Selection of fitness-associated substitutions in patients failing NS5A inhibitors based therapy: analysis of HCV full-length genome deep sequencing

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When treatment fails, NS5A inhibitors select for resistance associated substitutions (RASs) located in domain I of the NS5A protein. NS5A RASs persist for many years after failure.

By analogy to HIV or HBV resistance, it is thought that compensatory or fitness-associated substitutions are also selected during treatment.

These substitutions could at least partly explain the conserved fitness of resistant variants and/or explain the long-term persistence of NS5A RASs.
Objective

- Very limited information on compensatory mutations selected outside targeted genes has been generated thus far, principally due to the sequencing methods available that characterize short genome fragments only.

- Here, we used an in-house original shotgun metagenomics method to sequence full-length HCV genomes.

- The objective is to characterize AA substitutions selected by DAA therapy in regions targeted or not targeted by NS5A inhibitors in patients who fail to achieve SVR.
Methods

Population study

• The French National Reference Center for Viral Hepatitis B, C and D performs HCV resistance testing in patients failing DAA-containing regimens across the country.

-> 16 patients who failed to achieve SVR on SOF + NS5A inhibitor
   – GT-3 failing SOF + DCV+/- RBV 12W (n=7) or SOF/VEL 12W (n=2)
   – GT-1 failing SOF + DCV or SOF/LDV (GT1a, n=1; GT1b, n=2)
   – GT-2 failing SOF + DCV (n=1)
   – GT-4 failing SOF + DCV or SOF/LDV (n=3)
Shot-gun metagenomic procedures

Automated and standardized Pathogen and host DNA and RNA Extraction

Library preparation
Total RNA, Nextera XT kit

Sequencing
(Illumina NextSeq500)

Server calculation
(96Co/192Go RAM)

Automation

In-house MetaMIC® software

Filtering

Identification and genome reconstruction

Report and interpret
Methods

Generation of the full-length HCV genome sequences

-Sequences were analyzed using a 15% cutoff

-Candidate AA substitutions selected in at least two GT-3 infected patients were further characterized by phenotypic assays
Results
Patient LOMB
GT-3a failing SOF/DCV 12W

Patient BERT
GT-3a failing SOF/DCV 12W
Patient MESS
GT-3a failing SOF/DCV 12W

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List of amino-acid substitutions selected in at least two independent patients GT-3 failing NS5A inh + SOF

Changing AA 176 NS3 showed an increased replication level in con-1 replicon (GT-1b) in vitro \(^1\)

Changing AA 48 (NS4B-ER associated protein) (located at the start of the transmembrane domain) influence HCV RNA replication efficiency in vitro \(^3\)

AA substitutions NS3 98 compensate for the loss of fitness induced by NS3 RASs in vitro \(^2\)

Transient phenotypic assays of Gt-3 infectious prototype EC50 determination and fitness evaluation

Transfection

EC50
RT-qPCR 5 days post transfection (increasing concentration of drug)

Viral fitness
Ratio: viral RNA endpoint / viral RNA 4h (transfection efficiency)

Plasmids provided by Jens Bukh
Infectious viral prototypes GT-3

GT-3 WT, 120h following transfection

Estimation of % HCV-antigen positive culture cells following immunostaining with anti-NS5A Ab
**Results**

**Phenotypic characterization of GT-3 AA substitutions selected under SOF + NS5A-containing regimen (NS3, NS4B and NS5A DIII)**

EC50 DCV

**NS3**

![EC50 DCV graph for NS3](image1)

**NS4B**

![EC50 DCV graph for NS4B](image2)

**NS5A**

![EC50 DCV graph for NS5A](image3)

**Replication capacity**

**NS3**

![Replication capacity graph for NS3](image4)

**NS4B**

![Replication capacity graph for NS4B](image5)

**NS5A**

![Replication capacity graph for NS5A](image6)
• We used an original shotgun metagenomics method to generate full-length HCV sequences

-> AA substitutions independently selected in the NS2, NS3, NS4B or NS5A (domain III) regions in patients failing SOF + NS5A inh

• *In vitro* phenotypic assays revealed that many candidate AA substitutions increased viral fitness (including NS3 T98S, NS4b T48A, NS4b G114S)
Discussion

• Multiple mechanisms of action proposed in vitro:
  – Substitution(s) NS3 position 98 increase assembly efficacy
  – Substitution(s) NS4B position 48 located at the start of NS4B transmembrane domain is crucial for efficient NS4B function (formation of the membranous web) and HCV replication

• Further analysis are ongoing to characterize selected AA substitutions at treatment failure of other genotypes
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