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Viral Dynamic Modeling of Hepatitis C and Resistance-associated Variants in Hemophiliacs

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Abstract

Aim—Chronic hepatitis C virus (HCV) infection is an important source of morbidity and mortality among hemophiliacs. Limited data are available regarding treatment intervention using direct-acting antivirals (DAAs) and theoretical concerns regarding accumulation of drug-associated resistance variants (RAVs) remain. We conducted a pilot study of treatment with telaprevir/pegylated interferon-alfa/ribavirin to evaluate treatment response and the role of lead-in DAA therapy on mutational selection of resistance variants.

Methods—Ultra-deep sequence analysis was performed at baseline, 48 hours and 168 hours after treatment initiation.

Results—No dominant RAVs were identified at baseline but low level RAVs were noted at baseline in all subjects. Viral dynamic models were used to assess treatment responses. The efficacy parameter (ϵ) for lead-in ranged from 0 to 0.9745 (mean= 0.514). Subsequent addition of telaprevir resulted in a mean efficacy of more than 0.999. This was comparable to subjects who started all three medications simultaneously. 80% achieved SVR. While rapid shifts in the RAV population following DAA initiation were observed, treatment failure associated with A156V was observed in only 1 patient. Adverse event profiles were similar to that observed in non-hemophilia cohorts. There was no evidence of factor inhibitor formation. There was no evidence that lead-in provided benefit in terms of response efficacy.

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Conclusion—These data support DAA-based therapy in those with inherited bleeding disorders.

INTRODUCTION

Hepatitis C virus (HCV) infection is an important contributor to liver-related morbidity and mortality among patients with inherited bleeding disorders, including hemophilia, who received factor concentrates prior to 1987.¹ Treatment intervention has been limited in efficacy and acceptance by both patients and providers.² In 2011, the first generation of direct-acting antivirals (DAAs) was approved for use in the U.S. and Europe, but limited data are available regarding efficacy and safety among those with hemophilia. Theoretical concerns about treatment include decreased efficacy, poor tolerability, and risk of inhibitor formation. Concerns regarding emergent viral resistance led to clinical practices based upon the concept that a 4 week lead-in period in which pegylated-interferon/ribavirin were used to decrease viral load prior to DAA addition would mitigate failure due to resistance. A recently published model questioned this approach.³ To address these issues, a pilot study designed to carefully assess viral dynamics during treatment with a DAA-based therapy was performed and next generation deep sequence analysis was utilized to evaluate the effects on viral subpopulations.

METHODS

Patients with HCV and hemophilia A or B were offered participation in a clinical trial of treatment with telaprevir 750 mg po tid, pegylated interferon-alfa-2a 180 mg sq qd, and weight-based ribavirin (1000–1200) mg/day. Patients were randomized to receive a) initially all three drugs for 12 weeks, and then pegylated-interferon/ribavirin for an additional 12 or 36 weeks depending upon viral response at Week 4 and 12 as described in the product label, or b) a lead-in regimen with pegylated interferon-alfa /ribavirin for 4 weeks and addition of telaprevir followed by response-guided therapy. Key inclusion criteria included patients with chronic HCV genotype 1 infection, 18 years or older with liver disease staging by liver biopsy or non-invasive markers within 12 months of enrollment. Cirrhosis was not an exclusion factor, but those with hepatic decompensation were not enrolled. Patients were excluded if their hemoglobin was <11 g/dL, HIV+, hepatitis B or other active liver disease processes were present, or if their creatinine clearance was <50 ml/min. All patients provided informed consent. The study was performed under the auspices of the Institutional Review Board at the University of Cincinnati. (ClinicalTrials.gov Identifier: NCT01704521)

Intensive serum sampling was performed in the research unit at baseline, hours 0, 3, 6, 12, 24, 48, 72, and days 7 and 10. Intensive sampling was repeated in patients randomized to lead-in at week 4. HCV RNA was tested using the commercial COBAS 2.0 real-time PCR assay with a lower limit of quantitation of 43 IU/ml and a limit of detection of approximately 15 IU/ml. Sustained viral response (SVR₁₂), defined as HCV RNA being undetectable for twelve or more weeks after the end of treatment, was determined 12 weeks following completion of therapy. Factor inhibitors were tested at baseline, week 20 of treatment, and at end of follow-up.

Deep Sequencing Analysis of Resistance Associated Variants (RAVs)

Sequencing primers specific to the nonstructural-3 (NS3) region of HCV were used to amplify a partial sequence of the HCV NS3. SuperScript III one-step RT-PCR Platinum Taq HiFi (Invitrogen, Carlsbad, CA) was used to amplify all samples and the PCR-amplified product (600 bp) was then agarose gel-purified with final DNA concentrations ranging from a few ng/μl to 30 ng/μl, which is suitable for the DNA library preparation, which was performed by the University of Cincinnati Genomics, Epigenomics and Sequencing Core (GESC). To shear the DNA to proper size range for library preparation, 50 μl of DNA was aliquoted and sonicated with a Covaris S2 focused-ultrasonicator (Woburn, MA), following a protocol targeting the 150 bp peak. The sheared DNA was QC analyzed using the Bioanalyzer DNA 1000 chip (Agilent, Santa Clara, CA).

A PrepX DNA Library kit (WaferGen, Fremont, CA) and Apollo 324 NGS automatic library prep system (WaferGen) was used for the library preparation. ChIP-seq script was selected to capture all the sheared fragments over ~80 bp. First, the sheared DNA fragments with overhangs were converted into blunt ends in the end repair procedure, and adenylated at 3' ends for TA ligation to Illumina (San Diego, CA) sequencing adaptors. Next, the ligated library was enriched by 6 cycles of PCR using index-specific primers, followed by AMPure XP beads (Beckman Coulter, Brea CA) purification. Bioanalyzer DNA high sensitivity chip was then used to check the quality and yield of the purified library. To accurately quantify the library concentration for the clustering, the library was 1:10,000 diluted in dilution buffer (10 mM Tris-HCl, pH 8.0 with 0.05% Tween 20), and qPCR analyzed by Kapa Library Quantification kit (Kapabiosystem, Woburn, MA) using ABI's 9700HT real-time PCR system (Lifetech, Grand Island, NY).

Individually indexed libraries were proportionally pooled for clustering at final concentration of 8 pM. The pooled libraries were clustered onto a flow cell using Illumina's TruSeq SR Cluster kit v3 in cBot system (Illumina), followed by single read sequencing at 1X50 bp using Illumina's TruSeq SBS kit on HiSeq system (Illumina). Approximately three million reads were generated from each sample for HCV NS3 data analysis. The filtered, trimmed reads were aligned to H77 reference sequence, HCV genotype 1, from the Los Alamos HCV database (<http://www.hcv.lanl.gov>). The codons associated with resistance associated variants were identified and the mutation frequency calculated. The mean coverage was 372,324 and the threshold for variant frequency was 0.3% of the total population sample. Sequencing reads were processed using the following criteria: an exact match to primer sequences; no ambiguous bases; sequence reads pass all quality steps (no reads with unknown quality scores and no reads that failed the Illumina quality check).

HCV Kinetics Model

We constructed an HCV model that tracks the infected cell population (I) and the viral population (V), based on a previous work by Neumann et al.⁴ We assume that the level of target cells (T) stays constant, which should be a good approximation as the treatment time is short. The model for patients with lead-in treatment is:

$$\begin{aligned}\frac{dI}{dt} &= \beta TV - \delta I \\ \frac{dV}{dt} &= (1 - \varepsilon_1)(1 - \varepsilon_2)pI - cV \\ \delta &= \begin{cases} \delta_{DAA} & t \geq 28 \text{ days} \\ \delta_0 & \text{otherwise} \end{cases} \\ \varepsilon_1 &= \begin{cases} \varepsilon_{IFN} & t \geq t_{delay} \\ 0 & \text{otherwise} \end{cases} \\ \varepsilon_2 &= \begin{cases} \varepsilon_{TVR} & t \geq 28 \text{ days} \\ 0 & \text{otherwise} \end{cases} \end{aligned} \quad (1)$$

where β is the infectivity of the virus, T is the concentration of target cells, δ is the death rate of infected cells, ε_1 and ε_2 are the efficacy of peg-IFN+RBV and telaprevir therapy, respectively, p is the production rate of virions from infected cells, and c is the per virion viral clearance rate. In the model, we assume that peg-IFN + RBV therapy starts at time 0, but the effectiveness of interferon therapy is felt with a delay (t_{delay}), and the addition of telaprevir occurs at day 28 (4 weeks after peg-IFN lead-in treatment). The rate of the second phase viral load decline in patients undergoing peg-IFN+RBV treatment is different from the rates in patients with telaprevir treatment, possibly as a result of the loss of intracellular HCV RNA due to the action of TPV.⁵ Therefore, as in the work of Rong et al.³, we assume that the rate, δ , changes from δ_0 to δ_{DAA} once telaprevir is added to the treatment. For patients without lead-in treatment, we estimate the combined efficacy peg-IFN+RBV+TVR, ε_{Comb} . The model is:

$$\begin{aligned}\frac{dI}{dt} &= \beta TV - \delta I \\ \frac{dV}{dt} &= (1 - \varepsilon_{comb})pI - cV \\ \delta &= \begin{cases} \delta_{DAA} & t \geq 28 \text{ days} \\ \delta_0 & \text{otherwise} \end{cases} \end{aligned} \quad (2)$$

where we assume treatment starts at day 28. At the pre-treatment steady state, the number of target cells is given by

$$T = \frac{c\delta}{\beta p}.$$

Substituting this expression into Eqn. 1 and setting $Y = pI$, we obtain

$$\begin{aligned} \frac{dY}{dt} &= c\delta V - \delta Y \\ \frac{dV}{dt} &= (1-\varepsilon_1)(1-\varepsilon_2)Y - cV \\ \delta &= \begin{cases} \delta_{DAA} & t \geq 28 \text{ days} \\ \delta_0 & \text{otherwise} \end{cases} \\ \varepsilon_1 &= \begin{cases} \varepsilon_{IFN} & t \geq t_{delay} \\ 0 & \text{otherwise} \end{cases} \\ \varepsilon_2 &= \begin{cases} \varepsilon_{TVR} & t \geq 28 \text{ days} \\ 0 & \text{otherwise} \end{cases} \end{aligned} \quad (3)$$

Below, we fit Equation 3 to the viral load data from the clinical trial.

RESULTS

Seven treatment-experienced HCV infected patients with hemophilia A were enrolled. Two were screen failures due to spontaneous HCV clearance or loss to follow-up prior to the first dose. The five treated subjects were male, 4/5 were Caucasian and 1 was black, non-Hispanic. Four subjects were IL28B genotype CT, and one was CC. Three subjects were randomized to lead-in followed by addition of telaprevir after four weeks, and two received standard T/P/R therapy started at week 0. Ultimately, 4/5 subjects (80%) achieved SVR₁₂; the one non-responder (patient 112) was in the lead-in peg-IFN+RBV treatment group. The four responders had extended rapid viral response (eRVR, undetectable HCV RNA at weeks 4 and 12.) and received 24 weeks of total treatment.

Adverse event profiles were similar to that observed in non-hemophilia cohorts. One subject had treatment-emergent Grade 4 neutropenia, and one with mild hemophilia exhibited Grade 3 thrombocytopenia and anemia in association with gum bleeding. A rash attributed to telaprevir was observed in one patient leading to telaprevir discontinuation at week 8 of therapy. No serious bleeding was observed. There was no evidence of factor inhibitor formation.

The results of fitting the mathematical models to the data are shown in Table 1 and Fig. 1. Table 2 lists the baseline RAVs observed in the five HCV-infected hemophilic patients who underwent modeling and sequential mutational analysis. Baseline RAVs were rare except for one patient (121) who exhibited nearly complete representation by the Q80K mutation. Deep sequence analysis was also performed at 48 and 168 hours after initiation of treatment. In most patients there was a minor increase in RAVs seen but proportions tended to remain under 1–2% of the total population. However in patient 122, the baseline Q80L RAV present at 14.1% completely disappeared and Q80 wildtype was enriched from 85.8% to 99.8%. In patient 112, who was randomized to receive the lead-in regimen with pegylated interferon-alfa/ribavirin for 4 weeks, the A156 wildtype was reduced from 100% to 17.9% at day 7 after introduction of the DAA, and A156V became the dominant species at that site accounting for 82.1% of the total population. This patient had maintained A156 wildtype throughout exposure to interferon/ribavirin (lead-in) with selection occurring only after DAA exposure. No association with calculated treatment efficacy parameters was observed.

DISCUSSION

Though first generation protease inhibitors active against HCV have been available since 2011, published literature contains limited information regarding the use of direct acting agents in those with inherited bleeding disorders.^{6,7} Previous studies have suggested that patients with hemophilia who received multiple doses of human-derived factor concentrates may carry multiple genotypes⁸, exhibit more diverse quasispecies populations⁹, and have lower rates of response to therapy than non-hemophilic counterparts. Our group previously reported data describing the frequency of protease inhibitor mutations in hemophiliacs vs. HCV controls without hemophilia and found no difference in representation of baseline mutations using population sequencing methods.¹⁰

In this manuscript, we provide the first report in hemophiliacs of ultra-deep sequence analysis applied to the evaluation of the HCV NS3 coding domain. This region contains key mutations that have the potential to affect treatment outcomes in both first generation (telaprevir, boceprevir) and second wave/second generation serine protease inhibitors used to treatment HCV (simeprevir, paritaprevir, grazoprevir). Our ultra-deep sequence methodology is sensitive to population displays as low as 0.1%. In comparison, population (consensus) based methods typically detect RAVs at proportions of 25% or higher. Baseline ultra-deep sequence analysis shows that key mutations are indeed present at very low levels in most patients. However, the frequency of these mutations does not appear to be greater than that observed in non-hemophilic patients with recently acquired infections.¹¹ One subject had dominant Q80K mutations which confer a significant level of resistance to simeprevir containing regimens, even when those regimens are all oral DAAs.¹² In a study of naturally occurring serine protease RAVs in HCV mono and HCV/HIV coinfecting patients, dominant Q80K mutations were not found in the monoinfected patients and were rare (1.47%) in HCV/HIV coinfecting patients.¹³ Though our population is too small to draw any conclusions, evaluation of a larger cohort of hemophilic subjects may be warranted to determine if frequency may be higher for Q80 RAVs in this group, as this might influence treatment decisions regarding regimens that contain PIs. In our study of patients with inherited bleeding disorders, early selection of RAVs had a significant effect on treatment outcome in the one patient who failed therapy. In patient 112, who failed to achieve SVR₁₂, there was rapid emergence of an A156V mutation which was not detected at baseline but was seen as the dominate species by 7 days after DAA treatment initiation. This is similar to reports of RAV emergence and treatment failure described by Kieffer et al.¹⁴

Herein, we provide evidence using sensitive viral dynamic modeling techniques that these patients can exhibit excellent Phase 1 and Phase 2 responses to treatment intervention with a regimen containing a potent first generation protease inhibitor. Poor response to pegylated interferon and ribavirin alone can be mitigated by addition of telaprevir, and there was no apparent role for use of lead-in to drive down viral load as was postulated previously.³ The field of HCV treatment is moving forward rapidly, and we are now entering a stage of all oral HCV treatment with two or more agents active against different targets of the HCV replication schema. Based upon our findings, we believe that patients with hemophilia and other inherited bleeding disorders will respond well to these regimens, and we encourage

inclusion of these patients in clinical trials to avoid the delays that have been observed in treating the hemophilia community to date.

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Figure 1. Model fits to the viral load data from the 5 patients

Black lines denote model simulation using best-fit parameters; red circles denote viral load data. Dashed lines denote the limit of viral quantification. For patients 121 and 122, treated without lead-in, combination therapy is graphed as starting on day 28.

Table 1

Best-fit parameter values for each patient.

Pt ID	Lead-in	V_0	δ_0 (day ⁻¹)	δ_{DAA} (day ⁻¹)	c (day ⁻¹)	t_{delay} (days)	ε_{IFN}	ε_{TVR}	ε_{Comb}
111	Yes	6.49	0.14	0.12 ²	6.5	0.9	0.5677	0.9990	0.99957
112	Yes	6.20	0.14 ³	0.19	5.0	2.0 ³	0.0000	0.9995	0.99947
113	Yes	6.48	0.07	NA ⁴	8.3	0.6	0.9765	0.9960 ⁵	0.99991 ⁵
121	No	7.56	N/A	0.44	7.9	N/A	N/A	N/A	0.99989
122	No	7.20	N/A	0.29	7.7	N/A	N/A	N/A	0.99990

¹ ε_{Comb} for patient with lead-in treatment is calculated as $\varepsilon_{Comb} = 1 - (1 - \varepsilon_{IFN}) \cdot (1 - \varepsilon_{TVR})$.

² For Patient 111, the estimated clearance rate of infected cells under peg-IFN+RBV+TVR treatment is lower than peg-IFN+RBV lead-in treatment.

³ These values for Patient 112 cannot be reliably estimated, because the efficacy of lead-in is estimated to be 0, i.e., the patient is an IFN-nonresponder (see Fig. 1).

⁴ The value of δ_{DAA} for Patient 113 cannot be reliably estimated, since the viral load decreases to an undetectable level before the second phase decline begins (see Fig. 1).

⁵ The effectiveness of TVR estimated here is a lower boundary as the viral load falls below the level of quantification during the first phase decline induced by TVR in Patient 113 and we used the lower limit of quantification, i.e. 43 IU/mL, as the value of the first unquantifiable data point in our data fitting. If we use the mid-point between 0 and the lower level of quantification, i.e. 21.5 IU/mL instead, ε_{TVR} and ε_{Comb} become 0.9972 and 0.99993, respectively.

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Table 2

Percent Baseline RAVs in NS3 Detected by Deep Sequence Analysis

	V36A	T54A	V55A	Q80L	Q80R	Q80K	R155K	R155G	A156T	D168G/A
111	0	0	0	0.1	0.1	0	0	0	0	0.1
112	0.1	0.1	0.1	0	0.1	0	0	0	0	0
113	0	0.1	0.1	0	0	0	0	0	0	0
121	0	0	0	0	0	99.8	0	0	0	0.1
122	0	0	0	14.1	0	0	0	0	0	0.2