Published in final edited form as:

J Acquir Immune Defic Syndr. 2017 March 01; 74(3): 353–358. doi:10.1097/QAI.0000000000001256.

Characterization of HCV NS3 Protease Variants in HCV/HIV Coinfected Patients by Ultra-deep Sequence Analysis: Relationship with Hepatic Fibrosis

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Abstract

Background—Treatment of HCV/HIV coinfection is now largely based upon utilization of direct acting agents. Pre-treatment viral resistant-associated variants (RAVs) and host liver condition may affect the sustained virological response. In this study, we explored relative prevalence of protease resistance-associated mutations, the evolution of those RAVs following 12 weeks of pegylated interferon alfa exposure, and the role hepatic fibrosis might have on RAV display.

Methods—Thirty nonresponder HCV/HIV coinfecting subjects were evaluated pre- and post- 12 weeks of pegIFN treatment. Ultra-deep sequence analysis of NS3 RAVs was performed. Hepatic fibrosis was determined by sensitive computer-assisted histomorphometry determination.

Results—At baseline, protease inhibitor RAVs were present in 73.3% of patients and expanded to 83.3 % of patients after 12 weeks of pegIFN exposure. Q80K showed the highest prevalence before and after treatment at 46.7% and 56.7 %, respectively. The presence of Q80K is positively correlated to percent collagen content of the liver tissue.

Conclusions—Key RAVs for HCV protease inhibitors are present in a major portion of the HCV/HIV coinfecting population prior to therapy. Some variants get selected after exposure.

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Conflicts of Interest: K.E.S.: Research Support (to institution): AbbVie, BMS, Gilead, Merck, MedImmune, Innovio., The authors E.A.A.; S.D.R.; X.Z.; J.C.; M.M. and Z.D.G. have no conflicts of interest.

Part of this research was presented at conference venues as oral or poster presentations

“Characterization of HCV NS3 protease variants from patients enrolled in sustained long-term antiviral maintenance therapy in HCV/HIV-coinfecting patients (SLAM-C)”. The HIV and Liver Disease 2014 Conference proceedings (Sept 18–20, 2014, Jackson Lake Lodge, WY)

“HCV NS3 Variants in HIV/HCV Coinfecting Patients Before-After PegIFN/Ribavirin”, abstract number: 2100724. The Conference on Retroviruses and Opportunistic Infections, CROI 2015 proceedings, (February 23– 26, 2015, Washington State Convention Center, in Seattle, Washington)

Correlation of Q80K with collagen content of the liver suggests that compartmentalization within the liver may contribute to persistence of mutations less fit than wildtype.

Keywords

HCV/HIV coinfection; NS3 resistance associated variants; PegIFN/ribavirin maintenance therapy; Liver morphometric analysis

INTRODUCTION

Approximately 25% of persons infected with HIV are coinfecting with hepatitis C virus in the United States [1]. HCV/HIV coinfection is associated with increased risk of liver fibrosis, cirrhosis and more rapid progression to end-stage liver disease compared to those with either HCV or HIV mono-infection [2–7].

Treatment of HCV/HIV coinfection is now largely based upon utilization of direct acting agents, whose action targets the non-structural protein (NS) 3/4, a serine protease, the NS5A domain, a pleiotropic functions phosphoprotein and/or in the NS5B, an RNA-dependent RNA polymerase. Variation in key binding sites in each of these regions leads to drug resistance [8]. In this study, we explored a) the distribution and relative prevalence of resistance associated variants (RAVs) associated with protease inhibitor resistance discovered using ultra-deep sequencing methods, b) the evolution of these RAVs following long-term exposure to pegylated interferon alfa, and c) the effect hepatic fibrosis, determined by sensitive computer-assisted histomorphometry has on RAVs display.

SUBJECTS AND METHODS

Thirty HCV/HIV coinfecting subjects were evaluated. All subjects were previously enrolled/consented in ACTG 5178 (SLAM-C), a study designed to evaluate the effects of prolonged PegIFN on hepatic fibrosis ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study?term=ACTG%205178&rank=1) Identifier: NCT00078403). A detailed description of the study design and subject enrollment criteria is described and published elsewhere [9]. The thirty subjects included in this part of the study had failed to achieve an early viral response (<2 log drop in viral load) after 12 weeks of PegIFN/ribavirin treatment.

Ethical Approvals and Consent for Participation

This clinical study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice. The patients' consents and protocol of the study were approved by the University of Cincinnati Institutional Review Board. An independent Safety Monitoring Committee (SMC) provided oversight during the conduct of the trial.

Description of HCV amplicon-seq

The amplicon-seq targeting HCV genome variable region was performed by the Genomics, Epigenomics and Sequencing Core (GESCC) in the University of Cincinnati. Reverse transcription PCR HCV NS3 specific primers were used to amplify a partial sequence of the HCV NS3. The SuperScript III one-step RT-PCR Platinum Taq HiFi (Invitrogen, Carlsbad, CA) was used to amplify all samples according to manufacturer's recommendations.

Reverse transcription PCR-amplified product (600 bp) was agarose gel-purified with the final sample volume adjusted to 100 µl. Nanodrop results showed the concentrations of the purified DNA ranged from a few ng/µl to 30 ng/µl, which is suitable for the DNA library preparation.

To shear the DNA to proper size range for library preparation, 50 µl of DNA was aliquoted to a microTUBE (Covaris, Woburn, MA), and sonicated with Covaris S2 focused-ultrasonicator under the protocol targeting 150 bp peak. The sheared DNA was QC analyzed by Bioanalyzer DNA 1000 chip (Agilent, Santa Clara, CA).

Automatic DNA-seq library preparation

PrepX DNA Library kit (WaferGen, Fremont, CA) and Apollo 324 NGS automatic library prep system (WaferGen) was used for the library preparation. ChIP-seq script was selected to capture all the sheared fragments that were over ~80 bp. First, the sheared DNA fragments with overhangs were converted into blunt ends in the end repair procedure, and adenylated at 3' ends for TA ligation to Illumina (San Diego, CA) sequencing adaptors. Next, the ligated library was enriched by 6 cycles' of PCR using index-specific primers, followed by AMPure XP beads (Beckman Coulter, Brea CA) purification. A bioanalyzer DNA high sensitivity chip was then used to check the quality and yield of the purified library. To accurately quantify the library concentration for the clustering, the library was 1:10,000 diluted in dilution buffer (10 mM Tris-HCl, pH 8.0 with 0.05% Tween 20), and qPCR analyzed by Kapa Library Quantification kit (Kapabiosystem, Woburn, MA) using ABI's 9700HT real-time PCR system (Lifetech, Grand Island, NY).

Cluster Generation and HiSeq Sequencing

Individually indexed libraries were proportionally pooled for clustering at a final concentration of 8 pM. The pooled libraries were clustered onto a flow cell using Illumina's TruSeq SR Cluster kit v3 in cBot system (Illumina), followed by single read sequencing at 1×50 bp using Illumina's TruSeq SBS kit on HiSeq system (Illumina). About three million reads were generated from each sample for data analysis. RAVs were detected down to a proportional frequency of <1%.

Liver morphometric analysis

HCV RNA from Week 12 was used to determine response status. During this period a liver biopsy was performed if the subject was classified as a treatment non-responder (NR). Liver biopsies from Step 2 entry were transferred to a central site where they were de-identified and coded. Biopsies were then shipped to the central pathologist (ZDG) where they were evaluated in a blinded manner by two independent reviewers, using the Metavir and Ishak scoring criteria. In addition to standard microscopy, we employed computer generated assessment of liver tissue [10]. Sections of liver tissue were stained with picrosirius red for assessment of collagen. A digitized image of each section, including all tissue in the section, was acquired using an Aperio XT slide scanner at 20× magnification. The Aperio positive pixel count algorithm was used to identify and classify each red pixel (hue 0.95 ± 0.1) in the picrosirius red stained section as collagen. Each red pixel was compared to the total percentage of pixels in the sample (Fig 1). Accuracy of classification was confirmed by

visual inspection of the appearance under the microscope relative to the computer generated reading. Samples with poor stain uptake were re-stained or eliminated from the result. Results were expressed as a fraction of tissue positive for collagen.

Bioinformatics and statistical analysis

The filtered, trimmed sequencing reads were aligned to the H77C reference sequence, HCV genotype 1, from the Los Alamos HCV database (<http://www.hcv.lanl.gov>). The codons associated with resistance associated variants were identified and the mutation frequency calculated. The mean coverage was 372,324 and the threshold for variant frequency was 0.3% of the total population sample. Sequencing reads were processed according to the following standards: an exact match to primer sequences; no ambiguous bases; sequence base calls with Illumina low error rates quality scores.

Age, race, gender, HCV genotype, HIV RNA, HCV RNA, ALT, aspartate aminotransferase, platelet count, and bilirubin measures were collected. Frequency distribution of data was measured. Summary of mean $\pm$ SD is presented. Spearman r was used to measure correlation between parameters. For all comparisons, 2-sided P values, 0.05 were considered statistically significant.

RESULTS

Demographics and clinical characteristics

The study cohort was predominantly male (83.3%), with a median age of 47 years. 40% were white and 56.7% black. Baseline mean HCV log₁₀ viral load was 6.6 IU/mL and HIV viral load was 3365.1copies/mL. 56.7% had undetectable HIV due to effective cART. IL28B distribution for C/C, C/T, and T/T were 35, 45, and 20% respectively. Sixteen patients were genotype 1a, 2 were 1a1b and 12 were type 1 no subtype (Table 1).

Frequency and evolution of HCV NS3 variants

At baseline, protease coding domain RAVs was present in 22/30 patients (73.3%) and extended to 25/30 patients (83.3 %) after 12 weeks. At baseline, V36M/L, T54A/S, V55I and D168G, were each detected in 3.3% of patients. V55A was detected in 6.7% of patients. I170V was present in 36.7% of patients. Q80K showed the highest prevalence at 46.7% (Fig 2). Evolution of these protease RAVs after 12 weeks of treatment showed that V36M and V55I expanded to 6.7 % and 10% of the population respectively, while Q80K was selected in 56.7%. The proportion of subjects having detectable V55A and I170V declined to 3.3% and 23.3% of patients respectively. Over all amino acid positions studied, no statistical difference in relative abundance of RAVs within a patient before-after 12 weeks of treatment was seen. There was no relationship to IL28B, viral loads, age, gender or race.

Liver morphometric analysis

Liver biopsy was obtained from enrolled non-responder patients after 12 weeks of treatment. Computer-assisted assessment of liver tissue was determined. The mean $\pm$ SD of the liver biopsy length, area, and percent collagen content in the studied patients were 21.913 $\pm$ 9.74mm, 13.648 $\pm$ 8.61 mm³ and 10.913 $\pm$ 7.05 respectively. The percent collagen

content of the liver after 12 weeks of treatment showed a statistically significant correlation to the frequency of Q80K ($r=0.6160$, $p=0.0145$) in treatment non-responding HCV/HIV patients (Fig 3).

DISCUSSION

Naturally occurring resistance associated variances are important factors in several all-oral therapeutic regimens in HCV and HCV/HIV coinfecting populations. In this study, we determined the baseline representation of HCV protease inhibitor RAVs in thirty HCV/HIV coinfecting patients using a highly sensitive deep sequencing technique. We further explored changes in these populations over time when non-specific therapy (pegylated interferon) is applied.

The HCV virus explores virtually all possible combinations of sequence on a daily basis [11]. While most result in non-replicative virus, minor variations may result in relative resistance to antiviral therapy with virtual preservation of viral fitness. These resistant mutations may emerge during exposure to and pressure from extrinsic antiviral agents leading to overt viral resistance [12, 13]. At baseline the vast majority of patients harbor HCV protease RAVs. The proportion of RAVs that exist at baseline may represent an important predictor of emergence. Lok et al noted that R155K mutations present at baseline were associated with development of viral relapse after a course of treatment with a combination of daclatasvir (NS5A inhibitor) and asunaprevir (PI) [14]. This emergence was prevented if pegylated interferon and ribavirin were part of a quad therapy regimen. These observations suggest that the importance of minor variants may increase, as pegylated interferon is no longer part of the treatment regimen. The high level RAV, R155, was not detected in the HCV/HIV tested population either before or after 12 weeks of PegIFN. Only a few studies have looked at natural variability of NS3 in the HCV/HIV coinfecting patients using highly sensitive deep sequencing techniques [15–17,19]. The results of these studies showed a higher natural prevalence of protease resistance variance in the coinfecting population as compared to HCV monoinfection. In this study we further considered the evolution of the protease variants after 12 weeks of non DAA treatment. After 12 weeks of treatment, V55I and V36M expanded to 9% and 12% of the population respectively. The Q80K variant was present at baseline in 46.7% of the population but was detected in about 56.7% of HCV/HIV coinfecting patients after PegIFN exposure. The continued viral replication after 12 weeks of PegIFN may have put the patients at risk of developing or selecting for drug resistance viruses with high replicative capacities [18]. This implies that pressure from non-targeted therapies could lead to selection. In HCV monoinfecting patients; wide variation in Q80K representation is reported. In a European study, Q80K was present among HCV genotype 1, 1a and 1b at rates of 7.5%, 19.8% and 0.5%, respectively [19]. The prevalence of the Q80K variant is higher in the United States scaling to 47% in genotype 1a [20]. Among the two identified clades of HCV subtype 1a, Q80K associates strongly with clade 1 [21] and is thought to have originated in the United States during the 1940s [22]. The Q80K polymorphism has been shown to substantially reduce the efficacy of simeprevir, an FDA-approved protease inhibitor indicated for use with peg-IFN/RBV [23]. Thus, baseline Q80K screening is recommended prior to simeprevir treatment [24]. This study reports a natural prevalence of Q80K in 14 of 30 (46.7%) HCV/HIV coinfecting patients. Using

similar deep sequencing techniques, other studies reported similar Q80K high frequency in 17 of 40 (42.5%) [15], and in 12 of 21 (57.1%) of HIV/HCV coinfecting patients respectively [16]. The high proportion of Q80K observed in U.S. patients with HCV/HIV coinfection suggests that choice of therapeutic agents should be tailored to this finding. A limitation of the current study is that controls with HCV or HIV mono-infection were not available in the particular cohort described in this study for comparison.

Liver biopsies were obtained from enrolled HCV/HIV patients who did not show an early response after 12 weeks of non-targeted therapy. We found a significant correlation between the percentage of hepatic collagen content and the expanded Q80K frequency after 12 weeks of treatment but not with the Q80K variant frequency at baseline. In a proposed model by Talal et al., it was suggested that there are high and low level sites of HCV replication within the liver. This suggests that the majority of the plasma HCV RNA may have originated from the higher level replication foci within the infected liver before treatment. Early viral response to treatment may be a result of inhibition of those major replication foci. The advance in hepatic fibrosis can affect those higher HCV replicating sites with consequently lower decline rates in HCV viral load and intrahepatic drug concentration. This might affect interferon induced clearance of the infected cells from the liver [25]. This could explain the positive correlation between liver collagen content and the evolution of HCV NS3 Q80K variant after treatment in our coinfecting population. The distortion of the liver compartment with more collagen deposition and fibrosis progression might have led to lower blood flow to protected areas in the liver and thus to suboptimal concentration of the drug, higher viral replication rate within the liver, and emergence of variants such as HCV NS3 Q80K. We think that the effect of compartmentalization induced by the presence of cirrhosis is independent of the initial treatment in this cohort. However, different anti-HCV agents might have a differential effect on selection due to variable penetrance into protected fibrotic compartments. This will have to be explored further in ongoing studies.

In summary, this study is one of few that explore natural resistance protease variance in HCV/HIV patients using next generation sequencing. Key RAVs for HCV protease inhibitors are present in a large portion of the HCV/HIV coinfecting population prior to therapy. The prevalence of V36M, V55I and Q80K appears to be increased following prolonged therapy with Pegylated Interferon and ribavirin suggesting that pressure from non-targeted therapies could lead to selection. Correlation of Q80K frequency with collagen content of the liver after 12 weeks of non-targeted therapy suggests the possibility that compartmentalization within the liver may contribute to persistence of mutations less fit than wildtype.

Acknowledgments

We would like to acknowledge the ACTG 5178 team and patients for their efforts and participation in the parent study which provided samples for the analysis herein.

Source of Funding: This research was supported by National Institute of Allergy and Infectious Diseases funding to the AIDS Clinical Trials Group including U01 AI068634, U01 AI38858, U01 AI068636, an NIDDK K24 DK070528 to K.E.S.; and support to the Center for Environmental Genetics, NIEHS P30-ES006096. Also supported in part by the General Clinical Research Center Units funded by the National Center for Research Resources. Study drug and other support were provided by Roche Pharmaceuticals.

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Figure 1.

Liver biopsy analyzed for collagen by computer assisted morphometry. Image on the left is the biopsy stained with picrosirius red. Collagen is red and non-collagen is yellow. Image on right is the same biopsy after morphometric analysis. Collagen is represented by red pixels, while non-collagen pixels are colored blue. Red pixels, which are the area of collagen, are 19.5% of the total.

Figure 2.

Frequency of natural HCV NS3 Protease RAVs in HCV/HIV subjects who failed to achieve an early viral response (<2 log drop in viral load), N=30. At baseline twenty two coinfecting patients had natural HCV NS3 variants at baseline and 6 of them carried more than one variant. RAVs: V36m/L, T54S/A, V55A, and V55I were each detected in 1/30 (3.3%) of patients. The I170V variant was present in 11/30(36.7%) of patients. The Q80K showed the highest prevalence in 14/30 (46.7 %) coinfecting patients.

Figure 3. Collagen liver content correlation to Q80K variant frequency after 12 weeks of treatment. Positive significant correlation between liver collagen content and frequency of Q80K variant ($r = 0.6160$, $p = 0.0145$) after 12 weeks of non-targeted treatment in HCV/HIV subjects who failed to achieve an early viral response.

Table 1**Demographic, Virological and Clinical Characteristics of Patients**

N	30
Age, years	
Median (Range)	47 (33–60)
Gender, n (%)	
Female	5 (16.7)
Male	25 (83.3)
Race, n (%)	
Black non-Hispanic	17 (56.7)
White non-Hispanic	12 (40)
Unknown	1(3.3)
HCV Sub-genotype, n (%)	
1a	16 (53.3)
mixed 1a/1b	2 (6.7)
1 no subtype	12 (40)
Baseline mean HCV viral load (log ₁₀ IU/ml)	6.6
Baseline HIV viral load (log ₁₀ copies/ml)	3.53
Baseline HIV undetectable, n (%)	17 (56.7)
Baseline mean platelet count, (n ×10 ³ /μl)	191.53
Baseline mean ALT, UL	48.7
Baseline mean AST, UL	42.13
Baseline mean total bilirubin, UL	1.3
IL28B frequency, n (%)	
C/C	7(35)
C/T	9(45)
T/T	4(20)