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Prevalence of NS5A resistance associated substitutions in patients with hepatitis C virus genotypes 1a and 3: Impact on current therapeutic strategies

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Abstract
The presence of resistance-associated substitutions (RASs) at NS5A region might compromise the efficacy of Direct Acting Antiviral agents (DAAs). HCV resistance at NS5A region is mainly focused on patients with hepatitis C virus (HCV) genotypes 1a (G1a) and 3 (G3) with other factors of poor treatment response (ie cirrhosis, prior treatment-exposure, or HCV-RNA >800 000 IU/mL). Herein, we evaluated in a cohort of HCV G1a and G3 infected patients the prevalence of RASs at domain I NS5A using population-based sequencing and the impact of RASs on the optimization of current therapeutic strategies. The RASs considered as clinically relevant were: M28A/G/T, Q30D/E/H/G/K/L/R, L31M/V/F, H58D, and Y93C/H/N/S for G1a and Y93H for G3. A total of 232 patients naïve to NS5A inhibitors were included (166 G1a, 66 G3). The overall prevalence of NS5A RASs for G1a and G3 patients was low (5.5%) or null, respectively. A high proportion of patients harbored, at least, one factor of poor response (78.9% for G1a, and 75.8% for G3). Overall, the rates of patients harboring NS5A RASs in combination with any of the other factors were low and the vast majority of patients (G1a> 94% and G3 100%) could be treated with standard treatments of 12 weeks without ribavirin. In conclusion, testing NS5A RASs in specific HCV-infected populations (ie G1a & G3, cirrhosis, prior treatment experienced, HCV-RNA >800 000 IU/mL) might be useful to optimize current NS5A-based therapies avoiding ribavirin-related toxicities, and shortening treatment duration in the majority of patients.

Keywords
Genotype 1a, genotype 3, HCV-infection, NS5A, RASs

1. INTRODUCTION

Since the approval of Direct Acting Antiviral agents (DAAs), the face of hepatitis C infection (HCV) has dramatically changed. The combination regimens of DAAs are the new standard of treatment for HCV infection with high-sustained viral response (SVR) rates at 12 weeks for the majority of patients (90%) and almost without adverse events. However, some of these combination regimens require prolonging treatment duration (16 or 24 weeks) and/or the addition of ribavirin if some factors related with a poor treatment response are present. This is the case of HCV-infected patients with genotypes 1a (G1a) and 3 (G3) with cirrhosis, prior exposure to interferon-based regimens, high baseline HCV-RNA, and/or presence of resistance-associated substitutions (RASs) with a poorer response for NS5A inhibitors-based therapies.[1-3]

In the current treatment scenario, the relevance of HCV resistance is mainly focused on the NS5A region for G1a and G3. Several studies have shown that the presence of RASs at NS5A might compromise the virological response for HCV G1a and G3 patients.[4-13] The impact of these RASs on treatment efficacy varies according to the presence of other factors and can be countered by prolonging the duration of treatment or adding ribavirin for some DAA combinations.

In this context, current HCV treatment guidelines highlight that the presence of NS5A RASs before treatment initiation in combination with other factors of poor response previously cited might compromise the efficacy of specific DAA combinations. Therefore, testing NS5A resistance might help to select the optimal DAA combination among patients harboring predictors of poor response and avoid unnecessarily prolong treatment duration or the addition of ribavirin in those patients in whom RASs are not present. Therefore, this strategy might avoid the toxicity related to the use of ribavirin, allow optimize treatment duration, and afford costs to the Health systems. Moreover, it is known that NS5A RAS may persist for years, which could have implications for retreatment in case of failure.

This study analyzes, the prevalence of NS5A RASs for G1a and G3 patient's naïve to NS5A inhibitors and evaluates the benefits of performing a resistance test to optimize current therapeutic strategies.

2. MATERIALS AND METHODS

This is an observational study in patients with chronic hepatitis C (CHC) in clinical follow-up at two hospitals in Northwest Spain (Complejo Hospitalario Universitario de A Coruña and Complejo Hospitalario Universitario de Ferrol) between June-2014 and June-2016. All patients enrolled in the study had HCV G1a and G3 and were naïve to regimens containing NS5A inhibitors. This study was approved by the regional Ethics Committee (register code C.0003724). Only patients who had signed the informed consent and had HCV-RNA >1000 IU/mL were included. Liver stiffness was measured by transient elastography (FibroScan, EchoSense, París, France) and results expressed in kilopascals (kPa) were converted into the METAVIR scale as follows: F0-F1<7.5; F2 ≥7.5, and <9.5 kPa; F3 ≥9.5 and <12.5 kPa and F4 12.5 kPa (cirrhosis). Demographic and baseline characteristics were retrospectively recorded.

The HCV NS5A region was sequenced from plasma samples regularly stored at −80°C in the Biobank of A Coruña. NS5A Domain I (codons 1-213) was amplified with HCV genotype-specific primers through reverse transcription PCR (RT-PCR) and nested-PCR. NS5A amplification for HCV G1a was performed as previously described.[14] For HCV G3 NS5A amplification RT-PCR was performed using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA). The nested-PCR reaction was carried out using Hotstart Master Mix Kit (Qiagen, Hilden, Germany) with the following primers: NS5A-3a_F 5′CyGCATTGCTGAGTTCTCTA3′ and NS5A-3a_R 5′GYAGTCTCACYCCATCCACTTC3′.[15] Direct sequencing of all PCR products was performed using an automatic sequencer (ABI Prism 3130xl Genetic Analyzer [Applied Biosystems, Foster City, CA]) and BigDye Ready Reaction Kit V3.1 (Applied Biosystems, Foster City, CA). Nucleotide sequences were assembled using the Variant Reporter version 1.0 software (Thermo Fisher Scientific) and aligned with reference sequences (GeneBank accession numbers AF009606 for HCV G1a and D17763 for HCV G3).

NS5A sequences were analyzed using geno2pheno (HCV) algorithm for the identification of NS5A RASs16 considering the clinically relevant RASs for the analysis: M28A/G/T, Q30D/E/H/G/K/L/R, L31M/V/F, H58D, and Y93C/H/N/S for G1a and Y93H for G3 accordingly to the last updated guidelines of the American Association for the Study of Liver Diseases (AASLD) and the European Association for the Study of the Liver (EASL).[1, 2]

The statistical analysis was performed using the Statistical Package for the Social Sciences software (SPSS 19.0, Chicago, IL). Categorical variables are presented as number of cases or percentage. Continuous variables are expressed as mean ± standard deviation.

3. RESULTS

The study enrolled 232 CHC patients: 166 HCV G1a and 66 HCV G3. Clinical characteristics and prevalence of baseline NS5A RASs are detailed in Table 1. NS5A population sequencing was successfully performed for 162 of 166 patients G1a and for all G3 patients. The prevalence of clinically relevant RASs was 5.5% (9/162) in HCV G1a. The most frequent substitution recognized was Q30H/R (5/162, 3.1%). The remaining RASs were found with a prevalence below 1%: M28T (1/162, 0.6%), L31M (1/162, 0.6%), H58D (1/162, 0.6%), and Y93H (1/162, 0.6%). NS5A RASs were not detected among HCV G3 infected patients.

Table 1. Clinical characteristics and prevalence of baseline NS5A RASs of CHC patients infected with G1a and G3

	G1a (*N* = 166)	G3 (*N* = 66)
Male	132 (79.5)	50 (75.8)
Age, years	48.7 ± 8.3	49.4 ± 6.7
Time since HCV diagnosis, years	17 ± 6.9	16.1 ± 6.9
HIV/HCV co-infection	98 (61.3)	46 (69.7)
Factors of poor response		
HCV-RNA >800 000 IU/mL	120 (72.7)	42 (64.6)
Prior exposure to IFN-based therapies	47 (28.3)	13 (19.7)
Liver cirrhosis	35 (21.1)	17 (25.8)
Prevalence of baseline RASs[a]	9 (5.5)	0 (0)
M28T	1 (0.6)	-
Q30H/R	5 (3.1)	-
L31M	1 (0.6)	-
H58D	1 (0.6)	-
Y93H	1 (0.6)	0 (0)

Data are expressed as *n* (%) or mean ± standard deviation. Abbreviatures: HIV: Human Immunodeficiency Virus; HCV: Hepatitis C Virus; HCV-RNA: HCV viral load; IU/mL: International Units per mililiter; IFN: Interferon; RASs: Resistance-associated substitutions.

[a] Rates of baseline RASs in G1a were calculated with *N* = 162.

The presence of factors associated with poor response to treatment (liver cirrhosis, prior exposure to treatment and HCV-RNA >800 000 IU/mL) was evaluated in both genotypes. Regarding the fibrosis stage, 21.1% (35/166) of G1a and 25.8% (17/66) of G3 had liver cirrhosis. The proportion of patients with prior exposure to treatment was 28.3% (47/166) for G1a and 19.7% (13/166) for G3. According to the prior treatment status, all G3 patients were previously exposed to pegylated-interferon (peg-IFN) with ribavirin. Nevertheless for G1a patients, 83% (39/47) were previously exposed to peg-IFN with ribavirin, and 17% (8/47) were previously exposed to triple therapy with peg-IFN, ribavirin, and the first generation of protease inhibitors regimens (boceprevir or telaprevir). Furthermore, a high prevalence of patients with baseline HCV-RNA levels >800 000 IU/mL was observed for both genotypes (72.7% [120/166] G1a and 64.6% [42/166] G3).

Moreover, we evaluated in this cohort the impact of NS5A RASs on current therapeutic strategies to define the utility of resistance testing in patients with predictors associated with poor response to specific DAA therapies (Table 2). In this population, 71.6% (116/162) of G1a patients had HCV-RNA values >800 000 IU/mL. Considering current HCV treatment guidelines, treatment duration must be prolonged to 16 weeks with ribavirin for grazoprevir/elbasvir combination in case that testing RASs was not available. However, after performing NS5A RASs resistance testing in this population only 5.2% (6/116) of them had RASs and therefore would require prolonging treatment duration and the addition of ribavirin. The rest of patients could benefit of an optimized treatment for 12 weeks without ribavirin. Among G1a patients, 29% (47/162) were treatment-experienced with or without cirrhosis. Therapeutic guidelines recommend prolonging treatment duration to 24 weeks or adding ribavirin for 12 weeks for sofosbuvir/ledipasvir or sofosbuvir/daclatasvir regimens, in this subset of patients if baseline RASs conferring resistance to NS5A are present. In this population, only 4.3% (2/47) of patients harbored RASs and therefore would require reinforcing treatment. Finally, for G3 patients, 39.4% (26/66) were treatment-experienced (with or without cirrhosis) or naïve with cirrhosis for which prolonging treatment duration to 24 weeks or adding ribavirin for 12 weeks for sofosbuvir/velpatasvir combination if NS5A RAS Y93H is present. None of these patients with G3 had this mutation, hence, all of them could benefit from the standard sofosbuvir/velpatasvir treatment for 12 weeks without ribavirin.

Table 2. Rate of patients in whom baseline RASs might impact on treatment outcomes to specific DAA combinations affected by NS5A RASs

	Scenario for NS5A RASs testing		Rate of patients *n*/*N* (%)		
HCV genotype	DAA-combinations	Factors of poor response (FPR)	With FPR	With RASs[a]	Without RASs[b]
1a	GZR/EBV	Naïve or treatment-experienced with HCV-RNA >800 000 IU/mL	116/162 (71.6)	6/116 (5.2)	110/116 (94.8)
	SOF/LDV or SOF-DCV	Treatment-experienced with or without cirrhosis	47/162 (29.0)	2/47 (4.3)	45/47 (95.7)
3	SOF/VEL	Treatment-experienced (with or without cirrhosis) and naïve with cirrhosis	26/66 (39.4)	0/26(0)	26/26 (100.0)

Data are expressed as *n*/*N* (%). Abbreviatures: HCV: Hepatitis C Virus; DAA: direct acting antiviral agents; RASs: resistance-associated substitutions; HCV-RNA: HCV viral load; GZR/EBV: grazoprevir/elbasvir; SOF/LDV: sofosbuvir/ledipasvir; SOF-DCV: sofosbuvir-daclatasvir; SOF/VEL: sofosbuvir/velpatasvir; FPR: factors of poor response.
[a] Patients with RASs should reinforce treatment adding ribavirin and/or prolonging treatment duration.
[b] Patients without RASs should be treated with standard regimens of DAA.

Treatment outcome was retrospectively evaluated in the group of patients with baseline NS5A RASs ($N = 8$). Of them, 2 (2/8, 25%) are still naïve to HCV therapy; 2 (2/8, 25%) were treated without NS5A inhibitors; and 4 (4/8, 50%) were treated with NS5A inhibitors-based therapies (4/4, 100%, sofosbuvir/ledipasvir). Only one of these patients, presented factors of poor response associated with lower SVR rates (to be treatment-experienced). This patient had L31M mutation, which confers >100-fold change for ledipasvir. This patient was treated according to the guidelines recommendations in case of presence of RASs. Therefore, for this patient the treatment was reinforced by adding ribavirin during 12 weeks, so the effect of baseline RASs could be unmasked. All patients who presented baseline RASs (6/6, 100%) achieved SVR.

4. DISCUSSION

The present study evaluated the prevalence of RASs related with a poor response to NS5A inhibitors in 232 CHC infected patients with G1a and G3 (naïve to NS5A inhibitors), and the clinical implication of NS5A RASs testing on current HCV therapies.[4-11] A total of 166 patients with G1a (21.1% with cirrhosis, 28.3% treatment-experienced, 72.7% HCV-RNA >800 000 IU/mL) and 66 G3 (25.8% with cirrhosis, 19.7% treatment-experienced, 64.6% HCV-RNA >800 000 IU/mL) were included. The prevalence of baseline RASs to NS5A-inhibitors was 5.5% for G1a, being Q30H/R the most prevalent mutation identified (3.1%). The NS5A RAS Y93H was not recognized for any of G3 patients.

The prevalence of baseline NS5A RASs could vary according to the geographical region. In the United States, it has been described a prevalence ranging 9-12% for G1a.[5, 11, 17, 18] In Europe, recent studies have reported rates of prevalence ranging from 1.6% in Sweden to 12.5% in Italy.[19, 20] In Spain, Calleja et al[21] and Palladino et al[22] described rates of prevalence around 6%. These figures are similar to that observed in the present study. For G3, the scarce data available, show a rate of NS5A RASs ranging 3.8-8.8%.[6, 15] Nevertheless, the information available regard the prevalence of NS5A RASs should be carefully analyzed due to the lack of a standardized method for RASs detection, the selection of NS5A RASs considered for the analysis, and the number and clinical characteristics of the patient population included among the different studies.

Otherwise, the phenotypic resistance data generated from clinical trials indicate that only those RASs that confer medium- to high-level resistance might affect to the treatment response to DAAs. In this context, the most prevalent RAS identified in this study, Q30H/R, confers a high-level resistance (>100-fold change) to NS5A inhibitors.[1, 17]

In the current treatment scenario, the presence of clinically relevant NS5A RASs among G1a and G3 patients in combination with cirrhosis, prior treatment exposure, or high HCV-RNA >800 000 IU/mL would make necessary the use of ribavirin or extending treatment duration (16 or 24 weeks) for some regimens including NS5A inhibitors. This is the case of grazoprevir/elbasvir, sofosbuvir/ledipasvir, sofosbuvir/daclatasvir for G1a, and sofosbuvir/velpatasvir for G3.[2]

Herein, we analyzed the impact of the results obtained in this cohort of HCV G1a and G3 infected patients in the current clinical setting. In this cohort, many patients had, at least, one factor associated with lower SVR rates (78.9% in G1a, and 75.8% in G1b). However, since the proportion of HCV G1a and G3 patients harboring NS5A RASs is low (5.5% and 0.0%, respectively), the vast majority of them (>94%) could cure the HCV infection with 12 weeks of treatment without ribavirin when a NS5A RASs test was available.

The SVR was retrospectively analyzed in those patients with RASs, of them, half (4/8) had received a NS5A inhibitors-based therapy. Of note, only one of them had factors of poor response and therefore standard treatment was reinforced by adding ribavirin and the effect of baseline RASs was unmasked.

These figures indicate that the implementation of NS5A RASs resistance testing before the initiation of treatments including NS5A inhibitors seems to be helpful to optimize therapies and to avoid ribavirin-related toxicities, reduce treatment duration, and costs. However, NS5A RASs testing will be mainly focused on G1a and G3 patient's populations with characteristics associated with suboptimal SVR at 12 weeks.

There are scarce data regarding the prevalence of NS5A RASs in G1a and G3 CHC population outside clinical trials and none evaluating the impact of the presence of clinically relevant mutations for the current treatment options.[14, 15, 17, 18, 23, 24] Indeed, in the absence of a standard globally available methodology for testing HCV RASs, the latest AASLD and EASL recommendations highlight the usefulness of HCV resistance to NS5A inhibitors to guide physician's decisions in case of access to reliable resistance tests.[1, 2] Although deep sequencing might provide additional information, recent studies have shown that RASs present in low proportion (1-15%) do not significantly influence the response to DAAs therapies.[5, 11] Therefore, data obtained by population-based sequencing would be the appropriate to take clinical decisions.[1, 2] In this context and even in the absence of a standardized methodology for NS5A RASs testing, more data about the prevalence of NS5A RASs among HCV G1a and G3 patients and to evaluate their impact in the virologic response might be helpful for the design of optimized treatment strategies.

In summary, the prevalence of NS5A RASs in this cohort of patients with HCV G1a and G3 infection was low (5.5%) or null, respectively. However, there is a high proportion of patients harboring other factors associated with poor rates of virological response in combination with RASs, such as cirrhosis, prior exposure to treatment, and high HCV-RNA levels. For these patients, current guidelines recommended reinforcing treatment by adding ribavirin and/or prolonging treatment duration when these mutations are present. Therefore, testing NS5A RASs in these specific populations might be useful to optimize current NS5A-based therapies avoiding ribavirin-related toxicities and shortening treatment duration in the majority of patients.

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CONFLICTS OF INTEREST

All authors declare no conflicts of interest.

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