Naturally occurring, resistance-associated hepatitis C virus NS5A variants are linked to IL28B genotype and are sensitive to interferon-based therapy.

Running Head: Naturally occurring RAVs and IFN therapy

Jun Itakura [1], Masayuki Kurosaki [1], Hitomi Takada [1], Natsuko Nakakuki [1], Syuya Matsuda [1], Kouichi Gondou [1], Yu Asano [1], Nobuhiro Hattori [1], Yoshie Itakura [1], Nobuharu Tamaki [1], Yutaka Yasui [1], Shoko Suzuki [1], Takanori Hosokawa [1], Kaoru Tsuchiya [1], Hiroyuki Nakanishi [1], Yuka Takahashi [1], Syinya Maekawa [2], Nobuyuki Enomoto [2], Namiki Izumi [1]

[1] Division of Gastroenterology and Hepatology, Musashino Red Cross Hospital, 1-26-1 Kyonan-cyo, Musashino-shi, Tokyo, Japan

[2] First Department of Internal Medicine, University of Yamanashi, Yamanashi, Japan,

Corresponding author
Namiki Izumi, M.D.
Division of Gastroenterology and Hepatology
Musashino Red Cross Hospital
1-26-1 Kyonan-cho
Musashino-shi, Tokyo, 180-8610, Japan
Phone: 81-422-32-3111
Fax: 81-422-32-9551.
E-mail: nizumi@musashino.jrc.or.jp

This manuscript contains 2506 words, excluding reference.

This manuscript includes 4 tables and 2 figures.

There are no conflicts of interests for the study.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/hepr.12474

Abstract

BACKGROUND & AIMS: The presence of resistance-associated variants (RAVs) may attenuate the efficacy of direct acting antivirals (DAAs) in combination therapy for hepatitis C. The aim of this study was to characterize the NS3 and NS5A regions of hepatitis C virus (HCV) in naturally occurring RAVs.

METHODS: The NS3 and NS5A regions of HCV were amplified by nested PCR and their nucleotide sequences were determined by direct sequencing in 493 genotype 1b patients naive to DAA-based therapies. The effect of baseline RAVs on response to pegylated interferon and ribavirin therapy was analyzed in 65 patients after stratification by IL28B genotype.

RESULTS: The incidence of RAVs was 7.9% in NS3 (V36I/L: 1.2%, T54S: 2.8%, Q80K/R: 3.0%, A156S: 0.2%, and D168E/T: 2.4%), and 20.2% in NS5A (L31I/M: 2.2% and Y93H: 19.0%). The incidence in interferon experienced and naive patients was similar. The incidence of Y93H in NS5A was significantly higher in the IL28B TT genotype (rs8099917) than non-TT (27.1% vs. 9.5%, $p<0.001$). The virological response to peg-interferon plus ribavirin therapy was not affected by the presence of RAVs in IL28B TT genotype.

CONCLUSION: RAVs, especially Y93H in the NS5A region, were highly prevalent in DAA-naïve patients with genotype 1b HCV in Japan and were linked to IL28B TT genotype.

Interferon-based therapy could be an alternative for patients with RAVs because these variants did not attenuate the response to that therapy. The analysis of RAVs may impact the selection of the optimal treatment strategy.

Key words: direct acting antivirals, HCV, IL28B genotype, interferon-based therapy, resistance-associated variants

Abbreviations

HCV, hepatitis C virus; IFN, interferon; ISDR, interferon sensitivity determining region; NVR, non-virological response; Peg-IFN, pegylated interferon; PCR, polymerase chain reaction; PR therapy, Peg-IFN plus RBV combination therapy; RAV, resistance-associated variant; RBV, ribavirin; RNA, ribonucleic acids; SVR, sustained virological response;

Introduction

Interferon (IFN) has formed the basis of standard treatment for chronic hepatitis C since the 1990s. Combination therapy with pegylated IFN (Peg-IFN) and ribavirin (RBV) achieves a sustained virological response rate of 40-50% in genotype 1 and over 80% in genotype 2/3. The recent development of direct acting antivirals (DAAs), which specifically inhibit the activity of viral proteins essential for replication, has improved significantly the efficacy of therapy.

DAAs are classified according to the target HCV protein, NS3/4A, NS5A and NS5B (1-3). DAAs are highly potent but their efficacy is attenuated in the presence of HCV variants with resistance to their activity. Many such resistance-associated variants (RAVs) have been characterized and several hot spots for variation have been reported (4-9). Naturally occurring RAVs are present in a proportion of patients (10) but their prevalence has not been determined completely. The relationship between RAVs and response to interferon-based therapy is not known and their association with previously established factors that affect the efficacy of interferon-based therapy, such as mutations in the ISDR region of NS5A (11) and core protein (12) and SNPs in the human IL28B gene (13-15), also is not known.

Theoretically, the presence of RAVs could attenuate the efficacy of interferon-free combination therapy with DAAs. In fact, baseline RAVs involving amino acid position 168 of NS3 and amino acid positions 31 and 93 of NS5A significantly attenuated the sustained virological response (SVR) rates of interferon-free Asunaprevir (NS3 protease inhibitor) and Daclatasvir (NS5A inhibitor) combination therapy; the SVR rate was 50% in patients with D168E in NS3, 48% in interferon-ineligible/intolerant patients with L31M/V and/or Y93H in NS5A, and 29% in non-responder patients with L31M/V and/or Y93H in NS5A(16). In Simeprevir plus Peg-IFN and RBV combination therapy, Q80K in NS3 attenuated the efficacy in genotype 1a patients (17). On the basis of this evidence, the treatment guidance for hepatitis C released by the American Association for the Study of Liver Disease and the Infectious Diseases Society of America (IDSA), and recommendations on treatment of hepatitis C released by the European Association for the Study of the Liver, recommend that Simeprevir combination therapy is not indicated in patients with Q80K in NS3 (18,19). As seen above, the analysis of RAVs at baseline may be crucial in the era of DAA-based therapy.

The aim of this study was to characterize naturally occurring RAVs in the NS3 and NS5A regions of hepatitis C virus.

Patients and Method

Patients

Serum was obtained from a total of 493 HCV genotype-1b infected patients, who had not been exposed to DAAs. Of them, 308 had been treated previously by interferon-based therapy, 61 with standard IFN, 24 with standard IFN plus RBV, 23 with Peg-IFN and 190 with Peg-IFN plus RBV. The clinical backgrounds of patients are shown in Table 1. Fibrosis staging was categorized according to the METAVIR score: F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis with few septa; F3, numerous septa without cirrhosis; and F4, cirrhosis. Sequences of the ISDR and the core region of HCV were determined by direct sequencing after amplification by reverse-transcription and polymerase chain reaction, as reported previously. Genetic polymorphism in a SNP located near the IL28B gene (rs8099917) was determined by Taq-man PCR assay. Briefly, DNA was isolated from peripheral blood using the standard phenol-chloroform method. Genotyping was carried out using a predesigned TaqMan probe (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Written informed consent was obtained from each patient and the study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the institutional ethics review committee.

Amplification and sequencing of HCV genomes

We investigated the viral genome sequence by direct sequencing method. Viral RNA was extracted from serum using QIAamp Viral RNA Mini Kits (QIAGEN). The extracted RNA was reverse-transcribed and amplified by the PCR method using the SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen) with the pairs of primers as follows:, (sense, nucleotides 3302-3329) 5'-GGCAGACACCGCGGCGTGTGGGGACAT-3' and (antisense, 4286-4316) 5'-GCACTCATCACATATTATGATGTCATAGGC-3' for NS3 and (sense, 5872-5891) 5'-AAGAGGCTCCACCAGTGGAT-3' and (antisense, 6730-6749) 5'-CGCCGGAGCGTACCTGTGCA-3' for NS5A. The targeted HCV genome was amplified by nested PCR using PrimeSTAR Max DNA Polymerase ▪ (TaKaRa), with the pairs of primers as follows: (sense, 3305-3329) 5'-AGACACCGCGGCGTGTGGGGACAT-3' and (antisense, 4054-4074) 5'-AGACACCGCGGCGTGTGGGGACAT-3' for NS3 and (sense, 5893-5912) 5'-AATGAGGACTGCTCCACGCC-3' and (antisense, 6690-6709) 5'-GTGAAGAATTCGGGGGCCGG-3' for NS5A. The PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN) and sequenced using an automated DNA sequencer (3730xl DNA Analyzer, Applied Biosystems). Each sequence was confirmed for the sense and anti-sense strands. If minor sequences of RAV were detected in more than 10%

of the strength of the major sequence, it was regarded as RAV positive.

For analysis, the predicted HCV amino acid sequences from the patients were compared with the sequence of the HCV-J strain (GenBank Accession No. AJ238799, http://www.ncbi.nlm.nih.gov/nuccore/AJ238799.1) as a reference. RAV positions in the HCV gene were determined according to previous reports (4-7, 20-27).

Response to Peg-IFN plus RBV in terms of RAVs

The impact of RAVs on the treatment response to Peg-IFN plus RBV combination therapy (PR therapy) was analyzed in 65 patients. The virological response (VR) was defined as a greater than 2.0 log reduction of HCV RNA at 12 weeks after therapy. The rate of VR was compared between patients with and without RAVs after stratification by IL28B genotype.

Statistical analysis

Categorical data were compared using the chi-square and Fisher's exact test. Continuous variables were analyzed using the Student's *t* test. A p value of <0.05 was considered statistically significant. Factors associated with Y93H were determined by multivariable logistic regression analysis. Statistical analyses were performed using the

Statistical Package for the Social Sciences software version 18.0 (SPSS Inc, Chicago, IL, USA).

Results

Prevalence of RAVs

HCV from 69.8% of the patients had amino acid variations in the NS3 region (Table 2). Variations at positions Q80L (13.4%), S122G (18.9%) and V170I (48.9%) were detected at high frequency but these have not been reported to confer resistance to DAAs. Previously defined RAVs were detected in 7.9% of patients, including V36L (0.8%), T54S (2.8%), Q80K/R (3.0%), A156S (0.2%) and D168E/T (2.4%).

HCV from 83.9% of the patients had variations in the NS5A region (Table 3). Among them, F37L (50.2%) and Q54H (37.6%) were highly prevalent but these have not been reported to confer resistance to DAAs. Previously defined RAVs were detected in 20.2%, the frequency of the Y93H variant being the highest at 19.0%, followed by L31M (1.5%).

Multiple mutations within NS3 (T54S –D168E: 0.2%, T54S –A156S –D168E: 0.2%) or NS5A (L31I/M –Y93H: 0.6%) were infrequent. Dual mutations in NS3 and NS5A were also infrequent (T54S – Y93H: 0.2%, Q80K – Y93H: 0.2%, and D168E – Y93H: 0.9%).

Factors associated with the presence of RAVs

The frequency of variations in terms of prior experience of interferon-based therapy was analyzed. As shown in Table 1, interferon experienced patients were older, more likely to have the IL28B (rs8099917) non-TT genotype and had a higher incidence of wild type ISDR sequences than interferon-naive patients. The frequency of RAVs in NS3 or NS5A did not differ between patients with and without prior IFN therapy (Tables 2 and 3).

Clinical features associated with the Y93H variant, the most prevalent RAV, also were analyzed (Table 4). Comparison of patients with the Y93 wild type virus and Y93H RAV showed that the Y93H group was older (66 vs. 62 years old, $p=0.02$), had lower platelet counts (141 vs. 162 $x10^9/L$, $p<0.01$), higher serum HCV RNA levels (6.9 vs. 6.5 Log IU/mL, $p<0.01$, Figure 1) and a higher prevalence of the IL28B (rs8099917) TT genotype (81% vs. 53%, $p<0.01$). By multivariate analysis, lower platelet counts, higher HCV RNA levels and IL28B TT genotype were independent factors contributing to the presence of the Y93H RAV (Table 4).

Variations and early response of Peg-IFN / RBV combination therapy

The impact of RAVs on treatment response to Peg-IFN plus RBV combination therapy was analyzed in 65 patients. The virological response (VR), defined as a greater than 2.0 log reduction of HCV RNA at 12 weeks after therapy, did not differ between patients with or without Y93H RAV among IL28B TT genotype. For comparison, the impacts of the Q80L and S122 variants also were analyzed and these variants also did not affect the VR (Figure 2). The number of patients with IL28B non-TT genotype was too small to analyze the effect of Y93 RAVs on VR.

Discussion

Treatment of HCV has entered the era of DAA-based therapy. Early clinical trials and in vitro studies have shown that treatment with a single DAA can suppress the replication of HCV but the emergence of RAVs is rapid and this may negate the inhibitory effect of the drug. Several previous studies have reported the incidence of RAVs in patients naive to treatment with DAAs. Here, we studied a large number of patients and revealed that naturally occurring RAVs in NS3 and NS5A are not rare in genotype 1b HCV. The frequency of RAVs was 7.9% in NS3 and 20.2% in NS5A. Caution should be used because the presence of these RAVs could attenuate the efficacy of DAA-based therapy.

RAVs for the NS3 protease inhibitors vary for different DAAs and HCV genotypes (4-7, 20-24). RAVs common to first generation protease inhibitors include amino acid positions 36, 54, 155 and 156, while those for second generation protease inhibitors include positions 80,156, and 168 (6,8). The frequency of RAVs in NS3 was higher in the present study than about 1% in a previous report (8,9). The impact of these RAVs on treatment outcome should be evaluated separately for interferon-based therapy and interferon-free combination therapy using DAAs. Bartels et al. reported that the SVR rates of Peg-IFN, RBV and TVR triple therapy were similar in patients with or without TVR-associated RAVs at baseline (9). This observation suggested that RAVs are susceptible to interferon. The result of our study supports this conclusion because the VR to PR therapy did not differ between patients with or without RAVs. On the other hand, several reports have revealed that baseline RAVs significantly attenuated the SVR rate of interferon-free Asunaprevir (NS3 protease inhibitor) and Daclatasvir (NS5A inhibitor) combination therapy. Thus, the presence of RAVs in NS3 could impact the selection of optimal treatment. In cases with NS3 RAVs, interferon-based therapy or interferon-free combination therapy with DAAs other than against NS3 should be preferred for patients with RAVs.

In Japan, an NS5A inhibitor is now becoming a key drug for the interferon-free combination therapy using DAAs. The frequency reported for RAVs in NS5A is higher in

Japan than in Western countries (9,28,29). A strikingly high incidence of the Y93H mutation should influence the outcome of Asunaprevir and Daclatasvir combination therapy, the first interferon-free combination therapy using DAAs approved in Japan, if treatment is given without assessment of baseline RAVs. Previous reports clearly indicated that the rate of SVR decreased to below 50% in patients with baseline RAVs. Because susceptibility to interferon was not attenuated in patients with Y93H RAV, interferon-based therapy, such as with the NS3 inhibitor Simeprevir plus peg-interferon and RBV, may be preferable for these patients. It was shown recently that Daclatasvir plus peg-interferon and RBV combination therapy achieved a high rate of SVR in treatment naïve Japanese patients (30). Taking into account the high prevalence of NS5A RAVs at baseline, as shown here, the result of this report suggested that RAVs in NS5A may not impact the outcomes of interferon-based therapies. Furthermore, newer combination therapies with DAAs are expected to be effective against these naturally occurring RAVs (31-33).

The association between the IL28B genotype and Y93H mutation was an unexpected finding but is in accordance with a recent report of an independent cohort of Japanese patients (29). No other variations in NS3 or NS5A showed an association with IL28B. Prior exposure to interferon therapy was not associated with the presence of Y93H and the precise reason for the association is unclear. Another finding was an association between Y93H and a high HCV RNA titer. This finding and the high prevalence of Y93H suggest that the Y93H RAV may be replication competent. This is in contrast to RAVs in NS3, where the replication fitness of the variants is reduced compared to the wild type virus, a finding supported clinically by the observation that treatment induced RAVs in NS3 become undetectable after long term follow-up (34-36). However, this result should be considered carefully, since there is no *in vitro* data to support the enhancement of replication by Y93H mutations. Adaptive mutations other than Y93H may be linked to high serum HCV RNA titer. Further studies are necessary to confirm this.

Another factor associated with Y93H was a lower platelet counts. This finding may suggest a more advanced stage of disease in Y93H-infected patients. A prospective observational study is needed to confirm this relationship.

In conclusion, RAVs, especially Y93H in the NS5A region, were highly prevalent in DAA-naive patients with genotype 1b hepatitis C in Japan. The presence of RAV Y93H has been reported to attenuate the efficacy of interferon-free combination therapy with DAAs, while the present study revealed that this RAV may be linked to the IL28B TT genotype in Japanese and was susceptible to interferon-based therapy. Thus, the analysis of RAVs in NS3 and NS5A may impact the selection of optimal treatment strategies, where interferon-based therapy with or without combination of DAAs could be an alternative for patients with

RAVs.

Acknowledgements and disclosures

This study was supported by Grant-in Aid from Japanese Ministry of Health, Labor and Welfare.

References

1. Aghemo A, Francesco DR. New horizons in hepatitis C antiviral therapy with direct-acting antivirals. *Hepatology* 2013; 58: 428-438.

2. Chae HB, Park SM, Youn SJ. Direct-acting antivirals for the treatment of chronic hepatitis C: open issues and future perspectives. *ScientificWorldJournal* 2013; 2013: 704912.

3. Zhu Y, Chen S. Antiviral treatment of hepatitis C virus infection and factors affecting efficacy. *World J Gastroenterol* 2013; 19: 8963-8973.

4. Halfon P, Locarnini S. Hepatitis C virus resistance to protease inhibitors. *J Hepatol* 2011; 55: 192-206.

5. Sarrazin C, Zeuzem S. Resistance to direct antiviral agents in patients with hepatitis C virus infection. *Gastroenterology* 2010; 138: 447-462.

6. Salvatierra K, Fareleski S, Forcada A, López-Labrador FX. Hepatitis C virus resistance to new specifically-targeted antiviral therapy: A public health perspective. *World J Virol* 2013; 2: 6-15.

7. Pawlotsky JM. Treatment failure and resistance with direct-acting antiviral drugs against hepatitis C virus. *Hepatology* 2011; 53: 1742-1751.

8. 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: 1769-1778.

9. Bartels DJ, Sullivan JC, Zhang EZ, et al. Hepatitis C virus variants with decreased sensitivity to direct-acting antivirals (DAAs) were rarely observed in DAA-naive patients prior to treatment. *J Virol* 2013; 87: 1544-1553.

10. Robinson M, Tian Y, Delaney WE 4th, Greenstein AE. Preexisting drug-resistance mutations reveal unique barriers to resistance for distinct antivirals. *Proc Natl Acad Sci U S A* 2011; 108: 10290-10295.

11. Enomoto N, Sakuma I, Asahina Y, et al. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. *N Engl J Med* 1996; 334: 77-81.

12. Akuta N, Suzuki F, Sezaki H, et al. (). Predictive factors of virological non-response to interferon-ribavirin combination therapy for patients infected with hepatitis C virus of genotype 1b and high viral load. *J Med Virol* 2006; 78: 83-90.

13. Tanaka Y, Nishida N, Sugiyama M, et al. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat Genet* 2009; 41:1105-9.

14. Lange CM, Zeuzem S. IL28B single nucleotide polymorphisms in the treatment of hepatitis C. *J Hepatol* 2011; 55: 692-701.

15. Balagopal A, Thomas DL, Thio CL. IL28B and the control of hepatitis C virus infection. *Gastroenterology* 2010; 139: 1865-1876.

16. Izumi N. Efficacy of daclatasvir in hepatitis C virus. *Expert Rev Anti Infect Ther* 2014; 12:1025-31.

17. Forns X, Lawitz E, Zeuzem S, et.al. Simeprevir with peginterferon and ribavirin leads to high rates of SVR in patients with HCV genotype 1 who relapsed after previous therapy: a phase 3 trial. *Gastroenterology* 2014; 146:1669-79.

18. Recommendations for Testing, Managing, and Treating Hepatitis C 2014 by the American Association for the Study of Liver Diseases and the Infectious Diseases Society of America. *http://www.hcvguidelines.org*

19. Pawlotzky, JM., et.al. EASL Recommendations on Treatment of Hepatitis C 2014. *http://www.easl.eu/_newsroom/latest-news/easl-recommendations-on-treatment-of-hepatitis-c-2014*

20. Bartels DJ, Zhou Y, Zhang EZ, et al. Natural prevalence of hepatitis C virus variants with decreased sensitivity to NS3.4A protease inhibitors in treatment-naive subjects. *J Infect Dis* 2008; 198: 800-807.

21. Susser S, Welsch C, Wang Y, et al. Characterization of resistance to the protease inhibitor boceprevir in hepatitis C virus-infected patients. *Hepatology* 2009; 50: 1709-1718.

22. 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: 1878-1887.

23. Summa V, Ludmerer SW, McCauley JA, et al. MK-5172, a selective inhibitor of hepatitis C virus NS3/4a protease with broad activity across genotypes and resistant variants. *Antimicrob Agents Chemother* 2012; 56: 4161-4167.

24. Barnard RJ, McHale CM, Newhard W, et al. Emergence of resistance-associated variants after failed triple therapy with vaniprevir in treatment-experienced non-cirrhotic patients with hepatitis C-genotype 1 infection: a population and clonal analysis. *Virology* 2013; 443: 278-284.

25. McPhee F, Hernandez D, Yu F, et al. Resistance analysis of hepatitis C virus genotype 1 prior treatment null responders receiving daclatasvir and asunaprevir. *Hepatology* 2013; 58: 902-911.

26. Fridell RA, Wang C, Sun JH, et al. Genotypic and phenotypic analysis of variants resistant to hepatitis C virus nonstructural protein 5A replication complex inhibitor BMS-790052 in humans: in vitro and in vivo correlations. *Hepatology* 2011; 54: 1924-1935.

27. Nakamoto S, Kanda T, Wu S, Shirasawa H, Yokosuka O. Hepatitis C virus NS5A inhibitors and drug resistance mutations. *World J Gastroenterol* 2014; 20: 2902-2912.

28. Karino Y, Toyota J, Ikeda K, et al. Characterization of virologic escape in hepatitis C virus genotype 1b patients treated with the direct-acting antivirals daclatasvir and asunaprevir. *J Hepatol* 2013; 58: 646-654.

29. Miura M, Maekawa S, Sato M, et al. Deep Sequencing Analysis of Variants Resistant to the NS5A Inhibitor Daclatasvir in Patients with Genotype 1b Hepatitis C Virus Infection. *Hepatol Res* 2014; *In Press.*

30. Izumi N, Yokosuka O, Kawada N, et.al. Daclatasvir combined with peginterferon alfa-2a and ribavirin in Japanese patients infected with hepatitis C genotype 1. *Antivir Ther* 2014; *In Press.*

31. Afdhal N, Zeuzem S, Kwo P, et.al. Ledipasvir and sofosbuvir for untreated HCV genotype 1 infection. *N Engl J Med* 2014; 370:1889-98.

32. Afdhal N, Reddy KR, Nelson DR, et.al. Ledipasvir and sofosbuvir for previously treated HCV genotype 1 infection. *N Engl J Med* 2014; 370:1483-93.

33. Kowdley KV, Gordon SC, Reddy KR, et.al. Ledipasvir and sofosbuvir for 8 or 12 weeks for chronic HCV without cirrhosis. *N Engl J Med* 2014; 370:1879-88.

34. Schneider MD, Sarrazin C. Antiviral therapy of hepatitis C in 2014: Do we need resistance testing? *Antiviral Res* 2014; 105: 64-71.

35. Barnard RJ, Howe JA, Ogert RA, et al. Analysis of boceprevir resistance associated amino acid variants (RAVs) in two phase 3 boceprevir clinical studies. *Virology* 2013; 444: 329-336.

36. Sullivan JC, De Meyer S, Bartels DJ, et al. Evolution of treatment-emergent resistant variants in telaprevir phase 3 clinical trials. *Clin Infect Dis* 2013; 57: 221-229.