Transmission and evolution of hepatitis C virus in HCV seroconverters in HIV infected subjects

Chengli Shen a, Phalguni Gupta a,⁎, Xiaochuan Xu a, Anwesha Sanyal a, Charles Rinaldo a, Eric Seaberg b, Joseph B. Margolick b, Otoniel Martinez-Maza c, Yue Chen a,⁎⁎

a Department of Infectious Diseases and Microbiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, PA 15261, United States
b Department of Epidemiology, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD, United States
c Department of Epidemiology, UCLA School of Public Health, I.A., CA, United States

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HIV/HCV co-infection provides a model to determine the role of immunity on HCV transmission and evolution. In this study HCV transmission and evolution were evaluated in 6 HCV seroconverters in HIV-infected subjects with a wide range of CD4 cell count. The HCV envelope E1/E2 sequences were analyzed for transmission bottleneck, viral diversity/divergence, immune pressure, and mutations of HLA class I/II restricted epitopes. HCV infection started with transmission bottleneck in all HIV-infected individuals. During the 1.0–2.0 years of infection there was a shift of viral quasispecies in majority of the subjects from one to next visit. However, HCV diversity, divergence, mutations in HLA class I/II restricted and virus neutralizing epitopes were similar in all subjects regardless of CD4 cell count at the time of HCV infection. Our results suggest that HCV transmission and evolution in HIV-infected subjects may not be influenced by host CD4 cell count at the time of infection.

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Introduction

Hepatitis C virus (HCV) is a RNA virus that currently infects over 150 million people across the world. While a majority of infected subjects become chronically infected, which leads to chronic hepatitis, cirrhosis and hepatocellular carcinoma, approximately 25% of HCV-infected individuals spontaneously clear HCV during the first year of infection (Micallef et al., 2006). Since human immunodeficiency virus (HIV) and HCV share similar routes of transmission, such as exposure to contaminated blood and sexual activity, approximately 25% of HIV-infected persons are co-infected with HCV, which is much higher than the HCV prevalence in the general world population (Ghosn et al., 2006; Urbanus et al., 2009). HIV/HCV co-infection has been shown to decrease the probability of spontaneous HCV clearance and increase the rate of progression to cirrhosis, decompensated liver disease, hepatocellular carcinoma, and death (Lewden et al., 2005; Weber et al., 2006). Furthermore, HIV co-infection reduces efficacy of drug therapy against HCV (Rodriguez-Torres, 2012, 2013).

Because of lack of proofreading activity of viral RNA-dependent DNA or RNA polymerases, HIV and HCV replication results in a family of related genomic variants, or quasispecies. Recent studies reveal that after HCV transmission, a single or a few founder viruses are responsible for establishing productive HIV infection in 80% of the sexually transmitted recipients and 40% of the recipients exposed to HIV contaminated blood, even though a swarm of viral quasispecies is present in donor genital fluids and blood (Bar et al., 2010). Similar transmission bottleneck has been reported for HCV infection (Bull et al., 2011; Li et al., 2012). However, it is not clear whether the HCV transmission bottleneck still exists in immunocompromised HIV/HCV co-infected individuals. Moreover evolution dynamics of HIV and HCV in co-infected individuals pose a great challenge for host immunity to control these two viruses which could have important implications for the HCV pathogenesis in HIV-infected subjects. In HIV/HCV co-infected subjects, viral genome evolution is determined by the rates of viral replication, genomic mutation, and adaptive immune pressure to each of these two viruses. Currently it is unclear how host immunity controls respective viral replication in HIV/HCV co-infected subjects. The intricate mutual effect may propel the HCV disease progression in HIV/HCV co-infected subjects.

In this report we investigated transmission and evolution of HCV in six HIV-infected individuals with a wide range of CD4 cell count (13–807 cell/mm³) when HCV infection occurred.

⁎ Corresponding author. Fax: +1 412 383 8926.
⁎⁎ Corresponding author. Fax: +1 412 383 8926.
E-mail addresses: pgupta1@pitt.edu (P. Gupta), cheny@pitt.edu (Y. Chen).
Results

The genetic transmission bottleneck of HCV infection

A total of 19 plasma specimens from the six HCV early seroconverters were analyzed for HCV transmission and evolution for a period of 1–2 years. Five (subjects A, B, C, E, and F) of the six subjects were HIV positive for 3–10 years before HCV infection, and one (subject D) was infected with both HIV and HCV within a window of 6 months. At the time of HCV infection, 5/6 subjects were naïve to HAART, except subject B who was treated with HAART. All six subjects were naïve to anti-HCV treatment. The median plasma HCV load of the six subjects at the first HCV RNA positive visits was $5.2 \times 10^6$ copies/ml (ranging from $2.9 \times 10^4$ to $3.3 \times 10^5$), and the median plasma HIV load of five subjects at the same visits was $1.6 \times 10^5$ copies/ml (ranging from $1.8 \times 10^3$ to $6.5 \times 10^5$ copies/ml), while plasma HIV load was undetectable in one subject due to antiretroviral treatment (Table 1).

The sequences corresponding to HCV envelope E1/E2 region (corresponding to HCV H77 nt: 843–1868, 1026 bp) from the first HCV RNA positive samples of all six patients were subjected to neighbor-joining (NJ) phylogenetic analysis. To evaluate the number of the transmitted infectious founder viruses for HCV infection, the Poisson Fitter statistical model (http://www.hiv.lanl.gov/content/sequence/HIV/HIVTools.html) was used to examine whether the viral population had a star-like phylogeny with a Poisson distribution. Both the Poisson Fitter test and phylogenetic analyses indicated that four subjects (C–F) were initially infected by a single HCV variant (Fig. 1A for subject E), while subject A and subject B were infected by three closely related founder viruses (Fig. 1B for subject B). Figures for subjects F, C, D, and A are included in the supplement Fig. 1A–D. The highlighter plots of the sequences from these four subjects with a single founder virus showed that the mutated nucleotides were randomly distributed, and the phylogenetic tree showed only one cluster consistent with a star-like distribution of variants arising from a single founder, which is supported by Poisson Fitter analysis. The sequence maximum median distances were 0.25% (0.20–0.73%) in four subjects (subjects E, F, C, and D) with one founder virus and 1.32% (1.01–1.62%) for subjects A and B with three founder viruses (Table 1). These results indicate that limited number of viral variants initiated the clinical infection and there was a genetic bottleneck in HCV transmission in all subjects despite of a wide spectrum of CD4 cell count when HCV infection started.

HCV evolution over the course of infection

A total of 455 E1/E2 gene sequences, approximately 1000 bp long with a median of 20 sequences per sample (range 17–32), were used for evolutionary analysis. Phylogenetic analysis of longitudinal HCV sequences of five patients is shown in Fig. 2. Sequences from each individual subject formed subject-specific clusters with no intermixing of sequences among subjects. These five subjects were infected with HCV subtype 1a based on the braking results of the viral sequences with NCBI nucleotide data base. The other subject (subject B) was initially infected with HCV subtype 3a (Fig. 3A), and then one year later was re-infected with HCV subtype 1a (Fig. 3B). Therefore, all subsequent longitudinal sequence analysis for subject B was based on the sequential sequences of HCV subtype 1a following infection with subtype 1a.

In general, viral quasispecies sequences at the time of infection were more homogeneous compared to those at later time points. During 1–2 years of follow-up with 3 sequential visits for each patient, there was a temporal evolutionary trend with a shift of viral quasispecies in four of six subjects from one visit to next visit, regardless of whether the HCV infection started with low (subject A and subject C with CD4 cell count 274 and 13, respectively) or high CD4 (subject D and subject F with CD4 cell count 807 and 497, respectively) cell count (Fig. 2). In the other two subjects (subjects E with CD4 cell count 559 cells/mm$^3$ and subject B with CD4 cell count 171 cells/mm$^3$), the viral quasispecies at later time points intermingled with the sequences from previous time point (Figs. 2 and 3A). As described previously, subject B was on antiretroviral therapy with presumably improved immune system when HCV infection occurred. However, the HCV evolutionary pattern in subject B was similar to that of HCV from subject E. The dynamics of viral diversity and divergence of HCV quasispecies during the follow up period were also examined in six patients. Viral diversity was increased as the virus infection transitioned from acute to chronic phase during subsequent 1–2 years of infection. Regression analysis showed that there was a strong association between

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Table 1
Clinical and laboratory information of the six patients coinfected with HCV/HIV.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Age (years)</th>
<th>Length of HIV infection (years)</th>
<th>CD4 (cells/mm$^3$)</th>
<th>HIV Load (cp/ml)</th>
<th>Time of HCV infection (years)</th>
<th>HCV Load ($10^5$ cp/ml)</th>
<th>Star-like phylogeny</th>
<th>Maximum distance</th>
<th>ALT/AST</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-V1</td>
<td>42</td>
<td>8</td>
<td>274</td>
<td>647,299</td>
<td>0.83</td>
<td>2,350</td>
<td>Yes</td>
<td>1.01%</td>
<td>NA</td>
</tr>
<tr>
<td>A-V2</td>
<td>316</td>
<td></td>
<td>1,088</td>
<td>NA</td>
<td></td>
<td></td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>A-V3</td>
<td>451</td>
<td></td>
<td>7,934</td>
<td>NA</td>
<td></td>
<td></td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>B-V1</td>
<td>45</td>
<td>10</td>
<td>171</td>
<td>&lt; 40</td>
<td>0.25</td>
<td>33,021</td>
<td>No</td>
<td>1.62%</td>
<td>NA</td>
</tr>
<tr>
<td>B-V2</td>
<td>272</td>
<td></td>
<td>&lt; 40</td>
<td>0.25</td>
<td>33,021</td>
<td>603</td>
<td>Yes</td>
<td>0.73%</td>
<td>NA</td>
</tr>
<tr>
<td>B-V3</td>
<td>491</td>
<td></td>
<td>&lt; 40</td>
<td>0.25</td>
<td>21,65</td>
<td>603</td>
<td>Yes</td>
<td>0.73%</td>
<td>NA</td>
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<tr>
<td>B-V4</td>
<td>333</td>
<td></td>
<td>&lt; 40</td>
<td>0.25</td>
<td>21,65</td>
<td>603</td>
<td>Yes</td>
<td>0.73%</td>
<td>NA</td>
</tr>
<tr>
<td>C-V1</td>
<td>37</td>
<td>7</td>
<td>13</td>
<td>NA</td>
<td>0.47</td>
<td>8,059</td>
<td>Yes</td>
<td>0.73%</td>
<td>32/50</td>
</tr>
<tr>
<td>C-V2</td>
<td>8</td>
<td></td>
<td>630,703</td>
<td>15,510</td>
<td>10,208</td>
<td></td>
<td>NA</td>
<td>32/50</td>
<td>45/49</td>
</tr>
<tr>
<td>C-V3</td>
<td>18</td>
<td></td>
<td>111,854</td>
<td>15,510</td>
<td>10,208</td>
<td></td>
<td>NA</td>
<td>32/50</td>
<td>45/49</td>
</tr>
<tr>
<td>D-V1</td>
<td>41</td>
<td>0.5</td>
<td>807</td>
<td>1,806</td>
<td>0.23</td>
<td>29</td>
<td>Yes</td>
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<tr>
<td>D-V2</td>
<td>605</td>
<td></td>
<td>27,954</td>
<td>350</td>
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<td></td>
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<td>0.23%</td>
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<td>D-V3</td>
<td>585</td>
<td></td>
<td>119,982</td>
<td>687</td>
<td></td>
<td></td>
<td>Yes</td>
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<tr>
<td>E-V1</td>
<td>43</td>
<td>3</td>
<td>559</td>
<td>294,000</td>
<td>0.50</td>
<td>12,492</td>
<td>Yes</td>
<td>0.73%</td>
<td>101/74</td>
</tr>
<tr>
<td>E-V2</td>
<td>291</td>
<td></td>
<td>687,000</td>
<td>309</td>
<td></td>
<td></td>
<td>Yes</td>
<td>0.73%</td>
<td>101/74</td>
</tr>
<tr>
<td>E-V3</td>
<td>527</td>
<td></td>
<td>73,700</td>
<td>6,401</td>
<td></td>
<td></td>
<td>Yes</td>
<td>0.73%</td>
<td>101/74</td>
</tr>
<tr>
<td>F-V1</td>
<td>36</td>
<td>9</td>
<td>497</td>
<td>20,836</td>
<td>0.24</td>
<td>1,510</td>
<td>Yes</td>
<td>0.23%</td>
<td>NA</td>
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<tr>
<td>F-V2</td>
<td>407</td>
<td></td>
<td>NA</td>
<td>3,436</td>
<td></td>
<td></td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>F-V3</td>
<td>677</td>
<td></td>
<td>NA</td>
<td>4,886</td>
<td></td>
<td></td>
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<td>NA</td>
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</tbody>
</table>

NA, not available.
the infection time and overall viral diversity ($P=0.0024, R^2=44.6\%$) (Fig. 4A), indicating that the viral mutation and evolution were associated with the length of viral infection. In addition, the viral divergence from initial time point also showed the similar pattern (Fig. 4B), with the increasing of infection time, the viral divergence increased ($P=0.00149, R^2=46.3\%$). Moreover, there was no association between CD4 cell count at the onset of HCV infection and viral diversity and divergence after 1.0–2.0 years of HCV infection (Fig. 4C).
Immune pressure and selection of mutation

Viral random mutations arise from the lack of proofreading polymerase. However, the profiles of the mutation dynamics are shaped by viral fitness and immune selection. To evaluate immune selection of viral sequences in the six co-infected subjects, the non-synonymous (dN) to synonymous (dS) substitutions in sliding windows across E1/E2 envelope region were monitored as HCV infection proceeded from acute to chronic stages during the 2 years of follow up after infection. Sliding window analysis (Fig. 5) demonstrates an increase of synonymous over non-synonymous substitutions throughout the envelope regions in all six subjects. The median synonymous distance was greater than that of the non-synonymous distance from the first time point to the second time point (0.5 year apart, 0.1328 versus 0.0196, \( P < 0.001 \)). Similarly, the synonymous distance from the first time point to the third time point (1–2 years apart), was also greater than the non-synonymous distance during that period (0.126 versus 0.021; \( P < 0.001 \)). The greater synonymous distances compared to the non-synonymous distances suggests that host purifying selection dominated nucleotide changes during the first 1–2 years of acute HCV infection regardless of host CD4 cell count when the HCV infection started.

Assessment of host cellular immune pressure on viral genome evolution

Although the role of HCV specific immune response in controlling HCV replication at the initial phase of infection is still controversial, a strong cellular immune response is often detected in acute stages of HCV infection (Cooper et al., 1999; Gruner et al., 2000; Lechner et al., 2000; Takaki et al., 2000; Thimme et al., 2001). To evaluate viral genome mutations with the evasion of host T cell immunity against HCV in HIV-infected subjects with different CD4 cell count background, the predicted T cell epitopes in the E1/E2 region based on the binding affinity between the
amino acid sequences and respective host HLA class I and class II alleles (obtained from MACS data center) were analyzed in 5 of 6 subjects (HLA alleles of subject E were not available). For HLA class I restricted epitopes, the regions with predicted binding affinity < 50 nM were defined as strong binding epitopes, and the regions with the binding affinity between 50 and 500 nM

Fig. 3. Neighbor-joining tree of HCV E1/E2 sequences from patient B. Panel A: The tree was constructed based on the sequences from four consecutive visits of subject B. The first time point sequences (black empty square) clustered differently from the rest three follow-up visits by a high bootstrap support value (99%). The black, green and red circles indicate sequences amplified from three different visits. Panel B: The tree was constructed using viral sequences from subject B and HCV subtype 1 and 3 reference strains obtained from the Los Alamos HCV database. For clarity the patient sequence clusters with high bootstrap value were collapsed. There are two clusters, one is HCV subtype 1a and the other is HCV subtype 3a.
were defined as weak binding epitopes (Lundegaard et al., 2008a, 2008b). For each subject, the predicted HLA class I restricted epitopes at the first time point were compared with those at subsequent visits. In subjects D and patient F with CD4 cell count 807 and 497 cells/mm$^3$, 5–6 HLA class I restricted strong binding epitopes and 12–17 weak binding epitopes were detected. In subject A, subject B, and subject C with CD4 cell count of 274, 171, and 13 cells/mm$^3$, respectively, three class I strong binding epitopes and 11–20 weak binding epitopes were detected. The epitope changes from a representative subject D and subject A are shown in Fig. 6. The epitope changes in rest of the subjects are summarized in supplemental Fig. 2. As infection proceeded, none of the class I strong binding epitopes changed at subsequent time points. Only one weak binding epitope in subject A (CD4 cell count 274 cells/mm$^3$) lost its predicted binding affinity to HLA allele and one strong binding epitope changed to weak binding one at subsequent time points (Fig. 6B). These results suggest that there were no significant changes in the strong or weak binding epitopes during the initial 1–2 years of HCV infection in the five subjects with CD4 cell count ranging from 18–807 cell/mm$^3$ at the time of HCV infection.

For HLA Class II epitopes, the regions with predicted binding o$\leq$500 nM were defined as potential binding epitopes (Wang et al., 2010). Similarly, in each subject, the epitopes predicted on the viral amino acid sequences from the first time point were monitored at the subsequent visits. In subject D and subject F (CD4 cell count 807 and 497) 20–46 epitopes were detected. In subject A, subject B, subject C (CD4 cell count 274, 171 and 13) 25–50 epitopes were detected. However, no significant changes of the epitopes were detected in any of the five subjects as HCV infection progressed from acute to chronic phase. The list of predicted HLA class I and II restricted epitopes in E1/E2 region and corresponding mutations were shown in Supplemental Tables 1 and 2. It is possible that the immune pressure on the epitope regions was different comparing to the epitope-flanking regions. Therefore, we evaluated the ratio of non-synonymous versus synonymous mutations in the predicted epitope regions as well as the epitope-flanking regions as shown in Table 2. For all five patients, the ratios derived from these two regions were less than 1 suggesting no significant difference in immune selections on these two regions.

**Evaluation of neutralizing antibody epitopes in hypervariable region 1**

Hypervariable region 1 (HVR1) located at the beginning part of HCV E2 region is composed of 27 amino acids that include neutralizing antibody epitopes. Mutations in HVR1 are often associated with escape from neutralizing antibodies produced...
during acute HCV infection (Dowd et al., 2009; Guan et al., 2012). Examination of HVR1 regions in 6 subjects during first 1–2 years of HCV infection showed that there were no amino acid changes in five subjects during this period. However, a few amino acid changes were detected in subject A (CD4 cell count 274 cells/mm³) as the virus evolved from acute to chronic stages (Fig. 7). For subject A, 90% of proline at position 2 and 80% of arginine at position 21 at initial infection were changed to threonine within 6 months of infection. Additionally, after 2 years of infection, 70% of phenylalanine residues at position 16 were changed to leucine and 10% changed to isoleucine. The amino acid changes in remaining five subjects are not significant and summarized in the supplemental Fig. 3. These results indicate that there was limited immune pressure in HVR1 evolution during initial 1–2 years of infection regardless of the host CD4 cell count at the onset of HCV infection.

**HIV and HCV co-evolution in a dually infected subject**

In five of six HIV/HCV co-infected subjects in our study, HCV infection started in the hosts with pre-compromised immune system due to 3–10 years of HIV infection. However, we had accessed to one subject (subject D) who was infected with HCV within six months of HIV infection, which provided us an opportunity to evaluate HIV and HCV co-evolution from acute to chronic infection under the same presumably uncompromised immune system. As shown in Fig. 8, sequences encompassing envelope C2-V5 region of HIV (Fig. 8, left in panel A) and E1/E2 region of HCV (Fig. 8, left in panel B) at the first time point were quite homogeneous (sequences shown in blue dots in phylogenetic trees) indicating transmission bottleneck in both viruses. As the infection proceeded from acute to chronic phase, the diversity increased for both viruses, but the diversity of HIV (Fig. 8, middle in panel A) increased much faster than that of HCV (Fig. 8, middle in panel B). The HIV diversity observed here is compatible with HIV diversity reported in our previous study (Shankarappa et al., 1999). After one and half year of the co-infection, overall diversity of HIV was 2 times higher than that of HCV. In addition, there were more apparent amino acid changes in HIV than those of HCV (Fig. 8, right in panel A and B). Although viral mutations were controlled by respective rate of viral replication, characteristics of error-prone polymerases and virus fitness, our data suggest that the host may control these two viruses by different mechanisms.
Proinflammatory cytokine profiles in the plasma samples

Host immune system plays an important role in pathogenesis of HIV/HCV infection (Flynn et al., 2013; Nishitsuji et al., 2013). Inflammatory cytokines are thought to be directly involved in the immune responses against HIV/HCV replication. Therefore, levels of seven proinflammatory cytokines (INFγ, IL-10, IL-12p70, IL-1β, IL-6, IL-8 and TNF-α) were measured in the plasma samples from the six HCV-infected subjects at the first visit after HCV infection and subsequent visits. In most of the subjects, regardless of their CD4 numbers, low levels (less than 30 pg/ml) of plasma inflammatory cytokines were detected at all visits (data not shown). We also evaluated the levels of ALT/AST in plasma which could be useful indicators of liver inflammation and potentially an immune response against liver. Of the 6 patients, the AST/ALT data are available from 3 patients (2 patients with infection initiated with low CD4 counts and 1 with infection initiated with high CD4 count) (Table 1). All AST/ALT are below 150 IU/L. These preliminary data suggest that liver inflammation in these patients was mild.

Discussion

HCV evolution has been studied in detail in HCV-infected subjects. However, global rapid rise of acute HCV infection in HIV-infected subjects through sexual and intravenous drug abuse requires a detailed investigation of HCV evolution and the role of immunity in viral evolution in HIV/HCV co-infected subjects. The availability of plasma samples with defined onset of HCV infection and longitudinal follow up in highly characterized HIV-infected subjects from the Multicenter AIDS Cohort Study (MACS) provided us a unique opportunity for the first time to study HCV evolution from the onset of HCV infection under different pre-existing immune environment manifested by prior HIV infection.

In this study we measured HCV evolution in 6 HIV-infected subjects with a wide spectrum of CD4 cell counts at the onset of HCV infection. Analysis of 455 independent HCV E1/E2 sequences from initial infection up to 1–2 years after infection revealed that HCV infection in all six subjects was initiated with only 1 or 3 founder viruses, indicating that there was a significant transmission bottleneck in HCV transmission at all subjects. Longitudinal analysis of HCV sequences over a period of 1–2 years showed two modes of viral evolution as the infection progressed from acute to chronic phase. In 4/6 subjects there was a replacement of viral quasispecies from one visit with those from subsequent visits, while in 2/6 subjects viral quasispecies from later time points intermingled with those of earlier time points. However, such viral evolution was independent of host CD4 cell count at the time of HCV infection. In addition, both diversity and divergence from initial viral population at the time of infection increased with time and were very similar in all subjects regardless of their CD4 cell count at the time of HCV infection. These results suggest that preexisting CD4 levels in the host may not have effect on HCV evolution and hence replication.

Viral sequences were then used to compare evolutionary pressure in subjects with different CD4 cell count by measuring non-synonymous (dN) to synonymous (dS) substitution across E1/E2 region during 1–2 years as the infection proceeded from acute to chronic phase. Pairwise synonymous distance within this period of time was greater than non-synonymous distance, indicating that purifying selection was the major force modulating viral population at the beginning of the chronic phase of infection.

We then monitored the dynamic changes of predicted HLA class I and II restricted T cell epitopes in the HCV envelope region as a measure of CD8 and CD4 T cell mediated immune pressure on viral evolution. Except one change in the weak binding epitope for HLA class I, there were no significant changes in HLA class I and II binding epitopes in subsequent visits in all five subjects, which indicates there are limited immune pressure against HCV in these HIV infected individuals. Our results are consistent with recent studies, which showed that HIV infection greatly compromised the host immune system to control HCV (Flynn et al., 2012; Low et al., 2008; Rotman and Liang, 2009; Schnuriger et al., 2009) and contrasted with the changes of immune epitopes observed in HCV mono infected individuals (Bailey et al., 2012; Cox et al., 2005).

Since a number of studies have reported that virus neutralizing antibody response is the driving force for viral evolution and viral clearance (Pestka et al., 2007), we have examined virus neutralizing epitopes present in the hypervariable region 1 of the HCV envelope gene. In most (5/6) of the subjects we did not detect any
changes in neutralizing epitopes in the hypervariable region 1 within the 1–2 years of infection as it proceeded from acute to chronic phase regardless of subjects’ preexisting CD4 levels.

The cumulative results reported here have shown that transmission bottleneck and evolution pattern as determined by HCV diversity, divergence and occurrence of mutations important for adaptive immunity are independent of CD4 cell count at the time of HCV infection. Even in HIV-infected subjects with very low CD4 cell count, like 13 cells/mm³ (impaired immunity presumably due to prior HIV infection) and relatively high CD4 cell count, like 807 cells/mm³ (presumably near intact immunity), HCV transmission and evolution are very similar. These results led us to speculate that host immunity may handle these two viruses differently. This speculation is supported by our results in a subject who was infected with HIV and HCV within a very short period of time. Here we observed that viral diversity and divergence were much higher in the case of HIV as compared to HCV in the same immunocompetent host. In addition, in this subject there were more frequent mutations and amino acid changes in HIV than in HCV, suggesting that host might exert different immune pressure against these two viruses. There are several potential caveats to these conclusions: First: predicted immune response epitopes must be verified by actual T cell response data with CD8 and CD4 cells from co-infected subjects. Second: we examined T cell immune response in envelope region. We need to examine non-envelope region to make our conclusion more general, because there are reports indicating significant difference in HCV evolution between envelope and non-envelope region (Bailey et al., 2012; Liu et al., 2010). Third, sample size is relatively small. Further studies are needed in both envelope and non-envelope genes with more co-infected subjects in order to confirm this speculation.

Material and methods

Study subjects

Plasma samples from six HCV seroconverters (5 RNA+/Ab− and 1 RNA+/Ab+) with HIV infection and CD4 cell count ranging from 13 to 807 cells/mm³ at the time of HCV infection were obtained from the subjects enrolled in MACS. Semiannual or annual plasma samples starting from the earliest time of HCV RNA positivity up to 1.0–2 years after infection were used for this study and at least three time points were analyzed for each subject. The estimated time of infection relative to the first visit sequenced was calculated as half of the days between the first HCV positive visit and the last negative visit, which was converted to years. The median age of the 6 subjects was 41 years (range 37–45)

Fig. 8. HCV and HIV coevolution in HIV/HCV co-infected patient D. Panel A: Neighbor-joining tree of HIV C2-V5 sequences (left), HIV Viral diversity analysis (middle), and highlighter plot (right). Panel B. Neighbor-joining tree of HCV E1/E2 sequences (left), HCV Viral diversity analysis (middle), and highlighter plot (right).
when HCV infection occurred. All HCV infections were acquired by sexual transmission. Five of the patients were initially infected with HCV subtype 1a.

**Multiplex cytokine measurement**

Levels of eight inflammatory cytokines (IFN-γ, IL-10, IL-12p70, IL-6, IL-8 and TNF-α) in plasma were measured by Meso Scale Discovery (Meso Scale Discovery, Rockville, MD) assay following manufacture protocols.

**Nucleic acid extraction, viral load measurement and single genome amplification**

Total RNA was extracted from 200–500 μl plasma samples using the automatic NucliSens EasyMag nucleic acid extraction machine (bioMérieux, Durham, NC). To measure HCV load, extracted plasma RNA were subjected to reverse transcription (RT) followed by real time PCR using commercially available HCV primers/probe following manufacture’s instruction (Applied Biosystems, Foster City, CA). HIV viral load was measured by Amplicor assay (Roche, Indianapolis, IN). For single genome amplification (SGA) of E1/E2 region of HCV, total RNA extracted from the plasma was subjected to reverse transcription followed by single genome amplification as described previously (Salazar-Gonzalez et al., 2008; Shen et al., 2012). Briefly, each RT reaction included 1 × RT buffer, 0.5 mM of each of the four deoxynucleoside triphosphates, 5 mM dithiothreitol, 2 units/l of Superscript III reverse transcriptase, and 0.25 μM antisense primer, 100 μM outerR 5′-GGGCAGDBCARRGTTGTTGCC; and the primers for the first round of the nested PCR were: 100 μM outerF 5′-GGGCAGDBCARRGTTGTTGCC; and the primers for the second round of nested PCR were: 100 μM innerF 5′-AACCTCC-TGGTTGCTCTTTCTAT and 100 μM innerR 5′-GAAGCAATAYTGYGGRC-AACA. The single genome amplification of HCV C2-V5 region was performed as described previously (Salazar-Gonzalez et al., 2008; Shen et al., 2012).

**Sequencing and phylogenetic analysis**

Amplicons were directly sequenced in an ABI Prism 3700 DNA Sequencer. CLC main workbench 6.0 was used to inspect the chromatograms. The sequences without mixed bases were taken as an evidence of single genome amplification from a single viral RNA template. The median number of sequences analyzed per RNA template was 20 (range: 17–32). DNA alignments were constructed using CLUSTAL X alignment program and hand adjusted when necessary. The sequences were visually inspected by using neighbor-joining phylogenies implemented in MEGA5.0 to compute the viral diversity, divergence and construct phylogenetic tree. The sequence visualization tools, highlighter (www.HIV.lanl.gov) and Poisson Fitter program (www.HIV.lanl.gov) together with phylogenetic tree were employed to predict the number of founder viruses. Poisson Fitter program was based on the fit of the Poisson model to the frequency distribution of the Hamming distance observed in each sample. For the transmission genetic bottleneck analysis, only the HCV sequences obtained from the earliest time point after HCV infection from each subject were included in the founder virus analysis.

**Analysis of the regions important to immune functions**

Sliding window analysis of non-synonymous and synonymous changes was performed with VarPlot (v1.7; http://sray.med.umich.edu/SCSoftware/VarPlot), and ratios of dN and dS were calculated by the Nei-Gojobori method (Nei and Gojobori, 1986). The HLA Class I and Class II restricted epitopes in the amplified E1/E2 regions were predicted using available algorithms in http://www.immuneepitope.org based on individual patient's HLA types provided by MACS data center (Gao et al., 2010). For HLA Class I restricted epitopes, the results presented were derived from website NetMHC http://www.cbs.dtu.dk/services/NetMHC/, which used an artificial neural network for predictions. For HLA Class II restricted epitopes, the prediction results were derived using the software from website: http://tools.immuneepitope.org/analyze/html/mhc_II_binding.html#.

Furthermore, the ratios of synonymous versus non-synonymous mutations of the predicted epitope regions and the epitope-flanking regions were evaluated using DnaSP (5.10.1) (Librado and Rozas, 2009).

**Statistical analysis**

Significance of differences in diversity, divergence and amino acids evolution were calculated using Mann-Whitney U Test. For the longitudinal data, linear regression utilizing generalized estimating equations (GEEs) and an exchangeable correlation structure (Zeger and Liang, 1986) models were employed to analyze the relationship between viral divergence, diversity and duration of viral infection. The association between viral diversity, divergence and CD4 cell count at the onset of HCV infection were analyzed by using spearman correlation. The data were analyzed using the Stata version 11.0 statistical software (Stata Press, College Station, TX, USA).

**Nucleotide sequence accession numbers**

Sequences were deposited in GenBank under the accession no. KC614812–KC615266.

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**Appendix A. Supporting information**

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2013.11.001.

**References**


