CCR5Δ32 mutation does not influence the susceptibility to HCV infection, severity of liver disease and response to therapy in patients with chronic hepatitis C

Ankur Goyal, PV Suneetha, GT Kumar, Deepak K Shukla, Naveen Arora, Shiv K Sarin

Ankur Goyal, Department of Gastroenterology, G.B. Pant Hospital, New Delhi, Institute of Genomic and Integrative Biology, (Formerly CBT), Mall Road, Delhi, Dr. Ambedkar Center for Biomedical Research, University of Delhi, India
PV Suneetha, GT Kumar, Shiv K Sarin, Department of Gastroenterology, G.B. Pant Hospital, New Delhi, India
Deepak K Shukla, Division of Non-communicable Disease, Indian Council of Medical Research, Ansari Nagar, New Delhi, India
Naveen Arora, Institute of Genomic and Integrative Biology, (Formerly CBT), Mall Road, Delhi, India
Supported by National Task Force Project from the Indian Council of Medical Research; supported by Council of Scientific and Industrial Research (CSIR), New Delhi to Ankur Goyal, a Senior Research Fellow
Correspondence to: Dr. SK Sarin, MD, DM, FNA, Director, Professor and Head, Department of Gastroenterology, G.B. Pant Hospital, University of Delhi, New Delhi-110002 and Adjunct Professor, Molecular Medicine, Jawaharlal Lal Nehru University, New Delhi, India. sksarin@nda.vsnl.net.in
Telephone: +91-11-23232013 **Fax:** +91-11-23219710
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Abstract

AIM: To study whether CCR5Δ32 mutation was associated with viral infection and severity of liver disease.

METHODS: Two hundred and fifty two histologically proven, chronic HCV patients (mean age: 41 ± 14 years; M/F: 164/88) were genotyped. PCR based genotyping of 32 bp deletion at the CCR5 locus was done. Four-hundred and eight matched healthy controls were studied to assess susceptibility to HCV infection. To assess correlation of immune gene polymorphism with severity of HCV related liver disease, patients with chronic HCV infection were divided into those with a fibrosis score of ≤ 2 (mild) or > 2 (severe) and histological activity index (HAI) of ≤ 5 or > 5. For correlation between CCR5Δ32 mutations and response to therapy, 129 patients who completed therapy were evaluated.

RESULTS: The majority (89.4%) of the patients were infected with genotype 3. The frequency of homozygous CCR5Δ32 mutants was comparable to HCV patients as compared to the healthy controls (0.7% *vs* 0%, *P* = 0.1). Further more, the frequency of CCR5Δ32 mutation was comparable in patients with mild or severe liver disease.

(P = NS). There was also no association observed with response to therapy and CCR5Δ32 mutation.

CONCLUSION: CCR5Δ32 mutation does not have a role in disease susceptibility, severity or response to therapy in patients with chronic hepatitis C infection.

INTRODUCTION

HCV is the only known positive stranded RNA virus that causes persistent long-life infection in humans. Hepatitis C virus is an important cause for chronic liver disease and hepatocellular carcinoma in a proportion of patients with persistent infection. The mechanisms of chronicity and progression of liver disease are probably multifactorial and involve a rather complex interplay between the virus and the host. There has been a surge of interest in studying various immunomodulatory genes to answer these questions.

Hepatitis C virus being a hepatotropic virus requires the recruitment of virus specific T cells to the liver for viral clearance. Chemokines and interaction with their receptors may regulate the selective recruitment of primed Th-1 cells to the site of inflammation. It has been proposed that the immune response in chronic hepatitis C is compartmentalized, with a predominantly Th-2 or Th-0 response in the periphery and a Th-1 response in the liver[1-5]. In contrast, patients who clear the virus have a predominant peripheral Th-1 response. Moreover, differences in chemokine receptor expression between Th-1 and Th-2 cells might explain their selective recruitment to tissue. In particular, lymphocytes infiltrating HCV infected liver express high levels of the chemokine receptor (CCR5) and CXC

chemokine receptor (CXCR3). The CC chemokine macrophage inflammatory protein (MIP)-1α (CCL3), MIP-1β (CCL4) and RANTES (CCL5) are important in immune surveillance. These chemokines bind to their corresponding receptors CCR1 and CCR5. Both these receptors are preferentially expressed on lymphocytes with a Th-1 cytokine secretion pattern. Chronic hepatitis C virus infection has been shown to lead to reduced surface expression of CCR1 and CCR5 on peripheral blood T lymphocytes[6] and more so, on CD8 lymphocytes. While the mechanism of altered chemokine receptor expression and chemokine responsiveness is still not clear, reduced expression would probably lead to a decrease in T lymphocyte migration in response to MIP-1α, MIP-1β and RANTES in chemotaxis assays.

The Δ32 mutation of the CCR5 gene was reported to be associated with inflammatory bowel disease by some but not all authors[7]. Furthermore, a negative correlation with susceptibility to rheumatoid arthritis has been described[8,9]. In contrast to these weak or contradicting correlations, the Δ32 mutation was found to be of paramount importance for protection against HIV infection. The β-chemokine receptor CCR5 appears to be the major co-receptor for entry of macrophage-tropic and non-syncitium-inducing (NSI) variants of HIV[10]. A 32 bp deletion mutation (Δ32) of the CCR5 gene encodes a non-functional protein. Homozygosity for the Δ32 deletion makes the CD4 T cells resistant to infection from NSI virus strains or delay HIV-1 disease progression in CCR5 Δ32 heterozygotes.

There is a controversy related to the role of CCR5Δ32 mutation in susceptibility to HCV infection and disease progression. Woitas et al have suggested that a genetically determined loss of CCR5 gene expression is linked to chronic HCV infection and high viral load[11]. On the other hand, a recent study by Promrat et al has shown that reduced expression of CCR5 and RANTES may lead to reduced hepatic inflammation and 59029 -G/A to improved response to interferon therapy in chronic hepatitis C[12]. Hellier et al also suggested a possible role of CCR5Δ32 polymorphism in the outcome of HCV infection[13].

The present case-control association study was undertaken to identify whether CCR5Δ32 mutation is associated with HCV disease progression and severity. We also compared the allele frequencies defined by CCR5Δ32 mutation in HCV patients with a control group of healthy individuals in order to identify a possible association with susceptibility to disease.

MATERIALS AND METHODS

Patients

Two hundred and fifty two patients with histologically proven chronic HCV infection were studied. The *inclusion criteria* were evidence of chronic hepatitis on liver biopsy and HCV RNA positivity on two occasions at baseline. The HCV RNA was detected as described elsewhere[14]. HCV genotyping was performed with the reverse hybridization line probe assay (LIPA; Innogenetics, Ghent, Belgium). Patients were *excluded* if they had hepatitis B virus (HBV) or HIV infection, history of heavy alcohol consumption

(> 80 g/d for > 5 years), positivity for antinuclear or anti smooth muscle antibody (in 1:80 dilution), autoimmune liver disease, thyroid disease, diabetes mellitus or malaria. The Institutional Ethical Committee approved the study protocol. An informed consent was obtained for enrolling the patients. The clinical and biochemical assessment of the patients was done according to the study protocol. The histological examination of liver biopsies was done according to the modified Knodell scoring system[15].

Patients received either 3 million IU of IFN α 2b daily for 24 to 48 wk. A group of patients received pegylated IFN-alpha 2b (1 to 1.5 μg per wk) for either 24 or 48 wk depending on viral genotype. All patients received ribavirin given orally (patients with < 65 kg received 800 mg/d and 65-85 kg received 1000 mg/d). Outcome of treatment was classified as follows: End Therapy Response (ETR): patients with undetectable HCV RNA at the end of treatment; Sustained Virological Response (SVR): patients with undetectable HCV RNA 6 mo after the end-of-treatment; Non-Response (NR): failure to achieve viral clearance at the end-of-treatment or 6 mo there after.

Controls

Unrelated healthy adult subjects, with no previous history of liver disease and negative for HBV and HCV infection were included as controls. A total of 408 healthy subjects were included as controls. Both patients and control groups included Hindus, Muslims, and Christians. Patients and controls were prospectively matched for ethnic group.

DNA extraction and PCR- based genotyping of allelic variants

Genomic DNA was extracted from whole blood using a commercially available kit. The CCR5Δ32 deletion mutation was detected by PCR based techniques as mentioned elsewhere[16]. Primers amplified 2 fragments of 200 bp and 172 bp corresponding to wt and deleted CCR5 alleles. Primer sequences were CCR5L-5' TTA AAA GCC AGG ACG GTC AC 3' CCR5R-5' GAC CAG CCC CAA GAT GAC TA 3'. Cycling conditions were 96℃ for 5 min, followed by 30 cycles at 94℃ for 30 s, 58℃ for 30 s, and 72℃ for 30 s. A final extension step of 72℃ for 10 min was applied. Amplified fragments for the CCR5 locus were resolved in 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

DNA sequencing

The CCR5Δ32 mutation was confirmed by sequencing. For DNA sequencing, ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (ABI) was used.

Correlation of immune gene polymorphism and persistence of HCV infection

To study whether persistence of HCV infection is linked to immune gene polymorphism, patients with chronic HCV infection were compared with healthy controls.

Correlation of immune gene polymorphism and severity of HCV related liver disease

To study whether immune gene polymorphisms influence the course of HCV related liver disease, patients were

Table 1 Demographic profile of patients with chronic HCV infection and healthy controls

	Patients (*n* = 252)	Patients (Only genotype 3) (*n* = 187)	Healthy controls (*n* = 408)
Age (yr)			
Mean ± SD	40 ± 14	40 ± 14	33 ± 7
Male: Female	164:88	118:69	297:111
ALT (IU/L)			
Mean	112.3 ± 68.4	116 ± 70.1	29 ± 2
Range	(32-330)	(32-330)	
S. Albumin (g/L)	36 ± 4	34 ± 5	40 ± 3
HCV genotype (*n* = 209)			
I	17 (8.1%)		-
II	1 (0.5%)		
III	187 (89.4%)		-
IV	4 (1.5%)		-
Liver Biopsy			
Fibrosis score	2 ± 1	2 ± 1.2	-
HAI	5.5 ± 2.4	5.6 ± 2.5	-
Mode of Acquisition of HCV			
Blood transfusion	86		-
Needle stick injury	54		-
Intravenous drug use	10		-
Medical procedure	45		-
Unclear	57		

Table 2 Allele frequency of CCR5 variants in chronic HCV patients and healthy controls

CCR5	HCV Patients (*n* = 252)	Healthy Controls (*n* = 408)	*P* value
Wt/wt	247 (98%)	405 (99.3%)	0.07
∆32mt carrier	5 (1.9%)	3 (0.7%)	0.3
Wt/∆32	3 (1.2%)	3 (0.7%)	0.2
[a]∆32mt/∆32mt	2 (0.7%)	-	0.1

[a]The patients were genotype 1.

categorized on the basis of the stage of ALT levels (< 60 or ⩾ 60 IU/L), hepatic fibrosis and histological activity index (HAI). Patients were divided into those with a fibrosis score of ⩽ 2 (mild) or >2 (severe) and with a necroinflammatory score of ⩽ 5 (mild) or > 5 (severe).

Statistical analysis

The frequencies of CCR5∆32 mutant allele were compared between patients with chronic HCV infection and controls by Chi-square test. Categorical variables were analyzed with χ^2 or Fisher's exact test. Two-sample *t*-tests were used to compare means for continuous variables and for non-normally distributed continuous variables, non-parametric test, Wilcoxon Mann-Whitney-U test was used for comparison of median values. Univariate analysis was used to assess associations between the various allelic variants and severity of liver disease.

Hardy-Weinberg equilibrium was tested by comparing expected and observed genotype frequencies by χ^2-test. The distribution of genotypes between the patients and healthy controls were compared by contingency table analysis.

For all tests, a 2-tailed $P < 0.05$ was considered significant. The analysis was performed by using statistical software SPSS 12.0 software (SPSS, Chicago, IL).

RESULTS

Demographic profile

Two hundred and fifty two patients with chronic hepatitis C were studied. The baseline characteristics of the cases and controls in the study group are summarized in Table 1. The most common source of HCV infection was blood transfusion.

Viral genotype

HCV genotypes could be determined in 209 of the 252 (82.9%) patients. The predominant viral genotype was genotype 3a/3b. One hundred eighty seven (89.4%) patients had genotype 3, 17 (8.1%) had genotype 1, one patient had genotype 2 and 4 patients had genotype 4 (Table 1). No significant difference was observed in immune gene polymorphism and patients with different viral genotypes.

Distribution of CCR5 allelic variants

The frequency of CCR5∆32 mutations in HCV patients and controls is shown in Table 2. Two hundred and forty seven (96.6%) HCV patients and 405 (99.3%) healthy controls had CCR5 wt/wt homozygosity. 3 (1.2%) of 252 patients had heterozygous and 2 (0.7%) had homozygous ∆32 mutations; overall 5 (1.9%) of the HCV patients had ∆32 mutant alleles. On the other hand, none of the healthy controls had a homozygous ∆32mt/∆32mt pattern. Three healthy controls exhibited heterozygosity (Table 2). The difference between the patients and the controls was comparable but not significant. CCR5∆32/∆32 was present in HCV genotype 1 patients. Patients carrying CCR5∆32 (CCR5∆32 homozygote and heterozygote) mutation had no significant difference in the serum ALT level, degree of hepatic inflammation, fibrosis score and viral genotype compared with those patients who had wild type CCR5. All the allelic variants were in Hardy-Weinberg equilibrium.

Relation to serum alanine aminotranferase

We also used the mean serum ALT concentrations as an indicator of the activity of liver disease. However, no significant difference was observed for any of the genetic markers in patients with normal or without raised ALT (data not shown).

Relation to liver histology: Fibrosis and Histological inflammation

When patients were categorized based on the severity of hepatic fibrosis, 162 patients had mild and 90 patients had severe hepatic fibrosis. The frequency of CCR5∆32 mutations was comparable in patients with mild or severe liver disease (P = NS) and it did not correlate with the severity of the liver disease (Table 3). Similarly CCR5∆32 mutations did not correlate with histological severity

Response to therapy

Table 4 depicts the response pattern of the patients

Table 3 Correlation of Immune Gene Polymorphism and histological severity of HCV Related Liver Disease in Univariate analysis

Liver histology	CCR5-Genotype			P
	wt/wt	wt/Δ32	Δ32/Δ32	
Fibrosis				
≤ 2 (n = 162)	154 (98%)	1 (0.6%)	2 (1.2%)	
> 2 (n = 90)	88 (97.8%)	2 (1.2%)	0	NS
HAI				
≤ 5 (n = 162)	170 (98.3%)	1 (0.6%)	2 (1.2%)	
> 5 (n = 90)	77 (97.5%)	2 (2.5&)	0	NS

Table 4 Response patterns in HCV patients genotyped for CCR5

Response		CCR5-Genotype			P
		wt/wt	wt/Δ32	Δ32/Δ32	
IFN+ Riba/	Responders	86	1	-	NS
Peg IFN+ Rib	Non-responders	34	1	-	NS
	Relapsers	7	-	-	NS

enrolled in the study. When patients were categorized on the basis of the response pattern, no difference was observed in the distribution of alleles with respect to response rate. Of 252 patients, 129 patients received antiviral therapy. Eighty six patients had attained sustained virological response, 34 patients were non-responders while 7 patients relapsed after discontinuation of therapy and one patient developed decompensation and discontinued the therapy. Two patients who were CCR5Δ32/Δ32, did not receive therapy. There was also no difference in the response pattern among the heterozygotes.

DISCUSSION

Host genetic factors encoding for gene products, which are likely to be involved in the immune response following HCV infection, are likely to influence the disease susceptibility and progression. It has been demonstrated by studies which correlated polymorphisms or mutations of genes encoding for HLA subtypes[17-19], tumor necrosis factor[20-22], interleukin 10[23] or chemokine receptor 5 (CCR5) with disease susceptibility or treatment outcome in HCV.

In our population 1.9% (5/252) HCV patients were carriers of the CCR5Δ32 mutation as compared to 0.7% (3/408) of the general population. This refers to both homozygous and heterozygous mutations combined. Since the frequency of these mutations is rather low it is difficult to make definite statistical analysis between various groups. The reason for the variance in our results compared to the earlier studies is not easy to explain. This could probably be because the frequency of these mutations is rather low, thus it is difficult to make definite statistical analysis between various groups. An earlier study from India has reported a single heterozygote for CCR5Δ32 mutants[24]. However, the group had only genotyped 150 normal healthy individuals for the presence of Δ32 allele. We had adequate number of controls (n = 408). Woitas et al had observed a higher frequency of CCR5Δ32 homozygotes in chronic HCV patients[11]. In the present study, we observed a 0.7% occurrence of CCR5Δ32 mutants in chronic HCV patients but none in healthy controls, however the difference was not significant.

Apart from the sample size, a plausible explanation for these divergent results might be the differences in comorbidity of patients in the studies by Woitas and Nguyen[11,25], who had a substantial proportion of patients with haemophilia and/or concomitant hepatitis B. In the present study

patients with haemophilia, hepatitis B or HIV were carefully excluded. Moreover, we did not detect elevated aminotransferase levels in carriers of the Δ32 mutation, which might also be due to the small number of homozygous carriers of the Δ32 mutants in the present study[26].

CCR5Δ32 mutants did not increase the likelihood of a more severe liver disease in our patients due to HCV infection (Table 3). Table 5 gives an overview of the published data in chronic HCV patients genotyped for CCR5Δ32 mutation. As shown in Table 5, the correlation of CCR5 Δ32 mutations with histological severity is controversial. Significant associations were found between CCR5Δ32 and reduced portal inflammation and milder fibrosis[13]. Liver inflammatory activity was found to be significantly reduced in Jewish Israeli patients infected with the hepatitis C virus carrying the CCR5Δ32 allele[32].Heterozygosity for CCR5Δ32 has been shown to be significantly associated with lower hepatic inflammatory scores[26]. However, other studies failed to find an association of CCR5Δ32 mutations with the histological severity.

Table 5 also shows distribution of viral genotypes in the published data for CCR5Δ32 mutation. It is known that the distribution of genotypes could be dependent on geographical distribution and possibly susceptibility. In all the previous studies, the associations have been studied primarily in the population of HCV patients infected with genotype 1. It might be possible that this could influence the susceptibility and severity of liver disease and could have led to variations in the results. The present study is the first study which has looked into the role of CCR5Δ32 mutation in genotype 3.

In the study population, nearly 161 of 182 (88.4%) patients in whom genotyping could be done, had genotype 3 infection. Most studies from the Indian subcontinent have reported similar frequency of genotype 3[34]. HCV 3a is a hepatitis virus strain that responds better to interferon IFN-α therapy than other HCV strains. IFN-α induces the production of CCL3, a CCR5 ligand, in the liver. It is thus possible that CCR5 is involved in a cascade of events or recruitment of immune cells, which negatively regulate the production of IFN-α in the liver. Loss of CCR5 expression due to CCR5Δ32 mutation could affect the course of hepatitis C, probably by interfering with cellular immune response. It has been suggested that CCR5Δ32/Δ32 mutant has no expression of CCR5 on the cell surface and henceforth is an ineffective HCV-specific immune response resulting in an immune imbalance to Th-1→Th-2 response[27].

It is therefore important to study the role of CCR5Δ32 mutation with response to therapy. Ahlenstiel et al have shown that response rates to interferon-alpha monotherapy are reduced in hepatitis C virus infected patients

Table 5 Analysis of published data in chronic HCV patients genotyped for CCR5Δ32 mutation

Authors	Susceptibility	Histological Severity	Response to therapy	Genotype	Patients studied (*n*)
Ahlensteil G *et al*[27,28]	Yes	Not mentioned	Lower ETR[1]	Genotype 1	59
				Genotype 2	3
				Genotype 3	9
				Genotype 4	3
				Undetermined	4
Glas J *et al*[29]	No	Not mentioned	No	Genotype 1/4	37
				Genotype 2/3	25
Goulding C *et al*[26]	[2]Spontaneous viral clearance	[2]Lower hepatic inflammation	Not mentioned	Genotype 1b	283
Hellier *et al*[13]	No	Decreased portal inflammation	No	Stratification not mentioned	
Konishi *et al*[30]	No	No	No	Serotype 1	53
				Serotype 2	48
				Serotype 1 + 2	4
Mascheretti *et al*[31]	No	No	No	Genotype 1	358
				Genotype 2	14
				Genotype 3	79
				Genotype 4	14
Promrat *et al*[12]	No	No	No	Genotype 1	243
				Genotype 2	34
				Genotype 3	16
				Genotype 4	3
				Genotype 5	1
				Undetermined	42
Wald *et al*[32]	No	Significantly reduced	Not mentioned	Genotype 1	5
				Genotype 2	2
				Genotype 3	9
				Undetermined	31
Wasmuth *et al*[33]	No	No	No	Genotype 1	213
				Non-Genotype 1	117
Woitas *et al*[11]	Yes	Not mentioned	Not mentioned	Genotype 1	95
				Genotype 2	16
				Genotype 3	10
				Genotype 4	6
				Multiple genotype	5
				Undetermined	2
Present study Goyal *et al*	No	No	No	Genotype 1	16
				Genotype 2	1
				Genotype 3	161
				Genotype 4	4
				Undetermined	70

[1] All analysis mentioned w.r.t the CCR5Δ32 carriers. [2] Heterozygotes for CCR5Δ32 mutation.

carrying the CCR5Δ32 mutation[28]. It is difficult to comment on the role of CCR5 mutations in HCV infection with genotype 3 in our population, since this is the most common genotype. Also, we had 0.7% allelic frequency of CCR5Δ32 and since 98% of HCV patients had CCR5 wt/wt, speculating a higher SVR in 3a genotype is not possible. Our patients had received combination therapy of interferon/pegylated interferon and ribavirin. However it has been suggested that the interferon and ribavirin combination treatment may overcome this negative effect of CCR5Δ32[28]. Thus, in population a infected with genotype 3 HCV, CCR5Δ32 mutations do not influence the response to combination therapy with interferon and ribavirin.

In conclusion, our results indicate that CCR5Δ32 mutation does not influence the susceptibility and severity of liver disease in chronic hepatitis C patients. Moreover, CCR5Δ32 mutation does not influence the response to therapy.

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