Short communication

Natural prevalence of resistance-associated variants in hepatitis C virus NS5A in genotype 3a-infected people who inject drugs in Germany

Andreas Walker\textsuperscript{a}, Holger Siemann\textsuperscript{b}, Svenja Groten\textsuperscript{c}, R. Stefan Ross\textsuperscript{c}, Norbert Scherbaum\textsuperscript{b}, Jörg Timm\textsuperscript{a,}\textsuperscript{*}

\textsuperscript{a} Institute for Virology, Heinrich-Heine-University, University Hospital, Düsseldorf, Germany
\textsuperscript{b} LVR-Hospital Essen, Department of Addictive Behavior and Addiction Medicine, Faculty of Medicine, University of Duisburg-Essen, Germany
\textsuperscript{c} Institute of Virology, University of Duisburg-Essen, University Hospital Essen, Essen, Germany

ARTICLE INFO

Article history:
Received 30 April 2015
Received in revised form 30 June 2015
Accepted 6 July 2015

Keywords:
Hepatitis C virus
NS5A
Antiviral treatment
Genotype 3a

ABSTRACT

Background: People who inject drugs (PWID) are the most important risk group for incident Hepatitis C virus (HCV) infection. In PWID in Europe HCV genotype 3a is highly prevalent. Unfortunately, many of the recently developed directly acting antiviral drugs against HCV (DAAs) are suboptimal for treatment of this genotype. Detection of resistance-associated variants (RAV) in genotype 3a may help to optimize treatment decisions, however, robust protocols for amplification and sequencing of HCV NS5A as an important target for treatment of genotype 3a are currently lacking.

Objectives: The aim of this study was to establish a protocol for sequencing of HCV NS5A in genotype 3a and to determine the frequency of RAVs in treatment-naive PWID living in Germany.

Study design: The full NS5A region was amplified and sequenced from 110 HCV genotype 3a infected PWID using an in-house PCR protocol.

Results: With the established protocol the complete NS5A region was successfully amplified and sequenced from 110 out of 112 (98.2%) genotype 3a infected PWID. Phylogenetic analysis of sequences from PWID together with unrelated genotype 3a sequences from a public database showed a scattered distribution without geographic clustering. Viral polymorphisms A30K and Y93H known to confer resistance in a GT3a replication model were present in 8 subjects (7.2%).

Conclusions: A protocol for amplification of nearly all GT3a samples was successfully established. Substitutions conferring resistance to NS5A inhibitors were detected in a few treatment-naive PWID.

© 2015 Elsevier B.V. All rights reserved.

1. Background

During the last few years, several directly acting antivirals (DAAs) became available for the treatment of HCV [1], starting a new era of therapy of chronic hepatitis C. Most novel compounds were optimized for inhibition of viral replication of HCV genotype 1a and 1b and typically show no or much weaker activity against genotype 3a. At the moment, only the nucleoside analogue sofosbuvir and the NS5A inhibitors ledipasvir and daclatasvir are approved by the European medicines agency (EMA) for treatment of patients infected with HCV genotype 3a. If an NS5A inhibitor is used for treatment, a combination with sofosbuvir with or without ribavirin is currently recommended (guidelines of the German medical society) [2]. Although the number of patients that have been treated with this combination is still limited, sustained viral response rates between 63% and 96% have been reported, depending on the fibrosis stage, prior treatment experience and the duration of therapy.

Illicit intravenous drug use has become the most important risk factor for incident HCV infection. In 2013 more than 80% of the newly diagnosed HCV infections in Germany were most likely transmitted by intravenous drug use[3]. Notably, HCV genotype 3a is associated with injection drug use [4] and is the most frequent subtype in a single-center cohort of PWID collected in Essen, Germany (data not shown).

2. Objectives

The role of resistance associated variants (RAV) for treatment of patients infected with HCV genotype 3a is largely unclear. For
detection and monitoring of RAVs reliable protocols for amplification
and sequencing of NS5A are required. The aim of this study was
to establish a set of primers for amplification of genotype 3a and to
utilize the developed PCR protocol to determine the frequency of
RAVs in HCV genotype 3a in a cohort of treatment-naïve PWID in
Germany.

3. Study Design

Blood samples from patients with a history of injection drug
use were collected from the ward for inpatient detoxification
treatment of drug addicts or the clinic for opioid maintenance
 treatment (OMT) at the Department of Addictive Behavior and
Addiction Medicine, LVR-Hospital Essen, Hospital of the University
of Duisburg-Essen. Written informed consent was obtained from
all study participants and the study was approved by the ethics
committee of the Medical Faculty of the University of Duisburg-
Essen in accordance with the Declaration of Helsinki. Viral RNA
was extracted from all patients (n = 112) infected with genotype
3a (determined by sequencing of the core region [5]) with the
QIAamp viral RNA Kit (Qiagen, Hilden, Germany) according to
the manufacturer’s protocol. An alignment of all available GT3a
sequences from the HCV sequence database between position
nt5000 and nt8000 was used to design primers covering nearly all
GT3a variants (Table 1). RNA was transcribed with Superscript III
(Invitrogen) with the reverse primer GT3a-7544-R (final concen-
tration 0.5 pmol/µl). NS5A was amplified in a two-step nested PCR
using GoTaq Polymerase (Promega) with the primers as outlined in
Table 1 and the following PCR conditions: 120 s at 94 ◦C followed
by 35 cycles each 30 s 94 ◦C, 30 s 55 ◦C and 160 s 72 ◦C followed
by 10 min at 72 ◦C. The PCR products were directly sequenced and
sequences were aligned with the software Geneious 7.1.5 (Biomat-
ters, Auckland, New Zealand). All sequences were submitted to
GenBank (#KR082016–KR082126) Table 2.

4. Results

The PCR protocol was validated with samples of 112 HCV-RNA
positive treatment-naïve PWID with a broad range of viral load
(median 2.4 × 10⁵ IU/ml, range 615–3.4 × 10⁷ IU/ml). Amplification
of the expected 2 kb fragment was successful in 110 of 112 (98.2%)
samples. The samples for which the PCR has failed had a viral load of
Fig. 1. Phylogenetic tree of NS5A sequences from HCV genotype 3a. NS5A sequences
from PWID collected in Germany (blue or red) and from the HCV database (black)
were aligned and a phylogenetic tree was calculated with the neighbor-joining
method and the Jukes–Cantor distance model. Sequences with RAVs are shown in
red and are marked with red dots (Y93H) or black squares (A30K). (For interpreta-
tion of the references to color in this figure legend, the reader is referred to the web
version of this article).

3.502 and 930 IU/ml, respectively. Notably, 10 of 12 samples with a
viral load below 10.000 IU/ml were positive in the PCR including the
sample with the lowest viral load in the cohort (615 IU/ml). Bulk
sequencing of all 110 PCR products was performed with primers
listed in Table 1. A neighbor-joining phylogenetic tree of the result-
ning NS5A nucleotide sequences revealed that the sequences from
PWID in Germany are equally distributed among other unrelated
GT3a sequences (Fig. 1). Polymorphisms known to confer resistance
against NS5A inhibitors in genotype 3a were previously identified
in a subgenomic replication model and include the substitutions

---

**Table 1**

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Primer name</th>
<th>Primer sequence (5'→3')</th>
<th>Amplicon size</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT</td>
<td>GT3a-7544-R</td>
<td>ATCGCCCGGCTCYCCCTCGA</td>
<td>2403 bp</td>
</tr>
<tr>
<td>PCR-1</td>
<td>GT3a-5145-F</td>
<td>CCRAGYTGGGACGAGAYGTGGA</td>
<td>2175 bp</td>
</tr>
<tr>
<td>PCR-2</td>
<td>GT3a-7444-R</td>
<td>GTGTGTCRACCCCRGAGGATGA</td>
<td></td>
</tr>
<tr>
<td>Sequencing</td>
<td>GT3a-5275-F</td>
<td>TCATGGYATGCATGTCAGCYGA</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2**

The frequency of RAVs in GT3a infected, treatment-naïve PWID.

<table>
<thead>
<tr>
<th>Position</th>
<th>28</th>
<th>30</th>
<th>31</th>
<th>32</th>
<th>93</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prototype</td>
<td>M</td>
<td>(108)</td>
<td>A</td>
<td>(98)</td>
<td>L</td>
</tr>
<tr>
<td>Substitutions</td>
<td>V</td>
<td>(1)</td>
<td>K</td>
<td>(5)</td>
<td>T</td>
</tr>
</tbody>
</table>

a 44-fold IC50 change (6).
b 2154-fold IC50 change (6).
A30K, L31 V/M and Y93H [6]. Additional resistance associated positions identified in genotype 1 are 28, 32 and 58, however, it is not clear if substitutions at these positions in genotype 3a impact susceptibility to NS5A inhibitors. The residue L31 and P32 were fully conserved in our cohort and substitutions at position M28 were observed in 2 of 110 sequences (M28V and M28L). Residue A30 was more polymorphic with 12 of 110 sequences (10.7%) harboring substitutions (A30KTVSL). Of these polymorphisms, only the A30K substitution was shown to confer reduced susceptibility to the NS5A inhibitors daclatasvir and ledipasvir and was present in 5 of 110 (4.5%) subjects. The most important position for resistance to NS5A inhibitors is position 93. Variations from the prototype residue tyrosine (Y93HF) were observed in 4 of 110 patients (3.6%) including three patients (2.7%) with the substitution Y93H known to confer high-level resistance to NS5A inhibitors. Taken together, polymorphisms at positions associated with resistance to NS5A inhibitors were detected in 18 of 110 (16.4%) PWID infected with genotype 3a including two substitutions (A30K and Y93H) known to confer resistance to NS5A inhibitors in genotype 3a that were detected in 5 and 3 PWIDs, respectively.

5. Discussion

The clinical relevance of resistance mutations for treatment of HCV genotype 3a with DAAs is currently largely unclear. Although irrespective of the infecting genotype RAVs were detected in the majority of patients who fail to achieve sustained virological response (SVR) in clinical studies, the frequency of treatment failures with new combinations of DAAs is overall low [7,8]. Nevertheless, there is evidence from HCV genotype 3a infected patients treated with sofosbuvir and daclatasvir that pre-existing RAVs in NS5A were more frequent in the small number of patients experiencing viral relapse. In the ALLY-3 trial six of 13 patients (46%) who carried the Y93H substitution before treatment had a viral relapse whereas only ten of 134 (7.5%) of patients carrying the prototype residue tyrosine in this position failed to achieve SVR [8]. This strongly suggests that in genotype 3a presence of this substitution at baseline negatively impacts the response to combination therapy with an NS5A inhibitor and longer treatment duration may be considered. Importantly, it has been reported that RAVs in NS5A selected during treatment are associated with low fitness costs and can be detected for months after treatment cessation as the predominant variant of the quasispecies [9]. This is in line with the detection of NS5A RAVs in treatment naive patients. The Y93H substitution was reported in 13 of 147 (8.8%) in the ALLY-3 trial. Similar frequencies were previously reported in a study by Hernandez et al. (8 of 100; 8%) [6]. In PWIDs from Germany we observed the Y93H substitution as the dominant variant of the quasi species in 3 of 110 (2.7%) patients. Future studies specifically aimed to determine if detection of this substitution prior to therapy impacts treatment outcome or influences therapy decisions will be needed. The protocol presented here for amplification and sequencing of the NS5A region in genotype 3a may help addressing this question.

Competing interests

None declared.

Ethical approval

The study was approved by the ethics committee of the Medical Faculty of the University of Duisburg-Essen in accordance with the Declaration of Helsinki.

Acknowledgement

The study was supported by a grant of the German Ministry of Health to the National Reference Centre for Hepatitis C (Essen).

References