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RAPID COMMUNICATION

CCL2-2518 A/G and CCR2 190 A/G do not influence the outcome of hepatitis C virus infection in the Spanish population

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Abstract

AIM: To assess whether CCL2 or interactions between this chemokine and its receptor (CCR2) are associated with outcomes of chronic hepatitis C and with responses to antiviral therapy.

METHODS: Two hundred and eighty-four patients with chronic hepatitis C and 193 non-infected matched controls were included in this study. Patients were categorized according to their Scheuer score of hepatic fibrosis as F0-F2 (*n* = 202) or F3-F4 (*n* = 82) and according to their response to anti-Hepatitis C virus (HCV) therapy as sustained response (SR, *n* = 101) or non-sustained response (NSR, *n* = 98). Genotyping of the -2518 (A/G) CCL2 was performed using PCR-RFLP, genotyping of the 190 (A/G) CCR2 using a PCR-ARMS system, and genotyping of the rs3138042 (G/A) CCR2 using Taqman probes.

RESULTS: Univariate analyses identified 4 parameters (infection duration time, viral genotype, gender and AST levels) that tended to influence fibrosis and 7 parameters (CCL2G, CCL2ACCR2A, viremia levels, fibrosis stage, viral genotype, infection duration time and AST levels) that significantly influenced or tended to influence response to treatment. Multivariate analysis identified gender and AST levels as parameters that independently influenced fibrosis stage and viral genotype and infection duration time were the two parameters that independently influenced response to treatment.

CONCLUSION: Our results indicate that the mutations studied in the gene pair CCL2/CCR2 do not play a major role in the outcome and response to treatment for HCV infection in the Spanish population.

Key words: CCL2; CCR2; Polymorphism; Hepatitis C virus; Treatment; Fibrosis stage

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INTRODUCTION

Hepatitis C virus (HCV) infection results in chronic active hepatitis in more than 80% of infected patients, with 20%-30% developing progressive fibrosis and cirrhosis[1-3], whereas only about 10%-20% of the HCV infected people spontaneously eliminate the virus[3]. The morbidity and mortality associated with chronic infection is due to the development of cirrhosis and represents a high risk factor for hepatocellular carcinoma[1,4]. Different factors may influence both the ability to spontaneously clear the virus and the progression of chronic infection. Viral load, viral genotype, quasispecies, gender, alcohol consumption, iron load and age of infection have been evaluated and shown to be predictors of the natural course of HCV infection, with controversial results[5-7]. Currently, the most effective initial therapy for viral clearance is the combination of IFNα and ribavirin (RBV). Sustained response to antiviral therapy is also influenced by different viral and host factors[8,9].

Chemokines and chemokine receptor genes are candidates in HCV infection progression and in the response to anti-HCV therapy. Chemokines play important roles in leukocyte trafficking to the site of infection and in regulating T_H1/T_H2 balance. Chemokine-chemokine receptor interactions are likely to be important in chronic hepatitis C, in which T cells are recruited to the liver where they mediate virus clearance[10,11].

Monocyte chemoattractant protein-1 (MCP-1/CCL2) is a β-chemokine that has been suggested to be responsible

for monocyte and lymphocyte T recruitment in acute inflammatory conditions and may be an important mediator in chronic inflammation. In fact, it has been proposed that CCL2 is responsible for tissue inflammation in autoimmune diseases, as documented with tissue expression in human and experimental autoimmune animal models[12-15]. Additionally, CCL2 appears to be an important regulator of cytokine homeostasis within the liver[16,17]. Thus, genetic polymorphisms in the regulatory regions of the CCL2 gene could be involved in the outcome of HCV infection.

A biallelic G/A polymorphism at position -2518 of the CCL2 gene has been described[18]. This polymorphism seems to influence transcriptional activity since cells obtained from GG or AG individuals produced more CCL2 than those isolated from AA individuals[18], and it has been associated with the outcome of chronic hepatitis C[19-21]. Furthermore, higher CCL2 serum levels have been found in patients with chronic HCV infection[22]. Although CCL2 is a good candidate for influencing the course of HCV infection and response to treatment, there are few studies investigating the role of the functional -2518 CCL2 polymorphism in the progression of this viral infection[19-21].

The main receptor for CCL2 is CCR2, which plays an important role in the recruitment of cells and is highly expressed in chronic inflammation[23]. A 190-G/A transition has been described in the CCR2 gene, which leads to an amino acid change Val64Ile located in the first transmembrane domain of the protein. This polymorphism has been reported to be associated with a delay in disease progression in HIV-1 infected individuals[24]. Moreover, the CCR2-64Ile variant has been related to the lack of a capacity to clear the virus infection in individuals with HCV[25]. Association between fibrosis and CCR2-64Ile has been investigated in several studies[20,25-28]. In a previous study, with a limited cohort of patients, we could not find any relationship between this polymorphism and the outcome of the HCV infection[29]. However, a relationship between this polymorphism and the response to the treatment has not been investigated sufficiently[28,30,31].

In a murine model with a hepatotropic virus, such as CMV, a significant role for the CCL2/CCR2 pair in the recruitment of macrophages and NK cells to the liver, and in the regulation of cytokines, has been demonstrated. In the same model, authors demonstrated that CCL2 and CCR2 are critical factors in the control of virus replication and in the outcome of liver disease[17].

The aims of this study were to: (1) analyze the possible association of CCL2 with the outcome of chronic hepatitis after HCV infection, (2) investigate the influence of both CCL2 and CCR2 in the response to anti-HCV therapy, and (3) analyze the possible association of interactions between the chemokine and its receptor with the outcome of HCV infection.

MATERIAL AND METHODS

Patients

Two hundred and eighty-four Caucasian Spanish patients (166 males and 118 females, average age of 42.6 ± 11.3 years) who were suffering from biopsy-proven chronic hepatitis C with compensated liver disease and were followed in the outpatient clinic of the Hospital Universitario Virgen del Rocío and Hospital Universitario de Valme (Sevilla, Spain) were enrolled in this study. All patients were HBsAg and HIV negative, anti HCV positive and HCV RNA positive in serum. Anti-HCV, HbsAg and HIV were determined by commercially available methods (HCV 3.0 test, ORTHO, and Enzygnost HBsAg-5.0 and anti-HIV-1/2 plus, DADE Behring, respectively). Demographic data of patients and data from viral genotype, viral load and biochemical feature ALT, AST, GGT and AP levels are displayed in Table 1. Patients and controls agreed to a blood examination according to the guidelines of the Hospital Bioethics Committee. A total of 193 bone marrow donors were included as healthy controls and were considered as representative of the "normal" frequencies of the SNPs (Single Nucleotide Polymorphism) studied in the Spanish population.

Table 1 Demographic profile of patients with chronic HCV infection

Patients	*n* = 284	
Gender M/F	166/118	
	mean ± SD	Range
Age (yr)	42.6 ± 11.3	21-75
Age of Infection (yr)	24.3 ± 11.4	1-57
Age of liver biopsy (yr)	40.1 ± 11.8	16-70
ALT IU/L	121.3 ± 100.9	16-660
AST IU/L	67.6 ± 44.3	16-300
GGT IU/L	53.9 ± 53.8	10-472
AP IU/L	181.35 ± 65.6	73-637
Viral load (10^6) copies /mL	1.29 ± 1.3	0.0015-6.3
Viral genotypes	*n* = 205 (%)	
I	159 (77.6)	
II	7 (3.4)	
III	27 (13.2)	
IV	5 (2.4)	
I + another	7 (3.4)	

Genotyping

DNA from patients and controls was extracted from peripheral blood using standard methods. **-2518 CCL2 genotyping:** The regulatory region of the CCL2 gene (from -2746 at -1817) was amplified by polymerase chain-reaction (PCR) using the forward primer 5' CCGAGATGTTCCCAGCACAG 3' and the reverse primer 5' CTGCTTTGCTTGTGCCTCTT 3'[18]. PCR was performed using buffer 10 × (10 mmol/L Tris-HCl pH 9, 2.0 mmol/L $MgCl_2$, 50 mmol/L KCl), 200 μmol/L dNTPs (Pharmacia, Uppsala, Sweden), 2.5 pmoles of each primer, 0.5 μL of DNA, 0.5 U Taq polymerase (Pharmacia) and ddH_2O up to a final volume of 10 μL. The following thermal profiles were run: 94℃ for 1 min, 55℃ for 1 min, and 72℃ for 1.5 min. After a final extension of 10 min at 72℃, 7 μL of the PCR products were resolved in 2% agarose gels and stained with ethidium bromide previously diluted in blue juice buffer to detect the expected 930 bp band. After detection, 3 μL of PCR products were digested with 5 U of PvuⅡ (Boehringer Mannhein, Germany) in buffer 10 × (Boehringer Mannhein) and ddH_2O up to 10 μL of final volume incubating at 37℃ overnight. Digested products were resolved in 2% agarose gels. Samples with

a 930 bp band were assigned as AA, samples with two bands of 708 and 222 bp were typed as GG and samples with three bands of 930, 708 and 222 bp were assigned as AG[18].

190 CCR2 Genotyping: The genotyping at this position was performed with a PCR amplification refractory mutation system (ARMS) using the primers CCR2-F1 5' TTGTGGGCAACATGCTGGTCA 3', CCR2-F2 5' TTGTGGGCAACATGCTGGTCG 3' and CCR2-R 5' ACTGTGAATAATTTTGCACATTGC 3'[32]. For each sample, two separate PCR reactions were carried out, one with the primers CCR2-F1 and CCR2-R and the other with CCR2-F2 and CCR2-R. PCR reactions were performed in a Perkin Elmer 9600 Thermal Cycler with mixes consisting of 1 μL of genomic DNA, buffer 10 × (20 mmol/L Tris-HCl pH 8.3, 3.0 mmol/L $MgCl_2$, 100 mmol/L KCl, 0.005% gelatine), 400 μmol/L of each dNTP, 20 ng of cresol red, 10% of glycerol, 2 pmoles of each primer (Perkin Elmer, Foster City, CA), 0.4 U of Taq polymerase (Pharmacia, Uppsala, Sweden) and ddH_2O to a final volume of 10 μL. The following thermal profile was run: initial denaturation at 95℃ for 2 min, 10 cycles consisting of 94℃ for 10 s and 67℃ for 1 min, and finally 20 cycles consisting of 94℃ for 10 s, 62℃ for 50 s and 72℃ for 30 s. PCR products were resolved in 2% agarose. Samples showing the expected size fragment in only one tube were genotyped as homozygous whereas samples showing amplification in both tubes were assigned as heterozygous.

rs3138042 CCR2 genotyping: The genotyping of this SNP was performed using the TaqMan 5' allelic discrimination assay (Applied Biosystems, Foster City, CA, USA). The primers used were 5' CGT GTG GAA CCA CTG CAG AA 3' (forward) and 5' CCT TCC TGG CTC TCT CCA CTT A 3' (reverse), and the TaqMan MGB probe sequences were 5´ CTA TTT CCG AAA TCA AC 3', and 5' TTT CCG GAA TCA AC 3'. The probes were labeled with the fluorescent dyes VIC and FAM, respectively. PCR reactions were carried out in a total volume of 8 μL with the following amplification protocol: denaturation at 95℃ for 10 min, followed by 40 cycles of denaturation at 92℃ for 15 s and finished with annealing and extension at 60℃ for 1 min. Genotyping of each sample was automatically attributed by the SDS 1.3 software for allelic discrimination.

Liver biopsies

Percutaneous liver biopsies were performed under ultrasonographic control. A portion of the biopsy was used for the histology diagnosis. Disease staging was defined according to Scheuer *et al*[33] with ranking from F0 (absence of fibrosis) to F4 (cirrhosis stage). Patients were stratified into two groups: F0-F2, absence of fibrosis to moderate fibrosis, and group F3-F4 with advanced fibrosis-cirrhosis.

Treatment response

One hundred and ninety-nine patients were treated with recombinant IFNα three times per wk over a period of 1 year. One hundred and four patients received a standard schedule of combination therapy (IFNα 3 MU three times per week and ribavirin 100-1200 mg/d) over 1 year. Of these, 58 were naïve to treatment and 46 had relapsed after a previous IFNα treatment cycle. Based on therapy response, the patients were classified into two groups: those with sustained response (SR), when HCV RNA levels remained undetectable during 6 mo after therapy discontinuation, and those with non-sustained response (NSR), including non-responder patients (HCV RNA levels detectable during the completed period of the treatment) and relapsed responder patients (undetectable HCV RNA during the therapy but detectable after discontinuation).

Statistical analysis

Genotypic frequencies were obtained by direct counting. Statistical analysis was performed by the χ^2 test calculated on 2 × 2 contingency tables using the Statcalc program (Epi Info version 6.0; Centers for Disease Control and Prevention, Atlanta, GA). The odd ratio (OR) with 95% confidence intervals (95% CI) was calculated using the same program. Univariate and multivariate logistic regression analyses were used to determinate factors that significantly contribute to the development of advanced fibrosis-cirrhosis and those that contribute to NSR after treatment. Variables that achieved statistical significance ($P < 0.05$) or marginal significance ($P < 0.30$) on univariate analysis were entered into a multiple logistic regression analysis to identify significant independent factors (Epi Info version 6.0).

RESULTS

Variables included in the univariate analysis, except for genetic factors studied, are summarized in Table 1. HCV genotypes could be determined in 205 of the 284 patients. The predominant viral genotype was genotype 1b alone, which was present in 61.0% of cases. The genotypic frequencies for all the polymorphisms studied were not significantly different from those predicted by Hardy-Weinberg equilibrium in the healthy control group. The distribution of the -2518 CCL2 A/G genotypes was not significantly different when comparing patient and control groups. Since previous studies reported functional differences between bearing G and non-bearing G genotypes, we grouped GG and AG individuals for further analysis. We did not find differences in the distribution of bearing G and non-bearing G individuals for demographic features such as sex, age, genotype of HCV and route of infection (data not shown). No significant differences were observed among CCL2 genotypes and the mean serum AST concentration (mean AST in AA: 71.43 ± 48.28 *vs* mean AST in AG + GG: 61.62 ± 37.02, $P = 0.10$). When the patients were stratified according to their fibrosis stage, 202 patients were included in the F0-F2 group and 82 patients were included in the F3-F4 group. No statistically significant differences in the distribution of carriers of the G allele *vs* non-carriers were found between these fibrosis stage groups. Regarding the response to therapy, 199 patients received antiviral therapy. Among them, 101 (50.7%) presented SR to IFNα monotherapy or IFNα plus ribavirin combined therapy (Table 2). No significant differences in the distribution of CCL2 polymorphisms

Table 2 Genotypic and allelic distribution of the –2518 CCL2 (A/G) and CCR2 polymorphisms in Spanish patients with chronic hepatitis C (CHC) and healthy controls

Genotypes	CHC *n* = 284 (%)	F0-F2 *n* = 202 (%)	F3-F4 *n* = 82 (%)	SR *n* = 101 (%)	NSR *n* = 98 (%)	Healthy controls *n* = 193 (%)
-2518 CCL2 AA	168 (59.2)	123 (60.9)	45 (54.0)	64 (63.4)	55 (56.1)	119 (61.6)
-2518 CCL2 AG + GG	116 (40.9)	79 (39.1)	37 (46.0)	37 (36.6)	43 (43.9)	74 (38.4)
190 CCR2 GG	205 (72.2)	146 (72.3)	59 (72.0)	72 (71.3)	74 (75.5)	156 (80.8%)
190 CCR2 AG + AA	79 (27.8)	56 (27.7)	23 (28.0)	29 (28.7)	24 (24.5)	37 (19.1%)
CCR2 rs3138042 AA	134 (47.2)	93 (46.0)	41 (50.0)	48 (47.5)	46 (46.9)	102 (52.9)
CCR2 rs3138042 AG	124 (43.7)	91 (45.1)	33 (40.2)	42 (41.6)	45 (45.9)	72 (37.3)
CCR2 rs3138042 GG	26 (9.2)	18 (8.9)	8 (9.8)	11 (10.9)	7 (6.9)	19 (9.8)

F0-F2 = no fibrosis-mild fibrosis, F3-F4 = severe fibrosis-cirrhosis. SR: sustained response; NSR: no sustained response.

Table 3 Interactions between -2518 CCL2 G and 190 CCR2 A mutations

		Chronic hepatitis *n* = 284 (%)	F0-F2 *n* = 202 (%)	F3-F4 *n* = 82 (%)	SR *n* =101 (%)	NSR *n* =98 (%)	Healthy controls *n* = 193 (%)
CCL2G	CCR2A	31 (10.9)	22 (10.9)	9 (11.0)	10 (9.9)	11 (11.2)	13 (6.7)
CCL2G	CCR2G	85 (29.9)	57 (28.2)	28 (34.1)	27 (26.7)	32 (32.6)	61 (31.6)
CCL2A	CCR2A	48 (16.9)	34 (16.8)	14 (17.1)	19 (18.8)	13 (13.3)	24 (12.4)
CCL2A	CCR2G	120 (42.2)	89 (44.1)	31 (37.8)	45 (44.5)	42 (42.8)	95 (49.2)

F0-F2 = no fibrosis-mild fibrosis, F3-F4 = severe fibrosis-cirrhosis. SR: sustained response; NSR: no sustained response.

and response to anti-HCV treatment were observed. Nevertheless, a slight increase of G carriers was observed among the NSR group (Table 2).

Regarding the SNP CCR2 190 G/A, we had previously found no association between this polymorphism and susceptibility and histological damage in a group of HCV patients[29]. Data from the present cohort confirm previous results from a smaller cohort studied by our group. No statistically significant differences were found in the distribution of carriers of the A allele vs non-carriers of A with regards to AST levels (AA + AG 73.42 ± 52.31 *vs* GG 65.32 ± 40.83 $P = 0.20$) or to the fibrosis stage. In the previous study, we did not included response to treatment because data were available for only a few patients. While analyzing data from the current cohort, no significant differences in the response to treatment were found when comparing carriers of the A allele *vs* non-carriers of A (Table 2). Controversial results have been reported regarding association of CCR2 and the outcome of HCV infection. The CCR2 190A allele has a relatively low allelic frequency in our population. Additionally, CCR2 190 G/A SNP represents a conservative change (Val64Ile), and thus the molecular mechanism associated with the infection would be unclear. For these reasons, we investigated another possible area of association by genotyping another SNP in the same gene. The rs3138042 G/A SNP located within the intron 2 at 1.8 kb of the CCR2 190 G/A was selected because it is a SNP with a frequency that has been studied in different populations and it has a high heterozygosity index and thus may be informative. Results obtained for rs3138042 G/A are displayed in Table 2. No significant differences were found between patients with chronic HCV infection and healthy controls and no significant differences were found for any outcome parameter.

To explore possible interactions between -2518 CCL2G and 190 CCR2A, patients were categorized as having mutations in both genes, a mutation in only one of the genes or the absence of mutation in both genes. No statistical differences were observed in any comparison (Table 3). Noteworthy, a slight increase was observed in the frequency of individuals who had CCL2A CCR2A in the SR group.

Two different univariate analyses were performed. The first with fibrosis stage (F3-F4 yes/no) as a dependent variable and CCL2G (yes/no), CCR2A (yes/no), CCL2G CCR2A (yes/no), CCL2G CCR2G (yes/no), CCL2A CCR2G (yes/no), CCL2A CCR2A (yes/no), viral load, viral genotype 1 (yes/no), infection duration time, gender or AST levels as an independent variable. This analysis identified 4 parameters (infection duration time, viral genotype, gender and AST levels) that tended to influence fibrosis stage. The second univariate analysis included SR (yes/no) as a dependent variable and the variables described above plus fibrosis stage as independent variables. This analysis identified 7 parameters: (CCL2G, CCL2A CCR2A, viremia levels, fibrosis stage, viral genotype, infection duration time and AST levels) that significantly influenced the response or tended to influence the response to treatment. Multivariate analysis identified gender and AST levels as parameters that independently influence fibrosis stage; and the two parameters that independently influenced response to treatment were viral genotype and infection duration time.

DISCUSSION

The role of CCL2 in the pathophysiology of chronic liver diseases is well-documented and there is evidence

demonstrating that HCV is capable of inducing CCL2 gene expression in both nonhepatic and hepatic cells[16]. Moreover, it has been demonstrated in a murine CMV infection model that CCL2 expression in liver is dependent on IFNα/β. Also, the same study reported that CCL2 deficiency was associated with a dramatic reduction in the accumulation of macrophages and NK cells, as well as decreased production of MIP-1α and IFNγ[17]. Moreover, correlation between plasma chemokine levels and the outcome of antiviral therapy has been described in patients with chronic hepatitis C[34]. However, to our knowledge, there are only three studies investigating the role of functional -2518 CCL2 polymorphisms in the progression of HCV infection in Caucasian populations. These studies produced controversial results and do not include any analysis of the response to treatment[19-21]. In the present study no statistically significant differences between both liver damage and response to treatment with the presence of -2518 CCL2 G were found, although in the univariate analysis a trend to a higher frequency of -2518 CCL2 G individuals in the NSR group was observed. Nevertheless, no independent association for this mutation was found in the multivariate analysis. Thus, our results rule out a major role for -2518 CCL2 polymorphisms in the development of liver damage and in the response to antiviral therapy in HCV infection in the Spanish population.

Regarding CCR2 and susceptibility to develop chronic hepatitis and liver damage, the present study confirms data obtained by our group from a previous cohort with a more limited number of HCV patients. We did not find statistically significant differences to analyze the possible relationship between the presence of the mutation 190 CCR2 A with response to treatment. Controversial results in the association of CCR2 with progression of HCV infections have been reported[24-31]. This occurs frequently when the putative associated allele is relatively rare in the population under study (frequency under 15%), which is the case for the 190 CCR2 mutation in the Caucasian population. It has been proposed that the association of this variant with the progression of HCV infection may be due to linkage disequilibrium with the true causative variant[25]. Several SNPs have been described within the CCR2 gene, with six of them located in exons and only three as non-synonymous. Among the three that are non-synonymous, the most extensively studied is the SNP Ile64Val, whereas the other two have a low heterozygosity index. In order to explore another region of the gene, a SNP located in intron 2 with a high heterozygosity index was chosen for study. No association was found between this SNP and HCV infection outcome.

CCL2 and CCR2 interaction is a key factor in early liver inflammatory response and in cytokine regulation which is critical for an effective antiviral defense[17]. Thus, it is possible that alteration in the expression of both CCL2 and CCR2 genes modifies the course of HCV infection. We tested this hypothesis by analyzing the simultaneous presence of mutations in both genes, in only one of the two genes or absence of mutation in both. Although the univariate analysis detected a trend to a higher frequency of CCL2A CCR2A individuals among SR, no independent association for this parameter was detected in the multivariate analysis.

In conclusion, our results discard a major role for the mutations studied in the gene pair CCL2/CCR2 in the outcome and response to treatment for HCV infection in the Spanish population.

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