Tumor necrosis factor-α -G308A polymorphism is associated with liver pathological changes in hepatitis C virus patients

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Studied the association of tumor necrosis factor-α (TNFα) -G308A polymorphism with different liver pathological changes in treatment-naïve Egyptian patients infected with hepatitis C virus (HCV) genotype 4.

**AIM**
To investigate the association of tumor necrosis factor alpha (TNFα) -G308A polymorphism with different liver pathological changes in treatment-naïve Egyptian patients infected with hepatitis C virus (HCV) genotype 4.

**METHODS**
This study included 180 subjects, composed of 120 treatment-naïve chronic HCV patients with different fibrosis grades (F0-F4) and 60 healthy controls. The TNFα -G308A region was amplified by PCR and the different genotypes were detected by restriction fragment length polymorphism analysis. The TNFα protein was detected by enzyme-linked immunosorbent assay. The influence of different TNFα -G308A genotypes on TNFα expression and liver disease progression were statistically analyzed. The OR and 95% CI were calculated to assess the relative risk confidence.
RESULTS

Current data showed that the TNFα -G308A SNP frequency was significantly different between controls and HCV infected patients (P = 0.001). Both the AA genotype and A allele were significantly higher in late fibrosis patients (F2-F4, n = 60) than in early fibrosis patients (F0-F1, n = 60) (P = 0.05, 0.04 respectively). Moreover, the GA or AA genotypes increased the TNFα serum level greater than the GG genotype (P = 0.002). The results showed a clear association between severe liver pathological conditions (inflammation, steatosis and fibrosis) and (GA + AA) genotypes (P = 0.035, 0.03, 0.04 respectively). The stepwise logistic regression analysis showed that the TNFα genotypes (GA + AA) were significantly associated with liver inflammation (OR = 3.776, 95%CI: 1.399-10.194, P = 0.009), severe steatosis (OR = 4.49, 95%CI: 1.441-14.0, P = 0.010) and fibrosis progression (OR = 2.84, 95%CI: 1.080-7.472, P = 0.034). Also, the A allele was an independent risk factor for liver inflammation (P = 0.003), steatosis (P = 0.003) and fibrosis (P = 0.014).

CONCLUSION

TNFα SNP at nucleotide -308 represents an important genetic marker that can be used for the prognosis of different liver pathological changes in HCV infected patients

Key words: Hepatitis C virus immune response; Tumor necrosis factor alpha; Single nucleotide polymorphisms; Cytokine expression; Liver disease progression

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Core tip: Tumor necrosis factor alpha (TNFα) is a proinflammatory and antiviral cytokine that plays a major role in liver injury and initiation of the fibrosis process. We investigated the association of TNFα -G308A polymorphism with liver pathological changes in treatment-naïve Egyptian patients infected with hepatitis C virus (HCV) genotype 4. The results showed that the TNFα A allele produced high circulating TNFα in the body, which induces inflammatory response. The TNFα A allele was an independent risk factor for liver inflammation, steatosis and fibrosis. TNFα -G308A represents an important genetic marker that can be used for the prognosis of different liver pathological changes in HCV infected patients.
diseases are associated with TNFα -G308A polymorphism, such as systemic lupus erythematosus, inflammatory bowel disease, insulin-dependent diabetes mellitus, malaria and leishmaniasis\textsuperscript{[13,15-17]}. In chronic HCV patients, elevated levels of TNFα were detected in liver and serum\textsuperscript{[8]}. TNFα increases fat deposition in the liver by affecting the hepatic lipogenesis\textsuperscript{[18]}. Furthermore, Hooper \textit{et al.}\textsuperscript{[19]} showed that increased levels of TNFα and reduced anti-inflammatory cytokines correlate with the severity of liver injury in non-alcoholic steatohepatitis (NASH) patients.

The role of TNFα -G308A polymorphism in HCV pathogenesis has been examined in different studies, with divergent results\textsuperscript{[6,20-22]}. These variations may be due to racial background of the studied populations, who have different cytokine gene polymorphisms\textsuperscript{[23]}. Also, these studies focused on the role of TNFα -G308A in HCV susceptibility and treatment response rate but none of these studies examined the role of TNFα -G308A polymorphism in HCV liver steatosis and fibrosis progression rate. The current study was designed to investigate the association of TNFα -G308A polymorphism with liver inflammation, steatosis and different grades of fibrosis in treatment-naïve Egyptian patients infected with HCV genotype 4.

**MATERIALS AND METHODS**

**Subjects**

The study was approved by the Ethics Committee of the National Research Centre, Giza, Egypt according to the Helsinki Declaration of 1975 revised in 2008. A total of 180 subjects, including 120 treatment-naïve HCV infected patients with different fibrosis grades grade who did not receive any treatment and 60 healthy controls, were enrolled in this study. Informed consents were obtained from all study subjects before enrollment in the study and collection of blood samples. The healthy controls (mean age, 42.6 ± 9.4 years) had normal liver function tests, no history of liver injury and no viral hepatitis, particularly HCV (negative HCV Ab and negative viremia). The control subjects also did not suffer from any other metabolic dysfunctions or bacterial, viral or malignant diseases. The HCV patients (mean age, 43.1 ± 10.1 years) were admitted to the Endemic Medicine Department of Kasr El Ainy Hospitals at Cairo University. Patients were excluded if they had decompensated cirrhosis, metabolic liver disease, hepatitis B virus, schistosomiasis, alcohol, drug-induced hepatitis or any significant coexisting medical conditions. Laboratory tests, including measurements of alanine aminotransferase (ALT), aspartate transaminase (AST), bilirubin, alkaline phosphatase (ALP), creatinine, complete blood count, body mass index (BMI) and HCV viral load were performed, as well as liver biopsy, for all patients. The extents of liver inflammation, steatosis and different grades of fibrosis were determined according to the METAVIR scoring system\textsuperscript{[24]}.

**Detection of HCV virema by real-time-PCR**

Total RNA was extracted from serum using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) to detect the presence of HCV RNA. HCV RNA was detected quantitatively using the Artus HCV RT-PCR Kit (Qiagen) according to the manufacturer’s instructions. Genotype of HCV RNA was detected using the INNOLIPA HCV genotyping Kit (Innogenetics, Ghent, Belgium).

**Genotyping of TNFα -G308A polymorphism by PCR-restriction fragment length polymorphism analysis**

Total DNA was extracted from whole blood of all subjects using the QIAamp Blood Kit (Qiagen) according to the manufacturer’s instructions. The TNFα -G308A genotyping was performed using PCR-restriction fragment length polymorphism (RFLP) analysis. The TNFα -308 G/A promoter polymorphism was amplified by PCR using 5’-AGGCAATAGGTTTTGAGGGCCAT-3’ as forward primer and 5’-CCTCCTGCTCCGATTCCG-3’ as reverse primer, as previously described by Shmarina \textit{et al.}\textsuperscript{[20]} and Ho \textit{et al.}\textsuperscript{[21]}. These primers cover the TNFα -308 polymorphism region and produced a 107 bp PCR fragment. The PCR amplification was carried out in 25 µL, containing 5-10 µg DNA, 4 mmol/L MgCl\textsubscript{2} (Promega, Madison, WI, United States), 1 µmol/L of each primer, 200 µmol/L dNTPs (Promega), 1 × PCR buffer (Promega), and 1 U Go Taq DNA polymerase (Promega). The PCR thermal cycling was carried out in an MJ Research cycler instrument. The thermal cycling conditions consisted of initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min. The PCR fragments were detected by electrophoresis in a 3% agarose gel stained with ethidium bromide.

The G/A allelic polymorphism at TNFα -308 was detected by RFLP analysis. The PCR (107 bp) fragments from all subjects were digested with NcoI restriction enzyme. The digestion reaction was carried out in a total volume of 20 µL, containing 1 x restriction buffer, 8 µL PCR product, and 5 U NcoI (Promega) according to the manufacturer’s recommendations. The NcoI restriction digestion was performed at 37 °C for 4 h. Afterward, 10 µL of the digested products were run on a 4% agarose gel stained with ethidium bromide.

**Sequence analysis of TNF PCR products**

To confirm the results of TNFα -308 PCR-RFLP analysis, some TNFα -308 PCR products were sequenced using the Sanger dideoxynucleotide chain termination method. The TNFα -308 PCR products were purified using the QIAquick PCR Purification Kit (Qiagen). Then, the PCR products were sequenced with the TNFα reverse primer using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems Inc, Carlsbad, CA, United States). After the cycle sequencing reaction, the
Table 1  Clinical data of 120 chronic hepatitis C virus (F0-F4) patients

| HCV patients with different fibrosis grade (n = 120) | F0 (n = 30) | F1 (n = 30) | F2-3 (n = 30) | F4 (n = 30) |
|---|---|---|---|---|
| Age (yr) | 38.4 ± 7.1 | 41.5 ± 8.7 | 43.5 ± 8.4 | 48.3 ± 9.3 |
| Sex (female/male) | 15/17 | 12/18 | 10/20 | 9/21 |
| BMI (kg/m²) | 25.5 ± 2.3 | 27.6 ± 3.4 | 28.7 ± 2.7 | 29.5 ± 2.7 |
| ALT (U/L) | 23.4 ± 7.1 | 39.8 ± 23.7 | 51.1 ± 20.7 | 60.1 ± 20.3 |
| AST (U/L) | 22.4 ± 6.5 | 34.9 ± 20.9 | 48.9 ± 25.9 | 58.7 ± 18.6 |
| ALP (U/L) | 113.6 ± 32.8 | 122.5 ± 37.1 | 158.7 ± 47.7 | 153.8 ± 53.5 |
| Total bilirubin (mg/dL) | 0.53 ± 0.32 | 0.61 ± 0.23 | 0.96 ± 0.37 | 1.60 ± 0.65 |
| Albumin (g/dL) | 4.10 ± 0.33 | 4.00 ± 0.38 | 3.80 ± 0.45 | 3.50 ± 0.39 |
| Platelets (× 10³/μL) | 300.5 ± 56.2 | 250.2 ± 48.4 | 239.6 ± 55.6 | 159.4 ± 69.5 |
| HCV RNA (× 10⁶ IU/mL) | 684.3 ± 124.8 | 860.1 ± 1042 | 1241.5 ± 1286.7 | 1501.4 ± 1661.0 |
| Fibroscan (KPa) | 4.70 ± 0.75 | 6.30 ± 0.36 | 9.90 ± 1.78 | 23.27 ± 9.30 |
| Activity | A0-A1 | 20 (66.7) | 13 (43.3) | 10 (33.3) | 1 (3.3) |
| A2-A3 | 10 (33.3) | 17 (56.7) | 20 (66.7) | 29 (96.7) |
| Steatosis ≤ 33% | 28 (93.3) | 26 (86.7) | 16 (53.3) | 4 (13.3) |
| > 33% | 2 (6.7) | 4 (13.3) | 14 (46.7) | 26 (86.7) |

The clinical data was compared in HCV patients with different fibrosis grades: F0 (n = 30), F1 (n = 30), F2-F3 (n = 30), F4 (n = 30). The quantitative data were represented as mean ± SD while qualitative data were represented as n (%). HCV: Hepatitis C virus; BMI: Body mass index; ALT: Alanine aminotransferase; AST: Aspartate transaminase; ALP: Alkaline phosphatase.

Statistical analysis
The statistical methods of this study were reviewed by a specialized IT development and biomedical statistician. Data were collected, prepared and analyzed using the SPSS software, version 15. Data were expressed as number and percentage for qualitative parameters and as mean ± SD for quantitative parameters. The frequency of genotypes and alleles in patients and controls were analyzed by χ² test. Comparisons of the clinical parameters of different groups and genotypes were performed by t test. Then, stepwise logistic regression analysis was used to identify predictors associated with degree of fibrosis in chronic HCV patients. The OR and 95%CI were calculated to assess the relative risk confidence. P value ≤ 0.05 was considered significant, while was considered P < 0.01 highly significant.

RESULTS

General characteristics of HCV infected patients
The biochemical, virological and histopathological parameters of 120 HCV-infected patients with different fibrosis grades (F0: n = 30, F1: n = 30, F2-F3: n = 30, F4: n = 30) are summarized in Table 1. There were no significant differences within the different fibrosis groups for age, sex, BMI and HCV RNA viral load. Patients with liver fibrosis grades F2-3 or F4 had statistically significant higher levels of AST, ALT, ALP and total bilirubin and significantly lower platelet count and albumin level, as compared to patients with liver fibrosis grade F0 or F1.

TNFα -308 RFLP and sequence analysis
The amplified TNFα -308 PCR products were digested with NcoI restriction enzyme, and run on 4% agarose gel, as shown in Figure 1A. The homozygote AA genotype at TNFα -308 showed the original 107 bp fragment intact (lacking the NcoI site), while the homozygote GG genotype showed two fragments of 87 and 20 bp. The heterozygote GA genotype showed three fragments of 87, 20 and 107 bp. Moreover, the sequence results confirmed the integrity of the NcoI restriction site's surrounding area and verified the results of the TNFα -308 PCR-RFLP analysis. The sequence results for the TNFα -308 different genotypes are shown in Figure 1B.

Distribution of different TNFα -G308A genotypes in controls and HCV infected patients with different fibrosis grade
Distribution of TNFα -G308A genotypes in HCV infected patients and controls are shown in Table 2. The results showed that the TNFα -G308A SNP frequency was significantly different between controls and HCV infected patients (P = 0.001). The TNFα -G308A genotypes in the controls were 66.6% GG, 30% GA and 3.3% AA. The GG genotype in controls (66.6%) is higher than in chronic HCV patients (40%), while the chronic HCV patients had higher GA and AA genotypes (47.5% and 12.5%) compared to controls (30% and 3.3%). Moreover, the G allele is more frequent than the A allele in both controls (81.7% vs 18.3%) and HCV-infected patients (63.8% vs 36.3%), with statistically significant difference (P = 0.002). The distribution of TNFα genotypes in HCV patients with different fibrosis grade is shown in Figure 2. The TNFα GG genotype was 60% in F0, 43.3% in F1, 33.3% in...
Frequency of TNFα -308 genotypes in early and late HCV fibrosis patients

The 120 chronic HCV patients with different fibrosis grade were classified into 60 patients with early fibrosis grade (F0-F1) and 60 patients with late fibrosis grade (F2-F4). Analysis of the frequency of each TNFα genotype showed that early fibrosis patients have 26.7% of AA, 43.9% of AG and 64.6% of GG genotype, which indicates an increasing trend of having the G allele. The AA genotype was detected in 73.3% of late fibrosis patients vs 26.7% of early fibrosis patients (\( P = 0.05 \)), as shown in Figure 3. In general, the late fibrosis patients had a statistically significant higher rate of A allele than the early fibrosis patients.

Table 2  Distribution of tumor necrosis factor α -G308A polymorphism in controls and hepatitis C virus infected patients with different fibrosis grades (F0-F4) \( n (%) \)

| Polymorphism of TNFα -308 | Controls \( (n = 60) \) | HCV Patients \( (n = 120) \) | F0 patients \( (n = 30) \) | F1 patients \( (n = 30) \) | F2-3 patients \( (n = 30) \) | F4 patients \( (n = 30) \) |
|---------------------------|--------------------------|-----------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| GG genotype               | 40 (66.6)                | 48 (40.0)                   | 18 (60.0)                | 13 (45.3)                | 10 (33.3)                | 7 (23.3)                 |
| GA genotype               | 18 (30.0)                | 57 (47.5)                   | 10 (33.3)                | 15 (50.0)                | 16 (53.4)                | 16 (53.4)                |
| AA genotype               | 2 (3.3)                  | 15 (12.5)                   | 2 (6.7)                  | 2 (6.7)                  | 4 (13.3)                 | 7 (23.3)                 |
| GA + AA                   | 20 (33.3)                | 72 (60.0)                   | 12 (40.0)                | 17 (56.7)                | 20 (66.6)                | 23 (76.6)                |
| G allele                  | 98 (81.7)                | 153 (63.8)                  | 46 (76.7)                | 41 (68.3)                | 36 (60.0)                | 30 (50.0)                |
| A allele                  | 22 (18.3)                | 87 (36.3)                   | 23 (23.3)                | 19 (31.7)                | 24 (40.0)                | 30 (50.0)                |

Genotyping of TNFα -G308A was conducted and distribution of different genotypes and alleles was calculated as percentage and compared in 60 controls and 120 chronic HCV patients with different fibrosis grades: F0 \( (n = 30) \), F1 \( (n = 30) \), F2-F3 \( (n = 30) \), F4 \( (n = 30) \). TNFα: Tumor necrosis factor alpha; HCV: Hepatitis C virus.
patients (62.1% vs 37.9% respectively, \( P = 0.04 \)).

Relation between TNF\(\alpha\) genotypes and serum TNF\(\alpha\) level in HCV infected patients

The TNF\(\alpha\) serum levels were determined in healthy controls and HCV patients with different fibrosis grade. Compared with that in healthy controls, serum TNF\(\alpha\) level was gradually elevated in HCV infected patients with different fibrosis grade, as shown in Figure 4. The results showed that the TNF\(\alpha\) serum level was positively correlated to HCV liver fibrosis progression (\( P < 0.001 \)). Moreover, the TNF\(\alpha\) serum concentration was significantly higher in patients with A containing genotypes (GA or AA) than in those with GG genotype (10.9 ± 4.9 pg/mL vs 8.3 ± 2.6 pg/mL, \( P = 0.01 \)) in early fibrosis (F0-F1) patients and (14.4 ± 4.9 pg/mL vs 10.4 ± 2.1 pg/mL, \( P = 0.002 \)) in late fibrosis (F2-F4) HCV patients, as shown in Table 3.

Association of TNF\(\alpha\) genotypes and different liver pathological grades

The influence of different TNF\(\alpha\) genotypes on hepatic parameters and liver pathology (liver inflammation, steatosis, and fibrosis) is shown in Table 4. The results demonstrated that TNF\(\alpha\) AA genotype patients have
Table 4  Influence of tumor necrosis factor α -308 genotypes on the liver biochemical and pathological parameters

| TNF genotypes (n = 120) |  |  |
|-------------------------|------------------------|------------------------|
|                         | GG (n = 48)            | AG (n = 57)            | AA (n = 15) | GG vs AA | GG vs GA + AA |
| AST (U/L)               | 31.9 ± 20.6            | 44.7 ± 21.6            | 57.7 ± 28.7 | 0.0003   | 0.0003      |
| ALT (U/L)               | 34.5 ± 20.9            | 46.5 ± 20.5            | 62.9 ± 28.2 | 0.0011   | 0.0004      |
| ALP (U/L)               | 113.7 ± 36.8           | 138.3 ± 41.5           | 190.1 ± 46.5 | 0.0020   | 0.3500      |
| Albumin (g/dL)          | 3.9 ± 0.46             | 3.9 ± 0.39             | 3.6 ± 0.59  | 0.0100   | 0.2000      |
| Total bilirubin (mg/dL) | 0.73 ± 0.41            | 0.89 ± 0.51            | 1.3 ± 0.68  | 0.0002   | 0.0100      |
| Platelets (> 10^11/L)   | 254.9 ± 66.2           | 224.5 ± 72.3           | 205.8 ± 96.6 | 0.0360   | 0.0220      |
| HCV RNA (> 10^5 IU/mL)  | 862.6 ± 1295.6         | 1083.9 ± 1187.2        | 1675.5 ± 1477.6 | 0.0450   | 0.2200      |
| Activity                |                        |                        |             |          |             |
| A0-A1 (n = 44)          | 25 (56.8)              | 18 (40.9)              | 1 (2.3)     | 0.0290   | 0.0350      |
| A2-A3 (n = 76)          | 23 (30.3)              | 39 (51.3)              | 14 (18.4)   |          |             |
| Steatosis ≤ 33% (n = 74)| 36 (48.7)              | 34 (45.9)              | 4 (5.4)     | 0.0280   | 0.0300      |
| > 33% (n = 46)          | 12 (26.1)              | 23 (50.0)              | 11 (23.9)   |          |             |
| Fibrosis                |                        |                        |             |          |             |
| F0-F1 (n = 60)          | 28 (46.7)              | 30 (50.0)              | 2 (3.3)     | 0.0310   | 0.0400      |
| F2-F4 (n = 60)          | 20 (33.3)              | 27 (45.0)              | 13 (21.6)   |          |             |

The effect of different TNFα -308 genotypes (GG, GA, AA) on biochemical parameters (ALT, AST, ALP, albumin, total bilirubin), platelets count and pathological parameters (activity, steatosis, fibrosis) was statistically analyzed. The biochemical data were represented as mean ± SD, while pathological data were represented as n (%). P ≤ 0.05 is considered significant, while P ≤ 0.01 is highly significant. TNFα: Tumor necrosis factor alpha; HCV: Hepatitis C virus; ALT: Alanine aminotransferase; AST: Aspartate transaminase; ALP: Alkaline phosphatase.

Figure 5  Effect of different tumor necrosis factor α genotypes on liver pathological parameters (inflammation, steatosis and fibrosis). The effect of high expressing TNFα genotypes (GA + AA) were statistically compared with the low expressing TNFα genotype (GG), and the results showed strong effect of (GA + AA) genotypes on liver inflammation (P = 0.007), steatosis (P = 0.005) and fibrosis (P = 0.032). P ≤ 0.05 is considered significant while P ≤ 0.01 is highly significant. TNFα: Tumor necrosis factor alpha; HCV: Hepatitis C virus.

significantly higher levels of AST, ALT, ALP and total bilirubin than GG patients (P = 0.0003, 0.0001, 0.002 and 0.0002 respectively). Patients with AA genotype have significantly lower level of albumin and platelets count (P = 0.01 and 0.036 respectively). Moreover, the effect of serum TNFα level expressed by genotypes (GA and AA) on liver inflammation, steatosis and fibrosis is shown in Figure 5. The results showed that genotypes GA or AA which express higher TNF serum level were found in 69.7% of high liver inflammation (A2-A3) patients, in 73.9% of severe steatotic patients, and in 66.7% of late fibrotic (F2-F4) patients.

Table 5  Stepwise logistic regression analysis for the effect of tumor necrosis factor α -308 genotypes and alleles on liver disease progression

| TNF genotype       | Regression coefficient | SE  | OR (95%CI) | P value |
|--------------------|------------------------|-----|------------|---------|
| Activity           |                        |     |            |         |
| GG vs GA + AA      | 1.329                  | 0.507 | 3.776 (1.399-10.194) | 0.009   |
| G vs A allele      | 1.171                  | 0.400 | 3.226 (1.474-7.060)  | 0.003   |
| Steatosis          |                        |     |            |         |
| GG vs GA + AA      | 1.502                  | 0.580 | 4.491 (1.441-14.000) | 0.010   |
| G vs A allele      | 1.099                  | 0.373 | 3.000 (1.445-6.228)  | 0.003   |
| Fibrosis           |                        |     |            |         |
| GG vs GA + AA      | 1.044                  | 0.493 | 2.841 (1.080-7.472)  | 0.034   |
| G vs A allele      | 0.895                  | 0.566 | 2.446 (1.195-5.008)  | 0.014   |

P ≤ 0.05 is considered significant while P ≤ 0.01 is highly significant. TNF: Tumor necrosis factor.

Stepwise logistic regression analysis for the effect of TNFα -308 genotypes and alleles on liver disease progression

Different TNF genotypes were investigated to determine their significance in liver disease progression using stepwise logistic regression analysis. The outcome of liver progression was significantly influenced by different TNF genotypes and alleles, as shown in Table 5. Patients with TNFα GA and AA genotypes have increased risk of liver inflammation (A2-A3) (OR = 3.776, 95%CI: 1.399-10.194, P = 0.009), severe steatosis (> 33%) (OR = 4.49, 95%CI: 1.441-14.0, P = 0.010) and fibrosis progression (F2-F4) (OR = 2.84, 95%CI: 1.080-7.472, P = 0.034) than those with GG genotype. In general, patients with A allele have more risk of liver inflammation, steatosis and fibrosis than
those carrying the G allele ($P = 0.003, 0.003$ and $0.014$ respectively).

**DISCUSSION**

Cytokines represent a large family that includes the IFNs, ILs, chemokines and TNF proteins. Cytokines play important roles in the activation and regulation of human immune responses and their production are variable due to SNPs[26]. Several studies have reported the impact of different cytokines’ polymorphisms on the pathogenesis and outcomes of many diseases. Competent cytokine responses to HCV infection are very important to control virus replication, disease progression and treatment response.

TNFα is a diverse multifunctional, proinflammatory and immuno-mediator cytokine. Several studies have shown that the A allele at the TNFα promoter region -308 polymorphism affects the transcriptional level of TNFα gene[27,28]. There are few data regarding the role of TNFα polymorphism in disease progression and response to antiviral therapy in patients infected with HCV genotype 4. In this study, we examined the association between TNFα -308GA polymorphism and pathological parameters of liver such as inflammation, steatosis and fibrosis in 120 treatment-naïve HCV genotype 4 Egyptian patients.

Current findings showed that TNFα GG genotype is significantly higher in controls than in HCV infected patients (66.6% vs 40%, $P = 0.001$), while the A allele tends to be more frequent in HCV-infected patients than in controls (36.3% vs 18.3%, $P = 0.002$). The (GA + AA) genotypes exhibited gradual increase with progressive fibrosis, with 40% in (F0), 56.7% in (F1), 66.6% in (F2-F3), and 76.6% in (F4). In general, 62.1% of the A allele were found in late fibrosis patients (F2-F4) compared to 37.9% in early fibrosis patients (F0-F1) ($P = 0.015$). Similar results were found by Dogra et al[29], who reported that the frequency of TNFα -308 genotypes was significantly different between healthy controls and HCV patients. The association between TNFα -308A allele and HCV persistence and failure of response to IFN treatment was reported by Radwan et al[30] and Dai et al[31]. Nevertheless, other studies failed to find association between the TNFα -308A polymorphism and chronicity of HCV infection[6,20,32].

On the other hand, there were significantly high serum TNFα mean values in late fibrosis (F2-F4) patients, with higher levels in individuals with GA or AA genotypes than those with GG genotype. Similar findings were reported in patients with Crohn’s disease, where the TNFα -308A allele was associated with increased TNFα production and inflammatory activity[33]. Also, Roth-Isegiket et al[34] reported elevated TNFα serum levels in GA heterozygote individuals undergoing cardiac surgery. Further *in vitro* support of these findings was reported by Abraham and Kroeger[14], who found that A allele induced mRNA expression and TNFα production up to 5-fold more than did the G allele.

Several studies have shown that TNFα -308A polymorphism alters TNFα expression levels and changes the immune response. It was reported that TNFα -308A polymorphism can worsen the clinical outcome of many inflammatory and infectious diseases and can increase the risk of some autoimmune diseases. Abraham and Kroeger[14] defined TNFα as a genetic susceptibility factor for some autoimmune, inflammatory and infectious diseases. High TNFα expression level was also reported to inhibit insulin signaling and decrease adiponectin levels, leading to development of insulin resistance and liver steatosis. Furthermore, it was demonstrated that high circulating TNFα is a potent risk factor for steatosis[35].

In the present study, we examined the link between high TNFα serum levels and liver inflammation, steatosis and fibrosis. Our results showed that TNFα -308AA genotype patients have significantly higher levels of AST, ALT, ALP and total bilirubin than GG patients. Also, HCV patients with severe liver pathological conditions (inflammation, steatosis and fibrosis) have high frequencies of TNFα (GA or AA) genotypes. These results showed a clear association between severe liver pathological conditions (inflammation, steatosis and fibrosis) and (GA + AA) genotypes ($P = 0.035, 0.03$ and $0.04$ respectively). These results suggest that A containing genotypes (GA + AA) express higher TNFα levels than GG genotype and consequently induce inflammatory response that leads to liver inflammation, injury and severe fibrosis[36].

Our results confirmed the previous studies in which a significant elevation in TNFα serum level was detected in cirrhotic liver patients and was correlated with progression to hepatocellular carcinoma[22,27,38]. It was reported that TNFα -308AA genotype is associated with the HCV pathogenesis and viral persistence[21,31]. Aller et al[35] showed that patients with the TNFα mutant genotype (GA or AA) have moderate to severe portal inflammation and fibrosis, contrasting those patients with wild genotype (GG). Also, high TNFα plays a role in fatty liver disease pathogenesis[39] and leads to severe liver injury in NASH patients[49]. However, other studies were unable to find any association between TNFα genetic polymorphisms and severity of liver disease or response to antiviral therapy[6,26,39].

The stepwise logistic regression analysis showed that the TNFα genotypes (GA + AA) were significantly associated with liver inflammation (OR = 3.776, 95%CI: 1.399-10.194, $P = 0.009$), severe steatosis (OR = 4.49, 95%CI: 1.441-14.0, $P = 0.010$) and fibrosis progression (OR = 2.84, 95%CI: 1.080-7.472, $P = 0.034$). The A allele was an independent risk factor for liver inflammation ($P = 0.003$), steatosis ($P = 0.003$) and fibrosis ($P = 0.014$). Therefore, the current results confirm that HCV infected patients carrying one or two A alleles at TNFα -308 have a risk factor for development of severe liver pathological grades.
Several experimental studies showed that the inhibition of TNFα signaling by anti-TNFα antibodies or compounds could reduce inflammation, liver injury and improve fibrosis\(^\text{3,41}\). However, complete neutralization of TNFα in alcoholic hepatitis patients was associated with serious infectious complications\(^\text{42}\). Therefore, it was recommended to use pentoxifylline (PTX), which partially attenuates TNFα level and has lower infectious complication rates. It was demonstrated that PTX therapy effectively reduces the liver biochemical parameters and improves the histological injury in NASH patients\(^\text{43}\).

Although liver biopsy is the conventional way to determine the grade of liver fibrosis, it is an invasive method and there is too much sampling. Therefore, there is an increasing need for biomarkers that are specific, sensitive and respond quickly to changes in fibrosis and activity grades. Based on the results of the current study and our previous work on IL28B\(^\text{44-45}\), OAS1 and MaX\(^\text{7,46,47}\), we have presented some potential genetic markers that can be useful in the determination of liver disease progression. In conclusion, the TNFα SNP at nucleotide -308 represents an important genetic marker for developing strategies of prognosis for liver inflammation, steatosis and fibrosis in HCV infected patients.

**COMMENTS**

**Background**

Hepatitis C virus (HCV) infection is highly endemic in Egypt and the mortality rate of HCV-associated liver diseases reaches 67%. Both viral and host factors play important roles in controlling HCV liver disease progression and response to treatment. Host genetic factors and immunological response to HCV infection can affect the pathogenesis of liver diseases. The single nucleotide polymorphisms (SNPs) are responsible for inter-individual differences in cytokines' and cellular antiviral proteins' production and secretion. Tumor necrosis factor alpha (TNFα) is a proinflammatory and antiviral cytokine that plays a major role in liver injury and initiation of the fibrosis process. The TNFα expression is considered a key molecular link between liver inflammation, steatosis, and fibrosis. TNFα transcription level is affected by the TNFα -308GA polymorphism. In Egypt, more than 90% of patients are infected with HCV genotype 4. Therefore, we designed the current study to investigate the association of the TNFα -308GA polymorphism with liver pathological changes in treatment-naive chronic HCV genotype 4 patients with different fibrosis grades (F0-F4).

**Research frontiers**

Chronic hepatitis C infection can cause severe liver diseases, such as fibrosis, cirrhosis, liver failure and hepatocellular carcinoma. Liver fibrosis and cirrhosis could not be reversed but the liver scarring can be improved with HCV treatment. However, timing and rules for HCV genotype 4 treatment without major complications and relapse are under investigation by research studies and clinical trials. The research hotspot is currently how to determine and prediction of HCV liver disease progression, representing an approach that guides therapeutic interventions and prevents further spread of liver fibrosis and hepatic failure.

**Innovations and breakthroughs**

The TNFα SNP at nucleotide -308 represents an important genetic marker to predict the liver disease progression rate in treatment-naive chronic HCV genotype 4 infected patients. There is a demanding need for biological and genetic markers’ application for making a decision as to whether the HCV patient will have progressive liver disease or not. These data clearly improve the prediction by detecting the TNFα -308 polymorphism and assessing the TNFα serum level. The data presented in this study demonstrated that the TNFα expression level provide a predictive value for liver disease progression rate.

**Applications**

The data of this study will help hepatologists and gastroenterologists to better predict liver disease progression in chronic HCV infected patients, to make better decisions on liver disease treatment, and to design novel therapeutic interventions that will control HCV-associated liver disease complications.

**Terminology**

SNPs in HCV infected patients lead to different immune responses, which significantly influence the progression of chronic HCV infection and response to therapy. TNFα is a diverse multifunctional, proinflammatory and immuno-mediator cytokine. The A allele at the TNFα promoter region -308 polymorphism affects the transcriptional level of the TNFα gene. High TNFα level in HCV infected patients may accelerate liver disease progression and complications.

**Peer-review**

This study added new information regarding a subtyping (4) of HCV and its relation with the TNFα cytokine.

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