

Association of Genetic Variants of the Chemokine Receptor CCR5 and Its Ligands, RANTES and MCP-2, With Outcome of HCV Infection

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The effect of host genetic variation on the outcome of hepatitis C virus (HCV) infection and its treatment is poorly understood. The chemokine receptors CCR5, CCR2, and CCR3 and their ligands, RANTES, MCP-1, MCP-2, and MIP-1 α , are involved in the immune responses and the selective recruitment of lymphocytes to the liver in HCV infection. We studied 20 polymorphisms within these genes and investigated their association with persistent carriage of HCV, severity of liver disease, hepatic inflammation, and response to treatment in a large European cohort. Significant associations were found between CCR5- Δ 32 and reduced portal inflammation ($P = .011$, odds ratio [OR]: 2.3, 95% confidence interval [CI]: 1.09-4.84) and milder fibrosis ($P = .015$, OR: 1.97, 95% CI: 1.13-3.42). A promoter polymorphism at position -403 in the RANTES gene was associated with less severe portal inflammation ($P = .004$). An amino acid change in MCP2, Q46K, was associated with severity of fibrosis ($P = .018$, OR: 2.29, 95% CI: 1.14-4.58). In conclusion, our study suggests a possible role of the polymorphisms CCR5- Δ 32, RANTES -403, and MCP-2 Q46K in the outcome of HCV infection. (HEPATOLOGY 2003;38:1468-1476).

See Editorial on Page 1359

Hepatitis C virus (HCV) is a major global health problem with 1% to 2% of the world's population chronically infected. The prevalence of infection in the United Kingdom is approximately 0.5%,¹ but it is likely that this figure will continue to rise as more cases come to light. Persistent HCV infection has a very

variable outcome, with 25% of those infected developing cirrhosis within 20 years, of whom 3% to 5% per year will proceed to hepatocellular carcinoma. A further 5% per year will develop liver failure, and 2% per year will die of a liver-related death. There is also a very variable response to treatment because only 40% of those treated with interferon alfa and ribavirin successfully clear the virus.^{2,3} There are a number of epidemiologic and viral factors that influence susceptibility to persistent HCV infection, progression of HCV-related liver disease, and response to treatment, but host genetic factors are also influential. This study has examined 20 polymorphisms in the genes for the CC chemokine receptors (CCR) CCR5, CCR2, and CCR3 and their ligands, RANTES (regulated and normal T-cell expressed and secreted), MCP-1 (monocyte chemotactic protein), MCP-2, and MIP-1 α (macrophage inflammatory protein), to try and elucidate their role in the immunopathologic outcome of HCV infection.

Chemokines constitute a large family of small (8-10 kD) cytokines that are up-regulated in inflammation and whose effects are mediated by members of a family of 7 transmembrane domain, G protein-coupled receptors.⁴ Their major role is in leukocyte migration and dependent processes such as immune surveillance and innate and adaptive immune responses. Chemokines have been

Abbreviations: HCV, hepatitis C virus; CCR5, CC chemokine receptor 5; RANTES, regulated and normal T cell expressed and secreted; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory proteins; SNP, single nucleotide polymorphism; HSC, hepatic stellate cells; PCR, polymerase chain reaction; LDR, ligation detection reaction; OR, odds ratio.

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linked with several disease states including psoriasis, atherosclerosis, arthritis, and multiple sclerosis.

It is now known that chemokine receptors also have a role in infectious disease either because of over expression of receptors or by facilitating entry of pathogens into permissive cells. In 1996, the CCR5 was shown to act as a cofactor for entry of macrophage-tropic strains of HIV-1.^{5,6} Shortly after this, a series of reports described the defective CCR5- Δ 32 allele, which established the role of CCR5 in HIV pathogenesis.⁷⁻⁹ CCR5- Δ 32 is a 32-bp deletion in the CCR5 gene resulting in a nonfunctional protein,⁷ explaining the almost complete protection against HIV-1 infection in individuals homozygous for the defective allele and delayed progression to AIDS in heterozygotes.⁸⁻¹⁰

CCR5 is a strong candidate gene for the outcome of HCV infection and the course of HCV-related liver disease. The immune response in persistent hepatitis C is compartmentalized, with a predominant Th2 or Th0 response in the periphery¹¹ and a Th1 response in the liver¹² associated with progressive liver injury. Differences in chemokine receptor expression between Th1 and Th2 cells may influence their selective recruitment to tissues. *In vitro*, Th1 cells that express CCR5 migrate to their respective chemokines, RANTES and MIP-1 α ,¹³ which are largely confined to the portal regions.¹⁴ Portal inflammation is the predominant pattern of inflammation seen in persistent viral infection, including HCV, and is associated with a less aggressive course of disease.¹⁵ Antigen-presenting dendritic cells are located in the portal area and infiltrating T cells are exposed to infected hepatocytes in the periportal area. Thus, these areas could be sites of the initial immune response to hepatitis C viruses. Apart from T-cell migration, CCR5 and other chemokine receptors mediate cell activation, costimulation, and differentiation of T cells and monocytes during innate and adaptive immunity.¹⁶ These are all processes relevant to HCV clearance or persistence, which are likely to be modified by the loss of a functional CCR5 receptor. In addition to CCR5- Δ 32, 7 CCR5 promoter single nucleotide polymorphisms (SNP) were studied here.

CCR2 also serves as an HIV-1 coreceptor, and a role in modulating the immune response as well as recruiting monocytes/macrophages to sites of inflammation has been suggested.¹⁷ Only 1 variant has been reported in CCR2, which leads to a Valine \rightarrow Isoleucine substitution at amino acid position 64. This is within the first transmembrane region and has been shown to be associated with a delay in progression to AIDS^{18,19}; this protection is genetically independent of that conferred by CCR5- Δ 32.

Five polymorphisms have been identified in the CCR3 gene: 2 silent mutations (T51C, C240T) and 3 that en-

code amino acid changes, an arginine to glutamine at amino acid position 275 (G824A), a leucine to proline at amino acid position 351 (T1052C),²⁰⁻²² and a cysteine to serine substitution at amino acid position 218 (T652A).²³

Two polymorphisms have been described in the RANTES promoter region. It has been suggested that the RANTES -28G mutation increased RANTES expression in HIV-1-infected individuals and thus caused a delay in the progression of HIV-1 disease.²⁴ The second polymorphism is a G \rightarrow A substitution at position -403. There is evidence that the mutant A allele leads to increased transcription of RANTES and that the A allele is associated with an increased susceptibility to atopy, asthma,²⁵ and HIV.²⁶

MIP-1 α has both phagocyte stimulating and proinflammatory properties²⁷ and is a key ligand for CCR5. A biallelic dinucleotide microsatellite repeat has been identified within the MIP-1 α promoter region.²⁸ MCP-1 and 2 are active on multiple leukocyte populations showing chemotactic activity at low concentration *in vitro*.^{29,30} Hepatic stellate cells (HSC) have been shown to regulate leukocyte trafficking by secreting MCP-1³¹ and MIP-1 α , and it has been suggested that MCP-1 may have a direct profibrogenic action via HSC chemotaxis.³² Two polymorphisms have been identified in the distal regulatory region of the MCP-1 gene. The polymorphism at -2076 does not appear to affect MCP-1 transcription. However, *in vitro* cells from individuals who are heterozygous or homozygous for G at -2518 appear to produce more MCP-1 than cells from individuals homozygous for A at -2518.³³ Little is known about the role of MCP-2 in pathology, although it may act as an effector molecule in the inflammatory events occurring in multiple sclerosis.³⁴ One SNP has been identified in the MCP-2 gene to date. No significant difference in biologic activity has been observed between the 2 isoforms.³⁵

Tables 1 and 2 give an overview of the genes and polymorphisms investigated during the course of this study. Specifically, we report here on our findings of 20 polymorphisms in the chemokines CCR5, CCR2, and CCR3 and their ligands RANTES, MIP-1 α , MCP-1, and MCP-2 with respect to persistent HCV infection.

Patients and Methods

Patients. Participants for this study were recruited from several large hepatology clinics across Europe between October 1995 and February 2001. Patients were categorized on the basis of viral serology, liver histology, and response to treatment. Exclusion criteria included coinfection with HBV or HIV. All patients were positive for HCV antibody with no evidence of liver disease be-

Table 1. Summary of the Chemokines Analyzed in This Study and Their Receptors

Chemokine	Receptors	Responding Cells	Polymorphisms Studied	Polymorphism Functional Effect	Disease Association
RANTES	CCR1, CCR3, CCR5, DARC	Eosinophils, monocytes, activated T cells, NK cells, immature dendritic cells	-28 -403	↑ RANTES expression ↑ RANTES expression or no effect	HIV ²⁴ Asthma, atopy ^{25,26}
MCP-1	CCR2, CCR10	Basophils, monocytes, activated T cells, NK cells, immature dendritic cells	G-2518A A-2076T	↑ MCP-1 ³³ None known	
MCP-2	CCR1, CCR2, CCR3, CCR4, CCR5	Basophils, monocytes, activated T cells, NK cells, immature dendritic cells	Q46K	None known ³⁵	Multiple sclerosis ³⁴
MIP-1 α	CCR1, CCR5	Eosinophils, monocytes, activated T cells, NK cells, immature dendritic cells	Microsatellite	-	-

NOTE. The receptors shown in bold were studied.

cause of other causes. Patients classified as having a persistent infection were HCV antibody positive and PCR positive for more than 6 months postpresentation or likely time of infection. Patients with a self-limiting infection showed normal concentrations of liver aminotransferase enzymes; they were HCV antibody positive and PCR negative at presentation on at least 1 occasion more than 3 months later.

The response to combined interferon alfa and ribavirin or interferon alfa therapy alone was evaluated in patients who had received treatment for more than 3 months. Viraemia was measured using the Roche Amplicor test with a lower end sensitivity of 500 genomes per mL. Sustained responders (SR) were defined as individuals with normal concentrations of liver aminotransferases and no detectable viraemia for more than 6 months after the course of drug administration. Nonresponders (NR) were defined as patients with continuous viraemia more than 3 months into therapy. Relapsers (RL) were those patients with nondetectable viraemia during the course of treatment and detectable viraemia following the end of drug administration. Liver biopsy specimens were scored using Ishak's modification of the histology activity index (HAI)¹⁵ for the grading of inflammation (0-18) and fibrosis (0-6). Patients with persistent infection were divided into those with a fibrosis score of <2 (mild) or >3 (severe) at the time of presentation and those with a necro-

inflammatory score of <5 (mild) or >5 (severe). Further details of the patients' criteria are reported in Thursz et al.³⁶ Six hundred seventy-two patients were genotyped in total. Information on clinical and environmental factors was collected at recruitment. Unfortunately, complete data for all factors were not available for each subject, thus reducing the numbers available for analysis for some phenotypes (Table 3). All subjects were tested for each polymorphism.

Genotyping. Sample genotypes for SNP and the CCR5- Δ 32 were analyzed by the ligation detection reaction (LDR). PCR products containing the SNP of interest were generated under standard conditions in a 15- μ L reaction containing 1X reaction buffer, 1-2 mmol/L MgCl₂, 0.32 mmol/L dNTP (all reagents Perkin Elmer Applied Biosystems, Warrington, United Kingdom), 0.12 μ mol/L of forward (F) and reverse (R) primer, respectively, 1 U Taq Gold polymerase, 50 ng DNA, and dH₂O. Cycling parameters were 94°C for 14 minutes, followed by 35 cycles of 94°C for 30 seconds, the specific annealing temperature (see below) for 30 seconds, and 72°C for 30 seconds, followed by a final step of 72°C for 5 minutes. For PCR primer sequences, PCR annealing temperatures, and PCR product size, refer to Table 4.

For genotyping by the LDR method, 3 oligonucleotide probes are required for each SNP. These comprise 2 allele-specific probes labelled with a different fluorescent tag at

Table 2. Summary of the Chemokine Receptors Analyzed in This Study and Their Ligands

Chemokine Receptor	Ligands	Expression	Polymorphisms Studied	Polymorphism Functional Effect	Disease Association
CCR2	MCP-1, -2, -3, -4	Monocytes, T cells, basophils, dendritic cells	V64I	None known	HIV
CCR3	RANTES, Eotaxin, MCP-4	T cells, ²¹ basophils, ²¹ eosinophils, dendritic cells	T52C, C240T, T652A, G824A, T1052C	-	-
CCR5	MIP-1α, -1β, RANTES, MCP-2, -4	Monocytes, T cells, dendritic cells	Δ 32 A-2733G, G-2554T, G-2459A, T-2135C, C-2132T, A-2086G, C-1835T	↓ receptor function (see text)	HIV -2459 HIV

NOTE. The ligands shown in bold were studied.

Table 3. Demographic and Phenotypic Data for Cohort

		No.	Total
Sex	Male	349 (56%)	623 (93%)
	Female	274 (44%)	
Ethnicity	Caucasian	558 (92%)	607 (90%)
	Afro-Caribbean	30 (5%)	
	Asian	19 (3%)	
HCV carriage	Cleared	128 (19%)	672 (100%)
	Persistent	544 (81%)	
Fibrosis	Mild	318 (72%)	443 (66%)
	Severe	125 (28%)	
Interferon response	Sustained response	90 (26%)	346 (51%)
	Relapsed	125 (36%)	
	Nonresponse	131 (38%)	
Necroinflammation	<5	189 (62%)	307 (46%)
	>5	118 (38%)	
Portal Inflammation	0 + 1	56 (25%)	223 (33%)
	2, 3 + 4	167 (75%)	

NOTE. Fibrosis and inflammation scores are defined in the Patients and Methods section.

the 5' end and the allele-specific base at the 3' end as well as a phosphorylated unlabelled common probe. Details of the LDR method have been described elsewhere.³⁷ For genotype determination, 1 μL of each LDR amplification

product was run on an ABI prism 373 sequencer (Perkin Elmer Applied Biosystems), and sample genotypes were analyzed using GENESCAN and Genotyper software programmes (Perkin Elmer Applied Biosystems).

The MIP-1α microsatellite was genotyped by PCR using a fluorescein-labelled primer. The products were resolved by polyacrylamide gel and detected on an ABI prism 373 sequencer (Perkin Elmer Applied Biosystems).

Statistical Analysis. The distribution of genotype and allele frequencies was analyzed using standard 3 × 2 or 2 × 2 χ² tests or Fisher exact tests applying SPSS 10.0.5 for Windows (SPSS Inc.). A value of P < .05 was considered significant. The odds ratio (OR) was calculated to indicate the risk associated and presented with 95% confidence intervals (CI). The potentially confounding effects of ethnic group, age at infection, and sex were corrected for by logistic regression. Data for age at infection were only available for 60% of patients, thereby reducing the statistical power considerably. Therefore, power calculations have been performed for each SNP genotyped and presented as the probability of the study

Table 4. PCR and LDR Primers and Annealing Temperatures for all Polymorphisms Studied

Gene	PCR Primers	PCR Temp	Polymorphism	LDR Primers	LDR Size	LDR Temp
CCR5	For: CCTGGCTGTCTCCATGCTG Rev: CTGATCTAGAGCCATGTGCACAACCTCT	55°	CCR5-Δ32	Wild type: CAGTCAGTATCAATTCTGGAAGAATTTCCAGACA Deletion: AaaCATTACACCTGCAGCTCTCATTTCACATA Common: TTAAAGATAGTCATCATCTGGGGCTGTCTGCaaaataataataataataataa		62°
	For: TGGGGTCTCATTGCCCTCTTA Rev: AACTGTGACCCITTCCTTATCT			Com: ATTTGGCAACACCAAGTGCATACAAATATCTTAAATATATAAAAAATAT Allelic: GAGATGAGTAAAAGACTTTACAGGAAACCCATARAAGA T/C Common: GAAGAACYGTCTCTGATCTTTTCGCCTCAATACAAAATAAT Allelic: ACCAGAGATCTATTMTCTAGCTTATTTAAGCTCAACTAAAA G/A Common: AARKTTTATTACGGGCTTTCTCAGTGGATTATTTGAAAAAATAA Allelic: TTAAAGTGTGCTTAAAATAAGCTAGAKAATAGATCTCAGGTCT A/G Common: TTYAGACCAGAGATCTATTMTCTAGCTTATTTAAGCTCAACTAAAAAG Allelic: CAAAATAATCCAGTGAGAAARGCCCGTAAATAAAM C/T Common: CACAGGGTTAATGTGAAGTCCAGGATCCCaaaataataataataataataa Allelic: CGGGGAGAGTGGAGAAAAAGGGG A/G Common: TTTCCGTTACAGAGAAACAATAATTTGGGTGGTGAAA Allelic: TGAGCCATAGTAAAACCTTTAGACAACAGGTT T/G Common: ATCTCTGCCACCTATGTATCTGGCATAGTGTGAGTAAAA Allelic: AAATTTCTATAGCTTCAGATAGATTATATCTGGAGTGAAG G/A Common: TCCTCATCTAATAAATCTGCAAAAAGCTGAAGTCTTataataataataa Allelic: aGTTTTGTGGGCAACATGTCTGTC G/A Common: GATGACGTGGCCTGCTCTGTGAAAA Allelic: ACTAGATACAGTGTGACCTTTGTACCACATCCTACTA T/C Common: CTGCTCTCCTCGTACCCTTCCATTCTGATAAATAAATAAATAA Allelic: CTGTCAACCTGGCCATTCGGA C/T Common: GCTACACAGGAATCATCAAAACGCTGCTGAAAAAATAAATTTTT Allelic: TCTCCCTCTGCTCGTATGGCCATC T/A Common: GACGAAGCATCTGGACCTGGTCTGTAATAATAAATAA Allelic: AAATCCATCTATTGGAAATGACTGTGAGC G/A Common: TCTATTGTGTTTGGTGCAGATGCAGAAAATGCCCATTATAATAAATAA Allelic: TCCATCCACAGCAGCCGGAAC T/C Common: ACTTCCAGAAAGACTTTCTTCTGATTCATACCAATAAAAAAAAAAAT Allelic: TAAGCAGAAAGTGGGAGGCAGACAGCT G/A Common: GATTAGAGAGAGGTTCCCGGATATGAGGAAAATCAAAATATAATAAAT Allelic: ACACCAAGTTCATGTAAGGATGACTAAC A/T Common: AACGGGGCAAGGAGTCTGTGCTGTA Allelic: CTCTCTCCCCCAGCTTCAAGACC A/C		
CCR5 prom	For: TGGGGTCTCATTGCCCTCTTA Rev: AACTGTGACCCITTCCTTATCT	55°	-1835		92bp	66°
			-2086		89bp	66°
			-2132		86bp	71°
			-2135		83bp	71°
			-2459		77bp	66°
			-2554		74bp	64°
			-2733		80bp	64°
CCR2	For: CCAAGCAGAGCGGTGAAGAAGT Rev: AAGCAAACACAGCATGGACAATAG	53°				66°
CCR3	For: TTGTGGGATTGTATTTCTCTCT Rev: CCCCTGGCCTGTGGTAA	55°	T51C		67bp	68°
			C240T		70bp	68°
			T652A		73bp	68°
			G824A		76bp	68°
			T1052C		79bp	68°
MCP1-prom	For: GCTCCGGGCCAGTATCT Rev: GGCCATCTCACCTCATCTTCC	60°	-2518		83bp	68°
			-2076		85bp	68°
MCP2	For: TACGGTGGTCTTAATGTCT Rev: GGAGTTGGGAAAATAAAGC	53°	Q46K			
MIP-1α	For: CTGACCAGCATCGTTA Rev: AAGGCATGATTTCCAAGC	58°	-			
RANTES	For: GACCTCCTCAATAAACACT Rev: GCAGAGGCGAGTACCAATG	60°	-28	Common: AACTGGCCCTATAAataataCCTGAGCTGCAGAGTaa Allelic: AACTCCCTTAGGGGATGCCCT C/G	64bp	64°
			-403	Common: TAAGATCTGTAATGAATAAGCAGGAACCTTGAAGACTCAGTgtaata Allelic: a CCTTCATGTGATGAGGAAAGGAG G/A	74bp	64°

Table 5. Results Table

Gene	SNP	Persistence of Infection	Severity of Fibrosis	Interferon Response	NI	PI	Number Successfully Genotyped	Mutant Allele Frequency	Power
RANTES	-28	330	4%	10%
	-403	$P = .004$	321	15%	76%
CCR2	V64I	327	9%	36%
CCR5	$\Delta 32$.	$P = .015$.	.	$P = .011$	547	11%	72%
	2733	367	41%	100%
CCR5 prom	2554	364	36%	99%
	2459	212	23%	91%
	2135	234	25%	96%
	2132	$P = .048$.	$P = .023$.	.	234	8%	22%
	2086	260	32%	99%
	1835	302	8%	28%
MIP-1 α	ms	551	30%	99%
	2518	477	35%	100%
MCP-1	2076	517	17%	97%
MCP-2	K46Q	.	$P = .018$.	.	.	373	16%	87%
CCR3	51	347	7%	25%
	240	491	—	—
	652	457	—	—
	824	476	1%	3%
	1052	423	—	—

NOTE. P values given for significant results; a dot indicates no significant association. A power calculation is given for each polymorphism (see Patients and Methods) assuming that all genotyped samples were included in the analysis; however, in practice the number available for analysis for each phenotype varied.

detecting a relationship assuming a 5% significance level (Table 5).

Results

In this study, 20 polymorphisms were studied in 7 genes. All polymorphism frequencies were analyzed for an association with spontaneous clearance versus persistent carriage, mild versus severe fibrosis, interferon response, and overall necroinflammatory score. When appropriate, the overall necroinflammatory score was subdivided and analyzed in more detail. The significant associations are described below and summarized in Tables 5 and 6.

A significant association was found between severe fibrosis and carriage of CCR5 $\Delta 32$ ($P = .015$; OR: 1.97, 95% CI: 1.13-3.47). When looking at the subgroup comparison for portal inflammation, although the number of patients was markedly reduced to 124, there was a significant association between $-\Delta 32/-\Delta 32$ homozygotes and mild portal inflammation ($P = .011$, OR: 2.3, 95% CI: 1.09-4.84). The promoter polymorphism at position -2132 was found to be significantly associated with susceptibility to persistent HCV infection, with presence of the C allele increasing risk of persistent carriage ($P = .048$). Carriage of the C allele at -2132 was also associated with an initial response to interferon, *i.e.*, sustained responders and relapsers versus nonresponders ($P = .023$, OR: 3.15, 95% CI: 1.14-8.72).

The genotype frequency of the RANTES promoter polymorphism at position -403 was significantly associ-

ated with portal inflammation ($P = .015$). AA homozygotes are associated with milder portal inflammation than those carrying the G allele ($P = .004$).

For MCP-2 Q46K, a significant association was found between severity of liver fibrosis and genotype ($P = .027$). Carriage of the K variant is associated with more severe fibrosis ($P = .018$, OR: 2.29, 95% CI: 1.14-4.58).

When logistic regression was applied for the variables sex, ethnicity, and age at infection, the associations became nonsignificant because of the reduction in sample numbers and reduced statistical power. However, if each variable was assessed individually, there was no significant change in the OR, suggesting that these variables are not significant confounders.

There was no association between the polymorphisms analyzed in CCR2, CCR3, MIP-1 α , or MCP-1 and any of the phenotypes. Three of the SNP described in CCR3 (C240T, T652A, and T1052C) were not polymorphic in this population.

Discussion

This is a large case control study, looking at 20 polymorphisms in a group of 7 CC chemokines and their receptors. Interest in these genes was initially raised by the presence of the functional polymorphism CCR5- $\Delta 32$, which gives rise to a truncated protein that, in the context of HIV-1, does not act as a competent receptor. With the finding that CCR5 is partly responsible for the recruitment of T cells to the portal region, it was expected that an

Table 6. Details of Significant Associations Found in This Study

Polymorphism/ Phenotype	Genotype Distribution			HWE	P Value (χ^2 value)	Allele Presence/ Absence	P Value (χ^2 value)	OR (95% CI)
	Number (%)							
CCR5 Δ 32	ww	w Δ 32	Δ 32/ Δ 32					
PI <1	19 (63.3%)	8 (26.7%)	3 (10%)	0.378	0.045	ww vs Δ 32/ Δ 32	0.011	2.3
PI >2	73 (77.7%)	20 (21.3%)	1 (1.1%)	0.960	(5.722)		(6.410)	(1.09-4.84)
Fibrosis <2	193 (81.1%)	39 (16.4%)	6 (2.5%)	0.086	0.017	ww vs w/32 + Δ 32/ Δ 32	0.015	1.97
Fibrosis >3	61 (68.5%)	27 (30.3%)	1 (1.1%)	0.571	(7.583)		(8.365)	(1.13-3.47)
-2132	TT	TC	CC					
Cleared virus	23 (92%)	2 (8%)	0 (0%)	0.979	0.164	TT vs TC + CC	0.048	4.01
Persistent carriage	155 (74.2%)	47 (22.5%)	7 (3.3%)	0.368	(3.241)		(3.903)	(0.91-17.56)
SR + RL	37 (57%)	26 (40%)	2 (3%)	0.594	0.026	TT vs TC + CC	0.023	3.15
NR	25 (81%)	4 (13%)	2 (6%)	0.06	(7.334)		(7.539)	(1.14-8.72)
RANTES -403	GG	GA	AA					
PI <1	28 (68.3%)	10 (24.4%)	3 (7.3%)	0.359	0.031	GG vs AA	0.004	1.52
PI >2	82 (72.6%)	31 (27.4%)	0 (0%)	0.240	(7.122)		(8.433)	(0.78-2.96)
MCP-2 Q46K	QQ	QK	KK					
Fibrosis <2	107 (74.8%)	32 (22.4%)	4 (2.8%)	0.705	0.027	QQ vs QK + KK	0.018	2.29
Fibrosis >3	26 (56.5%)	16 (34.8%)	4 (8.7%)	0.802	(6.563)		(5.592)	(1.14-4.58)

Abbreviations: PI, portal inflammation; SR, sustained response to treatment; RL, relapse after initial response; NR, nonresponse to treatment.

incompetent receptor would result in the presence of less inflammation in this region. The finding that there is an association between CCR5- Δ 32 and lower levels of portal inflammation is in agreement with this hypothesis. The same finding was not found when CCR5- Δ 32 was assessed in the context of interface hepatitis, or with overall necroinflammatory score. How this observation can be put in context with the other finding that carriage of CCR5- Δ 32 is associated with more severe fibrosis is a matter of conjecture. It is likely that the recruitment of T cells to the liver is not dependent on the locally expressed chemokine receptors but that they are responsible for differential recruitment once the cells enter the liver. It is possible, therefore, that, if fewer cells are recruited to the portal regions, which are associated with a more benign fibrosis outcome, then more cells will be available for recruitment to areas of interface or lobular hepatitis, which are associated with a more severe outcome in liver fibrosis. However, in this study, an association between CCR5- Δ 32 and increased interface hepatitis was not found, although data were only available on a relatively small number of patients. Other groups have suggested that the presence of CCR5- Δ 32 may be associated with a less favorable outcome in HCV infection. A study in Ger-

many compared the frequency of this mutation among patients with HCV infection, HIV infection, and those with HIV/HCV coinfection. They found an increased frequency of Δ 32/ Δ 32 genotypes among patients with HCV infection and also increased viral loads, suggesting unfavorable effects of this mutation on the course of HCV infection.³⁸ Data on viral load were not available to make this comparison in our study. However, the frequency of Δ 32/ Δ 32 genotypes in those chronically infected with HCV was 1.6% close to the expected frequency in Caucasian subjects and much less than the 7.8% reported by Woitas et al.³⁸ This has also been shown in another recent study,³⁹ and the high prevalence of CCR5- Δ 32 homozygosity in the earlier study may reflect resistance to HIV in hemophiliacs rather than a susceptibility to HCV infection.

Of the 7 promoter polymorphisms studied in CCR5, only -2132 was found to have significant associations with HCV phenotypes, although it does not appear to have an effect on CCR5 expression. In this study, the C allele was weakly associated with the persistent carriage of HCV, although the numbers in the group of individuals who cleared the virus spontaneously are very small, so this could be a chance finding. There also appeared to be an

association between this allele and interferon response. In this instance, individuals who carried the C allele were more likely to have an initial response to interferon (*i.e.*, SR + RL) than individuals who were homozygous for the T allele.

The RANTES promoter polymorphism at position -28 showed no significant association with any of the phenotypes studied. However, this is a rare polymorphism in Caucasian subjects with a mutant allele frequency of only 4%, and a study of this size may miss an association. This is also the case for the CCR3 polymorphisms, CCR2 V64I, and the closely linked CCR5 promoter polymorphisms at position -1835.

The polymorphism at position -403 appears to be functional, increasing RANTES expression, and has been associated with increased susceptibility to asthma, atopy,²⁵ and HIV.²⁶ RANTES may have a role in the selective recruitment of T cells to portal and, in particular, periportal regions.⁴⁰ It might be expected, therefore, that the mutant allele would be associated with increased portal inflammation, interface hepatitis, and severity of fibrosis. A significant association was found with portal inflammation, suggesting that homozygotes for the less frequent allele (A) at position -403 had less severe portal inflammation than homozygotes for the wild-type allele (G). This is counterintuitive because the A allele has been associated with increased transcription of RANTES in some studies²⁵ and might, therefore, have been expected to lead to more severe portal inflammation. RANTES is not, however, a dedicated CCR5 ligand because it also binds CCR1 and CCR3; it is also expressed at sites of interface hepatitis.⁴⁰ It is possible, therefore, that, if RANTES expression is increased as a result of this polymorphism, T cells are not recruited specifically to the portal regions. No associations were found with the degree of interface hepatitis in this study, although only small numbers could be included in this subset analysis.

Although there is no published evidence of a role for MCP2 in the immunopathology of HCV, it is a reasonable candidate gene because it could potentially be involved in susceptibility to persistent carriage, inflammatory response, or fibrosis. The results from this study showed no significant association with susceptibility to persistent HCV carriage. They did, however, show a significant association between carriage of the K allele and severe fibrosis ($P = .018$, OR: 2.29, 95% CI: 1.14-4.58) but, interestingly, not with inflammation. In HCV, fibrosis is largely driven by the inflammatory response, and it would be expected that a chemokine involved in T-cell migration would lead to fibrosis through increased inflammation. Functional studies revealed no difference in chemotactic and calcium-mobilizing abilities of the 2 iso-

forms of MCP-2.³⁵ Critically, these functional assays did not assess the abilities of the 2 isoforms to block HIV-1 binding to CCR5, but it is unlikely that the polymorphism is directly responsible for associations seen in either HIV or HCV and may be acting as a marker for a functional polymorphism in MCP2 or a nearby gene. Given that MCP2 is on chromosome 17 within the β -chemokine gene cluster, this is a strong possibility, and it is, therefore, likely that the associations with HIV and HCV do not necessarily imply that the same gene is involved. Therefore, to elucidate the role of MCP2 in HCV, particularly that related to fibrosis, further functional studies will need to be performed.

A recent publication has looked for associations of these HCV phenotypes with polymorphisms in CCR5, CCR2, and RANTES.³⁹ An association was identified for RANTES -403 homozygotes and inflammation but with the overall necroinflammatory score rather than the portal inflammation subgroup. An association between CCR5- Δ 32 and fibrosis was not observed and possible association with portal inflammation not assessed. A marginal association between CCR5 promoter polymorphism at position -2459 (position 59029 from U95626) was noted, which we cannot confirm. As in this study, no associations were found with CCR2 -64I or RANTES -28.

In this study, 20 polymorphisms in 7 genes have been studied, raising the issue of multiple comparisons. A Bonferroni correction is too conservative in the domain of human complex trait genetic association, in which many genes are expected to be associated with the phenotype to some extent. Taking the limitations of our data as outlined above into account, we therefore believe that we have carried out the most appropriate evaluation of our results but acknowledge that our subgroup analysis is based on smaller sample numbers. Thus, the associations in this study should be reassessed in other studies and populations, but all of the positive results are with mutations that are known to be (CCR5- Δ 32, RANTES -403) or may well be (MCP-2) functional.

In conclusion, this study has attempted to elucidate the role of CC chemokines and their receptors in the immunopathologic outcome of HCV. This is a complex area because of the large number of chemokines in this group and the likelihood that there is an overlap in their roles and, therefore, a degree of redundancy. This may influence the observed effect of polymorphisms on individual chemokines or receptors. Importantly, the results add further weight to the role of CCR5 and its ligands in the recruitment of T cells to the portal regions of the liver and suggest that CCR5- Δ 32, RANTES, and MCP-2 may

have an effect on the outcome of HCV-related liver disease.

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References

- World Health Organisation. Hepatitis C: global prevalence. *Weekly Epidemiol Rec* 1997;72:341-343.
- Poynard T, Marcellin P, Lee SS, Niederau C, Minuk GS, Ideo G, Bain V, et al. Randomised trial of interferon α 2b plus ribavirin for 48 weeks or for 24 weeks versus interferon α 2b plus placebo for 48 weeks for the treatment of chronic infection with hepatitis C virus. International Hepatitis Interventional Therapy Group (IHIT). *Lancet* 1998;352:1426-1432.
- McHutchison JG, Gordon SC, Schiff ER, Shiffman ML, Lee WM, Rustgi VK, Goodman ZD, et al. Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. *N Engl J Med* 1998;339:1485-1492.
- Murphy PM. The molecular biology of leukocyte chemoattractant receptors [Review]. *Annu Rev Immunol* 1994;12:593-633.
- Choe H, Farzan M, Sun Y, Sullivan N, Rollins B, Ponath PD, Wu L, et al. The β -chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* 1996;85:1135-1148.
- Dragic T, Litwin V, Allaway GP, Martin SR, Huang Y, Nagashima KA, Cayanan C, et al. HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. *Nature* 1996;381:667-673.
- Liu R, Paxton WA, Choe S, Ceradini D, Martin SR, Horuc R, Mac Donald ME, et al. Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell* 1996;86:367-377.
- Zimmerman PA, Buckler-White A, Alkhatib G, Spalding T, Kubofcik J, Combadiere C, Weissman D, et al. Inherited resistance to HIV-1 conferred by an inactivating mutation in CC chemokine receptor 5: studies in populations with contrasting clinical phenotypes, defined racial background, and quantified risk. *Mol Med* 1997;3:23-36.
- Dean M, Carrington M, Winkler C, Huttley GA, Smith MW, Allikmets R, Goedert JJ, et al. Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study. *Science* 1996;273:1856-1862.
- Michael NL, Chang G, Louie LG, Mascola JR, Dondero D, Birx DL, Sheppard HW, et al. The role of viral phenotype and CCR-5 gene defects in HIV-1 transmission and disease progression. *Nat Med* 1997;3:338-340.
- Tsai SL, Liaw YF, Chen MH, Huang CY, Kuo GC. Detection of type 2-like T-helper cells in hepatitis C virus infection: implications for hepatitis C virus chronicity. *HEPATOLOGY* 1997;25:449-458.
- Napoli J, Bishop GA, McGuinness PH, Painter DM, McCaughan GW. Progressive liver injury in chronic hepatitis C infection correlates with increased intrahepatic expression of Th1-associated cytokines. *HEPATOLOGY* 1996;24:759-765.
- Bonocchi R, Bianchi G, Bordignon PP, D'Ambrosio D, Lang R, Borsatti A, Sozzani S, et al. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *J Exp Med* 1998;187:129-134.
- Shields PL, Morland CM, Salmon M, Qin S, Hubscher SG, Adams DH. Chemokine and chemokine receptor interactions provide a mechanism for selective T cell recruitment to specific liver compartments within hepatitis C-infected liver. *J Immunol* 1999;163:6236-6243.
- Ishak K, Baptista A, Bianchi L, Callea F, De Groote J, Gudat F, Denk H, et al. Histological grading and staging of chronic hepatitis [Review]. *J Hepatol* 1995;22:696-699.
- Strieter RM, Standiford TJ, Huffnagle GB, Colletti LM, Lukacs NW, Kunkel SL. "The good, the bad, and the ugly." The role of chemokines in models of human disease [Review]. *J Immunol* 1996;156:3583-3586.
- Kurihara T, Warr G, Loy J, Bravo R. Defects in macrophage recruitment and host defense in mice lacking the CCR2 chemokine receptor. *J Exp Med* 1997;186:1757-1762.
- Kostrikis LG, Huang Y, Moore JP, Wolinsky SM, Zhang L, Guo Y, Deutsh L, et al. A chemokine receptor CCR2 allele delays HIV-1 disease progression and is associated with a CCR5 promoter mutation. *Nat Med* 1998;4:350-353.
- Smith MW, Dean M, Carrington M, Winkler C, Huttley GA, Lomb DA, Goedert JJ, et al. Contrasting genetic influence of CCR2 and CCR5 variants on HIV-1 infection and disease progression. Hemophilia Growth and Development Study (HGDS), Multicenter AIDS Cohort Study (MACS), Multicenter Hemophilia Cohort Study (MHCS), San Francisco City Cohort (SFCC), ALIVE Study. *Science* 1997;277:959-965.
- Combadiere C, Ahuja SK, Murphy PM. Cloning and functional expression of a human eosinophil CC chemokine receptor. *J Biol Chem* 1995;270:16491-16494.
- Daugherty BL, Siciliano SJ, DeMartino JA, Malkowitz L, Sirofina A, Springer MS. Cloning, expression, and characterization of the human eosinophil eotaxin receptor. *J Exp Med* 1996;183:2349-2354.
- Zimmermann N, Bernstein JA, Rothenberg ME. Polymorphisms in the human CC chemokine receptor-3 gene. *Biochim Biophys Acta* 1998;1442:170-176.
- Kato H, Tsuchiya N, Izumi S, Miyamasu M, Nakajima T, Kawasaki H, Hirai K, et al. New variations of human CC-chemokine receptors CCR3 and CCR4. *Genes Immun* 1999;1:97-104.
- Liu H, Chao D, Nakayama EE, Taguchi H, Goto M, Xin X, Takamatsu JK, et al. Polymorphism in RANTES chemokine promoter affects HIV-1 disease progression. *Proc Natl Acad Sci U S A* 1999;96:4581-4585.
- Fryer AA, Spiteri MA, Bianco A, Hepple M, Jones PW, Strange RC, Makki R, et al. The -403 G \rightarrow A promoter polymorphism in the RANTES gene is associated with atopy and asthma. *Genes Immun* 2000;1:509-514.
- An P, Nelson GW, Wang L, Donfield S, Goedert JJ, Phair J, Vlahov D, et al. Modulating influence on HIV/AIDS by interacting RANTES gene variants. *Proc Natl Acad Sci U S A* 2002;99:10002-10007.
- Bluman EM, Bartynski KJ, Avalos BR, Caligiuri MA. Human natural killer cells produce abundant macrophage inflammatory protein-1 α in response to monocyte-derived cytokines. *J Clin Invest* 1996;97:2722-2727.
- Al-Sharif FM, Makki RF, Ollier WE, Hajeer AH. A new microsatellite marker within the promoter region of the MIP-1A gene. *Immunogenetics* 1999;49:740-741.
- Loetscher P, Seitz M, Clark-Lewis I, Baggiolini M, Moser B. Monocyte chemotactic proteins MCP-1, MCP-2, and MCP-3 are major attractants for human CD4+ and CD8+ T lymphocytes. *FASEB J* 1994;8:1055-1060.
- Taub DD, Proost P, Murphy WJ, Anver M, Longo DL, Van Damme J, Oppenheim JJ. Monocyte chemotactic protein-1 (MCP-1), -2, and -3 are chemotactic for human T lymphocytes. *J Clin Invest* 1995;95:1370-1376.
- Czaja MJ, Geerts A, Xu J, Schmiedeberg P, Ju Y. Monocyte chemoattractant protein 1 (MCP-1) expression occurs in toxic rat liver injury and human liver disease. *J Leuk Biol* 1994;55:120-126.
- Marra F, Romanelli RG, Giannini C, Failli P, Pastacaldi S, Arrighi MC, Pinzani M, et al. Monocyte chemotactic protein-1 as a chemoattractant for human hepatic stellate cells. *HEPATOLOGY* 1999;29:140-148.
- Rovin BH, Lu L, Saxena R. A novel polymorphism in the MCP-1 gene regulatory region that influences MCP-1 expression. *Biochem Biophys Res Commun* 1999;259:344-348.
- McManus C, Berman JW, Brett FM, Staunton H, Farrell M, Brosnan CF. MCP-1, MCP-2 and MCP-3 expression in multiple sclerosis lesions: an immunohistochemical and in situ hybridization study. *J Neuroimmunol* 1998;86:20-29.
- Van Coillie E, Proost P, Van AI, Struyf S, Polfliet M, DeMeester I, Harvey DJ, et al. Functional comparison of two human monocyte chemotactic protein-2 isoforms, role of the amino-terminal pyroglutamic acid and processing by CD26/dipeptidyl peptidase IV. *Biochemistry* 1998;37:12672-12680.

36. Thursz M, Yallop R, Goldin R, Trepo C, Thomas HC. Influence of MHC class II genotype on outcome of infection with hepatitis C virus. The HENCORE group. Hepatitis C European Network for Cooperative Research. *Lancet* 1999;354:2119-2124.
37. Hennig BJW, Hellier S, Frodsham AJ, Zhang L, Klenerman P, Knapp S, Wright M, et al. Association of low-density lipoprotein receptor polymorphisms and outcome of hepatitis C infection. *Genes Immun* 2002;3:359-367.
38. Woitas RP, Ahlensteil G, Iwan A, Rockstroh JK, Brackmann HH, Kupfer B, Matz B, et al. Frequency of the HIV-Protective CC Chemokine Receptor- $\Delta 32/\Delta 32$ Genotype is Increased in Hepatitis C. *Gastroenterology* 2002;122:1721-1728.
39. Promrat K, McDermott DH, Gonzalez, CM, Kleiner DE, Koziol DE, Lessie M, Merrell M, et al. Associations of chemokine system polymorphisms with clinical outcomes and treatment responses of chronic hepatitis C. *Gastroenterology* 2003;124:352-360.
40. Samson M, Labbe O, Mollereau C, Vassart G, Parmentier M. Molecular cloning and functional expression of a new human CC-chemokine receptor gene. *Biochemistry* 1996;35:3362-3367.