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HIV Variability in the Liver and Evidence of Possible Compartmentalization

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Abstract

There is growing evidence to suggest that HIV may interact with several hepatic cell types; however, evaluation of HIV variability in liver tissue has not been addressed to date. Among 16 HIV-positive individuals examined, nine (56%) had detectable HIV RNA in the liver. The mean CD4 cell count for these nine individuals was 337 cells/mm^3 (range: 0–601), while their mean plasma HIV RNA level was 106,974 copies/ml (range: 1200–320,740). Among individuals in this study with detectable HIV in both the plasma and the liver, the consensus *gag* nucleotide sequences for each tissue type were different for seven of seven (100%) individuals, while amino acid sequences were distinct for five of seven (71%). Consensus envelope (*env*) nucleotide and amino acid sequences were also distinct in the plasma and liver tissue for six of six (100%) individuals. Statistical evidence of compartmentalization between HIV in the plasma and in the liver was demonstrated, and multiple liver-specific amino acids were identified that may distinguish HIV variants replicating within the liver. These preliminary data demonstrate that HIV is frequently detectable in the liver of HIV-positive persons at various levels of immunosuppression. Possible compartmentalization may reflect tissue-specific selection pressures that drive viral adaptation to the liver microenvironment and may facilitate interactions with other hepatotropic viruses.

Introduction

Hepatic disease is increasingly recognized as a major cause of morbidity and mortality among HIV-positive individuals. Furthermore, HIV coinfection is associated with enhanced hepatitis C virus (HCV) replication, more advanced liver fibrosis and cirrhosis, higher rates of progressive liver disease and death, and decreased HCV treatment response.[1] While liver biopsies represent the gold standard for detecting liver damage, their utilization in individuals with HIV remains infrequent despite high frequencies of both hepatomegaly and liver enzyme abnormalities.[2–5] Even HIV-positive individuals with no evidence of viral hepatitis coinfection often exhibit mild-to-moderate increases in liver enzyme levels.[6,7] A recent study also found that HIV RNA levels were positively associated with liver fibrosis in HIV monoinfected persons even after controlling for other confounders,[8,9] thus supporting the involvement of HIV itself in hepatic disease.

Several lines of evidence suggest that HIV is present in the liver.[1] For instance, HIV RNA and proviral DNA have been detected in liver biopsies from persons with HIV infection.[10,11] Immunohistochemistry and *in situ* hybridization studies using liver specimens from HIV-infected individuals have also demonstrated HIV p24 protein and HIV RNA in Kupffer cells, inflammatory mononuclear cells, sinusoidal cells, and hepatocytes.[10,12,13] Efficient activation of the HIV long terminal repeat has also been reported in hepatocytes.[14,15] Importantly, we[16] and others[17–19] have demonstrated that HIV can infect hepatocyte-derived cell lines, as well as primary hepatocytes, although likely at lower levels than occurs during infection of lymphocytes. Collectively, these data would indicate that several distinct cell types within the liver might be permissive to HIV infection.

A hallmark of RNA viruses is their extreme variability. Within an individual, a population of viral variants termed the viral quasispecies exists. These variants may allow for the rapid, adaptive response of HIV to immunologic selection pressures and/or antiviral therapy.[20] Several studies have demonstrated an association between quasispecies diversity and HIV disease progression.[21–23] Importantly, HIV variability is not evenly distributed throughout the body, and distinct viral subpopulations may exist in different compartments within an infected individual.[24] For example, the blood and male genital tract may represent distinct HIV compartments as viral diversity and/or the majority sequences are often discordant in the blood compared to the genital tract.[25–29]

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Similarly, HIV compartmentalization may occur in the brain and cerebrospinal fluid,[30–32] suggesting that viral adaptation is frequently necessary for efficient infection of and replication within a particular cell/tissue type. Currently, there are no published reports on HIV diversity within the liver despite the link between HIV and liver disease. Thus, it is not clear if all variants of HIV present within an individual are equally capable of infecting the liver or if selection of particular HIV variants with tropism for the liver is occurring. Therefore, we investigated the presence of HIV RNA in liver biopsies and addressed whether HIV variability in the liver differed from that in the plasma.

Materials and Methods

Study participants

For this pilot study, a convenience sampling of 12 HIV-infected individuals was randomly selected from those receiving routine clinical care at the University of Cincinnati College of Medicine or those being evaluated for the initiation of antiretroviral therapy (ART). All subjects signed informed consents permitting collection of tissue and blood. Liver tissue and plasma collected at the time of autopsy were available for an additional four individuals through the National Disease Research Interchange.

Reverse transcriptase polymerase chain reaction amplification of HIV

Viral RNA was extracted from 140 μl of patient plasma using the QIAamp Viral RNA kit or from homogenized liver biopsies (typically 1–2 mm in length) using the RNeasy Mini kit. HIV RNA was detected by nested reverse transcriptase–polymerase chain reaction (RT-PCR) for HIV *gag* (p24) and *env* as described previously.[33] Briefly, to amplify a 485-nucleotide fragment of *gag* (nucleotides 1237–1721 of the HIV reference HXB2), first round primers were 5′–CCC TGR CAT GCT GTC ATC A–3′ and 5′–AGY CAA AAT TAY CCY ATA GT–3′ and second round primers were 5′–AGR ACY TTR AAY GCA TGG GT–3′ and 5′–TGT GWA GCT TGY TCR GCT C–3′. To amplify a 337-nucleotide fragment of *env* (nucleotides 7002–7338 of HXB2), first round primers were 5′–ATG GGA TCA AAG CCT AAA GCC ATG TG–3′ and 5′–ACT GCT TCC TGC TGC TCC CAA GAA CCC AAG–3′ and second round primers were 5′–CTG TTA AAT GGT AGT CTA GC–3′ and 5′–CAA TTT CTG GGT CCC CTC CTG AG–3′. All RT-PCR amplifications included one reaction containing no reverse transcriptase and a separate reaction containing no template as negative controls. The housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was also detected in liver tissues as previously described.[34]

PCR products were gel purified and ligated into a standard cloning vector (Promega; Madison, WI). Plasmids were propagated and purified prior to sequencing using dye terminator chemistry. Multiple plasmids per sample source were sequenced for *gag* (average 10.3 clones) and for *env* (average 10.8 clones) in the forward and reverse directions and edited using CodonCode Aligner 1.5.2 (CodonCode Corporation, Dedham, MA).

Phylogenetic and signature sequence analyses

All alignments were performed using the neighbor-joining (NJ) approach implemented in Clustal X.[35] By aligning compartment-specific viral variants from each tissue/cell type, consensus sequences were generated. References available through the HIV sequence database (http://www.hiv.lanl.gov/content/sequence/NEWALIGN/align.html) were used to identify the HIV subtype (data not shown). Subsequent analyses were performed using the prototype HIV-1 subtype B sequences HXB2 and NL4-3 as references, and the statistical robustness and reliability of the branching order within each phylogenetic tree were confirmed by bootstrap analysis using 1000 replicates.[36] Phylogenetic inference was also performed using a Bayesian Markov chain Monte Carlo (MCMC) approach as implemented in the BEAST v1.5.0 program[37] under an uncorrelated log-normal relaxed molecular clock using the generalized time reversible (GTR) or Hasegawa, Kishino, and Yano (HKY) model with nucleotide site heterogeneity estimated using a gamma distribution. The MCMC analysis was run for a chain length of 50,000,000 with sampling every 5000th generation. Results were visualized in Tracer v1.4 to confirm chain convergence, and the effective sample size (ESS) was calculated for each parameter. All ESS values were >500 indicating sufficient sampling. The maximum clade credibility tree was selected from the posterior tree distribution after a 10% burn-in using TreeAnnotator v1.5.0. Consensus sequences have been submitted to GenBank under accession numbers HM365303–HM365331.

Intrapatient genetic distances were calculated by pairwise comparison of nucleotide sequences at each time point using the Kimura method of MegAlign (DNASTAR, Inc., Madison, WI). Shannon entropy [$S_n = -\sum(p_i \ln p_i)/\ln N$], where p_i is the frequency of each distinct nucleotide sequence and N is the total number of sequences analyzed, was also calculated.[38] Entropy values vary from 0 (all sequences are identical) to 1 (all sequences are distinct). Nonsynonymous (dN) and synonymous (dS) mutations were calculated via the Nei–Gojobori method in MEGA.[39] Amino acid translations and manual editing to preserve the open reading frame were performed in MacClade version 4.08.[40] The Viral Epidemiology Signature Pattern Analysis (VESPA) program was used to determine the frequency of each amino acid in liver-derived versus serum-derived viral variants for each individual.[41] Only amino acid signatures above a 70% threshold were considered significant. Envelope coreceptor utilization was determined for consensus and clonal sequences using the WetCat program at http://genomiac2.ucsd.edu:8080/wetcat/v3.html.

Compartmentalization analyses

Compartmentalization of viral variants was assessed using Mantel's test as previously described to explore HIV compartmentalization.[42,43] Briefly, the Kimura two-parameter distance matrix that included all plasma and liver biopsy variants for an individual subject was compared to a similar matrix in which distances are replaced with 0 if the sequences are from the same compartment (e.g., plasma versus plasma or liver biopsy versus liver biopsy) and with 1 if the sequences are from distinct compartments (e.g., plasma versus liver biopsy or vice versa). The correlation coefficient was computed for the simple Mantel's test using the Permute! Software version 3.4α9 (www.bio.umontreal.ca/casgrain/en/labo/permute/index.html) with 9999 permutations. Compartmentalization was also assessed using the Slatkin–Maddison (S-M) test[44] for population gene flow as implemented in the

HyPhy program[45] using at least 5000 permutations. *p*-values <0.05 were considered statistically significant evidence that viral sequences derived from liver biopsies were compartmentalized compared to those derived from the corresponding plasma.

Statistical analysis

CD4 cell counts and plasma HIV viral loads were compared in persons with and without detectable HIV in the liver using two-sample *t* tests, while measures of diversity were compared using the Wilcoxon rank sum test (Statistix 9.0; Analytical Software, Tallahassee, FL).

Results

Patient demographics and HIV detection

A convenience sample of 16 HIV-positive individuals was utilized for this pilot study (Table 1). The average CD4 cell count was 401 cells/mm^3 (range: 0–764 cells/mm^3). Plasma HIV viral loads were detectable in 12 of 13 individuals (mean: 68,131 copies/ml; range 463–320,740 copies/ml) with available data. ART utilization was not an exclusion criteria; however, only two individuals were receiving ART at the time of sample collection. Thirteen of 16 individuals were HCV seropositive, while two individuals were hepatitis B virus (HBV) surface antigen positive. The median ALT and AST values were 58 U/liter and 77 U/liter, respectively.

Using RT-PCR, nine individuals (56.3%) had detectable HIV RNA in the liver. This included eight individuals with HIV *gag* detected in their livers, and six individuals with HIV *env* detected in their livers. To further confirm the quality of the extracted RNA, RT-PCR for GAPDH was attempted for 10 randomly selected liver biopsies included in the current study; all 10 were PCR positive (data not shown). The mean CD4 cell count for these nine individuals was 337 cells/mm^3 (range: 0–601), while their mean plasma HIV RNA level was 106,974 copies/ml (range: 1200–320,740). Seven individuals were also coinfected with HCV, while one individual was HBV surface antigen positive. CD4 cell counts were lower in those individuals with HIV detected in the liver compared to those without detectable HIV in the liver, although this difference did not reach statistical significance (336.6 cells/mm^3 versus 516.2 cells/mm^3; $p=0.080$). Interestingly, plasma HIV viral loads were significantly higher in those individuals with HIV detected in the liver compared to those without detectable HIV in the liver (106,974 copies/ml versus 5982 copies/ml; $p=0.033$).

Intrapatient HIV variability

We performed a phylogenetic analysis of HIV *gag* and/or *env* sequences for the nine individuals with detectable HIV RNA in the liver. All were infected with HIV-1 subtype B, and sequences clustered by individual suggesting that there were no epidemiologically linked infections. The consensus *gag* nucleotide sequences were different for seven of seven (100%) individuals with matched liver biopsies and plasma using the NJ approach (data not shown). Similar analyses and results were obtained using a Bayesian inference approach based on a GTR or HKY substitution model (Fig. 1A). Consensus *gag* amino acid sequences were distinct for five of seven (71.4%) individuals, while two individuals had synonymous nucleotide changes not affecting the resultant amino acid sequence. Similarly, the consensus *env* nucleotide sequences were different for six of six (100%) individuals with matched liver biopsies and plasma using both NJ (data not shown) and Bayesian approaches (Fig. 1B). The consensus *env* amino acid sequences were distinct for all six individuals.

To more accurately represent intrapatient diversity, individual viral variants derived from plasma and liver biopsies were compared. In individuals with available *gag* sequence

Table 1. Patient Demographic and Clinical Characteristics

Patient ID	*HIV viral load (copies/ml)*	*CD4 cell count (cells/mm^3)*	*HIV treatment at time of biopsy*	*Year of HIV diagnosis*	*ALT[a] (U/liter)*	*AST (U/liter)*	*HCV serostatus*	*HBV surface antigen*	*Risk factor*
Cin 01	56,335	408	No	1995	91	91	Positive	Negative	Sexual
Cin 02	218,106	423	No	1989	39	62	Positive	Negative	Sexual
Cin 03	320,740	338	No	2005	53	77	Positive	Negative	Sexual
Cin 04	98,919	265	No	2006	177	212	Positive	Negative	Sexual
Cin 05	29,638	346	No	1987	33	185	Positive	Negative	Sexual; drug use
1347	17,813	569	No	1988	NA	NA	Positive	Negative	Transfusion
1370	5,481	764	No	1997	NA	NA	Positive	Negative	Sexual
1493	6,126	397	No	1990	59	42	Positive	Negative	Hemophilia
1580	1,200	410	No	2001	NA	NA	Positive	Negative	Sexual; drug use
1627	463	547	NA	1990	58	47	Positive	Negative	Drug use
1724	<48	304	Yes	1999	56	54	Positive	Negative	Drug use
1756	66,370	601	No	1986	154	108	Negative	Negative	Sexual; drug use
HV52[b]	64,485	0	No	1992	NA	NA	Negative	Positive	NA
HV67[b]	NA	NA	NA	2005	NA	NA	Negative	Positive	NA
HV104[b]	NA	NA	Yes	1988	NA	NA	Positive	Negative	Hemophilia
HV106[b]	NA	238	No	2000	NA	NA	Positive	NA	Drug use

[a]Normal values for healthy individuals are defined as <19 U/liter for women and <30 U/liter for men.
[b]Denotes samples collected at autopsy.
PIDs in **bold** had detectable HIV *gag* and/or *env* sequences in liver biopsy. NA, not available; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCV, hepatitis C virus; HBV, hepatitis B virus.

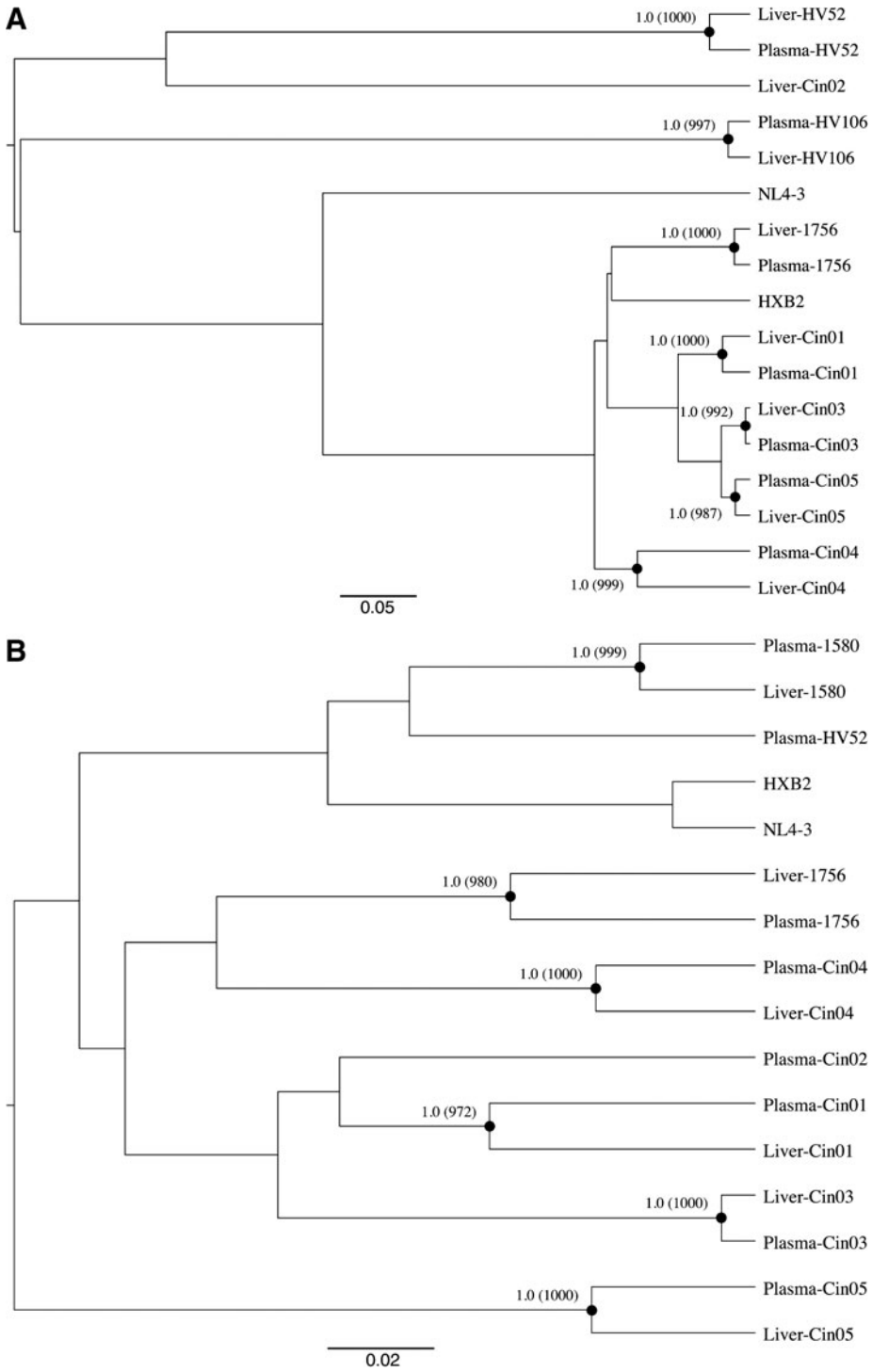

FIG. 1. Consensus nucleotide sequences in *gag* **(A)** and *env* **(B)** from plasma and liver biopsy tissue using a Bayesian inference approach. Relevant posterior probabilities greater than 90% are shown. Relevant bootstrap values >700 out of 1000 from a neighbor-joining approach are shown in parentheses. The reference sequences HXB2 (accession number K03455) and NL4-3 (M19921) are also included.

data, the median intrapatient genetic distance was 1.36% in the plasma (range: 0.80–3.12%) and 1.09% in the liver biopsy tissue (range: 0.00–2.77%) as shown in Supplementary Fig. S1A (Supplementary Data are available online at www.liebertonline.com/aid) [p = not significant (NS)]. Median *gag* entropy, which represents both the number of distinct variants as well as their frequencies, was 1.00 in the plasma (range: 0.84–1.00) and 0.83 in the liver biopsy tissue (range: 0.00–1.00) as shown in Supplementary Fig. S1C ($p = 0.021$). As an indicator of positive selection, dN-dS values were calculated for *gag* but were not greater than 0 for any plasma or liver biopsy tissue analyzed. Median dN-dS values were −0.045 (range: −0.082 to −0.026) and −0.30 (–0.053–0.00) in the plasma and liver biopsy tissue, respectively (Supplementary Fig. S1E; p = NS).

In individuals with available *env* sequence data, the median intrapatient genetic distance was 2.17% in the plasma (range: 0.00–6.47%) and 0.16% in the liver biopsy tissue (range: 0.00–1.85%) (Supplementary Fig. S1B; $p = 0.059$). Median *env* entropy was 0.77 in the plasma (range: 0.18 – 1.00) and 0.36 in the

liver biopsy tissue (range: 0.00–0.84) (Supplementary Fig. S1D; $p = \text{NS}$). Values for dN-dS were greater than 0 for one plasma sample (1580) and three liver biopsies (Cin05, 1580, and 1756). Median dN-dS values were −0.0032 (range: −0.0250–0.0234) and 0.0003 (–0.0205–0.0147) in plasma and liver biopsy samples, respectively (Supplementary Fig. S1F; $p = \text{NS}$).

Coreceptor utilization

Envelope coreceptor utilization was assessed with pretrained classifier algorithms using consensus and clonal envelope sequences. For viruses from individuals 1756, HV52, Cin01, Cin02, Cin03, Cin04, and Cin05, the consensus envelope sequences from plasma and liver were predicted to utilize the CCR5 coreceptor. However, for subject 1580, the envelope sequences from plasma and liver were predicted to utilize the CXCR4 coreceptor. When clonal sequences were analyzed, the majority (64–100%) of viruses from the plasma and liver were predicted to utilize CCR5 for subjects 1756, Cin01, Cin03, Cin04, and Cin05. In contrast, two algorithms (*C4.5* and *C4.5–only p8 and p12*) predicted CCR5 in all clones for subject 1580 regardless of the cell/tissue type, while three algorithms (*PART*, *SVM*, and the *Charge Rule*) predicted that only 0–22% of plasma-derived clones and 11–44% of liver-derived clones utilized CCR5. These findings are further supported by *in vitro* studies suggesting that liver cell infection may utilize CCR5 or CXCR4.[16,17,19]

Analysis of compartmentalization

Phylogenetic trees were also reconstructed for each of the individuals with both plasma and liver biopsy variants (Fig. 2). In the analysis of intrapatient *gag* variability, three

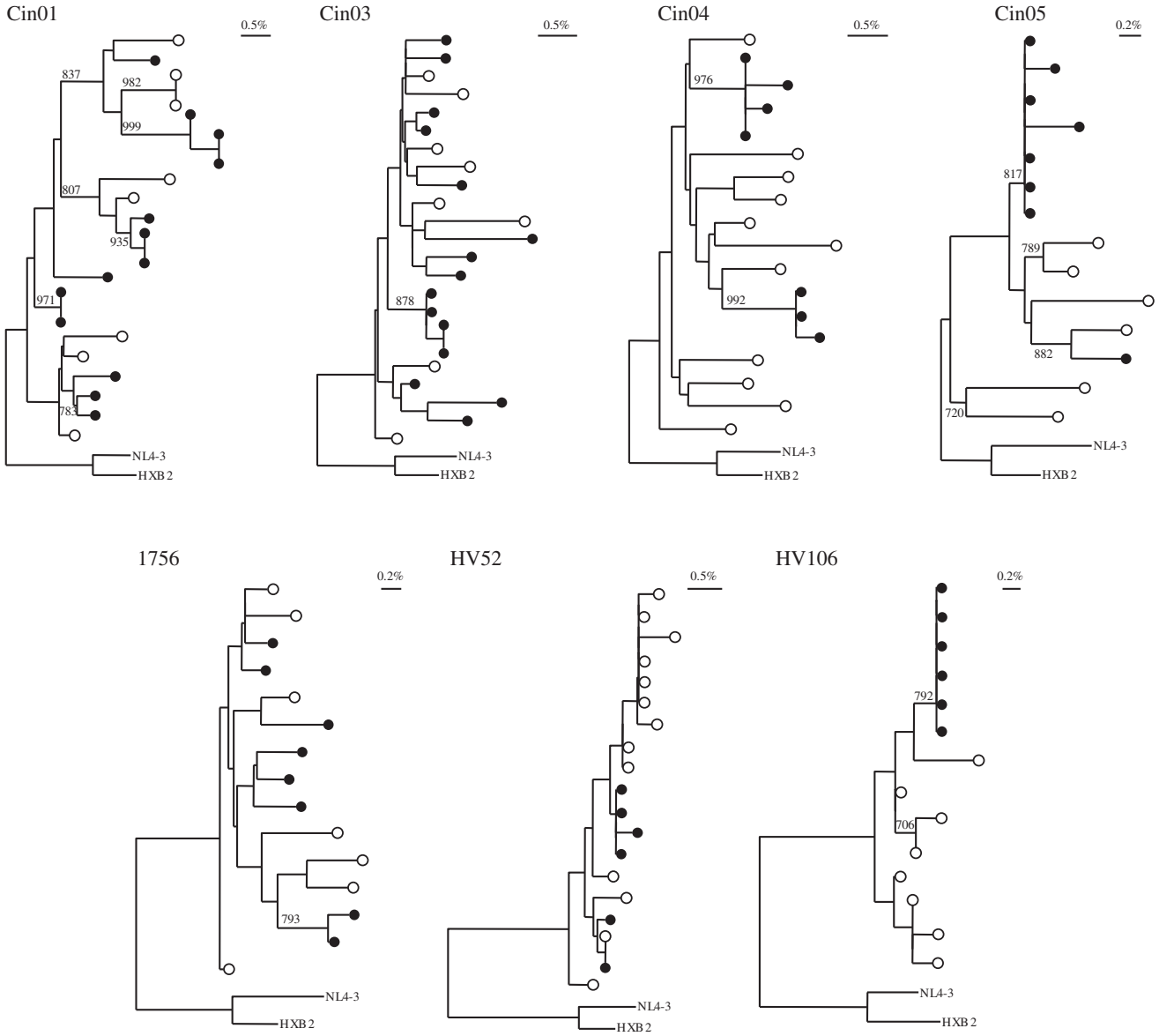

FIG. 2. Patient-specific phylogenetic trees with *gag* viral variants from plasma (open circles) and liver biopsy tissue (closed circles). Shown in the upper right corner is a bar depicting the percent genetic distance for each tree. Only relevant bootstraps greater than 700 out of 1000 are shown. HXB2 (accession number K03455) and NL4-3 (M19921) are included as references.

distinct patterns were observed. Pattern 1 included one individual (HV52) with plasma- and liver-derived variants that were intermingled with no statistically significant clustering of these variants by sample source. Pattern 2 included four individuals (Cin01, Cin03, Cin04, 1756) with plasma- and liver-derived variants that were intermingled but with at least one statistically significant clustering of variants by sample source. Pattern 3 included two individuals (Cin05 and HV106) with complete, or near complete, separation of plasma- and liver-derived variants into distinct groupings that were supported by high bootstrap values.

In the analysis of intrapatient *env* variability, only patterns 2 and 3 were observed (Fig. 3). For example, in individuals Cin03 and 1580, some intermingling of plasma- and liver-derived variants was observed, although there were also smaller groupings of variants that clustered by sample source. In contrast, for individuals Cin01, Cin04, Cin05, and 1756, viral variants from the liver clearly clustered separately from viral variants from the plasma and were supported by high bootstrap values. For 1756, *env* sequences from peripheral blood mononuclear cells (PBMCs) were also available for analysis and demonstrated clear clustering of PBMC- and plasma-derived variants that was distinct from the cluster of liver-derived variants. A replicate RT-PCR of *env* using a second RNA extraction from the same liver biopsy, plasma, and PBMCs samples of subject 1756 also demonstrated evidence of significant compartmentalization between the liver and plasma/PBMCs (data not shown).

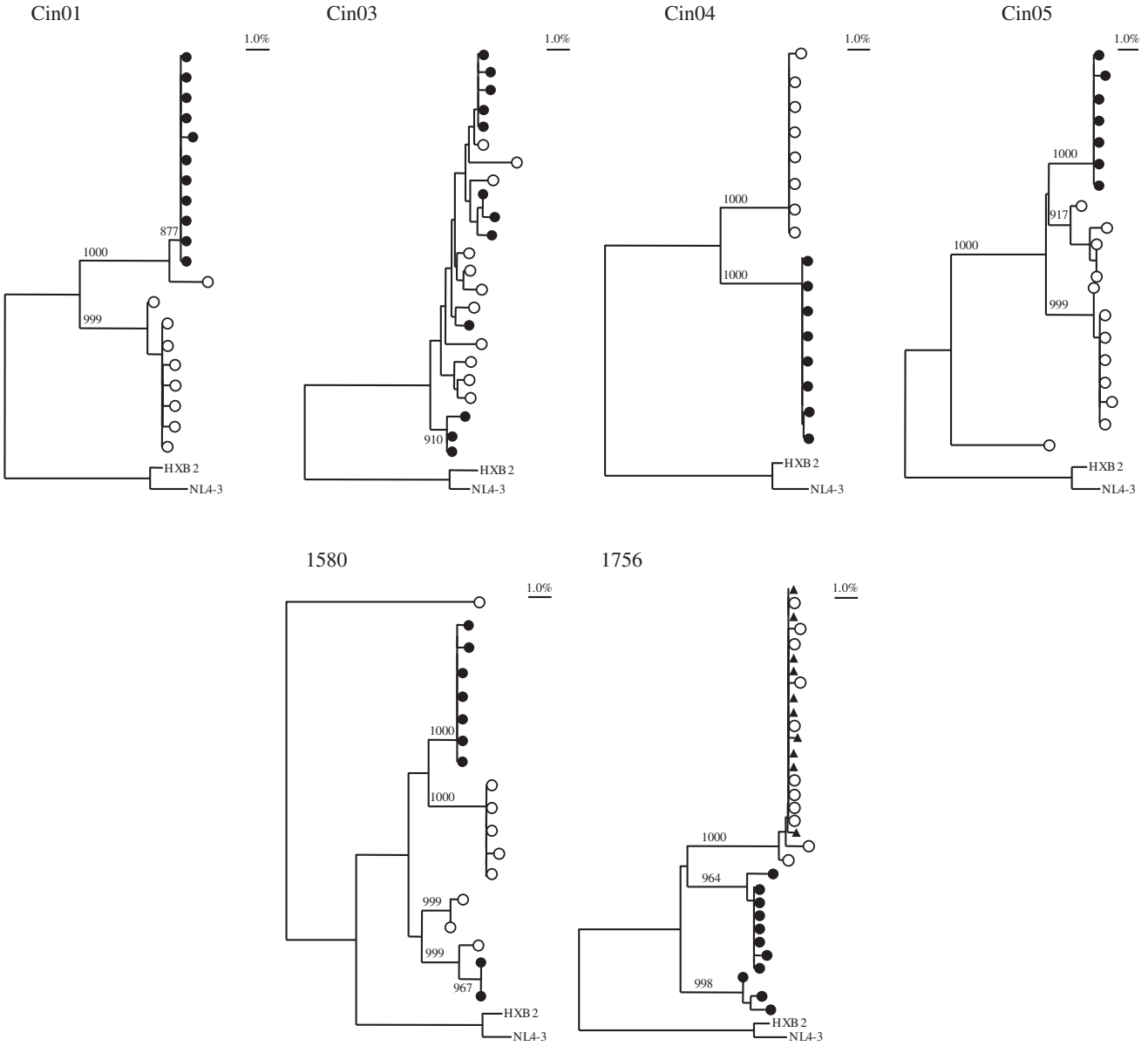

FIG. 3. Patient-specific phylogenetic trees with *env* viral variants from plasma (open circles) and liver biopsy tissue (closed circles). For subject 1756, viral variants from peripheral blood mononuclear cells (PBMCs) (closed triangles) were also included. Shown in the upper left corner is a bar depicting the percent genetic distance for each tree. Only relevant bootstrap values greater than 700 out of 1000 are shown. HXB2 (accession number K03455) and NL4-3 (M19921) are included as references.

We further explored the potential compartmentalization of HIV in the liver using Mantel's test as shown in Table 2. When comparing matched plasma- and liver biopsy-derived viral variants in the *gag* region of HIV, the results of Mantel's test were consistent with significant quasispecies compartmentalization for Cin05 ($p = 0.0009$) and HV106 ($p = 0.0027$), while Cin04 ($p = 0.066$) and HV52 ($p = 0.091$) showed a trend toward compartmentalization. No significant compartmentalization in *gag* was observed for 1756, Cin01, or Cin03. S-M test results demonstrated statistically significant evidence of *gag* compartmentalization for Cin04, Cin05, HV52, and HV106.

Evidence of significant compartmentalization in *env* was observed for 1580 ($p = 0.0043$), 1756 ($p = 0.0001$), Cin01 ($p = 0.0001$), Cin04 ($p = 0.0005$), and Cin05 ($p = 0.0002$) using Mantel's test, while Cin03 ($p = 0.060$) also showed a trend toward compartmentalization in *env*. S-M test results similarly demonstrated statistically significant evidence of *env* compartmentalization for 1580, 1756, Cin01, Cin02, Cin03, Cin04, and Cin05. Thus, all nine individuals with HIV detectable in the liver demonstrated evidence of *gag* and/or *env* compartmentalization between the plasma and liver biopsy with at least one statistical test of compartmentalization.

Signature sequence analysis of liver-specific amino acids

To identify specific amino acids associated with HIV in the liver, signature sequence analysis was performed using *gag* and/or *env* sequences for matched plasma-biopsy samples. A significant difference in amino acid frequency was identified in a single individual when analyzing *gag* variants in the liver biopsy compared to those in the corresponding plasma samples. For HV52, a 12 amino acid insertion was present in the liver biopsy that was present only at very low frequency among variants from the corresponding plasma. For the other five individuals with matched sequence data, no other liver-specific signature amino acids were identified in the region of *gag* analyzed.

In contrast, five of six individuals (Cin01, Cin04, Cin05, 1580, and 1756) had evidence of distinct amino acids frequencies when analyzing *env* data. For these five individuals, a total of 49 liver-specific signature amino acids (mean 9.8; range: 1–18) was identified (Fig. 4). Of these signature amino acids 14 (26.9%) were located within the V3 loop. Four amino acid signatures were shared by at least two individuals.

Discussion

To date, only one published study has examined HIV variability in the liver. Van't Wout *et al.* assessed HIV proviral DNA variability from a single individual who died of AIDS-related complications.[46] They concluded that the presence of HIV in nonlymphoid tissues was likely the result of the late disease stage of the samples examined, although samples collected from earlier disease stages were not included. Similarly, Donaldson *et al.* reported that infection of nonlymphoid organs, such as the liver, occurred only in individuals with AIDS-defining illnesses and not among asymptomatic individuals[11]; however, HIV variability was not explored. In contrast, in the current study, HIV RNA was detected in nine individuals with a wide range of CD4 cell counts (0 to 601 cells/mm^3) suggesting that HIV infection of the liver may occur at varying levels of immunosuppression.

Importantly, several lines of evidence suggest that the liver may represent a potential site of HIV compartmentalization. First, the presence of distinct consensus sequences in the liver compared to the corresponding plasma/PBMCs argues against simple contamination of biopsy tissues with peripheral lymphocytes and/or cell-free virions from the peripheral blood supply. Second, patient-specific clonal analysis frequently demonstrated distinct HIV variants in the liver compared to the corresponding plasma. These findings were further supported statistically by the results of both Mantel's and Slatkin–Maddison tests. Third, signature sequence analysis identified 49 amino acid residues in the region of *env* sequenced that were associated with HIV detection in the liver. These amino acids imply adaptation of HIV for infection of the liver and may impact HIV replication and/or cell tropism.

Despite frequent observation of liver enzyme abnormalities in those with HIV infection, HIV treatment providers rarely include liver biopsy in the evaluation process of these patients. Liver biopsy is more commonly employed in patients with HBV and/or HCV infection; therefore, few investigators have had access to samples that would permit assessment of the direct effects of HIV on the liver *in vivo*. While our methodology does not provide any information regarding the specific cell type(s) that may be infected by HIV or the overall level of HIV replication in the liver, there is considerable evidence suggesting that several liver cell types can support HIV replication as reviewed elsewhere.[1] Thus, it is reasonable to assume that there is at least one cell type in the liver that is capable of supporting HIV replication. Although we cannot

Table 2. *p*-Values from Mantel's Test and Slatkin–Maddison Test for HIV Compartmentalization in the Liver Compared to the Plasma

Patient ID	*Gag Mantel's test*	*Gag Slatkin–Maddison test*	*Env Mantel's test*	*Env Slatkin–Maddison test*
Cin01	NS[a]	NS	0.0001	< 0.0001
Cin03	NS	NS	0.060	0.0049
Cin04	0.066	0.0109	0.0005	0.0001
Cin05	0.0009	0.0103	0.0002	0.0001
1580	Not done	Not done	0.0043	0.0104
1756	NS	NS	0.0001	< 0.0001
HV52	0.091	0.0023	Not done	Not done
HV106	0.0027	0.0003	Not done	Not done

[a]NS, nonsignificant ($p > 0.10$).

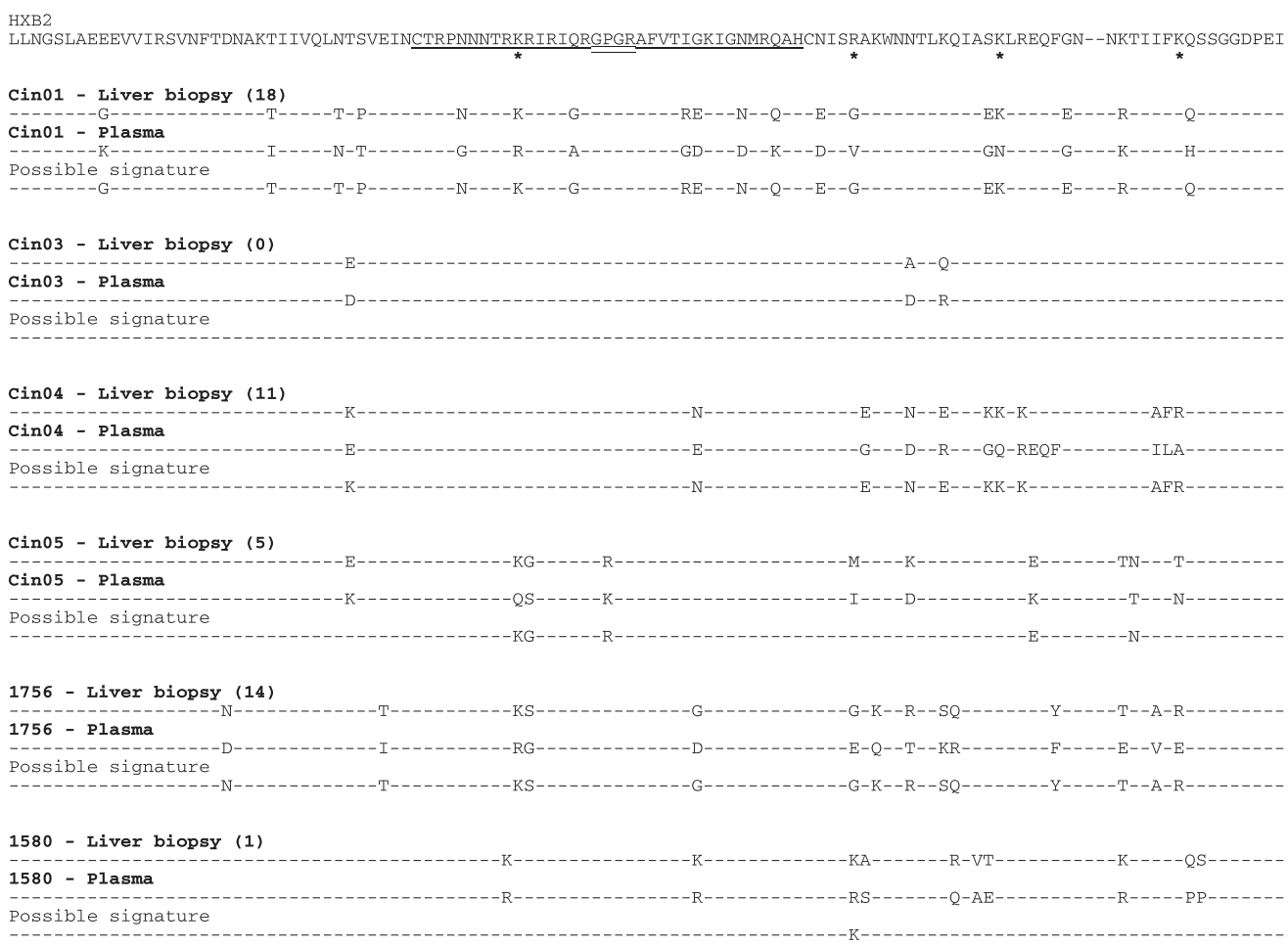

FIG. 4. Signature sequence analysis showing amino acid positions at which the distributions of liver- and plasma-specific envelope variants are significantly different. Numbers in parentheses indicate the number of amino acid signatures present in the liver. Shown are the consensus amino acids at each position. In several instances, a given amino acid may appear to be identical between the two compartments; however, the frequency distribution of the viral variants that make up that consensus is different between the two compartments. Asterisks denote amino acid signatures that were shared by at least two individuals. All sequences are shown relative to their position within HXB2 with the single underlined amino acid residues denoting the V3 loop (amino acids 296–330) and the V3 tip (amino acids 312–315) double–underlined.

definitively rule out contamination by PBMC-derived HIV, this is unlikely for two reasons. First, several studies have shown little or no compartmentalization, or similar mutational patterns, in PBMCs compared to plasma/serum.[47–49] Second, when PBMCs were included from subject 1756, PBMC- and plasma-derived viruses grouped together but were separate from liver-derived sequences.

Several limitations of the current study warrant further discussion. While the population size is modest, these data represent the largest study of HIV variability in the liver ever performed. Additionally, while several distinct methods to detect viral compartmentalization are available, there is no one gold standard or preferred approach. For example, Zarate *et al.* compared multiple methods for detecting HIV compartmentalization and found that discordant predictions by distinct methods may occur; therefore, utilizing several complementary methods, as performed here, provides the most reliable assessment of viral compartmentalization.[50] Furthermore, the cross-sectional nature of this analysis does not permit a detailed examination of liver-specific HIV variants over time or provide important data on the possible trafficking of liver-specific HIVs into the peripheral circulation. Similarly, it is possible that our cloning strategy may not have amplified all minor variants present in a given tissue/cell type.

Our study is the first to explore HIV RNA variability in the liver and to demonstrate distinct HIV variants in liver biopsy tissues. However, it is important to note that HIV was not amplifiable in all samples examined, although we did not directly quantify intrahepatic levels of HIV in the current study. Thus, identification of the virologic, immunologic, and genetic factors that impact HIV detection in the liver will require additional study. Moreover, exploring the variability of additional HIV genomic regions amplified from the liver, as well as detecting low-frequency viral variants by single-genome amplification, is warranted and may enhance our understanding of HIV pathogenesis. Finally, additional studies are currently underway to determine the relative contributions of distinct liver cell types to HIV pathogenesis and viral diversity, as well as the impact of liver-derived HIV on liver damage and fibrosis progression.

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Author Disclosure Statement

No competing financial interests exist.

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