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Deep sequencing of hepatitis C virus reveals genetic compartmentalization in cerebrospinal fluid from cognitively impaired patients

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Abstract

Background & Aims—Hepatitis C virus (HCV) causes neuropsychiatric impairment and fatigue with recent studies suggesting HCV invasion of the central nervous system (CNS). Our previous finding that endothelial cells from the blood-brain barrier support HCV infection warrants further investigation to elucidate whether the CNS can serve as a reservoir for independent HCV evolution.

Methods—Cerebrospinal fluid (CSF) and plasma from six HCV-infected patients without liver disease or co-morbidities together with plasma from six healthy subjects were profiled for markers of immune activation and viral quasispecies measured by deep sequencing. Unsupervised data analyses were used to identify any associations between cytokine activation markers and clinical outcomes.

Results—Four of six HCV-infected patients showed significant evidence of cognitive dysfunction and fatigue. Deep sequencing revealed independent viral evolution within the CNS of two cognitively impaired patients. Principal component analysis of peripheral cytokines demonstrated that individuals without cognitive impairment clustered together while a distinct cytokine pattern emerged with patients exhibiting cognitive dysfunction and fatigue.

Conclusions—Deep sequencing demonstrated unique viral variants in the CSF of two cognitively impaired patients consistent with CNS replication or sequestration. Meanwhile, compartmentalization was absent in infected patients with no neurocognitive impairment.

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Supporting information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1111/liv.13134/supinfo

Examination of cytokine profiles in HCV-infected patients with cognitive dysfunction revealed elevated peripheral cytokine levels resulting in a distinct cytokine profile that may be related to cognitive impairment or viral penetration into the CNS. Further studies to determine the significance of unique HCV variants within the CNS are warranted.

Keywords

brain; cytokine; hepatitis C virus; inflammation; quasispecies

Hepatitis C virus (HCV) infects approximately 180 million people worldwide. While the primary cellular reservoir for HCV infection is liver hepatocytes, infection is associated with extrahepatic symptoms including central nervous system (CNS) abnormalities, cognitive dysfunction, fatigue and depression (1). It is unclear whether HCV-associated CNS symptoms are a function of systemic disease, impaired hepatic function or infection of the CNS (2). Fatigue is the most commonly reported neuropsychiatric symptom associated with HCV infection, with 65–80% of infected patients complaining of fatigue that is independent of liver dysfunction (1). Recent studies reporting *ex vivo* infection of cells within the CNS and the detection of HCV RNA in brain biopsies (2) support a model where HCV infects the brain. This raises questions as to whether HCV can evolve within the CNS and if this is associated with neurological symptoms in the absence of confounding factors such as liver disease, HIV co-infection or prior interferon treatment.

Genetic compartmentalization of a virus within the brain has been observed for other infections, including HIV (3), and this has implications for treatment since antiviral therapies for HIV and HCV are substrates of p-glycoprotein, a multidrug transporter expressed at the blood–brain barrier (BBB) (4), that limits the penetration of many therapies into the CNS. Studies have reported evidence of independent HCV evolution in the CNS compared to the liver (5–7). However, these studies used tissue from patients diagnosed with cirrhosis, fibrosis or HIV co-infection: co-morbidities that are independently associated with cognitive dysfunction. Furthermore, genetic analyses of HCV variants were restricted to small fragments of the viral genome because of the difficulties in amplifying viral RNA from brain biopsies following RNA degradation with postmortem tissue combined with the reduced viral burden in the brain compared to liver (2, 5). Consequently, it has been difficult to establish a link between neuropsychiatric symptoms and HCV infection of the CNS. Furthermore, studies to date have been limited by Sanger sequencing with typically only a few virus clones selected. As a result the true genetic structure of the virus population could be obscured and minor variants not detected. To overcome this problem we have implemented a deep sequencing approach that allows for a sensitive and comprehensive understanding of the inpatient viral quasispecies. This sequencing technology coupled with a unique cohort of HCV-infected subjects without severe liver disease, HIV co-infection or prior interferon therapy allows us to investigate the impact of CNS invasion by HCV.

Materials and methods

Study subjects

Matched plasma and CSF was obtained from six patients enrolled in a study of cerebral function in hepatitis C infection. Patients were from an outpatient clinic at Aarhus University Hospital, Denmark. The patients were Caucasian, viraemic, non-cirrhotic patients infected with HCV genotypes 1 and 3 and did not have any significant neurological or psychotic disorders, previous history of head trauma, severe liver fibrosis or cirrhosis, history of drug abuse within the past two years, HIV or hepatitis B virus (HBV) co-infection or prior interferon treatment. Plasma was obtained from six non-HCV-infected age-, sex- and education-matched subjects free of somatic and psychiatric morbidity (Table S1). Four of six HCV-infected patients had significant fatigue (FSS average score 5) and cognitive dysfunction. A lumbar puncture was performed on the HCV-infected patients and peripheral blood-derived plasma was collected from all HCV-infected and control subjects.

Neuropsychological testing and fatigue assessment

Fatigue was assessed using the Fatigue Severity Scale (FSS). The psychometric properties of the FSS has been validated in HCV-infected subjects, and in accordance with Lerdal *et al.* (8) we defined clinically significant fatigue as an average item score of 5 or above. Neuropsychological testing included assessment of the following cognitive domains: Visuomotor speed (Symbol digit modalities test (SDMT) and trail making test A + B); attention (D2 test of attention and Stroop's test); perceptual organization (WAIS III matrix reasoning and Street completion test); memory and learning (WMS III logical memory I and II, Rey complex figure test (RCFT) and Rey auditory verbal learning test (RAVLT)), and executive function (WAIS III letter number sequencing, verbal fluency (s and d words, animals, street items) and Wisconsin card sorting test (WCST)). Performance was calculated as a z score based on the performance of the healthy controls, and z scores from each individual test were added to yield a domain z score. Subsequently, clinically significant cognitive impairment was defined as a cognitive performance 1 SD below healthy controls in 2 domains (9).

Cytokine measurement

Following collection of CSF, microscopy was performed to identify the presence of any contaminating cells. Patients 1–5 had no detectable contaminating cells in the CSF, and patient 6 had a low level of detectable leucocytes in the CSF (6 cells/ 10^{-6}). To further eliminate the presence of contaminating peripheral cells, the CSF was filtered through a 0.2- μ m filter before RNA was prepared or cytokine levels were measured by Luminex analysis. Cytokines were measured using a Luminex bead array consisting of a human 27-plex cytokine kit (Bio-Rad Laboratories Inc., Hercules, CA, USA) which included TNF- α , MCP-1, MIP-1 α , IL-8, IL-1 β , IL-6, VEGF, IL-1ra, IL-10, IL-2, IL-4, IL-5, IL-7, IL-9, IL-12 (p70), IL-13, IL-15, IL-17, IFN- γ , IP-10, G-CSF, GM-CSF, RANTES, FGF-basic, Eotaxin, PDGF-bb, and MIP-1 β . All samples were assayed concurrently in duplicate.

Ethics

All participants provided written informed consent, and the study was conducted in accordance with the ethical guidelines of the World Medical Association's Declaration of Helsinki. The study was approved by The Central Denmark Region Committees on Health Research Ethics (2008-0096), Danish Data Protection Agency (2009-41-2226) and monitored by the Good Clinical Practice Unit at Aarhus University Hospital. ClinicalTrials.gov identifier: NCT-00788918.

RNA amplification and Deep sequencing

RNA was isolated from 140 µl of plasma or CSF using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). An amplicon covering Core to NS2 was generated using the SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA) (primers available on request). Thermal cycling parameters were: 55°C for 30 min, 94°C for 2 min, followed by 40 cycles of 94°C for 15 s, 58°C for 30 s and 68°C for 4 min, with a final extension step at 68°C for 10 min. Purified PCR products were pooled in equimolar ratios, fragmented using the transposon-mediated Nextera DNA Sample Prep Kit (Illumina, San Diego, CA, USA) and purified with a DNA Clean and Concentrator Kit (Zymo Research, Freiburg, Germany). Adapters and barcodes were added by limited-cycle PCR (Nextera, Florida, USA). Small fragments were removed following Roche's recommended size selection protocol and quantified using a Promega Quantiflor-ST fluorometer. After quantification, barcoded samples were pooled at a final concentration of 10^7 molecules/µl to create the library for sequencing on a GS Junior 454 Genome Sequencer (Roche, Dublin, Ireland). Emulsion PCR, breaking, and DNA sequencing were performed according to the manufacturer's protocols for Lib-L (Roche). Genetic compartmentalization was assessed using the Slatkin-Maddison test (10) and the non-parametric test for panmixia described by Hudson *et al.* (11) with 10 000 permutations to generate a *P* value.

In the event that an amplification using the above strategy was unsuccessful, an alternative PCR amplicon-based ultra-deep sequencing approach was used to analyse a region encompassing the hypervariable region (HVR) in the N-terminus of E2 (primers available on request). Thermal cycling conditions were: 55°C for 30 min, 94°C for 2 min, followed by 40 cycles of 94°C for 15 s, 52°C for 30 s and 68°C for 30 s, with a final extension step at 68°C for 5 min. Sequencing was performed as described above except that Emulsion PCR, enrichment, breaking and DNA sequencing were performed according to the GS Junior FLX Titanium Series manuals for Lib-A (Roche).

Detection of minor HCV variants required a targeted coverage 250-fold per site. In this study, plasma and CSF samples were sequenced to an average depth of 354 and 314 reads per site respectively. The raw sequence outputs ('reads') were assembled by VICUNA *de novo* assembler software (12) and corrected for systematic 454 sequencing errors using the previously developed RC454 and V-Phaser algorithms (13, 14).

Statistical analyses

Principal component and hierarchical clustering analysis were performed using the JMP Pro version 12 statistical package.

Results

Hepatitis C virus RNA was detected in all plasma samples and in CSF from four of six patients (Patients 2, 4, 5 and 6) and subjected to deep sequencing. Despite failing to detect HCV RNA in the CSF from patients 1 and 3 low levels of virus may still be present but beneath the detection limit of standard assays. In the neurocognitive group the plasma viral burden was generally higher (median of 8.9×10^6 IU/ml) compared to the normal cognitive group (median of 1.93×10^6 IU/ml) although this was not statistically significant. Viral sequences recovered from the CSF were compared with their respective counterparts from plasma. The number of reads generated from plasma for all subjects was a median of 2807 (IQR: 2494–5620) while a median of 1690 (IQR: 1003–2228) reads were generated from CSF samples. Both sets of samples had similar sequencing depth for plasma ($394x \pm 165$) and CSF ($317x \pm 101$). Moreover, the mean reads lengths were $369 (\pm 28)$ bp for plasma and $379 (\pm 20)$ bp for CSF. The hypervariable region (HVR) of E2 is the most rapidly evolving region of the viral genome and provides a simple indicator of genetic variability. Significant genetic compartmentalization was detected between CSF and plasma-derived HVR populations in patient 4 ($P < 0.001$) where a genetically distinct CSF-derived virus population was observed (Fig. 1A). The CSF-derived viruses contained amino acid substitutions that were less charged and more hydrophobic than the major variant circulating in the periphery. Furthermore, additional genetic changes in E1 were found within the CSF that were absent in the plasma. Examination of viral sequences from patient 6 revealed that the blood contained five HVR variants of which two were detected in the CSF. Upon closer inspection a unique viral mutation was found in the C-terminus of E1 resulting in this variant being sequestered within the CNS ($P < 0.001$) (Fig. 1B). These data support the presence of an autonomous replicating viral population harboured within the CSF. In the normal neurocognitive group no independent viral evolution was detected in the CSF of one sequenced patient (patient 5) despite a sequencing depth to detect minority variants (Fig. 1C). In this instance the monotypic variant found within CSF comprised the major variant circulating in the blood quasispecies. Only in a single subject (patient 2) was a well-equilibrated viral population maintained between the periphery and CSF with a similar distribution of variants observed at both sites (Fig. 1D).

Quantification of 27 cytokines in plasma highlighted the levels of inflammatory markers in plasma which were elevated in cognitively impaired HCV-infected patients compared to non-cognitively impaired HCV-infected patients (Fig. S1). Using an unsupervised data reduction tool, principal component analysis (PCA), we attempted to explore the inflammatory profile that was associated with neurocognitive impairment. Principal component (PC1) described the vast majority of the variation in the data set with positive loadings (indicated by the right side of the quadrant) corresponding to HCV-infected patients, whereas negative loadings (indicated by the left side of the quadrant) correspond to healthy subjects (Fig. 2A). Strikingly, the patients demonstrating normal neurocognition

cluster together while those patients having neurocognitive impairment cluster independently. Unsupervised hierarchical clustering of all 12 patients revealed two major clusters linked to infected and normal status (Fig. 2B). While examination of the HCV-infected cluster revealed subclusters depicting subjects with normal neurocognition behaviour (patients 1 and 5), a significant similarity within neurocognitive impairment group was observed (Fig. 2B). Cytokine expression profiles in CSF were also measured with IP-10, MCP-1 and GM-CSF detected in CSF from HCV-positive patients (data not shown). However, we failed to detect increased cytokine expression in the CSF from HCV-infected patients with neurocognitive impairment compared to those with normal neurocognition (data not shown). While we did not measure cytokine levels in CSF from uninfected individuals we noted that the cytokine levels measured in the CSF from our HCV-infected cohort are comparable with previous reports of normal CSF, suggesting minimal perturbation (15). Although the expression levels of MCP-1 in CSF were significantly higher than in plasma ($P < 0.002$, Mann–Whitney test) the concentrations did not show any significant difference between the two neurocognitive groups.

Discussion

To our knowledge, this is the first study to use deep sequencing technologies to investigate HCV genetic compartmentalization between plasma and CSF from patients where the levels of fatigue and cognitive impairment are known. Importantly, virus was detected in the CSF from subjects without severe liver disease, HIV co-infection or prior interferon therapy, all of which are known to cause fatigue or neurological impairment. HCV RNA was previously reported to be present in the brain tissue from HCV/HIV co-infected patients (2, 5) and analysis of the HVR sequences demonstrated unique variants in 4 of 7 subjects (5). This study relied on bulk Sanger sequencing which is insufficient to detect low-frequency variants. Our study confirms and extends these observations and sampled the CSF from HIV-negative, cognitively impaired subjects with modest liver disease. Moreover, as a result of the intact nature of RNA obtained from the CSF, it was possible to amplify the structural half of the HCV genome and to deep sequence viral RNA species and to compare to peripheral circulating variants in the plasma. Without deep sequencing, it is likely that many of these variants would have gone undetected. Viral compartmentalization in CSF associated with a higher plasma viral load, suggesting that increased viral burden in the periphery may facilitate invasion of the CNS. These studies show clear evidence for genetic compartmentalization between plasma and CSF, supporting a model where HCV establishes a productive infection within the CNS. Although the number of patients studied was small, compartmentalization occurred in two of three subjects with cognitive impairment (patients 4 and 6) while patient 5 exhibiting normal cognitive dysfunction showed no evidence of viral evolution within the CSF. Schnell and colleagues reported transient HIV compartmentalization in CNS and blood (16) that may reflect asynchronous rates of virus seeding. Future studies involving larger sample sizes are required to replicate these findings and should include longitudinal sampling of CSF and plasma compartments to define the dynamics of HCV compartmentalization.

Quantification of 27 inflammatory cytokines revealed there was a trend towards higher cytokine levels in plasma from cognitively impaired compared with normal cognitive

patients, which warrants further investigation with larger cohorts. However, larger studies on chronically infected HCV patients examining the association between serum cytokine levels and the severity of neuropsychiatric symptoms have found conflicting results (17, 18). Furthermore, CSF cytokines do not appear to be elevated and parallel the perturbations in the periphery at least from the few cytokines where responses were detected. Inflammatory cytokines, including interferons, are known to enter the brain in a variety of disease states and induce fatigue and other neurological symptoms (19). Importantly, the patients enrolled in our study had not received interferon, which can complicate studies of cognitive impairment. Recent studies show that inflammatory cytokines, including IL-1 β and TNF- α , promote HCV particle entry into target cells (20), which may result in increased viral load in patients with high levels of circulating pro-inflammatory cytokines. These cytokines disrupt BBB integrity and may facilitate viral invasion of the brain (20).

In summary, this study demonstrates compartmentalization of HCV genetic variants between CSF and plasma in chronically infected patients with no significant liver disease or HIV co-infection. This study highlights that direct-acting antivirals (DAAs) for treating hepatitis C may need to penetrate the BBB and that drug levels in the brain may be subtherapeutic leading to development of viral ‘reservoirs’ that have the potential to seed resistance mutations into the periphery as previously reported in HIV infection (3).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

BBB	blood–brain barrier
CNS	central nervous system
CSF	cerebrospinal fluid
DAA	direct-acting antiviral
FSS	fatigue severity scale
HCV	hepatitis C virus
HVR	hypervariable region

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Keypoints

- Cognitive dysfunction was observed in four of six HCV-infected patients
- Deep sequencing showed evidence of unique HCV variants in the CSF of two cognitively impaired patients
- A distinct cytokine profile was observed in the plasma of HCV-infected subjects with cognitive impairment
- These data highlight a possible association between genetic compartmentalization and elevated peripheral cytokine levels in HCV-infected subjects with cognitive impairment

Fig. 1.

Evidence for hepatitis C virus (HCV) genetic compartmentalization in plasma and cerebrospinal fluid (CSF) from subjects with neurocognitive impairment. Phylogenetic analyses of the HCV E2 hypervariable region (HVR) are shown unless otherwise stated. Sequences from the CSF are labelled with solid red circles and sequences derived from plasma are labelled with solid blue squares. The genetic distance (0.01) represents the number of substitutions per site and is indicated by the horizontal line below each phylogeny. (A) One subject (Patient 4) demonstrates the presence of a statistically significant compartmentalized population within the CSF relative to the plasma as assessed using the Slatkin-Maddison test and supported by the test of panmixia. (B) Patient 6 shows evidence of compartmentalization where plasma and CSF populations are not uniformly equilibrated and a minor CSF subpopulation exists from the analysis of partial E1 sequences. (C) Patient 5 demonstrates a monotypic variant present within the CSF while multiple plasma variants are detected. (D) Comparison of HCV amino acid sequence diversity derived from a 3.2-kb fragment of blood and CSF for patient 2 illustrating a well-equilibrated population between blood plasma and CSF. Plotted is the percentage of amino acid diversity at each position spanning the HCV proteins Core to NS2 with respect to the dominant amino acid found within each sample.

Fig. 2.

Immune activation markers are elevated upon hepatitis C virus (HCV) infection and tend to associate with a state of impaired neurocognition. (A) Unsupervised analysis of cytokine expression by Principal Component Analysis (PCA) was used to reduce dimensionality and extract principal components composed of combinations of 27 different analytes. Principal component 1 (PC1) and 2 (PC2) are depicted in a two-dimensional scatter plot for each individual. Open red dots represent HCV-infected subjects with neurocognitive impairment; blue diamonds are HCV-infected subjects with normal neurocognition; green cross depicts healthy controls. (B) Unsupervised hierarchical clustering of HCV-infected samples analysed using Luminex. Cytokine expression is depicted with a colour scale; red denoting high expression level and blue denoting low expression level. Patient IDs are listed as HCV-infected (1–6) and healthy uninfected controls (7–12) with symbols as described in panel A.