

Clinicopathologic correlates of hepatitis C virus in brain: A pilot study

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Hepatitis C virus (HCV) has been detected in the brain tissues of 10 individuals reported to date; it is unclear what clinical factors are associated with this, and with what frequency it occurs. Accordingly, a pilot analysis utilizing reverse transcriptase–polymerase chain reaction (RT-PCR) to detect and sequence HCV in premortem plasma and postmortem brain and liver from 20 human immunodeficiency virus (HIV)-infected and 10 HIV-naïve individuals was undertaken. RNA encoding the first 126 amino acids of the HCV E1 envelope protein and the majority of the E1 signal sequence was analyzed in parallel with an 80-base-long segment of the 5' untranslated region (UTR). Liver HCV was detected only in subjects with premortem HCV viremia (10 HIV-infected and 3 HIV-naïve). Brain HCV was detected in 6/10 HCV/HIV-coinfected and 1/3 HCV-monoinfected subjects. In the setting of HIV, the magnitude of plasma HCV load did not correlate with the presence of brain HCV. However, coinfecting patients with brain HCV were more often off antiretroviral therapy and tended to have higher plasma HIV loads than those with HCV restricted to liver. Furthermore, premortem cerebrospinal fluid (CSF) analysis revealed that HCV/HIV-coinfected patients with brain HCV had detectable CSF HIV, whereas those without brain HCV had undetectable CSF HIV loads ($P = .0205$). Neuropsychologic tests showed a trend for hierarchical impairment of abstraction/executive functioning in HIV/HCV coinfection, with mean T scores for HIV monoinfected patients 43.2 (7.3), for liver-only HCV 39.5 (9.0), and for those with HCV in brain and liver 33.2 (5.1) ($P = .0927$). Predominant brain HCV sequences did not match those of the plasma or liver in 4 of the 6 coinfecting patients analyzed. We conclude that in the setting of HIV/HCV coinfection, brain HCV is a common phenomenon unrelated to the magnitude of HCV viremia, but related to active HIV disease and detectable CSF HIV. Furthermore, there is sequence evidence of brain compartmentalization. Differences in abstraction/executive function of HCV/HIV coinfecting patients compared to HIV monoinfected warrant further studies to determine if neuropsychiatric effects are predicated upon brain infection. *Journal of NeuroVirology* (2008) **14**, 17–27.

Keywords: brain; cognition; hepatitis C virus; HIV

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Introduction

Hepatitis C virus (HCV) is an important human pathogen, and is estimated to infect approximately 2% of the U.S. population (Kim, 2002). Its sequelae include progressive hepatic fibrosis, cirrhosis, and hepatocellular carcinoma, and it has been associated with a variety of extrahepatic syndromes, including cryoglobulinemia, glomerulonephritis, and porphyria cutanea tarda (Hoofnagle, 2002). In patients with poorly controlled human immunodeficiency virus (HIV) infection, HCV displays increased persistence and accelerated progression, and it is an important source of morbidity and mortality (Thomas, 2002).

HCV has been identified in a variety of nervous system tissues and fluids, both in monoinfected patients and those who are coinfecting with HIV (reviewed in Morgello, 2005; Forton *et al*, 2004b, 2006). There is evidence to suggest that it may replicate within the central nervous system (CNS), with detection of negative-strand replication intermediates and brain-specific quasispecies in a handful of patients reported to date (Forton *et al*, 2004a; Radkowski *et al*, 2002; Vargas *et al*, 2002). A separate body of work is growing to determine whether patients with clinical evidence of HCV (as determined by positive serology for anti-HCV antibodies or plasma viral load) have CNS dysfunction either caused or worsened by presence of the virus (Forton *et al*, 2004b; Hilsabeck *et al*, 2002; McAndrews *et al*, 2005; Ryan *et al*, 2004). However, although multiple lines of evidence are converging to delineate a neurobiology, it is currently unclear how commonly HCV can be found in brain, what factors promote brain invasion, and what the clinical sequelae of this brain invasion may be.

The Manhattan HIV Brain Bank (MHBB) and Manhattan Hepatology Brain Bank are well suited to the examination of these phenomena. Targeted to patients with either advanced-stage HIV or liver disease, there is a high rate of HCV infection in these studies (Ryan *et al*, 2004). Participants agree to be organ donors upon demise, allowing correlation of clinical phenomena with postmortem analysis of CNS tissues and fluids. In this paper, we describe pilot analyses of tissues originating from these cohorts.

Results

Patient characteristics

Demographics and laboratory assay characteristics of the subjects utilized for this study are displayed in Table 1. Of the 20 HIV-infected subjects, 10 had serologic evidence of past or current HCV infection (presence of antibody to HCV) prior to demise. Nine of these patients had chronic HCV infection as indicated by plasma HCV load. Ten HIV-infected patients did not have detectable HCV antibodies. One of these

10 HIV-positive, anti-HCV antibody-seronegative patients had chronic HCV infection, as determined by HCV plasma load. Of the 10 HIV-naïve patients, 5 were seropositive for anti-HCV antibodies, and 3 of these 5 had circulating HCV RNA.

None of the HIV/HCV-coinfecting patients had received therapy targeted to their HCV infection. Of the HIV-naïve, patient 40003 had received a 1-month course of interferon and ribavirin 3 months prior to demise, 40007 was untreated, and MHBB 584 had received multiple courses of interferon and ribavirin for unknown durations prior to demise. MHBB 553 had received an orthotopic liver transplant 5 years prior to demise for HCV-induced cirrhosis, with reinfection of the graft and ensuing recurrent cirrhosis; the medical treatment of this patient post transplantation was unclear (the liver tissue analyzed for this patient was the reinfected graft). The treatment history for MHBB 568 could not be elucidated.

Detection of HCV in autopsy tissues

Liver HCV was detected in all 13 HCV viremic patients (10 HIV-positive, 3 HIV-naïve); conversely, it was not detected in all 17 patients without pre-mortem HCV viremia. HIV/HCV-coinfecting patients were significantly older than those with HIV mono-infection (mean age HIV/HCV coinfecting 51.6 ± 8.0 [SD] years, HIV monoinfected 42.2 ± 4.8 years; $P = .0049$, student's *t* test), and more likely to have intravenous drug use (IVDU) as a risk for HIV ($P = .0014$, chi-square analysis). The gender and race composition of those with HIV/HCV coinfection was similar to those with HIV mono-infection.

Brain HCV was detected in 6 of the 10 HIV/HCV-coinfecting patients and 1 of the 3 HCV monoinfected patients. In the context of HIV, plasma HCV loads did not distinguish those with brain HCV from those with HCV in liver but not brain (Table 2). However, there was a tendency for those with brain HCV to more often have uncontrolled HIV disease involving the cerebrospinal fluid (CSF). Half of the brain HCV-positive patients (3/6) were not taking highly active antiretroviral therapy (HAART) at the time of demise; in contrast, all of the brain HCV-negative/liver HCV-positive patients (4/4) were on HAART. Correspondingly, there was a tendency for plasma HIV loads to be higher in those with brain HCV than in those with liver HCV only. Premortem CSF HIV loads were available in 7 of the 10 HIV/HCV-coinfecting patients; CSF HIV RNA was present in 4/4 patients with brain HCV and was undetectable in 3/3 patients without brain HCV. The mean log CSF HIV for individuals with brain HCV was 3.35 (0.83), and for individuals with liver HCV only, it was below the limit of detection: 1.70 (0.00) ($P = .0205$, student's *t* test). Finally, brain HCV-positive patients did not differ from liver restricted HCV with regard to CD4 counts or demographic variables.

Table 1 Demographic, therapeutic, and immunovirologic characteristics of study patients

| Patient ID | Age | Sex | Race | Risk for HIV | HIV status | HCV antibody serology | Liver HCV detected by RT-PCR 5'UTR/E1 | Brain HCV detected by RT-PCR 5'UTR/E1 | log plasma HCV viral load** | log plasma HIV viral load*** | log CSF HIV viral load** | cd4 count | Antiretroviral therapy at death | HCV therapy |
|------------|-----|-----|------|--------------|------------|-----------------------|---------------------------------------|---------------------------------------|-----------------------------|------------------------------|--------------------------|-----------|---------------------------------|-----------------------|
| 10066 | 54 | f | b | sex | pos | pos | yes +/+ | yes -/+ | 3.42 | 5.67 | 2.56 | 8 | none | none |
| 10016 | 58 | f | b | ivdu | pos | pos | yes +/+ | yes -/+ | 6.82 | 5.76 | 4.00 | 98 | none | none |
| 10086 | 48 | f | b | ivdu | pos | pos | yes +/+ | yes -/+ | 6.39 | 4.36 | nd* | 249 | combivir, kaletra | none |
| 20024 | 62 | m | w | ivdu | pos | pos | yes +/+ | yes +/+ | 7.88 | undetectable | 2.70 | 20 | d4t, 3tc, abacavir | none |
| 20028 | 49 | m | h | ivdu | pos | pos | yes +/+ | yes +/+ | 6.29 | 4.01 | nd* | 138 | none | none |
| 10034 | 49 | m | h | ivdu | pos | neg | yes +/+ | yes +/+ | 7.09 | 4.76 | 4.13 | 77 | combivir, nelfinavir | none |
| 10001 | 64 | f | b | ivdu | pos | pos | yes +/+ | no -/- | 7.67 | 2.56 | undetectable | 72 | d4t, 3tc, kaletra | none |
| 10027 | 39 | m | h | ivdu | pos | pos | yes +/+ | no -/- | 6.33 | 5.08 | nd* | 109 | d4t, kaletra, nevirapine | none |
| 20053 | 50 | m | w | sex | pos | pos | yes +/+ | no -/- | 6.91 | undetectable | undetectable | 606 | azt, abacavir, nevirapine | none |
| 20015 | 43 | f | h | ivdu | pos | pos | yes +/+ | no -/- | 6.03 | undetectable | undetectable | 69 | ddi, combivir | none |
| 20016 | 40 | m | h | sex | pos | pos | no -/- | no -/- | undetectable | 4.61 | undetectable | 1 | none | none |
| 10018 | 39 | m | b | sex | pos | neg | no -/- | no -/- | undetectable | 5.61 | undetectable | 1 | none | none |
| 10025 | 46 | m | h | sex | pos | neg | no -/- | no -/- | undetectable | 1.86 | nd* | 93 | azt, 3tc, indinavir | none |
| 10043 | 34 | f | b | sex | pos | neg | no -/- | no -/- | undetectable | 3.46 | nd* | 757 | none | none |
| 10065 | 46 | m | h | sex | pos | neg | no -/- | no -/- | undetectable | 5.88 | nd* | 18 | trizivir, efavirenz | none |
| 10095 | 42 | m | w | sex | pos | neg | no -/- | no -/- | undetectable | 5.88 | nd* | 13 | none | none |
| 10105 | 48 | m | h | sex | pos | neg | no -/- | no -/- | undetectable | 3.76 | 2.81 | 63 | d4t, nelfinavir, nevirapine | none |
| 10133 | 48 | m | w | sex | pos | neg | no -/- | no -/- | undetectable | 5.24 | nd* | 3 | d4t, kaletra, nevirapine | none |
| 10162 | 42 | m | w | sex | pos | neg | no -/- | no -/- | undetectable | 2.36 | undetectable | 298 | none | none |
| 20025 | 37 | m | h | sex | pos | neg | no -/- | no -/- | 4.63 | 5.18 | 2.16 | 21 | d4t, nelfinavir, nevirapine | none |
| 40003 | 54 | m | w | na* | neg | pos | yes +/+ | no -/- | 5.32 | na* | na* | na* | interferon, ribavirin | interferon, ribavirin |
| 40007 | 53 | m | w | na* | neg | pos | yes -/+ | yes +/[+] | 6.35 | na* | na* | na* | none | none |
| 553 | 46 | m | h | na* | neg | pos | yes +/+ | no -/- | 5.41 | na* | na* | na* | liver transplant | liver transplant |
| 568 | 48 | f | h | na* | neg | pos | no -/- | no -/- | undetectable | na* | na* | na* | unknown | unknown |
| 584 | 53 | m | w | na* | neg | pos | no -/- | no -/- | undetectable | na* | na* | na* | interferon, ribavirin | interferon, ribavirin |
| 528 | 52 | m | w | na* | neg | neg | no -/- | no -/- | nd* | na* | na* | na* | none | none |
| 531 | 44 | m | w | na* | neg | neg | no -/- | no -/- | nd* | na* | na* | na* | none | none |
| 543 | 31 | f | h | na* | neg | neg | no -/- | no -/- | nd* | na* | na* | na* | none | none |
| 556 | 54 | f | h | na* | neg | na | no -/- | no -/- | undetectable | na* | na* | na* | none | none |
| 555 | 35 | m | b | na* | neg | neg | no -/- | no -/- | undetectable | na* | na* | na* | none | none |

nd*: test not performed.

na*: risk for HIV not applicable, patients were HIV negative.

** : the lower limit for undetectable HCV plasma viral load was log value of 2.78; the upper limit was not achieved by dilution of the samples prior to assay.

***: lower limit for undetectable HIV plasma viral load was log value of 1.70; the upper limit was log value of 5.88.

”: antiretroviral therapy in this patient was stopped 4 days prior to death.

[+]: PCR product obtained, but insufficient material for accurate sequencing.

Table 2 Immunovirologic characteristics of HIV/HCV-coinfected patients

| | HCV in brain (n = 6) | HCV in liver not brain (n = 4) | P value |
|---------------------|----------------------|--------------------------------|---------|
| Plasma HCV load* | 6.32 (1.53) | 6.73 (0.72) | 0.6287 |
| Patients on HAART** | 3/6 | 4/4 | 0.0910 |
| Plasma HIV load | 4.38 (1.49) | 2.76 (1.60) | 0.1401 |
| CSF HIV load*** | 3.35 (0.83) | 1.70 (0.00) | 0.0205 |
| CD4 count | 98 (88) | 214 (262) | 0.3357 |

*All mean viral loads expressed as log₁₀ ± SD.

**Chi-square analysis; all other tests student's *t*.

***For patients with HCV in brain *n* = 4, for patients with HCV in liver *n* = 3.

Sequence analysis of 5' UTR and E1 regions

An amplicon representing the 5' UTR was obtained from 12/13 liver, 9/12 plasma, and 4/13 brain samples of individuals with HCV viremia. An amplicon representing the E1 region was obtained from 13/13 liver, 11/12 plasma, and 7/13 brain specimens from the same individuals (this was a nested polymerase chain reaction [PCR] technique, in contrast to the UTR analysis). Genotype could be deduced from E1 sequences, but not the more conserved UTR (Simmonds, 1999). All HCV sequences obtained in this study were of genotype 1 except for those from the liver and plasma of patient 40007, which were genotype 3a. The genotypes of brain HCV were 1a in five patients (10016, 10034, 10066, 10086, 20028), 1b in one patient (20024), and could not be determined in patient 40007, who had genotype 3a in systemic sites. Predominant sequences in brain were then compared to those obtained in liver and plasma, to determine if they were exact matches. Table 3 presents the results of sequence analysis, showing the number of nucleotide differences between brain and plasma and/or liver (whichever was the closest match). In the 5' UTR region, predominant brain sequences of three

Table 3 Comparison of predominant HCV brain sequences with closest related sequence in liver or plasma from HIV/HCV-coinfected subjects

| Region | Primary sequence [^] | Genotype | Closest Sequence in the Same Subject | Nucleotide Differences |
|---------|-------------------------------|----------|--------------------------------------|------------------------|
| 5' UTR | 10034B | NA | 10034P, 10034L | 1 |
| 5' UTR | 20024B | NA | 20024L | 1 |
| 5' UTR | 20028B | NA | 20028L | 0 |
| 5' UTR | 20028*B | NA | 20028L | 1 |
| Core-E1 | 10016B | 1a | 10016P | 0 |
| Core-E1 | 10034B | 1a | 10034P, 10034L | 0 |
| Core-E1 | 10066B | 1a | 10066P, 10066L | 0 |
| Core-E1 | 10086B | 1a | 10086P, 10086L | 1 |
| Core-E1 | 20024B | 1b | 20024L | 0 |
| Core-E1 | 20028B | 1a | 20028P | 58 |

NA: not applicable; the region of the 5' UTR that was analyzed is highly conserved and does not allow genotype to be assigned.

* 20028*B is a second sample of the same brain as 20028B.

[^]Suffix B for brain-derived sequence, P for plasma-derived sequence, and L for liver-derived sequence.

patients differed by one nucleotide from their closest match in liver or plasma (patients 10034, 20024, 20028—with two contiguous brain samples analyzed for patient 20028). In the core-E1 region, four patients displayed an identical match with predominant plasma and/or liver sequences (patients 10016, 10034, 10066, and 20024); one patient differed at one nucleotide (patient 10086), and one patient differed at 58 positions. In this latter patient, the HCV RNA of the plasma and brain clustered with genotype 1a, whereas the RNA from the liver clustered with 1b. In summary, four of six patients who could be analyzed had predominant brain HCV sequences that did not match either their predominant liver or plasma sequence.

Histology

Histologic findings in the brains and livers are given in Table 4. Although most liver HCV-positive patients displayed severe hepatic fibrosis or cirrhosis, this was not always the case. Hepatic fibrosis and cirrhosis were not limited to patients with HCV, as in this cohort there were multiple risk factors (such as other hepatotoxic infections and/or substance/alcohol abuse) for liver disease. Six of 7 patients with brain HCV, 6 of 6 patients with liver HCV only, and 10 of 17 patients with no autopsy evidence of HCV sequences displayed severe hepatic fibrosis or cirrhosis.

Reflecting the prevalence of significant liver fibrosis, the most frequent neuropathology in the patients analyzed was Alzheimer type 2 gliosis, an apparent enlargement of astrocyte nuclei thought to be indicative of reaction to metabolic abnormalities encountered in liver dysfunction. Alzheimer type 2 gliosis was more frequent in patients with HCV-positive liver fibrosis/cirrhosis (6 of 12 patients) than in those with HCV-negative fibrosis/cirrhosis (3 of 10 patients). It was found in one brain HCV-positive patient in the absence of cirrhosis. However, brain HCV patients were no more likely to have Alzheimer type 2 gliosis or necrotizing or overtly inflammatory neuropathologies by light microscopic analysis than those with liver HCV only. There was no evidence of HCV-associated encephalitis. HIV encephalitis was not present in any brain from an HCV-infected patient, and in only two patients without HCV infection. PCR for HIV DNA was negative in the brains of all patients with HCV.

Neuropsychologic assessments

Global and domain *T* scores for the HIV-infected patients are included in Appendix 1 (patient 20016 could not be tested), and group means for brain HCV-positive and brain HCV-negative in Table 5. There were high rates of impairment across many domains, consistent with the advanced nature of the cohort with regard to HIV disease. Even with this, patients with brain HCV tended to perform worse in abstraction and executive functioning than those without,

Table 4 Brain and liver autopsy pathology in study subjects

| Patient ID | Liver HCV detected by RT-PCR | Brain HCV detected by RT-PCR | Severe liver fibrosis/cirrhosis | Predominant neuropathology at autopsy |
|------------|------------------------------|------------------------------|---------------------------------|--|
| 10066 | yes | yes | no | Alzheimer type 2 gliosis |
| 10016 | yes | yes | yes | Focal neuronal loss |
| 10086 | yes | yes | yes | Alzheimer type 2 gliosis, aseptic leptomeningitis |
| 20024 | yes | yes | no | Normal brain |
| 20028 | yes | yes | yes | Alzheimer type 2 gliosis |
| 10034 | yes | yes | yes | Focal infarcts |
| 10001 | yes | no | yes | Mild perivascular inflammation |
| 10027 | yes | no | yes | Healed toxoplasmosis |
| 20053 | yes | no | yes | Optic nerve degeneration |
| 20015 | yes | no | yes | Hepatocerebral degeneration, focal candidal abscess |
| 20016 | no | no | yes | Focal infarct |
| 10018 | no | no | no | Normal brain |
| 10025 | no | no | yes | Minimal non diagnostic changes |
| 10043 | no | no | yes | Focal infarct, aseptic leptomeningitis |
| 10065 | no | no | no | HIV encephalitis, PCNSL |
| 10095 | no | no | no | PCNSL s/p radiation, meningioma |
| 10105 | no | no | no | Infarcts with anticardiolipin antibody syndrome |
| 10133 | no | no | no | HIV encephalitis |
| 10162 | no | no | no | Microglial nodule encephalitis and aseptic leptomeningitis |
| 20025 | no | no | yes | Alzheimer type 2 gliosis |
| 40003 | yes | no | yes | Alzheimer type 2 gliosis |
| 40007 | yes | yes | yes | Alzheimer type 2 gliosis |
| 553 | yes | no | yes | Alzheimer type 2 gliosis |
| 568 | no | no | yes | Minimal nondiagnostic changes |
| 584 | no | no | yes | Normal brain |
| 528 | no | no | no | Cortical microinfarct |
| 531 | no | no | yes | Normal brain |
| 543 | no | no | no | Midbrain microinfarct |
| 556 | no | no | yes | Alzheimer type 2 gliosis |
| 555 | no | no | yes | Alzheimer type 2 gliosis |

with decreasing domain *T* scores across mono-infected patients, those coinfecting without HCV brain sequences, and those coinfecting with HCV in brain. Mean abstraction and executive functioning *T* scores for mono-infected HIV patients were 43.2 (7.3), for coinfecting patients with HCV in liver but not brain

Table 5 Global and domain *t* scores for HIV-infected patients with and without HCV infection

| | Brain/liver HCV positive | Liver HCV positive | Brain/liver HCV negative | <i>P</i> * |
|-----------------------------------|--------------------------|--------------------|--------------------------|------------|
| Global | 31.0 (4.0) | 35.8 (8.5) | 35.3 (7.0) | .4078 |
| Motor | 28.0 (6.2) | 36.7 (2.5) | 34.7 (17.9) | .6007 |
| Speed of information processing | 31.2 (5.8) | 30.3 (11.9) | 35.9 (9.3) | .4776 |
| Working memory | 39.3 (8.4) | 39.3 (11.7) | 40.8 (7.2) | .935 |
| Memory encoding | 26.3 (7.9) | 32.7 (13.3) | 29.2 (6.5) | .5506 |
| Memory retrieval | 27.3 (6.3) | 33.0 (12.5) | 29.9 (8.3) | .6348 |
| Fluency | 40.3 (6.9) | 51.3 (14.6) | 41.8 (9.95) | .0852 |
| Abstraction executive functioning | 33.2 (5.1) | 39.5 (9.0) | 43.3 (7.3) | .0923 |

*Some domains could not be assessed in all patients due to inability to complete test batteries; *n* = 19 for global and speed of information processing domains; *n* = 18 for working memory, memory encoding, memory retrieval, and fluency domains; *n* = 16 for abstraction domain; and *n* = 15 for motor domain. All *P* values derived by ANOVA.

39.5 (9.0), and for coinfecting patients with HCV in both brain and liver 33.2 (5.1) (*P* = .0927, analysis of variance [ANOVA]). When patients with brain HCV were contrasted to all patients without (regardless of liver status), their impairment in abstraction and executive functioning was significantly worse (*P* = .0395, student's *t* test). Analysis of individual test *T* scores revealed that patients with brain HCV scored significantly worse in Trail Making Test Part B (brain HCV positive 31.2 (4.5), HCV brain negative 44.4 (12.2); *P* = .0380 student's *t* test). Fluency tended to be better in patients with liver HCV than those in other groups; this effect appeared to be mediated by two individuals with high normal performance in this test (see Appendix 1).

Discussion

The flavivirus family has many members (for example West Nile or Japanese B) with well-documented neurovirulence, capable of eliciting both significant neurologic and neuropathologic sequelae (McMinn, 1997; Sejvar *et al*, 2003). Typical of viral encephalitis, the pathogenesis of these disorders is often inflammatory, with acute to subacute neurologic progression. In contrast, the major clinical manifestation of HCV is chronic liver disease, with hepatitis evolving to cirrhosis in a variable proportion of individuals infected (Hoofnagle, 2002). However, although HCV's main clinical sequelae are hepatitis and cirrhosis, it is clearly not anatomically restricted to liver. The virus has been detected in multiple organs, including lymph node, bone marrow, pancreas, thyroid, adrenal, and spleen (reviewed in Gowans, 2000). Positive-sense HCV RNA has been detected in the

CSF and brains of limited numbers of chronically infected patients with variable clinical and neuropathological abnormalities (Laskus *et al*, 2002; Morsica *et al*, 1997; Maggi *et al*, 1999; Bagaglio *et al*, 2005). Evidence of replication in brain and CSF compartments, with negative-sense HCV RNA detection and sequence divergence between CNS and systemic quasispecies, has also been published in limited numbers of subjects (Forton *et al*, 2004a; Radkowski *et al*, 2002; Vargas *et al*, 2002). The present study adds 7 patients to the previous literature documenting HCV in brain tissues of 10 patients with chronic infection, and attempts to determine whether there are any clinical characteristics in the setting of HIV that are associated with or contribute to this localization. Unexamined is whether nervous system HCV infection is an isolated extrahepatic phenomenon, or part of a disseminated HCV disease process.

This study demonstrates that brain HCV sequences are commonly found in patients with HIV/HCV coinfection. They were detected in 60% of HIV-infected patients with untreated plasma/liver HCV. Prior studies of coinfecting patients from which CNS HCV prevalence can be estimated have been restricted to paired CSF and peripheral blood, and thus represent analysis of a different CNS compartment with regard to cellular trafficking and viral dynamics. Nevertheless, our rate of brain HCV sequence detection is the same as that found by Laskus and colleagues in CSF. In their study, HCV was found in 60% (6/10) of CSF samples from coinfecting individuals with advanced HIV (Laskus *et al*, 2002). In other prior studies of HIV/HCV-coinfecting patients, rates of CSF HCV detection have ranged from 24% (5/21 patients) to 100% (5/5 patients) (Morsica *et al*, 1997; Maggi *et al*, 1999; Bagaglio *et al*, 2005). The reasons for this variability are unclear, and underscore the need for larger numbers of patients to be studied, and an enhanced understanding of the factors that allow HCV penetration into CNS fluids and tissues. Such factors may include perturbations of blood-brain and blood-CSF barriers, potential neurotropic HCV variants, viral burden and duration, HCV RNA detection methods, immunologic and HIV virologic status of the coinfecting patients, or other comorbid risks or demographics.

With regard to factors that may be important for CNS pathogenesis, the present pilot is the first to suggest an association between poorly controlled HIV infection and brain localization of HCV. Although the magnitude of HCV plasma viremia did not correlate with its presence in brain, there was a trend for patients with brain HCV to be more frequently off antiretroviral therapy and have higher plasma HIV loads. A significant association was seen between brain HCV and detection of HIV in a premortem sample of CSF, consistent with the hypothesis that HIV may contribute to abnormalities favoring HCV brain penetration. Abnormalities of blood-brain barrier have been documented with HIV infection, and

literature is accumulating to demonstrate that HIV enhances the migration of leukocytes into the brain (Eugenin *et al*, 2006). HCV can be detected in peripheral blood mononuclear cells (PBMCs) (Laskus *et al*, 1998; for a review, see Dammacco *et al*, 2000), and HIV facilitates HCV infection of naive human macrophages *in vitro* (Laskus *et al*, 2004). Thus, uncontrolled HIV disease may be hypothesized to contribute to a state of enhanced brain mononuclear cell migration, facilitating cell-associated HCV brain entry. On the other hand, an alternative explanation for the association of brain HCV and uncontrolled HIV may also be postulated: it is possible that brain localization of HCV enhances HIV expression in the CNS. The concept of “synergistic copathogenicity”—where replication of one virus results in elevation of HIV—has been demonstrated for other organ systems (Corey, 2007). It would be possible to postulate that HCV may potentiate HIV in the CNS compartment. Further studies will be necessary to confirm our observation and determine its relevance towards HCV and HIV neuropathogenesis.

Prior studies have suggested that PBMCs comprise the pertinent blood fraction with regard to CNS penetration, with reports of two brain- and four CSF-derived HCV virotypes more comparable to PBMC or lymph node than plasma or serum (Forton *et al*, 2004a; Laskus *et al*, 2002). It may be that HCV brain penetration follows a mechanism similar to the “Trojan horse” monocyte-directed access of HIV. However, this has not been definitively established, and a comprehensive model of HCV neurobiology based on observation of *in vivo* phenomena is not extant. Furthermore, given the “Trojan horse” hypothesis, it is important to distinguish brain migration of leukocytes from brain accumulation. Neuropathologies were examined in the present study, and there was no indication at the level of hematoxylin and eosin (H&E) histology that inflammatory or necrotizing lesions were associated with the presence of brain HCV sequences. Indeed, CNS inflammation or necrosis was seen in three out of six patients with HCV in liver only (patients 10001, 10027, 20015), and in three out of seven with HCV in brain (patients 10016, 10086, 10034). This is consistent with prior literature, in which HCV has not generally been associated with encephalitis or myelitis. Only rare case reports have described individuals with acute inflammatory syndromes involving brain or spinal cord, in which HCV RNA has been demonstrated in brain or CSF (Bolay *et al*, 1996; Fujita *et al*, 1999; Gazzola *et al*, 2001; Sacconi *et al*, 2001). A caveat with our analysis is that it was performed only through light microscopic examination of routine H&E stains, and thus only pronounced accumulations of inflammatory cells would have been appreciated. It is possible that enhanced inflammatory cell infiltration or activation, detectable by expression of inflammatory cell markers, but not by increased nuclear density, could have been present. This has been

well demonstrated for patients with HIV, who can demonstrate elevations of brain inflammatory mediators in the absence of overtly encephalitic pathologies (Glass *et al*, 1993). Importantly, in some magnetic resonance spectroscopy (MRS) studies of HCV mono- and HIV/HCV-coinfected patients, there has been evidence of white matter inflammation as indicated by increased choline neurometabolites (McAndrews *et al*, 2005; reviewed in Forton *et al*, 2006).

The neuropathologic finding of Alzheimer type 2 gliosis tended to be present more often in patients with HCV than in those without, although this was not specific to those with brain localization. This pathology is typically seen in patients with decompensated liver disease, and is thought to reflect the profound metabolic CNS abnormalities induced by biochemical abnormalities in liver failure. Interestingly, one patient with brain HCV but without liver fibrosis or cirrhosis (10066) had Alzheimer type 2 gliosis. Further studies will be warranted to determine whether HCV contributes directly in some manner to this histopathology.

Although chronic HCV may lack a characteristic neuropathology, there is accumulating evidence that it is associated with cognitive dysfunction. In monoinfected as well as coinfecting cohorts, HCV has been associated with deficits in abstraction and executive functioning, speed of information processing, visuospatial construction, and concentration (Cherner *et al*, 2005; Forton *et al*, 2002; Hilsabeck *et al*, 2002; Perry *et al*, 2005; Richardson *et al*, 2005; Ryan *et al*, 2004). Individuals with chronic HCV may have significantly prolonged P300 latencies on electrophysiologic testing (Kramer *et al*, 2002). MRS abnormalities distinct from those encountered in liver failure have been noted in patients with HCV (Forton *et al*, 2001). In all these studies, nervous system function has been correlated with the presence or absence of HCV indicators or load in peripheral blood. However, in contrast to these studies, there are investigators who have been unable to demonstrate HCV-specific deficits that go beyond the cognitive perturbations accompanying hepatic decompensation (Edwin *et al*, 1999; Hilsabeck *et al*, 2002; Soogoor *et al*, 2006). This has left unresolved an important question: are the cognitive abnormalities detected in these patients specific to HCV in the nervous system, or a function of systemic disease and/or impairments in hepatic function?

The current pilot study does not resolve this question, as it is underpowered with regard to cognitive analysis. It does suggest that the presence of brain HCV is a factor in producing cognitive deficits in abstraction and executive functioning, with a descending hierarchy of performance from better to worse in those with HIV infection alone, HIV/HCV coinfection restricted to systemic locations, and HIV/HCV coinfection with brain HCV. Caveats must be considered: the brains analyzed derive from our larger clinical cohort, in which prior study of 116 HIV-infected

individuals showed deficits in abstraction and executive function correlated with positive HCV serology (Ryan *et al*, 2004). Furthermore, given numerical limitations, statistical analysis suggested trends but lacked significance. Analysis of larger numbers of brains from HIV-infected subjects with and without HCV will be necessary to confirm this suggestion of our pilot study.

Finally, the sequence analyses in the current study provide more evidence for an HCV subpopulation in brain. In four of six patients, predominant brain species did not match those in plasma or liver. The presence of a predominant sequence in brain that is different from the predominant sequence in plasma and liver suggests that the brain HCV is not produced in the liver; however, it is possible that the brain isolate is a minority population that is produced in the liver that has a propensity to accumulate in the brain. Further compartmental studies with quasispecies analysis are warranted.

In summary, the current pilot study adds seven patients to the literature documenting HCV sequences in brain, and begins an analysis of clinicopathologic correlates of this localization, demonstrating uncontrolled HIV infection in those with brain HCV. Future studies on larger numbers of subjects will be necessary to determine whether a clinically relevant phenotype can be related to this phenomenon, and to elucidate a neurobiology of HCV that is relevant not only to function, but also to general disease progression and treatment.

Materials and methods

Patient population

HIV-infected patients were obtained from the Manhattan HIV Brain Bank (R24MH59724), a longitudinal observational study of individuals who are willing to become organ donors upon demise. Eligibility criteria for the study have been published elsewhere (Morgello *et al*, 2004). These patients undergo semiannual neuropsychologic, psychiatric, and neurologic examinations; laboratory analyses are both recorded from extant clinical evaluations, as well as performed for research purposes. Included in the laboratory analyses are CD4 T-cell enumerations, HIV plasma viral loads, chemistries inclusive of liver function tests, and hepatitis virus serologies. Approximately two thirds of the cohort agree to pre-mortem lumbar puncture, and HIV loads are determined on CSF. Upon demise, autopsies and organ donation are performed as per the stipulation of study participants. For the current pilot, 20 autopsied MHBB patients were chosen: 10 who had pre-mortem documentation of anti-HCV antibodies, and 10 who were anti-HCV antibody seronegative. None of these patients had received therapy directed at HCV.

Ten patients who were HIV naïve were obtained from the Manhattan Hepatology Brain Bank

(R24MH59724), as well as from the Mount Sinai Department of Pathology autopsy service. The former were patients with advanced stage liver disease who had consented to be organ donors upon demise, and who had undergone evaluations similar to those of the HIV brain bank, without an option for lumbar puncture due to medical risk. The latter had been autopsied via consent of primary next-of-kin for the purposes of diagnosis, medical education, and research, and specimens utilized for this research were excess (“waste”) tissues not employed in diagnostic procedures. Hepatitis serologies for these patients were obtained from clinical record review. All patients underwent testing for HIV (enzyme-linked immunosorbent assay [ELISA]/Western blot) as part of the routine autopsy procedure, and these tests confirmed the negative serostatus of this group. For the current pilot, five HCV-seropositive and five patients without histories of HCV were examined.

Neuropsychologic assessment

The neuropsychologic battery used with MHBB patients has been described in prior publication (Ryan *et al*, 2004). Specific tests included the Trailmaking Test—Parts A and B (TMT-A and TMT-B), Grooved Pegboard Test—Dominant and Nondominant Hands (GPT-DH and GPT-NH), Hopkins Verbal Learning Test (HVLT), Brief Visual Memory Test-Revised (BVMTR), Paced Auditory Serial Addition Task (PASAT), Digit Symbol, Symbol Search, Letter Number Sequencing, Controlled Oral Word Association Test (FAS), Wisconsin Card Sorting Test-64 card version (WCST-64), and the reading subtest of the Wide Range Achievement Test-3 (WRAT-3). The individual tests were grouped according to the following domains as previously described (Woods *et al*, 2004): Motor: GPT-DH, GPT-NH; Psychomotor Speed: TMT-A, Digit Symbol, Symbol Search; Working Memory: Letter Number Sequencing, PASAT; Learning: HVLT Total Recall, BVMTR Total Recall; Memory: HVLT Delayed Recall, BVMTR Delayed Recall; Verbal Fluency: FAS; and Abstraction and Executive Functioning: WCST-64 Perserative Responses and TMT-B.

To investigate prevalence of impairment across domains, we assigned *T* scores using published norms (Woods *et al*, 2004). These normatively derived *T* scores corrected for neuropsychologic testing differences that could be attributable to age, race, and education. The *T* scores had a mean value of 50 with 1 standard deviation equal to 10; impaired scores were those less than 1 standard deviation below the mean (less than 40). Neuropsychologic assessments were performed at an average of 6 ± 3 months prior to patient demise (range 2 to 12 months).

Histologic assessment

At autopsy, a minimum of 57 sections were obtained from each brain. Formalin-fixed tissues were routinely processed for paraffin embedding, sectioning,

and staining with hematoxylin and eosin. The regions routinely sectioned have been previously published (Morgello *et al*, 2001). Sections were evaluated by a board-certified neuropathologist (SM) in the absence of knowledge about patient’s HCV serostatus. Sections of liver were processed routinely for paraffin histology, diagnoses of cirrhosis or extensive fibrosis recorded from autopsy evaluations, and confirmed by examination of a board certified anatomic pathologist (SM).

Plasma viral loads

Both HIV and HCV plasma viral loads were performed in the clinical microbiology laboratories of the Mount Sinai Medical Center. CSF HIV loads were performed at Johns Hopkins University. Plasma and CSF samples were obtained from pre-mortem clinical or research patient evaluations. HIV viral loads at both Mount Sinai and Johns Hopkins were determined via the Roche HIV1 Monitor Test Ultrasensitive Version 1.5, and HCV viral loads via the Roche Cobas Amplicor HCV Monitor Test, Version 2.0.

RNA extraction

Tissues for analysis were liver and brain frontal lobe that had been snap frozen at -80°C at the time of autopsy. RNA was recovered from 30 mg of tissue after homogenization in 350 μl of lysis buffer (RA1) and 3.5 μl of β -mercaptomethanol, as specified by the NucleoSpin RNA II protocol (BD Bioscience Clontech, Palo Alto, CA). Homogenization was carried out with a sterile hand pestle in a 1.5-ml Eppendorf tube and was followed by filtration through Nucleospin Filter units to reduce viscosity and remove insoluble material. Purified RNA was eluted in 50 μl of nuclease-free water. The yield was estimated by ultraviolet (UV) spectroscopy.

RNA was extracted from a plasma sample collected during a research study visit that was closest to the date of death. Purification of the viral RNA was done using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA). Extraction was performed on 140 μl of plasma treated with 560 μl of buffer AVL containing carrier RNA. Purified RNA was eluted and stored in 60 μl RNase-free water containing 0.04% sodium azide.

PCR amplification of HCV RNA

Reverse transcription of the 5’ UTR and E1 regions was performed using the external reverse E1 primer of (Corbet *et al*, 2003), (see table 1). The 20- μl final reaction mixture contained 0.5 μg of total tissue or plasma RNA, 2.5 μM primer, 1 \times Transcriptor RT reaction buffer, 20 U Protector RNase Inhibitor, 1 mM of each dNTP, and 10 U Transcriptor Reverse Transcriptase (Roche). The reverse transcription was performed under the following conditions: 60 $^{\circ}\text{C}$ for 10 min, 65 $^{\circ}\text{C}$ for 40 min, and 85 $^{\circ}\text{C}$ for 5 min. The cDNA was stored at -20°C .

Table 6 Oligonucleotide primers used in this study

| Region | Primer set | Sequence ^a |
|----------|-------------|--------------------------------------|
| 5' UTR | Reverse (-) | 184-CGTCCTGGCAATTCGGTGT-165 |
| | Forward (+) | 59-TGTCTTCACGCAGAAAGCGTCTAGC-83 |
| E1 outer | Reverse (-) | 1328-CGTAGGGGACCAGTTCATCATCAT-1305 |
| | Forward (+) | 834-GCAACAGGGAACCTTCCTGGTTGCTC-859 |
| E1 inner | Reverse (-) | 1316-GTTCATCATCATATCCCATGCCAT-1293 |
| | Forward (+) | 843-AACCTTCCTGGTTGCTCTTTCTCTAT-868 |
| GAPDH | Reverse (-) | 750-TTCTAGACGGCAGGTCAGGT-731 |
| | Forward (+) | 542-TCACTGCCACCCAGAAGACT-561 |
| HIV gag | Reverse (-) | 894-TTTGGTCCTTGTCTTATGTCCAGAATGC-857 |
| | Forward (+) | 777-ATAATCCACCTATCCCAGTAGGAGAAAT-804 |

^aHCV numbers relative to H77 (GenBank accession no. NC_004102). GAPDH numbers relative to coding sequence of human glyceraldehyde-3-phosphate dehydrogenase (GenBank accession no. BC083511). HIV gag relative to start of Gag coding region (GenBank accession no. AY314063).

PCR of the HCV 5' UTR was carried out using the 5' UTR forward and reverse primers presented in Table 6. The 20- μ l final reaction mixture contained 2 μ l of template cDNA (from the reverse transcription reaction), 1 \times Phusion HF buffer, 200 μ M of each dNTP, 0.5 μ M primers, 3% DMSO, and 0.4 U Phusion DNA polymerase (Finnzymes, Finland). Cycle conditions were 98°C for 10 s, followed by 35 cycles of 98°C for 10 s, 71°C for 30 s, and 72°C for 4 s, and a final elongation at 72°C for 10 min. This reaction generated a 126-base pair band.

The first round of the nested PCR amplification of the HCV E1 region was performed using external E1 primers previously described (Corbet *et al*, 2003) in a 20- μ l final reaction mixture containing 2 μ l of template cDNA (from the reverse transcription reaction), 1 \times Phusion HF buffer, 200 μ M of each dNTP, 0.5 μ M primers, 3% DMSO, and 0.4 U Phusion DNA polymerase (Finnzymes). Cycle conditions were 98°C for 30 s, followed by 35 cycles of 98°C for 10 s and 72°C for 20 s, and a final elongation at 72°C for 10 min. For the nested, second round of the process, 2 μ l of the first round reaction product were added to 18 μ l of new reaction mixture containing 1 \times Phusion HF buffer, 200 μ M of each dNTP, 0.5 μ M primers, 3% DMSO, and 0.4 U Phusion DNA polymerase (Finnzymes) for a final volume of 20 μ l. The internal E1 primers (Corbet *et al*, 2003) were used. The cycling conditions were 98°C for 30 s, followed by 35 cycles of 98°C for 10 s, 69°C for 2 s, and 72°C for 18 s, and a final elongation at 72°C for 10 min. The first reaction generated a 495-base pair band and the nested PCR generated a 474-base pair band. The negative control for each PCR assay was a reaction containing no cDNA and water to volume; the positive control was a reaction containing sequence-verified HCV cDNA.

PCR amplification of GAPDH mRNA

Amplification of GAPDH mRNA was used to verify the integrity of the RNA obtained from tissues and as a positive control for the HCV RNA assay. Primer design for GAPDH was performed by Dr. D. Zhang

(The Mount Sinai Medical Center). Reverse transcription was performed with the reverse GAPDH primer. The reaction mixture and conditions were the same as above. The PCR reaction mixture was the same as for the 5' UTR PCR assay, except the GAPDH forward and reverse primers were used. The cycling conditions were 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 45 s, and a final elongation at 72°C for 7 min. This reaction generated a 208-base pair band.

Detection of HIV DNA in brain

DNA was extracted from brain tissues using the DNeasy Tissue Kit from Qiagen (Valencia, CA) and the yield was estimated by UV spectroscopy. Purified DNA was then used with primers to a 115-base pair conserved region of HIV-1 gag (primers SK38 and SK39) as previously described (Ou *et al*, 1988). Products were electrophoresed on 3% NuSeive 1% agarose gels, and visualized with ethidium bromide. Products were then transferred to a nylon membrane by the technique of Southern, hybridized to digoxigenin-labeled probe SK 19, and detected by anti-digoxigenin antibodies as previously described (Aboody-Guterman *et al*, 1996; Ou *et al*, 1988).

Sequencing of 5' UTR and E1 amplicons of HCV

Amplicons were fractionated by size in 3% NuSieve/1% Seakem agarose gels. Gel bands were excised, eluted in Buffer QG at a 6-fold volume/volume excess relative to the gel band (100 mg of gel was taken to be equivalent to 100 μ l volume) and stored in 50 μ l of EB buffer (10 mM Tris/Cl, pH 8.5). The QIA quick gel extraction kit (Qiagen, Valencia, CA) was used to purify PCR products prior to sequencing. The internal primers were used to sequence the E1 region, and the PCR primers in Table 6 were used to sequence the 5' UTR. Sequencing was done on an Applied Biosystems 3730xl capillary electrophoresis DNA sequencing instrument at the Mount Sinai School of Medicine shared research core facility in the Department of Human Genetics. The tracings were manually

analyzed to resolve inconsistencies between the sequences obtained using forward and reverse primers. To correct for sequencing errors that can occur in the region immediately following primer binding sites, the 35 nucleotides at the 5' end of each sequence were obtained using the reverse primer and 35 nucleotides at the 3' end were obtained using the forward primer.

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Appendix 1 Individual domain T scores for patients in study

| Patient ID | Liver HCV | Brain HCV | Global | Motor | Speed of information processing | Working memory | Memory encoding | Memory retrieval | Fluency | Abstraction executive functioning |
|------------|-----------|-----------|--------|-------|---------------------------------|----------------|-----------------|------------------|---------|-----------------------------------|
| 010016 | yes | yes | 32 | 28 | 34 | 35 | 34 | 27 | 52 | 27 |
| 010034 | yes | yes | 28 | na | 24 | 37 | 16 | 36 | 34 | na |
| 010066 | yes | yes | 34 | 36 | 30 | 50 | 28 | 27 | 34 | 40 |
| 010086 | yes | yes | 36 | 32 | 38 | 47 | 33 | 32 | 40 | 36 |
| 020024 | yes | yes | 25 | 22 | 25 | 27 | 17 | 18 | 38 | 33 |
| 020028 | yes | yes | 31 | 22 | 36 | 40 | 30 | 24 | 44 | 30 |
| 010001 | yes | no | 42 | 37 | 41 | 48 | 36 | 34 | 64 | 43 |
| 010027 | yes | no | 27 | 39 | 25 | 26 | 18 | 20 | 33 | 36 |
| 020053 | yes | no | 30 | na | 16 | na | na | na | 46 | 29 |
| 020015 | yes | no | 44 | 34 | 39 | 44 | 44 | 45 | 62 | 50 |
| 010018 | no | no | 28 | 22 | 27 | 43 | 23 | 18 | 36 | 42 |
| 010025 | no | no | 28 | 22 | 29 | 31 | 24 | 20 | 42 | 36 |
| 010043 | no | no | 35 | 32 | 34 | 42 | 32 | 38 | 32 | 34 |
| 010065 | no | no | 41 | 38 | 50 | 50 | 26 | 30 | 33 | 49 |
| 010095 | no | no | 38 | 32 | 38 | 43 | 36 | 42 | 38 | 41 |
| 010105 | no | no | 33 | 24 | 40 | 32 | 27 | 27 | 30 | 46 |
| 010133 | no | no | 35 | na | 33 | 33 | 32 | 31 | 45 | na |
| 010162 | no | no | 30 | na | 23 | 50 | 22 | 25 | na | na |
| 020025 | no | no | 50 | 73 | 49 | 43 | 41 | 38 | 50 | 55 |

na: scores could not be computed due to patient inability to take these tests.