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Hepatitis C Virus NS3 Mutations in Hemophiliacs

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Abstract

Introduction—Hemophiliacs have high HCV exposure risk from blood products that did not undergo heat inactivation or disease-specific screening prior to 1987. Repeated exposure to infected factor concentrates predisposes hemophiliacs to higher likelihood of HCV from multiple sources. HIV coinfection could result in impaired clearance of less fit variants resulting in enrichment of quasispecies carrying resistance mutations.

Aim—We postulated that hemophiliacs demonstrate increased prevalence of baseline signature mutations in the HCV NS3/4 serine protease coding domain.

Methods—We examined the prevalence of putative HCV protease inhibitor mutations, mutations, sub-classified into dominant mutations if changes conferred resistance, and minor variants not associated with drug resistance, in patients with hemophilia A or B, infected with HCV or HCV/HIV, prior to HCV PI exposure.

Results—151 subjects were evaluated, including 22 hemophiliacs and 129 non-hemophilic controls. Of 58 mutations detected, 55 (95%) were resistance mutations and 3 (5%) were minor variants. Dominant mutations were detected in 10 (45.5%) hemophiliacs and in 43 (33.3%) controls (OR 1.67, 95% CI 0.67–4.16). There was no statistical difference in proportion of dominant mutations (p=0.27) or minor variants (p=0.47) between groups, despite adjustment for HIV status (p=0.44).

Conclusion—No significant differences in dominant or minor resistance mutations between hemophiliacs and non-hemophiliacs were observed. HIV presence or prior HAART exposure did not affect baseline distribution. We conclude that hemophiliacs are not at higher risk for pre-existing HCV PI mutations, and prospective studies of response to PI-based regimens with HCV activity are indicated.

Keywords

HCV; NS3; mutation; inherited bleeding disorders; protease

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INTRODUCTION

There are nearly 150 million individuals chronically infected with hepatitis C virus (HCV) worldwide.[1] A minority of those infected are able to eradicate infection without therapy,[2] while the remainder become chronically infected and are at increased risk of developing cirrhosis and hepatocellular carcinoma.[3–5]

Direct acting agents (DAA) are novel small molecules targeting specific viral proteins of the HCV life cycle, where they function as inhibitors to non-structural NS3/NS4A protease, NS5B polymerase, and NS5A phosphoprotein. Though drugs representing all classes are in development, only three NS3 protease inhibitors (PI), simeprevir, telaprevir and boceprevir, are currently approved by the FDA for HCV treatment. Use of these agents as monotherapy leads to rapid emergence of resistant virus populations; thus treatment regimens remain reliant on combination with a pegylated interferon alfa and ribavirin backbone. Primary NS3 protein mutations that have been identified as important in resistance development to NS3 PIs are: V36, T54, Q80, R155, A156, D168 and I/V170.

Hemophiliacs, who received clotting factor concentrates before 1987, were at risk for acquiring HCV infection from recurrent exposure to plasma pool concentrates prepared from blood donors that were not stringently screened.[6] It has been demonstrated that repeated exposure to infected blood products predisposes hemophiliacs to HCV genetic recombination.[7] Furthermore, the quasispecies complexity of hemophilic patients with HCV appears to be an independent predictor of treatment response.[8] However, it is unknown if this population possesses more frequent NS3 mutations that would limit PI use. HCV infection risk in hemophiliacs was compounded by HIV transmission in blood products during the 1980s. Data are conflicting on the presence of natural NS3 PI resistance mutations in coinfected individuals treated with Highly Active Anti-Retroviral Therapy (HAART) PIs. [9,10] With the need for more effective HCV anti-viral treatment of coinfected hemophiliacs, further assessment is essential to evaluate this potential for mutation development.

Our study aim was to assess differences in the presence of NS3 protease resistance mutations between hemophiliacs and non-hemophiliacs infected with HCV or HCV/HIV. We hypothesized that mutation rates are higher in hemophilic HCV/HIV coinfected individuals compared to non-hemophiliacs with monoinfection. We also aimed to compare NS3 mutation rates in coinfected patients who were exposed to HAART PIs with those who had no prior HAART PI exposure.

MATERIALS AND METHODS

Study Subjects

HCV monoinfected and HCV/HIV coinfected patients were identified sequentially in both hemophilic and non-hemophilic populations. Their serum samples were selected from the serum repository of the Hepatitis Research Group at the University of Cincinnati Digestive Diseases Division. Samples were collected from 03/1998 to 09/2010. Patients were excluded if they had incomplete demographic data, undetectable HCV, prior solid organ

transplantation, or exposure to any investigational HCV NS3 PIs. Demographic data included patient age, gender, and race. Clinical data included hemophilic status, HCV genotype and subtypes, HIV status, and current medication. In HCV/HIV coinfected patients, previous exposures or concurrent use of other classes of PIs (as part of HAART) were documented. These included lopinavir, ritonavir, nelfinavir, atazanavir, darunavir and indinavir. The study protocol was approved by the University of Cincinnati IRB and written informed consent was obtained from each study participant.

Direct Sequencing

Viral RNA was extracted from serum using the QIAamp Viral RNA Mini Kit (Qiagen). Primers were designed to flank the HCV NS3 region of interest for each genotype in the cohort (Table 1). A PCR reaction was run for each sample using the QIAGEN One-step RT-PCR kit (Qiagen). Appropriate PCR product size was confirmed by gel electrophoresis. A QIAquick PCR Purification kit (Qiagen) was used to purify PCR products, which were submitted to Massachusetts General Hospital DNA Sequencing Core for sequencing. Nucleotide sequences were analyzed for key NS3 mutations using CodonCode Aligner v3.7.1 (CodonCode Corporation, 2010) and Clustal X v2.0.12.[11]

Clonal Sequencing

At least 20 viral clones per sample were derived from six HCV monoinfected and six HCV/HIV coinfected PI-experienced patients using the pGEM-T Easy Cloning Protocol (Promega). DNA was isolated from clones using the QIAprep Spin Miniprep Kit (Qiagen) and submitted for sequencing. Sequences were analyzed for selected mutations as previously described.

Statistical analysis

Data were analyzed using Statistix 9.0 (Analytical Software, Tallahassee, FL). Frequency Distribution was used to compute frequency and relative frequency (percentage of total) of demographic and clinical data. Shapiro-Wilk Normality Test was used to determine data distribution. Kruskal-Wallis one-way ANOVA was used to assess statistical significance of dominant mutations between different genotypes amongst the two groups and to assess non-amplifiable rates at mutation site V36 between the groups. Two-sample T test was used to assess age difference between groups and Q80 mutations between HCV genotypes 1a and 1b. Two by Two contingency tables were constructed to determine the Odds Ratio of dominant mutations of hemophiliacs and controls, the different HCV subtypes, and the two coinfected groups. Logistic regression was used to assess independent predictors in dominant mutations. Wilcoxon Rank Sum Test assessed the association between dominant mutation and HIV protease exposure. Paired T test was used to compare the difference of mutations detected between clonal and direct sequencing. A statistically significant finding was defined by an alpha = 0.05 using a two-tailed hypothesis.

RESULTS

Demographics

151 serum samples were sequenced using the population sequencing method. These consisted of 22 hemophilia patients and 129 non-hemophiliacs (control group). Of the hemophiliacs, 13 (59%) had HCV monoinfection and 9 (41%) had HCV/HIV coinfection. Mean hemophilic age was 36 years (range 18–58) and there were 22 (100%) males. Twenty (91%) were white and 2 (9%) were black. There were 4 (18%) hemophiliacs with HIV coinfection who had exposures to HAART PIs. Of the controls, 71 (55%) were HCV monoinfected and 58 (45%) were HCV/HIV coinfected. Mean age in the controls was 46 years (range 19–63) (p=0.098). There were 94 (73%) males and 35 (27%) females. Seventy five (59%) patients were white and 52 (41%) were black. There were 27 (21%) controls with HIV coinfections who had exposures to HIV PIs as part of their antiretroviral regimen. 65 patients were infected with HCV genotype 1a. Distributions of HCV genotypes for both groups are listed in Table 2.

Dominant Mutations Analysis

There were 58 targeted mutations in 55 patients detected by population sequencing, of which 55 (95%) were dominant PI resistance mutations and 3 (5%) were minor variants. Dominant mutations were detected in 10 (45.5%) hemophiliacs and 43 (33.3%) in the controls (OR 1.67, 95% CI 0.67–4.2). There were 4 (40%) and 6 (60%) patients with dominant mutations in HCV monoinfection vs co-infection of hemophiliacs, while there were 23 (53%) and 20 (47%) patients with dominant mutations in HCV monoinfection vs. co-infection controls. (Figure 1). No minor variants were detected in the hemophiliacs while 3 (2.3%) minor variants were seen in the controls. One patient from each group had double resistance mutations (V36+Q80 in a monoinfected hemophiliac and T54+Q80 in a coinfected control). There was no statistical significance in the number of dominant mutations (p=0.272) or minor variants (p=0.473) between the two groups, despite statistical adjustment for HIV status (p=0.442). Of the independent predictors between the groups, (age, gender, race, HIV status, hemophilia status, prior HIV PI exposure, and HCV genotype), HCV genotype 1 was the only statistically significant predictor for a higher mutation rate (p=0.0025). In the coinfected population, 6 hemophiliacs had dominant mutations compared to 20 among non-hemophilic coinfected controls (OR 3.8, CL 0.86–16.8, p-0.079).

Dominant mutations occurred in 2 of 4 (50%) HCV genotype 1a hemophiliacs compared to 25 of 61 (41%) HCV genotype 1a controls (p=0.728). When assessing HIV status with regard to HCV genotype 1a and dominant mutations, there was 1 hemophilic coinfected patient who had dominant mutations compared to 10 coinfected controls (40%). In the monoinfected population, there was 1 hemophiliac (33%) who had dominant mutations compared to 15 (42%) in the controls. When controlled for either HIV or hemophilia status, the difference between the dominant mutations remained insignificant.

There were 35 patients with HCV genotype subtype 1b. Dominant mutations occurred in 2 of 8 (25%) HCV 1b hemophiliacs compared to 2 of 27 with genotype 1b controls (p=0.19)).

When comparing the proportions of dominant mutations between HCV 1a (27 patients) and HCV 1b (4 patients), the odds ratio was 5.51; CI 1.73 – 17.4; p=0.002) irrespective of hemophilia (p=0.27) or HIV status (p=0.16).

Of the 151 samples, there was 1 patient with R155K mutation (0.67%), 2 with V54S (3.2%) and 5 with V36L mutations (3.22%). The most common dominant mutation occurred at Q80 for both groups (31%). There were 7 (32%) Q80 mutations detected in the hemophiliacs compared to 40 (31%) in the controls (p=0.94). There were 25 HCV genotype 1a patients with Q80 mutations (16.6%) compared to 4 patients with HCV genotype 1b (2.6%) (p=0.001). Amongst the hemophiliacs, 4 patients (44%) had coinfection and 3 (23%) had monoinfection (OR 2.66, CI 0.42 – 16.8, p=0.30). Amongst the controls, 19 patients (33%) had coinfection and 21 (30%) had monoinfection (OR 1.16, CI 0.55–2.45, p-0.67

Impact of HIV protease inhibitor exposure

There were 31 patients who were exposed to HIV PIs, 117 patients had no prior PI exposure and 3 had unknown PI exposure. Dominant mutations occurred in 11 patients (35%) with PI exposure and 44 (38%) without PI exposure (p= n.s.). There were 4 (18%) hemophiliacs with prior PI exposure compared to 27 controls (21%)(p=n.s.). There was no significant association between dominant mutations and PI exposure between the two groups. (P=0.571)

Forty-one samples were non-amplifiable by direct sequencing at the V36 region and 1 sample in the Q41 region (Table 3). The majority of these were in the controls. There was a statistically significant higher non-amplifiable rate for V36 in the controls compared to the hemophiliacs (p=0.0097).

Direct vs. clonal sequencing methods

Twelve control patients were randomly selected for comparison of mutation prevalence between direct and clonal sequencing. There were 6 patients with HCV monoinfection and 6 with HCV/HIV coinfection. In the monoinfected group, 5 additional mutations (4 dominant mutations – V36E; Q80K; A156V/T and 1 minor variant – F43L) were detected by clonal sequencing compared to direct sequencing (p=0.05). In the coinfected group, 1 additional minor variant (Q41L) was detected by clonal sequencing. (p=0.34).

DISCUSSION

Standard-of-care for HCV infection in the past decade has been peginterferon-a (pegIFN) and ribavirin (RBV) which cures 40–50% of patients with chronic HCV genotype 1. With development of direct acting antivirals, sustained viral response (SVR) were reported to be as high as 70% and approaching 90–100% with some of the newer DAA agents in clinical trials. [12–19] However, currently there is no treatment response data reported among patients with hemophilia due to their exclusion from pivotal trials for currently approved PIs.

HCV exists in infected hosts as a quasispecies which are a result of mutations accumulated from rapid viral replication, and the lack of proof-reading with a low fidelity viral RNA-dependent RNA polymerase. These viral mutations give rise to amino-acid substitutions

within the targeted protein and produce conformational changes that can interfere with drug-target interactions. In this study, we analyzed baseline naturally occurring resistance mutations to NS3 PIs in hemophiliac and non-hemophilic populations.

We had a comparable rate of dominant mutations for R155K (0.67%), V54S (1.3%) and V36L (3.3%) to Kuntzen, et al, and a comparable Q80 mutations prevalence to an Italian study.[20,21] A Spanish study reported an overall prevalence of 67% NS3/4A mutations in HCV monoinfection and HCV/HIV populations, which is higher than what we observed.[22] There is no literature to date documenting the prevalence of naturally occurring dominant resistance mutations in hemophiliacs. Our study demonstrated a seemingly higher prevalence (OR 1.67) of signature resistance mutations in hemophiliacs compared to controls; however, it failed to reach statistical significance. We had hypothesized that intrinsic downregulation of the immune system (decreased IL8 and IL12) in the hemophilic population infected with HCV and repeated exposure to blood products with various HCV sub-species would result in increased accumulation and prevalence of naturally occurring mutations. [7,23] However, it has been shown that PI-resistant HCV mutants have reduced fitness as a consequence of impaired production of infectious virions, and the continuous process of selection for variants with the greatest fitness may extinguish the presence of minor species. [24] We did note a trend towards an increase (3.8 fold) in dominant mutation numbers in coinfected hemophiliacs compared to coinfected controls, suggesting decreased immune clearance in hemophiliacs with HIV infection. This may result in a reduced response rate to HCV therapy in this population for certain PIs, and could mandate greater stringency in the selection of eligible patients for HCV treatment.

With the introduction of interferon-free regimens, pre-existing mutations are becoming more closely scrutinized. R155 substitutions have been associated with resistance to most PIs. Linear PI resistance has been associated with substitution at V36, T54, V55 and V170. Substitutions at V36 in combination with R155 or A156 have been observed and increase the fold resistance synergistically. [25–27]

Our study identified Q80 mutations as the most common dominant mutation for both groups, accounting for an overall 31% in our population. A recent phase 2 study of on the use of simeprevir (second generation NS3 protease inhibitor), ribavirin and pegylated interferon showed a baseline prevalence of NS3 Q80K polymorphism of 10.4% and notably higher in HCV genotype 1a (22%) compared to genotype 1b (1.0%).[28] Although the overall prevalence is higher in our population, the breakdown prevalence according to the HCV genotype were similar, with a higher prevalence of Q80 mutations in HCV genotype 1a of 17% compared to genotype 1b of 2.6%. While isolated mutations/amino acid substitutions at residue Q80 conferred minimal to low levels of resistance to PIs, when combined with other variants such as R155 and D168, the resistance-fold increases dramatically, suggesting a compensatory role. [29,30] There was no significant difference in the number of Q80 mutations between hemophiliacs and controls, nor a difference between monoinfection and coinfection populations. However, it is important to characterize natural prevalence, as any enhancement in resistance with linkage of these variants to Q80K may have tremendous impact on treatment efficacy for next generation PIs. In addition, in the same Phase 2 study of simeprevir,, it was noted that patients without a Q80K polymorphism at baseline

experienced higher SVR 24 rate (70.6% to 85.5%) compared with patients with the Q80K polymorphism (55% to 66.7%).[28] This highlights the importance of baseline Q80K mutation screening prior to onset of treatment with simeprevir to identify those who are at risk of treatment failure and would otherwise benefit from an alternative treatment regimen.

HCV genotype was the only statistically significant predictor for the number of mutations identified by multivariate analysis. (p=0.0025). The majority of patients had HCV genotype 1. We noted a higher prevalence of dominant mutations in HCV genotype 1a compared to what has been reported in the literature by Kuntzen, et al. with a prevalence of 8.6%.[20] This is likely secondary to our more heterogeneous study population which also included, in addition to monoinfected patients, hemophiliac and HIV coinfected patients.[31] We also identified a four-fold increase in number of dominant mutations in the HCV genotype 1a subgroup as compared to genotype 1b (p=0.0028). This is consistent with the literature and pathogenesis whereby HCV genotype 1a possesses a low genetic barrier, i.e. the virus has a low threshold probability to mutate and escape from selective drug action, such that only a single nucleotide change is required to generate an amino acid change at the mutation site.[25,32]

Prevalence of natural resistance within the NS3 protease domain of HCV in HIV coinfected patients has been reported to be 7.9% to 16%.[10,33] Our study reported a higher prevalence of 39% in the coinfected patients. Overall, there was no significant difference in the risk of dominant mutations between coinfection and monoinfection in both hemophiliacs and controls. In our subgroup analysis of clonal vs. direct sequencing, we discovered additional mutations in both the monoinfected and coinfected groups. Mutation numbers were significantly higher in the monoinfected group compared to the coinfected, suggesting a robust immune system may play a role in virus replication dynamics and survival so rapid turn-over and elimination of unfit virus lead to mutations. Data are conflicting on HIV protease inhibitor exposure in coinfected patients and development of HCV NS3 natural resistance mutations secondary to selection pressure. Morsica, et al. demonstrated that HCV/HIV coinfected subjects on HIV PI regimen harbored HCV viral mutations at higher rates than GenBank published sequences for HCV monoinfected genotype 1. [9] In addition, they noted a difference in amino acid substitutions in coinfected patients who were exposed to PIs had more resistance mutations (R155K, A156T, V170E), compared to low or intermediate resistance in V170 domains.[9] In rebuttal,, Halfon, et al. did not find a difference in mutation rates observed in different positions in mono- vs. co-infected patients who were exposed to HIV PI (19% and 18% respectively).[9] However, it could be argued that they had too few patients (17 coinfected) to detect such a difference. Our findings are consistent with Halfon, et al. We had a larger number of coinfected patients and still failed to detect a difference in the number of dominant mutations between patients with prior HIV PI exposure and those who did not.

There are several limitations to our study. First, the number of hemophiliacs studied remains limited. Analysis of larger cohorts of hemophiliacs would be of interest. Secondly, we had a high proportion of non-amplifiable V36 regions. Both issues may have hindered our success in detecting a significant difference in the number of dominant mutations in hemophiliacs. Lastly, by using population/direct sequencing, we may have missed additional mutations.

The sensitivity of this technique is limited, requiring >20% variant species for detection compared to newer sequencing technology, which can detect up to <1–5% variant species. Next-generation or deep sequencing allows the entire sample to be sequenced and millions of small DNA fragments are sequenced in parallel with each sequence read, representing a single starting DNA molecule. [34] Detailed examination of intra-individual variations may provide additional information on the evaluation and importance of low-level drug resistance variants and allow identification of clinically relevant new variants.

While our study showed a positive trend towards higher baseline dominant mutations between hemophiliac and non-hemophiliac group (OR 1.67, 95% CI 0.67–4.2) and between the coinfected group and monoinfected patients (OR 3.8, CL 0.86–16.8, p- 0.079), the comparisons failed to reach statistical significance. Additional studies with larger patient sample size will be needed to elucidate whether this trend is real. In the era of direct acting anti-viral agents the presence of resistance mutations may represent a rate-limiting step in the success of HCV treatment in patients with mono- or co-infections. This may be particularly true when combinations of relatively low barrier agents of different classes are used together without interferon. Baseline resistance mutation screening may play a role in the near future as it may impact on the selection of the different HCV DAA drug combinations in HCV-infected patients with inherited bleeding disorders. Prospective studies of treatment response and mutational emergence following exposure to PI-based regimens with HCV activity are indicated.

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References

1. Organization WH. Hepatitis C. 2011.
2. Hoofnagle JH. Course and outcome of hepatitis C. Hepatology. 2002; 36:S21–9. [PubMed: 12407573]
3. Mehta SH, Cox A, Hoover DR, et al. Protection against persistence of hepatitis C. Lancet. 2002; 359:1478–83. [PubMed: 11988247]
4. Lagging LM, Westin J, Svensson E, et al. Progression of fibrosis in untreated patients with hepatitis C virus infection. Liver. 2002; 22:136–44. [PubMed: 12028408]
5. Saito I, Miyamura T, Ohbayashi A, et al. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. Proceedings of the National Academy of Sciences of the United States of America. 1990; 87:6547–9. [PubMed: 2168552]
6. Aach RD, Szmuness W, Mosley JW, et al. Serum alanine aminotransferase of donors in relation to the risk of non-A, non-B hepatitis in recipients: the transfusion-transmitted viruses study. N Engl J Med. 1981; 304:989–94. [PubMed: 6782484]
7. Moreno P, Alvarez M, Lopez L, et al. Evidence of recombination in Hepatitis C Virus populations infecting a hemophiliac patient. Virol J. 2009; 6:203. [PubMed: 19922637]
8. Shire NJ, Horn PS, Rouster SD, et al. HCV kinetics, quasispecies, and clearance in treated HCV-infected and HCV/HIV-1-coinfected patients with hemophilia. Hepatology. 2006; 44:1146–57. [PubMed: 17058240]
9. Halfon P, Bourliere M, Khiri H, et al. Mutation rate in hepatitis C virus NS3 protease is not influenced by HIV-1 protease inhibitor therapy. Aids. 2008; 22:1694–6. [PubMed: 18670239]

10. Morsica G, Bagaglio S, Uberti-Foppa C, Galli L, Lazzarin A. Detection of hepatitis C mutants with natural resistance to NS3/4A protease inhibitors in HIV/HCV-coinfected individuals treated with antiretroviral therapy. Journal of acquired immune deficiency syndromes. 2009; 51:106–8. [PubMed: 19390331]
11. Larkin MA, Blackshields G, Brown NP, et al. Clustal W and Clustal X version 2. 0. Bioinformatics. 2007; 23:2947–8. [PubMed: 17846036]
12. Sherman KE, Flamm SL, Afdhal NH, et al. Response-guided telaprevir combination treatment for hepatitis C virus infection. N Engl J Med. 2011; 365:1014–24. [PubMed: 21916639]
13. Zeuzem S, Andreone P, Pol S, et al. Telaprevir for retreatment of HCV infection. N Engl J Med. 2011; 364:2417–28. [PubMed: 21696308]
14. Kwo PY, Lawitz EJ, McCone J, et al. Efficacy of boceprevir, an NS3 protease inhibitor, in combination with peginterferon alfa-2b and ribavirin in treatment-naive patients with genotype 1 hepatitis C infection (SPRINT-1): an open-label, randomised, multicentre phase 2 trial. Lancet. 2010; 376:705–16. [PubMed: 20692693]
15. Manns MP, Markova AA, Serrano BC, Cornberg M. Phase III results of Boceprevir in treatment naive patients with chronic hepatitis C genotype 1. Liver Int. 2012; 32 (Suppl 1):27–31. [PubMed: 22212568]
16. Flamm SL, Lawitz E, Jacobson I, et al. Boceprevir With Peginterferon Alfa-2a- Ribavirin Is Effective for Previously Treated Chronic Hepatitis C Genotype 1 Infection. Clin Gastroenterol Hepatol. 2012
17. Sulkowski, MSGD.; Rodriguez-Torres, M.; Reddy, KR.; Hassanein, T.; Jacobson, I.; Lawitz, E.; Lok, AS.; Hinestrosa, F.; Thuluvath, PJ.; Schwartz, H.; Nelson, DR.; Everson, GT.; Eley, T.; Wind-Rotolo, M.; Huang, SP.; Gao, M.; McPhee, F.; Hernandez, D.; Sherman, D.; Hindes, R.; Symonds, W.; Pasquinelli, C.; Grasela, DM. AI444040 Study Group. Sustained virological response with daclatasvir plus sofosbuvir ± ribavirin (RBV) in chronic HCV genotype (GT) 1-infected patients who previously failed telaprevir (TVR) or boceprevir (BOC). 48th Annual Meeting of the European Association for the Study of the Liver; April 24–28, 2013; abstract 1417
18. Kowdley, KVLE.; Poordad, F.; Cohen, DE.; Nelson, D.; Zeuzem, S.; Everson, GT.; Kwo, P.; Foster, GR.; Sulkowski, M.; Xie, W.; Larsen, L.; Khatri, A.; Podsadecki, T.; Bernstein, B. Safety and efficacy of interferon-free regimens of ABT-450/r, ABT-267, ABT-333 +/− ribavirin in patients with chronic HCV GT1 infection: results from the AVIATOR study. 48th Annual Meeting of the European Association for the Study of the Liver; April 24–28, 2013; abstract 3
19. Lawitz, ERG.; Rodriguez-Torres, M. Suppression of Viral Load through 4 Weeks Post-Treatment Results of a Once-daily Regimen of Simeprevir + Sofosbuvir with or without Ribavirin in Hepatitis C Virus GT 1 Null Responders. 20th Conferenceon Retroviruses and Opportunistic Infections; March 3–6, 2013; 2013. abstract 155LB
20. Kuntzen T, Timm J, Berical A, et al. Naturally occurring dominant resistance mutations to hepatitis C virus protease and polymerase inhibitors in treatment-naive patients. Hepatology. 2008; 48:1769–78. [PubMed: 19026009]
21. Cento V, Mirabelli C, Salpini R, et al. HCV genotypes are differently prone to the development of resistance to linear and macrocyclic protease inhibitors. PloS one. 2012; 7:e39652. [PubMed: 22792183]
22. Franco S, Bellido R, Aparicio E, et al. Natural prevalence of HCV minority variants that are highly resistant to NS3/4A protease inhibitors. J Viral Hepat. 2011; 18:e578–82. [PubMed: 21914079]
23. Pimentel JP, Chaves DG, Araujo AR, et al. Anti-inflammatory/regulatory cytokine microenvironment mediated by IL-4 and IL-10 coordinates the immune response in hemophilia A patients infected chronically with Hepatitis C virus. Journal of medical virology. 2013; 85:1009–18. [PubMed: 23591975]
24. Shimakami T, Welsch C, Yamane D, et al. Protease inhibitor-resistant hepatitis C virus mutants with reduced fitness from impaired production of infectious virus. Gastroenterology. 2011; 140:667–75. [PubMed: 21056040]
25. Halfon P, Locarnini S. Hepatitis C virus resistance to protease inhibitors. J Hepatol. 2011; 55:192–206. [PubMed: 21284949]

26. Thompson AJ, McHutchison JG. Antiviral resistance and specifically targeted therapy for HCV (STAT-C). J Viral Hepat. 2009; 16:377–87. [PubMed: 19472445]
27. Romano KP, Ali A, Aydin C, et al. The molecular basis of drug resistance against hepatitis C virus NS3/4A protease inhibitors. PLoS pathogens. 2012; 8:e1002832. [PubMed: 22910833]
28. Fried MW, Buti M, Dore GJ, et al. Once-daily simeprevir (TMC435) with pegylated interferon and ribavirin in treatment-Naive genotype 1 hepatitis C: The randomized PILLAR study. Hepatology. 2013
29. Lenz O, Verbinnen T, Lin TI, et al. In vitro resistance profile of the hepatitis C virus NS3/4A protease inhibitor TMC435. Antimicrob Agents Chemother. 2010; 54:1878–87. [PubMed: 20176898]
30. McPhee F, Friborg J, Levine S, et al. Resistance analysis of the hepatitis C virus NS3 protease inhibitor asunaprevir. Antimicrob Agents Chemother. 2012; 56:3670–81. [PubMed: 22508297]
31. Paolucci S, Fiorina L, Piralla A, et al. Naturally occurring mutations to HCV protease inhibitors in treatment-naive patients. Virol J. 2012; 9:245. [PubMed: 23095680]
32. Luber AD. Genetic barriers to resistance and impact on clinical response. MedGenMed : Medscape general medicine. 2005; 7:69. [PubMed: 16369295]
33. Trimoulet P, Belzunce C, Faure M, et al. Hepatitis C virus (HCV) protease variability and anti-HCV protease inhibitor resistance in HIV/HCV-coinfected patients. HIV medicine. 2011; 12:506–9. [PubMed: 21410862]
34. Shendure J, Ji H. Next-generation DNA sequencing. Nature biotechnology. 2008; 26:1135–45.

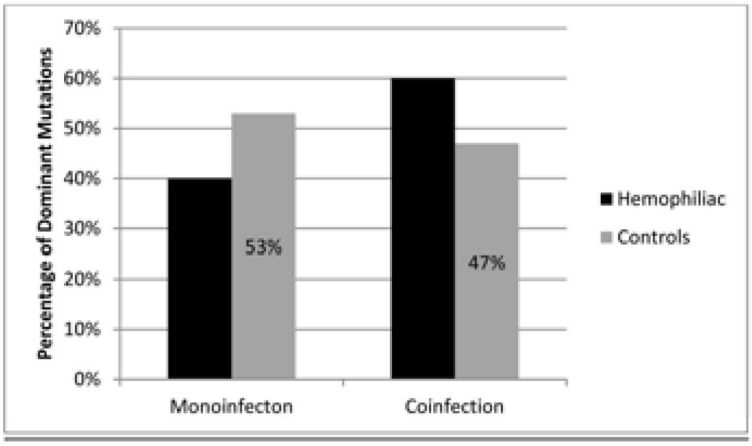

Figure 1.

Table 1

NS3 region Primers

Genotype 1a	Forward 5′-GGG ACA AAA ACC AAG TGG AG -3′ Reverse 5′-ACT GCT GGT GGA GAG GAG TT -3′
Genotype 1b	Forward 5′-GRC TCC TYG CGC CYA TCA CG -3′ Reverse 5′-CCR GTG GGR GCG TGT AGR TG -3′
Genotypes 2a/2b/2c	Forward 5′-GCY CCY ATY ACY GCH TAY R -3′ Reverse 5′-ACC TGR WAD GDY TGR GGY AC -3′
Genotypes 4c/4d	Forward 5′-TAY GCR CAR CAG ACC CGV GG -3′ Reverse 5′-GGC ACK GCA GGR GGW GTK GA -3′

Table 2

Demographic and clinical data

	Hemophiliacs (n=22)	**Control (n=129)**
Age, mean/range yr	36 (18–58)	48 (19–63)
Gender, n (%)	Male - 22 (100%) Female - 0	Male - 94 (73%) Female - 35 (27%)
Race, n (%)	White - 20 (91%) Black - 2 (9%)	White - 75 (59%) Black - 52 (41%)
HCV genotype, n	1a – 4 1b – 8 1 (no subtype) – 3 2b – 2 4c4d – 3 Unknown – 2	1a – 61 1b – 27 1a+1b – 4 1 (no subtype) - 37
HIV coinfection, n (%)	9 (41%)	58 (45%)
Prior PI exposure, n (%)	4 (18%)	27 (21%)

TABLE 3

HCV NS3 mutations- Direct Sequencing

	V36	Q41	F43	T54	Q80	S138	R155	A156	D168	I/V170
HCV/HIV Coinfected (58 total)	16 na	2 Q41H		2 T54S	18 Q80K 1 Q80L					
Hemophiliac HCV/HIV Coinfected (9 total)	2 V36L 1 na	1 na			1 Q80K 1 Q80G 2 Q80L					
HCV Monoinfected (71 total)	1 V36L 26 na	1 Q41H			21 Q80K		1 R155K			
Hemophiliac HCV Monoinfected (13 total)	2 V36L				2 Q80K 1 Q80G					

KEY:

na = non-amplifiable
key mutation
non-key mutation