Antiviral Therapy 2013; **18:**723–727 (doi: 10.3851/IMP2632)

Short communication

Dynamic and rapid changes in viral quasispecies by UDPS in chronic hepatitis C patients receiving telaprevir-based therapy

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Background: Telaprevir (TVR) is a protease inhibitor (PI) used in chronic hepatitis C treatment with pegylated interferon plus ribavirin. We analysed the prevalence and kinetic development of TVR resistance upon treatment.

Methods: A total of 24 cirrhotic patients (genotype 1a, *n*=8; genotype 1b, *n*=16) previously non-responders to standard therapy were treated with TVR-based therapy. The distribution of TVR-resistant variants was assessed at every HCV-RNA-positive time point by 454 ultra-deep pyrosequencing (UDPS) during a mean follow-up period of 9.4 months.

Results: A median of 6,837 reads/specimen was studied. Based on control UDPS, we considered mutations as real when present >0.4%. TVR-resistant variants were found at baseline in 8/24 patients (33.3%). Four of the 24 patients (16.7%), all genotype 1a, did not achieve HCV RNA<100 IU/ml between week (W)2 and W12 and stopped treatment. No statistical significant difference was observed in the prevalence of resistant mutants between responders and non-responders (25% [5/20] and 75% [3/4], respectively). The proportion of genotype 1a patients with R155K/T/Q at baseline was higher in non-responders than in responders (50% versus 0%). During treatment failure, significant enrichment in V36A/M and R155K/T/Q was observed but their frequency reverted back to baseline after TVR discontinuation.

Conclusions: TVR-resistant variants are widely present at baseline. The presence of TVR-resistant mutants at baseline, even in high abundance (>20%), did not always preclude TVR treatment success. The detection of R155K/T/Q at baseline may predict failure in genotype 1a patients. At failure, which occurred in genotype 1a patients, a significant enrichment in V36A/M and R155K/T/Q was observed.

Introduction

Telaprevir (TVR), a specific HCV NS3-4A protease inhibitor (PI), results in rapid emergence of drug-resistant strains when used as monotherapy [1], leading to the need for combination therapy with pegylated interferon (PEG-IFN) and ribavirin (RBV).

Recent reports have demonstrated that HCV PI-resistant variants may already be present in the quasispecies population in PI treatment-naive patients [2–4]. The eradication of drug-susceptible variants may result in the expansion of resistant viruses that were

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initially present as minority species and lead to treatment failure [4–7]. The extent to which pre-existing HCV variants and their dynamics under antiviral selection pressure may compromise treatment with direct-acting antivirals (DAAs) is not yet fully understood [8,9]. Ultra-deep pyrosequencing (UDPS) may be used to identify minority drug-resistant clones that are not detectable by population sequencing, leading to a better understanding of the composition and natural evolution of viral quasispecies in the presence of DAAs [9,10].

The aim of this UDPS study was to analyse viral sequences from cirrhotic patients treated with TVR-based therapy before treatment in order to define the baseline prevalence of resistant variants (V36A/M, T54A/S, R155K/T/Q, A156S/T/V), and to define the relationship between virological failure and the emergence of TVR-resistant variants during treatment.

Table 1. Primers used for HCV NS3 protease amplification

	Sequence (5′–3′)	H77 HCV genome position
RT-PCR		
Primer 5′	ACSGCRGRTGYGGGGACAT	3309–3328
Primer 3′	GTGCTCTTRCCGCTRCCRGT	4035–4054
Nested-PCR first amplicon		
Primer 5′	CCYGTCTCYGCYCGWAGGGG	3342–3361
Primer 3′	TCYACRTTRGTRTACATYTG	3636–3655
Nested-PCR second amplicon		
Primer 5′	CARATGTAYACYAAYGTRGA	3636–3655
Primer 3′	GTGCTCTTRCCGCTRCCRGT	4035–4054

RT, reverse transcriptase.

Methods

A total of 24 HCV-infected cirrhotic patients followed at the Hepatology Unit, Haut-Lévêque Hospital (Pessac, France) were consecutively enrolled in this prospective study. Patients infected with HCV genotype 1a or 1b who were previously non-responders to PEG-IFN and RBV and naive to any DAA treatment were included. They received TVR plus PEG-IFN and RBV for 12 weeks followed by PEG-IFN plus RBV for 36 weeks within the framework of the TVR access programme in France. Written informed consent was obtained from each participant. The study was registered at ClinicalTrials.gov (identifier: NCT01577069).

The protease amplification protocol used was adapted from the Agence Nationale de Recherches sur le SIDA et les Hépatites Virales consensus technique [3]. Two amplicons encompassing the HCV NS3 protease region spanning amino acids 1–205 were amplified using primers described in Table 1. Protease UDPS was performed using the Roche GS Junior apparatus (454 Life Sciences; Roche, Branford, CT, USA) according to the manufacturer's recommendations (threshold of detection 500 IU/ml). UDPS generated a median of 6,837 reads per sample and the median coverage was approximately 3,500 reads per nucleotide. Major established mutations associated with resistance to TVR were analysed using the GS Amplicon Variant Analyzer (AVA) software version 2.0.01 from Roche following the algorithms previously described [11,12].

The technical error rate was estimated by PCR amplification and UDPS in duplicate of the NS3 region of a genotype 1a H77 plasmid and a genotype 1b RNA replicon containing the full Con1 sequence. A median of 8,014 and 3,737 sequence reads was obtained for replicon and plasmid assays, respectively. Each position of the viral sequence was considered for mutation analysis. The mean error rate ±2 SD for plasmid control was 0.14% ±0.26 base substitutions per NS3 base position and 0.047% ±0.006 base substitutions per NS3 base position for replicon control. Therefore, mutations were accepted as real when present at a frequency of >0.4% among the total number of reads [13]. The UDPS results are available in GenBank under accession number SRA054409.

Statistical analyses

Qualitative variables were described as number (percentages) and quantitative variables as median (IQR) unless stated otherwise. Characteristics of responders and non-responders were compared using Fisher's exact test for qualitative variables and the Wilcoxon–Mann–Whitney test for quantitative variables. Fisher's exact test was used to assess the potential role of all baseline HCV TVR-resistant mutations on TVR virological response. All analyses were performed with SAS 9.13 (SAS Institute, Cary, NC, USA).

Results

Baseline characteristics and follow-up

The baseline characteristics of the 24 patients included in the study are described in Table 2. Four of the 24 patients (16.7%) who did not achieve HCV RNA<100 IU/ml at week (W)4, W8 and/or W12 of treatment were considered as non-responders and their treatment was stopped. Non-responders were younger (P=0.02) and were more often infected with HCV genotype 1a (P=0.01) than responders. At baseline, protease UDPS was performed in all patients. Regarding the follow-up of the 4 TVR non-responders, 8 samples could not undergo UDPS because of a lack of PCR amplification owing to low viral loads. Participants were followed up for an average of 9.4 months after starting treatment.

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Table 2. Demographic and clinical characteristics of the 24 cirrhotic patients at inclusion

Characteristic	All	Responder to TVR[a]	Non-responder to TVR[b]	*P*-value
Gender				1.0
Male	17 (70.8)	14 (70.0)	3 (75.0)	
Female	7 (29.2)	6 (30.0)	1 (25.0)	
Age, years	60 (49–65)	63.7 (50.9–66.0)	48.5 (47.3–50.1)	0.02
Genotype				0.01
1a	8 (33.3)	4 (20.0)	4 (100.0)	
1b	16 (66.7)	16 (80.0)	0 (0.0)	
Response to previous treatment				0.4
Relapse	6 (25.0)	5 (25.0)	1 (25.0)	
Partial response	10 (41.7)	9 (45.0)	1 (25.0)	
Null response	7 (29.2)	5 (25.0)	2 (50.0)	
Breakthrough	1 (4.2)	1 (5.0)	0 (0.0)	
HCV RNA, $\log_{10}$ IU/ml	6.4 (5.9–6.6)	6.3 (5.9–6.6)	6.7 (6.3–6.9)	0.14
IL28rs8099917 genotype[c]				0.54
TT	6 (26.0)	6 (31.5)	0	
GT/GG	17 (73.9)	13 (68.4)	4 (100)	
Delay between previous treatment and TVR start, years	3.7 (2.8–7.5)	3.7 (2.7–7.5)	3.8 (2.8–6.9)	0.97

Data are *n* (%) for categorical variables and median (IQR) for quantitative variables. [a]*n*=20. [b]*n*=4. [c]*n*=23. TVR, telaprevir.

Prevalence of TVR-resistant mutations at baseline and virological response

Overall, 8 of the 24 (33.3%) patients presented baseline TVR-resistant mutations. No significant difference was observed in the prevalence of these mutations between responders and non-responders to TVR-based therapy (25% [5/20] and 75% [3/4], respectively). None other mutation aside from known TVR-resistant mutations was observed (Q28H, Q41R, Y52C, H57L, P96H) [9].

Of the 5 TVR responders with TVR-resistant mutations, 2 showed resistant variants in high abundance (V36A/M, 35% and T54A/S, 49.5%), whereas in the other 3 responders, TVR-resistant mutations were present at a frequency <20% (T54A/S 2.8%, R155K/T/Q 0.43% and A156S/T/V 2%; data not shown).

In non-responders, all these mutations were present at a level <20% (range 0.44–2.3). Two patients harboured one resistant mutation at baseline (R155K/T/Q, 2.3% and A156S/T/V, 0.66%) and one patient had three resistant mutations (V36A/M, 0.44%; T54A/S, 1.67%; R155K/T/Q, 0.7%). While the overall presence of resistant variants did not appear to affect response to therapy, the presence of TVR-resistant mutations was evaluated separately. The proportion of genotype 1a patients with the R155K/T/Q mutation at baseline was higher in non-responders than in responders (50% versus 0%).

Virological failure

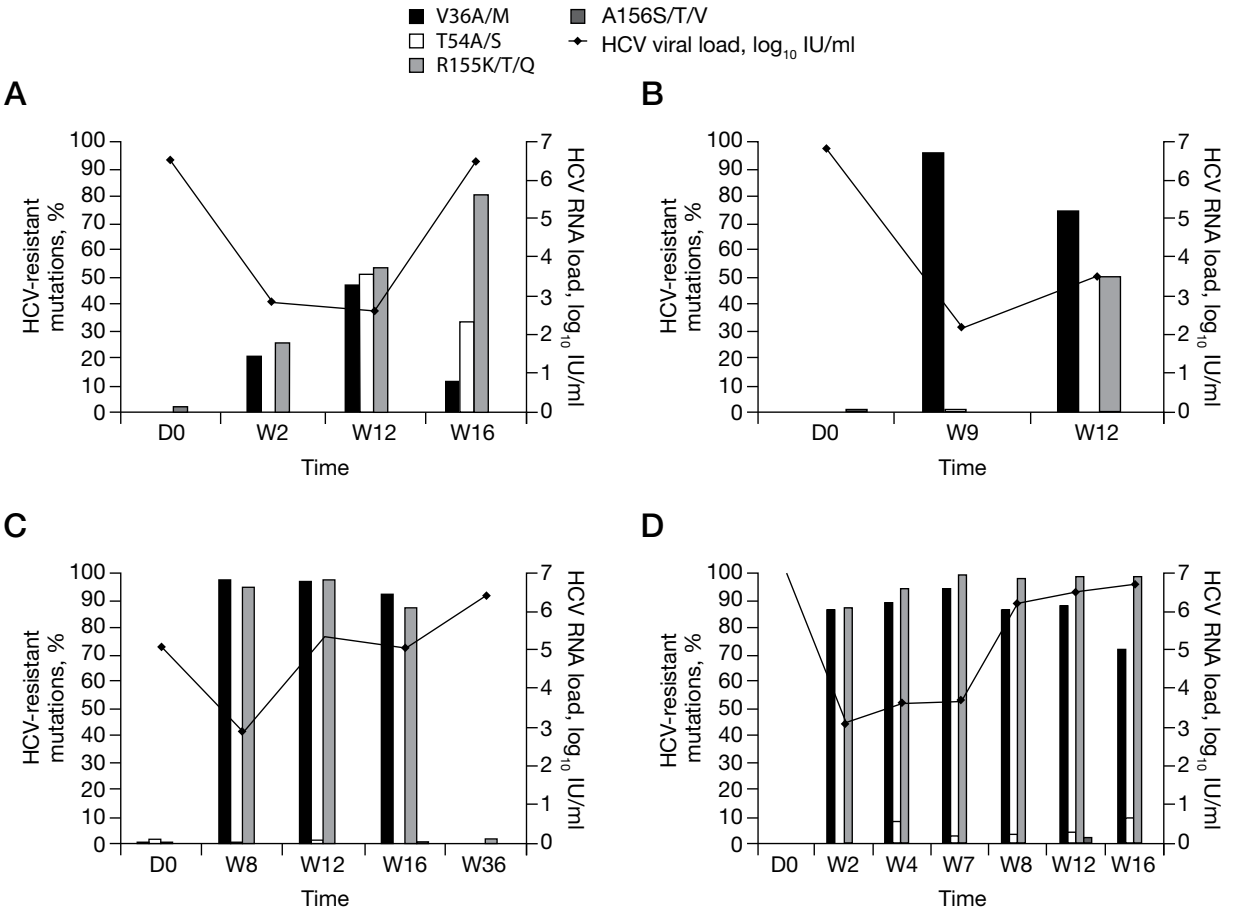
UDPS analysis of samples from the 4 genotype 1a non-responders revealed that treatment failure was associated in all cases with the presence of V36A/M and R155K/T/Q resistant variants (Figure 1). None of the variants selected during treatment failure bore additional amino acid changes together with the known TVR-resistant mutations, as previously described [9]. Two previous non-responders (NR1 and NR4) developed these variants in high abundance within the first 2 weeks of TVR-based treatment (Figure 1A and 1D). An increase in the prevalence of R155K/T/Q was observed during the entire follow-up, even after treatment has been stopped. The V36A/M mutation was also present in high abundance (>20%). The other two patients experienced treatment failure between W2 and W8 and developed the same profile of resistant variants detected at approximately 95% or more of the viral population (Figure 1B and 1C). In the previous relapser NR2 and the previous null responder NR3, both V36A/M and R155K/T/Q mutations decreased after treatment arrest. Regarding patient NR4, resistant mutations detected at high frequency at failure reverted back to baseline at W36 (ranging from 0.3% to 1.3%; Figure 1C).

Discussion

This study is one of the first using UDPS to analyse HCV protease quasispecies in routinely TVR-treated cirrhotic patients.

The findings demonstrate that HCV resistant variants are common at baseline in chronically HCV-infected, PI-naive patients, whether they are responders or not

Figure 1. Ultra-deep pyrosequencing analysis of the four NR patients



Ultra-deep pyrosequencing analysis of the four non-responder (NR) patients: **(A)** patient NR1 (treatment stop at week [W]9), **(B)** patient NR2 (treatment stop at W9), **(C)** patient NR3 (treatment stop at W6) and **(D)** patient NR4 (treatment stop at W6) before, during and after telaprevir (TVR)-based treatment are shown. Only mutants detected at positions known to confer TVR resistance are shown with the percentage of HCV resistant mutation indicated for each time point. D, day.

to TVR therapy. UDPS identified a significantly larger proportion of patients who harboured TVR-resistant variants at baseline than did standard genotyping [8,14]. Their presence, even in high abundance (>20%), did not always preclude TVR treatment success. However, the presence of certain baseline variants such as R155K/T/Q, which is present in 50% of TVR non-responders, may impact the response to TVR therapy. Although not statistically significant, the apparent difference between 0 and 50% of R155K/T/Q mutation in responders and non-responders indicates that this mutation may be an important factor in resistance development.

All the non-responders to TVR were infected with HCV genotype 1a. Virological failure was always associated with the resistant mutants V36A/M and R155K/T/Q, although other resistant variants were present at baseline but were not selected. These observations might be due to the higher genetic barrier to resistance with 1b versus 1a subtypes and the pattern of resistance mutations that is strikingly different between these subtypes [15]. In two patients in whom UDPS was performed at W2, the early detection of resistant variants correlated with the HCV viral load decrease, suggesting that clearance of wild-type viruses revealed the pre-existing resistant variants. Moreover, at each time point investigated during follow-up, resistant variants were present in high abundance, that is, above the limit of detection using standard sequencing. These data suggest that it may be relevant to perform UDPS in the first days of treatment, even if a decrease in viral load is observed, in order to rapidly differentiate responders and non-responders. The design of our study did not allow UDPS data on HCV genetic variation to be obtained during the first days following treatment, limiting our kinetic analysis to longer intervals.

UDPS results obtained during the 30 weeks after treatment stop in patient NR4 showed that HCV

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populations returned to the pretreatment state in the absence of drug-selective pressure [4,16,17].

In conclusion, UDPS demonstrated that TVR-resistant variants are widely present at baseline in chronically HCV-infected, PI-naive patients who were non-responders to previous treatment with PEG-IFN and RBV. Their presence, even in high abundance (>20%), did not always preclude TVR treatment success. The detection of R155K/T/Q at baseline may predict virological failure in genotype 1a patients. At treatment failure, which occurred exclusively in genotype 1a patients, there was almost 100% replacement of wild-type viruses by resistant strains harbouring V36A/M and R155K/T/Q mutations. Protease UDPS in the first days of treatment may therefore help to differentiate responders and non-responders.

Acknowledgements

This work was supported by Roche and Janssen–Cilag Laboratories. We thank the nurses (M Jonckeau, S Vandenabeele and M Phenix), physicians (N Messaoudi, C Fabères and P Couzigou) and patients involved in this study. We also thank R Bartenschlager for kindly providing us the replicon and T Astier-Gin and M Ventura for the NS3 plasmid. We thank Roche for providing the UDPS GS Junior equipment. The authors are indebted to Ray Cooke for linguistic assistance.

PT, PP, HF and VdL conceived and design the experiments. PP, JP and NC performed the experiments. PT, PP, JP, LW, WM, SR and MK analysed the data. JF, JV, FC and VdL monitored the patients. PT, PP, MM, MK, HF and VdL wrote the paper.

Disclosure statement

The authors declare no competing interests.

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Accepted 1 April 2013; published online 23 May 2013