APPENDIX A: HALT-C Ancillary Study PROPOSAL

Part I

Proposal Name: HCV Strain Variation and HCC

Proposal PI: Andrea Branch, Ph.D.

Co-Investigators: Francis Eng, Ph. D. (molecular virologist) and James Godbold, Ph. D. (biostatistician)

HALT-C PI: Adrian Di Bisceglie

Funding Agency and Review Body: NCI

I agree to follow HALT-C Policies and Procedures when conducting this study. I acknowledge that the data obtained from this 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 Ancillary Studies 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 that Protocols approved by the HALT-C Ancillary Studies Committee will be placed on the HALT-C Restricted Website.

Andrea Branch — Proposal Principal Investigator — July 26, 2010 — Date

HALT-C Principal Investigator — 7/22/10 — Date

Protocol Part II

Aims/hypotheses

Primary hypothesis: Mutations in the HCV *core* gene arise *prior* to the development of liver cancer and therefore are potential non-invasive biomarkers of elevated HCC risk.

Secondary hypotheses: Mutations in the HCV *core* gene are positively selected *during* IFN/RBV treatment; these mutations *increase* over time; and they resent in the *majority* of the patients at the time of HCC diagnosis.

Background/rationale

Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related mortality worldwide (21). The hepatitis C virus (HCV) is a major cause of HCC and therefore it is critical to determine the molecular basis of HCV-induced HCC. Expression of the HCV *core* gene is heavily implicated in the oncogenic potential of chronic HCV infection. Expression of the *core* gene can immortalize primary hepatocytes (22) and lead to cellular transformation and carcinogenesis (23-31). Significantly, clinical studies, mostly conducted in Japan, have revealed that mutations in codons **70** and **91** are associated with HCC (1-4; 16), interferon (IFN) treatment failure (5-14), and insulin resistance (15). These mutations enhance the IFN-resistance of JFH (20), an isolate of HCV that replicates efficiently in cell culture. We postulate that these, and additional *core* gene mutations, enhance virulence and block both the *anti-proliferative* *and* *anti-viral* activities of interferon, thereby promoting both hepatocellular carcinogenesis and IFN treatment failure.

Until recently, little was known about how mutations in the *core* gene might be altering HCV gene expression. We gained a key insight into this process when we discovered that mutations in codons **70** and **91** regulate the levels of two previously-unknown HCV proteins: **70** and **91 minicores** (Fig. 1). 70 and 91 minicores are members of a newly-identified family of HCV proteins that have the **C**-terminal portion of the classical p21 core protein, but lack the **N**-terminal portion (17). The discovery of minicores was made possible by the use of antibodies that bind to the C-terminal portion of the core protein.

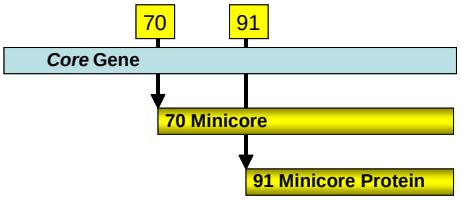

Fig. 1: The *core* gene expresses minicore proteins that have N-termini at codons 70 and 91

To further investigate mutations in codons 70 and 91 and to seek additional mutations associated with HCC, we carried out a comprehensive study using multivariable logistic regression to correlate *core* gene sequences with clinical events (16). We found that mutations in codons 12 and 182 have greatly increased odds ratios for HCC risk (OR > 10.0), establishing their likely biological significance. We hypothesize that mutations in codons 12, 70, 91, and 182 alter HCV function via changes in protein expression that lead to enhanced IFN resistance and increased oncogenic potential. This pilot project will explore the possibility that sequence analysis of the HCV *core* gene can help identify patients who have an elevated risk of developing HCC.

Relations to aims of HALT-C study

An important objective of the HALT-C trial was to identify factors associated with elevated HCC risk. Published data from the HALT-C Trial have analyzed host factors only (78). We hypothesize that viral factors—specifically mutations in the *core* gene—have prognostic and diagnostic value and can be used in combination with host factors to identify patients who have an elevated risk of developing HCC and to identify patients who have already developed early-stage HCC. If this hypothesis is correct, our study will advance the goals of the HALT-C Trial by providing a biomarker that may improve patient care by allowing more cases of HCC to be identified at an early stage when curative surgical interventions are possible. In addition, our project may yield new insights into the molecular

events leading to liver cancer and thus identify novel targets for pharmaceutical interventions to prevent the development of liver cancer and/or to delay progression.

Study design, experimental groups

Overview: We will determine whether *core* gene mutations are associated with incident HCC in the United States, as was found in Japan (1) by analyzing sera collected during the HALT-C Trial. We will use pyrosequencing to compare *core* gene sequences in patients with HCC (cases) and controls. This sequencing approach will enhance the sensitivity of our investigation because HCV RNA does not circulate as a single sequence, but rather as a "swarm" of closely related sequence variants called a quasispecies (67). Pyrosequencing will allow us to determine the *percentage* of the HCV RNA population that has a mutation at each position in the *core* gene. Based on published data and our previous results, we are especially interested in codons 12, 70, 91, and 182. The primary outcome measure will be the percentage of cases versus controls in which 50% or more of the viral RNA population has a codon **70** mutation at baseline. Secondary outcomes will include associations between mutations in other codons and clinical outcomes, and changes in the quasispecies over time.

Study design, experimental groups. A nested case-control study will be performed in which *core* gene sequences of all genotype 1b patients who later developed liver cancer (cases) will be compared to those of patients who did not (controls). Three controls will be used for each case. HCV RNA populations and clinical data from approximately 45 cases and 135 controls will be analyzed. The duration of follow up on controls will be at least as long as on cases. Because not all genotype 1 cases were subgenotyped during the HALT-C trial, this will be done as the first step in this study so that all possible cases can be identified and included. If possible, cases and controls will be matched for baseline histopathology (fibrosis/cirrhosis) and for assignment to one of the two arms of the HALT-C trial. Pyrosequencing will be carried out on samples collected at four time points: entry into HALT-C, at week-8 of IFN/RBV treatment, 9 months after the end of the lead-in phase, and at the time of HCC diagnosis (and at a similar time point for the control subjects).

Sample size and power calculation for the primary outcome measure. This study will include all subjects with genotype 1b HCV who developed HCC during the HALT-C Trial, with three controls for each case. The number of cases is expected to be about 45. The precise number is not yet known because not all specimens have been subgenotyped. To estimate the power of the study, data from our cross-sectional analysis of *core* gene sequences were used (16). The primary outcome measure in this study is the percentage of variants in the viral quasispecies with the 209A mutation (in codon 70) in cases versus controls in samples collected at the time of entry into the trial. In our cross-sectional analysis, 209A was present in 37 of 65 (56%) cases and 74 of 214 (35%) controls. Assuming these proportions, with 45 cases and 135 controls, we have a 63% power to detect a difference between cases and controls at the 5% significance level using a two-tailed chi square test and a 74% power using a one-tailed test, according to nQuery Advisor v.7.0. Multivariable logistic regression will be used to determine whether the codon **70** mutation is an independent predictor of HCC risk. Other variables entered into the regression model will include platelet count, age, gender, and race/ethnicity. We acknowledge that this pilot study may not achieve statistical significance; the data, however, will be valuable and provide the information needed to design a larger, definitive study.

Methods, data usage

For quasispecies analysis, HCV RNA will be purified from serum samples, as before, using the QiAmp viral RNA mini kit (17). The *core* gene region will be subjected to RT-PCR. Two amplicons will be prepared for each sample to allow the entire *core* gene and most of the adjacent 5' untranslated region (UTR) to be pyrosequenced on a 454/Roche GS FLX platform, which can analyze amplicons 400-500 bases in length (68). In order to multiplex samples in the pyrosequencing procedure, "barcoded" primers, each containing a unique 8-base sequence tag will be used during the RT-PCR step, as previously described (69). DNA primer design and data analysis will be done in collaboration with Dr. Omar Jabado (Mount Sinai DNAcore Facility). Reactions will be run at the DNA Sequencing

and Genotyping Laboratory at Cornell University by Dr. Peter Schweitzer, the laboratory Director. For data analysis, we will incorporate computational methods used in a deep sequencing analysis of HCV quasispecies (70). These methods help to distinguish authentic variants in viral populations from artifacts introduced during the isolation and sequencing procedures. These methods include the use of the PyroNoise program which removes homopolymer errors (71). A control HCV genome in a bacterial plasmid and HCV RNA transcripts will be used to quantify variation arising during the isolation and analytical procedure. We previously used the earlier generation of sequencing methods (cloning and sequencing) to characterize the HCV quasispecies in HCV-infected patients and thus are skilled in quasispecies data analysis (72).

Secondary outcome measures. Methods of quasispecies analysis (67; 70; 72-77) will be used to determine the association between clinical outcomes (diagnosis of HCC, disease progression, death) and mutations in codons 12, 70, 91, and 182 specifically and in other regions of the *core* gene and in the 5' UTR . Analysis will be done on samples obtained at entry into the trial, at week 8 during treatment, at 9 months after the end of the lead-in phase (18-19), and at the time of HCC diagnosis. Because this is an exploratory study, no correction for repeat testing will be made.

Anticipated results

We expect that *core* gene mutations will be more prevalent among patients who later developed HCC and we expect the percentage of the viral quasispecies with one or more *core* gene mutations to increase over time. If our results are similar to those obtained in Japan (1), we expect multivariable analysis to show that *core* gene mutations are independently associated with increased HCC risk. Because of the limited sample size, our results may not achieve statistical significance; however, they will provide the information needed to design a larger, definitive future study.

Statistical support

Statistical support will be provided by Dr. James Godbold, a Ph.D. biostatistician in the Department of Preventive Medicine at the Mount Sinai School of Medicine, and Erin Moshier, an MS-level biostatistician, in the same department.

HALT-C samples to be used in the study (complete Part III: Sample Requirements)

Financial issues (e.g., cost for data analysis and obtaining samples from Repository)

Costs for data analysis and for obtaining samples from the repository will be paid by NIH R21 CA152514

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Protocol Part III: Sample Requirements. (link to web site with actual sample availability)

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		180 0.5 ml			
Lead in Week 4					
Week 8		180 0.5 ml			
Week 12					
W16					
Week 20					
Week 24					
Randomized Month 9		180 0.5 ml			
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 features (age, date of birth, sex, race, ethnicity); baseline characteristics (fibrosis stage, platelet count, AST, ALT, smoking habits, esophageal bleeding history); HCV viral load at all time points

Comments (if any):

1. In addition to the samples indicated in the table above, we also need a sample from each case at the time of HCC diagnosis, or a sample collected as soon after the diagnosis as possible;

and we also need three control samples for each case (the control samples need to have a similar length of follow up as the cases).

2. The study is limited to subjects with genotype 1b HCV. Because the subgenotype was not determined for all subjects with genotype 1 HCV, we need the baseline sample from approximately 17 subjects so that we can determine the subgenotype.

3. To the extent that it is possible, we would like to match “cases” (subjects who developed HCC) with “controls” (subjects who did not develop HCC at any time during the trial) on the basis of fibrosis/cirrhosis stage at baseline and on the group assignment during the randomization phase.