Host Genes Snap Shot

Sites Participating: All sites and all patients in the HALT-C Trial

Principal Investigator: Timothy Morgan, MD (University of California, Irvine) and Herb Bonkovsky, MD (University of Connecticut)

Co-Investigators: Don Brambilla, PhD; Raymond Chung, MD; Karen Lindsay, MD; David Gretch, MD, PhD; Jules Dienstag, MD; Michael Dean, PhD; Mary Carrington, PhD; Tom O'Brien, MD, MPH

Study Name: Host Genes Influence the Course of Chronic Hepatitis C

Separate Consent Form: No

Withdrawal Form: Yes (Form# 9)

Eligible Patients: All Lead-in patients and Express patients who provide consent for genetic testing.

Visit Schedule:
Blood samples are obtained at baseline and during follow-up for the preparation of DNA from patients who provide consent for genetic testing. DNA is prepared by the repository and will be sent to the laboratories for this study.

Genetic Consent:
This consent is part of the main consent form for the HALT-C trial. Genetic consent is recorded on Form #4 and may be changed by the patient at any time, complete Form #9 Genetic Status Change. The current status of genetic consent is checked before DNA is prepared or samples are tested.

Host Genes Influence the Course of Chronic Hepatitis C

Principal Investigators: Timothy Morgan, MD, and Herb Bonkovsky, MD

HALT-C Co-Investigators: Don Brambilla, PhD; Raymond Chung, MD; Karen Lindsay, MD; David Gretch, MD, PhD; Jules Dienstag, MD

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Objective

There is increasing evidence that host genes influence the natural history of hepatitis C (see below). The overall objective of this ancillary study is to evaluate the association between several host genes/alleles and 1) rate of progression of liver fibrosis and 2) response to treatment. Finding genes that influence response to treatment and progression of fibrosis will lead to better understanding of the pathophysiology of hepatitis C and to better disease management.

Hypotheses

I. Genetic influences on liver fibrosis.

1. The following alleles/haplotypes are associated with an increased rate of liver fibrosis:

Transforming growth factor-beta 1 (TGF-β1) codon 25 arg/arg
Angiotensinogen gene promoter (-6) adenine/adenine
Tumor necrosis factor (TNF) promoter alleles –238A and –308A
Microsomal epoxide hydrolase exon 3 113 His/His homozygosity
The following HLA alleles are associated with an increased rate of liver fibrosis:
B54
DQB1*0401
DRB1*0405
DQB1*0502

3. Apolipoprotein E-epsilon4 allele is associated with a decreased rate of liver fibrosis.

The following HLA alleles/haplotypes are associated with a decreased rate of liver fibrosis:

a. alleles: DRB1*1302; DQB1*0604; DRB1*0901; DQB1*0303; DRB1*1101
b. haplotype: DQA1*0201-DQB1*0201
c. haplotype: DRB1*1104-DQA1*0501-DQB1*0301

Heterozygosity at HLA class I and/or class II loci is associated with a decreased rate of liver fibrosis.

II. Genetic influences on response to treatment with peginterferon plus ribavirin.

Patients with the following alleles/haplotypes are more likely to be PCR negative after 20 weeks' treatment with peginterferon plus ribavirin:
Interleukin-10 promoter alleles -592*A, -819*T or the haplotype ATA (-1082/-819/-592)
HLA haplotype DRB1*1301-DQA1*0103-DQB1*0603

Background

Most infectious diseases have a range of outcomes, from mild and benign to rapid and fatal. Host genes are among a variety of factors (e.g., patient age, concurrent medications, co-morbid conditions) that are accepted as influencing outcome in several infectious diseases. Hemoglobin S, for example, reduces the probability of developing severe disease following infection with *Plasmodium falciparium* [1]. Polymorphisms in the CCR5, CCR2 and various HLA genes influence HIV disease progression [2-5]. Furthermore, degree of HLA heterozytosity influences disease outcome in both HIV and in hepatitis B [2, 6].
Preliminary studies in hepatitis C suggest an association between several host alleles and disease outcome. In particular, host alleles in patients with hepatitis C have been associated with: 1) susceptibility to infection, 2) clearance of infection, 3) response to interferon treatment,

and 4) progression of disease (i.e., development of fibrosis). The purpose of this ancillary study is to further evaluate the role of host genetic factors in HCV disease progression and response to treatment.

Disease association studies

In disease association studies, a candidate gene polymorphism is studied in a group of individuals exposed to a particular infection. The frequencies of the polymorphism are compared between those with and without a defined study outcome. For example, the frequency of an HLA allele could be compared in HCV patients who have cirrhosis vs. those without cirrhosis. Although the development of cirrhosis is multi-factorial (e.g., duration of infection, alcohol use, gender?, genotype?), finding an increased (or decreased) allele frequency in patients with cirrhosis could suggest a role for the allele in the pathophysiology of cirrhosis. Genetic association studies are important because they may provide fundamental clues about the pathophysiology of a disease and/or lead to new therapeutic avenues.

HLA

The human major histocompatibility complex is located on the short arm of chromosome 6 and encodes for several protein products involved in immune function including complement, TNF-alpha, and the HLA molecules. The HLA genes include both class I (HLA-A, -B, and -C) and class II (HLA-DRB1, -DQA1, -DQB1, -DPA1, and BDPB1) alleles. HLA molecules are involved in immune response because they present foreign antigens to both the CD4+ helper T cells and the CD8+ cytotoxic T cells (CTL's). Class I molecules generally present antigens that are generated endogenously, including epitopes from viruses. To eliminate virally infected hepatocytes, CTLs must recognize the combination of viral peptide antigen and a class I molecule present on the surface of the hepatocyte. Class II molecules on antigen presenting cells present extracellularly derived antigens, including viral peptides, to CD4+ T cells to stimulate cytokine release driving both humoral and cell-mediated immune responses.

HLA in hepatitis C

Studies in HCV have examined associations of HLA alleles and 1) susceptibility to infection, 2) clearance of infection, and 3) progression of disease (i.e., development of fibrosis). Since the HALT-C study is restricted to patients chronically infected and who have significant liver fibrosis, we propose to examine HLA alleles as they relate to development of fibrosis.

Several studies have examined HLA alleles and progression of liver disease in patients with chronic hepatitis C (Table). Two Japanese studies compared HLA class II HLA allele frequency in HCV-positive patients with normal ALT to allele frequency in HCV-positive patients with elevated ALT, many of who had cirrhosis [7, 8]. Both studies found an association between cirrhosis and DRB1*0405 or DQB1*0401. An Italian study found DQB1*0502 to be associated with more rapid progression to cirrhosis in patients infected with genotype 1b [9]. Class I alleles were examined in one study. The risk of cirrhosis was 13-fold higher in patients with the extended haplotype B54-DRB1*0405-DQB1*0401 as compared to patients with DRB1*0405-DQB1*0401 but without B54 [8]. Thus, several specific class I and class II HLA alleles are associated with increased amount/rate of liver fibrosis.

In six studies a total of 7 HLA class II alleles have been associated with decreased hepatic fibrosis [7-12]. Of these 7 alleles, DRB1*1302 was associated with decreased fibrosis in 4 studies. DRB1*1101, an allele associated with increased rated of clearance of HCV, was associated with decreased fibrosis in two studies. DQB1*0301, another allele associated with increase clearance of HCV infection, was associated with decreased fibrosis in one study. Thus, several alleles have been associated with decreased risk of developing cirrhosis, including alleles that have also been associated with increased clearance of HCV infection.

These studies are suggestive of a potential role for HLA alleles in hepatitis C disease course, but

further studies need to be conducted. Each of these studies was relatively small (usually less than 100 patients) limiting the power of the study to find an association between alleles and disease course. Evaluation of HLA alleles was sub-optimal. Limitations included not evaluating class I loci or all class II loci, and using serologic typing rather than the more accurate genotyping of HLA loci. Finally, no study was performed in a US population. Thus, the findings should be considered preliminary when applied to the United States.

HLA may influence response to interferon treatment. HLA genotyping for class II DRB1, DQA1, DQB1, DPB1 and class I HLA-A locus was performed using PCR-SSO on 162 patients treated with varying doses of interferon alfa-2a [18]. The haplotype DRB1*1301-DQA1*0103-DQB1*0603 and the HLA allele DPB1*0401 were significantly associated with a SVR ($p<0.02$ for each).

In summary, several class I and class II HLA loci have been associated with altered rates of fibrosis in patients with chronic hepatitis C. One of the goals of the HALT-C study is to assess progression of fibrosis in patients with chronic hepatitis C. This ancillary study would support this goal by evaluating the contribution of several candidate HLA alleles to liver fibrosis.

HLA heterozygosity

It is generally believed that the great diversity and equal distribution of allelic frequencies observed in the class I and class II genes of the HLA system are maintained through selective forces, including infectious diseases [13]. Furthermore, it has been hypothesized that greater HLA diversity is a survival advantage. This hypothesis, known as overdominant selection or heterozygote advantage, proposes that individuals heterozygous at HLA loci are able to present a greater variety of antigenic peptides than are homozygotes, resulting in a more robust immune response and greater resistance to disease [14, 15].

Studies of HLA heterozygosity and disease outcome have been reported forHIV and hepatitis B [2, 6]. In each case, patients with greater HLA heterozygosity had a better outcome than did patients with less HLA heterozygosity.

The influence of HLA heterozygosity in hepatitis C has not been adequately examined. The single study examining this issue found more rapid development of cirrhosis in patients homozygous for HLA DRB [9]. The study, although limited by lack of evaluation of class I loci, was consistent with a selective advantage for patients with greater heterozygosity. Homozygosity at DQ*A1*, DQ*B1*, or DR*B1* was not associated with HCV persistence following acute infection [24].

Interleukin-10 promoter haplotype

Differentiated T-helper cells can be categorized into two groups (Th1 and Th2) based on the cytokines they produce. Interleukin-10 is a Th2 cytokine that inhibits Th1 cytokine response. As a modulator of Th1 response, IL-10 could affect intrahepatic CTL response to HCV-infected hepatocytes, potentially influencing clearance of viral infection.

IL-10 production is under genetic control; polymorphisms in the IL-10 promoter result in different IL-10 production rates [16]. Three biallelic polymorphisms at positions -1082, -819 and -592 from the transcription start site produce three different haplotypes: GCC, ACC, and ATA. Studies have demonstrated that patients with ATA haplotype have decreased IL-10 production in response to various stimuli when compared with patients with GCC or ACC haplotypes.

IL-10 promoter genotype is associated with response to interferon treatment in patients with chronic hepatitis C [17,19]. A study of 53 patients with chronic hepatitis C found that patients with the -592*A and -819*T alleles or the ATA haplotype were more likely to have an initial response to interferon treatment than were patients without these alleles/haplotype. Edwards-Smith and colleagues found that the -592*A and -819*T alleles were associated with initial response (normal ALT and/or loss of HCV at 12 weeks of treatment) to interferon-alpha treatment (odds ratio = 5.0; 95% CI: 1.4-19.0; P=0.01). Similarly, inheritance of the haplotype GCC was associated with non-responder status to interferon-alpha therapy (odds ratio = 0.22; 95% CI:

0.06-0.87; P=0.03). In a study of Caucasian Americans receiving treatment with interferon+ribavirn, Yee and colleagues reported improved SVR in patients with IL-10 promoter alleles -592A or -819T ($p<0.02$)[20]. Thus, there is suggestive evidence from two studies that IL-10 promoter alleles may be associated with response to interferon treatment in patients with chronic hepatitis C.

Although patients in the HALT-C study have failed an initial course of interferon (possibly with ribavirin) treatment, it may still be useful to evaluate IL-10 promoter polymorphisms. Approximately 1600 patients will be enrolled in the HALT-C study with an estimated 40% (~600 patients) responding to treatment with clearance of the virus after 20 weeks of treatment. Approximately half of these may have a SVR. Thus, there should be a large number of responding and non-responding patients, increasing the probability of finding a difference in allele frequency between groups.

Tumor necrosis factor, transforming growth factor-beta 1, and angiotensinogen II in hepatitis C liver fibrosis

Cytokines secreted in response to inflammation and liver injury stimulate liver fibrosis. Tumor necrosis factor, a cytokine whose plasma level is increased in patients with chronic hepatitis C, is one of the key cytokines stimulating hepatic inflammation. Transforming growth factor-beta 1, the dominant fibrogenic cytokine, is secreted by Kupffer cells in response to TNF-alpha stimulation. TGF b1 contributes to the activation of stellate cells and stimulates collagen production by stellate cells *in vitro*. TGF b1 mRNA is increased in the liver of patients with chronic HCV relative to healthy controls, and the level of expression correlates with expression of type 1 collagen mRNA in the liver[22]. Angiotensin II appears to influence the accumulation of fibrous tissue independently of its effects on blood pressure. This effect may be due to enhanced TGF b1 production by angiotensin II.

TNF alpha promoter contains bialleleic polymorphisms at positions –308 (G or A) and -238 (G or A) from initiation start site. The TNF alleles have been associated with differences in TNF alpha production in response to various stimuli and in altered disease outcome in patients with malaria, primary sclerosing cholagnitis and primary biliary cirrhosis. Several polymorphisms within the TGFb1 promoter and in codons 10 and 25 have been described and appear to influence TGF B1 production. Finally, functional polymorphisms have been described in the promoter region (-6 bp from transcription start site) of the angiotensinogen gene. These polymprphisms have been postulated to contribute to interindividual variability in the outcome of various renal and cardiovascular diseases. Their contribution to progressive fibrosis in other organs has not been evaluated.

Two studies report associations between TNF, TGF beta and angiotensinogen alleles and liver fibrosis in patients with chronic hepatitis C. Yee and coworkers evaluated TNF promoter polymorphism in114 HCV patients without cirrhosis and 30 HCV patients with cirrhosis[21]. Variability in progression of HCV cirrhosis was assessed in a multivariate model including gender, estimated duration of infection, alcohol consumption, viral genotype and gene polymorphism. TNF promoter variants –238A and –308A conferred a 3- and 5-fold risk for cirrhosis, respectively ($p<0.03$ for both). In another study, 128 biopsied patients with chronic hepatitis C were genotyped for TGF-beta 1 and angiotensinogen polymorphisms[22]. Presence of TGF-B1 codon 25 Arg/Arg and of the angiotensinogen promoter –6 A/A were highly associated with stage of fibrosis ($p<0.03$ for both). These relationships remained significant after adjusting for potential confounders (age, gender, alcohol intake, inflammation, steatosis). Although these findings are provocative, they have not been confirmed by all investigators, and larger studies are needed[23].

Miscellaneous candidate genes
Wozniak and coworkers reported a significantly decreased frequency of apolipoprotein E-epsilon4 allele frequency in HCV patients with advanced fibrosis as compared with HCV patients with mild fibrosis (4.3% vs 19.1%, $p<0.003$)[26]. Microsomal epoxide hydrolase is an enzyme that catalyzes the irreversible hydration of highly reactive epoxide intermediates to less active compounds that can be conjugated and excreted. The highest activity of microsomal epoxide hydrolases is found in the liver, kidney and testis. Clinical studies have documented an association between specific alleles in microsomal epoxide hydrolase and drug induced hepatoxicity, and susceptibility to liver cancer in hepatitis B. An initial study of microsomal epoxide hydrolase in hepatitis C has found an higher prevalence of very slow metabolizers in patients with cirrhosis or HCC as compared with HCV carriers (18% vs. 3.3%, $p<0.001$)[27]. These studies need to be confirmed in larger studies.

Future studies
Study of, and knowledge about, the human genome is advancing quickly. Along with increased knowledge of the human genome is a parallel interest in finding the genetic basis of human diseases, and the technological capability of performing more and faster genetic tests.

This study proposes to answer several specific questions concerning several HLA and non-HLA alleles/haplotypes and disease outcome in patients in the HALT-C study. Advances in understanding the pathophysiology of hepatitis C may suggest a possible role for other candidate genes in disease progression. Furthermore, techniques may become available in the future to perform fast, high-quality, genome-wide DNA searches for loci of interest. Consequently, it is likely that additional genetic studies will be proposed during the next decade to evaluate the role of host genes in hepatitis C disease progression. It is anticipated that the DNA samples from the HALT-C study would be available for future studies on the genetic influence on HCV disease outcome. Permission to use the HALT-C samples in future studies would need to be obtained from the HALT-C study group prior to such use.

Methods
All patients enrolled into the HALT-C will be asked to participate in this ancillary study. Alcohol consumption, a generally accepted contributor to liver fibrosis, will be determined using the Skinner questionnaire (already part of the HALT-C study).

Sixty milliliters (60 mL, approximately 4 tablespoons) of blood will be drawn into EDTA tubes (15 mL on 4 different occasions during the first year of participation in the HALT-C study). BBI will isolate DNA from blood and store DNA until it is needed for study.

HLA genotyping will be determined by Mary Carrington, PhD (National Cancer Institute) using molecular biology techniques. Each patient will be typed at both class I and class II loci.

Non-HLA allele status will be determined by Michael Dean, Ph.D (National Cancer Institute) using standard molecular techniques. IL-10 polymorphisms will be determined as described by Edwards-Smith [19]. Briefly, DNA is amplified by PCR using various specific probes followed by restriction enzyme digestion. The presence of the -592 (A/C) alleles is determined by *Rsa* 1 digestion (cuts the rarer A allele to generate 176- and 236-bp fragments). The -819 (T/C) alleles are determined by restriction digestion with *Mae* III (cuts the more common C allele to generate 125- and 84-bp fragments). Finally, the -1082 (G/A) alleles are determined by restriction enzyme digestion with *Mnl* I (cuts the G allele to generate 106- and 33-bp fragments). TNF-alpha –308 G/A polymorphism is determined using sequence-specific primers. A common reverse primer, 5' TCC TCC CTG CTC CGA TTC CG 3' was used to amplify specific forward primers: 5'CAA TAG GTT TTG AGG GGC ATG A 3' for the "A" allele, and 5' CAA TAG GTT TTG AGG GGCATG G 3' for the "G" allele. Angiotensinogen promoter polymorphism (G/A) is detected using PCR followed by restriction enzyme digestion. The primers used are 5'CTC AGT TAC ATC CTG AGA GAG

ACA AGA CC 3' and 5' GTC TCG CTT CTG GCA TCT GTC CTT CTG G 3'. The PCR product is digested with *BsiE* 1, which cuts the product when the G allele is present. TGF-beta polymorphisms are detected as described by Lympany and colleagues [25]. Apolipoprotein E-epsilon4 allele and the microsomal epoxide hydrolase alleles will be determine as described [26, 27].

Statistical analysis

A contingency table approach will be employed to compare rates of disease progression prior to study entry for different HLA alleles/haplotypes. Time from infection to study entry will be categorized into discrete groups. These groups may or may not contain intervals of equal length, depending upon the observed distribution of time since infection. For example, it would be counterproductive to define groups of equal length if most of the patients fall in one group and the rest are spread over the remaining groups. In that case, it may be better to categorize time since infection so that the categories contain roughly equal numbers of patients. The time categories will be cross-tabulated against fibrosis score and the number of patients with each HLA allele/haplotype in each cell will be determined. Differences in rates of disease progression will be inferred from differences in the distributions of alleles/haplotypes among cells. These distributions of alleles/haplotypes among cells will be compared using chi-square statistics.

A hypothetical example with 1,000 patients with each of two haplotypes is provided in Table 1 to illustrate this process. The numbers in italics represent a haplotype that is associated with rapid progression, while the other numbers represent a haplotype that is associated with slower progression. Each column represents a separate time interval. The rapid progressors tend to have higher fibrosis scores than the slow progressors in each column.

TABLE 1. DISEASE PROGRESSION IN TWO HYPOTHETICAL HAPLOTYPES

	Years since infection				
Fibrosis score	**10-15**	**16-20**	**21-25**	**25-30**	**TOTALS**
6			***50***	***50*, 20**	***100*, 20**
5		***100*, 25**	***100*, 75**	***50*, 60**	***250*, 160**
4	***50***	***175*, 150**	***150*, 150**	**100**	***375*, 400**
3	***200*, 200**	***75*, 125**	**75**	**20**	***275*, 420**
TOTALS	***250*, 200**	***350*, 300**	***300*, 300**	***100*, 200**	***1000*, 1000**

It may be important to take account of alcohol consumption in this analysis because alcohol consumption influences the rate of fibrosis. Therefore a stratified analysis will be employed. Using the data from the Skinner Lifetime Alcohol Questionnaire that will be collected at study entry, patients will be classified into groups reflecting different levels of lifetime alcohol consumption (e.g. low, medium, high). A separate contingency table, such as Table 1, will be constructed for each stratum of alcohol consumption and alleles/haplotypes will be compared separately in each table. Alcohol consumption may influence the rate of fibrosis within an allele or haplotype without influencing the difference between rates of disease progression in different alleles/haplotypes. This can be determined using a loglinear model, which will provide a chi-square test of differences in rates of disease progression in different HLA groups adjusted for the effects of alcohol consumption.

Differences in progression of fibrosis during the study among HLA groups will be assessed by comparing the distributions of fibrosis scores at 2 and 4 years among HLA alleles/haplotypes. It seems reasonable to expect that patients with HLA types that are associated with more rapid rates of disease progression will have higher fibrosis scores at study entry than will patients with HLA types that are associated with slower rates of progression. This could pose problems for the analysis if rate of progression over 2 or 4 years depends upon fibrosis score at study entry for reasons other than HLA type. Therefore, HLA type at study entry will be taken into account in the

analysis. This is easily accomplished using methods analogous to the contingency tables above: fibrosis score at 2 or 4 years is cross-tabulated against fibrosis score at entry and the distributions of HLA alleles/haplotypes among the cells of this table are compared. Again, alcohol consumption can be taken into account through a stratified analysis.

Finally, the association between patient genetics and treatment response will be examined simply by comparing the outcome of qualitative PCR at 20 weeks among patients with different alleles or haplotypes. Once again, chi-square statistics will be employed.

Burden to HALT-C Study

30 ug of DNA/patient should be sufficient to perform all the proposed analyses. Investigators at the National Cancer Institute will perform (and pay for) DNA analysis (HLA and other alleles)(no direct cost to HALT-C study).

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