

ORIGINAL RESEARCH

Increased Mitochondrial Genetic Diversity in Persons Infected With Hepatitis C Virus



David S. Campo,^{1,*} Ha-Jung Roh,^{1,*} Brian L. Pearlman,^{2,3,4} Daniel S. Fierer,⁵ Sumathi Ramachandran,¹ Gilberto Vaughan,¹ Andrew Hinds,² Zoya Dimitrova,¹ Pavel Skums,¹ and Yury Khudyakov¹

¹Laboratory of Molecular Epidemiology and Bioinformatics, Division of Viral Hepatitis, Centers for Disease Control and Prevention, Atlanta, Georgia; ²Center for Hepatitis C, Atlanta Medical Center, Atlanta, Georgia; ³Medical College of Georgia, Augusta, Georgia; ⁴Emory School of Medicine, Atlanta, Georgia; ⁵Division of Infectious Diseases, Icahn School of Medicine at Mount Sinai, New York, New York

SUMMARY

Hepatitis C virus infection strongly affects mitochondrial DNA genetic diversity, which can be used to discriminate recent from past infections.

BACKGROUND & AIMS: The host genetic environment contributes significantly to the outcomes of hepatitis C virus (HCV) infection and therapy response, but little is known about any effects of HCV infection on the host beyond any changes related to adaptive immune responses. HCV persistence is associated strongly with mitochondrial dysfunction, with liver mitochondrial DNA (mtDNA) genetic diversity linked to disease progression.

METHODS: We evaluated the genetic diversity of 2 mtDNA genomic regions (hypervariable segments 1 and 2) obtained from sera of 116 persons using next-generation sequencing.

RESULTS: Results were as follows: (1) the average diversity among cases with seronegative acute HCV infection was 4.2 times higher than among uninfected controls; (2) the diversity level among cases with chronic HCV infection was 96.1 times higher than among uninfected controls; and (3) the diversity was 23.1 times higher among chronic than acute cases. In 2 patients who were followed up during combined interferon and ribavirin therapy, mtDNA nucleotide diversity decreased dramatically after the completion of therapy in both patients: by 100% in patient A after 54 days and by 70.51% in patient B after 76 days.

CONCLUSIONS: HCV infection strongly affects mtDNA genetic diversity. A rapid decrease in mtDNA genetic diversity observed after therapy-induced HCV clearance suggests that the effect is reversible, emphasizing dynamic genetic relationships between HCV and mitochondria. The level of mtDNA nucleotide diversity can be used to discriminate recent from past infections, which should facilitate the detection of recent transmission events and thus help identify modes of transmission. (*Cell Mol Gastroenterol Hepatol* 2016;2:676–684; <http://dx.doi.org/10.1016/j.jcmgh.2016.05.012>)

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Hepatitis C virus (HCV) is a major public health problem in the United States and worldwide. HCV infects at least 3.5 million persons in the United States and 180 million persons worldwide.¹ HCV infection is the most common chronic blood-borne infection and the leading cause for liver transplantation.² Since 2007, HCV has surpassed human immunodeficiency virus (HIV) as a cause of death in the United States.³ Approximately 80% of patients who become infected with HCV develop chronic hepatitis and are at risk for advanced liver disease, with 15%–30% of these patients progressing to liver fibrosis and cirrhosis and up to 5% dying from liver failure or hepatocellular carcinoma (HCC).² The outcome of HCV infection is determined by a complex interplay between host genetic, immunologic, and viral factors.⁴ The host innate and adaptive immune responses are key determinants of viral clearance and persistence. Several host genetic factors have been associated with efficiency of immune response against HCV and with antiviral therapy outcome. The most well-known host genetic factors affecting HCV infection are ethnic background, interleukin 28B polymorphisms, and alleles of the inhibitory natural killer cell receptor and HLA classes I and II.⁵ Recent genome-wide association studies identified several new factors influencing treatment efficacy or clinical course of HCV infection such as variants of interferon (IFN) L3-IFNL4, IRAK2, DEPDC5, MICA, RNF7, TULP1, and MERTK.^{6,7}

It is well established that viruses, which are simple biological entities with high mutation rates, adapt rapidly to the host genetic environment, producing distinct genetic variants. In human beings, viral infections are not generally

*Authors share co-first authorship.

Abbreviations used in this paper: AUC, area under the curve; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HVS, hypervariable segment; IFN, interferon; mtDNA, mitochondrial DNA; NGS, next-generation sequencing; PCR, polymerase chain reaction; pegIFN, peginterferon; ROC, receiver operating characteristic.

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considered to be able to induce genetic changes in the host beyond those related to adaptive immune responses or rare mutations associated with cancer,⁸ owing to the stability of nuclear DNA. Mitochondrial DNA (mtDNA), however, varies in copy number and has a high mutation rate.⁹

Chronic HCV infection has been associated strongly with prominent mitochondrial injury of the liver.¹⁰⁻¹³ These effects include mitochondria swelling, loss of mitochondrial cristae, reduction in number of mitochondria, calcium-mediated mitochondrial depolarization and dysfunction, and reactive oxygen species generation. Under normal circumstances, these impaired mitochondria would be eliminated. However, in infected cells, HCV attenuates apoptosis by cleaving mitochondrial antiviral signaling protein¹⁴ and promoting mitochondrial fission and mitophagy of the functionally compromised organelles,^{12,13} which contribute to persistence of infection. These results are consistent with the low mtDNA copy number found in the blood of persons with chronic HCV infection.¹⁵ Oxidative stress and reactive oxygen species generated by distressed mitochondria now are recognized as a major procarcinogenic cofactor in chronic HCV infection.¹⁰ In addition, mtDNA mutations were found in the liver of patients with HCC¹⁶⁻²² and were linked to outcomes of antiviral therapy.²³

In this study, we used next-generation sequencing (NGS) to evaluate the intrahost mtDNA variants from HCV-uninfected persons and cases with seronegative acute and seropositive chronic HCV infection. We show that the mtDNA genetic diversity is higher in HCV-infected persons and decreases after completion of therapy.

Materials and Methods

Human Subjects

A total of 130 serum samples from 116 individuals belonged to 4 groups. Groups 1 and 2 were obtained from commercially available seroconversion panels (Seracare, Milford, MA), and groups 3 and 4 were obtained through clinical studies with Institutional Review Board approval, with all patients providing written consent to participate. The protocol was approved by Atlanta Medical Center's Institutional Review Board and the Icahn School of Medicine at Mount Sinai (study ID: 06-0974 ME).

Group 1 comprised 50 HCV-uninfected (anti-HCV-seronegative) plasma donors (control group). The samples also were negative for anti-HIV and anti-hepatitis B virus. No demographic or clinical information was available for this group.

Group 2 comprised 18 plasma donors with seronegative, polymerase chain reaction (PCR)-positive, acute HCV infection. In 12 cases, we analyzed 2 specimens obtained an average of 10.2 days apart, both before seroconversion. No demographic or clinical information was available for this group.

Group 3 comprised 46 treatment-naïve patients with chronic HCV infection (anti-HCV-seropositive with PCR-positive HCV infection for > 6 months). African American (n = 19), Caucasian (n = 27); female (n = 23), male (n = 23); noncirrhotic (n = 28), cirrhotic (n = 10); age at time of specimen (n = 45; range, 28–70 y; median, 53 y); steatosis

absent (n = 24), steatosis present (n = 13); genotype 1a (n = 28), genotype 1b (n = 9), genotype 2 (n = 7), genotype 3 (n = 2); and HCV viral load quantified (n = 39).

Group 4 comprised 2 HIV-infected men (designated patient A and patient B) with a recent HCV infection who underwent successful therapy with peginterferon (pegIFN) plus ribavirin. Two samples from each man were evaluated, the first from treatment baseline and the second from the last HCV-positive sample during treatment. Patient A had not seroconverted at baseline and patient B was anti-HCV-positive at baseline, having seroconverted 0–7 days before.

Nucleic Acid Extraction

Blood was incubated in an upright position at room temperature for 30–45 minutes to allow clotting. Then the sample was centrifuged for 15 minutes at 2000 relative centrifugal force to obtain serum. Total nucleic acid was extracted from serum samples using the automated Roche MagNA Pure LC robot and the MagNA Pure LC Total Nucleic Acid Isolation kit (Roche Diagnostics, Indianapolis, IN), and eluted with 50 μ L of elution buffer according to the manufacturer's instructions. Complementary DNA was generated using the high-temperature capability SuperScript VIL0 complementary DNA Synthesis Kit (Invitrogen, Life Technologies, Carlsbad, CA) on an ABI PRISM19700 PCR system (Thermo Fisher Scientific, Waltham, MA).

mtDNA Amplification

Hypervariable segment (HVS)1 and HVS2 in the D-loop domain of mtDNA were analyzed. Two sets of primers (external and nested) were used to amplify the HVS1 and HVS2 regions of the mitochondrial D-loop: (1) for HVS1: HVS1-H16401 (TGATTTACACGGAGGATGGTG),²⁴ HVS1-L15900 (TAAACTAATACACCAGTCTTGTAACCC),²⁵ HVS1-H16391 (GAGGATGGTGGTCAAGGGAC), and HVS1-L15997 (CACCATTAGCACCCAAAGCT)²⁶; (2) for HVS2: HVS2-F-16449 (CGCTCCGGGCCATAACACTT), HVS2-R-722 (GAAC TCACTGGAACGGGGATGCT), HVS2-F-44 (CATGCATTTGG TATTTTCGTCTGG), and HVS2-R-638 (GGTGATGTGAGCC CGTCTAA). For external primers, the following cycling conditions were used: 95°C for 150 seconds, 40 cycles of 94°C for 1 minute, 55°C for 30 seconds, and 72°C for 1 minute. For the nested amplicons, PCR conditions were as follows: 95°C for 9 minutes, 36 cycles of 95°C for 10 seconds, 60°C for 30 seconds, and 72°C for 30 seconds (HVS1) and 95°C for 9 minutes, 36 cycles of 95°C for 10 seconds, 52°C for 30 seconds, and 72°C for 30 seconds (HVS2). For separation of PCR products, E-gel imager and E-gel size select gel (2%) (cat# G661002; Invitrogen) were used. After gel purification, samples were run in a bioanalyzer (Agilent 2200 tape station, Agilent Technologies, Santa Clara, CA) to prepare for emulsion PCR.

NGS

PCR products were pooled and subjected to pyrosequencing using the GS FLX Titanium Sequencing Kit (454 Life Sciences, Roche Applied Sciences, Indiana, IN). Low-quality reads were removed using the GS Run Processor

v2.3 (Roche). Initial reads were processed by matching to the corresponding multiplex identifier. The files then were analyzed with the error correction algorithms KEC and ET,²⁷ which have been validated to be highly accurate in finding true haplotypes, estimating their frequencies and removing false ones.

Data Analysis

For each mtDNA amplicon, a multiple sequence alignment was created by means of MAFFT,²⁸ and then the primer sequences were trimmed, yielding a final amplicon of 433 base pairs for HVS1 and 502 base pairs for HVS2. The level of genetic diversity in each sample was examined by unbiased estimates of nucleotide diversity, calculated according to Nei and Li²⁹ using MATLAB.³⁰ All other statistical analyses were performed with IBM SPSS statistics for windows.³¹ Comparison among groups was performed with the independent samples median test, which tests the null hypothesis that the medians are equivalent across groups. To test the median of differences between the 2 time points of acute samples, we used the related samples Wilcoxon signed-rank test. Box plots show the minimum, first quartile, median, third quartile, and maximum of the distribution of values. A receiver operating characteristic (ROC), or ROC curve, was built, which illustrates the performance of a binary classifier (eg, acute vs chronic samples) because the mtDNA nucleotide diversity threshold varied, plotting the true-positive rate against the false-positive rate at various threshold settings.

Monte-Carlo Sampling

To establish the effect of sampling depth on the mtDNA nucleotide diversity, a Monte-Carlo sampling method was used. For each sample, 5% of the total number of reads were sampled randomly from the original file, and the nucleotide diversity of the subsamples was calculated. This procedure was repeated 1000 times for each sample, also with a different number of reads (from 5% to 100%, in increments of 5%).

Network Visualization

We built a k-step network as previously described by Campo et al³² using MATLAB.³⁰ The k-step network contains all possible minimum spanning trees and allows efficient visualization of the genetic relatedness among all haplotypes present in the sample. The networks were drawn with GEPHI.³³

Results

NGS of mtDNA

A total of 258,503 and 75,515 NGS reads were obtained from HVS1 and HVS2, respectively, the 130 serum specimens. On average, 2039.49 HSV1 reads (SEM, 278.21) were sampled from each specimen. Although the number of reads varied among persons (Table 1), no statistically significant difference in the median number of reads ($P = .069$) was found among the study groups ($P = .069$). For HVS2, however, the average number of reads per sample was much lower ($n = 638.55$; SEM, 74.20), and there were statistically significant differences in the median number of reads among groups ($P < .001$).

Nucleotide diversity, which is defined as the average number of nucleotide differences per site between any 2 DNA sequences chosen randomly from the sample population, was calculated. Although estimates of nucleotide diversity of intrahost variants are robust to the sample size variations,^{34,35} reliability of the estimates also may vary depending on the genetic heterogeneity of tested populations. Considering the observed variation in the number of reads obtained from each group, the effect of the read sample size on the measured nucleotide diversity was evaluated using Monte-Carlo sampling for both HVS1 and HVS2 for each group. The data show that the NGS sampling depth has very little effect on the measured nucleotide diversity, with even 10% of the reads providing estimates that were very similar to those obtained from full-size samples (Figure 1A and B). Samples with low mtDNA nucleotide diversity (controls and cases with seronegative acute HCV infection) were less affected by changes in sample size, whereas estimates of nucleotide diversity for more heterogeneous populations (cases with chronic HCV infection) did not converge, indicating that a greater sample size is required for accurate estimates. However, the value of nucleotide diversity in the last group rapidly increased with the increase in sample size and achieved 75%–80% of the value of the full-size sample at only 25% of sampled sequences.

mtDNA Is More Heterogeneous in HCV-Infected Persons Than in Healthy Controls

A predominant major variant was found in all samples that corresponds to the germline mtDNA sequence. However, the frequency of the major variant differed among the groups. Controls and acute cases had the highest values,

Table 1. Mean Values for All Samples of Each Group

	Samples, n	Nucleotide diversity, mean (SEM)	Nucleotide diversity, median	Sequences, n (SEM)	Reads, n (SEM)	Major sequence, % (SEM)	Mutants, % (SEM)
Controls HVS1	50	1.99E-05 (6.08E-06)	0.00E+00	1.52 (0.19)	865.20 (187.65)	99.57 (0.13)	0.43 (0.13)
Acute HVS1	18	8.25E-05 (2.10E-05)	7.15E-05	2.11 (0.28)	1092.61 (126.43)	98.25 (0.43)	1.75 (0.43)
Chronic HVS1	46	1.96E-03 (2.04E-04)	2.16E-03	559.22 (87.46)	3686.41 (579.88)	64.76 (3.26)	35.24 (3.26)
Controls HVS2	50	2.67E-04 (3.22E-05)	2.33E-04	3.06 (0.20)	352.98 (40.97)	93.07 (0.85)	6.93 (0.85)
Acute HVS2	18	1.23E-04 (4.61E-05)	7.77E-05	2.00 (0.20)	202.06 (45.07)	96.79 (1.21)	3.21 (1.21)
Chronic HVS2	46	1.07E-03 (1.29E-04)	7.05E-04	86.37 (21.35)	1119.76 (152.32)	76.59 (2.66)	23.41 (2.66)
Total	228	6.90E-04 (6.92E-05)	2.32E-04	131.58 (23.08)	1339.02 (150.99)	86.16 (1.24)	13.84 (1.24)

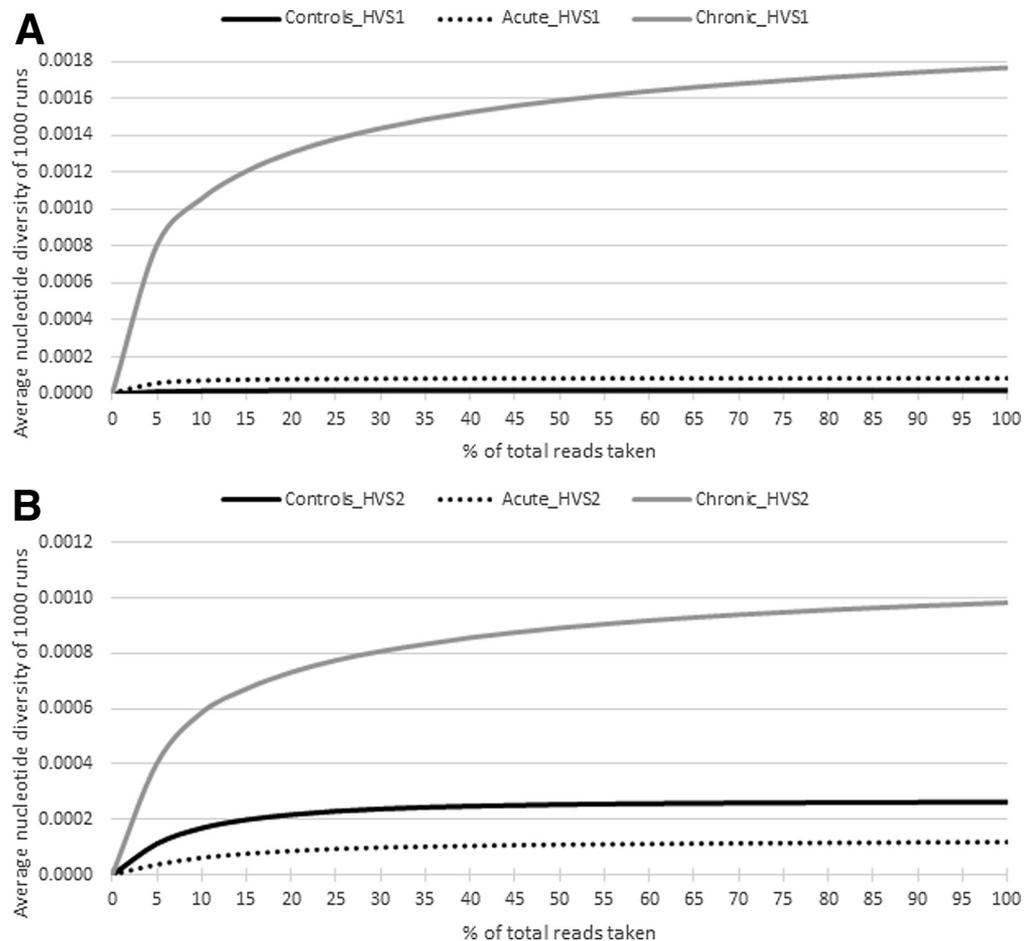


Figure 1. Monte-Carlo sampling. (A) HVS1 and (B) HVS2 samples.

whereas chronic cases had significantly lower values (Table 1 and Figure 2). In concordance with the frequency of major variants, it was found that the average level of HVS1 mtDNA nucleotide diversity in chronic cases was 98.5 times higher than in controls ($P < .001$) and was 4.2 times higher in cases with seronegative acute HCV infection than in controls ($P = .001$) (Table 1). Higher nucleotide diversity of HVS2 in chronic cases also was found, with the average level of HVS2 mtDNA nucleotide diversity 4 times higher than in controls ($P < .0001$). The average diversity of acute cases was numerically but not significantly lower than in controls ($P = .054$) (Figure 3), although the distributions were markedly different (independent samples Mann-Whitney test, $P = .005$).

High mtDNA Diversity in Cases With Chronic HCV Infection

The average mtDNA HVS1 nucleotide diversity among chronic cases was 23.7 times higher than among acute cases ($P < .001$) (Table 1). However, the average total number of reads obtained was not statistically different between these 2 groups ($P = .120$). The average HVS2 mtDNA nucleotide diversity among chronic cases was 8.7 times higher than among acute cases ($P < .001$). The correlation between nucleotide

diversity values for HVS1 and HVS2 obtained for all patients was highly significant but moderate ($r = 0.441$, $P = .0001$), indicating that both mtDNA regions experienced genetic diversification during HCV infection but at different rates.

The differences in mtDNA heterogeneity suggest that the degree of HVS1 nucleotide diversity could be used to discriminate between acute and chronic HCV infection. The ROC curve in Figure 4 shows performance of a simple binary classifier based on the mtDNA nucleotide diversity threshold, which allows for distinguishing acute from chronic samples with 100% sensitivity and 84.8% specificity (area under the curve [AUC], 0.9577) (Table 2). A similar approach can be used to distinguish controls from chronic samples (AUC, 0.9887) and controls from acute samples (AUC, 0.7172).

There was no significant difference in nucleotide diversity of either HVS1 or HVS2 over the short time (mean, 10.2 days; range, 2–21 days) between the paired specimens from 12 acute cases. Although the average nucleotide diversity was found to be 3.2 and 1.4 times higher for HVS1 and HVS2, respectively, at the second time points, these differences were not statistically significant for both regions (HVS1, $P = .327$; HVS2, $P = .695$). There was not a significant correlation between the differences in nucleotide diversity and time for HVS1 ($r = 0.075$, $P = .816$) as well as for HVS2

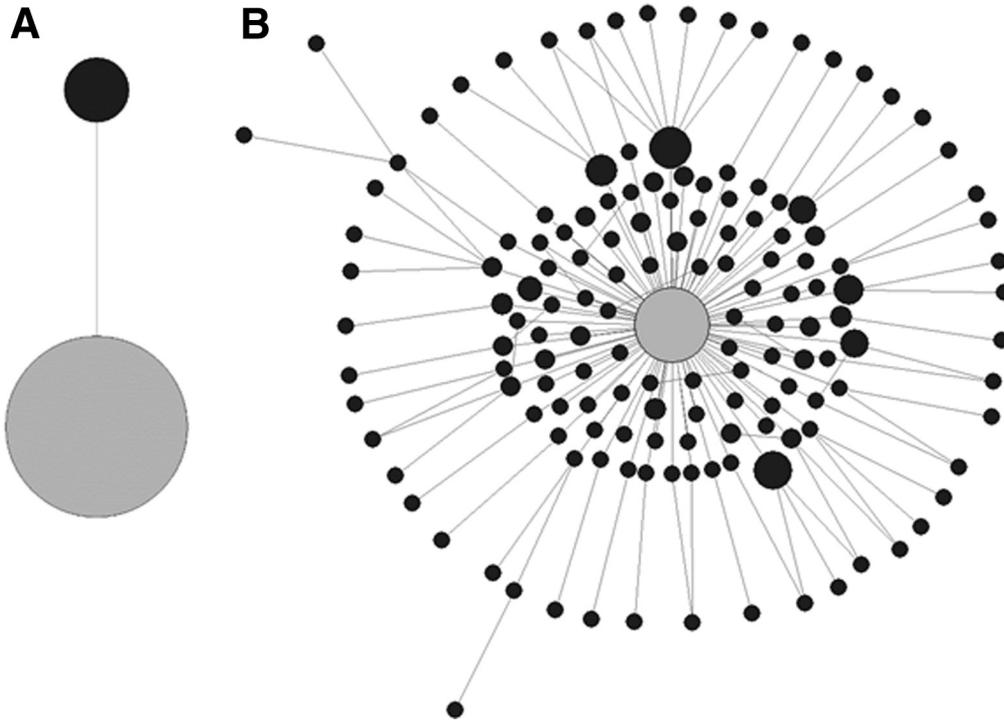


Figure 2. K-step networks of 2 HCV samples. Each node is a different mtDNA HVS1 variant and 2 nodes are linked if they differ in a single nucleotide. The size of the node is proportional to the square root of the frequency of that particular variant. The grey node is the major variant and the black nodes are mutant variants. (A) Acute sample from an individual with mtDNA nucleotide diversity close to the median of the acute group (7.15E-05). (B) Chronic sample from an individual with mtDNA nucleotide diversity close to the median of the chronic group (2.16E-03).

($r = -0.028, P = .931$), suggesting insufficient time for accumulation of detectable genetic heterogeneity in serum mtDNA. Nonetheless, the threshold-based binary classifier correctly classified all of the follow-up specimens as acute.

Analysis of the HVS1 nucleotide diversity from 46 chronic cases, for whom clinical and demographic data were available, showed no association with ethnicity ($P = .194$), sex ($P = .111$), HCV genotypes (1 vs 2; $P = .099$), HCV subgenotypes (1a vs 1b; $P = .926$), steatosis ($P = .863$), or cirrhosis ($P = .182$). However, for all variables the sample

sizes were smaller than the minimum needed to find significant differences ($\alpha = .05$; power = 0.80). In addition, there was no significant correlation with age (Pearson correlation, $r = 0.072, P = .636$) or viral load (Pearson correlation, $r = 0.114, P = .491$).

Reduction of mtDNA Diversity After Antiviral Therapy

To assess the effect of a pegIFN+ ribavirin treatment-associated decrease in HCV viremia on mtDNA nucleotide diversity, paired specimens from 2 HIV-infected men with

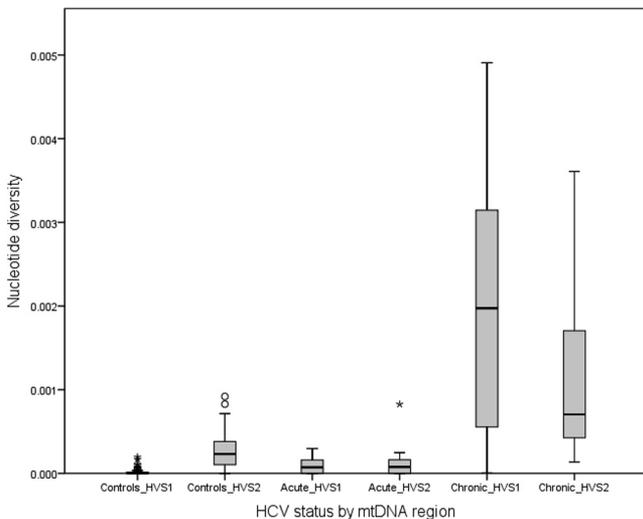


Figure 3. Box plot of the nucleotide diversity values of mtDNA samples.

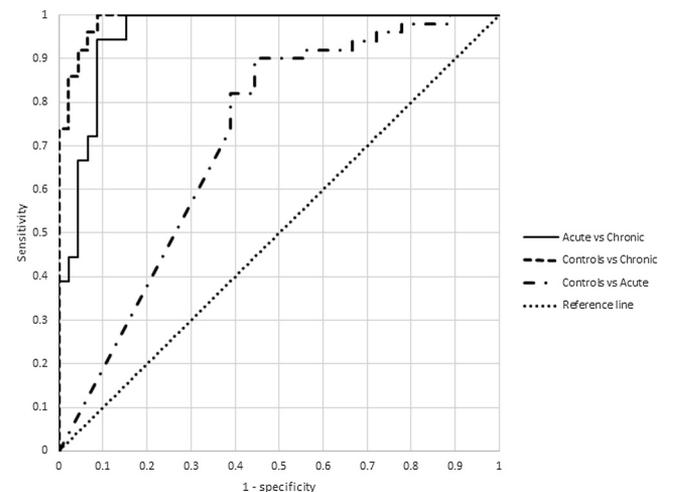


Figure 4. ROC curve of the mtDNA nucleotide diversity binary classifier.

Table 2. Classification Performance of a Nucleotide Diversity Cut-Off Value

	Ratio of the means	Independent samples, median test (<i>P</i> value)	AUC	Sensitivity (%)	Specificity (%)	Nucleotide diversity cut-off value
Chronic HVS1/ acute HVS1	23.71	.0001	0.9577	100	84.78	3.20E-04
Chronic HVS1/ controls HVS1	98.53	.0001	0.9887	100	91.3	2.20E-04
Acute HVS1/ controls HVS1	4.16	.001	0.7172	82	61.11	5.00E-05

acute HCV infection were analyzed. A different number of reads was obtained from each sample: 3954 and 1222 from patient A and 6163 and 2853 from patient B. The effect of the sample size on robustness of the nucleotide diversity using Monte-Carlo sampling showed that the sample of sequences from each specimen was adequate for representation of the nucleotide diversity (Figure 5A). The mtDNA nucleotide diversity decreased dramatically after therapy in both patients: by 100% in patient A after 54 days and by 70.51% in patient B after 76 days (Figure 5B), which can be seen in the sequence networks (Figure 5C and D).

Discussion

There are many reports on the significant role of human genetics in determining the outcomes of HCV infection, antiviral therapy, and the genetic composition of intrahost HCV populations.⁴⁻⁷ However, little is known about any effects of HCV infection on the host genetics beyond any changes related to adaptive immune responses, although interacting genetic systems should affect each other mutually. It is assumed that nuclear DNA is genetically stable and does not change genetically in HCV-infected cells, although mtDNA is known to vary genetically, owing to a high mutation rate. We show through NGS that the genetic diversity of 2 mtDNA regions, HVS1 and HVS2, is higher in sera of HCV-infected persons than in healthy controls.

Sampling Depth and Nucleotide Diversity

Application of NGS to sequencing intrahost genetic variants presents several new challenges, including the representational depth of sequence samples. In our experiments, the number of reads varied among groups of specimens. However, sampling differences were statistically significant only for HVS2. Such variation potentially may affect estimates of genetic diversity. To reduce the effect of sampling variation, we used nucleotide diversity, which was shown to be minimally dependent on the sample size.^{34,35} For instance, estimates of HCV nucleotide diversity were found to be almost identical between samples of dozens or thousands of reads.³⁴ This finding was confirmed in this study for the NGS samples of both HVS1 and HVS2 using the Monte-Carlo sampling method (Figure 1). The sampling depth was shown to have a limited effect on the measure of nucleotide diversity, with only 10%–25% of reads from full samples closely approximating all observations made in this study for acute and chronic groups of specimens.

Acute and Chronic Stages of HCV Infection

Currently, seroconversion and detection of HCV RNA in serial specimens are the only reliable markers of acute HCV infection. The avidity index of IgG anti-HCV³⁶ and patterns of IgG anti-HCV responses to several structural and nonstructural HCV proteins³⁷ recently were evaluated for discrimination of acute from chronic infection. Genetic diversity of the hypervariable region 1^{38,39} and nonstructural 5B protein⁴⁰ also were used to detect acute infections. However, none of the developed approaches detects stages of HCV infection with high accuracy. Unfortunately, the application of HCV genetic diversity to the detection of acute infection is not straightforward because of the complex dynamics of intrahost HCV populations, which are organized into several communities,³² each varying in density during infection.⁴¹ Although in general the degree of HCV genetic heterogeneity increases over time during infection, these genetic changes vary among patients, depending on many factors such as the number of founders and communities, genetic distances among communities, strength and specificity of immune responses, and host genetics, which usually are unknown or difficult to establish.

In contrast to the complex intrahost HCV population dynamics, genetic changes in the intrahost mtDNA population easily can be traced to a single known germline mtDNA sequence. mtDNA variants are organized in a single cluster, with genetic heterogeneity of the cluster being much higher in patients with chronic HCV infection. This property significantly facilitates application of the mtDNA genetic diversity to the discrimination of acute and chronic stages of HCV infection. Indeed, we have shown that a simple binary classifier based on the mtDNA nucleotide diversity threshold allows for accurate distinguishing of acute and chronic samples, indicating its utility for timing HCV infection and for the detection of recent transmission events, thus improving chances for timely public health interventions to disrupt HCV dissemination, especially in health care settings.

Analysis of the mtDNA nucleotide diversity in follow-up serum specimens from 2 patients who cleared HCV infection after combined treatment with pegIFN and ribavirin showed that the mtDNA diversity detectable in serum may decrease as fast as it increases after inception of infection. This finding supports the earlier observations of reduction of somatic mtDNA mutations in liver tissue of chronically HCV-infected patients with HCC after interferon treatment.^{23,42} Although reduction of mtDNA heterogeneity was attributed to interferon treatment,^{23,42} the increased level of mtDNA genetic diversity found in cases with HCV infection suggests a direct role of HCV in the presentation of mtDNA

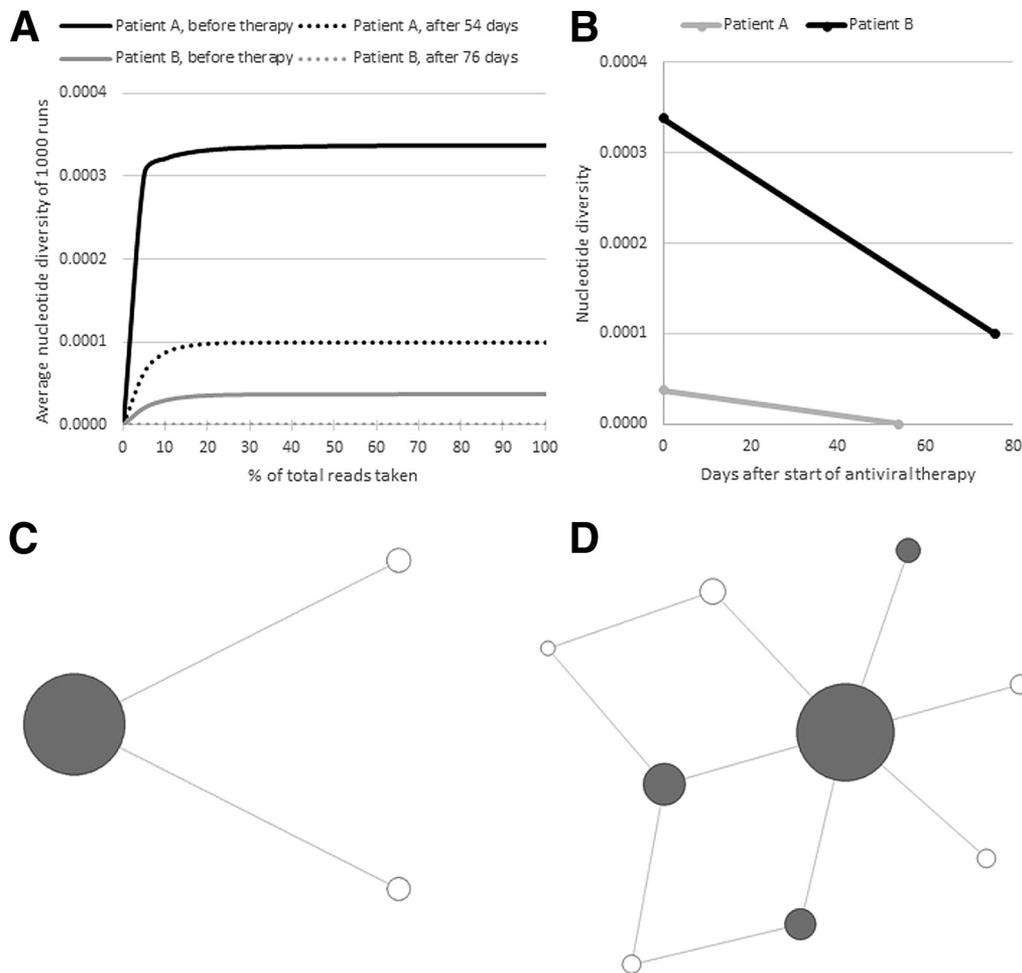


Figure 5. mtDNA diversity of 2 followed up HCV samples. (A) Monte–Carlo sampling and nucleotide diversity. (B) Nucleotide diversity on 2 patients who were followed up. The values of patient A are shown in black, whereas the values of patient B are shown in grey. (C and D) K-step networks. Each node is a different mtDNA HVS1 variant and 2 nodes are linked if they differ in a single nucleotide. The size of the node is proportional to the square root of the frequency of that particular variant. The *gray nodes* correspond to mtDNA variants that were found before and after therapy. *White nodes* correspond to mtDNA variants that were present only before therapy.

mutations, implying an association of a decrease in mtDNA diversity with the success of anti-HCV treatment. However, the number of mtDNA mutations detectable in serum and liver may differ considerably,^{23,42} indicating that comparison between observations made in different tissues should be interpreted with caution.

Study Limitations

Nucleotide diversity estimated for short genomic regions may not represent the genetic diversity of the entire mtDNA accurately. Hence, all observations presented here should be extrapolated with caution to the whole genome of mitochondria. However, the selection of specific primers for short-amplicon sequencing has an advantage of limiting the contribution of nuclear mtDNA insertions to the measured nucleotide diversity. Although more extensive analysis of the mitochondrial genome may show greater complexity of genetic associations with HCV infection, the findings made for HVS1 and HVS2 are statistically significant and can be explored for the identification of stages of HCV infection.

All findings made in this study reflect the accumulation of mutant mtDNA species in serum and may not be completely applicable to samples of liver tissue. The origin of mtDNA and its genetic variants in serum is not well understood, beyond the fact that both particle-associated and free mtDNA are known to be present in plasma,⁴³ a phenomenon that has been exploited since the early efforts to detect cancer-related mitochondrial mutants in serum.⁴⁴ Further studies on the origin of mtDNA variants detected in serum during HCV infection are needed, knowledge that can be used to ascertain if the increase in nucleotide diversity observed here is caused by either the variation in frequency among different pre-existing mitochondrial populations, mtDNA of which becomes represented most abundantly in the blood during HCV infection; and/or HCV-induced reduction in mitochondria-mediated apoptosis, resulting in accumulation of impaired mitochondria and their mtDNA variants in infected hepatocytes, which eventually can be found in blood.

Conclusions

HCV infection strongly affects mtDNA genetic diversity, with the effect being most pronounced at later stages of HCV infection. A rapid decrease in the mtDNA genetic diversity observed after the therapy-induced HCV clearance suggests that the effect is reversible, emphasizing dynamic genetic relationships between HCV and mitochondria. These findings suggest applications of mtDNA genetic diversity as a novel noninvasive biomarker to stage HCV infection and to monitor therapy effectiveness.

References

- Edlin BR, Eckhardt BJ, Shu MA, et al. Toward a more accurate estimate of the prevalence of hepatitis C in the United States. *Hepatology* 2015;62:1353–1363.
- Alter M. Epidemiology of hepatitis C virus infection. *World J Gastroenterol* 2007;13:2436–2441.
- Ly KN, Xing J, Klevens RM, et al. The increasing burden of mortality from viral hepatitis in the United States between 1999 and 2007. *Ann Intern Med* 2012;156:271–278.
- Bengsch B, Thimme R, Blum HE. Role of host genetic factors in the outcome of hepatitis C virus infection. *Viruses* 2009;1:104–125.
- Schmidt J, Thimme R, Neumann-Haefelin C. Host genetics in immune-mediated hepatitis C virus clearance. *Biomark Med* 2011;5:155–169.
- Matsuura K, Tanaka Y. Host genetic variants influencing the clinical course of hepatitis C virus infection. *J Med Virol* 2016;88:185–195.
- Wang H, El Maadidi S, Fischer J, et al. A frequent hypofunctional IRAK2 variant is associated with reduced spontaneous hepatitis C virus clearance. *Hepatology* 2015;62:1375–1387.
- Levero M. Viral hepatitis and liver cancer: the case of hepatitis C. *Oncogene* 2006;25:3834–3847.
- Wallace DC. Mitochondrial DNA variation in human radiation and disease. *Cell* 2015;163:33–38.
- Bouchard MJ, Navas-Martin S. Hepatitis B and C virus hepatocarcinogenesis: lessons learned and future challenges. *Cancer Lett* 2011;305:123–143.
- Khan M, Syed GH, Kim SJ, et al. Mitochondrial dynamics and viral infections: a close nexus. *Biochim Biophys Acta* 2015;1853:2822–2833.
- Kim SJ, Syed GH, Khan M, et al. Hepatitis C virus triggers mitochondrial fission and attenuates apoptosis to promote viral persistence. *Proc Natl Acad Sci U S A* 2014;111:6413–6418.
- Kim SJ, Syed GH, Siddiqui A. Hepatitis C virus induces the mitochondrial translocation of Parkin and subsequent mitophagy. *PLoS Pathog* 2013;9:e1003285.
- Li XD, Sun L, Seth RB, et al. Hepatitis C virus protease NS3/4A cleaves mitochondrial antiviral signaling protein off the mitochondria to evade innate immunity. *Proc Natl Acad Sci U S A* 2005;102:17717–17722.
- Yen HH, Shih KL, Lin TT, et al. Decreased mitochondrial deoxyribonucleic acid and increased oxidative damage in chronic hepatitis C. *World J Gastroenterol* 2012;18:5084–5089.
- Wong LJ, Tan DJ, Bai RK, et al. Molecular alterations in mitochondrial DNA of hepatocellular carcinomas: is there a correlation with clinicopathological profile? *J Med Genet* 2004;41:e65.
- Nishikawa M, Nishiguchi S, Shiomi S, et al. Somatic mutation of mitochondrial DNA in cancerous and noncancerous liver tissue in individuals with hepatocellular carcinoma. *Cancer Res* 2001;61:1843–1845.
- Tamori A, Nishiguchi S, Nishikawa M, et al. Correlation between clinical characteristics and mitochondrial D-loop DNA mutations in hepatocellular carcinoma. *J Gastroenterol* 2004;39:1063–1068.
- Wheelhouse NM, Lai PB, Wigmore SJ, et al. Mitochondrial D-loop mutations and deletion profiles of cancerous and noncancerous liver tissue in hepatitis B virus-infected liver. *Br J Cancer* 2005;92:1268–1272.
- Nomoto S, Yamashita K, Koshikawa K, et al. Mitochondrial D-loop mutations as clonal markers in multicentric hepatocellular carcinoma and plasma. *Clin Cancer Res* 2002;8:481–487.
- Zhang R, Zhang F, Wang C, et al. Identification of sequence polymorphism in the D-loop region of mitochondrial DNA as a risk factor for hepatocellular carcinoma with distinct etiology. *J Exp Clin Cancer Res* 2010;29:130.
- Shawky R, Abdel-Gaffar T, Eladawy M, et al. Mitochondrial alterations in children with chronic liver disease. *Egypt J Med Hum Genet* 2010;1:143–151.
- Nishikawa M, Nishiguchi S, Kioka K, et al. Interferon reduces somatic mutation of mitochondrial DNA in liver tissues from chronic viral hepatitis patients. *J Viral Hepat* 2005;12:494–498.
- Vigilant L, Pennington R, Harpending H, et al. Mitochondrial DNA sequences in single hairs from a southern African population. *Proc Natl Acad Sci U S A* 1989;86:9350–9354.
- Parson W, Bandelt HJ. Extended guidelines for mtDNA typing of population data in forensic science. *Forensic Sci Int Genet* 2007;1:13–19.
- Moore J, Isenberg A. Mitochondrial DNA Analysis at the FBI Laboratory. DNA Analysis Unit II. Federal Bureau of Investigation. Washington, DC.
- Skums P, Dimitrova Z, Campo DS, et al. Efficient error correction for next-generation sequencing of viral amplicons. *BMC Bioinformatics* 2012;13(Suppl 10):S6.
- Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol* 2013;30:772–780.
- Nei M, Li W. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc Natl Acad Sci U S A* 1979;76:5269–5273.
- Mathworks. Matlab. Natick, MA: 2010.
- IBM SPSS Statistics for Windows. 21.0 ed. Armonk, NY: IBM Corp, 2012.
- Campo DS, Dimitrova Z, Yamasaki L, et al. Next-generation sequencing reveals large connected networks of intra-host HCV variants. *BMC Genomics* 2014;15(Suppl 5):S4.

33. Bastian M, Heymann S, Jacomy M. Gephi: an open source software for exploring and manipulating networks. International AAAI Conference on Weblogs and Social Media. San Jose, CA; May 17–20, 2009.
 34. Dimitrova Z, Campo DS, Ramachandran S, et al. Evaluation of viral heterogeneity using next-generation sequencing, end-point limiting-dilution and mass spectrometry. *In Silico Biol* 2011;11:183–192.
 35. Fumagalli M. Assessing the effect of sequencing depth and sample size in population genetics inferences. *PLoS One* 2013;8:e79667.
 36. Klimashevskaya S, Obriadina A, Ulanova T, et al. Distinguishing acute from chronic and resolved hepatitis C virus (HCV) infections by measurement of anti-HCV immunoglobulin G avidity index. *J Clin Microbiol* 2007;45:3400–3403.
 37. Araujo A, Astrakhantseva I, Fields H, et al. Distinguishing acute from chronic hepatitis C virus (HCV) infection based on antibody reactivities to specific HCV structural and nonstructural proteins. *J Clin Microbiol* 2011;49:54–57.
 38. Astrakhantseva IV, Campo DS, Araujo A, et al. Differences in variability of hypervariable region 1 of hepatitis C virus (HCV) between acute and chronic stages of HCV infection. *In Silico Biol* 2011;11:163–173.
 39. Campo D, Xia G, Dimitrova Z, et al. Accurate genetic detection of hepatitis C virus transmissions in outbreak settings. *J Infect Dis* 2016;213:957–965.
 40. Montoya V, Olmstead AD, Janjua NZ, et al. Differentiation of acute from chronic hepatitis C virus infection by non-structural 5B deep sequencing: a population-level tool for incidence estimation. *Hepatology* 2015;61:1842–1850.
 41. Ramachandran S, Campo DS, Dimitrova ZE, et al. Temporal variations in the hepatitis C virus intrahost population during chronic infection. *J Virol* 2011;85:6369–6380.
 42. Hayashi T, Tamori A, Nishikawa M, et al. Differences in molecular alterations of hepatocellular carcinoma between patients with a sustained virological response and those with hepatitis C virus infection. *Liver Int* 2009;29:126–132.
 43. Chiu RW, Chan LY, Lam NY, et al. Quantitative analysis of circulating mitochondrial DNA in plasma. *Clin Chem* 2003;49:719–726.
 44. Fliss MS, Usadel H, Caballero OL, et al. Facile detection of mitochondrial DNA mutations in tumors and bodily fluids. *Science* 2000;287:2017–2019.
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- Correspondence**
Address correspondence to: David S. Campo, PhD, Centers for Disease Control and Prevention, 1600 Clifton Road, MS A33, Atlanta, Georgia 30329. e-mail: fyv6@cdc.gov; fax: (404) 639-1563.
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