Evolutionary pathways to NS5A inhibitor resistance in genotype 1 hepatitis C virus

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ABSTRACT

Direct-acting antivirals (DAAs) targeting NS5A are broadly effective against hepatitis C virus (HCV) infections, but sustained virological response rates are generally lower in patients infected with genotype (gt)-1a than gt-1b viruses. The explanation for this remains uncertain. Here, we adopted a highly accurate, ultra-deep primer ID sequencing approach to intensively study serial changes in the NS5A-coding region of HCV in gt-1a- and gt-1b-infected subjects receiving a short course of monotherapy with the NS5A inhibitor, elbasvir. Low or undetectable levels of viremia precluded on-treatment analysis in gt-1b-infected subjects, but variants with the resistance-associated substitution (RAS) Y93H in NS5A dominated rebounding virus populations following cessation of treatment. These variants persisted until the end of the study, two months later. In contrast, while Y93H emerged in multiple lineages and became dominant in subjects with gt-1a virus, these haplotypes rapidly decreased in frequency off therapy. Substitutions at Q30 and L31 emerged in distinctly independent lineages at later time points, ultimately coming to dominate the virus population off therapy. Consistent with this, cell culture studies with gt-1a and gt-1b reporter viruses and replicons demonstrated that Y93H confers a much greater loss of replicative fitness in gt-1a than gt-1b virus, and that L31M/V both compensates for the loss of fitness associated with Q30R (but not Y93H) and also boosts drug resistance. These observations show how differences in the impact of RASs on drug resistance and replicative fitness influence the evolution of gt-1a and gt-1b viruses during monotherapy with an antiviral targeting NS5A.

1. Introduction

New all-oral, combination direct-acting antiviral (DAA) therapies are highly effective against hepatitis C virus (HCV) and result in sustained virological response (SVR) in most (> 95%) persons with chronic hepatitis C (Bartenschlager et al., 2013). Most DAA combinations used today include an inhibitor of the nonstructural (NS) 5A protein combined with an NS3/4A protease inhibitor and/or an NS5B RNA-dependent RNA polymerase inhibitor. While treatment failure is uncommon, sequencing of HCV RNA after failure typically suggests the presence of drug-resistant viral variants in patients who do not achieve SVR. NS5A inhibitors are a highly potent class of DAA (Gao et al., 2010; Ivanenkov et al., 2017). NS5A is unique to HCV and closely related viruses with multiple essential functions during the viral life cycle, but no known enzymatic activity (Ross-Thriepland and Harris, 2015). NS5A inhibitors have dual modes of action, both impeding viral RNA synthesis by blocking the formation of replicase complexes, and also preventing the intracellular assembly of infectious virions (Benzine et al.,

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2017; Berger et al., 2014; Boson et al., 2017; McGivern et al., 2014).

HCV is characterized by a high degree of inter- and intra-host diversity, which likely plays an important role in the response to DAAs and development of resistance (Jabara et al., 2014; Smith et al., 2014). There are 7 HCV genotypes that differ by more than 30% at the nucleotide level, with each further divided into subtypes that differ by 20% (Smith et al., 2014). Genotype 1 (gt-1) is the most common globally and in the U.S. Early phase 1 clinical trials of NS5A inhibitors suggested that the antiviral efficacy of NS5A inhibitors was greater in patients with gt-1b versus gt-1a infection (Lawitz et al., 2012; Liu et al., 2015). Amino acid substitutions in NS5A associated with antiviral resistance ('resistance-associated substitutions' or RASs) also differ between gt-1a and gt-1b viruses (Issur and Gotte, 2014; Lontok et al., 2015; Zeuzem et al., 2017). Amino acid substitutions conferring at least a 2.5-fold reduction in susceptibility to an NS5A inhibitor (daclatasvir, ledipasvir, pibrentasvir, or elbasvir) have been identified at residues 24, 28, 30, 31, 32, 38, 58, 92, and 93 within domain I of NS5A (Gottwein et al., 2018; HCV Drug Development Advisory Group, 2012; Zeuzem et al., 2017). However, the diversity of RASs appears to be greater in gt-1a than gt-1b viruses (Zeuzem et al., 2017). More importantly, while pre-existing NS5A RASs may negatively impact the SVR rate in gt-1a patients treated with some NS5A inhibitors, such substitutions have limited impact in gt-1b infections (AASLD-IDSA; Zeuzem et al., 2017).

To better understand the evolutionary pathways leading to NS5A inhibitor resistance in gt-1a and gt-1b infection, we used next generation sequencing (NGS) with tagged cDNA primers ("primer ID sequencing") (Jabara et al., 2011; Zhou et al., 2015) to intensively characterize HCV diversity in small cohorts of gt-1a and gt-1b infected patients receiving a 5 day course of elbasvir monotherapy. Primer ID sequencing overcomes several limitations of conventional NGS by eliminating multiple types of PCR-related errors, thereby allowing unprecedented sequencing depth and accuracy (overall error rate ~0.01%) (Jabara et al., 2011; Zhou et al., 2015). Each template consensus sequence (TCS) generated by primer ID sequencing represents an original viral RNA template queried at the very first cDNA synthesis step, making primer ID sequencing an excellent tool to study highly diversified viral populations such as HIV-1 and HCV (Barnard et al., 2016; Jabara et al., 2014; Keys et al., 2015; Zhou et al., 2016). Coupling this approach with a unique set of serial serum samples collected before, during and after five days of elbasvir monotherapy, we show here that gt-1a and gt-1b HCV acquire antiviral resistance through distinct selection pathways, and that genotype-specific differences in RAS linkage and frequency can be linked to differences in cell culture assays of viral fitness.

2. Materials and methods

2.1. Study participants, clinical samples, and primer ID sequencing

Serial plasma samples were collected from subjects participating in a phase 1b clinical trial in which they received either elbasvir (10 mg or 50 mg q.d.) monotherapy or placebo for 5 days (MK-8742 P002 trial, ClinicalTrials.gov Identifier: NCT01532973) (Liu et al., 2015). Seven subjects were infected with gt-1a HCV (4 receiving 50 mg of elbasvir, 2 10 mg of elbasvir, and 1 placebo) and 6 subjects were infected with gt-1b virus (5 receiving 50 mg elbasvir and 1 placebo). RNA encoding domain I of NS5A (amino acids 23–123) was sequenced in an average of 6 serum samples from each subject, collected between 0 (pre-treatment) and 60 days after the initiation of therapy (Table S1). Primer ID libraries were constructed using previously published protocols and primer sequences shown in Table S2 (Zhou et al., 2015, 2016). Multiplexed libraries were sequenced using Illumina MiSeq (Illumina, San Diego, CA) 300 bp paired-end sequencing.

2.2. Bioinformatics and phylogenetic analysis

The Illumina bcl2fastq pipeline (v 1.8.4) was used for initial de-

multiplexing of the data and TCS pipeline (v 1.3.2) to create primer ID template consensus sequences (TCSs) (available at https://github.com/SwanstromLab/PID). In-house scripts were generated to derive abundances of substitutions at each nucleotide and amino acid position in each sample. The primer ID approach allowed identified linkages within each TCS, and neighbor-joining trees were built with MUSCLE (v 3.8.31) (Edgar, 2004a; b).

2.3. In vitro replicative fitness assays

The impact of RASs on replicative fitness and drug resistance were determined using recombinant cell culture-adapted gt-1a H77S.3/GLuc2A (Shimakami et al., 2011) and gt-1b N.2/Gluc2A (Yamane et al., 2014) reporter viruses expressing *Gaussia princeps* luciferase (GLuc). RASs were introduced into plasmid DNAs using Quikchange XL site-directed mutagenesis (Agilent). Plasmids were linearized with XbaI and transcribed in vitro to produce HCV genomic RNA with the T7 Megascript kit (Ambion, Austin, TX). Huh7 2-3c cells were electroporated with the RNA and replication fitness assessed by monitoring secreted GLuc activity as described previously (Mauger et al., 2015; Shimakami et al., 2011).

3. Results

3.1. Primer ID MiSeq sequencing of HCV RNA in serial plasma samples

Approximately 3000 to 12,000 TCSs were obtained from each clinical sample, providing a sampling depth of 0.1%–0.03% (defined as the abundance of minor variants with a 95% chance of being detected) (Table S1). However, extremely low or undetectable levels of viremia precluded sequencing of virus in samples collected between 1 and 13 days after starting elbasvir therapy in all gt-1b and some gt-1a-infected subjects.

3.2. Pre-therapy resistance-associated substitutions and intra-host diversity

We considered that differences in the genetic diversity of gt-1a and gt-1b viruses could contribute to differences in the development of NS5A inhibitor resistance and thus treatment outcome. We thus examined the frequency of pre-treatment polymorphisms at the most relevant 16 amino acid positions between codons 23 and 123 of NS5A (Fig. 1A). Significant baseline intra- and inter-host variation existed at codons 28, 30 and 93 (positions of several important RASs), but the intra-host average pairwise diversity (π) of the baseline nucleotide sequences of the viral populations did not differ significantly between gt-1a and gt-1b subjects (π = 0.87 vs. 0.82, p = 0.30). We also calculated estimated genetic distances using the model-based Tamura-Nei method (TN93 distance) which assesses the distribution of pairwise distances between sequences (Tamura and Nei, 1993). Three of 7 gt-1a subjects (subjects 1, 2, and 5) but only 1 of 6 gt-1b subjects (subject 12) showed a high frequency of TCSs with a TN93 distance greater than 0.04 substitutions/site (p = 0.24 by χ^2 test) (Fig. 1B and C). The existence of multiple peaks in the frequency distributions of TN93 distance suggest that these subjects had several distinct viral lineages before therapy.

3.3. Elbasvir-induced changes in sequence encoding NS5A domain I

We calculated Shannon's entropy as a measure of the variability of amino acid residues at each position within the NS5A protein (Korber et al., 1994). Significant elbasvir-induced changes occurred between baseline and day 28 in gt-1a virus infected subjects at codons 28, 30, 31, 58, and 93 (Fig. S1), and in gt-1b subjects at codons 28, 31, and 93 (Fig. S2). Significant changes in the frequency of amino acid polymorphisms were evident by day 1, but not 4hr after the start of elbasvir therapy. In contrast, no changes were observed in Shannon's entropy at any position in subjects (7 and 13) receiving placebo.