

VIRAL HEPATITIS

The CCR5-Δ32 mutation: impact on disease outcome in individuals with hepatitis C infection from a single source

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Background and aims: Chemokines are small polypeptides, a major function of which is lymphocyte recruitment and trafficking. The aim of this study was to assess the involvement of inherited variations in CCR2, CCR5, and the ligand RANTES in determining disease outcome in hepatitis C virus (HCV) infected individuals.

Methods: A total of 283 women, all exposed to HCV genotype 1b from a single donor, and including those who had spontaneously cleared the virus and those chronically infected, were genotyped for CCR2, CCR5, and RANTES polymorphisms. The frequencies of these polymorphisms were then compared with disease activity and severity.

Results: CCR5, CCR2, and RANTES genotypes were compared with HCV polymerase chain reaction (PCR) status, alanine aminotransferase levels, and liver histology. There was no significant relationship between CCR2 or RANTES polymorphisms and disease outcome or severity. However, CCR5Δ32 heterozygotes were more likely to have spontaneous clearance of the virus than those without the mutation (42% PCR negative v 28.3% negative; $p=0.044$, odds ratio 1.83 (95% confidence interval 1.1–3.6)). Among the subgroup of DRB1*03011 negative individuals, previously found to be associated with more severe inflammation, the difference in histological inflammatory score (CCR5WT/WT=4.9 v CCR5Δ32/WT=3.53; $p=0.043$) was significant.

Conclusion: Heterozygosity for CCR5Δ32 was shown to be significantly associated with spontaneous hepatitis C viral clearance and with significantly lower hepatic inflammatory scores in subgroups within this cohort. Both controls and the HCV population had similar heterozygosity frequencies.

Chronic hepatitis C virus (HCV) infection has a prevalence of approximately 2% worldwide.¹ Up to 70% of those exposed to HCV remain chronically infected. Many aspects of response to HCV are poorly understood, including the reasons for the wide variation in disease severity and spontaneous clearance versus chronic infection. Viral genotype, age at exposure, and alcohol consumption are known determinants of disease severity but the role of genetic predisposition to chronic infection remains unclear. The population in this study, comprising 283 women infected by contaminated anti-D immunoglobulin in 1977, represent a unique opportunity to assess a large HCV population with minimal confounding issues.² The anti-D immunoglobulin was contaminated from a single source and therefore all women were infected with the same genotype (that is, genotype 1b), minimising the varying effect that genotype could have on the study.

Chemokines are small polypeptides with a significant role in leucocyte recruitment and trafficking. Leucocyte recruitment during inflammation requires intercellular communication between infiltrating leucocytes, endothelial cells, and parenchymal cells, mediated by early response chemokines. Migration of T cells is modulated in vitro and in vivo by conditioning with chemokines.³ CCR5 is a receptor for the proinflammatory chemokines macrophage inflammatory protein 1α (MIP-1α), MIP-1β, and RANTES, which have key roles in host responses to viruses in both human and murine disease.⁴ A 32 base pair (bp) deletion in CCR5 results in a protein that is not detectable at the cell surface. Homozygosity for this deletion is found in 1% of

Caucasians and has been shown to be protective against human immunodeficiency virus (HIV) infection, while the heterozygote state, which is found in 10% of Caucasians, leads to slower disease progression. Its role in other viral infections remains to be determined.

CCR5 and other chemokines play an important role in T cell differentiation. CD4+T cells can differentiate into Th1 or Th2 cells depending on their exposure to chemokines. CCR5 is expressed on Th cells and also on memory and activated T cells. Migration of antigen primed T cells is facilitated by CCR5.⁵ When human T cell clones were analysed, CCR5 appeared to be expressed at higher levels on Th1 cells, whereas many Th2 clones had no expression of CCR5. Sallusto *et al* demonstrated that CCR5 expression depends on the activation state of T cells and that its expression is upregulated by IL-2.⁶ It has been proposed that in HCV, there is predominantly a Th1 response in the liver.^{7,8} Indeed, progressive liver damage in HCV is associated with upregulation of Th1 cytokines (interferon (IFN) and interleukin (IL)-2), as shown by Napoli *et al*, where increased expression of IFN-γ and IL-2 correlated with both fibrotic and portal inflammatory histological scores.⁷

CCR5 along with CCR2 are two of a cluster of six chemokine receptor genes mapped to 3p21. CCR2 codes for a minor HIV receptor, for which a G to A coding sequence

Abbreviations: HCV, hepatitis C virus; MIP-1α, macrophage inflammatory protein 1α; bp, base pair; IFN, interferon; IL, interleukin; RT-PCR, reverse transcription-polymerase chain reaction; ALT, alanine aminotransferase; HAI, histological activity index; HIV, human immunodeficiency virus

polymorphism resulting in a valine to isoleucine substitution, designated CCR264I, has been described. It appears that CCR2WT is in complete linkage disequilibrium with CCR5 Δ 32, which is 10 kb away. Thus in a study of 3000 individuals, Smith and colleagues⁹ demonstrated that the Δ 32 mutation is never seen on the same haplotype as the CCR264I mutation. The distribution of CCR2 and CCR5 in cells and tissues is very similar. CCR2 signalling also promotes Th1 development in infection models and studies using a CCR2 knockout mouse have shown that these mice have a 46% reduction in lymphocyte recruitment to sites of infection and inflammation and an 80% reduction in CD4⁺T cells locally.¹⁰ RANTES, the CCR5 ligand, is also critical for lymphocyte recruitment, as it attracts memory and activated T cells. An A to G mutation of RANTES has been described at position -403, resulting in an additional GATA transcription factor binding site, with the mutant promoter having up to an eightfold higher constitutive transcriptional activity than the wild-type.¹¹

In normal liver, RANTES expression is restricted to a few scattered hepatocytes. However, in HCV infected livers its expression was significantly elevated, especially in periportal and lobular areas that had the most lymphocytic infiltration.¹² In view of this, we postulated that genetic variation in either CCR5 receptors or in the chemokines binding to such receptors might have an impact on outcome of hepatitis C infection. The impact of such variation might be difficult to detect in populations in which there was heterogeneity in terms of ethnicity, viral genotype, and source and dose of infection. Hence we have undertaken a study of the genetic impact of both the CCR5 Δ 32 mutation and the RANTES position -403 mutation on the outcome of hepatitis C infection in a genetically homogenous population infected through a single source.

PATIENTS AND METHODS

Study population

The study population of 283 women was recruited from the outpatient hepatology units in St James, St Vincent's, and the Mater Misericordiae Hospitals in Dublin. All women attending these units who had been exposed to HCV via contaminated anti-D immunoglobulin in 1977 were invited to participate. The group included those who were chronically infected, persistently HCV RNA positive as determined by reverse transcription-polymerase chain reaction (RT-PCR), and those who had cleared the virus (that is, remained HCV antibody positive but RNA negative). None of the participants had any other risk factors for acquisition of viral hepatitis (for example, blood transfusion or past history of intravenous drug abuse). All had an alcohol consumption of less than 14 U/week and other forms of chronic liver disease were excluded in all cases. Of the 283 initially exposed to contaminated anti-D immunoglobulin, 196 remained chronically infected (that is, RT-PCR (RNA) positive) and 87 were anti-HCV antibody positive but persistently RNA negative (that is, they had cleared the HCV infection). RNA negative individuals had an average of six RT-PCR reactions carried out at different time points to confirm spontaneous viral clearance. The majority of these subjects had already been genotyped for both class I and class II HLA polymorphisms, and a significant association was found between viral chronicity and the presence of DRB1*03011 and DQB1*0201.¹³ All subjects gave informed consent prior to participating in the study, which received ethics approval from the research and ethics committees at all three institutions.

Controls

To estimate the frequency of the CCR5 Δ 32 allele in the Irish population, a control group of 120 unselected unrelated

healthy volunteers were genotyped. These were health care workers and all of Irish descent.

Diagnosis of HCV infection HCV antibodies

A third generation enzyme immunoassay (ELISA; Abbott Diagnostics, Germany) was used to test all subjects for HCV specific antibodies and a third generation recombinant immunoblot assay (RIBA 3; Chiron Corp., Emeryville, California, USA) was then used as a confirmatory test.

RT-PCR testing

An RT-PCR assay (Amplicor; Roche Diagnostic Systems, New Jersey, USA) was used to test for HCV RNA in all subjects.

DNA extraction

A salting out technique was used to extract DNA from whole blood.¹⁴ DNA was also extracted using the QIAmp DNA midiprep kit, (Qiagen Ltd., Crawley, UK). During this process, all RNA was removed by incubating the digested preparation with 1.5 μ l ribonuclease A (Boehringer Mannheim UK Ltd, East Sussex, UK) per 400 μ l of nuclear lysate, according to the manufacturer's instructions.

CCR5- Δ 32 genotyping

A PCR reaction consisting of PCR 1 $\times$ buffer (as supplied by the manufacturer), 25 μ M MgCl₂, 200 μ M deoxynucleotide triphosphates, 0.5 μ M of both forward and reverse primers, 15 ng of extracted DNA, 1 unit of Qiagen DNA polymerase Taq, and 14.3 μ l of H₂O was used to genotype both the subject and control groups. The primers flanking the CCR5-32 mutation (sense 5'-CAA AAA GAA GGT CTT CAT TAC ACC-3'; antisense 5'-CCT GTG CCT CTT CTC ATT TCG-3') were used to amplify 189 bp (wild-type) and 157 bp (32 bp deletion) fragments of the CCR5 gene, respectively. Following amplification the fragments were visualised on a 3% agarose gel.

CCR264I genotyping

Genotyping was performed by restriction digest of amplified fragments following electrophoresis as follows: a PCR reaction with the forward primer 5'-TTG GTT TTG TGG GCA ACA TGA TGG-3' and the reverse primer 5'-CAT TGC ATT CCC AAA GAC CCA CTC-3' was performed to give a 173 bp amplicon. This was then digested with the enzyme Bsa BI (New England Biolabs, Beverly, Massachusetts, USA) to yield restriction fragments of 149 and 24 bp. When the wild-type sequence is present, the fragment remains uncut, thus giving a band of 173 bp.

RANTES genotyping

Applied Biosystems Ltd (Foster City, California, USA) designed a 5' exonuclease assay using the Assay by Design service for TaqMan analysis to genotype the RANTES 403 polymorphism. Forward primer 5'-GAG GAC CCT CCT CAA TAA AAC ACT TTA TAA AT-3', reverse primer 5'-ACT GAG TCT TCA AAG TTC CTG CTT-3' and the probes VIC CAT TAC AGA TCT TAC CTC CTT T and FAM CAT TAC AGA TCT TAT CTC CTT T were used. As a quality control measure, 10% of the samples were repeated in all of the above genotyping and all were in concordance. All of the above were read by one reader who was blinded to the identification of the sample until all genotyping was completed and recorded.

Histological evaluation

All 196 RT-PCR positive women had undergone liver biopsy as part of their initial clinical evaluation (that is, prior to the commencement of any treatment) and between 17 and 20 years post infection. Liver biopsies were all scored by a

Table 1 Summary of polymorphism distribution and hepatitis C virus (HCV) polymerase chain reaction (PCR) status

Genotyping	HCV PCR +ve (%)	HCV PCR -ve (%)	p Value	OR (95% CI)
CCR5WT/WT	167 (85.2%)	66 (75%)	0.044	1.9 (1.1–3.6)
CCR5Δ32/WT	29 (14.8%)	21 (23.9%)		
CCR5Δ32/Δ32	0	1 (1.1%)		
CCR2WT/WT	125 (90.6%)	73 (93.6%)	0.327	0.66 (0.23–1.6)
CCR264I/WT	11 (7.8%)	5 (6.4%)		
CCR264I/64I	2 (1.6%)			
RANTESWT/WT	90 (63.8%)	54 (63.5%)	0.441	1.01(0.58–1.7)
RANTES403/WT	45 (32%)	29 (34.1%)		
RANTES403/403	6 (4.2%)	2 (2.4%)		

Number of individuals with each genotype for the three different polymorphisms tested, divided according to their HCV PCR status. Values in parentheses are percentage of those HCV PCR positive or negative, according to their genotype.

p values were calculated using the χ^2 test, and odds ratio (OR) by Epi-Info.

95% CI, 95% confidence interval.

single histopathologist in each centre, blinded to the CCR5 genotype of the individual. Biopsies were scored according to the modified histological activity index (HAI 0–18 for inflammation, 0–6 for fibrosis). HCV RNA negative subjects did not have a liver biopsy performed.

Statistical analysis

The Mann-Whitney U test was used to compare histological, inflammatory, and fibrotic scores, and alanine aminotransferase (ALT) levels, between the different subgroups classified according to patient genotypes. The association between viral clearance and polymorphism was assessed by the χ^2 and Fisher's exact test. A p value of 0.05 was deemed as significant for all of the above tests. The odds ratio (OR) for each of the different polymorphisms and disease association was calculated by Epi-Info.¹⁵ The results were also analysed taking the previous genotyping for HLA DR, DQ loci into account. All data were entered into the statistical package SPSS (SPSS Inc, Chicago, Illinois, USA) and Epi-Info.

RESULTS

The results of the genotyping for the three different polymorphisms were compared with HCV PCR status (table 1), histological scores, and ALT levels.

Genotypes associated with viral clearance

Heterozygote frequency for the CCR5Δ32 mutation in the general population was similar to that of the HCV study group (17.9% and 17.6%, allele frequency 0.193 and 0.186, respectively).

There was only one CCR5Δ32/Δ32 individual in each group. The presence of the CCR5Δ32/WT (wild-type) genotype was significantly associated with spontaneous viral clearance: 42.0% of those who were CCR5Δ32/WT were HCV PCR

negative versus only 28.3% of CCR5WT/WT ($p = 0.044$, one sided Fisher's exact test, OR 1.9 (95% confidence interval (CI) 1.1–3.6)). Only one patient was homozygotic CCR5Δ32/Δ32 and she was HCV PCR negative. Allele frequency was in Hardy-Weinberg equilibrium for both patient and control groups. When the association of CCR5 genotype and viral clearance was looked at in the DRB1*03011 and DQB1*0201 negative groups, none was found ($p = 0.563$ and 0.68 , respectively). Analysis of the CCR264I ($p = 0.327$, OR 0.66 (95% CI 0.23–1.6)) and RANTES ($p = 0.441$, OR 1.01 (95% CI 0.58–1.7)) genotypes failed to reveal any relationship with HCV clearance.

Relationship between genotypes and histological severity

There was no significant difference in hepatic inflammatory scores between heterozygotes for the Δ32 mutation and those without a copy of this mutation (HAI 3.82 v 4.53; $p = 0.098$) in this cohort. Furthermore, in the DRB1*03011 positive group, previously found to be associated with less severe inflammation, CCR5Δ32 had no further additive impact on histological severity, with a mean HAI of 4.16 for non-CCR5WT/WT and 3.80 for CCR5Δ32 heterozygotes ($p = 0.78$). In contrast, within the DRB1*03011 negative group, associated with more severe inflammation, CCR5Δ32 heterozygotes had significantly lower inflammatory scores than the CCR5WT/WT group (mean inflammatory score 3.53 v 4.91; $p = 0.043$) (table 2).

Relationship between genotypes and ALT levels

ALT levels were slightly higher in the CCR5 and CCR2 wild-type group compared with the heterozygotes, while the opposite was observed for the RANTES group. However, none of these differences reached statistical significance (table 3).

Table 2 Summary of CCR5 results

	CCR5WT/WT (%)	CCR5Δ32/WT (%)	CCR5Δ32/Δ32	p Value
HCV PCR +ve	167 (71.7%)	29 (58%)	0	0.044
HCV PCR -ve	66 (28.3%)	21 (42%)	1	
Inflammation	4.53	3.82		0.098
Fibrosis	1.20	1.05		0.503
DRB1*03011 -ve inflam (n=81)	4.91	3.53		0.043
DRB1*03011 +ve inflam (n=55)	4.16	3.80		0.78

Impact of the CCR5Δ32 mutation on hepatitis C virus polymerase chain reaction (PCR) status, histological activity index (HAI) scores for the entire group, and also for the subgroups of DRB1*03011 positive and negative individuals.

The p value for the affect of PCR status was given by χ^2 , and for HAI scores as per the Mann-Whitney U test.

Table 3 Summary of mean alanine aminotransferase (ALT) levels and the polymorphisms examined

1	ALT levels	p Value
CCR5WT/WT	52.76	0.419
CCR5Δ32/WT	44.69	
CCR2WT/WT	51.03	
CCR264I/WT	40.67	
RANTESWT/WT	49.07	0.535
RANTES403/WT	54.46	

Mean ALT levels for all individuals with the above genotypes, and the associated p values. p values were calculated using the Mann-Whitney U test.

DISCUSSION

This study showed significantly higher spontaneous HCV viral clearance in the CCR5Δ32/WT over the CCR5WT/WT group ($p = 0.044$). This association was not found in Hellier's¹⁶ or Promrat's¹⁷ studies, both of which had several confounding issues in relation to HCV genotype, sex, and ethnicity. Specifically, Hellier's study comprised individuals from multiple European populations and contained a lower percentage of viral negative patients. HCV genotype was not specified in this study. In addition, the infection came from multiple sources, suggesting a high degree of HCV genetic heterogeneity. Hence a number of confounding variables are inherent in these studies that may have hindered the ability to detect changes in viral clearance. Our study population had a number of relatively unique features, principally that (i) all subjects were female and Caucasians of Irish descent and (ii) all were infected by a single inoculum of HCV genotype 1b through anti-D immunoglobulin in 1977.² They had no other risk factors for liver disease and no other significant comorbid illnesses.

The CCR5Δ32 mutation arises from a 32 bp deletion causing a frame shift mutation and premature termination of the protein. The resultant CCR5 mutant protein is likely to be functionally inert as it not only lacks the last three of seven putative transmembrane regions but also the domains involved in G protein coupling and signal transduction.¹⁸ Indeed, Liu *et al* showed that the resultant protein was not detectable on the surface of cells that would normally express it, and therefore cannot act as a receptor.¹⁹ However, the role of the CCR5 mutation in HCV is not the same as for HIV as the method by which HCV gains entry into the cell is unknown, but unlike HIV, it is generally not believed to be related to the CCR5 receptor.

The heterozygous genotype was present in 17.6% of the HCV population and in 17.9% of the control population, with the CCR5Δ32/Δ32 genotype found in 0.32% and 0.69%, respectively, one homozygote in each group. This is one of the highest carrier rates reported for any European population and is consistent with the results of Libert *et al* who investigated gene frequency in 18 European countries and found a North/South gradient, the highest frequencies being in Finland (16%) and the lowest in Sardinia (4%).²⁰ Controversially, in 2002, Woitas *et al* reported that CCR5Δ32 homozygosity occurred three times more frequently in anti-HCV antibody positive HIV negative individuals.²¹ The fact that this group remained HIV negative, despite multiple exposure, would suggest that they were a selected population, most probably on the basis of the CCR5Δ32 genotype. Hence the increased CCR5Δ32 homozygosity most likely reflected resistance to HIV, rather than increased risk of HCV infection.

In explaining how the CCR5Δ32 polymorphism could alter HCV clearance, we must consider that the effect of CCR5 heterozygosity in acute HCV may not be representative of what happens in chronic HCV infection. In acute HCV

infection, clearance is associated with a strong T cell response to a wide range of HCV specific antigens.²² Counterintuitively, lack of CCR5 may actually lead to increased T cell expansion. This was demonstrated using an acute lymphocytic choriomeningitis infection model in CCR5 knockout mice where clonal expansion of antigen specific T cells was increased, not decreased, among CD8+ and CD4+ T cells.²³ Likewise, lack of CCR5 has been associated with increased T cell production of IFN- γ , leading to the suggestion that CCR5 might be part of a negative regulatory feedback loop on acute T cell activation.²⁴ In CCR5 deficient mice infected with mouse hepatic virus there was reduced T cell infiltration at day 7, but by day 12 T cell infiltration was similar to wild-type and this study also suggested that IFN production may have been increased in the CCR5-/- group.²⁵ Infection with leishmania donovani showed a shift from an initial low to an exaggerated antigen specific IFN response at eight weeks post infection in CCR5-/- mice, suggesting that perhaps the impact of CCR5 alters during the course of an infection.²⁶ In contrast with the above, a study by Belnoue *et al* showed that CCR5-/- mice infected with cerebral malaria had significantly reduced T cell cerebral infiltration.²⁷ These contrasting results may reflect CCR5 interaction with parasitic rather than viral infection.

This study also showed a trend towards less severe hepatic inflammatory scores in CCR5WT/Δ32 versus CCR5WT/WT individuals. In a previous study, we identified HLA DRB1*03011 positivity as being associated with reduced hepatic inflammation in this cohort. We did not observe an additive effect of CCR5Δ32 in DRB1*03011 positive individuals, suggesting a dominant role for this HLA allele. However, we observed significantly lower hepatic inflammatory scores for the CCR5Δ32/WT groups who were DRB1*03011 negative ($p = 0.043$). In a recent publication by Hellier *et al*, a significant decrease in portal inflammation, but not in overall necroinflammatory score, was found among CCR5Δ32 heterozygotes.¹⁶ CCR5Δ32 is associated with reduced migration of circulating lymphocytes in response to ligands such as MIP-1 α ¹⁹ and we also observed this in vitro in CCR5Δ32 heterozygotes (data not shown).

In HCV there is predominantly a Th1 response in the liver. CCR5 is expressed on Th1 cells and facilitates the migration of T cells primed by antigen. Although the number of HCV specific cytotoxic lymphocytes in the liver is low during infection, there are many activated/memory T cells present, most of which express CCR5. It has been reported that in HCV patients, liver infiltrating lymphocytes showed increased expression of CCR5,²⁸ which correlated with histological severity.²⁹ Similarly, animal studies have shown a key role for CCR5 in hepatic lymphocyte migration.³⁰ Hence reduced expression of CCR5, associated with heterozygosity for CCR5Δ32, could be mechanistically associated with less hepatic inflammation, due to reduced migration of CCR5 expressing cells.

Both Hellier and Promrat found an association with the RANTES -403 promoter polymorphism and reduced hepatic inflammation in a subgroup of patients, which was not found in this study. It is possible that ethnic variation in the RANTES polymorphism and patient numbers may partly explain differences between these studies.

There is clearly much work yet to be done in this very exciting area, particularly detailed functional studies. While there is a detectable effect on HCV clearance seen in this study, further studies are required to determine whether such data are generalisable to the broader HCV infected population. Such studies will require large numbers of patients and will also require either genetic homogeneity regarding ethnic origin or stratification given the wide diversity in allele frequency for this polymorphism even in Caucasian European populations. The effect that this mutation may have on HCV

clearance and severity may be not only important in relation to those solely infected with HCV, but also of vital importance to the vast numbers who are coinfecting with HIV, particularly as anti-CCR5 directed medications are already being investigated for the treatment of HIV.^{31 32}

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