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Polymorphisms of IL-10 gene in patients infected with HCV under antiviral treatment in southern Brazil

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ABSTRACT

Interleukin-10 (IL-10) is a cytokine that plays an important role in the regulation of the immune system. Gene polymorphisms of IL-10 have been associated with the different expression levels of this cytokine. In hepatitis C virus infection, IL-10 appears to interfere with the progression of disease, viral persistence and the response to therapy. This study investigated genetic variability in the IL-10 gene promoter between patients infected with hepatitis C virus (HCV) and healthy individuals, associating the frequency of polymorphisms with different aspects of viral infection. This is a case-control study with 260 patients who were infected with HCV and 260 healthy individuals. Genotyping of the polymorphisms was performed using the technique of amplification refractory mutation system PCR (ARMS-PCR) for regions of the IL-10 gene promoter (-1082 G/A, -819 C/T, -592 C/A). The frequencies of alleles and genotypes related to polymorphisms in the IL-10 gene promoter showed a higher frequency of the G allele and genotype GG in the -1082 region between the infected group and the control group ($p = 0.005$ and $p = 0.001$, respectively), whereas the AA genotype was significantly more frequent in the control group. The frequencies of the haplotypes GTA and GCC were higher in the group of infected individuals, whereas the haplotype ATA was more frequent in the healthy group ($p < 0.006$). It was also observed that the genotypes GG and AG in the region -1082 were significantly more frequent among patients infected with HCV who were in advanced stages of fibrosis and cirrhosis ($p = 0.042$). No association was observed between polymorphisms of IL-10 and sustained virologic response (SVR).

1. Introduction

Approximately 2–3% of the world's population is chronically infected with HCV, which is considered a serious public health and economic problem [1,2]. The prevalence of HCV in Brazil ranges from 1.5% to 10% of the population, according to data from the World Health Organization [3].

HCV induces chronic liver disease, which is characterized by a persistent hepatic parenchyma inflammatory process that may progress to cirrhosis and hepatocarcinoma [4]. Viral clearance occurs in a minority of patients with viral hepatitis C, whereas chronic infection is established in 60–80% of all cases, 20–40% of which may evolve to cirrhosis and hepatocarcinoma [5,6]. Genetic differences among the infected hosts can determine the progression of the infection, causing different individuals to respond in different ways to the viral infection [7,8].

Cytokines have become the focus of many scientific studies, as there is evidence of the contribution of genetic factors to the imbalance of the inflammatory profile of the patient, which directly affects the clinical outcome and the severity of hepatitis C and other infectious diseases [9–12]. Experiments have shown that pro- and anti-inflammatory cytokines can modulate the benefits of antiviral therapy, affecting the evolution of liver disease and viral clearance after the acute phase and causing rapid progression to decompensated liver disease [6].

IL-10 is produced by Th2 lymphocytes, B lymphocytes, hepatocytes, Kupffer cells, epithelial cells and keratinocytes, among others. This cytokine seems to play an important role in the immune response against HCV, as it interferes with the balance of Th1/Th2, negatively regulating the response of Th1 lymphocytes

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and suppressing the action of pro-inflammatory cytokines such as interferon gamma [13]. The IL-10 gene contains five exons that are separated by four introns and occupies approximately 5.1 kb on chromosome 1, between 1q31 and 1q32 [14]. Three biallelic single-nucleotide polymorphisms (SNPs) were described in the promoter region -1082 (G/A), -819 (C/T) and -592 (C/A). There is linkage disequilibrium between the SNPs -819 and -592 because the C allele of -819 is always present when there is a C allele in -592, whereas the T allele is always present in -819 when the A allele is in -592 [15,16].

Recent studies indicate that genetic variability in the promoter region of the IL-10 gene is related to different levels of expression of this cytokine [9,17]. These levels of expression seem to be involved in the immune response of patients infected by HCV, in progression of liver disease and in the response of patients to antiviral therapy [17,18]. The alleles -1082/G, -819/C and -592/C (haplotype GCC) have been linked to high production of IL-10 [19], while the haplotype ACC and ATA are associated with lower levels of expression. Such polymorphisms are directly involved in the control of the expression levels of IL-10, which plays an important role in the depuration of HCV [20].

In view of the contradictory results already published, this study investigated genetic variability in the IL-10 gene promoter among patients infected with HCV and healthy individuals, associating the frequency of polymorphisms with different aspects of HCV infection.

2. Material and methods

2.1. Patients

This study was conducted between October 2012 and May 2013. Among the patients involved, 260 were chronic carriers of HCV genotypes 1, 2 and 3 who were receiving antiviral drugs for HCV at the Center for Application and Monitoring of Injectable Drugs (CAMMI) in Pelotas and Rio Grande, RS, Brazil. The healthy group was composed of 260 healthy volunteers at the University Hospital. These volunteers showed normal liver enzymes, negative serology for HCV, HBV and HIV and no history of viral hepatitis or endocrine disorders. This study was approved by the Ethics Committee at the Federal University of Rio Grande (Approval No.: 182.257). All patients signed an informed consent form and responded to a questionnaire for sociodemographic and behavioral data. Biochemical variables were obtained by reviewing the patients' medical records.

2.2. Treatment

All chronic HCV patients received treatment with subcutaneous injection of conventional interferon (IFN) or pegylated interferon (PEG-IFN) and oral doses of ribavirin (RBV), adjusted according to their body weight. Among treated patients, 25 received triple therapy with Ribavirin, PEG-IFN and a protease inhibitor (Boceprevir). The patients were divided into two groups, according to their virologic response: SVR, with undetectable HCV-RNA 24 weeks after the end of treatment; and nonresponder patients (NR), with detectable HCV-RNA after the same time period.

2.3. Blood samples and laboratory techniques

Venous blood samples (10 mL) were collected from the patients in tubes containing ethylenediaminetetraacetic acid (EDTA). The DNA was extracted with a PureLink® DNA Genomic Mini Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The extracted samples were stored at −20 °C for further molecular assays. The amplification refractory mutation system PCR (ARMS-PCR) technique was used for SNP genotyping, as described by Perrey [21]. According to Afzal [22], two reactions containing an antisense generic primer and a sense allele-specific primer were performed for each polymorphism. The primers used in this study were antisense generic primer 5′-CAGTGCCAACTGA GAATTTGG-3′ and sense allele-specific primer 1 (G) 5′-CTACT AAGGCTTCTTTGGGAG-3′ and 2 (A) 5′-ACTACTAAGGCTTCTTTGG GAA-3′ for the SNP -1082 (G/A); antisense generic primer 5′-AGG ATGTGTTCCAGGCTCCT-3′ and sense allele-specific primer 1 (C) 5′-CCCTTGTACAGGTGATGTAAC-3′ and 2 (T) 5′-ACCCTTGTACAG GTGATGTAAT-3′ for the SNP -819/-592. All PCR reactions had a final volume of 25 μL, containing 2 μL of DNA, 1X reaction buffer, dNTPs (1.5 mM), $MgCl_2$ (25 mM), 10 pmol of each primer and 0.4 units of Taq polymerase (Life Technologies, Carlsbad, USA). The cycling conditions were as follows: 3 min at 95 °C, followed by 35 cycles of 95 °C for 45 s, 58 °C for 40 s and 72 °C for 1 min, and a final extension of 7 min at 72 °C. The sizes of the fragments were 258 bp and 233 bp for the -1082 and -819/-592 regions, respectively. Human growth hormone was used as an internal control to confirm the success of the reaction, with primers 1 (5′-GCC TTCCCAACCATTCCCTTA-3′) and 2 (5′-TCACGGATTTCTGTTGTGT TTC-3′) generating a 429 bp product. The PCR products were stained in a 2% agarose gel with Blue Green Loading Dye I (LGC Biotechnology, São Paulo, Brazil).

2.4. Statistical analysis

Statistical analyses were performed using the program STATA 8.0, with Fisher and chi-squared tests. Values were considered significant when $p < 0.05$.

3. Results

The sociodemographic variables of the individuals involved in this study are described in Table 1. The average age of patients infected with HCV was 54.1 ± 11.5 years, significantly higher than 48.9 ± 15.1 years in the healthy group ($p = 0.0001$). The skin color also showed statistically significant differences between patients with HCV and healthy patients ($p = 0.047$). As expected, the average alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were significantly higher in patients with HCV ($p < 0.001$).

Analysis of the alleles and genotypes related to polymorphisms in the IL-10 gene promoter between the study groups showed an increased frequency of both the G allele in the -1082 region of the infected group and the A allele in the healthy group ($p = 0.01$ in both cases). The genotype GG in the same region was significantly more frequent in the infected patients ($p = 0.01$). Conversely, the AA genotype was significantly associated with the control group ($p = 0.01$) (Table 2). There were no significant differences between alleles or genotypes in the polymorphisms of the -819/-592 region, which shows linkage disequilibrium. In relation to haplotypes, as shown in Table 3, GTA and GCC were more frequent in the infected patients, although there was no significant association. However, the ATA haplotype was significantly more frequent in the healthy group ($p < 0.01$). The genotypes GG and AG were significantly more frequent in the -1082 region in infected individuals with advanced stages of fibrosis and cirrhosis. No statistically significant association was observed for the region -819/-592 between different haplotypes. As noted, the genotype of HCV and the stages of hepatic fibrosis were statistically related because genotype 3 of HCV was significantly associated with advanced fibrosis and cirrhosis ($p < 0.033$).

Table 1
Sociodemographic and clinical variables in patients with chronic hepatitis C and in healthy patients ($n = 520$).

	Chronic hepatitis C ($n = 260$)		Control group ($n = 260$)		p Value[a]
	N	%	*N*	%	
Sex					0.313
Female	112	43.1	123	47.3	
Male	148	56.9	137	52.7	
Skin color					0.047
White	215	83.0	196	75.7	
Non-white	45	16.9	63	24.3	
	Mean	(sd)	Mean	(sd)	
Age	54.1	(11.5)	48.9	(15.1)	<0.001[b]
AST	48.2	(35.3)	21.2	(8.1)	<0.001[b]
ALT	51.2	(45.2)	19.7	(7.6)	<0.001[b]

[a] Chi-squared test.
[b] Student's T test.

Table 2
Frequency of alleles and genotypes in the regions -1082 and -819/-592 of the IL-10 gene promoter in patients with chronic hepatitis C and in healthy patients ($n = 520$).

Locus		Chronic hepatitis C ($n = 260$)		Healthy group ($n = 260$)		p Value[a]
		N	%	*N*	%	
	Genotypes					
-1082						**<0.001**
	AA	23	8.8	40	15.4	0.031
	GA	191	73.5	202	77.7	0.307
	GG	46	17.7	18	6.9	<0.01
-819/-592						**0.161**
	TT/AA	58	22.3	48	18.5	0.327
	CT/CA	171	65.8	190	73.1	0.087
	CC/CC	31	11.9	22	8.4	0.113
	Alleles					
-1082						**0.005**
	A	237	45.6	282	54.2	<0.01
	G	283	54.4	238	45.8	<0.01
-819/-592						**0.950**
	T/A	287	55.2	286	55	0.999
	C/C	233	44.8	234	45	0.999

[a] Chi-squared test.

Table 3
Haplotypes in the regions -1082 and -819/-592 of the IL-10 gene promoter in patients with chronic hepatitis C and in healthy patients ($n = 520$).

IL-10 haplotype	Chronic hepatitis C ($n = 260$)		Healthy group ($n = 260$)		p Value[a]
	N	%	*N*	%	**0.006**
ATA	16	6.1	38	14.6	$p < 0.01$
ACC	7	2.7	2	0.8	$p = 0.176$
GTA	213	82.0	200	76.9	$p = 0.193$
GCC	24	9.2	20	7.7	$p = 0.637$

[a] Chi-squared test.

Between viral and host parameters, only genotype 1 of HCV was significantly associated with the absence of sustained virologic response ($p = 0.001$). Among the 261 patients infected with HCV who were attending reference centers to receive antiviral treatment, 232 (88.9%) were undergoing treatment with PEG-IFN and RBV, 25 (9.6%) were receiving triple therapy (PEG-IFN, RBV and Boceprevir) and 3 (1.1%) were receiving conventional IFN. One patient (0.4%) did not adhere to the treatment or return to the center. Thus, of the 260 treated patients, 160 (61.5%) presented RVS, 147 (91.9%) of whom were treated with PEG-IFN and RBV, and 13 (8.1%) received triple therapy. Only one of the three patients treated with IFN presented SVR. These data showed no association between IL-10 gene polymorphisms and SVR but did show differences in the genotypic profile of IL-10 between infected and healthy patients.

According to Table 4, it is noted that patients with genotype 1 and 3 presents the most advanced degree of fibrosis as compared genotype 2 ($p < 0.033$). Degree of Inflammation was no significant association between HCV genotypes.

4. Discussion

This study was performed in reference centers for the treatment of HCV in southern Brazil, allowing us to map the demographic, clinical and immunogenetic profiles of patients infected by HCV.

Table 4
HCV genotypes associated with the degree of hepatic fibrosis and inflammation (according to the METAVIR scale) ($N = 260$).

	G1		G2		G3		*p*-Value[a]
	N	%	*N*	%	*N*	%	
Fibrosis							**0.033**
F0	8	6.6	4	25.0	5	4.1	
F1	28	23.1	4	25.0	38	30.9	
F2	51	42.2	5	31.3	42	34.1	
F3	27	22.3	2	12.5	22	17.9	
F4	7	5.8	1	6.2	16	13.0	
Inflammation							**0.238**
A0	6	5.0	2	12.5	4	3.3	
A1	41	33.9	8	50.0	52	42.3	
A2	68	56.2	6	37.5	65	52.8	
A3	6	4.9	0	0.0	2	1.6	

[a] Chi-square test.

Viral factors, such as genotype and viral load, and host factors, such as gender, age and ethnicity, contribute to the progression of hepatitis C virus infection and to the response to antiviral therapy among infected individuals [19]. In recent years, the immunogenetics of the hosts have been studied in an attempt to understand predictive factors for a cure or disease progression at the onset of an HCV infection. The relationship between polymorphisms in the promoter of IL-10 and the outcome of infection and response to treatment against HCV has been discussed because the results obtained thus far are conflicting [6,17,18].

IL-10 is an anti-inflammatory cytokine that plays an important role in the regulation of the immune response. The expression levels of this cytokine are associated with viral persistence due to the direct action of IL-10 on the mechanisms of innate and adaptive immunity [23,24]. Variations in these levels have been directly related to the influence of IL-10 on the organization of the immune response and are associated with the presence of polymorphisms in the IL-10 gene [25–33]. Most published studies demonstrate a relationship between chronic HCV infection and high levels of IL-10 production, confirming that some polymorphisms in the IL-10 gene promoter are crucial to determine the course of infection and eliminate the viral agent [34–36].

The analysis of polymorphisms in the IL-10 gene promoter regions -1082 A/G and -819 C/T (-592 C/A) in patients in this study demonstrated that the genotype -1082 GG is more frequent in individuals infected with HCV than in healthy individuals. The genotype -1082 GG is responsible for high levels of IL-10 production, which then undermine the antiviral cellular immune response [20]. These results are in agreement with previously published data [37,38] and corroborate the study by Afzal et al. [22], who reported a significant frequency of the genotype -1082 GG in 17% of patients with chronic HCV infection and 3% of patients from the healthy group. Conversely, Knapp et al. [39] reported an association of the genotype -1082 AG with viral infection. Other authors have not observed a significant difference in the polymorphisms of the -1082 and -819/-592 regions of the IL-10 gene promoter between infected and healthy patients [6,40–44]. The genotype -1082 AA showed a higher frequency in the healthy group, in contrast to other studies that associated this genotype with patients infected with HCV [37,38,44].

Significant differences were not observed in the analysis of polymorphisms in the regions -819/-592 between infected and healthy patients in our study, which is in accordance with the data published by other authors [7,22,37,40,42,45–47]. In contrast, a few studies have shown the association of this polymorphism with HCV infection [35,48].

The increased frequency of the ATA haplotype in the control group that was observed in our study was related to a lower risk of HCV infection and may be considered a protective factor in this sample. The haplotypes GTA and ACC presented at a higher frequency in HCV patients compared with healthy patients, as reported in the study conducted by Afzal [22].

Regarding SVR, our data showed no significant association with the polymorphisms -1082 A/G, -819 C/T and -592 A/C, corroborating the study by Yee et al. [18,40,49]. However, an association between polymorphisms of the IL-10 promoter and SVR was observed, and the genotype -592 CC was more frequent in patients who responded to antiviral therapy [48]. With regards to the SNP -1082 AG, our results showed no significant difference between the groups of RVS and NR patients, as also observed by Pasha [44] and Chuang [40]. However, these results contradict other studies that demonstrated that the genotype -1082 GG was more common in NR patients [39]. In fact, several studies have reported that genotypes, haplotypes and serum levels of IL-10 have no significant effects on SVR and NR patients [40,43,50,51].

Considering the side effects and cost of antiviral therapy, the determination of new genetic markers becomes crucial in therapeutic decisions prior to the onset of therapy in patients with chronic hepatitis C. Our results contribute to the establishment of possible genetic markers of response to the hepatitis C treatment. This knowledge has a prognostic significance in patients chronically infected and may suggest a more aggressive therapy for those with increased risk of disease progression. Our findings about the HCV genotypes are notable. Although our results were expected and similar ones have been extensively discussed in the literature, they showed a smaller SVR in patients infected with HCV genotype 1 and more advanced stages of fibrosis in patients infected with genotype 3 [52].

Because HCV infection involves numerous factors related to both the host and the virus, the ambiguous and conflicting results involving polymorphisms of IL-10, the progression of infection and the response to antiviral therapy are within reason. It is important to consider that the frequency of polymorphisms -1082 A/G, -819 C/T and -592 A/C was strongly related to ethnic and regional factors in the studied population. It has been shown that people of European and Asian origin demonstrate drastically different production levels of IL-10 [53]. Other studies have shown significant differences regarding the frequency of genotypes producing high levels of IL-10 among black, Hispanic, Asian and Caucasian individuals [54].

Despite the conflicting results found in our study, we showed an association of the -1082 GG genotype with patients infected with HCV and an association of the ATA haplotype with healthy individuals. We hope that this study will contribute to the knowledge and characterization of host restriction factors in HCV infection.

Authors' contributions

NMOS and FNG conducted all experimental procedures. NMOS, BMVB and DMS performed the statistical analyses. NMOS, BMVB and FNG provided reagents and infrastructure for the experimental procedures. AMBM conceived and financed the study. RL and BMVB helped in samples collections. All authors read and approved the final manuscript. NMOS and FNG wrote the manuscript. All authors have read and approved the final version of the manuscript.

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the acquisition of material and reagents and did not take part in patient selection, analysis of results or the writing of this manuscript.

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