APPENDIX A: HALT-C Ancillary Study PROPOSAL

Part I (1 page)
Proposal Name: Contribution of telomere length and telomerase gene variants to fibrosis progression in chronic hepatitis C virus infection

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HALT-C PI: James Everhart, MD, NIDDK

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

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

   a) To investigate whether in chronic hepatitis C virus infection, telomere length at the time of randomization correlates with progression to cirrhosis by the two-point increase in fibrosis score.

   b) To test whether hypomorphic genetic variants in the telomerase complex genes correlate with the two-point increase in fibrosis score in chronic hepatitis C.

2. Background/rationale

   Some patients with chronic hepatitis C progress to cirrhosis, but the risk factors for fibrosis development are poorly understood.

   Telomeres are structural elements in elements that cap the ends of chromosomes, protecting them from recombination, end-to-end fusion, and recognition as damaged DNA (Calado & Young, Blood 2008). In human somatic cells, telomeres typically consist of hundreds to thousands of

TTAGGG tandem repeats, which are gradually lost with cellular replication. Attrition eventually shortens telomeres critically; the result is arrested proliferation and senescence, shortened life span, apoptosis, and genomic instability of the cell. Telomere maintenance requires telomerase, which consists of telomerase reverse transcriptase (TERT) and its integral RNA template (TERC), in addition to other proteins (dyskerin, NOLA1, NOLA2, and NOLA3). TERT copies a short region of TERC into telomeric DNA to extend the 3' end of the telomeres.

Deficient telomerase function resulting in excessive telomere shortening has been implicated in the pathophysiology of certain human diseases (Calado & Young, NEJM 2009). Dyskeratosis congenita is an inherited bone marrow failure syndrome characterized by a triad of ectodermal dysplasia: nail dystrophy, leukoplakia, and reticular skin hypopigmentation. An increased prevalence of liver disease, mainly cirrhosis, is observed in patients with dyskeratosis congenita (Dokal, BJH 2000). X-linked dyskeratosis congenita (Online Mendelian Inheritance in Man [OMIM] number 305000) is caused by mutations in the *DKC1* gene, which encodes dyskerin, a protein associated with telomerase, while mutations in *TERT* and *TERC* also are etiologic in autosomal recessive or autosomal dominant dyskeratosis congenita. Telomerase mutations also may have marrow failure as the sole clinical manifestation, clinically translating into apparently acquired aplastic anemia in the absence of other clinical features of dyskeratosis congenita (Fogarty et al., Lancet 2003; Yamaguchi et al., NEJM 2005).

More recently, TERT and TERC mutations in patients with severe liver disease, identified within kindreds of patients presenting with aplastic anemia (Calado et al. PLoS ONE 2009). The most common diagnoses were cirrhosis and liver nodular regenerative hyperplasia, which occurred in the absence of marrow failure or other physical stigmata associated with dyskeratosis congenita.

In a subsequent analysis of patients with cirrhosis due to various etiologies (chronic hepatitis C virus infection, alcohol, cryptogenic), we and others have found an increased prevalence of loss-of-function telomerase mutations in cirrhotic patients compared to healthy controls, suggesting that telomerase mutations predisposed to cirrhosis (Calado et al.; Rudolph et al., unpublished data). Patients with cirrhosis and carrying mutations had short telomeres, and mutations led to decreased telomerase enzymatic activity. However, both independent studies were cross-sectional and did not address whether mutation status or short telomeres might contribute to progression to fibrosis in chronic hepatitis C virus infection or other etiologies.

In a murine model of telomerase deficiency (*mTR*$^{-/-}$), short telomeres associated with defects in liver regeneration and accelerated the development of liver cirrhosis after chronic liver injury, while telomerase over-expression restored telomerase activity and telomere function, alleviated cirrhotic pathology, and improved liver function, indicating that short telomeres contribute to liver cirrhosis and that telomerase may modulate liver regeneration capacity (Rudol[h et al., Science 2000).

3. Relations to aims of HALT-C study

   The aim of our proposed study is to investigate whether telomere length and telomere shortening are risk factors and biomarkers for progression to fibrosis in chronic hepatitis C virus infection. While HALT-C study aims to evaluate the safety and efficacy of pegylated interferon to prevent progression to cirrhosis, our proposal aims to identify those patients who are at higher risk of progression and who might especially benefit from early intervention.

4. Study design, experimental groups

   Our study would consist of a single study group of 220 patients who did not receive pegylated interferon (untreated control arm), in which we will measure telomere length at baseline (before randomization) and correlate telomere length to fibrosis progression. Sample size justification is given in Section 7 below.

5. Methods, data usage

   Telomere length will be assessed using a quantitative polymerase chain reaction (qPCR) method in DNA samples extracted from peripheral blood leukocytes (Cawthon, NAS 2002; Brouilette et al., Lancet 2007). Genetic screening will be performed by direct bi-directional sequencing (Yamaguchi et al., NEJM 2005).

6. Anticipated results

   Based on our previous results indicating that telomerase mutations are associated with cirrhosis and that in a mouse model, telomerase deficiency may precipitate liver cirrhosis while telomerase over-expression may ameliorate it, our hypothesis is that shorter telomeres will correlate with a higher chance to fibrosis progression in chronic hepatitis C virus infection.

   The analyses will be performed by the authors (NSY, RTC, and CW), who have long term expertise on genetic case-control studies and clinical trials. Once clinical variables are provided to the authors, they will be transformed into numerical results along with telomere length data and computed using the S-PLUS software package (TIBCO Software Inc., Palo Alto, CA).

7. Statistical support

   A subject's "predicted telomere length" will be computed using ($a + b \times age$) based on a sample of healthy control subjects, where a and b are the least squares estimates of the slope and intercept for the linear model of telomere length versus age. Age-adjusted telomere length for each subject will be computed by subtracting the subject's "predicted telomere length" from his/her observed telomere length. Quartiles of the age-adjusted telomere length will be computed, and used as the categorical age-adjusted telomere length for evaluating the probabilities of time to fibrosis progression and overall survival. Multivariate logistic regression models will be used to evaluate the continuous and binary age-adjusted telomere length and other baseline variables on the rates of progression to fibrosis at 3.5 years.

   Sample Size Justification: From a preliminary sample of 175 healthy control subjects and 35 cirrhosis patients, we observed that the age-adjusted telomere lengths for the healthy control subjects have approximately mean 0 and standard deviation 0.275, while the age-adjusted telomere lengths for the cirrhosis patients have mean -0.145 and standard deviation 0.148. Let n1= # of patients without progression, n2= # of patients with progression, T1= mean age-adjusted telomere length for patients without progression, T2= mean age-adjusted telomere length for patients with progression. Assume that the standard deviations for both cohorts (with and without progression) are 0.148. The following table shows the statistical power of two-sample t-tests under 0.05 significance level and a series of scenarios for n1, n2, T1 and T2:

n1	n2	T1	T2	Power
100	100	0.001	-0.145	>0.999
100	100	0.001	-0.060	0.830
100	100	-0.080	-0.160	0.969
150	50	0.001	-0.070	0.084
150	50	-0.070	-0.145	0.874
150	50	-0.080	-0.160	0.912

Based on these results, we anticipate that our sample size of at least 200 patients would yield sufficient power for a number of preliminary analyses.

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

   One microgram of DNA collected at baseline from 200 untreated patients (randomized to the untreated control group).

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

   The costs of experiments in our laboratory are paid by the NIH Intramural Research Program (IRP).

10. References

Brouilette SW, Moore JS, McMahon AD, Thompson JR, Ford I, Shepherd J, et al. Telomere length, risk of coronary heart disease, and statin treatment in the West of Scotland Primary Prevention Study: a nested case-control study. Lancet. 2007 Jan 13;369(9556):107-14.

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Calado RT, Regal JA, Kleiner DE, Schrump DS, Peterson NR, Pons V, et al. A spectrum of severe familial liver disorders associate with telomerase mutations. PLoS ONE. 2009;4(11):e7926.

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Cawthon RM. Telomere measurement by quantitative PCR. Nucleic Acids Res. 2002 May 15;30(10):e47.

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Rudolph KL, Chang S, Millard M, Schreiber-Agus N, DePinho RA. Inhibition of experimental liver cirrhosis in mice by telomerase gene delivery. Science. 2000 Feb 18;287(5456):1253-8.

Yamaguchi H, Calado RT, Ly H, Kajigaya S, Baerlocher GM, Chanock SJ, et al. Mutations in TERT, the gene for telomerase reverse transcriptase, in aplastic anemia. N Engl J Med. 2005 Apr 7;352(14):1413-24.

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					220 patients, frozen PB cells
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): demographic data on patients, progression to fibrosis, and survival.
Comments (if any): Samples from 200 patients randomized to the untreated group are requested.