APPENDIX A: HALT-C Ancillary Study PROPOSAL

Part I (1 page)
Proposal Name:
Epidermal growth factor gene functional polymorphism and the risk of hepatocellular carcinoma in the HALT-C cohort.

Proposal PI: Ray Chung

Co-Investigators:
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HALT-C PI: Ray Chung

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

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

We hypothesize that: HALT-C subjects with the EGF G/G or G/A genotypes will have increased odds for developing HCC after controlling for age, sex, ethnicity, and the severity of liver disease by histopathology. Also, they will have higher serum levels of EGF.

2. Background/rationale

Hepatocellular carcinoma (HCC) is the sixth most common solid tumor world-wide, and the third leading cause of cancer related deaths (1,2). Cirrhosis is the strongest and the most common known risk factor for HCC, particularly cirrhosis related to hepatitis C virus (HCV) and hepatitis B virus (HBV) infections (3, 4). Given current limited therapies for HBV and HCV infections, identification of populations at high risk for the development of HCC for targeted screening and chemoprevention is of a vital importance. The current screening strategies for HCC are targeted at high-risk patients with cirrhosis irrespective of etiology and some HBV-infected patients irrespective of cirrhosis, and utilize α-fetoprotein (AFP) and abdominal ultrasound (US) imaging (5). Both of these screening modalities

are limited. US has a reported sensitivity around 60% with lower sensitivities reported in advanced cirrhosis (6, 7). AFP has reported sensitivities in the range of 25-65%, and can be falsely elevated in patients infected with HCV (8, 9).

Mounting evidence from animal studies support a role for the epidermal growth factor (EGF) in the pathogenesis of HCC and malignant transformation of hepatocytes (10, 11, 12). Transgenic mice with liver-targeted overexpression of the secreted EGF fusion protein develop hepatocellular carcinoma (13), and blockade of EGF receptor (EGFR) activity by gefitinib (an EGFR-tyrosine kinase inhibitor) halt the development of HCC in diethylnitrosamine exposed rats (14). Single nucleotide polymorphism involving *A* to *G* transition at position 61 in the 5' untranslated region of the *EGF* gene, causes higher EGF levels in individuals with the G/G genotype and is associated with the development of malignant melanoma compared to individuals with the A/A genotype (15).

Recently, our group demonstrated in a case-control study of 207 patients with cirrhosis a four fold increase in the odds of developing HCC in patients with the G/G genotype compared to the A/A genetype (16). Furthermore, patients with the G/G genotype had significantly higher serum and hepatic levels of EGF due to a 2-fold increase in its half-life compared to patients with the A/A genotype (16). These results were further validated in a separate cohort of French patients with alcoholic cirrhosis (16).

Thus, based on the results of the above study, using the EGF gene single-nucleotide polymorphism (A to G) to identify cirrhotic patient with high risk of developing HCC to target for a more aggressive screening and chemoprevention approach appears to be feasible. However, our current study was retrospective in design with no control for the severity of the underlying liver disease by liver histopathology, and lacked ethnic variability as most of the patients were white. Thus, the HALT-C trial cohort represents an ideal, large, and ethnically diverse cohort to evaluate the correlation between the EGF gene single-nucleotide polymorphism and the risk of HCC, while controlling for the severity of the liver disease by histopathology (Ishak score).

3. Relations to aims of HALT-C study

As stated above, the HALT-C trial cohort represents an ideal, large, and ethnically diverse cohort to evaluate the correlation between the EGF gene single-nucleotide polymorphism and the risk of HCC, while controlling for the severity of the liver disease by histopathology (Ishak score).

4. Study design, experimental groups

We will select our cases and controls from all HALT-C patients with genetic consent who are Caucasian (non-Hispanic) or African American with known liver histopathology and available liver tissue/serum for EGF genotyping and protein expression levels who will have complete genomic sequencing performed by Celera Diagnostics. From the approximately 837 subjects who meet these criteria, there are about 56 subjects with confirmed HCC; these will be designated as cases. From the pool of 837 subjects, we will then match the cases in a 1:2 ratio to non-HCC controls by age, sex, ethnicity, and severity of liver disease on histopathology (Ishak score) to report the odds ratio of each EGF genotype A/A, A/G, and G/G for association with HCC. There will therefore be about 112 matched controls in the HALT-C cohort. Sub-group analysis of Caucasians (non-Hispanics) and African Americans will be performed. We have already secured AS Committee approval for use of DNA in cases and controls. In the second part of the analysis, we will perform multivariate analysis with logistic regression to determine which of the following variates (age, sex, ethnicity, severity of liver disease on histopathology, EGF serum level, and the number of copies of the EGF *G allele)* contribute significantly to the development of hepatocellular carcinoma after adjusting for the other co-variates.

In the event that the primary analysis reveals differences in EGF genotype between the two groups, we will then return to the AS Committee with a request to analyze the corresponding sera (0.5 mL) to correlate EGF protein levels with EGF genotype.

5. Methods, data usage

- *EGF* polymorphism will be analyzed using restriction fragment-length polymorphism.
- EGF protein level from the serum will be quantified using an enzyme-linked immunosorbent assay (ELISA).
  Outcome variable (development of HCC) will be analyzed using Cox regression. The covariates (Age, sex, ethnicity, severity of liver disease on histopathology (Ishak score), serum EGF level, EGF genotype (A/A, A/G, G/G) and/or the number of copies of the EGF G allele) will be entered one at a time in a univariate model. The multivariate model will include the covariates that were significant in the univariate model.
- Comparisons of serum EGF level by number of copies of the *G allele* will be made using a Jonckheere-Terpstra test.
- Depending on the results of these studies, confirmatory assays on available unstained liver biopsy slides and/or frozen tissue will be performed

6. Anticipated results

HALT-C subjects with the EGF G/G or G/A genotypes will have increased odds for developing HCC after controlling for age, sex, ethnicity, and the severity of liver disease by histopathology. Also, they will have higher serum levels of EGF.

7. Statistical support: NERI

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

See Part III Table – we anticipate use of about 168 DNAs and, in second phase, serum samples (about 56 HCCs and up to 112 controls)

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

The procedures of sample collection and preparation will include following steps, and the cost required at each site of operation will be appropriately reimbursed after the procedure is accomplished.

a. Sample selection (by NERI)
   1. Selecting HCC cases according to the criteria described in Section 4.
   2. Matching controls to the HCC cases according to the criteria described in Section 4.
b. Serum and DNA acquisition (by SeraCare)
   1) Pulling of these samples
   2) Sample shipping by FedEx to MGH
c. EGF sequencing and serum testing
d. Data analysis
   1. Provision of dataset and/or analyses (by NERI).

The cost of selection, shipping, experimentation and data analysis will be assumed by investigator funds.

10. References

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(11) Blanc P, Etienne H, Daujat M, et al. Mitotic responsiveness of cultured adult human hepatocytes to epidermal growth factor, transforming growth factor alpha, and human serum. *Gastroenterology*. 1992; 102:1340-1350.

(12) Giannetti G, Sgarra C, Porcelli L, et al. EGFR and VEGFR as potential target for biological therapies in HCC cells. Cancer Letters 2008; 262:257–264.

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(14) Schiffer E, Housset C, Cacheux W, et al. Gefitinib, an EGFR inhibitor, prevents hepatocellular carcinoma development in the rat liver with cirrhosis. Hepatology 2005; 41:307-314.

(15) Shahbazi M, Pravica V, Nasreen N, et al. Association between functional polymorphism in *EGF* gene and malignant melanoma. *Lancet* 2002; 359: 397-401.

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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		ca 168 (2nd phase)	ca 168 (Celera to do genotyping)		
Screen 2					
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 Π X density of tissue)

Data needed (please specify):

- DNA from patients for EGF gene genotyping.
- Serum from patients to measure EGF protein level (0.5mL) (2nd phase request)
- Liver biopsy unstained slides (1 per pt): desirable but not essential

Comments (if any):