Natural NS5A inhibitor resistance associated substitutions in hepatitis C virus genotype 1 infected patients from Italy

TITLE PAGE

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Natural NS5A inhibitor Resistance Associated Substitutions in HCV genotype 1 infected patients from Italy

Running title: Natural HCV genotype 1 NS5A RASs in Italy

Authors name and affiliations

Cinzia Caudai[1], Angelo Materazzi[2], Francesco Saladini[2], Simona Di Gianbenedetto[3], Carlo Torti[4], Barbara Ricciardi[5], Barbara Rossetti[3,6], Paolo Almi[7], Andrea De Luca[6], Maurizio Zazzi[2].

1 Microbiology and Virology Unit, Siena University Hospital , Siena, Italy.
2 Department of Medical Biotechnologies, University of Siena, Siena, Italy.
3 Infectious Diseases Clinic, Catholic University of Sacred Heart, Rome, Italy, Italy.
4 Infectious Diseases Unit, Department of Medical and Surgical Sciences, "Magna Graecia" University, Catanzaro, Italy.
5 Infectious Diseases Unit, Misericordia Hospital, Grosseto, Italy.
6 Infectious Diseases Unit, Siena University Hospital, Italy.
7 Unit of Infectious Diseases and Hepatology, Siena University Hospital, Siena, Italy.
8 Infectious Diseases Clinic, Catholic University of Sacred Heart, Rome, Italy, Italy.

Corresponding Author

Cinzia Caudai
Microbiology and Virology Unit, Siena University Hospital,
Viale M. Bracci,16, Siena, Italy 53100
Phone +39 0577 233874
Fax +39 0577 233870
E-mail cinziacaudai@gmail.com

ABSTRACT

ObjectivesGenetic variability in NS5A is associated to different levels of resistance to the currently licensed NS5A inhibitors. The aim of this study was to detect NS5A inhibitor resistance associated substitutions (RASs) in HCV genotype 1 (GT1) patients naïve to direct acting HCV antivirals.

Methods

Amplification, Sanger sequencing and phylogenetic analysis of the HCV NS5A region were performed on plasma obtained from 122 consecutive patients with HCV chronic infection attending four different clinics in Italy.

Results

NS5A inhibitor RASs were detected in 14/61 (23.0%) HCV GT1b and 3/61 (4.9%) HCV GT1a infected patients (P = 0.007). The pan-genotypic RAS Y93H was detected in 1 (1.6%) GT1a and 4 (6.6%) GT1b cases. GT1a sequences clustered into two different clades with RASs detected in 1/34 (2.9%) clade I and 2/27 (7.4%) clade II sequences.

Conclusions

Although the impact of naturally occurring NS5A RASs might be limited with upcoming pan-genotypic treatment regimens, this information is still useful to map naturally occurring HCV variants in different geographic areas in the context of current HCV therapy.

KEYWORDS

HCV, NS5A, Drug resistance, DAAs, Peg-IFN/RBV.

ABBREVIATIONS

HCV: Hepatitis C Virus; NS5A: non-structural protein 5A; GT1: genotype 1; DAAs: Direct-acting antivirals; Peg-IFN/RBV: pegylated interferon alpha and ribavirin; SVR: sustained virological response; RASs: resistance-associated substitutions; PCR: polymerase chain reaction.

INTRODUCTION

The standard treatment of chronic HCV infection has been based on pegylated interferon alpha and ribavirin (Peg-IFN/RBV) up to few years ago. This therapy lasted up to 48 weeks, was associated with severe adverse effects and resulted in low sustained virologic response (SVR) rates in patients infected with HCV genotype 1 (GT1) [1]. The recent introduction of all-oral regimens of direct acting antiviral agents (DAAs) has resulted in significantly shorter treatment duration, better tolerability and higher SVR rates. Since 2014, new DAA generations have been approved enabling interferon-free antiviral treatments with SVR rates >90% [2]. The currently approved DAAs are directed against three viral targets: the NS3/4A protease, the NS5B RNA-dependent RNA-polymerase and the NS5A protein. NS5A is an optimal target because of its multiple roles in HCV life cycle including viral RNA binding, replication and assembly as well as down-regulation of the antiviral IFN response [3]. Anti-NS5A compounds are the most potent DAAs ever discovered showing a remarkable ability to inhibit HCV RNA replication with minimal toxicity *in vivo* [4]. Although the antiviral potency of most DAAs is impressive, the issue of rapid emergence of drug resistance associated substitutions (RASs) remains not completely solved. In addition, the effectiveness of most drugs is genotype/subtype-dependent and potentially influenced by the presence of natural RASs [5]. Indeed, natural NS5A variability has been shown to influence the success of different DAA regimens at least under specific circumstances [5].

In this study, we report the prevalence of naturally occurring NS5A inhibitor RASs in an unselected DAA-naïve HCV genotype 1 patient population with and without previous Peg-IFN/RBV treatment from Italy.

METHODS

A total of 122 consecutive plasma samples from distinct patients with HCV chronic hepatitis were analyzed. All patients were infected with HCV GT1a (n=61) or GT1b (n=61) as defined by sequencing of the 5'UTR/core region [6]. Viral load was determined by the commercial kit ABBOTT RealTime HCV (Abbott Diagnostics Inc., Abbott Park, IL, USA). All patients were naive to all DAAs and 49 out of 122 had previously failed Peg-IFN/RBV therapy. The patients were followed at four different clinics and participated to the Antiviral Response Cohort Analysis [www.dbarca.net] which was authorized by the South-East Tuscany Ethics Committee. Written informed consent was obtained from all patients before participated in.

Amplification of the whole NS5A region was obtained by random hexamer directed reverse transcription of HCV RNA and PCR using primers P665 5'-ACTAYGTGCCKGAGAGCGA-3' (fwd nt. 6139-6157) and P663 5'-GTCCAGRACYTGCAGTCTGTCAA-3' (rev nt. 7762-7784) (ref. H77 AF009606) for 40 cycles. Direct Sanger sequencing of the 1645-bp PCR product was performed by using the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies Corp., Carlsbad, CA, USA). Sequencing products were run in an ABI3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and the sequences were aligned to the H77 HCV 1a and to the Con1 HCV 1b prototypes by using Geneious v. 7.1. A phylogenetic tree was constructed with all the NS5A sequences by the neighbour-joining method using the PHYLIP software package version 3.69 (http://evolution.genetics.washington.edu/phylip.html) and edited with FigTree version 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/). Sequences are available at GenBank under accession codes KX688325 through KX688446.

Specific HCV GT1a and GT1b NS5A inhibitor RASs were retrieved based on the latest subtype-specific drug resistance mutation list, including only those mutations labelled as relevant *in vivo* for at least one of the licensed NS5A inhibitors [7]. The association between dichotomous variables was analyzed by the Fisher exact test or the chi-square test, depending on cell frequencies. The association between the presence of RASs and continuous variables was analyzed by the Mann-Whitney test. All the analyses were performed by SPSS 20 (IBM Corp., Armonk, NY, USA).

RESULTS

Overall, NS5A inhibitor RASs were detected in 17 (13.9%) cases, including 3 (4.9%) genotype 1a and 14 (23.0%) genotype 1b cases (P = 0.007) (**Tab. 1**). Phylogenetic analysis of the NS5A sequences revealed two distinct GT1a clades (34 clade I and 27 clade II) (**Fig. 1**). However, RASs were not significantly associated with clade (2.9% in clade I vs. 7.4% in clade II; P = 0.579). The most frequently detected RASs were at codon 58 and 93. When considering RASs affecting susceptibility to at least two of the five currently licensed NS5A inhibitors (28T/V, 30H and 93H for GT1a; 28M, 31M and 93H for GT1b), there were only 3 (4.9%) and 7 (11.5%) cases in GT1a and GT1b, respectively. There were two cases (1.6%) with double mutants expected to be highly resistant to NS5A inhibitors: 30H/93H in one GT1a clade II and 31I/58S in one GT1b.

There were no significant associations with age, HIV status, gender and median HCV RNA load but RASs were more prevalent in patients previously exposed than non-exposed to Peg-IFN/RBV (12/49 vs. 5/73, P = 0.008).

DISCUSSION

Currently licensed NS5A inhibitors such as daclatasvir, ledipasvir, ombitasvir, elbasvir and velpatasvir are included in virtually all potent treatment regimens and upcoming NS5A inhibitors will be a cornerstone of the future three-class anti-HCV regimens [8]. Nevertheless, NS5A inhibitors do select for drug-resistant variants at treatment failure and such isolates can persist as dominant or minority species for months to years after treatment cessation, making retreatment challenging [9]. In addition, NS5A inhibitor activity may be decreased by the presence of naturally occurring RASs, at least under specific circumstances [5]. While analysis of baseline HCV DAA RASs has not been integrated into the general clinical practice, two recommendations to test for the presence of RASs before treatment have been issued including the Q80K mutation prior to treatment with the NS3 inhibitor simeprevir and NS5A RASs prior to elbasvir treatment, although both limited to patients with HCV GT1a infection [10]. Thus, investigating the prevalence of naturally occurring NS5A inhibitor RASs in different geographic areas is still relevant.

The present study included 122 DAA-naive patients infected with HCV GT1, half GT1a and half GT1b. Similar to what reported with full-length HCV genome and with our previous NS3 analysis [11], GT1a sequences clustered into two different clades but we did not detect any NS5A inhibitor RAS preferentially associated with one clade. NS5A inhibitor RASs were detected in 13.9% of cases and were significantly more frequent in GT1b (23.0%) than in GT1a (4.9%). However, the latest global analysis of approximately one thousand sequences available worldwide through GenBank [7] indicates a less pronounced higher prevalence of NS5A inhibitor RASs in GT1b than GT1a (15.0% vs. 11.2%, P = 0.081), when the same RAS set is considered. Notably, the only previously

published study on a small number of NS5A sequences from Italian patients also detected a larger frequency of RASs in GT1b than in GT1a [12]. In that paper, the prevalence of NS5A inhibitor RASs in GT1b was reported as high as 53.3% but Q54H, a mutation no longer considered to affect NS5A inhibitor activity, accounted for half of the GT1b RAS cases. Thus, the results obtained in these two Italian studies are consistent and depict a distribution of naturally occurring NS5A inhibitor RASs which appears to be different from that derived from worldwide analysis.

The association between RASs and previous exposure to pegIFN/RBV detected in our case file is of potential interest but the sample size is underpowered to fully elucidate any potential causative association. A role of NS5A variability has been suggested in response to pegIFN/RBV, but there would seem to be no evidence that pegIFN/RBV does impact on the evolution of NS5A [13,14]. Since we could not compare pre and post treatment sequences, we cannot either confirm or deny this concept.

Although future three-class pan-genotypic anti-HCV therapy is expected to be minimally affected by HCV natural variability, the current treatment scenario is based on several NS5A inhibitor including regimens with a potential role for baseline RASs, particularly for difficult-to-treat patients. While genotype 1 is the most prevalent worldwide, other genotypes, particularly genotype 3, may also require thorough investigation of the role of natural NS5A polymorphisms due to a generally lower response to anti-HCV treatment [15]. In addition, the availability of upcoming most potent three-drug regimens will probably be significantly delayed in low-middle income countries, further supporting a role for natural NS5A polymorphisms.

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CONFLICT OF INTERESTS
The authors declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Table 1. Characteristics of the patient population and NS5A inhibitor resistance associated substitutions (RASs) detected.

FEATURE	All patients (n = 122)	Genotype 1a (n = 61) [34 clade I; 27 clade II]	Genotype 1b (n = 61)
Age, median (IQR)	54 (50-59)	53 (50-57)	54 (48-62)
Gender, males (%)	83 (68.0)	45 (73.8)	38 (62.3)
Previous pegIFN/RBV treatment failure, cases (%)	49 (40.1)	23 (37.7)	26 (42.6)
HIV infection, cases (%)	43 (35.2)	24 (39.3)	19 (31.1)
HCV RNA, median IU/ml [IQR]	5.86 (5.07-6.30)	6.01 (5.25-6.51)	5.76 (5.00-6.20)
RAS, cases (%)[a]			
28M	1 (0.8)	NA[c]	1 (1.6)
28T	1 (0.8)	1 (1.6) [1; 0]	0
28V	1 (0.8)	1 (1.6) [0; 1]	NR[d]
30H	1 (0.8)	1 (1.6) [0; 1]	0
30Q	2 (1.6)	NR[d]	2 (3.3)
31I	1 (0.8)	0	1 (1.6)
31M	2 (1.6)	0	2 (3.3)
58S	5 (4.1)	NR[d]	5 (8.2)
93H	5 (4.1)	1 (1.6) [0; 1]	4 (6.6)
Any RAS[b]	17 (13.9)	3 (4.9) [1; 2]	14 (23.0)

[a]For genotype 1a, numbers in square brackets indicate cases in clade I and clade II

[b]The total count does not match the sum of individual cases because there were two double mutant cases (one 30H/93H in genotype 1a, clade II; one 31I/58S in genotype 1b).

[c]Not applicable (28M is the consensus amino acid in genotype 1a; 30Q is the consensus amino acid in genotype 1a).

[d]Not reported because the variant is not a RAS in the specific genotype.

FIGURE LEGENDS

Figure 1. - Phylogenetic tree of HCV NS5A sequences belonging to 122 subjects infected with HCV genotype 1. Subtype 1c reference are included as an outgroup. Reference sequences are labelled by an asterisk (US H77-H21 AF011753 and H77 NC_004102 for genotype 1a clade I, DE BID_V25EU482831 and CH BID-V271 EU482858 for genotype 1a clade II; CH BID_V272 EU482859, AU HCV_A AJ 000009 and JP HCV_BK M58335 for genotype 1b; ID HC G9 D14853 and IN AY051292 for genotype 1c).

Figure 1