Quasispecies Analysis of HCV Breakthroughs Occurring During the HALT-C Trial Lead-In Treatment Phase Revision 3.1

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Rationale: Response to interferon therapy is multi-factorial in hepatitis C infection. However, one component of interferon resistance undoubtedly resides in the virus itself. Thus, the hepatitis C virus found in certain patients appears to possess unique sequences that are capable of resisting interferon-based therapy. The purpose of the proposed study is to identify unique sequences in the HCV viral genome that are associated with interferon resistance by targeting a specific small group of patients—those who develop viral breakthrough while fully compliant with interferon-based therapy. In this group that initially is responsive to therapy, comparing viral sequences at baseline to those that are observed at the time of breakthrough (BT) may point to unique differences associated with viral resistance.

Background: The hepatitis C virus (HCV) remains a major public health problem and a leading cause of chronic liver disease. The current standard of care for chronic hepatitis C is the combination of Peg-IFN with ribavirin which leads to a sustained viral response (SVR) in more than 50% of patients[1]. The likelihood of achieving an SVR can be predicted by certain pretreatment patient characteristics, of which viral genotype is the strongest[2].

Responsiveness to interferon is thought to depend in large part on characteristics of the particular virus infecting the host. A rapidly mutating virus, HCV persists in its host as several genetically distinct viral variants called collectively 'quasispecies'. Each separate variant may have different sensitivity to IFN therapy and different ability to affect the innate host response. It seems likely that patients who are capable of achieving an SVR are infected with viral quasispecies that in aggregate are more sensitive to IFN than those found in patients who fail to respond to IFN[3]. This assumption has been confirmed in studies showing that patients with higher number of mutations in the interferon sensitivity-determining region (ISDR) have higher rates of SVR compared with those with lower number of mutations in this region. The importance of ISDR has been hotly debated[4-6] but supported by the recent meta-analysis[7, 8]. Some studies also show that mutations downstream of the ISDR region, in the V3 region, are associated with the response to IFN therapy[9]. We have recently shown that interferon responsiveness is associated with a narrowing of quasispecies diversity either at baseline or shortly thereafter[10]. If confirmed by further work, quasispecies diversity determined by cloning and sequencing could possibly be used as a very early marker to predict an SVR, but should also help in determining resistant from responsive viral strains.

Most studies of responders and non-responders (NR) have focused only on the baseline viral characteristics rather than the evolving virus under treatment. While many patients are non-responders (~30%) or relapse after end of treatment response (~10%), a smaller number of patients demonstrate viral breakthrough while on therapy[11]. A study focused on determining viral factors involved in non-responders is complicated by the

heterogeneous nature of such patients' therapy courses and the role that compliance plays in determining outcome. Withdrawal of medication or reduction of IFN dose may be responsible for non-response or relapse in many patients[12]. By contrast, viral breakthrough usually occurs later in the treatment course when initial intolerance to therapy is over and dosing is relatively constant. Thus, breakthrough may represent the natural evolution in a small fraction of patients of a mutant viral strain less responsive to the effects of interferon. In the HALT-C study, viral breakthrough was observed during the lead-in phase in 43 (4%) HCV patients. However, when we reviewed compliance in this patient group (since that would greatly influence likelihood of breakthrough) there were only 12 patients who claimed to have taken full dose interferon throughout their course. Eight of the twelve had taken full dose of ribavirin as well during the study period. We propose to study this special group of 12 compliant patients who had become HCV RNA negative and later reverted to positive despite full dose therapy, since this analysis might provide hints as to the locus of interferon responsiveness in the HCV genome. We hypothesize that *comparison of baseline samples* with those obtained at the time of breakthrough will demonstrate unique differences in viral sequence (escape mutations) that will identify HCV interferon-resistant quasi-species that emerge under interferon pressure.

Aims:

Specific Aim 1: Analyze the HCV sequences both at baseline, week 4 and week 12, and at the time of viral breakthrough when patients are still on the peg-IFN therapy by clone analysis assay. Changes in the sequence in the E2 and NS5a regions will be analyzed to determine whether either specific mutations or the number of mutations in some regions are responsible for viral breakthrough. These analyses will be performed on the BT patients as well as patients with NR and SVR.

Specific Aim 2: Further comparisons of HCV sequence diversity will be determined using heteroduplex assays on sera from the same 12 compliant patients, at baseline, at 4 weeks into therapy, week 12 and at the time of return of virus (breakthrough) while under treatment. The dynamic changes in sequence diversity and complexity will be noted. This work will allow us to see whether there is a change in sequence diversity if more IFN resistant quasi-species were selected by the augmented immune pressure.

Specific Aim 3: Cloning and sequencing will be performed on samples at baseline, at 4 weeks into therapy, week 12 and at the time of return of virus (breakthrough) to further elucidate the changes in complexity and diversity over time.

Relationship of the present study to the HALT-C Study: HALT-C has targeted non-responder patients who are most in need of therapy[13]. The availability of a lead-in phase that allowed all patients to test their responsiveness to treatment with the best therapy currently available served to identify factors associated with response, non-response and breakthrough. In the present study, we seek to capitalize on the availability of well-characterized patients for whom carefully collected samples are available. One virtue of our study is that it requires only minimal samples at first. If results with the initial 12 patients (48 serum samples maximum) prove promising then we would ask for

additional samples on the SVR and NR patients. In the overall aims of HALT-C, the further elucidation of the viral basis of non-response seems an important area to explore.

Patients and Methods

Strategy: 500 µL samples obtained at baseline prior to treatment, at the time of last positive sample and at the time of first reappearance of virus (breakthrough) will be requested from the NIDDK repository from the 12 selected compliant patients in the HALT-C trial lead-in phase. Our initial pilot project will focus only on the 12 compliant patients at baseline and at the time of breakthrough. For comparison purposes, if the initial study is promising, the same volume samples will be requested from 24 age-, gender-, genotype-, and viral-load-matched HALT-C patients with non-response to Peg-interferon therapy at baseline and at week 24 to validate the findings in the initial patient cohort. Finally, 500 µL baseline serum samples only will be requested from 24 age-, gender- and genotype-matched HCV patients with sustained responses.

We will proceed to amplify the NS5A region and E2 region by RT-PCR and determine the sequence change by comparison after direct sequencing. Each region will be analyzed using NTI Vector 9.0 software to determine differences in sequence diversity and specific sequence differences between baseline and point of breakthrough. Following analysis of these initial samples, we will only proceed to phase 2 if there are promising differences observed in these 12 patients between their own baseline and later samples. If initial results look promising we will request samples from 24 NR patients who were eventually enrolled in the long term study and who have samples available at baseline and at week 24 who can be matched to the 12 phase 1 breakthrough patients in regard to a) received at least 80% of therapy, b) had similar genotype breakdown, and c) similar viral loads to the original 12 patients. In addition, we will perform parallel assays on up to 24 SVR patients who have week 12 samples positive for HCV RNA so that sequences can be obtained. We will further consider sequencing the entire HCV genome if the results in NS5A and E2 are not helpful.

If promising leads are forthcoming, then in the second phase of the study we will analyze the degree of HCV quasi-species diversity during HCV viral breakthrough using the heteroduplex tracking assay with reference to the dominant sequence at the baseline. We will also consider more a detailed analysis of clonal sequences if there is evidence of unique sequences present in the compliant breakthrough group. This should not require any further samples beyond the control groups of non-responder and responder patients.

Data needed from HALT-C: We will need viral load data on all 12 patients for their course of therapy and drug dosages during therapy. Only if there is promise in the analysis of the first 12 patients will the study proceed to the request of the 24 NR patients matched for compliance, genotype and approximate viral load. We will also request 24 SVR patients who are HCV RNA positive at week 12 as another group of control patients. For choosing the matched controls compliance was defined as reported taking at least 80% of peginterferon and ribavirin treatment.

Other data requested from HALT-C are: age, gender, race, ethnicity, BMI, genotype and viral load.

Anticipated Results: We suspect that we will find differences in the initial 12 patients within NS5A that impact response to therapy. These patients' samples can then be used in a replicon system (in the laboratory of Dr. Michael Gale) to confirm the presence of resistance to interferon in vitro as well compared to pre-treatment samples. Identification of unique viral sequences associated with resistance might allow the prediction of non-response prior to interferon therapy, a unique breakthrough in understanding of hepatitis C and its interaction with interferon therapy.

Statistical Support: Analysis of the initial sequences will be done by alignment and comparison of mutations to identify regions associated with significant amino acid mutations that might confer resistance. Continuous variable data will be analyzed by the Mann Whitney Wilcoxon U test. The Categorical data will be analyzed using the X2 test with Yate's correction or Fisher's exact test. All statistical analyses will be done using SPSS Inc for Windows (release 11.0; Chicago, IL).

Financial Issues: The cost of the pcr, heteroduplex analysis and sequencing at UT Southwestern will be underwritten by the Jeanne Roberts Fund for Hepatitis Research of the Southwestern Medical Foundation. No additional costs to HALT-C are anticipated beyond identification of the serum samples and development of patient and serum lists.

References

1. Strader, D.B., et al., *Diagnosis, management, and treatment of hepatitis C.* Hepatology, 2004. **39**(4): p. 1147-71.
2. Feld, J.J. and J.H. Hoofnagle, *Mechanism of action of interferon and ribavirin in treatment of hepatitis C.* Nature, 2005. **436**(7053): p. 967-72.
3. Gale, M., Jr. and E.M. Foy, *Evasion of intracellular host defence by hepatitis C virus.* Nature 2005. **436**(7053): p. 939-45.
4. Enomoto, N., et al., *Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection.* N Engl J Med, 1996. **334**(2): p. 77-81.
5. Zeuzem, S., J.H. Lee, and W.K. Roth, *Mutations in the nonstructural 5A gene of European hepatitis C virus isolates and response to interferon alfa.* Hepatology, 1997 **25**(3): p. 740-4.
6. Chung, R.T., et al., *Mutations in the NS5A region do not predict interferon-responsiveness in american patients infected with genotype 1b hepatitis C virus.* J Med Virol, 1999. **58**(4): p. 353-8.
7. Pascu, M., et al., *Sustained virological response in hepatitis C virus type 1b infected patients is predicted by the number of mutations within the NS5A-ISDR: a meta-analysis focused on geographical differences.* Gut, 2004. **53**(9): p. 1345-51.
8. Schinkel, J., W.J. Spoon, and A.C. Kroes, *Meta-analysis of mutations in the NS5A gene and hepatitis C virus resistance to interferon therapy: uniting discordant conclusions.* Antivir Ther, 2004. **9**(2): p. 275-86.

9. Nousbaum, J., et al., *Prospective characterization of full-length hepatitis C virus NS5A quasispecies during induction and combination antiviral therapy.* J Virol, 2000. **74**(19): p. 9028-38.
10. Jain, M.K., et al., *Sequence diversity in the Non-Structural 5A region of hepatitis C virus and early response to peginterferon.* Hepatology, 2005. **42**(4, Suppl.1): p. 565A.
11. Shiffman, M.L., et al., *Peginterferon alfa-2a and ribavirin in patients with chronic hepatitis C who have failed prior treatment.* Gastroenterology, 2004. **126**(4): p. 1015-23; discussion 947.
12. Shiffman, M.L., *Retreatment of chronic hepatitis C virus infection in patients who failed to achieve sustained virologic response.* Minerva Gastroenterol Dietol, 2004. **50**(1): p. 37-49.
13. Lee, W.M., et al., *Evolution of the HALT-C Trial: pegylated interferon as maintenance therapy for chronic hepatitis C in previous interferon nonresponders.* Control Clin Trials, 2004. **25**(5): p. 472-92.

Protocol Part III: Serum Requirements <u>For Phase I study of 12 compliant patients who developed HCV viral breakthrough</u>

Visit	Liver # patients, mm*	Blood # patients, ml	DNA # patients, ug	Liver Biopsy Slides # patients, slides/patient	Other (describe) # pts, amount
Screen 1					
Screen 2					
Baseline		0.5ml× 12pts			
Lead in Week 4					
Week 8					
Week 12		0.5ml× 12pts			
W16					
Week 20					
Week 24					
Randomized Month 9		0.5ml× 12pts (or at the time of breakthrough)			
Month 12					
Month 15					
Month 18					
Month 21					
Month 24					
Month 27					
Month 30					
Month 33					
Month 36					
Month 39					
Month 42					
Month 45					
Month 48					
Post-treatment					
Responders W30					
W36					
W42					
W48					
W60					
W72					

* Assume 1 mm tissue weighs about 0.75 mg (= 0.5 mm^2 X Π X density of tissue)

Data needed (please specify): demographic data (age, sex, race), viral load and genotype)

Protocol Part III: Serum requirements for phase II study of 24 Non-responder and SVR patients matched for genotype and approximate viral load, if indicated by early results in breakthrough patients.

Visit	Liver # patients, mm*	Blood # patients, ml	DNA # patients, ug	Liver Biopsy Slides # patients, slides/patient	Other (describe) # pts, amount
Screen 1					
Screen 2					
Baseline		0.5ml× 24pts NR 0.5 ml x 24 pts SVR			
Week 4					
Week 8					
Week 12		0.5ml× 24pts NR 0.5 ml x 24 pts SVR			
W16					
Week 20					
Week 24		0.5ml× 24pts NR 0.5 ml x 24 pts SVR			
Randomized Month 9					
Month 12					
Month 15					
Month 18					
Month 21					
Month 24					
Month 27					
Month 30					
Month 33					
Month 36					
Month 39					
Month 42					
Month 45					
Month 48					
Post-treatment					
Responders W30					
W36					
W42					
W48					
W60					
W72					

Data needed (please specify): demographic data (age, sex, race), viral load and genotype)

Protocol Part III: Serum requirements for phase III study of 12 Breakthrough, 24 Non-responder and SVR patients matched for genotype and approximate viral load.

Visit	Liver # patients, mm*	Blood # patients, ml	DNA # patients, ug	Liver Biopsy Slides # patients, slides/patient	Other (describe) # pts, amount
Screen 1					
Screen 2					
Baseline					
Lead in Week 4		0.5ml× 12pts BT 0.5ml× 24pts NR 0.5 ml x 24 pts SVR			
Week 8					
Week 12					
W16					
Week 20					
Week 24					
Randomized Month 9					
Month 12					
Month 15					
Month 18					
Month 21					
Month 24					
Month 27					
Month 30					
Month 33					
Month 36					
Month 39					
Month 42					
Month 45					
Month 48					
Post-treatment					
Responders W30					
W36					
W42					
W48					
W60					
W72					

Data needed (please specify): demographic data (age, gender, race, ethnicity), viral load, genotype and BMI.

APPENDIX A

HALT-C Ancillary Study PROPOSAL

Part I (1 page)

Proposal Name: Quasi-species Analysis of HCV Breakthroughs Occurring During the HALT-C Trial Lead-In Treatment Phase

Proposal PI: He-Jun Yuan, MD, PhD

Co-Investigators: Mamta Jain, MD, William M. Lee, MD

HALT-C PI: William M. Lee, MD

Funding Agency and Review Body (e.g., NIDDK; my university/GAC): N/A; we will fund study with unrestricted research funds.

I agree to follow HALT-C Policies and Procedures when conducting this study. I acknowledge that the data obtained from my ancillary study will belong to the NIH and will be placed in the HALT-C database for use by other investigators. I understand that I cannot begin experiments using HALT-C specimens/data until I receive approval from the HALT-C Steering Committee and funding from the Scientific Review Body for my proposal. I also understand that the data analysis for this proposal will be performed by NERI (unless otherwise approved by the HALT-C study) and the Protocol will be placed on the HALT-C Website if approved by the HALT-C Steering Committee.

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Proposal Principal Investigator Date

_____ _____

HALT-C Principal Investigator Date

Part II (4 page limit, single space)
Aims/hypotheses
Background/rationale
Relations to aims of HALT-C study
Study design, experimental groups
Methods, data usage
Anticipated results
Statistical support
HALT-C samples to be used in the study (complete Part III: Tissue Requirements)
Financial issues (e.g., cost for data analysis and obtaining samples from Repository)