Virus Research 223 (2016) 140–146

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

# Naturally occurring NS3 resistance-associated variants in hepatitis C virus genotype 1: Their relevance for developing countries

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ARTICLE INFO

*Article history:*
Received 6 July 2016
Received in revised form 15 July 2016
Accepted 18 July 2016
Available online 19 July 2016

*Keywords:*
HCV variability
DAAs
Resistance-associated variants

ABSTRACT

Hepatitis C virus (HCV) is a major cause of global morbidity and mortality, with an estimated 130–150 million infected individuals worldwide. HCV is a leading cause of chronic liver diseases including cirrhosis and hepatocellular carcinoma. Current treatment options in developing countries involve pegylated interferon-α and ribavirin as dual therapy or in combination with one or more direct-acting antiviral agents (DAA). The emergence of resistance-associated variants (RAVs) after treatment reveals the great variability of this virus leading to a great difficulty in developing effective antiviral strategies. Baseline RAVs detected in DAA treatment-naïve HCV-infected patients could be of great importance for clinical management and outcome prediction. Although the frequency of naturally occurring HCV NS3 protease inhibitor mutations has been addressed in many countries, there are only a few reports on their prevalence in South America. In this study, we investigated the presence of RAVs in the HCV NS3 serine protease region by analysing a cohort of Uruguayan patients with chronic hepatitis C who had not been treated with any DAAs and compare them with the results found for other South American countries. The results of these studies revealed that naturally occurring mutations conferring resistance to NS3 inhibitors exist in a substantial proportion of Uruguayan treatment-naïve patients infected with HCV genotype 1 enrolled in these studies. The identification of these baseline RAVs could be of great importance for patients' management and outcome prediction in developing countries.



## 1. Introduction

Hepatitis C virus (HCV) is a significant human pathogen affecting nearly 3% of the world's population, and is a leading cause of chronic liver diseases including cirrhosis and hepatocellular carcinoma. Infections with HCV have become a major cause of liver cancer and one of the most common indications for liver transplantation (Hoofnagle, 2002; Martin et al., 2013; Pawlotsky, 2003; Simmonds, 2004; World Health Organization, 2015).

HCV belongs to the family *Flaviviridae* and has a single stranded positive sense RNA genome that is 9.6 kb in length. This genome contains a single open-reading frame and encodes a unique polyprotein that is processed to yield ten structural (core, E1 and E2) and non-structural (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) proteins (Scheel and Rice, 2013).

The high error rate of the viral RNA-dependent RNA-polymerase and the pressure exerted by the host immune system, have driven the evolution of HCV towards the development of a global diversity that reveals the existence of seven genetic lineages (genotypes 1–7) and more than 67 subtypes (Smith et al., 2014). Subtypes 1a, 1b and 3a are widely distributed and account for the vast majority of infections in Western countries including the South American region (World Health Organization, 2015).

HCV NS3 protein is responsible for processing the non-structural region of the viral polyprotein. NS3 is a bifunctional protein with an amino-terminal domain exhibiting a zinc-dependent serine protease activity, and a carboxyl-terminal one with helicase activity (Bartenschlager and Lohmann, 2000).

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http://dx.doi.org/10.1016/j.virusres.2016.07.008
0168-1702/

With the advent of direct antiviral agents (DAAs) for the treatment of HCV infections, there is a need to monitor the emergence of resistance-associated variants before and after treatment (Fonseca-Coronado et al., 2012). Although the most recent DAAs increase response rates and allow shortened and simplified regimens, the emergence of genetic variants that could induce changes in the drug binging properties of these compounds, and by doing so, generate resistant phenotypes, is a matter of concern and may jeopardize the effectiveness of DAAs (Wyles, 2013). These resistance-associated variants (RAVs) usually emerge after a few days of DAA treatment and have been accounted responsible for the failure or hypo responsiveness to treatment. Moreover, they can also be detected in HCV-infected treatment-naïve patients (Applegate et al., 2015).

The circulation of DAAs resistance mutations in treatment-naïve patients has not been reported in depth in South America, with the exception of Brazil (de Carvalho et al., 2014; Lisboa-Neto et al., 2015; Peres-da-Silva et al., 2012; Zeminian et al., 2013) and Argentina (Sede et al., 2015).

It is extremely important for low income countries, like Uruguay and other South American countries, to determine the presence of naturally occurring RAVs prior to the incorporation of these expensive DAAs regimens to treat HCV-infected patients.

In this study, we investigated the presence of resistance variants to protease inhibitors (PIs) in the HCV NS3 serine protease region, by analysing a cohort of Uruguayan patients with chronic hepatitis C who had not been treated with any DAAs.

## 2. Materials and methods

### 2.1. Patients and clinical samples

Serum samples were obtained from 20 patients with serological markers for HCV which attended either the Hospital de Clínicas or the Asociación Española (Montevideo, Uruguay). All of the patients were DAA treatment-naïve at the time of blood extraction. Written informed consent was obtained from all patients. The studies have been performed according to the World Medical Association Declaration of Helsinki and approved by appropriate institutional boards.

### 2.2. RNA extraction, cDNA synthesis and NS3 amplification

Viral RNA was extracted from serum using the QIAamp Viral RNA mini Kit (QIAgen). cDNA synthesis and PCR amplification of the complete NS3 genome region was carried out as previously described (Chusri et al., 2015; Vicenti et al., 2012). Amplicons were purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Lifescience).

### 2.3. NS3 sequencing

Full-length NS3 sequencing was performed by Macrogen, Korea, with previously described primers (Vicenti et al., 2012).

### 2.4. NS3 genotype determination and sequence analysis

HCV NS3 sequences obtained from Uruguayan patients were aligned with sequences from strains corresponding to representatives of all HCV genotypes, isolated in different geographic regions of the world. Sequences were obtained from Los Alamos HCV Sequence database (Kuiken et al., 2005). For strains enrolled in these studies, see Supplementary material Table S1. Sequences were aligned using the CLUSTAL W software (Thompson et al., 1994). Once aligned, the best evolutionary model that described our sequence data was assessed using Modelgenerator program (Keane et al., 2006). Using the T92 plus gamma plus invariant sites model, Maximum Likelihood Phylogenetic trees were constructed using the MEGA 5.0 software (Tamura et al., 2011). As a measure of the robustness of each node, we employed the bootstrapping method (500 pseudo-replicates).

In order to properly identify substitution changes in NS3 region from HCV strains circulating in Uruguayan patients, we generated consensus sequences for 1a and 1b subtypes using a wide range of NS3 sequences from HCV strains isolated from all regions of the world. For this purpose, we downloaded 110 genotype 1a and 100 genotype 1b NS3 sequences from Los Alamos HCV Sequence database (Kuiken et al., 2005) and aligned them using the CLUSTAL W program (Thompson et al., 1994). Once aligned, we generated each genotype consensus sequence by using the Lasergene application SeqMan, Version 7.0.0 (DNASTAR. Madison, WI), and each NS3 sequence derived from Uruguayan patients was compared to the corresponding generated consensus sequence in order to determine the substitutions present.

### 2.5. Signature pattern analysis of HCV genotype 1a

Viral Epidemiology Signature Pattern Analysis (VESPA) identifies particular sites in amino acid or nucleic acid alignments of a set of sequences that are distinctly represented between a query set and a background set (Korber and Myers, 1992).The query set included 26 sequences corresponding to HCV NS3 region subtype 1a isolated in South America (Brazil and Uruguay). The background set included 146 sequences corresponding to HCV NS3 region subtype 1a isolated elsewhere, which were downloaded from Los Alamos HCV Sequence Database (Kuiken et al., 2005).

### 2.6. Mapping amino acid substitutions in NS3 3D structure

The crystallographic structure of HCV NS3 protein was determined by Schiering, and co-workers (Schiering et al., 2011) and deposited in the Protein Data Bank (PDB) under accession ID: 4A92. In order to map amino acid substitutions found in Uruguayan strains, crystallographic data were imported from PDB database and amino acids substitutions found in HCV NS3 protein from Uruguayan patients were mapped using Pymol (Seeliger and De Groot, 2010).

## 3. Results

### 3.1. Genetic variability of NS3 genes from HCV circulating in uruguayan patients

To gain insight into the genetic variability of NS3 region of HCV strains circulating in Uruguayan patients, sequences of this region were aligned with corresponding sequences from 59 HCV strains isolated elsewhere, representing all genotypes and main sub-types. For strains included in these analyses, see Supplementary material Table S1. Once aligned, maximum likelihood phylogenetic trees were generated. The results of these studies are shown in Fig. 1.

All strains in the tree are assigned according with their genotype and each cluster is supported by very high Bootstrap values (see Fig. 1). All the strains isolated from Uruguayan patients (n = 20) were assigned to genotype 1 (15 are subtype 1a and 5 are subtype 1b, see Fig. 1).

### 3.2. NS3 substitution analysis

In order to study the amino acid substitutions in NS3 protein, NS3 sequences obtained from all patients enrolled in this study were aligned using the CLUSTAL W program (Thompson et al., 1994) and amino acid substitutions at positions previously found to be potentially associated with resistance to both first and second