**APPENDIX A: HALT-C Ancillary Study PROPOSAL**

**Part I** (1 page)

Proposal Name: Occult HBV Infection and HCC in HALT-C Patients

Proposal PI: Anna Lok

Co-Investigators: Timothy Morgan and Adrian Di Bisceglie

HALT-C PI: Anna Lok

Funding Agency and Review Body (e.g., NIDDK; my university/GAC): NIDDK

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.

_____ _____

Proposal Principal Investigator Date

_____ _____

HALT-C Principal Investigator Date

**Protocol Part II** (4 page limit, single space)

## 1. Aims/hypotheses

Hypotheses: A substantial proportion of HALT-C patients have previous HBV infection because of shared risk factors for HBV and HCV infection. Past HBV infection increases the risk of HCC development among HALT-C patients.

Aims: To compare the prevalence of (a) past and (b) occult HBV infection in HALT-C patients who developed HCC and those who did not and to determine the factors associated with the presence of (a) past and (b) occult HBV infection in HALT-C patients.

## 2. Background/rationale

HBV DNA remains detectable in the liver in many patients and in the serum in some patients who have serological recovery from past HBV infection. The presence of HBV DNA in persons who are HBsAg negative is often referred to as occult HBV infection. Occult HBV infection has been defined as the presence of HBV DNA in the liver (with or without detectable HBV DNA in the serum) of individuals testing negative for HBsAg by currently available assays [1]. The molecular basis for occult HBV infection is related to the stability and long-term persistence of covalently closed circular (ccc) HBV DNA in infected livers. Occult HBV infection may be classified as seropositive (anti-HBc and/or anti-HBs positive) or seronegative (anti-HBc and anti-HBs negative). A recent expert conference on occult HBV infection stated that the gold standard for the diagnosis of occult HBV infection is the testing of snap frozen liver for HBV DNA using real-time PCR technique [1]. When liver samples are not available, testing for HBV DNA in serum using a highly sensitive PCR assay or for anti-HBc using enzyme immunoassays may be used as surrogates for the diagnosis of occult HBV infection but it should be recognized that testing of blood samples is far less sensitive than testing of liver samples.

The prevalence of occult HBV infection is higher in countries where HBV infection is prevalent and in patients who have risk factors for HBV infection such as those with HIV or HCV infection [2]. Several studies, mostly from Europe and Japan, have found a higher rate of occult HBV infection in HCV patients who have HCC compared to HCV patients with no HCC [2-5]. In some of these studies, the frequency of detection of occult HBV infection in HCV patients with HCC was as high as 60-70%. Data on the prevalence of occult HBV infection in HCV patients with HCC in the United States is limited. Occult HBV infection may increase the risk of HCC by causing more severe inflammation and more rapid progression to cirrhosis. Alternatively, HBV may increase the risk of HCC via direct oncogenic effects.

HALT-C study with its very well characterized cohort of patients prospectively followed provides important data on the incidence of HCC in American patients with advanced HCV. The study also provides an opportunity to examine risk factors associated with HCC development. It is possible that one explanation for the somewhat lower incidence of HCC among American patients with advanced HCV compared to Japanese patients is related to the lower prevalence of occult HBV infection in the American patients.

## 3. Relations to aims of HALT-C study

HCC is one of the primary outcomes of the HALT-C study. An important goal of the HALT-C study is to determine the factors associated with HCC development in patients with advanced hepatitis C. This study will make use of serum and liver tissue samples collected in the HALT-

C study to determine the role of occult HBV infection in HCC development in patients with advanced hepatitis C in the United States, a country with low endemicity for HBV infection.

## 4. Study design, experimental groups

This will be a case-controlled study.

HCC cases – will include patients that meet HALT-C criteria for presumed or definite HCC diagnosed at any time after enrollment into HALT-C. Patients with presumed HCC who did not receive any cancer treatment and had no evidence of tumor progression after ≥2 years of follow-up will be excluded.

Controls – 2:1 with matching for fibrosis strata, treatment assignment (for randomized patients), duration of follow-up, and no HCC for at least 12 months beyond censoring will be selected.

Total no. of HCC cases = 96 (71 definite and 25 presumed), 90 after randomization (68 definite and 22 presumed), and 6 during lead-in/responder phase (3 definite and 3 presumed).
No. of HCC cases with flash frozen liver at screening = 34

Stored sera at screening are available on all HCC cases and controls. For HCC cases with snap frozen liver – an additional selection criterion for controls would include availability of snap frozen liver to ensure that liver samples will be available for the controls of the 34 HCC patients in whom flash frozen liver at screening is available.

No. of controls for blood testing = 192
No. of controls for liver tissue testing = 68

## 5. Methods, data usage

All cases and controls will be tested for anti-HBs, anti-HBc and HBV DNA in serum. HBsAg will not be tested because HBsAg positivity was an exclusion criterion in the HALT-C study. HCC cases and matched controls with snap frozen liver will be tested for HBV DNA in the liver. Cases and controls will be identified by NERI. Deidentified coded serum samples will be sent to University of California-Irvine and deidentified coded liver samples will be sent to University of Michigan for testing.

Testing of blood samples - Stored serum during screening visit (S00 or S02) prior to interferon treatment will be tested for anti-HBs and anti-HBc with enzyme immunoassays [Simens (Bayer) Anti-HBs (aHBs) ADVIA Centaur System and Diasorin ETI-AB-COREK PLUS, respectively] and for HBV DNA with a quantitative real-time PCR assay [Roche COBAS® AmpliPrep / COBAS® TaqMan® HBV Test] that has a lower limit of detection of 30 IU/mL. To ensure reproducibility of results, all blood samples that test positive for HBV DNA and 20% of randomly selected blood samples that test negative for HBV DNA will be retested. Serum samples that are consistently positive for HBV DNA will be sent to University of Michigan for sequencing.

Testing of liver samples - Snap-frozen liver tissue from baseline biopsy will be tested for HBV DNA using an in-house real-time PCR assay which has a lower limit of sensitivity of approximately $2 \times 10^{-6}$ copies/cell [5,6]. This assay was developed in Dr. Lok's lab and had been applied to study liver biopsy samples. Positive (liver samples from HBsAg-positive patients who had been previously found to have detectable HBV DNA in serum as well as liver) and negative (liver samples from liver donors who are seronegative for HBV and who

had been previously found to have undetectable HBV DNA in serum as well as liver) controls will be included in each assay. To verify reproducibility of assay results, all liver samples that test positive and 20% of liver samples that test negative will be randomly selected for repeat testing. To verify specificity of assay results, two regions of the HBV DNA genome will be amplified, and all samples with positive results will be sequenced. A house keeping gene, β-actin, will be amplified in parallel to confirm integrity of the liver samples and to estimate the amount of genomic DNA in each liver sample such that the results of the HBV DNA assay can be expressed as copies/liver cell.

Data on the following variables will be analyzed:
*Baseline/screening*

- Demographics
- Risk factors for and duration of HCV infection
- Metabolic factors: BMI, diabetes
- Labs: CBC, liver panel, INR, AFP, HCV genotype, HCV RNA
- HFe genotype
- Liver histology: iron, steatosis, inflammation, and fibrosis scores
- Alcohol and smoking history

*Randomization/post-randomization* [for randomized patients only]

- Treatment assignment
- Esophageal varices on endoscopy at W24 or R00
- Ishak fibrosis scores on subsequent biopsies: year 1.5, year 3.5
- Lead-in response: Non-responder vs. Breakthrough/relapser
- Clinical outcome: CTP ≥7, ascites, SBP, encephalopathy, liver-related death

6. Anticipated results

Occult HBV infection will be defined as the presence of HBV DNA in the liver (with or without detectable HBV DNA in the serum) of individuals testing negative for HBsAg. Presence of HBV DNA will be determined by duplicate assays that amplify 2 different regions of the HBV genome. Samples that are positive in at least 3 of 4 assays (duplicates of 2 PCR) will be considered positive.
Because liver samples are available in only 1/3 of patients with HCC, past HBV infection defined as the presence of anti-HBc in serum (with or without detectable HBV DNA or anti-HBs) of individuals testing negative for HBsAg will also be analyzed.

A high proportion (>30%) of HALT-C patients will have evidence of past HBV infection as evidenced by the presence of anti-HBc in serum. Most of these patients will have undetectable HBV DNA in serum. The rate of HBV DNA detection in the liver will be higher than that in the serum. The vast majority of patients with detectable HBV DNA in the liver will be anti-HBc positive. Sequencing of HBV DNA in the serum and liver will not reveal major changes indicating that the HBV is capable of replicating and that absence of detectable HBsAg in serum is not a result of mutations in the S gene or S promoter.
Occult HBV infection will be present in a higher proportion of patients who developed HCC, in patients with risk factors for HBV infection such as injection drug use and sexual promiscuity, and in racial/ethnic groups that have a higher prevalence of HBV infection, e.g. African Americans.

7. Statistical support

NERI will provide statistical support

8. HALT-C samples to be used in the study (complete Part III: Sample Requirements) – see table

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

A) Testing of blood samples at UCI
   a. Unit cost of anti-HBs = $10
   b. Unit cost of anti-HBc = $10
   c. Unit cost of HBV DNA = $87
   d. Total no. of samples to be tested = 96+192 = 288
   e. Quality control and repeat testing for HBV DNA = all positive samples [estimated to be <20%] and 20% of negative samples = 58
   f. Direct cost of testing = $30,816 + $5,046 = $35,862
   g. Indirect cost @55% = $19,724
   h. Total cost = $55,586

B) Testing of liver samples at UMich – DNA extraction, PCR amplification of 2 regions of HBV DNA genome, and sequencing of positive samples (~40 samples)
   a. Unit cost of reagents x DNA extraction and PCR = $50
   b. Unit cost of tech labor x DNA extraction and PCR = $250
   c. No. of samples = 34+68 = 102
   d. Quality control - repeat test of all positive samples (~50% of samples initially positive) and 20% of negative samples = 51 + 10 = 61 samples
   e. Unit cost of sequencing (automated sequencing + interpretation) – 2 amplicons/sample = $50
   f. Direct cost of testing = $300 x (102+61) + $50 x 40 = $50,900
   g. Indirect cost @ 54.5% = $27,740
   h. Total cost = $78,640

   Sequencing of serum samples with detectable HBV DNA – PCR needs to be repeated prior to sequencing unless primers used for amplification in Roche assay is known. Only 1 region will be amplified and sequenced
   a. Unit cost of reagents for DNA extraction and PCR = $30
   b. Unit cost of tech labor x DNA extraction and PCR = $150
   c. No. of samples = 30
   d. Unit cost of sequencing (automated sequencing + interpretation) = $30
   e. Direct cost of testing = $30 x 210 = $6,300
   f. Indirect cost @54.5% = $3,433
   g. Total cost = $9,733

   Total cost of liver and serum testing at U Michigan = $88,373

C) Additional work
   a. NERI – covered under current contract
      i. Identification of cases and controls
      ii. Pulling samples by Seracare and shipment of samples
      iii. Data analyses
   b. UCI – PI supervision = no cost
   c. UMich – PI supervision = no cost

**10. References**


1. Raimondo G et al., Statements from the Taormina expert meeting on occult hepatitis B virus infection. J Hepatol 2008; 49: 652-7.
2. Raimondo G et al., Occult hepatitis B virus infection. J Hepatol 2007; 46: 160-170.

3. Brechot C et al., Persistent hepatitis B virus infection in subjects without hepatitis B surface antigen: clinically significant or purely occult? Hepatology 2001; 34: 194-203.
4. Marrero JA and Lok AS. Occult hepatitis B virus infection in patients with hepatocellular carcinoma: innocent bystander, cofactor or culprit? Gastroenterol 2004; 126: 347-350.
5. Shetty K, Prevalence and significance of occult hepatitis B in a liver transplant population with chronic hepatitis C. Liver Transpl 2008; 14: 534-540.
6. Hussain M et al., Presence of intrahepatic (total and ccc) HBV DNA is not predictive of HBV recurrence after liver transplantation. Liver Transpl 2007; 13: 1137-1144.

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

| Visit | Liver<br># patients, mm* | Blood<br># patients, ml | DNA<br># patients, ug | Liver Biopsy Slides<br># patients, slides/patient | Other (describe)<br># pts, amount |
|---|---|---|---|---|---|
| Screen 1 | | 96 cases + 192 controls<br>1.5 mL each at S00 or S02 | | | |
| Screen 2 | 34 cases + 68 controls, minimum 2 mm | | | | |
| Baseline | | | | | |
| Lead in Week 4 | | | | | |
| 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 | | | | | |

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

Data needed (please specify): See section 5
Comments (if any):