

ANALYSIS OF POLYMORPHISMS IN THE INTERLEUKIN 18 GENE PROMOTOR (-137 G/C AND -607 C/A) IN PATIENTS INFECTED WITH HEPATITIS C VIRUS FROM THE BRAZILIAN AMAZON

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ABSTRACT - Background - The hepatitis C virus has been recognized as the leading cause of chronic liver disease in the world. Host genetic factors have been implicated in the persistence of hepatitis C virus infection. Single nucleotide polymorphisms at positions -607 C/A (rs1946518) and -137 G/C (rs187238) in the IL-18 gene promoter have been suggested to be associated with delayed hepatitis C virus clearance and persistence of the disease. **Objective** - Identify these polymorphisms in a population infected with hepatitis C virus from the Brazilian Amazon region. **Methods** - In a cross-sectional analytical study conducted in Belém, Pará, Brazil, 304 patients infected with hepatitis C virus were divided into two groups: group A, patients with persistent infection; group B, patients with spontaneous clearance. The control group consisted of 376 volunteers not infected with hepatitis C virus. Samples were analyzed by RT-PCR for the detection of viral RNA and by RFLP-PCR to evaluate the presence of the -137 G/C and -607 C/A IL-18 gene promoter polymorphisms. **Results** - Comparison of polymorphism allele frequencies between the patient and control groups showed a higher frequency of allele C at position -607 among patients ($P=0.02$). When the association between the polymorphisms and viral infection was analyzed, patients carrying genotype C/A at position -607 were found to be at higher risk of persistent hepatitis C virus infection ($P=0.03$). **Conclusion** - the present results suggest a possible role of the -607 IL-18 gene promoter polymorphism in the pathogenesis of hepatitis C virus infection.

HEADINGS - Single nucleotide polymorphism. Interleukin-18. Hepatitis C. Hepacivirus.

INTRODUCTION

Since its discovery in 1989, hepatitis C virus (HCV) has been recognized as the leading cause of chronic liver disease in the world. Approximately 3% of the world population suffers from chronic hepatitis C^(3,22).

Viral replication in the liver for a prolonged period of time can have severe clinical consequences such as fibrosis, cirrhosis, and hepatocellular carcinoma^(5,13). Host genetic factors have been implicated in the persistence of HCV infection. In this respect, two polymorphisms in the promoter region of the interleukin 18 (IL-18) gene have been shown to influence the expression of this gene⁽¹⁾.

IL-18, also called interferon gamma (IFN- γ) inducing factor, is a proinflammatory cytokine that

plays an important role in both Th1 and Th2 responses^(13,15). Immune responses and genetic factors are known to influence the prognosis of HCV infection⁽⁸⁾. Cytokines play a critical role in the pathogenesis of HCV infection, with different clinical outcomes. Evidence indicates that genetic variants of IL-18 can influence host susceptibility, viral persistence or clearance, tissue injury, and the response to antiviral treatment⁽²³⁾. Two single nucleotide polymorphisms (SNP) identified at positions -607 C/A (rs1946518) and -137 G/C (rs187238) in the IL-18 promoter region seem to influence the promoter transcription activity of IL-18 and, potentially, of IFN- γ , and have been associated with delayed clearance of HCV and persistence of the disease⁽¹⁾.

To our knowledge, no Brazilian studies have

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investigated the association between the -607 C/A (rs1946518) and -137 G/C (rs187238) SNPs in the IL-18 gene promoter and the outcomes of HCV infection. The objective of the present study was to identify these polymorphisms in a population infected with HCV from the Brazilian Amazon region.

METHODS

Sample characterization

A total of 304 patients of Tropical Medicine Center of the viral hepatitis program, were selected and divided into 2 groups: Group A - 174 patients with persistent infection, with anti-HCV and HCV polymerase chain reaction (PCR) positive for more than 6 months. Group B - 130 patients with spontaneous clearance, characterized by positive anti-HCV and HCV PCR negative. Viral clearance was defined when anti-HCV serology was positive and HCV RNA was undetectable in serum over a period of 6 months or less in the absence of specific HCV treatment (Figure 1). For the control group, 376 anti-HCV-negative volunteers with negative HCV PCR were selected at random.

None of the patients was co-infected with hepatitis B virus (HBV surface antigen negative) or Human Immunodeficiency Virus (HIV), none of the patients consumed alcoholic beverages, and all were treatment naive. All patients received detailed information about the study and signed a consent form.

All results of serology confirmed by at least these parated serological tests within consecutive 6 months during the follow-up. All subjects were diagnosed by experienced physicians on the basis of clinical and laboratory findings and internationally accepted criteria⁽¹⁸⁾.

All subjects included (patients and control) were of the same socioeconomic status and had similar cultural habits. In addition, all subjects were born in Pará state and had the same ethnic origin, approximately 50% Portuguese, 40% Amerindian, and 10% African⁽²⁰⁾.

The study was approved by the Ethics Committee of the Tropical Medicine Center, Federal University of Para (Núcleo de Medicina Tropical, Universidade Federal do Pará - NMT/UFGPA), Belém, PA, Brazil (Permit No. 042/2011). All patients gave their informed consent to participate in the study.

Serological testing and HCV genotyping

Peripheral venous blood (5 mL) was collected from each participant into EDTA tubes. Plasma was isolated by centrifugation and stored at -80°C until assayed. Serological markers of HCV infection (anti-HCV antibodies) were investigated using the ETI-ABHCVK-4 kit from Diasorin (Saluggia, Italy).

RNA was extracted from all samples using the QIAamp Viral RNA kit (Hilden, Germany). HCV RNA was investigated by nested PCR using primers that target the 5'-UTR region. The first reaction consisted of the synthesis and amplification of cDNA in a single step using 1 mL of the k10

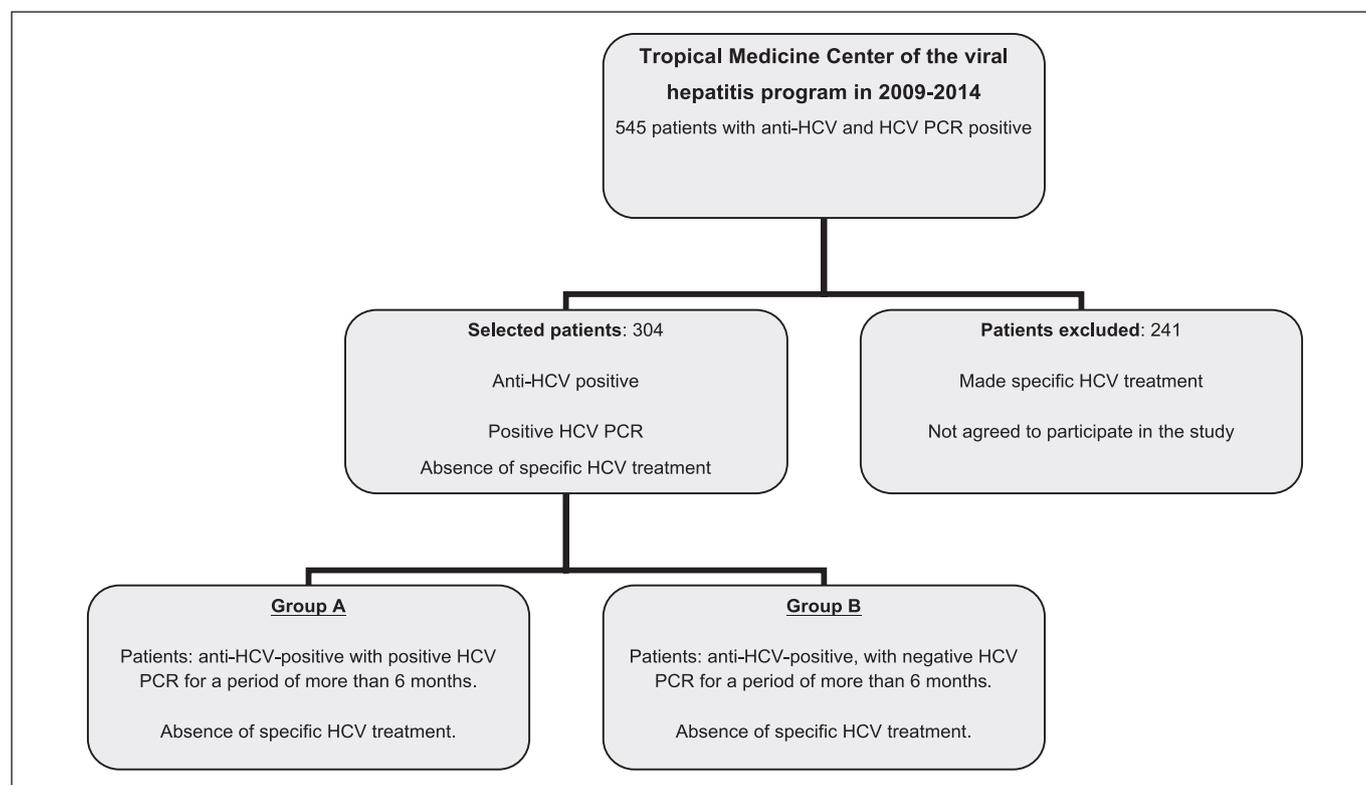


FIGURE 1. Flowchart of inclusion and exclusion criteria of the patients hepatitis C virus (HCV) carriers participating in the study.

(5'-GGC GAC ACT CCA CCA TRR-3') and k11 (5'-GGT GCA CGG TCT ACG AGA CC-3') primers, 5 mL DNase-free ultrapure water, 5 mL RNase, and 1 mL One-Step Taq DNA Polymerase (Invitrogen, São Paulo, Brazil).

The samples were incubated in a thermocycler at 42°C for 45 min. The amplification conditions were initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 45 s. A final extension was performed at 72°C for 7 min and the samples were cooled to 4°C.

The second reaction mixture contained 2.5 mL buffer, 4 mL dNTPs, 1.5 mL MgCl₂, 1 mL k15 (5'-ACC ATR RAT CAC TCC CCT GT-3') and k16 (5'-CAA GCA CCC TAT CAG GCA GT-3') primers, 12.5 mL DNase- and RNase-free ultrapure water, and 0.5 mL Platinum Taq DNA Polymerase (Invitrogen).

The virus was genotyped by the restriction fragment length polymorphism (RFLP) technique using Ava II and Rsa I restriction enzymes. Positive and negative controls were included in all reactions⁽¹²⁾.

Detection of the IL-18 gene polymorphisms (-137 G/C and -607 C/A)

Human DNA was isolated from whole blood samples (patients and controls) using the PureLink™ Genomic DNA kit (Invitrogen).

Specific primers were used for the polymorphisms at position -137 G/C: (forward) 5'-TGCTTCTAATGGACTA-AGGAGGTG-3' and (reverse) 5'-CTTCTTTTAATG-TAATACTACTATTTTCATGAGA-3', and at position -607 C/A: (forward) 5'-TTCTGTTGCAGAAAGTGTA-AAAATTTT-3' and (reverse) 5'-AAAGGATAGTTGATA-CAGGCCATT-3'.

The IL-18 gene polymorphisms were identified as described by An et al.⁽²⁾. For RFLP-PCR, the restriction enzymes Bgl II and Dra I were used to detect the -137 and -607 polymorphisms, respectively. Digestion with Bgl II produces a single band of 141 bp for allele C and two bands of 105 and 36 bp for allele G (Figure 1). Digestion with Dra I produces a single band of 154 bp for allele C and two bands of 125 and 28 bp for allele A.

Histopathology analysis

A liver biopsy were obtained from all patients anti-HCV positive. The biopsies were fixed in 10% buffered formalin, submitted to routine processing, and embedded in paraffin. The paraffin blocks were cut and the sections were stained with hematoxylin-eosin and analyzed by a pathologist.

Fibrosis staging and inflammatory activity grading were used for the classification of chronic hepatitis⁽²³⁾. Fibrosis was staged as follows: 0: no fibrosis, 1: portal fibrosis without septa, 2: few septa, 3: numerous septa delineating nodules without cirrhosis, and 4: cirrhosis. Inflammatory activity was graded taking into account activities in the portal tract and in the periportal and lobular regions: 0: no histological activity, 1: minimal damage, 2: mild activity, 3: moderate activity and 4: severe activity.

Statistical analysis

The results were analyzed using the BioEstat 5.0 program⁽⁴⁾. The chi-square test was used to compare frequencies between groups. The frequency distribution of the variables is expressed as percentage and odds ratios (OR) were calculated. A level of significance of 95% was adopted.

RESULTS

Table 1 shows the demographic data of each group. No significant difference in mean age or gender distribution was observed between the three groups studied. However, comparison of the frequency of HCV genotypes showed a higher prevalence of genotype 1 in patients with persistent infection (group A) compared to the group with spontaneous clearance (group B).

Histological analysis revealed the presence of chronic hepatitis in all patients. Grade 0 or 1 necroinflammatory activity was observed in 93% (284/304) of these patients and grade 2 or 3 in 7% (20/304). Regarding the stage of fibrosis, 90% (275/304) of the patients had stage 0 or 1 and 10% (29/304) stage 2. Analysis of histopathological alterations between the groups A and B showed no statistically significant difference ($P>0.05$).

TABLE 1. Demographic and clinical characteristics of HCV-infected patients with persistent infection and spontaneous clearance and controls

Variable	Group A	Group B	Group C	P
	n (%)	n (%)	n (%)	
Mean age (years)	37.5	38	36	0.6251 ^a
Gender				
Male	120 (69)	92 (70)	240 (64)	0.1561 ^b
Female	54 (31)	38 (30)	136 (36)	
HCV genotype				
1	140 (80)	91 (70)	-	0.0481 ^c
Non-1	34 (20)	39 (30)	-	

Group A: patients with persistent infection; Group B: patients with spontaneous clearance; Group C: control group. a: one-way ANOVA; b: Kruskal-Wallis test; c: X² test.

Distribution of the IL-18 gene promoter polymorphisms in patients and controls

Table 2 shows the genotype frequencies of the -137 G/C and -607 C/A polymorphisms in the IL-18 gene in patients and in the control group. A higher frequency of genotypes C/A and C/C of the -607 polymorphism was observed in patients when compared to controls (Table 2).

TABLE 2. Distribution of the IL-18 gene promoter polymorphisms (-137 G>C, -607 C>A) in patients and controls

SNP/ genotype	Patients (n=304) n (%)	Controls (n=376) n (%)	OR	95% CI	P
-607 C/A					
A/A	36 (11.8)	68 (18.1)	-	-	-
C/A	156 (51.3)	192 (51.1)	1.53	0.97-2.42	0.08
C/C	112 (36.9)	116 (30.8)	1.82	1.12-2.94	0.01
C/A+C/C	268	308	1.64	1.06-2.54	0.03
Alleles					
A	228 (37.5)	328 (43)	-	-	-
C	380 (62.5)	424 (57)	1.28	1.03-1.6	0.02
-137 G/C					
C/C	100 (32.9)	128 (34)	-	-	-
G/C	120 (39.5)	132 (35.1)	1.16	0.81-1.66	0.46
G/G	84 (27.6)	116 (30.9)	0.92	0.63-1.36	0.77
GC +GG	204	248	1.05	0.76-1.45	0.81
Alleles					
C	320 (52.6)	388 (51.5)	-	-	-
G	288 (47.4)	364 (48.5)	0.95	0.77-1.18	0.74

OR: odds ratio; 95% CI: 95% confidence interval.

Association of IL-18 gene polymorphisms with the resolution of HCV infection

To evaluate the influence of the IL-18 gene promoter polymorphisms (-137 G/C and -607 C/A) on the course of HCV infection, we compared allele frequencies between the patient groups: group A (persistent infection) and group B (spontaneous clearance). The frequency of allele C at position -607 was higher in patients with persistent infection than in those with spontaneous clearance (Table 3). No significant difference between patients was observed for the allele distribution at position -137.

TABLE 3. Association between IL-18 gene polymorphisms and PCR results

IL-18 polymorphism	Group A n (%)	Group B n (%)	OR	95% CI	P
-607 C/A					
A/A	14 (8)	22 (17)	-	-	-
C/A	96 (55)	60 (46)	2.51	1.19-5.28	0.02
C/C	64 (37)	48 (37)	2.09	0.97-4.51	0.06
C/A+C/C	160	108	2.32	1.14-4.70	0.02
-137 G/C					
C/C	62 (35.6)	38 (29.2)	-	-	-
G/C	64 (36.8)	56 (43.1)	1.42	0.83-2.45	0.24
G/G	48 (27.6)	36 (27.7)	1.22	0.67-2.21	0.60
G/C+G/G	112	92	1.34	0.82-2.18	0.29

Group A: patients with persistent infection; Group B: patients with spontaneous clearance. OR: odds ratio; 95% CI: 95% confidence interval.

DISCUSSION

Progression of hepatitis C varies between patients. There is sufficient evidence suggesting that the cause of progression is multifactorial and includes viral, immunological and genetic factors⁽¹³⁾.

Among viral factors, the infecting HCV genotype seems to play an important role. In the present study, a higher frequency of genotype 1 was observed in the population studied. In addition, the prevalence of genotype 1 was higher among patients with persistent infection compared to those with spontaneous clearance.

Differences in the distribution of HCV genotypes between different geographic areas have been demonstrated. In Brazil, genotype 1 is the most frequent, followed by genotypes 3 and 2⁽⁷⁾. Similar results have been reported for the state of Pará^(17,19).

Studies conducted in different countries suggest that the genomic heterogeneity of HCV has a significant impact on the severity of liver disease and on the response to treatment. In this respect, genotype 1 has been associated with severe liver damage^(14, 16, 21).

Cytokines play a crucial role in the differentiation, maturation and functional activation of immune cells, as well as in the regulation of the immune response that controls the clearance or persistence of HCV^(1, 13). The main genes involved in the progression of disease and persistence of HCV are related to viral replication and to triggering the immune response⁽¹³⁾. Host genetic factors have been implicated in the persistence of HCV infection.

The inflammatory cytokine IL-18 has been indicated as a marker of inflammation and liver damage^(6, 9). Two SNPs have been described in the promoter region of the IL-18 gene at positions -607 C/A (rs1946518) and -137 G/C (rs187238).

These polymorphisms are associated with the transcription activity of the IL-18 promoter and cause a reduction in serum IL-18 levels^(2, 10).

Some research groups suggested that these polymorphisms influence the clinical evolution of HCV infection. In the present study, comparison of the -607 C/A and -137 G/C polymorphisms between HCV-infected patients and controls showed a higher frequency of allele C at position -607 in the patient group. In addition, genotype C/A at position -607 was associated with a higher risk of persistent infection with HCV (OR=2.51).

Similar studies conducted in America⁽¹⁾, India⁽¹³⁾, and Europe⁽¹¹⁾ also showed that the mutation from C to A at position -607 was associated with viral clearance and protection against severe liver disease in the populations studied.

CONCLUSION

The importance of -607 and -137 polymorphisms in the IL-18 gene promoter for HCV infection should be further studied, but our studies suggest an association between the polymorphism -607 and the persistence of viral infection.

Authors' contributions

Santos KN e Almeida MKC: realization of tests for molecular detection of IL-18 polymorphism. Fecury AA: realization of serological and molecular testing for HCV infection. Costa CA: clinical monitoring of patients. Martins LC: writing and statistical analysis of the article.

Santos KN, Almeida MKC, Fecury AA, Costa CA, Martins LC. Análise do polimorfismo na região promotora do gene da interleucina 18 (-137 G/C e -607 C/A) em pacientes portadores do vírus da hepatite C na Amazônia Brasileira. *Arq Gastroenterol.* 2015;52(3):222-7.

RESUMO - Contexto - O vírus da hepatite C é reconhecido como a maior causa de doença hepática crônica no mundo. Fatores genéticos do hospedeiro têm sido implicados na persistência da infecção pelo vírus da hepatite C. Polimorfismos de nucleotídeos únicos na posição -607 C/A (rs1946518) e -137 G/C (rs187238) na região promotora do gene da IL-18 têm sido evidenciados em alguns estudos que sugerem sua associação ao atraso na depuração do vírus da hepatite C e na persistência da doença. **Objetivo** - O presente estudo pretende identificar esses polimorfismos em uma população da região da Amazônia Brasileira infectada pelo vírus da hepatite C. **Métodos** - Estudo do tipo transversal analítico no município de Belém-PA foi realizado em 304 pacientes infectados pelo vírus da hepatite C, divididos em: grupo A, pacientes com infecção persistente; grupo B, pacientes que apresentaram clareamento viral. Como grupo controle participaram 376 voluntários não infectados pelo vírus da hepatite C. As amostras foram submetidas à RT-PCR, para detecção do RNA viral e, posteriormente, à RFLP-PCR para avaliação do polimorfismo na região promotora do gene da IL-18, nas posições -137 G/C e -607 C/A. **Resultados** - A comparação das frequências dos polimorfismos no grupo de pacientes com o grupo controle demonstrou uma maior frequência do alelo C na posição -607 entre os pacientes ($P=0,02$) que no grupo controle. Na análise da associação dos polimorfismos com a infecção viral foi obtido que portadores do genótipo C/A na posição -607 possuíam maior risco de infecção persistente pelo vírus da hepatite C ($P=0,03$). **Conclusão** - Nossos resultados sugerem uma possível implicação do polimorfismo da região promotora -607 do gene IL-18 na patogenia da infecção pelo vírus da hepatite C.

DESCRITORES - Polimorfismo de nucleotídeo único. Interleucina-18. Hepatite C. Hepacivirus.

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