other than HBsAg, against which host immune reactions occur (Ehata et al., 1992). Thus, it is possible that the clinical course of chronic HBV infection will vary according to the genotype of HBV. Nevertheless, it is not clear whether the genotypes are closely related to the clinical features of HBV carriers (Kikuchi et al., 2000). On the other hand, it has been suggested that the viral genotype may correlate with differences in clinical features of the infection. For example, recent data suggest that patients with genotype C are more likely to have severe liver disease, whereas those with genotype B are more likely to develop HCC (Kao et al., 2000; Fujie et al., 2001). Interestingly, there also appear to be geographic factors involved in these observations because a recent study in India (where genotypes A and D are prevalent) shows that genotype D is associated with more severe disease and may predict occurrence of HCC in younger patients (Thakur et al., 2002). Therefore, genotyping of HBV appears to be important for better patient management.

Current approaches to genotyping involve sequencing and phylogenetic analysis (Ohba et al., 1995; Bartholomeusz and Schaefer, 2004), differential hybridization (Stuyver et al., 2000; Bartholomeusz and Schaefer, 2004), hemi nested and multiplex polymerase chain reaction (PCR) (Kirschberg et al., 2004; Bartholomeusz and Schaefer, 2004; Kato et al., 2001), and restriction fragment length polymorphism (RFLP) (Mizokami et al., 1999, Bartholomeusz and Schaefer, 2004). The other methods are subtyping and genotyping using antibodies (Bartholomeusz and Schaefer,

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doi:10.1016/j.diagmicrobio.2005.04.007

2004) and INNO-LiPA HBV genotyping (Bartholomeusz and Schaefer, 2004). However, the most accurate way to provide detailed molecular analysis on these viruses is by accumulation of full genomic sequence information. We have performed a DNA cycle-sequencing analysis of an important part of the S region of HBV and compared it with 18 reported HBV clones.

2. Materials and methods

2.1. Patients

Serum samples were obtained in Mersin and Cukurova University Hospitals from 50 patients who live in 8 different cities and their towns in the south of Turkey (Adana, Mersin, Gaziantep, Osmaniye, Hatay, Malatya, and Kahramanmaras cities). All patients were seropositive for HBsAg, positive for HBV DNA, their ALT and AST levels were 740 ± 443 and 465 ± 305 U/L, respectively. Chronic hepatitis B was diagnosed by HBsAg positivity with or without the presence of HBsAg, and their histological diagnosis was chronic hepatitis B. All patients were regularly following and having antiviral therapy at least for 1 year.

2.2. Extraction of HBV DNA

One hundred microliters of serum was mixed with 300 μL of lysis buffer (13.3 mmol/L Tris-HCl, pH 8.0, 6.7 mmol/L ethylenediaminetetraacetic acid, 0.67% sodium dodecyl sulfate, 133 μg/mL proteinase K) and incubated at 55 °C for 4 h. Two phenol-chloroform extractions were followed by one chloroform extraction, and DNA was precipitated with ethanol. The precipitate was dissolved in 20 μL of TE buffer (10 mmol/L Tris–HCl, pH 8.0, 1 mmol ethylenediaminetetraacetic acid).

2.3. Nested PCR amplification

The first round of PCR was performed with 5 μL of DNA, 2 U of Taq polymerase (Promega, Madison, WI), 200 μmol/L dNTP (mix), 100 pmol of each primer, and the reaction buffer. The primers were HBs1-1 (sense, 5′-TCG-TGTTACAGGCGGGGTTT-3′, nt 192–211) and HBs1-2 (antisense, 5′-CGAACCACTGAACAAATGGC-3′, nt 685–704). The reaction was allowed to produce at 94 °C for 1 minute, 55 °C for 1 minute, and 72 °C for 1 minute in each cycle. We have also used a hot start (at 95 °C for 2 minutes) and a postelongation (at 72 °C for 5 minutes) before and after the reaction. The amplification was performed for 30 cycles in a thermal cycler (Gene Amp PCR system 9700). In the second round of PCR, we used 5 μL of first PCR product and internal primers at the same conditions and same thermal cycles described above. The primers for the second PCR were GRE2-1 (sense, 5′-GAGTCTAGACTCGTGG-TGG-3′, nt 247–264) and GRE2-2 (antisense, 5′-ACCACTGAACAAATGGCA-CTA-3′, nt 683–702). All primers were chosen from the research article of Kikuchi et al. (2000).

2.4. DNA cycle-sequencing of HBV isolates

The second-round PCR products were 436 bp. We have purified these products by Wizard PCR preps DNA purification system (Promega). We have used internal sense primer as sequencing primer. Purified PCR products were cycle-sequenced by fmol DNA cycle-sequencing system (Promega) on thermal cycler according to manufacturer's instructions. We have direct incorporated ^{35}S-dATP into sequencing products as a labeling reagent. The cycle-sequencing reaction was performed for 30 cycles in a thermal cycler with the same parameters used for PCR amplification (as described above). The cycle-sequencing products were separated by electrophoresis through a polyacrylamide gel with urea. The gel was dried, placed in an X-ray film cassette and a layer of X-ray film was placed on it. After 12–24 h, the film was developed and analyzed.

2.5. Analysis of the sequences

We compared the isolated sequences with the 18 reported HBV clones. The names of the clones and GenBank accession numbers of HBV sequences used for analysis were as follows: HBVADW,V00866 (genotype A);HBVADW2, X02763 (genotype A);HPBADW1, D00329 (genotype B);HPBADW2, D00330 (genotype B);HBVADR, V00867 (genotype C);HHVBC, X75665 (genotype C);HBVDNA, X68292 (genotype D);HBVGEN1, Z35716 (genotype D);HPBMUT, L27106 (genotype D);HPBHBVVAA, M32138 (genotype D);HPBAYW, J02203 (genotype D);XXHEPAV, X02496 (genotype D);HBVAYWMCG, X59795 (genotype D);HHVBBAS, X75657 (genotype E);

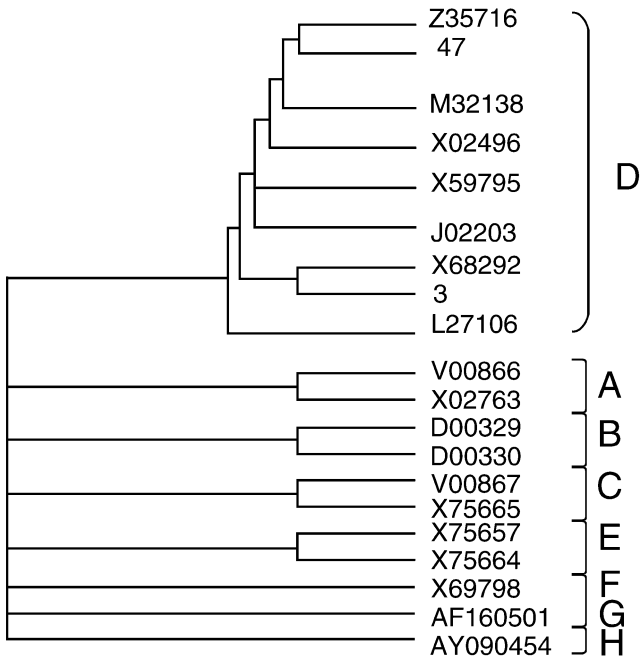

Fig. 1. The phylogenetic analysis of S region sequences from 18 reference strains and 50 isolates. The numbers of HBV DNA clones belonging to a branch are indicated. The capital letters represent each genotype.