VIROLOGY

Analysis of HIV Quasispecies Suggests Compartmentalization in the Liver

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Abstract

Liver disease is now a major cause of morbidity and mortality among persons infected with the human immunodeficiency virus (HIV). An increasing body of evidence suggests that HIV infection is associated with exacerbated liver fibrosis and that HIV has the ability to infect several hepatic cell types. Despite the recognized existence of genetically distinct subpopulations of HIV in the central nervous system and genital tract, viral diversity and compartmentalization in the liver have not been explored extensively. Therefore, phylogenetic analysis was performed on full-length *env* and *nef* sequences for four patients. Distinct clustering of viral variants was observed for all patients in both areas of the genome. Statistical evidence of HIV compartmentalization in the liver was demonstrated in 85.4% of comparisons. Signature sequence analysis identified several liver-specific amino acids in all patients. Thus, the current study demonstrates statistically significant evidence for HIV compartmentalization in the liver. Additionally, these data suggest that the hepatic microenvironment harbors unique selective pressures that drive viral adaptation.

Introduction

CHRONIC LIVER DISEASE is a major cause of non-AIDS-related mortality in HIV-infected individuals.[1–3] This can partially be attributed to coinfection with hepatitis C virus (HCV) and HIV, which share similar transmission routes. An estimated seven million people are HIV/HCV coinfected worldwide.[4] Coinfection is associated with acceleration of liver fibrosis and cirrhosis, elevated HCV RNA levels, and decreased HCV treatment response rates compared to HCV monoinfected individuals.[5] Additionally, HIV infection—with and without viral hepatitis coinfection—is associated with increased liver enzyme levels and markers for liver fibrosis.[6,7] Increasing evidence indicates a direct correlation between HIV RNA levels and liver fibrosis in persons infected with HIV, even in the absence of viral hepatitis, antiretroviral therapy (ART), and alcohol use.[8,9] A number of other clinical presentations of liver disease are associated with HIV monoinfection, including noncirrhotic portal hypertension and nonalcoholic fatty liver disease.[10] These studies suggest that HIV itself may play a role in liver disease, despite other potential confounders.

Emerging data suggest that HIV has the ability to infect cells of the liver, including endothelial sinusoidal cells, stellate cells, Kupffer cells, and hepatocytes.[11–16] Collectively, these data indicate that HIV is present in several intrahost microenvironments, each harboring its own unique selective pressures, thus contributing to the extensive sequence diversity of HIV. However, to our knowledge, HIV diversity in the liver has been explored in only one previous study.[17] Therefore, the current analysis utilized complementary phylogenetic and statistical approaches to examine intrapatient HIV variability and compartmentalization in the liver.

Materials and Methods

All sequence data from patients HIV$_{Ams}$198, AM, AZ, and DY were previously published without phylogenetic or statistical evaluation of viral compartmentalization.[18,19] All tissue samples were obtained postmortem from patients who died of AIDS-related causes. For the current analysis of viral compartmentalization, alignments were performed using the neighbor-joining (NJ) approach as implemented in Clustal X2.[20] The statistical robustness and reliability of the branching order within each phylogenetic tree were confirmed by bootstrap analysis using 1,000 replicates.[21] The Viral Epidemiology Signature Pattern Analysis (VESPA) program was used to determine the frequency of each amino acid in liver-derived viral variants versus viral variants from other tissues for each individual (www.hiv.lanl.gov/content/sequence/VESPA/vespa.html).[22] Only amino acid signatures above an 80% threshold were considered significant. All nucleotide sequences

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were translated to amino acids using Molecular Evolutionary Genetics Analysis software version 5 (MEGA5).[23] Compartmentalization of viral variants from each tissue type was assessed using Mantel's test as previously described for HIV.[24,25]

Results

Phylogenetic analyses were performed on full-length HIV gp120 and *nef* sequences for patients AM, AZ, and DY, and on V3 loop sequences for patient HIV$_{Ams}$198. A single intrapatient neighbor-joining tree was compiled including sequence data from all tissues for subject HIV$_{Ams}$198 (Fig. 1A). Interestingly, liver-derived sequences formed a single monophyletic cluster that contained no non-liver-derived sequences. Peripheral blood mononuclear cell (PBMC)-derived sequences were interspersed throughout the phylogenetic tree but were not closely related to the liver sequences, suggesting that liver-derived sequences were not the result of peripheral lymphocyte infiltration. Neighbor-joining phylogenetic trees including sequence data from all tissues were created for HIV gp120 and *nef* sequences for patients AZ and DY (Figs. 1B and C and 3A and B). Due to tree size constraints, phylogenetic trees for HIV gp120 and *nef* sequences from patient AM are shown as paired comparisons of the liver and one other tissue (Figs. 2 and 4). Distinct clustering of liver variants was observed in patients AM, AZ, and DY as well. Evidence of viral compartmentalization was supported further by Mantel's tests results, with a *p*-value < 0.05 for 41 of 48 comparisons involving the liver (85.4%) (Table 1).

Analysis of V3 loop sequences

Phylogenetic analysis demonstrated that V3 loop sequences from the brain and cerebral spinal fluid (CSF) exhibited distinct phylogenetic clustering. Likewise, V3 loop variants from the liver formed a distinct monophyletic group supported by a high bootstrap value (962 out of 1,000). No further compartmentalization was observed for any V3 loop sequences from the kidney, lung, lymph node, bone marrow, spleen, and PBMCs (Fig. 1A). Thus, HIV quasispecies compartmentalization was observed in the liver and brain/CSF only for patient HIV$_{Ams}$198. Evidence for significant V3 loop compartmentalization was further supported by Mantel's test, in which all pairwise genetic distance comparisons of V3 loop sequences involving the liver had a *p*-value < 0.05 (Table 1). Signature sequence analysis identified specific amino acid differences between V3 loop variants from the liver and all other tissues (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/aid). The frequency of amino acid differences ranged from 2 in the liver–spleen comparison to 4 in liver–bone marrow, liver–brain, liver–lung, and liver–lymph node comparisons (mean = 3.4) (Supplementary Table S1). The most notable residue change occurred in the conserved GPGR tip of the V3 loop, where a single mutation (R315K) was found only in the liver, brain, and CSF variants (Supplementary Table S1). Two other possible function-altering mutations that serve as coreceptor binding sites were found in the V3 loop—I309M in the brain and N325D in the brain, CSF, and lung.[26]

Analysis of gp120 sequences

Intrapatient phylogenetic trees including gp120 sequences from all available tissues were created for patients DY and AZ

(Fig. 1B and C). Both patients exhibited distinct phylogenetic clustering of gp120 variants from the liver supported by high bootstrap values (1,000 for DY and 819 for AZ). Similar clustering was observed with temporal cortex variants from patient DY and frontal cortex variants from patient AZ. Phylogenetic trees containing paired comparisons involving the liver were created for patient AM. Viral variants from the liver and diaphragm were particularly distinct from one another, as complete separation between these tissues was observed (Fig. 2). All pairings, except liver–right lymph node and liver–spleen showed evidence of compartmentalization supported by high bootstrap values (Fig. 2). Despite the lack of visible phylogenetic separation between the liver and spleen variants from patient AM, Mantel's test comparing these two tissues was statistically significant (*p* = 0.0071) (Table 1). In the analysis of gp120 sequences, Mantel's test confirmed significant evidence of compartmentalization for 17 of 20 comparisons involving the liver (85%) (Table 1). Signature sequence analysis highlighted multiple amino acid differences in HIV gp120 from the liver versus all other tissues for patient DY (range = 21–41, mean = 30.3), AZ (range = 42–46, mean = 43.3), and AM (range = 2–50, mean = 29.3) (Supplementary Tables S2–4). Liver sequence divergence was most apparent in patient AZ, as 42 of 48 total residue differences between liver-derived variants and variants derived from other tissues were found in all three paired comparisons. The liver–diaphragm comparison from patient AM had 50 amino acid substitutions, the highest of all paired comparisons (Supplementary Table S4). Several amino acid differences between gp120 variants from the liver and other tissues occurred in functional areas, including the LDI/LDV tripeptide in the V2 loop, several CD4 contact residues and coreceptor binding sites, the GPGR tip of the V3 loop, the CD4 binding loop, a lectin DC-SIGN binding site, monoclonal antibody glycosylation binding sites, and gp120 contact residues with gp41.[27–30]

Analysis of nef sequences

Complete or near complete separation of *nef* variants from the liver was observed in phylogenetic analyses of sequences from patients DY and AZ (Fig. 3). The statistical robustness of liver variant branching order was supported by high bootstrap values (970 for DY and 730 for AM). Distinct phylogenetic clustering was also demonstrated by *nef* variants from the meninges of patient AZ. No further phylogenetic evidence of compartmentalization was observed for any other tissue evaluated from patients DY and AZ (Fig. 3). Neighbor-joining trees were completed for paired comparisons involving the liver for patient AM (Fig. 4). Complete or near complete separation of viral variants was observed for liver–diaphragm, liver–kidney, liver–prostate, and liver–liver tumor comparisons. Although there was no visible phylogenetic separation of variants between the liver and left lymph node variants from patient AM, Mantel's test suggested that sequences from the liver shared more genetic identity with each other than with sequences from the left lymph node (*p* = 0.0108) (Table 1). Furthermore, Mantel's test provided statistically significant evidence of viral compartmentalization for 16 of 20 HIV *nef* comparisons involving the liver (80 %) (Table 1). Signature sequence analysis of *nef* identified multiple amino acid substitutions between liver-derived variants and variants from all
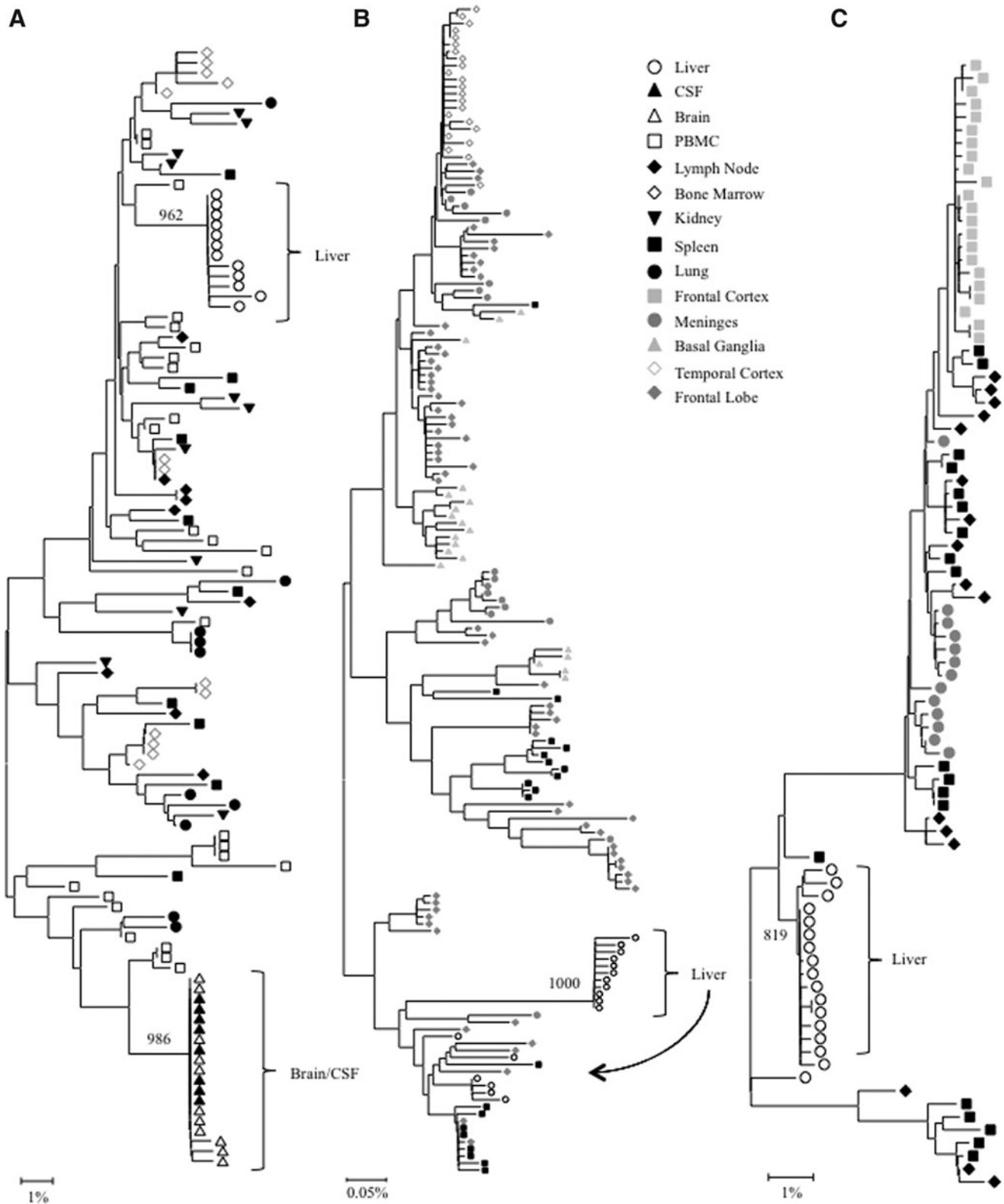
FIG. 1. Intrapatient phylogenetic trees of **(A)** V3 loop variants from patient HIV$_{ams}$198, **(B)** gp120 variants from patient DY, and **(C)** gp120 variants from patient AZ. Shown in the lower left corner is a bar depicting the percent genetic distance for each tree. Compartmentalization is observed only in the liver and brain tissues. PBMC, peripheral blood mononuclear cells; CSF, cerebrospinal fluid. Only relevant bootstrap values greater than 700 out of 1,000 are shown. Sequence data for patient HIV$_{ams}$198 were originally reported in Van't Wout et al.[19] Sequence data for patients AZ and DY were originally reported in Lamers et al.[18]

FIG. 2. Intrapatient phylogenetic trees of gp120 variants from patient AM. Shown in the lower left corner is a bar depicting the percent genetic distance for each tree. Only relevant bootstrap values greater than 700 out of 1,000 are shown. Sequence data were originally reported in Lamers *et al.*[18]
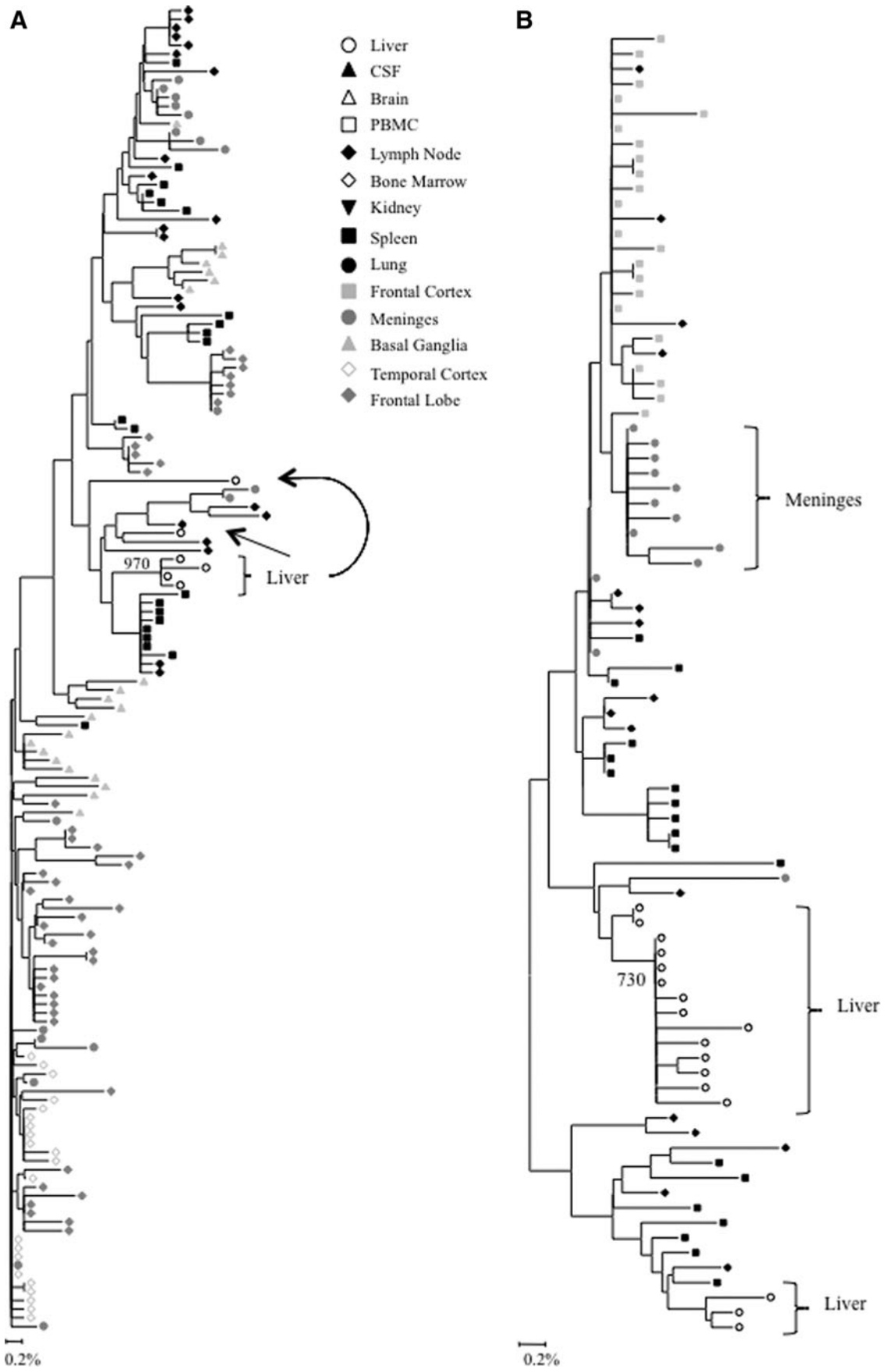
FIG. 3. Intrapatient phylogenetic trees of **(A)** *nef* variants from patient DY and **(B)** *nef* variants from patient AZ. Shown in the lower left corner is a bar depicting the percent genetic distance for each tree. Only relevant bootstrap values greater than 700 out of 1,000 are shown. Sequence data were originally reported in Lamers *et al.*[18]

FIG. 4. Intrapatient phylogenetic trees of *nef* variants from patient AM. Shown in the lower left corner is a bar depicting the percent genetic distance for each tree. Only relevant bootstrap values greater than 700 out of 1,000 are shown. Sequence data were originally reported in Lamers *et al.*[18]
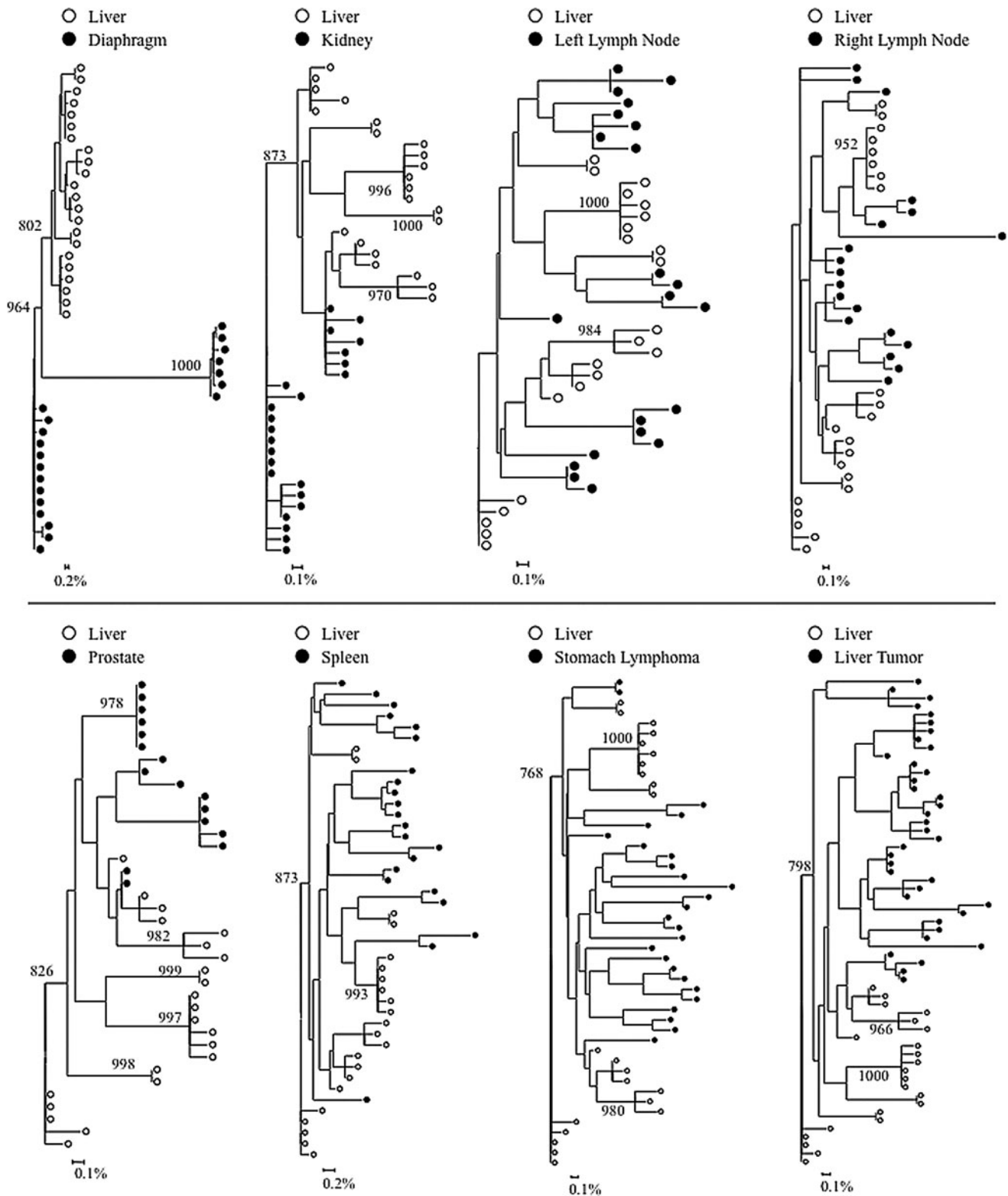
TABLE 1. MANTEL'S TEST FOR COMPARTMENTALIZATION OF V3 LOOP, GP120, AND *nef* VARIANTS COMPARED TO THE LIVER

Tissue being compared to liver	V3 loop HIV$_{ams}$198	gp120 DY	AZ	AM	nef DY	AZ	AM
Basal ganglia	—	0.0001	—	—	0.0002	—	—
Bone marrow	0.0002	—	—	—	—	—	—
Brain (unspecified)	0.0001	—	—	—	—	—	—
CSF	0.0001	—	—	—	—	—	—
Diaphragm	—	—	—	0.0062	—	—	0.0001
Frontal cortex	—	—	0.0001	—	—	0.0001	—
Frontal lobe GM	—	0.0001	—	—	0.0002	—	—
Frontal lobe WM	—	0.0001	—	—	0.0001	—	—
Kidney	0.0014	—	—	0.0001	—	—	0.0091
Lung	0.0001	—	—	—	—	—	—
Lymph node	0.0002	0.0002	0.0001	—	NS	0.0001	—
Left lymph node	—	—	—	0.0001	—	—	0.0108
Right lymph node	—	—	—	NS	—	—	NS
Liver tumor 1	—	—	—	0.0001	—	—	0.0027
Liver tumor 2	—	—	—	0.0001	—	—	0.0009
Meninges	—	0.0001	0.0001	—	0.0010	0.0001	—
PBMC	0.0050	—	—	—	—	—	—
Prostate	—	—	—	NS	—	—	0.0011
Spleen	0.0017	0.0001	0.0012	0.0071	0.0046	0.0042	NS
Stomach lymphoma	—	—	—	0.0001	—	—	NS
Temporal cortex	—	0.0001	—	—	0.0001	—	—

Sequence data for patient HIV$_{ams}$198 were originally reported by Van't Wout *et al.*[19] Sequence data for patients AM, AZ, and DY were originally reported in Lamers *et al.*[18]

CSF, cerebrospinal fluid; PBMC, primary blood mononuclear cells; GM, gray matter; WM, white matter; NS, not significant; dash, no data available; *p*-values < 0.05 were considered statistically significant.

other tissues for patients DY (range = 2–5, mean = 3.1), AZ (range = 1–2, mean = 1.5), and AM (range = 1–8, mean = 2) (Supplementary Table S5A–C). The two comparisons with the highest frequency of amino acid differences were the liver–diaphragm comparison from patient AM (eight substitutions) and the liver–frontal lobe white matter comparison from patient DY (five substitutions). Several residue changes occurred in the *nef* core domain structure and the conserved SH3 binding pocket.[31]

Discussion

A very limited number of studies have examined HIV variability in the liver. Van't Wout *et al.* performed a phylogenetic analysis on HIV proviral DNA from multiple tissues, including the liver, of one patient who died of AIDS-related causes and observed that HIV variants from the liver clearly clustered separately from variants of all other tissues analyzed. As well, there was no phylogenetic clustering observed between PBMC-derived and liver-derived HIV sequences, suggesting that HIV compartmentalization in the liver was not due to infiltrating lymphocytes. Despite this, the authors concluded that the monophyletic grouping of liver variants was the result of low proviral copy numbers, as all liver sequences were generated from a single PCR.[19] Only one other study to date has predicted liver-specific amino acid variations in *env*.[17] However, that study included only partial *env* sequences and, therefore, was unable to identify liver-specific signature sequences outside of the region amplified. Thus, in the current study, we sought to confirm these findings in an independent dataset, analyze the entire *env* gene, and examine compartmentalization of HIV *nef* sequences in the liver. Our results demonstrate that phylogenetically distinct HIV variants from the liver exist in three other patients who have died of AIDS-related causes. These findings were further supported statistically by Mantel's test. Collectively, these data suggest that viral compartmentalization may occur more frequently in the liver than previously recognized.

Signature sequence analysis identified specific amino acids in both gp120 and *nef* that were unique to the liver. While a bioinformatics approach cannot specifically determine if the predicted amino acid variations are relevant in the context of a replicating virus, several of the predicted amino acid variations were identified only in liver-derived HIV sequences, thus strengthening the body of evidence that suggests viral compartmentalization in the liver. Importantly, many amino acid substitutions were found in conserved areas of gp120. This suggests that they did not simply occur as a result of random sequence variability; rather, there may be unique selective pressures acting on HIV in the liver that are not found elsewhere in the body. For example, CD4-independent HIV interaction with cells is known to occur through gp120 binding of C-type lectin receptors, such as the mannose receptor or the DC-SIGN receptor on macrophages and dendritic cells.[32,33] Indeed, even minor mutations in gp120 can drastically alter the requirement for entry receptors and cell tropism. Such mutations may be the result of strong selective pressures on HIV resulting in liver-specific viral variants.

Compartmentalization can also occur without adaptive mutation, when the selective pressures in separate organs

differ such that the fitness of a particular preexisting viral subpopulation is greater in one organ than another. The predicted amino acid variations discovered in this study may have arisen from adaptive mutations or selective pressures on an existent viral subpopulation, as both mechanisms can result in viral compartmentalization. The lack of *in vitro* analysis in the current study does not allow for the determination of specific cell types that may support HIV replication in the liver. However, compelling evidence indicates that HIV has the ability to infect endothelial sinusoidal cells, stellate cells, Kupffer cells, and hepatocytes.[11–16] Studies also demonstrate that the gp120 antigen may engage apoptotic pathways without direct viral infection.[34] Both direct infection of liver cells and antigen activation of liver cells may contribute to viral compartmentalization in the liver. Future *in vitro* studies are warranted to determine the exact mechanisms by which HIV interacts with specific liver cell types and to confirm the role of organ-specific signature sequences.

Recent data suggest a role for HIV in liver disease.[35,36] This study provides evidence of HIV compartmentalization in the liver and identifies signature amino acids that may characterize liver-specific quasispecies. These data strongly suggest that HIV variants differ in their ability to infect various cell types. Furthermore, HIV mutation may be required for attachment, entry, and replication within a given cell type. Thus, these investigations may provide additional insight into the immune response and selective pressures unique to the hepatic microenvironment.

There are several limitations to the current analysis including the small number of patients, the lack of sequence data outside the *env* and *nef* genes, and limited information of whether other comorbid conditions may impact HIV diversity and/or compartmentalization. As noted in our previous study,[17] cross-sectional analyses do not permit a detailed examination of liver-specific HIV variants over time. Similarly, the cloning strategy utilized—while robust–may not have amplified all minor variants present in a given tissue/cell type. Thus, additional studies are now warranted in larger patient populations at varying stages of liver disease progression. It is recommended that future studies concerning HIV compartmentalization in the liver compare viral variants from (1) HIV monoinfected individuals with no history of liver complication, (2) HIV monoinfected individuals with liver disease, and (3) HIV/HCV coinfected individuals. However, it should be noted that due to the rapid adaptation of HIV to its host, organ-specific (including liver-specific) signature sequences may differ between patients. The phenotypic implications of liver-specific HIV mutations should be further examined, as they may aid in the development of liver-specific treatment modalities.

Acknowledgments

Author Disclosure Statement

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