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World J Gastroenterol 2005;11(11):1658-1662
World Journal of Gastroenterology ISSN 1007-9327
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• *BRIEF REPORTS* •

"Defective" mutations of hepatitis D viruses in chronic hepatitis D patients

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Supported by grants from the National Science Council (NSC90-2314-B-010-016, NSC91-2314-B-010-080-MH), Taiwan, China
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Received: 2004-10-19 Accepted: 2004-11-29

Abstract

AIM: To verify whether "defective" mutations existed in hepatitis D virus (HDV).

METHODS: Hepatitis delta antigen (HDAg)-coding sequences were amplified using Pfu DNA polymerases with proof-reading activities from sera of five patients with chronic hepatitis D. Multiple colonies were sequenced for each patient. Pfu analyzed a total of 270 HDV clones. Three representative defective HDV clones were constructed in expression plasmids and transfected into a human hepatoma cell line. Cellular proteins were extracted and analyzed by Western blot.

RESULTS: Four of five cases (80%) showed defective HDV genomes in their sera. The percentage of defective genomes was 3.7% (10/270). The majority (90%) of the defective mutations were insertions or deletions that resulted in frameshift and abnormal stop translation of the HDAg. The predicted mutated HDAg ranged from 45 amino acids to >214 amino acids in length. Various domains of HDAg associated with viral replication or packaging were affected in different HDV isolates. Western blot analysis showed defected HDAg in predicted positions.

CONCLUSION: "Defective" viruses do exist in chronic HDV infected patients, but represented as minor strains. The clinical significance of the "defected" HDV needs further study to evaluate.

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Key words: Defective virus; Hepatitis D virus; Hepatitis B virus; Polymerase chain reaction; Hepatitis delta antigen

Wu JC, Hsu SC, Wang SY, Huang YH, Sheen IJ, Shih HH, Syu WJ. "Defective" mutations of hepatitis D viruses in chronic hepatitis D patients. *World J Gastroenterol* 2005; 11(11): 1658-1662
http://www.wjgnet.com/1007-9327/11/1658.asp

INTRODUCTION

The hepatitis D virus (HDV) is about 1.7 kb in genomic length and encodes a single protein, the hepatitis delta antigen (HDAg)[1,2]. The delta antigen exists in two forms: the large and the small HDAg. Both forms are identical in the 195 amino acids (AA) at the amino end, but the large form contains an additional 19 AA at the C-end due to RNA editing[1,2]. There are various domains in the HDAg. The coiled-coil structure (CCS; AA 31-52) is essential for small or large HDAg to form homo-dimers, hetero-dimers, or oligomers; the nuclear localization signal (NLS; AA 68-88) is essential for the nuclear translocalization of HDAg; the RNA-binding domain (RBD; AA 95-146) is essential for HDV RNA binding and subsequent replication. Lastly, the packing signal found in the large form of HDAg (PAS; AA 196-214) is crucial for the packaging of HDV virions. Changes or deletions of these domains usually result in impairment of HDV replication, packaging, or both[1,2].

HDV infection may induce fulminant hepatitis and exacerbations of chronic hepatitis B infection[3-8]. The great majority of patients with HDV superinfection progress to chronic disease[9]. The cause for the high chronicity rate is not completely clear. Recently, we found that selection of escape mutants from quasi-species may be an important mechanism for evasion of virus from immune-system attack[10]. In addition, recombination of HDV RNA sequences may play a role in the evolution and diversity of HDV sequences, which in turn, may contribute to evasion from immune selection and progression to chronic disease[10].

Defective viruses carry mutations in the wild type viral genome. They cannot replicate or package without help from coexisting wild type virus[11]. Defective viruses have been found in many viral infections, and they are associated with modulation of disease course and viral persistence[11]. There were several reports about the stability and heterogeneity of nucleotide sequence of HDV genome[12-15], but there have been no reports of the detection of defective viruses unable to replicate or assemble in human HDV infection or the interactions of wild type and defective HDV in cell

culture system. In the current study, we detected defective viruses from patients with chronic HDV infection.

MATERIALS AND METHODS

Patients

Serum samples were obtained from five patients with chronic hepatitis type D who had been under examination for several years[8-10,12]. Two patients were infected with genotype I HDV and the remaining three were infected with genotype IIa HDV[12,16]. They were positive for serum HBsAg and antibody to HDV antigen (anti-HDV), and were negative for immunoglobulin M antibody to hepatitis B core antigen (Ausria II-125, anti-Delta and CORAB-M; Abbott Laboratories, North Chicago, IL). Serum alanine aminotransferase (ALT) levels were measured by a sequential multiautoanalyzer (Technicon SMAC; Technicon Instruments, Tarry Town, NY).

Reverse transcription polymerase chain reaction

Viral RNA was extracted from 50 μL of serum. Reverse transcription polymerase chain reaction (RT-PCR) using primer #120 (homologous to a sequence from nt 889 to nt 912) and #88 (complementary to a sequence from nt 1663 to nt 1684) was performed as reported previously[10,17,18]. The cDNA was generated in the presence of reverse transcriptase (^{TM}GIBCO BRL, Life Technologies, Rockville, MD) according to the manufacturer's instructions. Each 100 μL of PCR reaction mixture contained 5 μL cDNA, 0.5 μL (5 units/μL) Pfu DNA polymerase (Promega, Madison, WI), 10 μL 10× PCR buffer, 8 μL dNTP mixture (2.5 mmol/L each), 4 μL primer (10 pmol/μL each) and 72.5 μL of water. The PCR was performed in a thermal cycler (Perkin Elmer Cetus Corp., Norwalk, CT). It was started at 95 ℃ for 2 min, followed by 35 cycles (each cycle: 95 ℃ for 20 s, 55 ℃ for 40 s, 72 ℃ for 1 min) of amplification and ended at 72 ℃ for 10 min. The RT-PCR products were analyzed in 2% agarose gel, followed by staining with ethidium bromide. Strict procedures were followed to avoid false positive results[19]. Negative control sera from normal control subjects without viral infection and chronic hepatitis B patients without HDV infection were included in experiments. And the results of the controls were negative.

PCR cloning and sequencing

The amplified PCR products were ligated into the plasmid pCR2 vector (Original TA Cloning Kit, Invitrogen Corporation, Carlsbad, CA) or pCR-Blunt II-TOPO (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's instructions. The ligation mixture was used to transform the competent *E.coli* strain DH5α (Gibco BRL, Life Technologies, Gaithersburg, MD)[10,17]. The colonies were lysed by heating at 95 ℃ for 5 min, followed by direct sequencing using primers #88 and #120[10,17]. Sequencing was performed with a dye terminator cycle sequencing kit (Dye terminator cycle sequencing core kit #402117, Perkin Elmer Cetus Corp., Norwalk, CT) according to the manufacturer's instructions, and sequencing products were analyzed in an ABI 373A sequencer (Perkin Elmer Cetus Corp., Norwalk, CT).

Plasmids for HDAg expression

The cDNA fragments encoding HDAg were obtained by RT-PCR of HDV genomes from one of the five patients. The PCR products were recovered from gel after electrophoresis and then cloned into a commercial TA cloning vector, pCRII (Invitrogen Corp., Carlsbad, CA). The inserted segments in the pCRII were completely sequenced and then cloned into *Xba*I/*Pst*I-digested pCMV-EBNA (Clontech, Laboratories, Palo Alto, CA). In order to be concise, the small and large forms of wild type HDAg derived from TW2479-12S and TW2697-51L are referred to in this paper as HDAg-S and HDAg-L, respectively. The letters TW indicate a Taiwanese origin and the Arabic numerical following TW is the serum sample number of the patient. The numbers 2 479 and 2 697 represent samples collected at different time points from the same patient with chronic hepatitis D. Similarly, HDAg-L-18d, HDAg-S-53d, and HDAg-S-13d represent the corresponding defective HDAgs derived from the TW2479-18, TW2697-53, and TW2479-13 isolates, respectively. HDAg-L-18d had a nucleotide guanine insertion in the HDAg-coding region that resulted in a frameshift and premature stop translation of HDAg due to the generation of a novel stop codon. HDAg-S-53d had a deleted segment (nt 1255 to 1329) that was substituted by a segment (nt 337 to 355) from a different region of the HDAg-coding sequence. This mutation also resulted in a frameshift and premature stop translation of HDAg. HDAg-S-13d had an insertion of two cytosines between the first and the second stop codons of the HDAg. This mutation resulted in the correct translation of a wild type small HDAg, but frameshift translation of the large HDAg. The predicted amino acid sequences of the wild type and defective HDAgs are shown in Figure 1. HDAg-S (24S) is a small HDAg isolate derived from sample TW2476, which came from another patient with genotype IIa HDV infection.

Transfection of cells

The human hepatoma cell line Huh-7 was used for DNA transfection[20,21]. Maintenance of cells and transfection of DNA by the calcium phosphate-DNA co-precipitation method were carried out as previously described[20,21]. In general, cells were seeded onto a 60 mm-diameter dish at 70% confluence one day prior to transfection. After transfection with a total of 10 μg DNA, the cells were incubated for an additional 20 h. The medium was then replaced at 3-d intervals thereafter. To produce virion-like particles (VLPs), the expression plasmid pS1X encoding the three forms of HBsAg was co-transfected with expressing plasmids of whole HDV genome[22], HDAg-S and HDAg-L. VLPs harvested from media 3 and 6 d after transfection were concentrated by centrifugation through a 20% sucrose cushion.

Western blot analysis of HDAg

To detect expression of HDAg, immunoblotting was performed as previously described[23-25]. Transfected cells were lysed in NET buffer containing 50 mmol/L Tris-HCl

Figure 1 Predicted amino acid sequences of the defective or wild type HDAg expressed by the plasmids. Dashes indicate conserved amino acids. The terminations of the wild type and defective HDAgs are indicated by asterisks (*). The coiled-coil domain is doubly underlined. The nuclear localization signal is marked by a thin line. The RNA-binding domain is marked by a thick line. The large HDAg package signal is marked by a hatched bar. The small and large forms of wild type HDAg are referred to as HDAg-S and HDAg-L, respectively. Similarly, HDAg-L-18d, HDAg-S-53d and HDAg-S-13d represent the defective HDAgs derived from the same patient. HDAg-L-18d had an insertion of a nucleotide G in the coding region of HDAg and resulted in frameshift and premature stop translation of HDAg due to the generation of a novel stop codon. HDAg-S-53d contained a segment (nt 1 255 to 1 329) of deletion and substituted by a segment (nt 337 to 355) from different regions of the HDAg-coding sequence. This mutation also resulted in frameshift and premature stop translation of HDAg. HDAg-S-13d had an insertion of two cytosines between the first and the second stop codons of HDAgs. This mutation resulted in translation of a wild type small HDAg, and a frameshift translation of large HDAg.

(pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 1% deoxycholate, and 0.1% SDS. Human polyclonal antiserum with a high titer against HDAg was diluted 1:2 000 and used as primary antibody. The secondary antibody was HRP-conjugated goat anti-human antibody (Sigma, St. Louis, MO). To detect HDAg in released particles in either serum or culture medium, 1 mL culture medium from transfected cells was ultracentrifuged and particles were pelleted. Then pellets were treated and immunoblotted as cellular proteins. Membranes were finally developed with a Western blot chemiluminescence reagent (NEN Life Science, Boston, MA).

RESULTS

Detection of defective HDV genomes in human sera

In order to determine if PCR procedures will generate defective HDV genomes observed in this study, RT-PCR cloning followed by direct sequencing of serum HDV from the five patients were conducted using Pfu DNA polymerase with proofreading activity. In this study, defective mutations were focused on the HDAg-coding sequence. Variants with mutations that created novel stop codons or frameshift translation of HDAg were defined as defective HDV genomes. HDV genomes that encoded complete large or small HDAgs without frameshift or premature stop translation were defined as wild type. A total of 270 HDV clones from the five patients were analyzed. As shown in Table 1, all except one case showed defective HDV genomes in quasi-species of HDV in their sera. The percentage of defective genomes amplified was 3.7% (10/270). Four of the five patients had detectable defective HDV genomes in their sera. One of the wild type HDV plasmids had been amplified using Pfu, and the PCR products were transformed and cloned to serve as control. None of the 75 HDV clones showed defective mutations that resulted from PCR.

Of the 10 defective HDV mutations based on the amplification by Pfu DNA polymerase, only one (10%) was a transition that resulted in a novel stop codon and the majority (90%) were insertions or deletions that resulted in frameshift and abnormal stop translation of HDAg. The predicted mutated HDAg ranged from 45 amino acids to >214 amino acids in length. Various domains of HDAg including CCS, NLS, RBD and PAS were affected in different defective HDV isolates (Figure 1). Most defective mutations occurred randomly within the HDAg-coding region, but identical mutations could be found in two patients (patients D and E). In the patient (case no. E in Table 1) whose long-term follow-up sera were available for analysis, identical defective HDV clones were found at different time points. Because this patient had relatively higher percentage of defective HDV clones, the dominant defective HDV clones could be reproducibly obtained using Pfu. This patient cleared both HBV and HDV and went into biochemical remission after an increase of defective HDV clones up to 50% (2/4) of viral populations. This patient had not received anti-viral treatment before remission.

Table 1 Percentages of defective viral genomes in HDV genomic populations in the sera of patients with chronic hepatitis D

Patients	HDV genotype	Number of clones defective/analyzed (%)
A	IIa	0/36 (0)
B	I	1/52 (1.9)
C	I	2/60 (3.3)
D	IIa	3/58 (5.2)
E	IIa	4/64 (6.2)
Total		10/270 (3.7)

Expression of defective HDAg in cell culture

Of the viral genomic variants analyzed, three representative defective HDV clones and their wild type partners were cloned in expression plasmids and co-transfected into Huh-7 human hepatoma cells to observe viral interactions. To study if HDAg proteins were expressed by the defective HDV genomes, wild type and defective expression plasmids of HDAg were transfected into the Huh-7 hepatoma cell line. Cellular proteins were extracted, electrophoresed, and immunoblotted. As shown in Figure 2, wild type small and large HDAg proteins (abbreviated as HDAg-S and HDAg-L, respectively) were expressed with the expected electrophoretic sizes. Defective HDAg proteins (abbreviated as HDAg-L-18d and HDAg-S-53d, respectively) were also expressed, but in different molecular weights due to the underlying mutations. HDAg-S-13d had a mutation between the stop codons for the small and large HDAg-S. Therefore, wild type small HDAg was expressed and moved to an expected size by electrophoresis.

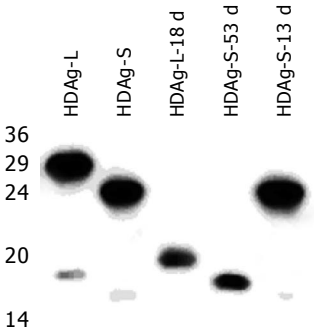

Figure 2 Western blot analysis of wild type and defective HDAgs. The wild type and defective expression plasmids of HDAg (see Methods and Figure 1) were transfected into the Huh-7 hepatoma cell line. Cellular proteins were analyzed by Western blotting. Wild type small and large HDAgs (HDAg-S and HDAg-L) were in proper positions and served as controls. Defective HDAgs (HDAg-L-18d and HDAg-S-53d) were of different molecular weights due to mutations. HDAg-S-13d had a mutation between the stop codons for the small and the large HDAg. Therefore, the small HDAg was expressed and was electrophoretically correct.

DISCUSSION

Defective viruses have been reported in many viral infections[11,26-29]. Defective hepatitis B and C viruses have also been reported[30,31]. Defective viral genomes may interfere with replication and suppress the direct cytotoxic effect of the wild type viruses, or they may induce immunity that protects hosts[11,26-30]. Defective genomes may also be a factor contributing to persistent viral infection[11]. However, there has been no report of defective viral genomes in human HDV infection. This study showed that defective viral genomes are very common (at least 80%) in HDV infections. If more HDV clones were analyzed, we suspect defective HDV genomes might be found in all cases with HDV infection. However, the amounts of defective viral genomes are rather low (usually <10% of viral population) compared to the amounts of wild type in HDV quasi-species. Therefore, defective viral genomes cannot be detected by screening only a few HDV clones. Direct sequencing of RT-PCR products of serum HDV genomes can only discover variants for more than 20% of viral populations[14].

One may argue that these mutations may result from fidelity problems in PCR due to the lack of proofreading activity of the polymerase[32,33]. To test this possibility, we conducted our experiment using Pfu DNA polymerase with proofreading activity. Pfu is currently reported to be the enzyme with the lowest error rate[32,33]. The average error rates (mutation frequency/bp/duplication) of various DNA polymerases have been reported as follows: Pfu (1.3×10^{-6}) <Deep Vent (2.7×10^{-6}) <Vent (2.8×10^{-6}) <Taq (8.0×10^{-6})[33]. Previous studies reported defective genome of HBV and HCV were using Taq DNA polymerases[30,31]. In the current study, defective viral genomes of HDV were detected using Pfu DNA polymerases. The percentages of defective HDV genomes are much higher than those that could be attributed to PCR error rates[32]. Moreover, the 75 HDV clones amplified and cloned from a wild type HDV plasmid did not show any defective mutation resulted form PCR. Therefore, the defective HDV genomes detected in this study were not likely due to PCR errors. In the study of HDV sequence stability by Netter *et al*[14], neither insertions nor deletions were detected in the finally cloned HDV sequences amplified by Taq polymerase from woodchuck serum after transfection of a single cDNA clone of HDV into a human hepatoma cell line followed by the innoculation of the assembled HDV particles in the medium into woodchucks for a total of six passages in different woodchucks. In previous studies evaluating the errors generated by PCR based on different DNA polymerases, most of the PCR errors were found to be transitions or transversion of nucleotides[32,33]. And the Taq polymerase has a higher single nucleotide substitution rate, especially a transition rate, compared to Pfu. While in the current study, the great majority (90%) of the defective mutations were insertions or deletions that resulted in frameshift and abnormal stop translation of the HDAg. This is also an evidence to support the existence of defective HDV genomes in human infection, which gradually accumulated after long history of evolution. Another finding to support the existence of defective HDV is the isolation of a unique defective clone, HDAg-S-53d, with two deleted segments (nt 1 255 to 1 329 and nt 1 627 to 1 635, respectively) and a replaced segment (nt 337 to 355) from a different region of the HDAg coding sequence. A frank mutation like this is unlikely to result from PCR error reported previously[32,33].

HDV is a RNA virus that replicates using a RNA polymerase lacking proofreading activity, a fact that contributes to a fast evolutionary rate. Mutations are expected to occur after several runs of replication in a large population of the HDV genome. In a recent report of transfection of HDV RNA into HuH7 hepatoma cells, they found that the polymerase was able to make an intramolecular template switch[34]. Furthermore, this switch produced small deletions of template sequences and in some cases even insertion of non-templated sequences. Although their experiments are not completely comparable to human infection, the results are at least supportive to the finding of naturally occurring defective viral genomes in human HDV infection. In the experiment of "serial passage of

hepatitis delta virus in chronic hepatitis B virus carrier chimpanzees", an effective infectious dose was estimated to be about 10 HDV genomes per mL. Although, defective HDV mutations were not described in that report, it is possible that some HDV genomes in the inoculum may be defective and a higher concentration of viral inoculum containing sufficient wild type HDV may be needed to induce effective infection in chimpanzees.

From another point of view, the detection rate of defective HDV may have been underestimated in the current work because some defective HDV genomes may not be secreted into serum. In addition, this study focused only on the HDAg-coding sequence. If more clones of whole HDV genome had been analyzed, the detection rate might have increased. The actual incidence and percentages of defective viral genomes in HDV quasi-species may vary depending on individual patients and the experimental conditions used. Nevertheless, the current study clearly indicates that defective HDV does exist in chronic HDV infection.

In summary, defective HDV genomes do exist in chronic HDV infection of humans. The clinical implication of the "defective" HDV needs further study.

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Science Editor Li WZ **Language Editor** Elsevier HK