Myxovirus-1 and Protein Kinase Haplotypes and Fibrosis in Chronic Hepatitis C Virus

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Candidate genes, including *myxovirus resistance-1 (Mx1), protein kinase (PKR), transforming growth factor-β1 (TGF-β), interleukin-10 (IL-10), and interferon-gamma (IFN-γ),* were evaluated for associations with liver fibrosis in 374 treatment-naive patients with genotype-1 chronic HCV infection [194 Caucasian Americans (CAs) and 180 African Americans (AAs)], using a genetic haplotype approach. Among the 18 haplotypes that occurred with a frequency ≥5% in the cohort overall, the *Mx1-(-123C)-(+6886A)-(+19820G(379V))-(+38645T)* (abbreviated *Mx1-CAGT),* and *PKR-(+110T)-(+7949G)-(+13846A)-(+22937T)-(+40342T)* (abbreviated *PKR-TGATT)* haplotypes were independently associated with less severe hepatic fibrosis (Ishak ≥ 3 versus <3). These associations persisted after adjustment for potential confounders such as alcohol use, sex, age (which is strongly correlated with the estimated duration of HCV infection [Spearman's correlation coefficient (r_s) = 0.6)], and race (for *Mx1-CAGT*: OR = 0.33; 95% CI: 0.16-0.68; $P = 0.0027$; and for *PKR-TGATT*: OR = 0.56; 95% CI: 0.32-0.98; $P = 0.0405$). Population structure was evaluated using the structured association method using data from 161 ancestry-informative markers and did not affect our findings. We used an independent cohort of 34 AA and 160 CA in an attempt to validate our findings, although notable differences were found in the characteristics of the two patient groups. Although we observed a similar protective trend for the *Mx1-CAGT* haplotype in the validation set, the association was not statistically significant. *Conclusion:* In addition to other factors, polymorphisms in cytokine genes may play a role in the progression of HCV-related fibrosis; however, further studies are needed. (HEPATOLOGY 2007;46:74-83.)

Abbreviations: AA, African Americans; CA, Caucasian Americans; IFN-γ, interferon gamma; Mx1, myxovirus resistance-1; NIH, National Institutes of Health; PKR, protein kinase; SNP, single-nucleotide polymorphisms; tagSNP, haplotype-tagging single-nucleotide polymorphisms; TGF-β, transforming growth factor beta.

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The histological progression of chronic HCV infection is characterized by inter-individual variability.[1] In particular, variability in fibrosis progression has been associated with race in retrospective, cross-sectional studies, with African Americans (AA) showing slower disease progression compared with Caucasian Americans (CA).[2,3] However, the biological mechanisms contributing to this variability are not understood, and confirmatory prospective studies involving large numbers of patients are lacking.

The human genome comprises regions of high linkage disequilibrium, and a few common haplotypes may represent the majority of total variation within a region of the genome. As a result, one allele at a particular locus is often more likely to be found along with other specific alleles at adjacent polymorphic loci than would be expected by chance.[4-6] Accordingly, individuals inherit specific combinations of alleles, termed haplotypes. Therefore, a few haplotype-tagging single-nucleotide polymorphisms (SNPs) (tagSNPs) may represent a majority of variation within an extended region. Determining associations between haplotypes and disease outcomes, and subsequently fine mapping associations between individual SNPs within haplotypes and disease outcome, has represented an efficient approach to conducting disease association studies by reducing the overall cost of genotyping without compromising the amount of genetic information obtained.

Immune mechanisms also may play an important role in mediating the histological progression of chronic HCV infection.[7] Imbalances between Thelper 1 (Th1) and Th2 cytokines that lead to a predominance of a proinflammatory interferon gamma (IFN-γ) phenotype may increase necroinflammatory activity and histological progression in chronic HCV.[8-10] Also, tissue repair after the immune response to infected hepatocytes along with stimulation of hepatic stellate cells after cytokine activation resulting from chronic necroinflammatory activity also may mediate histological progression. Therefore, pro-inflammatory cytokines such as IFN-γ and anti-inflammatory cytokines such as interleukin (IL-10), which is known to function as a down-regulator of IFN-γ and Th1 activity,[11] may play a significant role in mediating the histological progression of HCV. In addition, cytokines such as transforming growth factor-β1 (TGF-β) also may play an important role in HCV progression by stimulating hepatic stellate cells and increasing collagen deposition.[12] The native host immune response to HCV, including the interferon system, is believed to be an important component of the immune response to HCV. For example, a number of genes, including *myxovirus resistance-1* (*Mx1*), and *protein kinase* (*PKR*), are induced by interferon and have antiviral effects, and are believed to be important in the host response to HCV infection.[13,14] Given the importance of immunomodulatory cytokines as well as interferon-induced genes in HCV infection, we used a candidate gene, haplotype-tagging (tagSNPs)-approach to determine whether polymorphisms in the genes that encode *IL-10*, *IFN-γ, TGF-β, Mx1,* and *PKR* are associated with liver fibrosis in chronic HCV.

Methods

Study Population and Clinical Data. This study used patients from the Study of Viral Resistance to Antiviral Therapy of Chronic Hepatitis C (Virahep-C), a multicenter study sponsored by the National Institutes of Health (NIH) aimed at understanding the mechanisms of resistance to antiviral therapy for chronic HCV genotype-1 infection. The details of this study have been described elsewhere.[15] All subjects were required to have been born in the United States, and race was determined by a self-administered questionnaire.

All participants were interferon treatment naïve and had undergone a liver biopsy within 18 months of enrollment. All biopsies were scored by a single pathologist (D.E.K.), who was blinded to patient outcome and clinical status at the time of biopsy scoring. Hepatic fibrosis severity was scored using the Ishak modified HAI scoring system, and inflammation was scored using the HAI score.[16] Because the distribution of the full spectrum of biopsy scores did not fulfill the proportional odds assumption, we were unable to use ordered logistic regression to examine the effects of genetic markers on fibrosis. Instead, we dichotomized fibrosis scores as "high" ($\geq$3) versus "low" ($<$3) for analyses. Multiple logistic regression using the SAS software system (the *proc logistic* procedure) was used to conduct multivariable analyses. The inflammation score, which represents the sum of the portal, periportal, and lobular inflammation scores, was dichotomized at the median ($\geq$8 vs. $<$8) in our analyses.

Data Analyses. We dichotomized variables for data analyses: self-reported alcohol consumption was analyzed as "high" ($\geq$7 drinks per week) versus "low" ($<$7 drinks per week). Estimates of the duration of infection were determined from exposure to parenteral risk factors by the patient's physician, and were available in 276 individuals (143 CAs and 133 AAs). Because we observed a strong correlation between estimated duration of HCV infection and patient age [Spearman's correlation coefficient (r_s) = 0.6], we used patient age as a surrogate marker for the estimated duration of HCV infection in our multivariable analyses (Supplementary Fig. 1). Ethical approval for this study, including the study of host genetics, was provided by the Institutional Review Boards of all of the participat-

Table 1. Primers and Probes Used in the Present Study. Chromosomal Locations of Each of the Genes, Along With the Relative Gene Sizes, Are Also Provided

Locus	Polymorphism & Location in Gene	Oligo Name	Oligo Sequence (5′ → 3′)	Probe Name	Probes for Allelic Discrimination
***Interleukin-10 (IL10)*; Chromosome 1; 4.86kb**					
rs222202	C(+430)T	Forward	CAGATGATACAGTAAATGTGCAGGAA	Probe 1	ATGCGTTCTCTCTCGT
	Exon 1	Reverse	AGCGCCAGCAGGATCTTATAAG	Probe 2	AATGCGTTCTTTCTC
rs1554286	T(+1578)C	Forward	GGCTACGGCGCTGTGTAAGT	Probe 1	AGATCAGTTTTTTCCCTTG
	Intron 3	Reverse	TGGTCTGTAGGAGATGGTATTTTGG	Probe 2	ATCAGTTCTTTCCCTTGC
rs3024496	T(+3947)C	Forward	CCAGCCCCTTGAGAAACCTT	Probe 1	TGTACCTCTCTTATAGAAT
	Exon 5 3′-noncoding	Reverse	CAGAGAAGCTCAGTAAATAAATAGAAATGG	Probe 2	TGTACCTCTCTCATAGAA
***Transforming growth factor-β1 (TGFβ)*; Chromosome 19; 23.5kb**					
rs2241716	G(+5703)A	Forward	TGGGTCGGCTGGTTACAAGGT	Probe 1	CTGAAGGATAGAAGAA
	Intron 2	Reverse	GCAACAGAGTGAGACACTGTCTCAA	Probe 2	CCTGAAAGATAGAAGAA
rs2278422	G(+14031)C	Forward	ACCAGATCTTAGCGCCATCAG	Probe 1	CAGCAACGCTGTCAA
	Intron 5	Reverse	TCTCAAAAAAGACCTCAATTTGTTC	Probe 2	AGCAACCCTGTCAAT
***Protein kinase (PKR)*; Chromosome 2; 43.7 kb**					
rs2254958	C(+110)T	Forward	AGCATAGAAACATCCTAAACAATCTTCA	Probe 1	CGAGGCATCGAGGT
	Exon 1	Reverse	CGAGTGATACCAGCGAAGACTAAGG	Probe 2	TCGAGGCATTGAGGT
rs2287350	A(+7949)G	Forward	CTCTCTTGGGTGTTCCACAGACT	Probe 1	AACCATCCAGCTACT
	Intron 4	Reverse	GGAACCCATTTACCATAATTGATCA	Probe 2	AACCACCCAGCTAC
rs2307479	A(+13846)C	Forward	CGGTGGATGTTTTTGGTAGTTG	Probe 1	CTGTGAAATGGCC
	Intron 9	Reverse	TTACCCTGCGTAGTTAATCTTATTCAAAT	Probe 2	TCTGTGCAATGGC
rs2307478	T(+22937)C	Forward	TGGCCAAGTTTTCAAAGCAAA	Probe 1	ACGGAAAGACTTACGTTAT
	Exon 10	Reverse	TCAGCGTACCTTGCCATTCA	Probe 2	ACGGAAAGACCTACGTTA
rs2372443	T(+40342)C	Forward	TCCCTGGAAGGTAGTACATCTCA	Probe 1	TGCTTTGTGTAAGACAC
	Intron 14	Reverse	TGCAACCCTTACAGCACTTAGC	Probe 2	CTTTGTGCAAGACAC
***Interferon-γ (IFNg)*; Chromosome 12; 4.9 kb**					
rs2069707	C(-765)G	Forward	GCCTCAACCTCCCAAAGTG	Probe 1	TGGGAATATTCTCTACACTG
	-765 bp upstream	Reverse	TAAACCAATATATCCCAAATAGTACTTCAG	Probe 2	TGGGAATATTCTGTACACTG
rs1861493	T(+2325)C	Forward	GCTCAGTTTCCTCATCTTAAAAGGA	Probe 1	ACTGTTAGGTTCACTGTAT
	Intron 3	Reverse	GGAGCAAAGAAGGTCATCAAACTT	Probe 2	CTGTTAGGCTCACTGTA
rs2069718	C(+3360)T	Forward	GGCAGAGCCAAGAGGAAGGT	Probe 1	CCACATCTTATGAAGCA
	Intron 3	Reverse	CACTCTAACCAATAGGGCCATTTAG	Probe 2	CCACATTTTATGAAGCAT
***Myxovirus-resistance 1 (Mx1)*; Chromosome 21; 33kb**					
rs17000900	-123 bp upstream	Forward	CCTGGGAGGGAACTGAAGAC	Probe 1	N/A
		Reverse	CGCTCCTAGCGCGGTTACTG	Probe 2	N/A
rs455816	A(+6886)G	Forward	AGCTTGGCCTTTTCCAGTCA	Probe 1	TGTGTCTCGTGATCTG
	Intron 5	Reverse	CACACTTTGAGAACCACAGTCTATCC	Probe 2	TGTGTCTCATGATCTG
rs469390	A(+19820G)	Forward	AGTCTATCCACTTATACTGATGTTTTTCTTCT	Probe 1	TGACAGAAAATTAATGCCT
	Exon 13	Reverse	CATGAGAGCAGTGATGTCCTGATT	Probe 2	TGACAGAAAGTTAATGCC
rs456298	A(38642)T	Forward	CACAGACAGATCCTGCAAATGG	Probe 1	ATTGCATGACTTTCCATT
	3′-noncoding	Reverse	TGATTCTACAGCTAGGACTTAACCTTGA	Probe 2	ATTGCAAGACTTTCCATTT

N/A, not applicable. Genotyping was conducted by RFLP and not by allelic discrimination.

ing centers. All patients provided written informed consent.

SNP Selection and Genotyping. SNPs were selected from the HapMap and NCBI databases for the following candidate genes: *IFN-γ, Mx1, PKR, TGF-β*, and *IL-10*. The SNPs were selected to cover the entire gene (from promoter to 3′ untranslated region), in both introns and exons, at 2- to 3-kb intervals for small genes (*IFN-γ* and *IL-10*), and 5- to 9-kb intervals for larger genes (*Mx1* and *PKR*), and with a minor allele frequency of 10% or greater among AAs and CAs. To ensure coverage of both the regions upstream and downstream of a gene, we also included an SNP within 5 kb of each of the flanking regions of a gene. Seventeen of 23 evaluated SNPs were polymorphic and successfully genotyped in our sample. In addition, we genotyped SNPs that were previously reported to be associated with HCV severity, such as *-88Mx1-(C/A)* and *-123Mx1-(C/A)*. After genotyping 100 samples for *-88Mx1-(C/A),* we did not find the locus to be polymorphic in our cohort, and discontinued genotyping the remaining samples for this locus.

With the exception of the C(-123)A polymorphism in the *Mx1* gene, allelic discrimination was used to genotype haplotype-tagging SNP variants in the *IFN-γ, Mx1*, *PKR*, *TGF-β,* and *IL-10* genes. Table 1 lists the primers used in this study, along with the chromosomal location and relative sizes of the targeted genes. The allelic discrimination assay was performed on an ABI 7000 Sequence Detection System using TaqMan technology (American Biosystems Inc., Foster City, CA). The reaction mix was in a total

volume of 10 μl containing 1× TaqMan Universal PCR Master Mix (ABI part 4326708), 2 pmole of each of 2 probes, and 9 pmol of each of 2 PCR primers (Table 1) and 10 ng sample DNA. PCR cycling conditions included an initial 2-minute incubation step at 50°C, and 10 minutes at 95°C for DNA denaturation and enzyme activation. This was followed by 40 cycles of amplification that included 15 seconds at 95°C for DNA denaturation and 60 seconds at 60°C for primer annealing and DNA synthesis extension. The *C(-123Mx1)A* polymorphism was genotyped by restriction fragment length polymorphism. Amplification was conducted using AmpliTaq Gold PCR Master Mix (ABI, Foster City, CA) in a total volume of 15 μL, containing 1 × Master Mix, 7.5 pmol of each primer (Table 1), and 10 ng DNA. Amplification conditions included 9 minutes at 95°C for DNA denaturation and enzyme activation step, which was followed by 35 cycles at 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 1 minute, and final extension step at 72°C for 5 minutes. The resulting 445–base pair (bp) amplicon was then digested using 3 units of *Pst I* restriction enzyme for at least 4 hours at 37°C. Allelic variants were determined through resolution of digestion products on a 1% agarose gel. Products either remained uncut (which corresponds to the AA genotype) or were digested into 2 fragments of 239 bp and 206 bp (which corresponds with the CC genotype), or with both uncut and cut DNA bands at a band intensity of 1:1 ratio (heterozygous). All haplotypes were determined using the PHASE software.[17,18] The haplotypes were constructed using PHASE 2.0.2 in AA and CA populations separately. PHASE was run for 10 times with 10 different random seeds. The best estimates of haplotypes from each run were compared with each other. The haplotypes of each individual were assigned only if the same haplotypes came up in at least 5 of the 10 runs.

Evaluation of Population Structure. To evaluate the population structure, we genotyped 161 unlinked, ancestry-informative SNPs using the Illumina system (Illumina Inc., San Diego, CA) (Supplementary Table 1). Briefly, the markers selected are believed to vary significantly in frequency between CAs and AAs and are spread throughout the human genome. The markers used in the current study do not allow for the evaluation of admixture from different African subpopulations. The Structure version 2 computer program was used to assess the population structure, using the structured association method developed by Pritchard et al.[19] Estimations were conducted assuming uncorrelated alleles, the presence of admixture, and two to five random-mating ancestral populations (termed *k*) using a burn-in length of 100,000 and a run length of 100,000 repetitions. Three repetitions were conducted for each level of *k*. From this analysis, we obtained a probabilistic estimate of each individual's membership in each of the ancestral populations. The most informative value of *k* was selected using the variance of the log likelihood score averaged from each of the three runs at each level of *k*. The value of k with the smallest variance (which was $k = 2$) was selected, and the proportion of membership in the first ancestral population from the $k = 2$ simulation was used as a covariate in logistic regression models.

Statistical Methods. Haplotypes with a frequency ≥5% in the cohort overall were analyzed for their association with fibrosis stage. Standard univariable analyses were calculated using contingency tables or logistic regression, whereas bivariable adjustments for race were conducted using logistic regression, using both self-reported race (CA or AA) and the probabilistic racial makeup as determined by estimation using the Structure version 2 program.[19,20] Multivariable logistic regression (*proc logistic* procedure) was used to adjust for potential confounders and included models using self-reported race as well as estimated race as a covariate. Statistical calculations were conducted using the SAS version 8 software package. Using *P*-values from the race-adjusted bivariable regression models, Holm's step-down method was used to adjust for multiple comparisons.[21] Also known as the Bonferroni step-down method, Holm's procedure is a less conservative method of adjusting for multiple comparisons than the Bonferroni correction.

Validation Cohort. To replicate our initial observations for associations of particular *Mx1* and *PKR* haplotypes with fibrosis observed in the Virahep-C Study, we examined patients attending the NIH Clinical Center. For this study, we included individuals who were interferon treatment naïve and had a baseline biopsy available. Available demographic data including self-reported race, sex, and patient age were used for multivariable analyses. Genotyping was conducted as described previously; however, because of the very small numbers of AAs in this cohort ($n = 34$ AAs), PHASE calculations for haplotypes were conducted on the cohort as a whole.

Results

Cohort Characteristics. Among 401 individuals treated for chronic HCV in the Virahep-C Study, 374 (194 CA, and 180 AA) agreed to participate in genetics studies (Table 2). Briefly, CAs and AAs did not significantly differ with respect to sex, the estimated duration of HCV infection, or level of fibrosis. However, a trend toward increased patient age was seen in AAs compared with CAs ($P = 0.062$).

Table 2. Baseline Characteristics of the 374 Virahep-C Study Participants Who Consented to Participate in Genetics Studies

Factor	African Americans (n = 180)*	Caucasian Americans (n = 194)	P
Age (years) mean (±SD)	48.7 (±7.1)	47.0 (±8.6)	0.062†
Sex			
Male	65.60%	65.00%	
Female	34.40%	35.00%	0.902
Est. duration of HCV (years)‡	24.5 (±9.3)	25.5 (±9.9)	0.261
Ishak score			
<3	64.20%	60.30%	
≥3	35.80%	39.70%	0.410
Self-reported alcohol use			
<7 drinks per week	87.80%	88.70%	
≥7 drinks per week	12.20%	11.30%	0.791
Summary inflammation score			
<8	30.60%	35.10%	
≥8	69.40%	64.90%	0.355

*A baseline fibrosis score was not available for 1 AA participant. †Wilcoxon rank-sum test.

‡Estimations of duration of HCV infection were obtained for only 143 CA and 133 AA individuals.

Population Structure. In our study population, we observed a strong correlation between self-reported race and the racial makeup obtained through simulation (Supplementary Fig. 2), which supports observations from other studies.[22-24] Because all models using estimated race yielded results similar to those using self-reported race, we opted to report only the results for self-reported race. Three individuals in the study population had an estimated genetic makeup that was different from that of their self-reported racial group. We conducted the analyses with these 3 individuals excluded and did not detect any significant differences in results (data not shown). Accordingly, we present the results of analyses that included the three individuals.

Associations of Haplotypes and SNPs with Fibrosis Scores in Virahep-C. We successfully genotyped all SNPs targeted in the current study in at least 372 of the 374 participants at all targeted loci, except for MxA rs17000900 and PKR rs2254958, where we successfully genotyped 368 and 366 of the 374 individuals, respectively. As a result, we were able to successfully assign haplotypes with a high probability using PHASE. The common haplotypes that occurred at a frequency ≥5% in the cohort overall and their associations with fibrosis adjusting for race are provided in Table 3. Observed haplotype frequencies approximated those reported for ethnically similar populations in the public databases. Both the *Mx1-CAGT* and *PKR-TGATT* haplotypes were associated with lower fibrosis scores. Adjustment for the number of comparisons made yielded a *P*-value of P_c = 0.1350 for *Mx1-CAGT* and P_c = 0.1904 for *PKR-TGATT*. Examination of the associations of individual SNPs within both of these genes and fibrosis in the cohort overall as well as by race are provided in Table 4. In multivariable models that adjusted for potential confounders such as alcohol use, and sex, along with self-reported race, *Mx1-CAGT* (OR = 0.33; 95% CI: 0.16-0.68; $P = 0.0027$) and *PKR-TGATT* (OR = 0.56; 95% CI: 0.32-0.98; $P = 0.0405$), were both independently associated with lower fibrosis scores (Table 5). Higher levels of inflammation were also associated with increased fibrosis (OR = 5.96; 95% CI: 3.29-10.78; $P < 0.0001$).

Validation Cohort. Among the 490 individuals in the NIH validation cohort at the time this study was conducted, 194 fit our criteria for inclusion and were included in the current study. The basic demographics of the cohort are presented in Table 6. Unlike Virahep-C, however, only 17.5% were AA. In addition, the 34 AAs in this cohort tended to have higher fibrosis than the 180 AAs in Virahep-C. The association between *Mx1-CAGT* in this cohort was in the same direction as that observed in Virahep-C; however, it did not achieve statistical significance after adjustment for potential confounding variables (OR = 0.64; 95% CI: 0.23-1.81; $P = 0.3999$) (Table 7). The presence of the *PKR-TGATT* haplotype was not detected through our calculations using PHASE.

Discussion

We investigated the hypothesis that polymorphisms in cytokine genes are associated with the severity of fibrosis in chronic HCV infection, and that host genetic factors may contribute to the racial differences that have been reported with respect to HCV-related histological progression.

Among the tagSNPs and haplotypes examined, an association of the *Mx1-CAGT* and the *PKR-TGATT* haplotypes with lower fibrosis was observed in the Virahep-C cohort. The protective effects for both haplotypes seen in individual race-adjusted bivariable models (Table 3) were also observed in a multivariable regression model that adjusted for other potential confounding variables (Table 5). Significant differences by race were not observed. Previous studies have reported associations between polymorphisms in the *Mx1* gene and the natural clearance of HCV as well as the responsiveness to interferon therapy for HCV.[25-27] In addition, *in vitro* functional studies of a polymorphism of the *Mx1* gene have suggested that the *Mx1(-88G)+Mx1(-123C)* haplotype produces lower levels of Mx1.[26] The functional characteristics of the *Mx1-CAGT* haplotype that we studied are not known.

However, a significant association for carriage of the *Mx1-CAGT* haplotype and fibrosis was not observed in

Table 3. Haplotypes, Haplotype frequencies and Disease Association in Virahep-C

A. Haplotype	(Alleles Within the Haplotypes)	B. Haplotype Frequencies (%) Cohort (%) (2n = 748)	AA (%) (2n = 360)	CA (%) (2n = 388)	C. Among Entire Cohort* OR	95% CI	P-Value	D. Among AA Only OR	95%CI	P-Value	E. Among CA Only OR	95%CI	P-Value
Myxovirus resistance-1 (Mx1)													
Global test chi-square P-value=0.0809													
Mx1-CAAA	(−123C, +6886A, +19820A(379I) +38642A)	13.5	21.4	6.2	0.79	0.47–1.32	0.3598	0.78	0.42–1.47	0.4474	0.79	0.32–1.96	0.6089
Mx1-CGAA	(−123C, +6886G, +19820A(379I), +38642A)	25.7	13.9	36.6	1.14	0.73–1.77	0.5979	1.22	0.61–2.43	0.5797	1.07	0.60–1.92	0.8227
Mx1-CAGA	(−123C, +6886A, +19820G(379V), +38642A)	25.9	18.3	33	1.01	0.66–1.56	0.9500	0.85	0.44–1.64	0.6315	1.17	0.65–2.08	0.6075
Mx1-CAGT	(−123C, +6886A, +19820G(379V), +38642T)	9.2	17.5	1.5	0.41	0.21–0.79	0.0075	0.42	0.21–0.85	0.0152	0.30	0.03–2.57	0.2691
MX1-AAAA	(−123A, +6886A, +19820A(379I), +38642A)	6.6	6.4	6.7	0.8	0.42–1.55	0.5135	1.59	0.65–3.92	0.3137	0.38	0.14–1.08	0.0687
MX1-AAGT	(−123A, +6886A, +19820G(379V), +38642T)	6.8	14.2	0	1.31	0.66–2.60	0.4371	1.31	0.66–2.60	0.4371	–	–	–
MX1-CGAT	(−123C, +6886G, +19820A(379I), +38642T)	5.2	1.1	9	1.79	0.88–3.64	0.1059	0.90	0.08–10.09	0.9297	1.93	0.91–4.06	0.0851
Protein kinase (PKR)													
Global test chi-square P-value=0.0163													
PKR-TGATT	(+110T, +7949G, +13846A, +22937T, +40342T)	23.7	9.2	37.1	0.53	0.33–0.87	0.0112	0.37	0.14–0.96	0.0416	0.61	0.34–1.11	0.1039
PKR-CAATT	(+110C, +7949A, +13846A, +22937T, +40342T)	58.2	67.5	49.5	1.63	0.93–2.84	0.0863	1.21	0.49–2.99	0.6732	1.92	0.95–3.87	0.0683
Transforming growth factor-β (TGFB)													
Global test chi-square P-value=0.4870													
TGFB-GC	(+5703G, +14031C)	36.6	30	42.8	1.34	0.86–2.08	0.1976	1.19	0.64–2.19	0.5853	1.53	0.80–2.91	0.1989
TGFB-GG	(+5703G, +14031G)	61.2	65.6	57.2	1.07	0.58–2.01	0.8245	0.74	0.28–1.95	0.5423	1.38	0.61–3.14	0.4402
Interleukin-10 (IL10)													
Global test chi-square P-value=0.0768													
IL10-CCT	(+430C,+1578C, +3947T)	26.7	18.3	34.5	1.42	0.92–2.19	0.109	1.63	0.86–3.09	0.1344	1.27	0.71–2.28	0.4185
IL10-TCC	(+430T, +1578C, +3947C)	39.4	28.6	49.5	0.72	0.46–1.13	0.1505	0.79	0.43–1.46	0.4556	0.65	0.35–1.24	0.1931
IL10-CTT	(+430C, +1578T, +3947T)	27.8	40.6	16	0.85	0.54–1.33	0.4738	0.72	0.38–1.36	0.3112	1.0	0.53–1.87	0.9947
IL10-CCC	(+430C, +1578C, +3947C)	5.8	11.9	0	1.57	0.77–3.19	0.2169	1.57	0.77–3.19	0.2169	–	–	–
Interferon-γ (IFNG)													
Global test chi-square P-value=0.2172													
IFNG-CCT	(−765C, +2325C, +3360T)	20.5	10.8	29.4	1.79	0.72–4.42	0.2081	–	–	–	1.6	0.63–4.04	0.3229
IFNG-CTC	(−765C, +2325T, +3360C)	45.2	41.4	48.7	0.94	0.61–1.45	0.7731	0.86	0.42–1.62	0.6302	1.02	0.56–1.85	0.9562
IFNG-CTT	(−765C, +2325T, +3360T)	29.9	46.4	14.7	0.93	0.58–1.47	0.7418	1.09	0.58–2.07	0.7850	0.78	0.40–1.51	0.4553

NOTE. Part A lists the abbreviations for the haplotypes targeted in the present study, along with the individual alleles that constitute each of the haplotypes. Part B presents the haplotype frequencies in the cohort overall, among African Americans (AA) and Caucasian Americans (CA). Part C presents adjusted associations of haplotype carriage with liver fibrosis (Ishak ≥ 3 versus < 3). Parts D and E present association with Ishak fibrosis among AA only and CA, respectively–Univariable logistic regression within each race. Only haplotypes ≥5% are presented.

*Bivariable logistic regression adjusting for self-reported race.

the NIH validation cohort. Although we restricted our analyses to a subset of patients in the NIH group that approximates the Virahep-C study, the NIH cohort did have some substantial differences (Table 6) compared with the Virahep-C cohort. First, the NIH cohort consisted of a small proportion of AAs (17.5%), compared with the Virahep-C cohort (48.1%). In addition, co-factors for fibrosis progression such as estimates of alcohol use were not available in the validation cohort. The NIH cohort was also smaller (n = 194) than Virahep-C (N = 374). Additionally, the fact that we did not observe the *-88Mx1* to be polymorphic in our study may further contribute to the lack of consistent results, particularly given the previous findings of the functional relevance of this locus.[26,27] Our findings highlight the need for additional replication in other cohorts of sufficient size for host genetics studies. These validation cohorts should also contain a sufficient number of AAs to allow for meaningful statistical comparisons. In addition, replication of our observations should be extended to other racial groups as well.

Polymorphisms in the *PKR* gene have been previously implicated in the natural clearance of HCV, with the heterozygous *PKR(-168)-C/T* genotype associated with self-limiting infection. In addition, a microsatellite repeat in the *5′-UTR* region of *PKR* has been associated with self-limiting HCV infection as well as the response to interferon therapy.[25] We observed a protective association between the *PKR-TGATT* haplotype and fibrosis in the Virahep-C cohort. However, the *PKR-TGATT* haplotype was not present in the replication cohort, and this may be a reflection of chance differences between the 2 cohorts.

IFN-γ is an important cytokine that is involved with many aspects of the immune as well as inflammatory responses to infectious diseases, including the differentiation of T and B cells, macrophages, natural killer cells, endothelial cells, as well as fibroblasts.[28] In the current study, we did not observe any significant associations between *IFN-γ* haplotypes and fibrosis severity, similar to findings reported in several previous studies.[29,30] Studies that have examined the relationship between polymorphisms in the *IFN-γ* gene and cytokine production have

Table 4. Associations of Individual SNPs Within MxA and PKR with Liver Fibrosis (Ishak ≥3 versus <3) in Virahep-C

	B. Allele Frequencies (%)			C. Among Entire Cohort*			D. Among AA Only			E. Among CA Only		
A. Allele	Cohort (%) (2n = 748)	AA (%) (2n = 388)	CA (%) (2n = 360)	OR	95% CI	*P*-value	OR	95% CI	*P*-value	OR	95% CI	*P*-value
Myxovirus resistance-1 (Mx1)												
Mx1 rs17000900 C	83.3	76.7	89.3	0.90	0.31–2.60	0.8449	0.55	0.15–1.98	0.3619	2.74	0.30–24.98	0.3717
Mx1 rs17000900 A	16.7	23.3	10.7	0.96	0.60–1.55	0.8802	1.63	0.87–3.03	0.1258	0.43	0.19–0.98	0.0440
Mx1 rs455816 A	66.8	83.2	51.6	0.66	0.35–1.23	0.1865	0.55	0.08–3.99	0.5533	0.67	0.35–1.29	0.2317
Mx1 rs455816 G	33.2	16.8	48.5	1.05	0.66–1.65	0.8502	1.00	0.52–1.93	0.9940	1.09	0.58–2.07	0.7870
Mx1 rs469390 G	46.4	53.6	39.7	0.89	0.56–1.41	0.6169	0.53	0.26–1.08	0.0810	1.27	0.70–2.32	0.4341
Mx1 rs469390 A	53.6	46.4	60.3	1.12	0.67–1.86	0.6697	1.12	0.57–2.19	0.7457	1.12	0.51–2.44	0.7817
Mx1 rs456298 A	75	62.4	86.6	0.56	0.25–1.26	0.1576	0.55	0.23–1.28	0.1653	0.66	0.04–10.63	0.7662
Mx1 rs456298 T	25	37.6	13.4	0.84	0.53–1.33	0.4660	0.47	0.25–0.89	0.0193	1.59	0.83–3.04	0.1649
Protein kinase (PKR)												
PKR rs2254958 C	71.0	85.0	57.9	2.02	0.93–4.39	0.0769	0.56	0.08–4.06	0.5652	2.47	1.05–5.81	0.0375
PKR rs2254958 T	29.0	15.0	42.1	0.56	0.35–0.90	0.0164	0.47	0.23–0.99	0.0482	0.64	0.35–1.18	0.1493
PKR rs2287350 A	71.8	84.9	59.6	1.34	0.63–2.85	0.4423	0.27	0.02–3.06	0.2919	1.62	0.72–3.62	0.2441
PKR rs2287350 G	28.2	15.1	40.4	0.57	0.36–0.90	0.0149	0.45	0.22–0.94	0.0335	0.66	0.36–1.20	0.1751
PKR rs2307479 A	93.3	88.5	97.7	–	–	–	–	–	–	–	–	–
PKR rs2307479 C	6.7	11.5	2.3	0.63	0.31–1.27	0.1942	0.82	0.38–1.77	0.6162	0.18	0.02–1.46	0.1086
PKR rs2307478 T	98.0	95.8	100.0	–	–	–	–	–	–	–	–	–
PKR rs2307478 C	2.0	4.2	0.0	0.89	0.29–2.73	0.8381	0.89	0.29–2.73	0.8381	–	–	–
PKR rs2372443 T	96.6	98.0	95.4	0.63	0.09–4.50	0.6405	–	–	–	0.32	0.03–3.63	0.3600
PKR rs2372443 C	3.4	2.0	4.6	1.50	0.62–3.63	0.3746	1.87	0.37–9.53	0.4532	1.36	0.47–3.92	0.5666

NOTE. **Part A** lists the alleles for each SNP, whereas **Part B** presents the allele frequencies. **Part C** presents race-adjusted associations between allele carriage and fibrosis score (Ishak ≥3 versus <3). **Parts D** and **E** present the associations between allele carriage and fibrosis among AA only and CA only, respectively.

*Bivariable logistic regression adjusting for self-reported race.

failed to show any direct functional relationships.[29,31] However, other polymorphisms not targeted in the current study may play a stronger role in mediating IFN-γ expression, and the haplotypes targeted may not reflect the influence of this locus.

IL-10 is an important pleiotropic, anti-inflammatory (Th2) cytokine that is believed to be a suppressor of the immune response and downregulator of IFN-γ.[11] Studies have reported varying levels of IL-10 between different individuals, and evidence suggests a strong genetic and environmental component affecting this expression.[32-36] In a trial of recombinant IL-10 for the treatment of HCV-related cirrhosis, histological improvement was observed, suggesting an important role for IL-10 in liver fibrogenesis,[37] although this effect was not duplicated in a subsequent larger, randomized controlled trial. We failed to detect a significant association between haplotypes within the IL-10 gene and fibrosis. Our findings support those of several previous studies, which have failed to show any relationship between IL-10 promoter polymorphisms and fibrosis stage.[29,30,38] In a study by Knapp and colleagues,[25] a higher frequency of the *IL10 (-1082A/A)* genotype along with homozygosity for the *IL10 (-1082A)+(-819C)+(-592C)* and *IL10 (-1082A)+(-819T)+(-592A)* haplotypes was found, which have been shown in previous *in vitro* functional studies to re-

Table 5. Multivariable Analyses Adjusting for Potential Confounding Variables in the Virahep-C Cohort

Variables in Model	OR	95% CI	*P*-Value
Mx1-CAGT	0.33	0.16–0.68	0.0027
PKR-TGATT	0.56	0.32–0.98	0.0405
Alcohol use (high versus low)	1.31	0.63–2.72	0.4629
Self-reported race* (CA versus AA)	1.43	0.81–2.54	0.2166
Gender (Male versus Female)	1.36	0.82–2.24	0.2333
Patient age (per 1 year increase)	1.08	1.05–1.12	<0.0001
Summary inflammation score (≥8 versus <8)	5.96	3.29–10.78	<0.0001

Table 6. Characteristics of the NIH Validation Cohort

Factor	African Americans (n = 34)	Caucasian Americans (n = 160)	*P*
Age (years) mean (±SD)	52.7 (±8.42)	48.0 (±7.83)	0.008*
Sex			
Male	76.5%	53.1%	0.125
Female	23.5%	46.9%	
Ishak score†			
<3	45.5%	72.0%	0.003
≥3	54.6%	28.0%	
Summary inflammation‡			
<8	33.3%	40.0%	0.474
≥8	66.7%	60.0%	

*Two-sample *t* test; otherwise, chi-square test used. †Ishak scores were available on 33 AAs and 157 CAs.

‡Inflammation scores were available on 33 AAs and 160 CAs.

Table 7. Multivariable Analysis in the NIH Validation Cohort

Variables in Model	OR	95% CI	*P*-Value
Mx1-CAGT carrier	0.64	0.23–1.81	0.3999
Self-reported race (CA versus AA)	0.44	0.17–1.13	0.0885
Gender (Male versus female)	1.46	0.83–3.74	0.1436
Patient age (per 1-year increase)	1.08	1.03–1.13	0.0010
Summary inflammation score (≥8 versus <8)	11.72	4.38–31.35	<0.0001

sult in lowered IL-10 production among individuals with faster progression of HCV-related fibrosis, although this relationship was not statistically significant.[39] However, *in vivo* IL-10 production is likely much more complex, with environmental factors affecting IL-10 production. Recently, decreasing body mass index and smoking were reported to decrease IL-10 production.[33] These complexities may explain, at least in part, the conflicting results presented in functional studies of promoter alleles and IL-10 production.

Previous studies have also yielded conflicting results with respect to associations of *TGF-β* polymorphisms with HCV-related fibrosis.[30,38,40-42] TGF-β is a potent activator of hepatic stellate cells, which may contribute to the production of excess extracellular matrix proteins and consequently enhance fibrogenesis.[12] In addition to elevated TGF-β levels among individuals with chronic HCV compared with healthy controls, TGF-β levels have been shown to correlate with the expression of type 1 collagen mRNA.[43] In the current study, we did not observe any associations between *TGF-β* variants and liver fibrosis. However, the patients enrolled in the current study tended to have milder fibrosis, and TGF-β variants may play a different role among individuals with more severe liver disease.

The current study represents the first of 2 stages, in which we identified important genes associated with fibrosis. Future studies will focus on high-density genotyping of variants in these genes to identify specific variants that are associated with fibrosis. In summary, we identified associations between the *Mx1-CAGT* and *PKR-TGATT* haplotypes and lower fibrosis scores among patients with genotype-1 chronic HCV infection in the Virahep-C Study. However, the associations were not statistically significant in a second, independent validation cohort. We also did not observe significant associations between haplotypes in *IL-10*, *TGF-β,* and *IFN-γ* and fibrosis.

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