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Original Article

Prevalence of mutations within major hydrophilic region of hepatitis B virus and their correlation with genotypes among chronically infected patients in Egypt

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ABSTRACT

Background and study aims: Mutations within the major hydrophilic region (MHR) of the hepatitis B surface antigen (HBsAg) have been reported in relation to viral persistence by evasion from vaccine and immunotherapy, severity of liver disease and lack of detection by commercial kits. The aim of this study was to elucidate the circulation of hepatitis B virus (HBV) genotypes, subgenotypes and serotypes in Egypt, with recognition of the pattern and prevalence of MHR mutations possibly occurring during the course of the disease.

Patients and methods: Eighty-eight samples from patients with chronic HBV infection were included in the study. The surface protein-encoding gene (S gene) in the HBV genome was subjected to amplification and partial sequencing.

Results: Based on phylogenetic analysis, only genotype D was found circulating among patients. The majority of isolates belonged to subgenotype D3 (86.3%), followed by D7 (8%), then D5 (3.4%) and lastly D1 (2.3%). Two subtypes were identified: *ayw2* (97%) and *ayw3* (2%). The '*w*' sub-determinant was not defined in one isolate (1%). A significant proportion of patients (13/88, 14.8%) exhibited mutations in the MHR, 10 of whom harboured mutations in the 'a' determinant region and three outside. The first loop comprised four patients with three mutations (P127S, P127T and Y134F). The second loop contained six patients, all with one mutation, S143L, which was most frequently encountered in this study (6.8%).

Conclusions: We conclude that genotype D, subgenotype D3 and HBsAg subtype *ayw2* are the most common types circulating in Egypt, which account for 100%, 86.3% and 97% of the population, respectively, with a moderate degree of MHR mutations.

Introduction

Hepatitis B virus (HBV) infection represents a major health burden in Egypt, which is constantly at a risk of progressing to cirrhosis and hepatocellular carcinoma (HCC), given the poor healthcare service in the country [1]. According to the World Health Organization (WHO), the prevalence of hepatitis B surface antigen (HBsAg) ranges from 1% to 10% in the Eastern Mediterranean region, which extends from North Africa through the Middle East to Pakistan, making it a region of intermediate to high endemicity [2]. The exact burden of hepatitis B in Egypt is difficult to assess due to inaccurate records and underreporting, however; the estimated prevalence rate has decreased from 10.1% 30 years ago [3] to 4.2% [4] and 1.3% [5] 25 years later.

Lack of proofreading activity of DNA- and RNA-dependent DNA polymerase (reverse transcriptase) results in mis-incorporations of nucleotides during viral replication, which lead to a high degree of genetic heterogeneity [5,6]. Thus, several HBV genotypes and subgenotypes have emerged. At least eight different genotypes (A-H) and several other subgenotypes have been identified, which differ by at least 8% and 4% of the genome, respectively [7–10].

The clinical significance of HBV genotypes is becoming crucial. The identification of HBV genotypes may help in the early identification of potential sequelae following chronic HBV infection as well as in the implementation of appropriate therapeutic regimens [11]. Patients with HBV genotype D (HBV-D) infection typically

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show seroconversion from hepatitis B e-antigen (HBeAg) to anti-HBe in adolescence or early adulthood, which is frequently associated with the precore mutant [12]. Although many patients enter and remain in the inactive carrier phase [13,14], some develop HBeAg-negative/anti-HBe-positive chronic hepatitis B infection, which can lead to cirrhosis and HCC [14–17]. Genotype D was found to be an independent risk factor for fulminant hepatitis in patients suffering from acute liver failure [18]. As reported, genotype D was linked to more severe liver disease and HCC in younger patients compared to genotype A [19].

The central region of HBsAg, called the major hydrophilic region (MHR) extending from amino acids (aa) 100–169, is highly immunogenic and is potentially under the selective pressure of the immune system. Antibodies detected in vaccinated subjects and in immunoassays for HBsAg particularly target a cluster of neutralizing B-cell epitopes called the 'a' determinant, which is located between aa 124 and 147 within the MHR. The 'a' determinant region is composed of two loops of aa [20,21]. Mutations within MHR might arise naturally in chronic HBV carriers. These mutations were reported in relation to the severity of liver disease, lack of detection by commercial kits and viral persistence as vaccine- and immunotherapy-escape mutants are produced [22].

Data on HBV genotypes in Egypt are scarce. Furthermore, as long-term prognosis, initial clinical pictures and response to antiviral treatment may differ with the HBV genotype, the aim was to elucidate the circulation of HBV genotypes, subgenotypes and subtypes in Egypt with recognition of the pattern and prevalence of MHR mutations that might have occurred during the course of the disease.

Patients and methods

This cross-sectional study included 96 HBV DNA-positive samples with persistent HBsAg levels over 6 months. They were collected at the Molecular Biology Unit of Theodor Bilharz Research Institute during the period from June 2012 until June 2014. The study was approved by the ethics committee of the hospital. Anti-hepatitis C virus (HCV)-positive samples were excluded. All samples were subjected to the following tests.

Liver function tests

The alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured using a Beckman Coulter AU680 autoanalyser (Brea, CA, USA).

Extraction of HBV DNA

This was performed on 500 μl of samples using a manual DNA extraction kit (Abbott mSample Preparation System$_{DNA}$, Abbott, Santa Clara, CA, USA).

Amplification of HBV surface gene

Enzymatic amplification was performed by polymerase chain reaction (PCR) using Taq polymerase enzyme with a Bio-Rad T100 Thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). The HBV surface (S) gene was amplified by a nested PCR, using the universal outer primers HBV POL1 (5′-TTC CTG CTG GTG GCT CCA GTT C-3′, nucleotide position 54–75) and HBV POR4 (5′-TAC CCA AAG ACA AAA GAA AAT TTC-3′, 826–810) for the first round [23], and the inner primers HBV 123s (5′-TCG AGG ATT GGG GAC CCT G-3′, 123–141) and HBV 778r (5′-GAG GTA TAA AGG GAC TCA AG-3′, 778–758) for the second round [24]. All primers were manufactured by Bioneer Corporation, Daejeon, Republic of South Korea.

The first round of PCR was performed with an initial denaturation step at 95 °C for 5 min followed by 40 cycles of 95 °C for 20 s, 56 °C for 20 s and 72 °C for 2 min, and a final extension step at 72 °C for 4 min. This was carried out in a reaction volume of 25 μl containing 50 ng of the extracted DNA quantified by NanoDrop, 1× PCR master mix and 25 pmol/μl of each outer primer. The PCR master mix was composed of 10 mM Tris–HCl containing 1 mM molecular-grade ethylenediaminetetraacetic acid (EDTA). Na_2 (pH 8.0), 200 μM of each deoxynucleotide triphosphate (dNTP) (Fermentas Corporation, Ontario, Canada), 1.5 mM $MgCl_2$ and 1.25 U Taq DNA polymerase (Promega Corporation, Madison, WI, USA).

One microlitre of the first-round PCR product was then subjected to a second round of PCR under the same conditions using the inner primers. A suitable negative control (consisting of the abovementioned components except DNA) was included in each assay.

Detection of PCR amplification products

After the second round, a 750-bp fragment was obtained and detected by electrophoresis in 2% agarose gel.

Post-amplification purification of the resulting product

This was achieved with an EZ-10 Spin Column PCR Products Purification Kit (BS363, BIO BASIC Inc., Ontario, Canada).

Sequence analysis of the S gene

Direct DNA sequencing was performed using the Sanger method [25], with the primer HBV 123s. The PCR products were sequenced using BigDye Terminators Version 3.1 on an Applied Biosystems 3730xl DNA Analyzer (Applied Biosystem, Grand Island, NY 14072, USA).

Nucleotide sequence and phylogenetic analysis

The resulting DNA sequences were aligned with known sequences of different HBV genotypes retrieved from GenBank (HBV genotypes A–H), using a biological sequence comparison software. Genetic distance was estimated and the phylogenetic tree constructed. The nucleotide sequences were translated into aa sequences according to the open reading frame (ORF) of the S gene; then, the HBsAg subtypes were predicted from the aa sequence. For identifying HBsAg variants, the deduced aa sequences were aligned and compared with the consensus aa sequence of similar genotype.

With the aid of bioinformatics tools, the following programs were used to define the genotypes, subgenotypes and subtypes of the resulting isolates: (1) the online tool of Clustal Omega [26], (2) BLASTX 2.2.30 [27] and (3) the ExPASy online translation tool [28].

Multiple alignments and phylogenetic trees were created based on the differences in MHR using Clustal Omega.

Statistical analysis

Quantitative data are presented as mean ± standard deviation (SD) in a parametric distribution and as median (25th–75th) in a non-parametric distribution. Comparison between groups was performed using the *Mann–Whitney* test. Qualitative data are presented as number (percent). The tests were two-tailed and were considered statistically significant at a *P*-value <0.05. Statistical analysis was performed with SPSS for Mac, release 20.0 (SPSS Inc., Chicago, IL, USA).