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ORIGINAL ARTICLE

Combined effect of pro- and anti-inflammatory cytokine gene polymorphisms on susceptibility to liver cirrhosis in Tunisian HCV-infected patients

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Abstract
Purpose Chronic hepatitis C progression is commonly attributed to the continuous activation of the immune response with an increased production of pro-inflammatory cytokines, leading to fibrosis and ultimately to cirrhosis. On the contrary, anti-inflammatory cytokines, mainly interleukin (IL)-10 have a modulatory effect on hepatic fibrogenesis. The association between individual polymorphisms within cytokine genes and hepatitis C outcome is often weak and non-informative. Interestingly, it has been demonstrated that a combination of specific genotypes may be a more significant and powerful approach for predicting disease risk.
Aim This study is aimed at investigating the combined effect of single nucleotide polymorphism (SNP) in IL-18 (−607C/A, −137G/C), interferon (IFN)-γ (+874T/A) and IL-10 (−1082G/A) genes on cirrhosis risk in HCV-infected patients.
Methods Seventy-seven chronic hepatitis C Tunisian subjects were included in this study. The patients were divided into two groups: the first included 31 non-cirrhotic patients, and the second included 46 liver cirrhosis patients. IL-18 genotyping was performed using the PCR amplification and the restriction fragment length polymorphism analysis (RFLP). IFN-γ and IL-10 polymorphisms were analyzed using the allele-specific PCR (AS-PCR).
Results The combined high-risk genotype (IL-18 −607C/*, IL-18 −137G/*, IFN-γ +874T/*, IL-10 −1082A/A) frequency was compared between patients with and those without cirrhosis. Individuals were classified according the number of high-risk genotypes as follows: (0–2), patients with at most two high-risk genotypes; (3–4), patients with at least three of the high-risk genotypes. The logistic regression analysis showed that patients harboring 3–4 putative high-risk genotypes have a fivefold higher risk for developing cirrhosis in comparison to those harboring at most two high-risk genotypes (OR = 5.19; 95% CI = 1.49–18.05; $p = 0.009$).
Conclusion Our study showed that the co-inheritance of IL-18, IFN-γ and IL-10 specific high-risk genotypes is associated with a greater risk for liver cirrhosis.

Keywords Hepatitis C · Cirrhosis · Cytokine · Polymorphism · Combined analysis

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Introduction

Today, there is an increasing evidence that the immune response against hepatitis C virus (HCV), in particular, the cell-mediated response and the host genetic factors contribute to the natural history of HCV infection [1–6]. Clinical research showed the prominent role of T helper cell type 1 (Th1) or pro-inflammatory and type 2 (Th2) or anti-inflammatory cytokines in the pathogenesis of chronic hepatitis C. Indeed, a positive correlation between Th1

cytokine levels or Th1-type cell percentage and histological fibrosis or progressive liver injury in chronic hepatitis C was detected [7]. On the contrary, Th2 cytokines modulate self-inflicted injury by suppressing the Th1 response leading to milder forms of chronic hepatitis C [8–11]. Actually, in the absence of an efficient eradication of the infected cells, the continuing inflammation is responsible for liver damage leading to fibrosis and ultimately cirrhosis [12, 13]. Accordingly, it was supposed that polymorphisms in genes that potentially influence the immune response are candidates for influencing hepatitis C outcome.

One of the most important Th1 cytokines is IFN-γ, a pro-inflammatory cytokine already implicated in hepatic pathology [13–16]. IFN-γ synthesis is downregulated by IL-10, a potent anti-inflammatory Th-2 cytokine that also has a modulatory effect on hepatic fibrogenesis [17–20]. Similarly, the involvement of IL-18, well known for its contribution to the stimulation of Th1 response and the induction of inflammatory cytokines, has already been thoroughly investigated [21, 22]. Several studies including ours have reported a positive correlation between IL-18 level and hepatitis C severity [10, 23–26].

Cytokine production is regulated at the genetic level [27]. Therefore, the majority of studies related to associations between polymorphisms within immune genes and hepatitis C outcome have focused on cytokine genes, and particularly on those participating to Th1 and/or Th2 immune responses [28–33]. We have previously reported association studies of the IL-18, IFN-γ, and IL-10 single nucleotide polymorphisms with chronic hepatitis C outcome [25, 34]. In accordance with our findings, some significant associations with regard to IL-18 and IFN-γ polymorphisms have been observed [30, 35]. However, other studies revealed opposing results [36, 37]. These contradictory results could be partly explained by the extreme complexity in the cytokine network related to the intricate interactions of cytokines by which they induce or suppress their own synthesis or that of other cytokines, and antagonize or synergize with each other in many different and often redundant ways. On the other hand, it is now obvious that interactions between host genetic polymorphisms should be taken into account in defining complex and multifactorial disease risk, such as hepatitis C. Although the risk attributed to an individual polymorphism is often very small, it has been repeatedly demonstrated that a combination of specific genotypes may be a more significant and powerful approach in predicting disease risk [38–41].

So far, a few studies have examined multiple loci simultaneously for determining hepatitis C progression risk. Accordingly, this study is aimed at investigating the combined effect of single nucleotide polymorphisms in IL-18 (−607C/A, −137G/C), IFN-γ (+874T/A) and IL-10 (−1082G/A) genes on liver cirrhosis risk in Tunisian HCV-infected patients.

Materials and methods

The following points have been discussed under the above head.

Patients

This study included 77 Tunisian chronic hepatitis C subjects who were hospitalized or who visited the gastroenterology unit of La Rabta Hospital during the study period from May 2005 to March 2008 (21 male, 56 female; mean age 56.4 years, range 37–77). The diagnosis of all patients was made by biochemical and molecular assays, including the detection of anti-HCV antibodies using the third generation commercial enzyme immunoassays (INNOTEST HCV Ab III, Innogenetics-Belgium and Murex anti-HCV, Murex Diagnostics, Chatillon, France) and the detection of HCV RNA in serum (Amplicor HCV assay, Roche Diagnostics, Mannheim, Germany). Patients presenting with other causes of chronic hepatitis (i.e., alcohol or autoimmune hepatitis), testing positive for other hepatotropic viral antigens and treated with antiviral drugs prior to liver biopsy or echography were excluded.

Liver cirrhosis was diagnosed from the histological examination of liver biopsy samples by two blinded anatomopathologists or from clinical and ultrasonographic analysis. The patients were divided into two groups: the first included 31 non-cirrhotic patients with chronic hepatitis, and the second included 46 patients with liver cirrhosis.

Approval for the study was given by the National Ethical Committee, and informed consent was obtained from each patient.

DNA extraction

Peripheral blood (5 ml) was collected in ethylenediaminetetraacetic acid (EDTA) tubes. Genomic DNA was extracted from peripheral leukocytes using the standard method (salting out procedure) [42] and stored at −20°C. Briefly, blood cells were mixed with Triton lysis buffer (0.32 M sucrose, 1% Triton X-100, 5 mM $MgCl_2$, 10 mM Tris–HCl, pH 7.5). The leukocytes were spun down and washed with sterilized H_2O. The pellet was incubated with proteinase K at 56°C and subsequently salted out at 4°C using a 5 M NaCl solution. The precipitated proteins were removed by centrifugation. DNA in the supernatant fluid was precipitated with ethanol. The DNA pellet was dissolved in 400 µl sterilized H_2O and stored at −20°C until use.

Genotyping

The IL-18 −607C/A, IL-18 −137G/C, IFN-γ +874T/A and IL-10 −1082G/A genotypes were determined as previously described [25, 34].

Briefly, IL-18 polymorphisms were genotyped by PCR amplification and RFLP analysis using the primers: 5′-GC CCTCTTACCTGAATTTTGGTAGCCCTC-3′ (forward) and 5′-AGATTTACTTTTCAGTGGAACAGGAGTCC-3′ (reverse) for IL-18 −607C/A genotyping and 5′-ATG CTTCTAATGGACTAAGGA-3′ (forward) and 5′-GTAAT ATCACTATTTTCATGAATT-3′ (reverse) for IL-18 −137G/C genotyping.

For the IL-18 −607C/A genotyping, a 171 bp PCR amplification fragment was digested with the restriction enzyme Tru9I. The −607A allele was cut into two fragments of 101 and 70 bp while the −607C allele remained uncut (171 bp). For the IL-18 −137G/C genotyping, a 131 bp PCR amplification fragment was digested using the restriction enzyme EcoRI. The −137G allele was cut into two fragments of 107 and 24 bp while the −137C allele remained uncut (131 bp). All products were electrophoresed on 3% agarose gel containing ethidium bromide.

Genotyping for the polymorphisms IFN-γ +874T/A and IL-10 −1082G/A was carried out by the AS-PCR assay. Three specific primers were used for each polymorphism [43, 44]. For IFN-γ +874T/A genotyping, forward primer specific to IFN-γ +874T allele (5′-TTCTTACAACA CAAAATCAAATCT-3′) or IFN-γ +874A allele (5′-TTC TTACAACACAAAATCAAATCA-3′) and a common reverse primer (5′-TCAACAAAGCTGATACTCCA-3′) were used. In IL-10 −1082G/A polymorphism genotyping, the specific primer sequences used were as follows: the forward primers IL-10 −1082G: 5′-CTACTAAGGCTT CTTTGGGAG-3′ or IL-10 −1082A: 5′-ACTACTAAGG CTTCTTTGGGAA-3′ and the reverse primer 5′-CAGTGC CAACTGAGAATTTGG-3′.

In each reaction, an internal control primer pair which amplifies a human growth hormone (HGH) sequence (forward: 5′-GCCTTCCCAACCATTCCCTTA-3′; reverse: 5′-TCACGGATTTCTGTTGTGTTTC-3′) was used to check for successful PCR amplification.

Statistical analysis

The statistical analyses were made using the Epi-info statistical program (version 5.01a; 1991; Centers for Disease Control and Epidemiology Program office, Atlanta, Georgia, USA) and SPSS 13.0 software (SPSS, Chicago, IL, USA). The differences in combined genotype frequencies between patient groups were analyzed by 2 × 2 tables using χ^2 analysis or Fisher's exact test if one or more variables in 2 × 2 tables were less than five. Odds ratio (OR) and 95% confidence intervals (CI) were calculated to assess the relative disease risk conferred by a combination of high-risk genotypes. Adjusted OR for sex, age, and the number of high-risk genotypes was estimated by logistic regression analysis. All statistical tests were two tailed and $p < 0.05$ values were considered statistically significant.

Results

In the present study, our analysis was focused on the joint contribution of IL-18 (−607C/A and −137G/C), IFN-γ (+874T/A) and IL-10 (−1082G/A) polymorphisms to the increased risk of liver cirrhosis. Table 1 summarizes the demographic, virological and clinical characteristics of the 77 included patients genotyped for the four loci. Among them, 31 subjects (40%) presented with liver cirrhosis. The HCV genotypes were determined in 62 patients. The distribution was characterized by the predominance of HCV-1 genotype which was detected in 54 patients (70%).

Genotype frequency analyses of IL-18, IFN-γ, and IL-10 gene polymorphisms with regard to chronic hepatitis C severity were previously published [25, 34]. Polymorphism genotype distribution in Tunisian HCV-infected patients with or without cirrhosis was summarized in Table 2.

The mean age for cirrhotic and non-cirrhotic patients at enrolment was 60.5 ± 9.2 and 50.7 ± 7.6, respectively ($p < 0.0001$). No significant differences with regard to sex distribution were noticed (Table 3).

To investigate the joint effect of the above-mentioned genetic polymorphisms, we compared the combined high-risk genotype frequencies (IL-18 −607C/*, IL-18 −137G/*, IFN-γT/*, IL-10A/A) between chronic hepatitis C patients with and without cirrhosis. The patients were classified according to the number of putative high-risk genotypes as

Table 1 Baseline characteristics of patients with chronic HCV infection

	Chronic hepatitis C patients $n = 77$ (f)
Age	56.4 ± 9.8
Gender	
Male	21 (0.27)
Female	56 (0.73)
HCV genotypes	
HCV-1	54 (0.70)
HCV non-1	8 (0.10)
Not done	15 (0.20)
Cirrhosis	
With	31 (0.40)
Without	46 (0.60)

Table 2 Summary of genotype frequencies of polymorphisms in IL-18, IFN-γ, and IL-10 genes in Tunisian chronic hepatitis C patients with or without liver cirrhosis [25, 34]

Genotypes (*n*)	Liver cirrhosis (−) (*f*)	Liver cirrhosis (+) (*f*)
IL-18 −607C/A ($n = 81$)	($n = 34$)	($n = 47$)
CC	0.27	0.32
CA	0.38	0.53
AA	0.35	0.15
CC + CA	0.65	0.85
IL-18 −137G/A ($n = 81$)	($n = 34$)	($n = 47$)
GG	0.44	0.49
GC	0.32	0.43
CC	0.24	0.08
GG + GC	0.76	0.92
IFN-γ +874T/A ($n = 100$)	($n = 42$)	($n = 58$)
TT	0.24	0.34
TA	0.24	0.36
AA	0.52	0.30
TT + TA	0.48	0.70
IL-10 −1082G/A ($n = 100$)	($n = 42$)	($n = 58$)
GG	0.22	0.18
GA	0.45	0.41
AA	0.33	0.41
GG + GA	0.67	0.59

follows: (0–2), patients with at most two high-risk genotypes; (3–4), patients with at least three of the high-risk genotypes. We observed that the frequency of having 3–4 putative high-risk genotypes was significantly increased in cirrhotic patients compared to non-cirrhotic patients (0.78 vs. 0.42, respectively; $p = 0.002$). Patients with at least three high-risk genotypes showed a fivefold risk of developing liver cirrhosis in comparison to those with at most two high-risk genotypes (OR = 4.98; 95% CI = 1.65–15.46). In a logistic regression model that included age and sex, the adjusted OR for the number of high-risk genotypes was statistically significant (5.19; 95% CI = 1.49–18.05; $p = 0.009$) (Table 3).

Discussion

Hepatitis C virus infection is the leading cause of chronic liver disease worldwide and may, in some patients, develop into liver cirrhosis and hepatocellular carcinoma [45, 47]. The progression of chronic hepatitis is highly variable among individuals as a result of several factors including quasispecies diversity, viral genotype, gender, age at infection, alcohol intake, diabetes, and duration of infection [48, 49]. However, these viral, demographic, and environmental factors account for only a small proportion of the variability [50, 51]. Nowadays, it is well established that the host immune response, which is regulated by the host genetic background, plays a prominent role in the natural history of the disease [3, 8, 52]. A positive correlation between a high level of pro-inflammatory cytokines secreted in response to HCV-related liver injury, and an increased necroinflammatory activity, liver fibrosis or cirrhosis was previously reported [8, 10, 11, 53].

Cytokine production varies among individuals and is associated with certain mutations within the coding and the regulatory regions [27]. In a previous study, we reported that IFN-γ +874T allele or a pro/anti-inflammatory response imbalance mediated by polymorphisms in IFN-γ and IL-10 genes may influence the outcome of chronic HCV infection [34]. Furthermore, we previously suggested that the carriage of at least one allele C at position −607 or G at position −137 of IL-18 gene seems to be a risk factor for developing more severe forms of chronic hepatitis in

Table 3 Hepatitis C-related liver cirrhosis estimated relative risk associated with the number of putative high-risk genotypes of IL-18, IFN-γ, and IL-10 genes

	Liver cirrhosis (−) $n = 31$ (*f*)	Liver cirrhosis (+) $n = 46$ (*f*)	OR (95% CI)[b]	p^b	OR (95% CI)[c]	p^c
Age	50.7 ± 7.6	60.5 ± 9.2	–	<0.0001	–	0.001
Sex						
Male	5 (0.16)	16 (0.35)	2.77 (0.80–10.12)	0.07	1.54 (0.38–6.25)	0.539
Female	26 (0.84)	30 (0.65)	1			
No. of high risk genotypes[a]						
0–2	18 (0.58)	10 (0.22)	1		1	
3–4	13 (0.42)	36 (0.78)	4.98 (1.65–15.46)	0.002	5.19 (1.49–18.05)	0.009

[a] IL-18 −607C/*, IL-18 −137G/*, IFN-γT/*, IL-10AA were considered as putative high-risk genotypes

[b] Crude odds ratios

[c] Odds ratios adjusting by sex and age

Tunisian HCV-infected patients [25]. Similarly, −607AA genotype of IL-18 has been associated with mild HCV-related liver disease in a larger Indian cohort [30]. However, there are contradictory reports about the implication of IFN-γ +874T/A polymorphism to liver fibrosis in HCV-infected patients. In line with our finding, a large scale study conducted by Dai et al. [35] on Taiwanese HCV-infected patients described a significant increase of IFN-γ +874T allele in cirrhotic patients. Nevertheless, other studies failed to demonstrate any association between T allele and hepatitis C severity [36, 37].

In reality, in multifactorial diseases like HCV infection, where the host, the pathogen and the environmental factors all contribute to disease outcome, genetic association studies focused on a single polymorphism are often weak and non-informative. Therefore, studying the joint contribution of multiple loci that interact in the same biological pathway, such as inflammation, fibrogenesis, angiogenesis, etc., seems to be more significant in predicting disease risk by enhancing the major effects of a single gene [39, 40, 54, 55]. In this regard, Ahluwalia et al. [56] reported a tenfold increased risk of nephropathy conferred by the co-occurrence of risk-associated genotypes of three inflammatory genes, CCL2, CCR5, and MMP9 in type 2 diabetes patients. Similarly, Chen et al. [54] found that the risk of hepatitis B-related hepatocellular carcinoma increased with the number of high-risk genotypes of IL-1B, TNF-α, hMLH1, and XRCC1 genes.

In HCV infection, the immune disorder characterized by an imbalance between pro- and anti-inflammatory responses plays a major role in disease outcome. Different pro-inflammatory cytokines including IL-18 and IFN-γ have been associated to fibrosis severity and cirrhosis [10, 14–16, 23–26]. IL-18 and IFN-γ synthesis is downregulated by IL-10, a potent anti-inflammatory cytokine that also has a modulatory effect on hepatic fibrogenesis [17, 57, 58]. Louis et al. [18] have reported the antifibrogenic property of IL-10 in carbon tetrachloride (CCl_4)-treated mice. More interestingly, a decrease in liver fibrosis has been observed in chronic hepatitis C patients treated with recombined IL-10 [19, 20]. Polymorphism at position −1082 of IL-10 gene was reported to have an independent influence on IL-10 production [59–62]. The −1082AA genotype was associated with a low IL-10 production and with a higher risk of a worse clinical progression of HCV or HBV infection [29, 31].

Taken together, this study is aimed at investigating the joint effect of high-risk genotypes of IL-18, IFN-γ, and IL-10 genes on liver cirrhosis risk in Tunisian HCV-infected patients. Individuals were classified according to the number of high-risk genotypes (IL-18 −607C/*, IL-18 −137G/*, IFN-γT/*, IL-10A/A) as follows: (0–2), patients with at most two high-risk genotypes; [3, 4], patients with at least three of the high-risk genotypes. The logistic regression analysis showed that, in chronic hepatitis C, a predominant pro-inflammatory profile stemming from the co-inheritance of 3–4 putative high-risk genotypes of IL-18, IFN-γ, and IL-10 is an independent factor associated with a greater risk for liver cirrhosis. Interestingly, this result seems to be more relevant compared to those we reported previously on each individual polymorphism [25, 34]. It underscores the hypothesis that the pro/anti-inflammatory response imbalance toward a pro-inflammatory cytokine profile is associated with HCV-related fibrosis aggressiveness and the risk of cirrhosis [8–11, 26, 53, 63].

Our finding provides further evidence concerning the role of inter-individual genetic variations within pro/anti-inflammatory cytokine genes in hepatitis C pathogenesis. We believe that it could be possible to define a specific genetic profile associated with the highest risk for cirrhosis development and therefore identify those patients who require a more aggressive therapeutic management.

In conclusion, we suppose that some additional genetic studies using new multi-locus analysis approaches, such as the multiple-dimensionality reduction approach, should be considered for a deeper analysis of the epistatic interaction of the pro-inflammatory molecules toward hepatitis C progression. It is hoped that a better understanding of the genetic factors that influence hepatitis C progression will provide a scientific basis for the development of new immunomodulatory treatments for chronic hepatitis C patients.

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