Original Article

High genetic diversity of hepatitis delta virus in eastern Turkey

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Abstract

Introduction: Hepatitis delta virus (HDV) is a serious cause of liver-related mortality in patients infected with hepatitis B virus (HBV). Determination of genotypes of HDV and phylogenetic analysis are important for better understanding the pathogenesis of the liver diseases associated with HBV infection. The aim of this study was to determine the genotype or genotypes of HDV among chronically infected patients with HBV in eastern Turkey.

Methodology: A group of 113 patients infected with HBV and HDV were included in this study. The samples taken from the patients were analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR) and restriction enzyme cleavage.

Results: According to the results of the restriction enzyme analysis, all of the RT-PCR products were determined to be HDV genotype I. Furthermore, for phylogenetic analysis and genotyping, 40 of HDV RT-PCR positive products were sequenced. Phylogenetic analysis of the sequences showed that all of the samples were infected with HDV genotype I. In addition, the results of the alignment analysis showed that the sequences of clinical samples were 82%-95% similar.

Conclusion: These results indicate that high genetic diversity of the virus is possible in endemic areas such as Turkey.

Key words: hepatitis delta virus; hepatitis B virus; genotyping; restriction enzyme analysis; phylogenetic analysis

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Introduction

Hepatitis delta virus (HDV) is a hepatotropic virus that has a very small RNA genome (approximately 1700 bases) [1]. In order to form the infectious virion, the HDV ribonucleoprotein structure is packed within the envelope proteins of the hepatitis B virus (HBV) during the viral replication process. Thus, HDV reproduction is possible only in people infected with HBV, and HDV has therefore been accepted as a defective or a satellite virus affecting people with HBV [2,3].

Hepatitis delta virus was first discovered in patients with severe liver disease infected with HBV in the mid-1970s [3]. Many of the studies conducted since then have demonstrated that co- or super-infection with HDV and HBV have caused more severe liver diseases compared to infection with HBV alone [2-5]. In cases with cirrhosis or liver carcinomas, the prevalence of co-infection with HBV and HDV has been shown to be higher than the prevalence of infection with HBV alone. Thus, with regard to the community health, it has been suggested that HDV should be cautiously monitored in patients with HBV infection [2,3,6].

Although the prevalences of HBV and HDV have recently decreased due to comprehensive vaccination programs for HBV and strict control of blood samples, both viruses are still endemic in many regions of Turkey, including the region of this study [7-10]. The phylogenetic analysis and genotyping of HDV is important in understanding the pathogenesis and epidemiology of HBV-related liver diseases. The aim of this study was to perform genotyping and phylogenetic analysis of HDV in patients chronically infected with HBV in eastern Turkey.

Table 1. The primers used from semi-nested RT-PCR

Primers	Nucleotides
Primer I	5'ATG CCA TGC CGA CCC GAA GAG GAA 3' (Nucleotide 884-907 of HDV)
Primer II	5'GGC CTC TCA GGG GAG GAT TCA C 3' (Nucleotide 1.334-1.313 of HDV)
Primer III	5' CTC AGG GGA GGA TTC ACC GAC A 3' (Nucleotide 1.329-1.308 of HDV)

Methodology

Patient population

Among the patients who had been followed up at Fırat University Hospital Internal Diseases Gastroenterology Department and Infectious Diseases Outpatient Clinics since 2006, those from whom samples had been obtained between February 2011 and March 2012 were included in the study. The Fırat University Hospital is located in eastern Turkey, providing services primarily to the city of Elazığ, which has a population of 400,000, and to a wide region including Muş, Tunceli, Bitlis, and Bingöl. The study was approved by the Ethical Committee of Fırat University. Blood samples were obtained from a total of 113 patients (35 female, 78 male) between 22 and 82 years of age (mean 46, 97) who had a confirmed diagnosis of chronic HBV and chronic HDV infections, and who had been confirmed to not use drug or alcohol. The diagnoses of chronic hepatitis (68 patients) and cirrhosis (45 patients) in the study groups were made according to the clinical, biochemical and histopathological findings as described in previous studies [8].

The presence and quantity of HBV DNA was determined via a quantitative PCR method (Roche Molecular Systems, Branchburg, USA). The quantification of HDV RNA was performed using the quantitative real-time reverse transcriptase-PCR (Real-time RT-PCR, Light Cycler) method (Roche Molecular Systems, Branchburg, USA).

RT-PCR

Semi-nested RT-PCR was performed as described by Theamboonlers et al. [11]. The primers used in the study are presented in Table 1. The synthesis of cDNA was achieved using primer II, clinical RNA samples, and the M-MuVL reverse transcriptase enzyme (Fermentas-Thermo Fisher Scientific Inc., Waltham, USA). The primers used in the first step of the semi-nested PCR were primer-I and primer-II, and those used in the second step were primer-I and primer-III. The products of the RT-PCR were visualized in 2% agarose gel containing ethidium bromide.

Restriction enzymes analysis

Prior to cleavage by restriction enzymes, the semi-nested RT-PCR-positive products were purified using a DNA purification kit (Wizard Gel and PCR Clean-Up System, Promega Corp., Fitchburg, USA). The restriction procedure was performed using Sma I and Xho I (Promega Corp., Fitchburg, USA) restriction enzymes according to the protocol recommended by the manufacturer.

Phylogenetic analysis

For HDV genotyping, in-house RT-PCR was performed on the RNA samples of 22 patients with chronic hepatitis and on the samples of 18 patients with cirrhosis who had been determined to be RT-PCR-positive [11]. The amplified products visualized in agarose gel electrophoresis at 441 base pair (bp) long were sequenced. This was performed through bidirectional sequencing of each product using F and R primers and an automatic sequencing instrument (Iontec Corp., Istanbul, Turkey).

The alignment of these sequences was performed with ClustalW from a MEGA5.1 program. The phylogenetic relation (phylogenetic tree) of these sequences was investigated using a MEGA5.1 program (PHYLIP) according to the neighbor-joining method. The maximum difference of alignment in the phylogenetic program was calculated according to 0.05.

Results

In the 113 patients co-infected with chronic HBV and HDV, the HDV RNA level was between 2.3 $x10^2$ and $7.8x10^6$ copies/mL, and the median HDV RNA level was 9.6 $x10^3$ copies/mL.

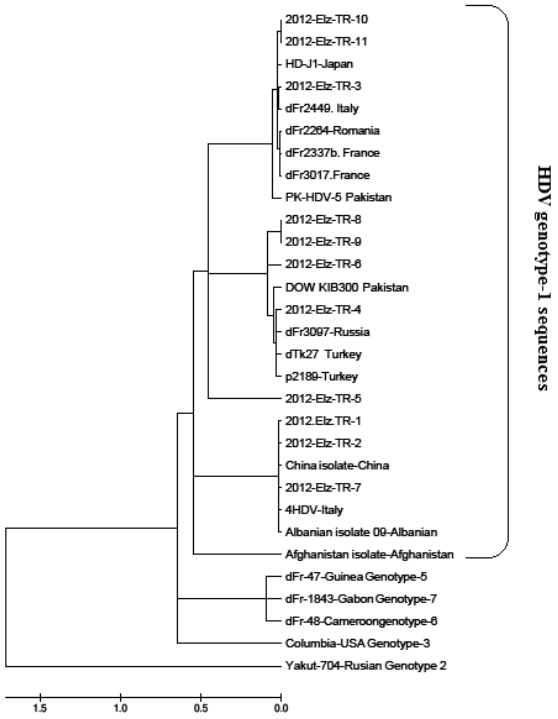
All the patients were HBsAg-positive, with a titer of 1-3803 IU/mL. The median HBsAg titre was 315 IU/mL.

Of the 113 clinical sample studies, approximately 441 base pair-long PCR products were determined in 2% agarose gel electrophoresis of the semi-nested PCR products (data not shown).

In the results of the cleavage of the semi-nested RT-PCR-positive purified products with *Sma I* restriction enzymes, two DNA bands of approximately 219 and 222 bp long were determined. However, these two bands were observed as a single band due to their close proximity to each other (data not shown). As a result of the cleavage of the semi-nested RT-PCR-positive purified products with *Xho I* restriction enzymes, two DNA bands of approximately 382 and 59 bp long were determined. According to the results of the restriction enzyme analysis, all of the semi-nested PCR products were determined to be HDV genotype I.

The results of the alignment analysis of the sequences of 40 products amplified using the semi-nested RT-PCR method revealed a similarity rate of 82%-95%. Furthermore, the comparison between the sequential arrangements in these clinical samples and the HDV genomes present in the gene bank demonstrated 80%-93% similarities according to the BLAST program. The highest sequence similarities (93%) were observed in the HDVs obtained in another in the same region (GeneBank ID: AM779589.1). According to the phylogenetic tree of the sequences that belonged to the clinical samples and the present sequences in the GenBank, it was determined that all of the clinical samples belonged to the HDV genotype I (Figure 1).

Figure 1. The phylogenetic tree constructed by the neighbor-joining method with Kimura two-parameter model distances using the MEGA5 program. The phylogenenetic tree was performed by comparing of the nucleotide sequences of the same gene region of HD viruses obtained from GenBank database and the Turkey isolates (2012-Elz-TR-1 to 2012-Elz-TR-11) amplified by RT-PCR in this study.



Discussion

More than 350 million individuals worldwide are presumed to have chronic HBV infection and more than 15 million of these are presumed to be infected with HDV [10]. This indicates an important global health problem [2,3]. The prevalence of HDV was demonstrated to be high in Turkey in previous studies [7-10]. Considering the number of patients included in the study and taking into account that the study hospital was a regional hospital, it may be concluded that the HDV prevalence is endemic, particularly in the eastern cities of Turkey.

It has been determined that different HDV genotypes are endemic to different geographical regions [12-15]. The studies on this subject are quite limited in Turkey [7,9,16,17]. The HDV genotype has been determined to be genotype I in different regions of Turkey [7,9,16].

The primary aim of this study was to determine the potential different genotypes of HDV in our region by investigating larger patient populations.

The rates of genetic variation between the HDV isolates were 30%-40% between the genotypes and 10%-15% between the isolates of the same genotype. Although the significance of the differences between sequences is still indefinite, it may be responsible for the pathogenic variations related to different genotypes [1]. Amplification of the R0 region within the HDV genome was targeted in both restriction enzyme cleavage and phylogenetic analyses [11]. In this study, a genetic variation of 5%-18% was demonstrated in the sequencing of the R0 regions of HDV genomes of the samples collected from the same period in the same area. The results of alignment analysis shown that high genetic diversity of the virus is a possible in endemic areas such as Turkey. In another recent study [17], three different samples, including samples from the regions of our study, were analyzed, and it was concluded that this difference was quite significant for the sequences obtained in the same region. Therefore,

further studies are planned with regard to the possible reasons of this high genetic diversity and its relationship with viral pathogenesis or epidemiological importance. Furthermore, not all of the genome was sequenced in the present study. Thus, future complete genome studies are planned.

The impact of HDV genotype on disease progression and the success of the therapy are known [12,13,18]. However, since only genotype I had been detected in patients in our study, no comparison of the contribution to the pathogenesis could be made with other genotypes.

Genotyping of the HDV genome using restriction enzymes has been reported in several studies [11,19]. In this study, it was observed, with the restriction enzyme analysis of the R0 region, that all of the patients included in this study were infected with the HDV genotype I. However, it was also observed in the sequence analysis results that two different recognizing sites were present for *Smal I* enzyme in one sample, although one had been expected. Nevertheless, it was concluded that the restriction enzyme analysis is an easy and reliable method when there is no opportunity for sequencing or when not all the RT-PCR samples can be sequenced.

Conclusion

It was observed that HDV is common among patients with HBV in eastern Turkey. The viruses belonged to genotype I in these patients and demonstrated a high genetic diversity in this region. There was no convincing evidence that there was a potential relationship between the genetic variations and the two subgroups (chronic hepatitis and cirrhosis) of patients. Therefore, further studies to determine the possible relationship between HDV genotypes or subtypes and genetic diversity are warranted.

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