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Original Article

Determination of Hepatitis Delta Virus Genotype among HBV Carriers in Southwest of Iran

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Abstract

Background and Aims: HDV is a defective satellite virus and classified in genus Deltavirus. Its disease is related and limited to HBV-infected patients. Acute infection of delta agent occurs in two different patterns; simultaneous infection with both HBV & HDV or super infection of chronically HBV infected patients that lead to more sever type of hepatitis. According to genetic diversity of genomic RNA of HDV, 8 clades have been classified. The aim of this study was to determine the delta virus genotype among the HBV & HDV infected patients in Ahvaz city.

Materials and Methods: Sera sample collected from 31 seropositive HDV patients including 21 male and 9 female mean age 46±13.5 suffering from chronic hepatitis and 1 male patient with acute hepatitis. The encoding region for C terminal half of the Delta anti gene of the HDV's genomic RNA reverse transcribed and then amplified by nested PCR. The HDV PCR positive samples were sequenced, and the sequences were compared with reference sequences on GenBank. All samples were tested for HBV DNA, HCV RNA and HCV anti body.

Results: Only 15(48%) out of 31 anti HDV seropositive patients were positive for HDV RNA by nested RT-PCR. Alignment and phylogenic analysis of the present HDV sequences revealed that all sequences belong to clade 1. Only 1 HDV RNA positive patient was positive for HBV DNA by nested PCR.

Conclusion: According to previous studies the clade 1 (genotype 1) is the predominant clade of HDV in our country. However some (2-HDV-R, 4-HDV-R &11-HDV-R) of our isolates show extensive differences from the two previously isolated HDVs from Iran. Most of the our isolates were closely related to the Iranian HDVs, but Egyptian HDV still remains the most relevant foreign isolate. Suppression of HBV replication by delta virus is common but mutual suppression of HDV and HCV remains unclear.

Keywords: Hepatitis delta; Hepatitis B; Genotype; Phylogenetic Analysis

Introduction

Hepatitis delta virus (HDV) is the only defective pathogen virus in human (1). Since a novel antigen discovered in worldwide but its prevalence among HBsAg carriers varied (3) approximately 5% of chronic HBV infected individuals are also infected with HDV worldwide (9). Prevalence of antibody against HDV in HBsAg carriers ranges from 2.4% in center of Iran and up to 10% in southern part of our country (12). Seroprevalence of HDV in Khuzestan province (south west of Iran) is 3.59% in inactive

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HBsAg carriers and 45.5% in patients with chronic active hepatitis B virus infection (13).

HDV's genomic nucleic acid is a 1.7 kilo base negative sense circular single stranded RNA (4).According to the nucleic acid sequence diversity of HDV genome at least 8 clades have been defined. The most prevalent genotype of HDV is clade 1(genotype 1) (4). Clade1 is related to severe hepatitis leading to liver cirrhosis and hepatocellular carcinoma (5). While clade 2 & 4 are restricted to eastern Asia and cause milder disease compared to clade 1 & 3. Clade 3 was found in north South America and related to severe disease with more frequency of fulminant hepatitis or chronic progressive hepatitis in indigenous population of Indians in this region (6). Recent isolates of HDV from different parts of Africa were classified in clades 5 to 8 (7).

Little is known about replication of viral genome, evidences suggest that it is performed by RNA pol II (8).

Coinfection occurs in two different patterns, acute infection with both HDV and HBV patients that lead to a more severe form of acute hepatitis than HBV alone but usually results in a self-limited hepatitis and the second pattern is superinfection of HBsAg carriers that leads to more sever disease with higher incidence of fulminant hepatitis and usually leads to chronic hepatitis (3, 9 and10). The aim of this investigation was to determine HDV's genotype in seropositive patients in Ahvaz capital city of Khuzestan province located in south west of Iran (11).

Methods

Patient selection

Patients sufferings from chronic hepatitis B with elevated levels of liver functional enzymes checked for HDV antibody and were positive selected for the study. The sera samples were collected and stored at -80 C˚, 22 male and 9 female patients with mean age of 45 ±13.5 ranging from 22 to 77 year old were our study population. The positive control was kindly provided by Keyvan Diagnostic Laboratory in Tehran. Seropositive samples were tested for HDV RNA. The nucleic acid extracted from 200 µl of serum of each individual using High Pure Viral RNA Kit (Roche, Germany) and then immediately cDNA was synthesized with a cDNA Kit (Fermentas AB, Vilnius, Lithuania) in a final volume of 20µl as mentioned in manufacturer's instruction. Amplification of HDV was done by 5 µl of cDNA into PCR master mix containing 20 pmol(0.4 µl) of external sense primer (5'-GCC CAG GTC GGA CCG CGA GGA GGT- 3' nucleotide 858-881) and 20 pmol (0.4 µl) of external anti sense primer (5'-ACA AGG AGA GGC AGG ATC ACC GAC-3' nucleotide 1289-1312) and 0.3 µl (5 U) *Taq* DNA polymerase 1 µl dNTPs (10 mM) 5 µl 10x PCR buffer (Roche Germany) the total volume of reaction was adjusted to 50 µl by adding ddH_2O. The mixture was amplified by 35 cycles of PCR (94˚ C 5 min initial denaturation, 94˚ C f or 1 min, 55˚ C for 1 min, 72˚ C for 65 sec) followed by a 5-min final extension at 72˚ C amplifying 458 bp fragment. Five micro litter of first round PCR product was used as template in the second round of PCR using (0.3 µl) 15 pmol of internal sense primer (5' GAG ATG CCA TGC CGA CCC GAA GAG 3' nucleotide 883- 906), (0.3 µl) 15 pmol of internal anti sense primer (5' GAA GGA AGG CCC TCG AGA ACA AGA 3' nucleotide 1265-1288) and 0.3 µl (5 U) U *Taq* DNA polymerase 1 µl dNTPs (10 mM) 5 µl 10x PCR buffer (Roche Germany) the total volume of reaction was adjusted to 50 µl by ddH_2O. The first round of thermo cycle program was as follow (94˚ C 5 min initial denaturation, 94˚ C 1 min 56˚ C 1 min 72˚ C 55 sec in 35 cycle). The sera samples negative for anti HDV IgM & IgG were used as negative controls in the extraction, cDNA synthesis and PCR steps. The final products analyzed by electrophoresis on 2% agarose gel following DNA safe stain (Sinagene Iran) staining of the gel and visualization under UV trans illuminator (VILBERANT LUMART, France). A 405 bp amplified fragment was visible in HDV positive cases (17). The positive samples were sequenced directionally by ABI genetic analyzer 3110 (USA) and big dye terminator kit (Invitrogen USA) and Raw data were analyzed by mega software version

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Table 1. The result of PCR and ELIASA tests of the samples.

Patient ID	Patient age (year)	Gender F: female M: male	HDV RNA status P: positive N: negetive	HBV DNA status P: positive N: negetive	HCV RNA status P: positive N: negetive	Anti HBc IgG P: positive N: negetive	Anti HBc IgM P: positive N: negetive
1-HDV-R	70	M	P	N	N	P	N
2-HDV-R	41	M	P	N	N	P	N
3-HDV-R	40	M	P	N	N	P	N
4-HDV-R	62	M	P	N	N	P	N
5-HDV-R	50	F	P	N	N	P	N
6-HDV-R	49	F	P	N	N	P	N
7-HDV-R	53	M	P	N	N	P	N
8-HDV-R	39	M	P	N	N	P	N
9-HDV-R	43	M	P	N	N	P	N
10-HDV-R	61	M	P	N	N	P	N
11-HDV-R	73	M	P	N	N	P	N
12-HDV-R	45	F	P	N	N	P	N
13-HDV-R	37	M	P	p	N	N	P
14-HDV-R	50	F	P	N	N	P	N
15-HDV-R	32	M	P	N	N	P	N
16-HDV-R	55	M	N	N	N	P	N
17-HDV-R	27	F	N	N	N	P	N
18-HDV-R	36	M	N	N	P	P	N
19-HDV-R	40	M	N	P	N	P	N
20-HDV-R	51	F	N	P	N	P	N
21-HDV-R	22	F	N	N	N	P	N
22-HDV-R	37	M	N	P	N	P	N
23-HDV-R	77	M	N	N	N	P	N
24-HDV-R	38	M	N	N	N	P	N
25-HDV-R	43	M	N	P	N	P	N
26-HDV-R	36	M	N	N	N	P	N
27-HDV-R	48	F	N	N	N	P	N
28-HDV-R	25	M	N	N	P	P	N
29-HDV-R	26	M	N	N	N	P	N
30-HDV-R	38	F	N	P	N	P	N
31-HDV-R	54	M	N	N	N	P	N

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5.05. Phylogenetic tree obtained after boot strapping 1000 replicates with neighbor joining method and the results are shown in figure 1.

HBV detection

All samples tested for presence of IgG and IgM antibodies against hepatitis B core antigen using two separate ELISA kit (Diapro Italy) which could distinguish acute and chronic infection from each other. Samples were also tested to detect HBV DNA by nested-PCR as previously described (21).

HCV detection

HCV anti body detected by using an ELISA kit (Diapro, Italy) according to manufacturer's instruction. For the positive HCV Ab samples RNA extraction and cDNA synthesis was done as mentioned for the HDV and the nested PCR for detection of HCV was carried out as previously described (15).

Results

Fifteen out of 31 samples were positive for HDV RNA, 2 for HCV RNA and 6 for HBV DNA and the results are shown in tables 1. None of HDV or HBV positive samples were positive for HCV RNA. Only one patient showed simultaneous presence of HDV RNA& HBV DNA in his blood who was HBc IgM positive. This pattern shows the acute coinfection of patient with HBV and HDV. The absence of HBV DNA in anti HBc IgG positive patients demonstrates the super infection of HBV carriers by HDV and further suppression of HBV replication in these patients. The absence of HBV DNA and HDV RNA in patient with HCV viremia can show mutual suppression of these viruses by HCV; However these results can be validated by further follow up of these patients.

The sequencing was successful in thirteen HDV samples. Obtained sequences from these patients were aligned with deposited sequences of HDV genome in GenBank representing all clades. Accession numbers, clade and place of isolation of reference sequences represented in figure 1in front of each branch. It was obviously clear in our Phylogenetic tree that all of the HDV sequences we isolate belonging to clade 1 but tree of these isolates (2-HDV-R, 4-HDV-R &11-HDV-R) show extensively difference with other isolates from Iran and other countries still the Pakistan isolate beside Egyptian isolates remain the most relevant foreign HDV isolates further informations are presented in figure 1. The Phylogenetic tree compares present HDV genotypes with previously reported genotypes from Gene Bank representing all known HDV genotypes.

Discussion

Previous studies in Iran showed that all HDV sequences belong to clade 1(1, 6, 15, and 19). Sequence diversity of C- terminal half of HDAg encoding region among one clade sequences was 15%, and up to 34% between different clades sequences (12). The sequence of HDV genome detected in Ahvaz compared with reported sequences from other part of Iran shows less than 16% for intra genotype (clade1) and less than 36% inter genotype variability and they are at some degree different from previously reported sequences of HDV genome from central Iran. Our results showed that HDV particles in this study belong to clade 1 as other part of Iran reported by Esmaeili *et al* (1). The mean pair wise nucleotide distance among our HDV sequences was 0.108 and nucleotide distance between our sequences and previous Iranian HDV sequences ranges from 0.085 up to o.164. These figures indicate that a tendency for alteration of HDV sequences in different geographical regions of our country therefore, in future it is expected to encounter more diversity in HDV nucleotides sequences detected in Iran, especially considering the nucleotide sequence diversity of HDVs in Turkey, the neighbor of Iran (18).

In the present study only one case has active replication of both HBV and HDV simultaneously, it was due to suppressive effect of HDV on HBV replication in hepatocytes. We assume that presence of HCV has suppressed replication of HBV and HDV. In this project genotypes of HCV were not determined, but in a previous study reported from our city shows that most of HCV isolates belong to genotype 1a (14). Since HBV

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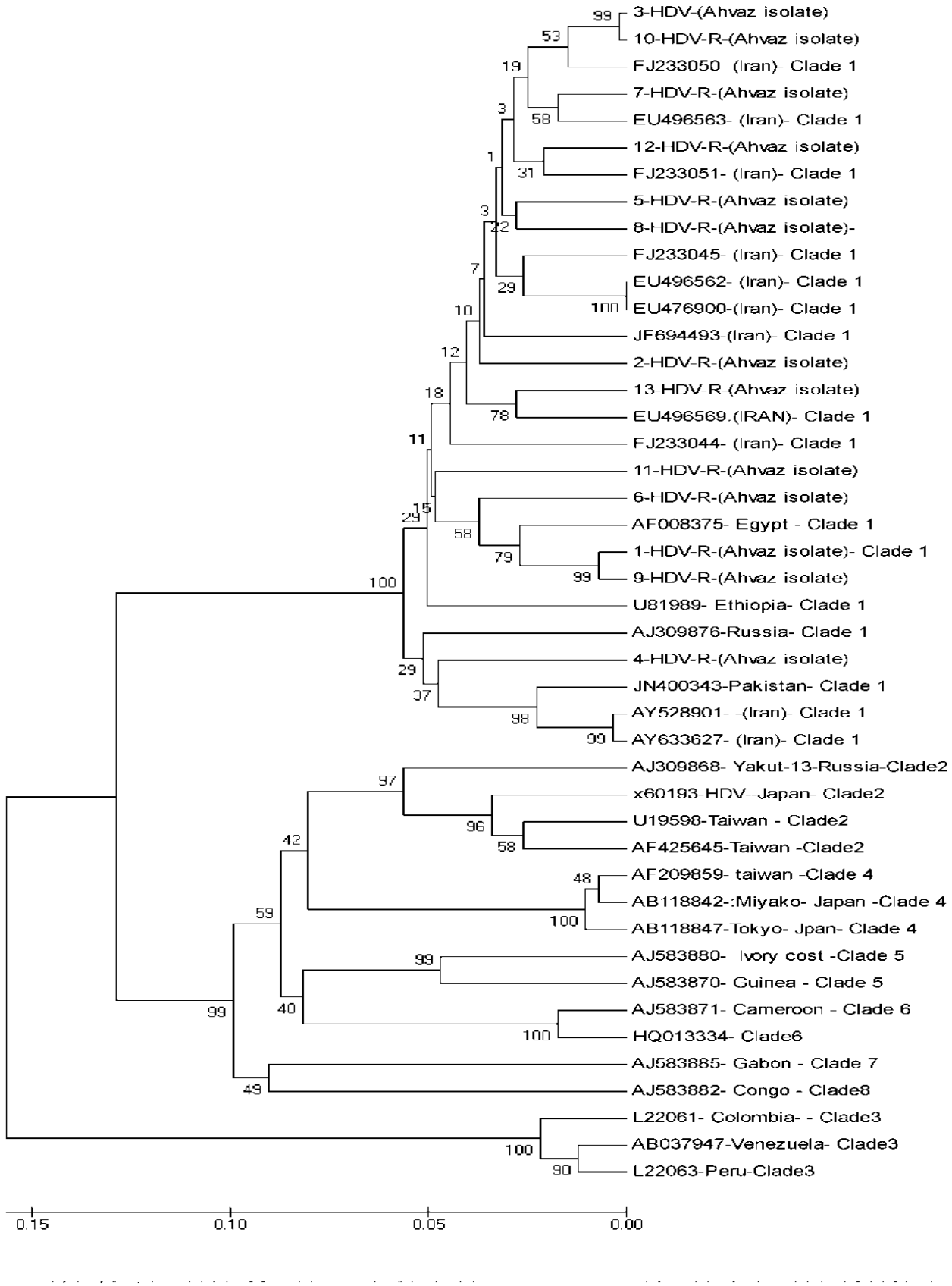

Fig. 1. Phylogenetic Tree

genotype in our city has been reported as genotype D (16) and clade 1 of HDV usually

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present alongside with genotype D of HBV, so molecular epidemiology of HDV is similar to HBV (1).

Conclusion: Our investigation demonstrates that the HDV clade 1 is dominant genotype in Ahvaz similar to other part of Iran (1). This genotype is involved in chronic progressive hepatitis and necessitates regular screening of HDV in HBV infected patients furthermore 2 (6.5%) of patient have triple infection of HBV, HDV and HCV that shows risk of triple infection should not underestimated.

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