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Detection of anti-protease inhibitors resistance mutations in HCV strains infecting treatment-naïve chronic patients from Romania

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Abstract

***Background**: Severe complications of chronic hepatitis C – i.e. cirrhosis and hepatocellular carcinoma – are important causes of morbidity and mortality worldwide. Despite the overwhelming rates of sustained virologic response achieved after therapy with different combinations of direct-acting antiviral drugs (DAAs), treatment failure is still recorded, and is due to the mutations harboured by hepatitis C virus (HCV) resistance associated variants (RAVs) selected during therapy. Baseline RAVs testing was found significant for guiding treatment in the cases of treatment failure and, sometimes, in naïve patients.*

***Methods**: Romanian chronic hepatitis C patients unexposed to DAAs and infected with subtype 1b HCV were studied. Serum samples were used for Sanger population sequencing of a fragment containing NS3 viral protease, known to harbour resistance mutation against protease inhibitors (PIs).*

***Results**: Catalytic triad and zinc-binding site in the studied sequences were conserved. Low-intermediate resistance mutations to first generation PIs were detected either alone or in conjunction with resistance substitutions associated with second generation PIs. Cross-resistance and reduced susceptibility to certain DAAs were observed.*

***Discussion**: This study focused on HCV patients infected with subtype 1b strains, the most prevalent in Romania. The rate of RAVs found in this work is consistent with the results reported by similar studies from other countries. Noticeably, numerous polymorphisms of unknown significance to DAAs resistance, but reflecting the*

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high genetic variability of HCV, were found in the studied sequences. Testing for RAVs can be a useful method for guiding treatment in a cost-efficient manner in developing countries where access to DAAs is limited.

***Keywords**: NS3 protease, direct-acting antivirals, resistance mutations, naïve to treatment patients*

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Introduction

World Health Organization estimated for 2015 that 71 million persons were chronically infected with hepatitis C virus (HCV). A fraction of these patients will further develop severely decompensated cirrhosis and/or hepatocellular carcinoma, the main indications for liver transplantation (1).

HCV is an enveloped, positive single-stranded RNA virus (*Flaviviridae* family, *Hepacivirus* genus, *Hepacivirus C* species) characterised by a high genetic diversity, reflected at intra-host level by quasispecies, and respectively, at inter-host level by the worldwide circulation of seven genotypes and more than 60 subtypes (2-5).

The advent in 2011 of first direct-acting antivirals (DAAs) targeting NS3-NS4A viral serine protease has raised the chances of obtaining sustained virologic response (SVR) for up to 70% of genotype 1 infected patients (6, 7). However, these first generation protease inhibitors (PIs), namely boceprevir and telaprevir, were efficient only when administrated as triple therapy in combination with interferon and ribavirin, and exhibited side effects (8). Treatment failure was recorded in cases of triple therapy and was due to resistance associated variants (RAVs) selected during therapy (9). The need for interferon-free regimens, with pan-genotypic activity, displaying high genetic barrier to resistance (GB), and with minimal side effects led to development of second wave DAAs. These highly efficient compounds (SVR rates above 90%) were targeted against NS3, NS5A and NS5B viral proteins and are administered in different combinations. However, it has been shown that the presence of RAVs at baseline and their expansion during the antiviral therapy contribute to treatment failure in the case of second generation DAAs, also. For some regimens, testing for RAVs at baseline was found significant for guiding the treatment and is now recommended or even mandatory (10).

A small number of HCV infected patients in Romania were exposed to DAAs, since such regimens were only used there in clinical trials (11), limited studies (12, 13) and for the treatment of chronic patients with advanced fibrosis (F3 and F4 scores) (14). A recent autochthonous study showed 100% SVR among subtype 1b chronic Romanian patients undergoing DAAs treatment with ombitasvir/paritaprevir/ritonavir, dasabuvir with and without ribavirin for 12 weeks and that the quantitative regression of liver stiffness was inversely correlated with the duration of the HCV infection (15).

We aimed to characterise the pattern of resistance to PIs displayed by the subtype 1b HCV strains, the most prevalent in Romanian population (92.6%) (16).

Methods

Study lot

Forty-four 1b subtype HCV chronically infected Caucasian patients unexposed to any antiviral therapy were selected for study. Viral subtyping was assessed elsewhere by sequencing a fragment of core region (17). The study was approved by the Bioethics Committee of Cantacuzino National Medico-Military Institute for Research and Development and conducted in accordance with the ethical principles stated in the Declaration of Helsinki. Each patient provided informed consent.

Detection of mutations in NS3 protease viral region

Viral RNA was extracted from 140 µL of serum using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) and used for reverse transcription followed by nested-PCR amplification of a fragment spanning codons 1-181 of NS3 protease region (18). PCR products were gel-purified (Wizard SV Gel and PCR Clean-up System, Promega, Madison, WI, USA) and sequenced with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Austin, TX, USA) on a 3130 Genetic Analyzer (Applied Biosystems, Tokyo, Japan). Sequences were visually inspected and proofed with BioeEdit version 7.2.5 (19). Substitutions associated with DAAs resistance were detected using Geno2pheno [hcv 0.92] online tool (20).

Results

No substitutions affecting the catalytic triad of NS3 protein (H57, D81 and S139 amino acid residues) were found. Degeneracy in codons for all three amino acid residues of the catalytic triad was observed for a limited number of samples. Zinc binding site (*i.e.* amino acid residues C97, C99, C145 and H149) was found to be conserved in all the studied samples.

Four of the analysed HCV samples (4/44, 9%) harboured at least one substitution conferring resistance to first or second generation PIs (Table).

The mutated isolates were found either exclusively or in mixture with the wild type variants. The quasispecies infecting one patient (*i.e.* no. 5, Table) harboured three substitution (41H, 168E and 174F) responsible for cross-resistance or conferring reduced susceptibility to first and

Table. Resistance pattern to direct- acting antivirals in patients infected with 1b subtype HCV strains. Analysis was conducted using Geno2pheno [hcv 0.92] online tool (20). Bold: resistance mutations; normal font: substitution conferring reduced susceptibility.

	Boceprevir	Telaprevir	Simeprevir	Paritaprevir	Grazoprevir	Voxilaprevir	Asunaprevir
1.	-	-	-	-	56F	-	-
2.	-	-	-	-	56F	-	-
3.	-	-	-	-	56F	-	-
4.	-	-			56F	-	-
5.	**41H**, **168E**, **174F**	174F	**168E**	168E	56F, 168E	-	**41H**, **168E**
6.	**54S**, **55A**, **170I**	54S, 55A, 170I	-	-	-	-	-
7.	-	117H, 170I	-	-	56F	-	-
8.	-	-	-	-	56F	-	-
9.	-	-	-	-	56F	-	-
10.	-	-	-	-	56F	56F, 122N	-
11.	-	-	-	-	56F	-	-
12.	-	-	-	-	56F	-	-
13.	-	-	-	-	56F	-	-
14.	**36L**	36L, 117C	36L	-	36L	-	36L
15.	-	-	-	-	56F	-	-
16.	-	-	-	-	56F	-	-
17.	-	-	-	-	56F	-	-
18.	-	-	80H	-	56F	-	-
19.	-	117H, 170I	-	-	56F	-	-
20.	**55A**	55A	-	-	-	-	-

second generation PIs, respectively resistance to boceprevir, simeprevir, and asunaprevir and susceptibility to telaprevir, paritaprevir and grazoprevir. Substitutions responsible only for resistance to boceprevir and susceptibility to telaprevir (36L, 54S, 55A and 170I) – the two compounds from the first generation PI class – were detected in the case of three patients (*i.e.* no. 6, 14 and 20, Table). Noticeably, substitution 56F responsible for reduced susceptibility to grazoprevir – a second generation PI – was found in a significant number of isolates (17/44, 39%). Other substitutions related to reduced susceptibility to first wave and second wave PIs were found (Table). Numerous polymorphisms of unknown relevancy for DAAs resistance were detected, also.

Discussion

The advent of the new interferon-free DAAs regimens has drastically changed the course of chronic hepatitis C infection, making it a curable disease for an impressive number of patients (>95%) (21). DAAs are targeted against NS3, NS5A, and NS5B viral proteins display different GB and, in some cases, can induce the selection of pre-existing resistance mutants (22). Currently, DAAs are recommended to be administered as double/triple combination regimens, mainly due to the relatively low GB displayed by NS5A and NS3-4A targeting compounds. Specific combinations of DAAs are addressed to each genotype/subtype, to special populations or are used for patients with different comorbidities. Furthermore, pan-genotypic formulations are available, also. Indeed, NS5A RAVs are frequently found prior to the initiation of treatment and were associated with lower rates of SVR. Therefore, the highly efficient anti-NS5A compounds must be used together with anti-NS3 and/or anti-NS5B specific agents. In the case of pre-existing RAVs, ribavirin may be used to enhance the effect of DAAs combination (23, 24).

The viral serine protease NS3-NS4A is responsible for polyprotein processing and its substrate binding site is blocked by PIs. Substitutions at specific positions conferring different levels of resistance to a certain PI are well described and are genotype/subtype specific (22). Regardless of genotype and exposure to treatment, resistance mutations against DAAs were found in HCV isolates worldwide, as proofed by studies conducted on sequence databases (25, 26). Furthermore, studies have shown the presence of DAAs resistance mutations in different unexposed populations (27, 28). However, there are no clear indications for baseline screening of NS3 PIs resistance mutations in the absence of prior PIs exposure, nevertheless clinicians should be aware of Q80K polymorphism in 1a subtype isolates and resistant variants at positions 155, 156 and 168 in genotype 1 isolates (29).

The present study focused on a population of chronic hepatitis C patients from Romania, unexposed to DAAs. All the patients selected for this study were infected with subtype 1b HCV strains, the most prevalent in our country (16). Isolates of this subtype are prone to severe complications and display intrinsic resistance to interferon and ribavirin regimen (30), which is still widely used in Romania. It has been shown that frequency of RAVs in 1b subtype isolates is lower comparing to 1a subtype isolates (31). No substitutions were found in the catalytic triad and zinc binding site – important structures for the functionality of NS3 protein (32, 33). Polymorphisms responsible for low-intermediate resistance to first generation PIs, second generation PIs, or for conferring cross-resistance were found in our study lot at a rate of 9%. This finding is consistent with previous reports (34, 35).

A recent study found variants at position 168 at high frequency (41%) in subtype 1b-infected

patients not achieving SVR after simeprevir-sofosbuvir treatment. Particularly, substitution 168E – found also in one patient included in the present study – was frequently identified after simeprevir- or paritaprevir-based treatment failure (36). Moreover, substitutions at position 168 (V/E/T) were associated with high frequency of failure (84.2%) after daclatasvir-asunaprevir therapy in 1b-infected patients initially treated with simeprevir (37).

Substitutions 54S and 55A, detected in our samples also, are known to give low-medium level of resistance to boceprevir (38) and were found at frequencies of 6.85%, and respectively 3.42% in a Scottish cohort of treatment naïve patients (39). These mutations were found at high rates (37% for 54S and 24% for 55A) in 1b-infected patients not achieving SVR after boceprevir treatment (40). Our previous studies have shown the presence of 54S and 55A substitutions in a naïve patient from Romania (18) and the presence of 54S mutation in four boceprevir-interferon-ribavirin relapse cases (13).

Sanger-based population sequencing method used for identification of mutations in this study has a poor sensitivity, polymorphisms at frequencies below 20% not being detected (41). Furthermore, in the case of the detection of multiple polymorphisms it is impossible to establish if a single viral variant harbours all the substitutions or if they are scattered across the swarm of quasispecies. Given the fact that there is a consensus of 15% cut-off for studies assessing the impact of quasispecies on treatment outcome (24), new methods based on deep-sequencing are more suitable for a sensitive detection of low-frequency RAVs (42).

Our limited study focused only on the mutations harboured by NS3 viral protease, which, along with NS5A, are the most prone to mutation proteins targeted by DAAs. Of course, an approach investigating all three targets of DAAs, using deep-sequencing, and on a larger number of samples of different genotypes/subtypes would be desirable.

Testing for RAVs, at least for patients not achieving SVR, can represent an important tool for guiding the treatment in a cost-efficient manner in a developing country, such as Romania, where DAAs are available only for a limited number of chronic hepatitis C patients.

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Authors' contribution

SD performed the PCRs, sequencing and sequence analysis and drafted the manuscript; GT enrolled the patients, collected the samples and revised the manuscript; EC, SYF, LM, AME and MM enrolled the patients and revised the manuscript; GO coordinated the study and revised the manuscript.

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