Lack of effect of tumor necrosis factor-alpha -308 G/A polymorphism on severity of liver fibrosis in Tunisian hepatitis C virus (HCV)-infected patients

Recherche d’association entre le polymorphisme -308 G/A du gène du facteur nécrosant des tumeurs de type alpha (TNF-α) et la sévérité de la fibrose hépatique chez des patients Tunisiens infectés par le virus de l’hépatite C

N. Bouzgarrou, E. Hassen, S. Gabbouj, E. Schvoerer, N. Ben Mami, H. Triki, L. Chouchane

Molecular Immuno-Oncology Laboratory, Faculty of Medicine, 5019 Monastir, Tunisia
Institute of virology, Strasbourg, France
Gastroenterology B Unit, La Rabta Hospital, Tunis, Tunisia
Clinical Virology Laboratory, Pasteur Institute, Tunis, Tunisia
Genetic Medicine Department, Weill Cornell Medical College, Qatar

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Summary
Objectives. — Tumor necrosis factor alpha (TNF-α) plays a key role in the immune response. An elevated plasma level of TNF-α was repeatedly observed in patients with active liver injury or cirrhosis regardless of the aetiology. The G/A transition at position -308 in the promoter region have been shown to influence TNF-α expression. In this study, we aimed to evaluate the impact of TNF-α -308 G/A functional polymorphism on fibrosis severity in Tunisian Hepatitis C Virus (HCV)-infected patients.

Methods. — TNF-α -308 G/A polymorphism was evaluated by polymerase chain reaction (PCR) amplification followed by Restriction Fragment Length Polymorphism (RFLP) method in 53 chronic hepatitis C patients. Single-nucleotide polymorphism (SNP) frequencies were compared with regard to liver fibrosis severity as assessed by the META VIR scoring system (F1—F2; n = 22 versus F3—F4; n = 31).

Results. — The genotype distribution of the TNF-α -308 G/A polymorphism among the HCV-infected patients was as follows: GG: 67.9%, GA: 32.1%, AA: 0%. With regard to fibrosis score, no significant differences in TNF-α genotype distribution were observed between F1—F2 and F3—F4 patients (p = 0.15).

* Corresponding author.
E-mail address: nadia.bouzgarrou@lycos.com (N. Bouzgarrou).

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Conclusion. — No significant association between TNF-α -308 polymorphism and and the severity of liver fibrosis was found in our Tunisian cohort.

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Résumé
Objectifs. — Le facteur nécrosant des tumeurs de type alpha ou tumor necrosis factor alpha (TNF-α) représente un élément clé de la réponse immunitaire. Quelle que soit l’étiologie, des taux sériques élevés ont souvent été observés dans des cas de fibroses hépatiques sévères, voire même de cirrhoses. La transition G/A en position −308 du gène semble influencer l’expression du TNF-α. Le but du présent travail est d’étudier l’implication du polymorphisme fonctionnel −308 G/A du gène TNF-α dans la sévérité de la fibrose hépatique chez des patients tunisiens chroniquement infectés par le virus de l’hépatite C (VHC).

Méthodes. — L’étude du polymorphisme −308 G/A du TNF-α a été réalisée par la technique PCR-RFLP chez 53 patients infectés par le VHC. La fréquence génotypique du polymorphisme étudié a été comparée entre les patients regroupés selon le score de fibrose METAVIR (F1–F2 versus F3–F4).

Résultats. — La distribution du polymorphisme −308 G/A du TNF-α chez les sujets infectés par le VHC est la suivante: GG : 67,9 %, GA : 32,1 %, AA : 0 %. L’analyse statistique des fréquences génotypiques n’a montré aucune différence significative entre les patients F1–F2 et F3–F4 (p = 0,15).

Conclusion. — Nos résultats suggèrent l’absence d’association entre le polymorphisme −308 G/A du gène TNF-α et la sévérité de la fibrose hépatique dans notre population tunisienne.

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Introduction

Hepatitis C virus (HCV) infection is the major causative agent of chronic liver disease characterized by the persistence of necroinflammatory damage that can progress to cirrhosis and hepatocellular carcinoma (HCC) at a rate of 1–4% per year [1–3]. HCV infection pathogenesis is not fully understood. Today there is increasing evidence that immunological and host genetic factors contribute to the natural history of HCV infection [4,5]. Immune response against HCV, in particular the cell-mediated response by the action of the cytokine system, is reported to contribute to both viral clearance [6,7], treatment response and disease severity [8]. Indeed, in the absence of an efficient eradication of the infected cells, the continuing inflammation is responsible for liver damage resulting in fibrosis development [9,10].

Tumor necrosis factor (TNF)-α, a pro-inflammatory cytokine with direct antiviral effects [11,12], plays a key role in the regulation of the cellular immune response in HCV infection [13,14]. It also directly contributes to the process of hepatic fibrosis by activating Kupffer cells, which in turn stimulate hepatic stellate cells, the principal cells implicated in liver fibrogenesis [15]. Some previous studies have reported a correlation between baseline TNF-α level and either histological grading score of hepatitis or response to IFN-α based therapy [8,16,17]. Nelson et al. described a decrease in serum TNF-α levels and an improvement of liver histology or a reduction of fibrosis in chronic hepatitis C “non responder” patients after a 12-week course of recombinant IL-10 therapy [18].

Accordingly, it was supposed that the genetic variation affecting TNF-α expression might influence chronic hepatitis C outcomes. In fact, cytokine genes are polymorphic at specific sites, and some of these mutations within the coding and regulatory regions (i.e., promoter sequences), have been associated with a different expression level of the specific cytokines [19].

The TNF-α gene is located in the HLA class III region in the 6p21.3 band of the short arm of chromosome 6. The production of TNF-α is regulated at the transcriptional level by modulating the transcription factor binding. The single nucleotide polymorphism (SNP) consisting of a G-to-A transition at position −308 (TNF1 allele to TNF2 allele) was associated with an increased TNF-α expression as demonstrated by a reporter gene assay [20,21].

TNF-2 allele was associated with poor prognosis of several auto-immune and infectious diseases such as leishmaniasis [22] and HIV infection [23]. Nevertheless, the data about its implication in hepatitis C progression and severity are inconsistent. This study is aimed at determining whether TNF-α polymorphism is associated with fibrosis severity as suggested by liver biopsy in Tunisian HCV infected patients.

Material and methods

Patients and controls

This study included 53 Tunisian chronic hepatitis C subjects who attended the Gastroenterology Unit of La Rabta hospital from May 2005 to March 2008 (14 men, 39 women; mean age 53.1 years, range 37–74). One hundred and three healthy controls testing negative for HCV infection were selected randomly from blood donors (42 men, 61 women, mean age, 46 years, range 30–75). Patients and controls were matched for ethnicity and for geographical area. The diagnosis of all patients was made by biochemical and molecular assays, including the detection of anti-HCV antibodies using the 3rd generation commercial enzyme immunoassays (INNOTEST...
Tumor necrosis factor-alpha -308 G/A polymorphism and liver fibrosis

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) [24] and stored at −20 °C. Briefly, blood cells were mixed with Triton lysis buffer (0.32 M sucrose, 1% Triton X-100, 5 mM MgCl₂, 10 mM Tris-HCl, pH = 7.5). The leukocytes were spun down and washed with sterilized H₂O. 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 H₂O and stored at −20 °C until use.

The G to A transition at −308 position of TNF-α gene was determined using a PCR followed by digestion with the endonuclease Ncol. Two sequence specific oligonucleotide primers were used for the polymerase chain reaction (PCR): the 3’ primer (5’-TCCTCCCTGCTCCGATTCCG-3’) was used in combination with the 5’ primer (5’-AGGCAATAGGTTTTGAGGGCCAT-3’). A twenty-five-microliter PCR mixture was composed of genomic DNA samples (100 ng), 200 mM dNTPs, 1.5 mM MgCl₂, 1 × Taq polymerase buffer, 50 pmol of each primer, and 0.5 U of Taq DNA polymerase (Amersham, Paris, France). Thermal cycling was performed in a Biometra thermal cycler (Göttingen, Germany). The reaction conditions were as follow: 95 °C for 5 minutes; 29 cycles of 95 °C for 30 seconds, 60 °C for 30 seconds and 72 °C for 45 seconds all followed by final extension step at 72 °C for 10 minutes.

The amplified fragments (107 bp) were digested with Ncol and analyzed by a 3% agarose-gel electrophoresis. The wild-type allele G or TNF1 was indicated by the cleavage of the 107-bp amplified product by Ncol resulting in products of 87 and 20 bp. The mutant allele A or TNF2 resisted the digestion (fragment length 107 pb).

Statistical analysis

The allele frequencies were tested for the Hardy-Weinberg equilibrium using the X² test. The same test was used to evaluate any significant differences in the TNF-α genotype frequencies among subgroups of patients. The Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated to estimate the relative disease risk. The data were analyzed using the Epi-Info statistical program (version 5.01a-1991; Centers for Disease Control and Epidemiology Program office, Atlanta, GA). Group means were compared using the student’s t-test. All statistical tests were two tailed and p < 0.05 values were considered statistically significant.

Table 1 summarizes the main features of the patients studied. HCV genotype distribution was as follows: genotype 1 was found in 40 patients (75.5%), genotype 2 in four patients (7.5%), genotype 4 was found in one patient (1.9%), mixed genotype in one patients (1.9%) and the genotype was undetermined in seven patients (13.2%). As for liver fibrosis, 41.5% of patients presented with mild to moderate fibrosis (F1 or F2) and 58.5% with severe fibrosis (F3 or F4). Concerning the pre-treatment plasma HCV RNA load, 67.9% patients presented with an elevated RNA load (≥ 400,000 UI/mL) and 32.1% with a low RNA load (< 400,000 UI/mL). The mean pre-treatment alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were 104 ± 70.4 and 82.5 ± 49.4, respectively.

Table 1: Demographic, virologic and clinical characteristics of the 53 chronic hepatitis C patients.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>%</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>53.1±8.94</td>
<td></td>
</tr>
<tr>
<td>AST (UI/L)</td>
<td>82.5±49.4</td>
<td></td>
</tr>
<tr>
<td>ALT (UI/L)</td>
<td>104±70.4</td>
<td></td>
</tr>
<tr>
<td>HCV genotype</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4 (75.5%)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>4 (7.5%)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1 (1.9%)</td>
<td></td>
</tr>
<tr>
<td>Mixed</td>
<td>1 (1.9%)</td>
<td></td>
</tr>
<tr>
<td>Undetermined</td>
<td>7 (13.2%)</td>
<td></td>
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<tr>
<td>Plasma HCV RNA load</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥ 400,000 UI/L</td>
<td>37 (67.9%)</td>
<td></td>
</tr>
<tr>
<td>&lt; 400,000 UI/L</td>
<td>16 (32.1%)</td>
<td></td>
</tr>
<tr>
<td>Fibrosis score</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1/F2</td>
<td>22 (41.5%)</td>
<td></td>
</tr>
<tr>
<td>F3/F4</td>
<td>31 (58.5%)</td>
<td></td>
</tr>
<tr>
<td>Cirrhosis (n,%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>With</td>
<td>16 (30%)</td>
<td></td>
</tr>
<tr>
<td>Without</td>
<td>37 (70%)</td>
<td></td>
</tr>
</tbody>
</table>
| ALT: alanine aminotransferase; AST: aspartate aminotransferase; HCV: hepatitis C virus.
Relationship between patient characteristics and the severity of liver fibrosis

As shown in Table 2, the two patient groups (F1/F2 and F3/F4) were comparable in terms of age, gender, ALT and AST/ALT ratio, HCV RNA load and HCV genotype distribution. However, patients with severe fibrosis (F3/F4) presented a higher AST level than those with moderate fibrosis (F1/F2) (61.4 ± 27.3 UI/L vs. 95.8 ± 55.8, respectively; p = 0.03).

Differential distribution of TNF-α genotypes according to hepatitis severity

Genotype distributions of the −308 G/A polymorphism of the TNF-α gene were consistent with the Hardy–Weinberg equilibrium. No statistically significant differences in genotype distribution were observed between HCV infected patients and controls (GG: 67.9, GA: 32.1, AA: 0 and GG: 67, GA: 31, AA: 2%; respectively).

To evaluate the association of this functional polymorphism with hepatitis severity, the differential distribution of TNF-α genotypes was analysed with regard to the stage of liver fibrosis. As indicated in Table 3, our results showed no significant differences between patients with moderate fibrosis (GG: 59.1%, GA: 40.9%, AA: 0%) and those with severe fibrosis (GG: 77.4%, GA: 22.6%, AA: 0%) (p = 0.15; OR = 0.42; 95% CI: 0.11–1.62). In addition, TNF-α −308G/A polymorphism did not seem to have any significant effect on AST level (Table 3).

Discussion

HCV infection is the leading cause of chronic liver disease worldwide and may, in some patients, develop into liver cirrhosis and HCC [1–3]. Nowadays, it is well established that host-associated rather than virus-associated factors are involved in the pathogenesis of hepatitis C [25–28]. It is believed that the immune response, which is regulated by the host genetic background, plays a prominent role in the natural history of the disease. A positive correlation between a high level of pro-inflammatory cytokines, secreted in response to HCV-related liver injury, and an increased necroinflammatory activity or liver fibrosis was previously reported [29–31].

Hepatic fibrosis is driven by inflammatory responses to liver injury in most types of chronic liver diseases [9,10], with TNF-α as a key cytokine in this process [15,32–34]. Indeed, it has been demonstrated that TNF-α helps to activate hepatic stellate cells and to get into their activated myofibroblast-like phenotype, leading to extracellular matrix accumulation and fibrosis development [15,35]. Accordingly, an elevated plasma level of TNF-α was observed in patients with active liver injury or cirrhosis regardless of the aetiology [36–39]. In the case of HCV infection, an increased TNF-α level was correlated with the severity of hepatic inflammation and fibrosis [8,17]. In considering the strong relation between the TNF-α promoter genotype at position −308 and the cytokine expression level, this study is aimed at investigating the...
genotype distribution of TNF-α gene polymorphism and its possible effect on the HCV-related fibrosis severity in Tunisian HCV-infected patients.

Several of studies have reported a significant association between the high TNF-α production allele (A) and advanced liver fibrosis or the risk of cirrhosis in chronic HCV infection [40–42]. An accelerated graft injury was further observed in HCV-seropositive liver transplant patients who received donor liver with one or two A alleles at position −308 of TNF-α gene [43]. To confirm a possible association between TNF-α −308 polymorphism and fibrosis severity in Tunisian patients, the genotype frequency at position −308 was compared between patients with mild to moderate fibrosis (F1 to F2) and patients with severe fibrosis (F3 to F4). Both groups had approximately the same mean age (51.3 ± 9.08 vs. 54.3 ± 8.78).

Among the 53 Tunisian patients included in this study, 58.4% had severe fibrosis, 75.5% were infected with HCV genotype 1 and 67.9% presented an elevated HCV RNA load (> 400,000 UI/ml).

The TNF-α genotype frequency in Tunisian HCV-infected patients was quite different from that observed in other studies of Caucasian HCV-infected patients from Germany and Italy [41,44]. It was characterized, unlike the healthy subjects, by the absence of the mutant genotype (AA). However, this finding should be confirmed in a large cohort.

The comparison of the genotype distribution of TNF-α −308 polymorphism with regard to fibrosis severity revealed no significant differences. As shown in Table 4, our finding, although based on a small cohort, is concordant with other results from large and different ethnic populations that exclude the influence of TNF-α −308 polymorphism on the severity of HCV-related liver fibrosis [44–49]. No association between TNF-α −308 polymorphism and liver fibrosis stage was further observed in a large cohort of 273 Chinese chronic hepatitis B patients [50].

Investigations about the role of TNF-α −308 polymorphism in chronic hepatitis C outcome have actually produced inconclusive results [41,42,44–49]. The main reason of these discrepancies could be the possible modulation of the functional effect of TNF-α −308 polymorphism by interaction with other functional polymorphisms in the same gene or in other inflammatory pathway genes located either within or far away from the MHC locus [47,48], since gene-gene interactions may play a role in the mechanisms of complex diseases by weakening or enhancing the major effects of a single gene [51]. Goyal et al. reported a linkage between TNF-β NcoI AA and TNF-α −308 GG alleles, which seems to confer a 4.9-fold risk of HCV persistence [47]. The same team failed to demonstrate any association between TNF-α −308 polymorphism and susceptibility to HCV infection. Considering that TNF-β NcoI AA and GG genotypes were associated with a lower and a higher TNF-α expression levels respectively [52,53], and that TNF-β NcoI AA was associated with HCV-related fibrosis severity [47], it is obvious that other TNF system loci take concerted action for controlling TNF-α expression.

Furthermore, it is noteworthy that a strong linkage disequilibrium exists between TNF-α −308 alleles and HLA alleles. The latter was also incriminated in the worst outcome of hepatitis C [28]. TNF2 or A allele were found to be strongly associated with A1, B8 and DR3 alleles, whereas TNF1 or G allele were significantly associated with DR4 and DR6 alleles [54], implying that ethnicity may largely contribute to the differences in the relation between TNF-α −308 polymorphism and hepatitis C outcome [41,42,44–49]. Indeed, in a meta-analysis conducted by Chen and Pei investigating the possible effect of TNF-α −308 polymorphism on susceptibility to HCV infection, Asian subjects carrying A allele were found approximately 20% more likely to have HCV infection than non-Asian patients [55]. Besides, Mitchell et al. reported a linkage between the increased frequency of TNF 2 in primary sclerosing cholangitis patients and the presence of DRB1*0301 allele [56].

The conflict around the implication of TNF-α −308 polymorphism in hepatitis C progression may also be explained by the differences observed in the study design which particularly concerns the patient’s stratification strategy and/or the fibrosis scoring system, as summarized in Table 4.

Additionally, although liver histology is frequently considered as the gold standard method for assessing hepatic fibrosis, it is evident that it is associated with sampling error resulting from irregular distribution of liver fibrosis and interobserver or intraobserver variability [57,58]. At present, non-invasive serum markers such as AST/ALT ratio (AAR), age-platelet index (API) and AST/ PLT ratio index

### Table 4: Summary of the published studies on the relationship between −308 TNF-α genotype and the liver fibrosis severity in hepatitis C virus (HCV) infected patients.

<table>
<thead>
<tr>
<th>Country [References]</th>
<th>Number of subjects</th>
<th>−308 TNF-α genotype frequency (%)</th>
<th>Study design</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GG</td>
<td>GA</td>
<td>AA</td>
</tr>
<tr>
<td>Taiwan [43]</td>
<td>250</td>
<td>74.8</td>
<td>22.8</td>
<td>2.4</td>
</tr>
<tr>
<td>Italy [42]</td>
<td>186</td>
<td>82.0</td>
<td>13.0</td>
<td>5.0</td>
</tr>
<tr>
<td>India [48]</td>
<td>52</td>
<td>75.0</td>
<td>25.0</td>
<td>0</td>
</tr>
<tr>
<td>Tunisia [The present study]</td>
<td>53</td>
<td>67.9</td>
<td>32.1</td>
<td>0</td>
</tr>
<tr>
<td>USA [47]</td>
<td>110</td>
<td>74.0</td>
<td>24.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Spain [49]</td>
<td>242</td>
<td>73.1</td>
<td>26.9</td>
<td>0</td>
</tr>
<tr>
<td>Germany [45]</td>
<td>153</td>
<td>74.2</td>
<td>21.8</td>
<td>4.0</td>
</tr>
<tr>
<td>Australia [46]</td>
<td>128</td>
<td>72.0</td>
<td>22.0</td>
<td>6.0</td>
</tr>
<tr>
<td>Pakistan [50]</td>
<td>40</td>
<td>95.0</td>
<td>5.0</td>
<td>0</td>
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</table>
(APRI), GGT/PLT ratio index (GAPI) and AST to GGT ratio (AGR) are being tested to determine fibrosis score as an alternative to liver biopsy [59–61]. In chronic viral hepatitis, AST/ALT ratio (AAR) has been controversially associated with the extent of liver fibrosis and cirrhosis [62–66]. In our study only AST was associated with the severity of fibrosis, and this finding could be due to the impairment of AST clearance by the injured sinusoidal cells in advanced fibrosis [67]. AAR did not show any significant differences between fibrosis stage groups (F1–F2 vs. F3–F4). However, in accordance with the previous studies [62,64,68], we found a trend to a significant association between AAR and fibrosis severity when comparing chronic hepatitis C patients without cirrhosis to cirrhotic patients (data not shown). Accordingly, the diagnostic accuracy of the AAR for prediction of cirrhosis should be investigated in a large cohort. In the light of our findings, the relationship between TNF-α –308 polymorphism and hepatitis severity as suggested by AST level was analyzed, and the results were non-significant.

**Conclusion**

Our investigation of the relationship between the TNF-α –308 G/A polymorphism and the severity of HCV-related liver fibrosis demonstrated a lack of effect of such a polymorphism on disease aggravation. The combination of TNF-α gene polymorphisms and HLA haplotype analysis on larger different cohorts with regard to the severity of hepatitis C outcome should be considered.

**Conflict of interest**

The authors declare no conflict of interest.

**Acknowledgments**

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