Molecular Epidemiology of Hepatitis D Virus Infection among Injecting Drug Users with and without Human Immunodeficiency Virus Infection in Taiwan

Sui-Yuan Chang,1,2 Chia-Ling Yang,3 Wei-Shin Ko,1 Wen-Chun Liu,4 Chi-Ying Lin,3 Cheng-Hsin Wu,4 Yi-Chin Su,1 Shu-Fang Chang,1 Mao-Yuan Chen,4 Wang-Huei Sheng,4 Chien-Ching Hung,4,5 and Shan-Chwen Chang4

Department of Clinical Laboratory Sciences and Medical Biotechnology, National Taiwan University College of Medicine, Taipei, Taiwan1; Departments of Laboratory Medicine2 and Internal Medicine,4 National Taiwan University Hospital and National Taiwan University College of Medicine, Taipei, Taiwan; and Department of Internal Medicine, National Taiwan University Hospital Yun-Lin Branch, Douliou City, Yun-Lin County, Taiwan3

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An outbreak of human immunodeficiency virus (HIV) infection occurred among injecting drug users (IDU) in Taiwan between 2003 and 2006, when an extremely high prevalence of hepatitis C virus (HCV) infection was also detected. To determine whether clusters of hepatitis D virus (HDV) infection occurred in this outbreak, 4 groups of subjects were studied: group 1, HIV-infected IDU (n = 904); group 2, HIV-infected non-IDU (n = 880); group 3, HCV-uninfected IDU (n = 211); and group 4, HCV-uninfected non-IDU (n = 1,928). The seroprevalence of hepatitis B virus (HBV) was 19.8%, 18.4%, 17.1%, and 6.7%, and HDV seroprevalence among HBV carriers was 75.4%, 9.3%, 66.7%, and 2.3%, for groups 1, 2, 3, and 4, respectively. Ninety-nine of 151 (65.6%) HDV-seropositive IDU had HDV viremia: 5 were infected with HDV genotype I, 41 with genotype II, 51 with genotype IV, and 2 with genotypes II and IV. In the phylogenetic analysis, only one cluster of 4 strains within the HDV genotype II was identified. Among patients with HCV viremia, a unique cluster within genotype 1a was observed; yet, patients within this cluster did not overlap with those observed in the HDV phylogenetic analysis. In summary, although IDU had a significantly higher HDV seroprevalence, molecular epidemiologic investigations did not support that HDV was introduced at the same time as HCV among IDU.

Hepatitis D virus (HDV) is a defective, satellite virus that requires a helper function provided by hepatitis B virus (HBV) (25). It has been estimated that approximately 5% of HBV carriers are coinfected with HDV, leading to an estimated 15 million persons infected with HDV worldwide (10). HDV is divided into 8 major genotypes: genotype I, distributed worldwide; genotype II, mainly in Asia; genotype III, in South America; genotype IV (genotype Ib by old nomenclature), in Taiwan and the Okinawa islands; and genotypes V to VIII, in west and central Africa (9, 35). Most studies suggest that the majority of HDV infection is acquired through parenteral and sexual routes (16, 22, 28), which are also important routes for human immunodeficiency virus (HIV) transmission. In HIV-uninfected patients with chronic HBV infection, HDV coinfection may suppress HBV replication, with subsequent clearance of HBsAg (6, 13, 17), by exerting an inhibitory effect on the host DNA-dependent RNA polymerase that is involved in HBV transcription (12, 20); however, HDV coinfection may lead to exacerbation and rapid progression of chronic liver disease, hepatic failure, and deaths in patients with HBV infection (22, 28).

It is estimated that 6 to 10% of HIV-infected patients in Western countries have HBV coinfection (8, 33). Coinfection with HBV has been shown to increase the risk of acute hepatitis, hepatic decompensation, liver-related mortality, and virological failure in HIV-infected patients receiving highly active antiretroviral therapy (HAART) (8, 26, 33). For patients with HIV infection, clinical studies of the impact of HDV infection on patients with HBV and HIV coinfection were limited and yielded inconsistent results before the introduction of HAART (2, 11, 21, 24). Some investigators suggested that HIV coinfection might worsen chronic liver damage by HDV (11, 22) and that patients with chronic HDV infection were more likely to develop cirrhosis than patients with HBV monoinfection (1), while others showed that the course of chronic HDV infection was not influenced by concomitant HIV infection (2, 21, 24). These discrepancies may be related to patient selection and the shorter survival of patients before the introduction of HAART. With the introduction of HAART in 1996, the improved survival of HIV-infected patients may have allowed complications and liver deaths related to chronic hepatotropic virus infections to emerge (27, 31).

In Taiwan, the prevalence of chronic HBV infection is estimated to be 15 to 20% among individuals born before the implementation of a nationwide HBV vaccination program in 1984. In contrast to what has been observed in Western countries, we have found that the HBV seroprevalence among injecting drug users (IDU) is similar to that of other groups at risk for HIV transmission (32). Between 2003 and 2006, an outbreak of HIV infection and hepatitis C virus (HCV) infection occurred among IDU who shared needles and diluent in...
Taiwan (4, 7). In this outbreak, the seroprevalence of HCV infection was as high as 98% in HIV-infected IDU (19), and several HCV genotypes that had never been described previously were introduced into Taiwan. With the preexisting high HBV seroprevalence in Taiwan, we postulated that a high HDV seroprevalence would be observed with the HIV-infected IDU and clusters of HDV infection may have occurred among IDU with chronic HBV infection who became HIV infected in this outbreak. In this study, we aimed to investigate the molecular epidemiology of HCV and HDV infection among persons at risk for HIV transmission in Taiwan.

(Preliminary analyses of the data from this study were presented as abstract no. PE13.3/2 at the 12th European AIDS Conference, Cologne, Germany, 11 to 14 November 2009.)

MATERIALS AND METHODS

Setting and study subjects. The first case of HIV infection in Taiwan was detected in 1984, and several programs of screening for HIV were implemented subsequently. Mandatory HIV testing has been provided to inmates in Taiwan since 1990, while programs of free anonymous HIV counseling and testing were implemented in 1997. As of 31 December 2009, 18,378 cases of HIV infection since 1990, while programs of free anonymous HIV counseling and testing were subsequently. Mandatory HIV testing has been provided to inmates in Taiwan since 1997. As of 31 December 2009, 18,378 cases of HIV infection were reported to the Taiwan Centers for Disease Control. Following the implementation of a harm reduction program in 2005 that we observed the numbers of cases of HIV-infected IDU increased rapidly, and it was not until the introduction of a harm reduction program in 2005 that we observed the numbers of cases of HIV-infected IDU increased rapidly, and it was not until the implementation of a harm reduction program in 2005 that we observed the rapid decline of the numbers of cases. However, the cumulative proportion of IDU has increased from less than 2% before 2002 to 34.4% in 2009. Between April 2006 and April 2009, blood samples were collected from four groups of subjects for determinations of serore prevalences of HBV and HDV infections: group 1, HIV-infected IDU; group 2, HIV-infected non-IDU; group 3, HIV-uninfected IDU; and group 4, HIV-uninfected non-IDU. The IDU of group 1 were newly diagnosed with HIV infection upon entry into prisons between 2003 and 2006, when the HIV outbreak occurred among IDU in Taiwan. The IDU of group 1 were newly diagnosed with HIV infection upon entry into prisons between 2003 and 2006, when the HIV outbreak occurred among IDU in Taiwan. Most of the individuals were not continuously in prison, although re-cidivism and reincarceration were frequent. After the IDU were released from the prison, they were enrolled in an outreach program and case management program for HIV care, after giving informed consent, in which blood sampling and basic HIV care, including the provision of clean needles and condoms and determinations of plasma HIV RNA load and CD4 cell counts, were provided. Non-IDU were enrolled at the HIV clinic and a voluntary counseling and testing (VCT) site for HIV in the National Taiwan University Hospital located in northern Taiwan. A total of 130 (80.2%) HIV-infected non-IDU were receiving HAART when blood sampling was performed. For HIV-infected IDU, only 27 (19.4%) were receiving HAART during their incarceration, but HAART was interrupted after they were released from the prisons. A standardized case collection form was used to record data for demographics and clinical characteristics. This study was approved by the Institutional Review Board of the hospital, and subjects gave informed consent.

Laboratory investigations. (i) Determinations of CD4 count and plasma HIV RNA load. In Taiwan, HIV-related care, including HAART and laboratory tests to monitor virological and immunological status before or after initiation of HAART, is provided free of charge to patients in designated hospitals around Taiwan since HAART was introduced into Taiwan in 1997. For HIV-infected individuals, plasma HIV RNA load and CD4 cell counts were quantified by the Cobas Amplicor HIV-1 monitor test, version 1.5, (Roche Diagnostics Corporation, Indianapolis, IN) and FACSFLOW (Becton Dickinson), respectively.

(ii) Serologic tests for hepatotropic viruses. Hepatitis B surface antigen (HBsAg) and anti-HDV antibody were determined using the HBsAg enzyme-linked immunosorbent assay (ELISA) kit and AUSAB-EIA kit (Abbott Laboratories, Abbott Park, IL), respectively. Anti-HCV antibody was determined by the anti-HCV ELISA kit (AxSYM HCV III; Abbott Laboratories, North Chicago, IL).

(iii) PCR. Viral RNA was extracted using the QIAamp viral RNA minikit (Qiagen, CA). The purified RNA was subjected to nested reverse transcription-PCR. The HDV delta gene fragment (nucleotides [nt] 856 to 1275 relative to HDV reference strain JA-M27), HCV NS5B fragment (nt 8260 to 8615 relative to HCV reference strain H77), and HIV partial genome fragment (nt 864 to 1275 relative to HIV reference strain 9129-M) were amplified by PCR. A standard PCR condition for HIV was briefly described below. The first primer pair used was HDV850 as the forward primer and HDV1275 as the reverse primer.
FIG. 1. Seroprevalence (%) of HBV and HDV among the four groups of subjects. The seroprevalences of HBV (gray bar) and HDV (black bar) among the four groups of subjects between April 2006 and April 2009 are individually displayed. The proportions of patients seropositive for HDV among the HBV carriers are indicated above the column for each of the four groups.

TABLE 2. Clinical characteristics of patients with HDV viremia

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Genotype I</th>
<th>Genotype II</th>
<th>Genotype IV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HIV⁺</td>
<td>HIV⁻</td>
<td>HIV⁺</td>
</tr>
<tr>
<td>No. of patients</td>
<td>3</td>
<td>2</td>
<td>39</td>
</tr>
<tr>
<td>Age, median (range), yr</td>
<td>44 (43–45)</td>
<td>43 (42–43)</td>
<td>35 (26–46)</td>
</tr>
<tr>
<td>Male gender, no. (%)</td>
<td>3 (100)</td>
<td>2 (100)</td>
<td>36 (92.3)</td>
</tr>
<tr>
<td>Risk behavior (no.)</td>
<td>3</td>
<td>0</td>
<td>37</td>
</tr>
<tr>
<td>IDU</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>MSM</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Heterosexual</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Others</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CD4, median (range), cells/µl</td>
<td>288 (252–324)</td>
<td>NA</td>
<td>333 (67–654)</td>
</tr>
<tr>
<td>HIV-1 RNA viral load, median (range), log₁₀ copies/ml</td>
<td>4.29 (3.68–4.53)</td>
<td>NA</td>
<td>4.26 (2.60–4.86)</td>
</tr>
<tr>
<td>HCV status, no. (%)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>7 (17.9)</td>
</tr>
<tr>
<td>HCV genotype, no.</td>
<td>1a</td>
<td>1b</td>
<td>3a</td>
</tr>
<tr>
<td>1a</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1b</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3a</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>6a</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6n</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>1a/6n</td>
<td>0</td>
<td>0</td>
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</table>

Abbreviations: IDU, injecting drug users; MSM, men who have sex with men; NA not applicable.
seroprevalence than non-IDU in this study; 74.0% of IDU compared to 6.2% of non-IDU were seropositive for HDV ($P < 0.001$). Of the 151 IDU who were seropositive for HDV, none received interferon therapy for HDV infection and 99 (65.6%) had HDV viremia by PCR. Among HIV-infected IDU, those with HDV viremia were significantly younger than those without HDV viremia (33 versus 38 years, $P = 0.04$). HCV PCR was performed, and for those with HDV viremia, more HIV-infected IDU had HCV viremia than HIV-uninfected IDU (22.1% versus 15.4%, $P = 0.58$).

**HDV phylogenetic analysis.** Of the 99 IDU subjects with HDV viremia, 5 were infected with HDV genotype I, 41 with genotype II, 51 with genotype IV, and 2 were coinfected with genotypes II and IV (Table 1). The baseline characteristics of the patients with HDV viremia were stratified based on HDV genotypes (Table 2). There were no statistically significant differences in terms of age, gender, risk behavior, and CD4 and plasma HIV RNA load between HIV-infected patients who were coinfected with HDV genotype II and those with HDV genotype IV. The patients who were infected with HDV genotype I were older than those infected with genotype II (43 versus 35.5 years, $P = 0.01$) and genotype IV (43 versus 32.5 years, $P = 0.02$). None of the HDV genotype I-infected individuals had HCV viremia. In the phylogenetic analysis, only one cluster of four strains within HDV genotype II was identified (Fig. 2), and these sequences did not cluster with those previously described for Taiwan. All of the 4 patients in this cluster were IDU. No cluster was observed for the strains of HDV genotypes I and IV.

When the results of population sequencing were analyzed, 3 of 41 genotype II (7.3%) and 8 of 50 genotype IV (16.0%) HDV-positive specimens from IDU, but none from non-IDU, were found to contain variant sequences. PCR products of the specimens were subjected to TA cloning, and at least 4 clones from each specimen were sequenced for comparison. Except for one specimen (YL-329), which exhibited sequences in different clusters within genotype IV, others had cloned sequences close to each other and of the same origin in the phylogenetic tree (Fig. 2).

**HCV phylogenetic analysis.** Of the specimens collected from the subjects who had HDV viremia, 21 of 109 specimens (19.3%) had detectable HCV viremia, and all of them were from IDU. Of these HCV-positive specimens, 8 were HCV

![Phylogenetic analysis of hepatitis D virus](http://jcm.asm.org/)

FIG. 2. Phylogenetic analysis of hepatitis D virus. A 419-bp fragment covering the HDV delta gene fragment (nt 856 to 1275 relative to HDV reference strain JA-M27) was used to construct the phylogenetic tree to analyze the relationships between the HDV isolates in this study. The study and reference sequences were aligned using the Clustal W program with minor manual adjustment. The tree was constructed by the neighbor-joining method based on the Kimura 2-parameter distance matrix listed in the MEGA software (version 3.0) (14). The horizontal branches are drawn in accordance with their relative genetic distances. Bootstrap values greater than 700 of 1,000 replicates were considered significant and are indicated at the nodes of the corresponding branches. The brackets at the right indicate the major sequence genotypes. All the sequences from Taiwan are labeled with filled diamonds. A hepatitis D virus, genotype III, isolated among Yuca Indians in Venezuela (AB037949) was used as an out-group strain for phylogenetic analysis.
genotype 1a, 4 were genotype 1b, 4 were genotype 3a, 3 were genotype 6a, 1 was genotype 6n, and 1 was coinfected with genotype 1a/6n. The genotype 1a sequences formed a unique cluster in the phylogenetic tree (Fig. 3), while their corresponding HDV sequences did not cluster (4 were genotype II, and 3 were genotype IV) (Fig. 2). Two other HCV clusters were observed for 6a and 6n, but the corresponding HDV sequences from the two patient pairs did not cluster in the HDV phylogenetic tree either (Fig. 2).

**DISCUSSION**

In this study, we have found that IDU, regardless of HIV serostatus, had a significantly higher HDV seroprevalence than non-IDU in Taiwan. A lower frequency of genetic relatedness of those HDV strains compared with HCV strains from IDU suggests that in areas where HBV infection is hyperendemic, such as Taiwan, HDV may have been introduced into the population at risk at different time points before or after transmission of HIV infection occurred when IDU continued to share needles and diluent.

In Taiwan, sexual contact and needle sharing are two major routes for HDV transmission. Compared with patients with HBsAg-positive chronic liver disease, for which the prevalence of HDV infection was 5 to 8% in Taiwan (5), prostitutes (39.6%) and IDU (78.9%) had much higher prevalences of HDV infection (15, 38). In this study, we also found that HDV seroprevalence among IDU was significantly higher than that among subjects who were at risk for HIV transmission through sexual contact (74.0% versus 6.2%, \( P < 0.001 \)). Because of the pre-existing high seroprevalence of chronic HBV infection in Taiwan, it is not unexpected that HDV seroprevalence will be high in our study populations. Given the similar seroprevalences of chronic HBV infection between IDU and HIV-infected non-IDU, the findings of a higher HDV seroprevalence among IDU suggest that sharing needles and diluent is much more efficient in HDV transmission than sexual contact or that a higher frequency of exposure to HDV among IDU may contribute to a higher cumulative prevalence of HDV infection.

Our study is the first one to describe the molecular epidemiology of HDV among IDU in Taiwan. In Taiwan, genotype II accounted for 85.4% (35/41) of the patients with HDV superinfection in 1995 (37). The genotype distribution reported by Su et al. in 2006 appeared to have changed, with genotypes I (26.3%, 51/194) and II (38.1%, 74/194) being dominant and genotype IV accounting for a small proportion (4.1%, 8/194) (29). In this current study, HDV genotype II (39.4%, 43/109) and genotype IV (54.1%, 59/109) became the two major genotypes, and genotype I accounted only for a matrix listed in the MEGA software (version 3.0) (14). The horizontal branches were drawn in accordance with their relative genetic distances. Bootstrap values greater than 700 of 1,000 replicates were considered significant and are indicated at the nodes of the corresponding branches. The brackets at the right indicate the major sequence genotypes. All the sequences from Taiwan are labeled with filled diamonds. West Nile virus sequence (NC001563) was used as an out-group strain for phylogenetic analysis.

**FIG. 3.** Phylogenetic analysis of hepatitis C virus. A 380-bp fragment covering the partial NS5B gene (nt 8260 to 8615 relative to HCV reference strain H77) was used to construct the phylogenetic tree to analyze the relationships between the HCV isolates in this study. The study and reference sequences were aligned using the Clustal W program with minor manual adjustment. The tree was constructed by the neighbor-joining method based on the Kimura 2-parameter distance
small proportion of HDV infection (4.6%, 5/109). The different genotype distributions between our study and a previous study from Taiwan could be due to different study populations (29). In Su’s study, those subjects who were seropositive for HCV and HIV were excluded from enrollment, while the majority of HDV-seropositive individuals were also seropositive for HCV and/or HIV in our study.

It has been suggested that HDV genotypes may influence the outcome of disease progression. In one previous study from Taiwan, patients infected with HDV genotype I had a lower remission rate and poorer outcomes than patients infected with genotype II (29). Genotype IV was also believed to cause a relatively mild disease like genotype II (36). Whether the predominance of genotypes II and IV in our study subjects may indicate a better outcome remains to be closely monitored because the influence of HCV and HIV coinfection cannot be overlooked.

In the outbreak of HIV-1 and HCV coinfection among IDU in Taiwan between 2003 and 2006 (4, 7, 18), the great majority of these newly diagnosed HIV-infected IDU were infected by the CRF07_BC strain, which circulates mainly in northern China (23, 30). The CRF07_BC strain is most likely disseminated among IDU, because it was not detected in HIV-infected patients of other risk groups in Taiwan in the first 2 decades of the HIV epidemic (3). Several new HCV genotypes, such as 3a, 6a, and 6n, and clusters of HCV in phylogenetic analyses have been detected among HIV-infected IDU in this outbreak (19), suggesting that, similar to HIV, some of the HCV strains have also been introduced into Taiwan along the drug trafficking routes. Based on these previous findings, we aimed to determine whether HDV was also introduced to IDU with HIV CRF07_BC/HCV. We found that, although several sequence clusters in the HDV phylogenetic tree were observed, the corresponding HCV sequences did not cluster in the HCV phylogenetic tree and vice versa. In addition, no clustering of the corresponding HIV in the HCV phylogenetic tree was observed (see Fig. S1 in the supplemental material). Such findings suggest that HDV infection was not introduced at the same time as HCV among IDU in Taiwan.

There are several limitations in our study. First, it is a cross-sectional study, and without analyzing sequential blood specimens of the four groups of patients with chronic HBV infection, we are not able to know when HDV infection may have occurred and whether the absence of HDV viremia in patients seropositive for HDV was due to recovery from acute infection. Second, quantifications of the plasma viral loads for HBV, HCV, and HDV were not performed, which precluded us from delineating the interactions among the three hepatotropic viruses, in terms of changes in viral load. Third, with the use of population sequencing, we may not be able to detect the HDV strains that were minor strains, if concurrent or sequential infections due to multiple HDV strains ever occurred among IDU or non-IDU.

In conclusion, the seroprevalence of HDV infection among HBV carriers was significantly higher in IDU compared to the non-IDU population. Phylogenetic analyses suggest that HDV was not introduced to the IDU population simultaneously during the HIV and HCV outbreak among IDU between 2003 and 2006 in Taiwan. Because HDV/HIV/HCV coinfection might accelerate progression of chronic viral hepatitis to cirrhosis of the liver, public health measures are urgently needed to prevent further dissemination of HDV among IDU in Taiwan.

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REFERENCES


