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Genetic divergence of HCV: The role of HIV-related immunosuppression

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Abstract

Background—We tested the hypothesis that HIV-related immunosuppression alters the host-hepatitis C virus (HCV) interaction, resulting in fewer amino acid changing substitutions in HCV viral variants. Higher HCV RNA levels in persons coinfected with HIV compared with HCV infection alone suggests increased viral replication. If this increase is dependent on decreased immune selective pressure, then a reduced rate of nucleotide changes resulting in amino acid replacements (nonsynonymous changes, d_N), would be expected.

Methods—We investigated HCV envelope sequences over time in 79 persons with chronic HCV infection that were HIV negative (group 1), or HIV positive without (group 2) or with (group 3) severe immunodeficiency. We amplified a 1026 nucleotide region of the HCV genome, which encodes a portion of the envelope glycoproteins E1 and E2, including HVR-1 for direct sequence analysis.

Results—The overall divergence between paired sequences, d_S, d_N, and d_N/d_S all showed no significant differences among the three groups.

Conclusions—By measuring nucleotide substitutions in HCV sequences over time, we found no significant differences in the genetic divergence between HCV monoinfected control subjects and HIV/HCV coinfected subjects with various levels of immunodeficiency as measured by CD4+ T cell counts.

INTRODUCTION

Hepatitis C virus (HCV) is a major etiological agent of liver disease in most regions of the world, with approximately 170 million infected individuals [1;2]. Coinfection with Human immunodeficiency virus (HIV) is found in approximately one third of HCV infected persons [3]. This is important because HIV coinfection increases HCV-related liver diseases [4].

HCV is a single-stranded RNA virus, which encodes a polyprotein of approximately 3000 amino acids, which is cleaved into three structural (Core, E1, and E2) and several nonstructural (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins [5]. HCV, like HIV, exists in each infected person as a quasispecies, or “swarm” of closely related but divergent

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genetic sequences [6;7]. These divergent sequences are generated by an error-prone polymerase (the NS5B protein), driven by the highly dynamic nature of HCV replication[8]. HCV quasispecies arise by nucleotide substitutions that either preserve protein structure (synonymous substitutions) or substitutions that change the amino acid composition (nonsynonymous substitutions). Whereas both types of substitutions arise from error prone viral replication, their relative proportions may be informative due to different selective forces. Synonymous changes are generally well-tolerated by the virus except in regions of RNA secondary structure,[9] and thus are thought to be nearly neutral in the envelope gene region. In contrast, nonsynonymous substitutions may be deleterious due to effects on protein structure and function, or may be advantageous when they mediate immune escape. Thus, a reduced ratio of nonsynonymous to synonymous changes (d_N/d_S) is an indication of decreased immunologic pressure [10;11].

There is conflicting information on the effect of HIV on the course of HCV evolution. It has been shown that HCV RNA levels rise approximately 0.5 $\log_{10}$ in HIV coinfected subjects, suggestive of increased viral replication[12–15]. If this increased replication is due to reduced immunologic pressure, then a decrease in d_N/d_S would be anticipated. Therefore, we hypothesized that HIV infection would alter the host-HCV interaction resulting in lower d_N/d_S. To test this hypothesis, we examined HCV envelope sequences over time in persons with chronic HCV infection that were either HIV negative or HIV positive, with or without severe immunodeficiency.

MATERIALS AND METHODS

Subjects

We studied subjects participating in a longitudinal study of the natural history of HCV infection among drug users that were enrolled between November 2, 1999 and December 13, 2000 in the Bronx, New York [14;16]. Subjects underwent semiannual standardized interviews and phlebotomy for several tests including HIV-1 viral loads, HIV antibody, T-lymphocyte subset assays and HCV RNA assays as previously described [14;16]. In order to assess the possible relationship of HIV infection and immunodeficiency to changes in HCV sequences over time, we chose a nested sample of 115 individuals having chronic HCV viremia who could be classified at the time of their last study phlebotomy into one of the following three groups: Group 1 consisted of HIV-negative, HCV RNA positive subjects; Group 2 consisted of subjects with a persistently high CD4+ T cell count of >350 cells/mm^3, and Group 3 consisted of subjects with a persistently low CD4+ lymphocyte count of <200 cells/mm^3.

HCV RNA analyses

To characterize the HCV RNA sequence we amplified a 1026 nucleotide (nt) region of the HCV genome, which encodes the envelope glycoproteins E1 and E2, including hypervariable region 1 (HVR1), as previously described [17;18]. Prior work has indicated that the consensus sequence is an excellent approximation of the master sequence, or most-commonly-observed sequence.[19] Therefore, RT-PCR PCR products were purified and directly sequenced using the reverse primer 1868a21 (positions 1868–1848 relative to HCV-H77, Genbank accession number AF009606) 5′-GAAGCAATAYACYGGRCCACA-3′[17]. Sequences generated in this study are available in GenBank, with accession numbers ____ through _____.

Phylogenetic analysis

Sequences were aligned from raw tracings using Aligner version 2.0.4 (CodonCode Corporation), and trimmed to 756 nucleotides (positions 1041–1796 relative to HCV-H77)

encompassing approximately most of E1 and a third of E2 including HVR1, using BioEdit [21]. Sites containing a gap in any sequence were stripped during analysis. Genetic distance (divergence) between paired sequences was calculated using the distance matrix calculation scripts provided with HyPhy version 0.99,[22] with model selection and parameter estimation by maximum likelihood (inferred model parameters available on request from the authors). Synonymous and nonsynonymous genetic distances were determined using the MG94custom model as implemented in HyPhy.[23] To determine the specific regions of the HCV genome where these changes were occurring we used the VarPlot program (available from S.C.R. at http://sray.med.som.jhmi.edu/SCRoftware),[18] which uses the Nei and Gojobori method.[24] Using this software we calculated values for d_S, d_N and the d_N/d_S ratio in a sliding window of 20 nucleotides [18]. This process was then repeated for an overlapping segment of 20 bp, which was shifted by 1 bp (the step size), and continued across the alignment. At each step all pairwise comparisons for a subject were performed and values averaged. The mean values for all subjects were then averaged, ensuring that each subject was given equal weight. The Jukes-Cantor correction was used to correct for underestimation of distance due to multiple substitutions at the same site [25].

Statistical Analysis

The chi-square test was used to analyze categorical data and a Wilcoxon signed rank test was used to compare HIV viral loads and CD4+ T cell counts nonparametrically. The Kruskal-Wallis rank test was used for multiple comparisons among the 3 groups of subjects.

RESULTS

Initially 115 subjects were chosen for analysis at two time points. Twenty two subjects were excluded because HCV-specific RT-PCR failed at both time points, and 8 were excluded because only one of two specimens was successfully amplified. Two subjects were excluded because sequencing of RT-PCR products yielded sequence product length less than 80% of the mean length of other subjects and four subjects were excluded because of possible HCV reinfection. Among the excluded subjects twenty were from group 1, fourteen from group 2 and two from group 3. The primers used in this study were designed to amplify genotype 1, which is the most common genotype in the United States and as expected a majority of the pairs that we failed to amplify, were non-genotype 1. Therefore, a total of 79 subjects (74.7%) at two time points were evaluable (Table 1). The median time between visits was 933 days (range 360–1303). Thirty eight subjects were HIV negative, 21 had CD4+ T cell counts > 350 cells/mm^3 at both time points and 20 had CD4+ counts < 200 cells/mm^3 at both time points. No significant differences were observed in the gender or age between the 3 groups. The subjects were primarily Hispanic and a significant difference in race composition was detected between the three groups. By design, the median CD4+ T cell count was significantly higher in group 2 compared to group 3; in addition, a significantly higher HIV viral load was observed for group 3 compared to group 2. No significant differences were detected in the HCV viral load or the estimated duration of HCV infection among the three groups at either visit.

Effective antiretroviral therapy (ART) has been shown to alter the complexity and diversity of HCV quasispecies. Among the 41 HIV infected individuals in our study 20 had reported ART. Seven of the 21 subjects in Group 2 reported ART, 3 at both time points and 4 at one time point. Thirteen of the 20 subjects in Group 3 reported ART, 2 at both time points and 11 at a single time point. Over the time course of this study only 4/20 subjects on ART maintained HIV RNA levels less than 2.6 $\log_{10}$ copies/mL, which precluded an analysis of the effect of ART on HCV sequence divergence.

Overall, divergence between paired sequences in the three groups was similar (Table 2). In addition, no significant differences in divergence were detected between groups when the four excluded subjects (possible reinfection) were included in the analysis. Because a significant difference in racial composition was detected between groups we analyzed divergence controlling for race. However, since our study only had 7 Caucasian subjects, and group 2 did not contain Caucasian subjects they were not included in this analysis. No significant differences in divergence stratified by race were detected ($P > 0.50$, Kruskal-Wallis Test).

The median number of days between specimens was significantly different among the three groups ($P = 0.036$, Kruskal-Wallis Test), with group 1 having a significantly longer interval between the specimens compared to group 3 (Dunn's pairwise multiple comparison). The rate of divergence/year was also calculated and was not significantly different among the groups (Table 2). Divergence rate was also stratified by race but no statistical differences were detected ($P >0.50$ Kruskal-Wallis Test).

To specifically address our hypothesis that increased immunosuppression would alter the host-HCV interaction by decreasing amino acid changing mutations, we determined synonymous and nonsynonymous substitution frequencies. The median d_S, d_N substitution frequencies and rates, and d_N/d_S ratiosare listed in Table 2. Analysis of these measures for the three groups showed no significant differences (Kruskal-Wallis Test).

Since we found no global differences between the three groups in selective pressure, a high-resolution analysis was performed to determine if specific regions within the genome segment we analyzed were under greater selective pressures (Figure 1). Using the program VarPlot we calculated values for d_S, d_N and the d_N/d_S ratio in a sliding window. Comparison of the three groups using this method revealed little difference within d_S or d_N (Figure 1A and B). However, in general synonymous distances were greater than non-synonymous distance with median values for the three groups being 0.436 versus 0.081, respectively. Interestingly, synonymous changes were suppressed in the region centered on codon 362. The highest rate of nonsynonymous mutations was observed for the HVR-1 region centered on codon 398 in the E2 protein, as has been described previously[18].

Based on our initial assumptions we would expect that increasing immunosuppression as a result of HIV infection would tend to decrease immune selection (d_N) and thus decrease the d_N/d_S ratio particularly in the group with the lowest CD4 counts. The HVR-1 d_N/d_S ratios in this study reached a maximum of approximately 0.9. However, the three groups essentially had overlapping d_N/d_S curves with the highest ratio centered on HVR-1.

DISCUSSION

In this study we examined the hypothesis that immunosuppression resulting from HIV infection would modify the host-HCV interaction resulting in decreased HCV sequence evolution over time. By measuring genetic divergence, synonymous and nonsynonymous nucleotide substitutions in HCV sequences over time, we found no significant differences in the master sequence between HCV monoinfected control subjects and HIV/HCV coinfected subjects with various levels of immunodeficiency as measured by CD4+ T cell counts.

Most previous studies have primarily analyzed complexity and diversity of the quasispecies. These analyses are efficient in determining the temporal composition of the HCV population and have sensitivity to allow determination of both minor and major variants. We decided to examine evolution (divergence) by directly sequencing RT-PCR products at two time points. This approach gives a more global or average weight to the quasispecies, but is insensitive for detection of minor variants of the population. Based on our approach, our data suggest

that the shift in the quasispecies master sequence was similar among the three groups. However, a shift in minor variants could be occurring, but would not be detectable by our approach.

In our study we analyzed HCV sequence variability over a 2–3 year interval in essentially two groups, HIV positive and HIV negative subjects. One possible explanation for our inability to detect differences among these groups is that our assay was insensitive. However, the median d_S value observed in our subjects was similar to synonymous distances found when analyzing full length genomes in this region and the HVR-1 d_N/d_S ratios, which reached a maximum of approximately 0.9 were also similar to another study [26]. The synonymous and nonsynonymous substitution rates that were observed in our study were also consistent with previous reports of HCV sequence evolution [27]. Furthermore, the highest rate of nonsynonymous mutations in our subjects was observed for the HVR-1 region centered on codon 398 in the E2 protein as expected. In addition, synonymous changes in our subjects were suppressed in the region centered on codon 362, which has been previously described and may represent RNA secondary structure [26]. Therefore, our results are consistent with previous reports and validates that our methodology was sufficient to detect earlier recognized HCV sequence variability.

In our analysis we looked at other factors that could influence HCV sequence evolution such as race, age, duration of HCV infection, sampling interval and HCV viral load. Keenan and colleagues examining HCV infected subjects found significantly higher frequencies of d_N and d_N/d_S ratios in the HVR-1 region for Caucasian versus African American subjects[28]. However, no significant differences were detected in complexity and diversity among these groups. A limitation of our study was that the group of HIV seropositive persons with CD4+ counts >350 cells/mm^3 had no Caucasian subjects and we therefore could not examine our data for differences between this group and African Americans. However, our HIV negative group contained 6 African Americans and 5 Caucasian but no significant differences were detected in divergence, d_S, d_N or the d_N/d_S ratio (data not shown). We therefore stratified our results based on race and omitted the data from Caucasian subjects in all groups based on the small sample size, and found no significant differences between Hispanics and African Americans.

Previous HCV quasispecies analyses of HIV/HCV coinfected subjects have produced conflicting results. In one report examining HCV HVR-1 diversity in HIV negative versus HIV/HCV positive subjects no differences were detected until the CD4+ T cell count decreased below 50/μL [29]. Still others have detected either an increase [30;31] or decrease in HCV sequence variability [17;32;33]. The role of HAART in this setting has also been examined with some groups finding increased HCV quasispecies diversity after immune restoration [33;34], and some finding no difference [35]. It is difficult to reconcile the differences in these studies because they used different study designs (cross-sectional versus longitudinal), and variable duration of HCV infection existed among study subjects. In addition to the above mentioned factors the HCV sequence analysis methodology also differed. A majority of these studies utilized gel shift patterns to determine the diversity and complexity of the quasispecies while some utilized direct sequencing of PCR products. The gel shift analyses are used to examine viral heterogeneity (eg. complexity and diversity among clones) which has been shown to be influenced by the factors listed above. Since we did not examine viral heterogeneity we could have missed changes in minor quasispecies variants on which evolution could be acting. While we agree these changes in minor variants are likely occurring, we found in our study the major or most fit variant seems to be stable over the time period studied. Since the interaction of HIV and concomitant immunosuppression on HCV is likely a multifactorial process, small differences in

experimental designs with small and diverse study populations make it difficult to precisely model this interaction.

There were limitations to our study. Antiretroviral treatment has been shown to influence complexity and diversity of the quasispecies [3433]. Since only 4 subjects in our study had effective ART we could not assess its role in HCV sequence divergence. Similarly, we did observe higher median rates of divergence in subjects with HIV and CD4 depletion compared with those without HIV infection that were not statistically significant, suggesting that future studies using similar methods may need to be larger than the one described here to determine whether there may be small significantly different divergence rates. Another potential limitation is that we only examined approximately 600 nucleotides in the E1 and E2 regions of the HCV genome and could have missed changes in other B and T cell epitopes outside of this region. Data on liver histology and aminotransferase levels were not available, precluding an examination of the effect of liver disease activity on HCV sequence divergence.

A possible explanation for our results could be that amino acid substitutions in the envelope glycoproteins, especially HVR-1, have saturated. By studying HCV sequence evolution in a cohort of Irish women infected by contaminated anti-D immunoglobulin, McAllister et.al. found that amino acid substitutions become saturated over short durations of divergence. Therefore, it is plausible with the greater than 20 years of estimated HCV infection that the subjects in the current study have reached their divergence or evolutionary plateau.

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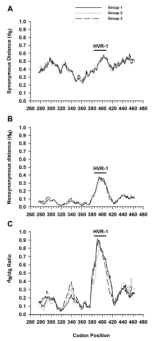

Figure 1.

Variability plots of HCV envelope region. For each subject the intersample d_S (A), d_N (B) or d_N/d_S ratio (C) variability was calculated for each position along the alignment, for a sliding window of 20 codons. The graph shows the mean values for each group (HIV negative, group 1; HIV positive absolute CD4+ T cell count >350 cells/mm^3, group 2 or HIV positive CD4+ T cell count <200 cells/mm^3, group 3). The indicated amino acid positions in the HCV polyprotein are according to HCV-H77 Genbank accession number AF009606. The position of hypervariable region 1 (HVR-1) is indicated.

Table 1

Demographic, immunologic and virologic characteristics

Characteristic	Group 1 (n=38)	Group 2 (n=21)	Group 3 (n= 20)	*P*
HIV status	Negative	Positive	Positive	NA[a]
Age				
median (IQR[b])	47 (43–50)	45 (41–50)	44 (39–52)	0.315[c]
Estimated years of HCV infection[d]	27[g]	25[g]	29[g]	0.633[c]
Gender (% male)	74	67	85	0.395[e]
Race (%)				0.037[e]
Hispanic	71	52	75	
African American	16	48	15	
Caucasian	13	0	10	
CD4+ T cell count, median (IQR), cells/mm^3				
Time point (TP) 1	ND	680 (476–754)	84 (28–138)	<0.0001[f]
TP 2	ND	681 (510–804)	54(34–83)	<0.0001[f]
HCV RNA, median (IQR), log$_{10}$ IU/mL				
TP 1	5.7 (5.4–5.9)[h]	5.6 (5.4–5.9)[h]	5.8 (5.6–5.9)[h]	0.404[c]
TP 2	6.3 (5.7–6.6)	6.6 (6.0–6.7)[h]	6.7 (5.9–6.9)[h]	0.112[c]
HIV RNA median (IQR), log$_{10}$ copies/mL				
TP 1	NA	2.3 (1.7–3.3)	3.9 (2.6–4.3)	0.009[c]
TP 2	NA	3.2 (1.9–3.9)	4.6 (4.0–4.6)	0.003[c]

[a] NA, not applicable

[b] IQR, interquartile range

[c] Kruskal-Wallis test

[d] Estimated from the time of first injection drug use

[e] Chi-Square test

[f] Wilcoxon Signed-rank test

[g] Time of first injection drug use unknown for n=5 (group1), n=2 (group 2), n = 2 (group 3)

[h] HCV RNA unknown for n=1 (group 1, TP1), n=1 (group 2, TP1), n=1 (group 3, TP1), n=3 (group 2, TP2), n=2 (group 3, TP2)

Table 2

Phylogenetic analysis of paired sequence data

Characteristic	Group 1 (HIV -)	Group 2 (HIV+)	Group 3 (HIV+)	*P*[a]
Divergence (range)				
Median (range)	0.0054 (0 – 0.094)	0.0044 (0 – 0.049)	0.0053 (0 – 0.071)	0.903
Interval between specimens				
Median months (range)	36 (13–40)	31 (12–43)	27(13–43)	0.036[b]
Rate of divergence/year	0.0018 (0 – 0.050)	0.0022 (0 – 0.033)	0.0024 (0 – 0.022)	0.470
Nonsynonymous (d_S)				
Distance Median (range)	0.0014 (0 – 0.065)	0.0026 (0 – 0.024)	0.0027 (0 – 0.031)	0.916
Rate Median (range)	0.00067 (0 – 0.034)	0.00092 (0 – 0.016)	0.0010 (0 – 0.0096)	0.647
Synonymous (d_N)				
Distance Median (range)	0.0016 (0 – 0.034)	0.0031 (0 – 0.024)	0.0035 (0 – 0.044)	0.533
Rate Median (range)	0.00055 (0 – 0.024)	0.0014 (0 – 0.016)	0.0013 (0 – 0.014)	0.288
d_N/ d_S ratio				
Median (range)	0.94 (0 – 13)	0.79 (0 – 15)	0.69 (0 – 23)	0.379

[a] Sequence results compared using the Kruskal-Wallis Test

[b] Group 1 significantly longer interval between visits compared to group 3 using Dunn's multiple comparison test.