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# Tumour Necrosis Factor-Alpha Gene Expression in Chronic Hepatitis C Virus Infection.

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## Abstract

**Objective:** Tumour necrosis factor (TNF)-alpha, a prototype proinflammatory cytokine, has been implicated as an important pathogenic mediator in a variety of liver conditions. Some genetic polymorphisms in the human TNF-alpha promoter region, such as the G-A transitions -308 and –238, have been shown to influence TNF-alpha expression in chronic hepatitis C virus infection.

**Aim of the work:** The present study was to investigate the influence that the – 308 and – 238 TNF- alpha promoter polymorphisms have on the response to interferon and ribavirin therapy in chronic hepatitis C virus infection.

**Patients and methods:** One hundred forty patients with chronic hepatitis C virus infection, their age ranges between (20-56) years, selected from the National Hepatology and Tropical Medicine Research Institute were included in this study, during interferon and ribavirin therapy and thirty five healthy individuals were included to serve as controls, the patients and controls were divided into two groups the first group forty patients and fifteen controls for the detection of TNF-alpha -308, -238 genotypes polymorphisms, the second group were one hundred patients and twenty healthy controls for the detection of serum levels of TNF-α. All the patients and controls were subjected to the following history, clinical examination, abdominal ultrasonography and collection of blood samples for routine laboratory investigation, CBCs and serological assay, genotyping of 308, 238 TNF-alpha promoter polymorphism and serum levels of TNF-α.

**Results:** There was no statistically significant difference between chronic HCV patients and healthy controls as regarding TNF-alpha -238 different alleles.

The frequencies of TNF-alpha gene polymorphism with A/G and G/G mutation at – 308 were significantly higher in chronic HCV patients than those in the controls.

The serum level of TNF-alpha was markedly higher in the chronic HCV patients than in the healthy controls.

There were significant association between TNF- alpha gene polymorphism in the – 308 A/G, G/G alleles and increased serum TNF- alpha in CHCV infection.

**Conclusion:** The results indicate that the TNF-alpha gene polymorphism at position -308 is associated with susceptibility of chronic HCV infection.

**Recommendations:** Our major concern was to improve the response to treatment in patients with chronic HCV infection, whether the disadvantage of having the TNF-alpha -308 allele became more apparent after interferon and ribavirin therapy is unclear and needs further study that detecting the polymorphism of the -308 TNF-alpha allele before administering interferon therapy may be valuable for predicting the treatment response, especially in difficult- to treat patients.

**Key Words:** TNF-alpha, a promoter polymorphism at position 238, 308, chronic hepatitis C virus infection therapy.

## Introduction

The hepatitis C virus (HCV) is a major public health problem and a leading cause of chronic liver disease (1). An estimated 180 million people are infected worldwide (2). Approximately thirty percent of patients treated with pegylated interferon and ribavirin are unable to clear virus from the serum (3,4). When host genetic factors are considered in disease and treatment response, the role of single-nucleotide polymorphisms (SNPs) becomes increasingly important (5). Proinflammatory

cytokines including tumour necrosis factor (TNF) mediate the pathogenesis of hepatitis C (HCV) infection (6). TNF-α may play a role in the pathogenesis of acute and chronic HCV infection, the persistence of the virus, and the response to IFN-α therapy (7).

TNF-α and IFN-γ were upregulated in chronic HCV infection (8). Baller et al (9) noticed that polymorphisms in genes encoding immunoregulatory proteins, proinflammatory cytokines, and fibrogenic factors may affect the production of these factors and influence disease progression in patients with chronic liver disease due to alcohol, primary biliary cirrhosis or hepatitis C. Polymorphisms in the promoter of the TNF-α gene have been reported to affect the transcription rate and the release of this cytokine (10). The G-A transition at positions -308 and -238, have been shown to influence TNF-α expression (11,12). Activation of the tumour necrosis factor (TNF)-alpha system has a pivotal role in the inflammatory process of chronic hepatitis C, and TNF-α levels correlate with degree of inflammation (13).

**Patients and Methods:**

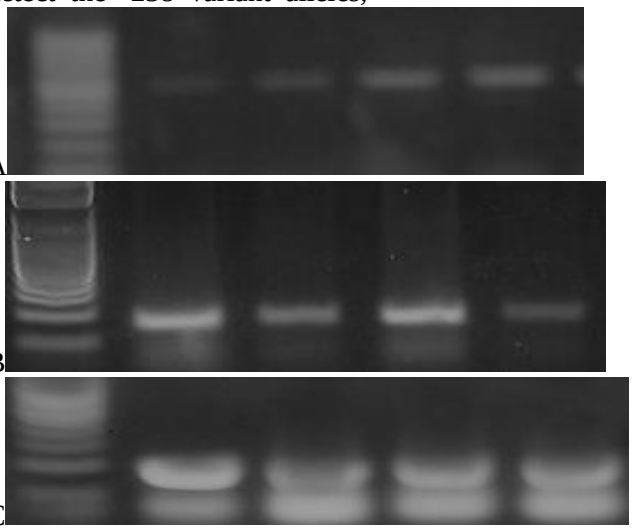
One hundred forty patients with chronic hepatitis C virus (CHCV) infection, their age ranges between (20-56) years, selected from the National Hepatology and Tropical Medicine Research Institute, were included in this study during interferon and ribavirin therapy, and thirty five healthy individuals were included to serve as controls. All patients have anti-HCV antibodies, HCV RNA in serum , evidence of chronic hepatitis on liver biopsy, elevated levels of aminotransferase above the upper limit, serum albumin, bilirubin, and prothrombine time within normal limit with negative history of drug abuse, non reactive HBsAg, with exclusion of other chronic disease and pregnancy no clinical signs of decompensated liver disease. All the patients were subjected to the following history and through clinical examination, abdominal ultrasonography and collection of blood samples. About 5 mL of peripheral venous blood was collected under aseptic conditions, divided into 2 parts one part was clotted that seprated immediately into serum for determination of TNF-α , determination was done by using a commercial Sandwitch ELISA Kit (Orgenium, Cat. No. 881786-GammmaTrade).

The other part was added into sodium-citrated tubes and processed within the same day for detection of genotyping of 308, 238-TNF-α promoter polymorphism.

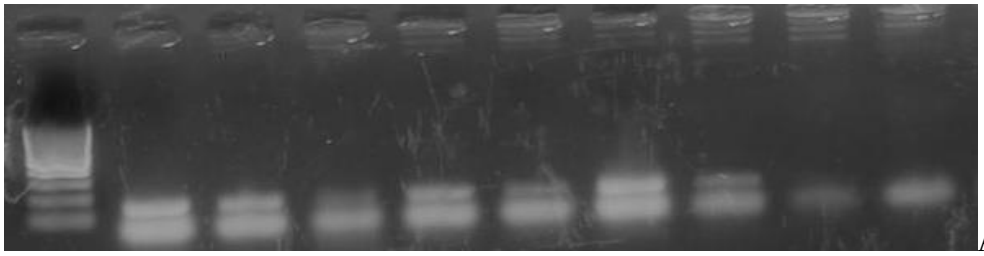
The genomic DNA was extracted using a Qiagen amp DNA mini kit (USA) extraction kit with lot no (139289002) according to manufacturer instruction. The purity and concentration of DNA was determined using spectrophotometry. Following deproteinisation, the quality of DNA was reflected by a consistent ratio of 1.8 to 2.0. The coded genomic DNA solution was stored at 4°C. Genotyping for G–308A was performed using polymerase chain reaction (PCR) followed by restriction fragment length polymorphism. The TNF-α polymorphism was amplified using an upstream primer with a mismatch that introduced an artificial Sty 1 restriction site into the wild-type allele (allele 1), but not in the variant allele (allele 2). The forward primer for the –308 polymorphism used was 5'-AGGCAATAGGTTTTGAGGGCCATG' and the 3' reverse primer was '5-ACACACAAGCATCAAGGATACC-3'. and (5-ATCTGGAGGAAGCGGTAGTG-3) and (5-AGAAGACCCCCCTCGGAACC-3) for the -238 variant allele. A 143-bp fragment was amplified with this primer set. Each 25-mL PCR reaction contained 2.5 uL of 10 X PCR buffer, 1.5 m Mol of MgCl2, 10 p mol of each primer, 0.2 mM of the dNTPs, 15 uL of deionized water, 1 µL of Taq DNA polymerase, and 40-50 ng of genomic DNA as a template. The mixture was denatured at 95ºC for 5 minutes and underwent 35 cycles in a thermocycler PCR system under the following conditions: denaturation at 95ºC for 1 minute, annealing at 60ºC for 45 seconds, extension at 72ºC for 1 minute, and a final extension for 10 minutes at 72ºC. The amplified fragments were detected on 2 % agarose gel (Invitrogen, Carlsbad, California, USA).

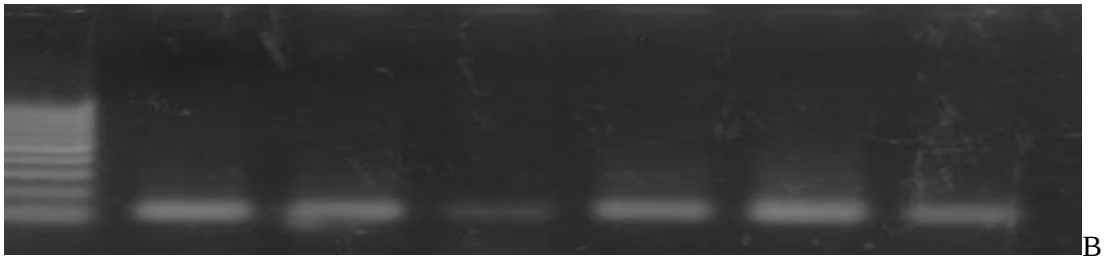
Restriction enzyme digestion of PCR products for 308 genotyping was carried out by adding 2 uL of Buffer O and 1 uL (10U) of restriction enzyme Nco1 (Fermentas, Hanover, Maryland, USA lot no: (0421105). Digested fragments were observed on 3 % agarose gels according to (R1) and to detect the -238 variant alleles,

The PCR product was digested with MspI at 37 ºC for an hour, leaving a 152 bp fragment when the variant was present and products of 133 and 19 bp fragments for the normal allele visualized on 3 % according to R2 (14,15).




An agarose gel electrophoresis show PCR products of TNF alpha (308 site) genotype Genotype A ( fig.A ) and genotype T ( fig.B ) genotype A & T ( fig.C ).




An agarose gel electrophoresis show PCR products of 238 TNF genotyipng

Figure (A1) Lane M:DNA marker with 100 bp Lane (1-7) show GA TNF 238 allele ,Lane (8,9) show AA allele .

Figure (B1) Lane M: DNA marker with 100 bp, Lane (1-6) show GG allele.

**Statistical analysis:** Analysis of data of all patients was done by IBM computer using SPSS (statistical program for social science version 12) as follows: Description of quantitative variables as mean, SD and range. Description of qualitative variables as number and percentage. Chi-square test was used to compare qualitative variables between groups. Mann Whitney Willcoxon U test was used instead of t-test in non parametric data, Unpaired t- test was used to copmpare quantitative variables, in parametric data (SD > 50 % mean) (16).

**Results**

This study included 140 patients with CHCV infection and 35 healthy volunters.

We found that the TNF-alpha promoter genotypes polymorphism -238 shows no statistically significant difference between CHCV patients and controls as regarding different alleles by using chi-square test ( $p > 0.05$ ) table (1). TNF- alpha – 238 GG = ( 52.5 % ), - 238 AA = ( 15 % ), - 238 GA = (32.5 %) in patients and – 238 GG = ( 73.3 % ) , GA = (26.7 %) in the controls.

Table (2) shows that the TNF-alpha promoter genotypes polymorphism -308 shows that -308 AG was more frequent among cases = (45 %) together with, -308 GG = (50 %) , while the – 308 AA was more frequent among controls = (33.3 %) with statistically significant difference in between by using chi-square test.

Table (3) shows that the majority of CHCV patients had A1F1 biopsy results and A1F2 while A3F2 was among 2.5 %.

Table (4) shows that 87.5 % of the CHCV patients had response to interferon therapy.

Graph (1) illustrates the variants polymorphisms in CHCV patients and controls as regarding TNF- alpha gene promoter region at – 308 alleles genotypes.

Table (5), (6) are showing that there's no statistically significant difference in the comparison between responders and non responders to interferon and ribavirin therapy as regarding TNF-alpha – 238 or 308 alleles genotypes polymorphism after 6 months of the comparison of the treatment by using chi-square test.

Table (7) illustrates the mean ± SD of the polymerase chain reactions (PCR) of the studied cases before starting interferon therapy = ( 1367855 ± 3271795 ) Iu / mL and the range was ( 150000 – 2000000 ) Iu / mL.

Table (8) shows that there's no significant difference in the comparison between responders to interferon therapy and non responders as regarding PCR by using Mann Whitney test.

Table (9) illustrates that serum TNF- alpha was ( 79.3) in patients and was (52) in the controls.

Table (10) shows that serum TNF-alpha was higher among CHCV patients as compared to controls with statistically highly significant difference in between by using unpaired t – test.

Fig (A,B,C), Fig (A1,B1) an agarose pictures to detect the -238, -308 polymorphisms by the polymerase chain reaction (PCR) and restriction fragement length polymorphism (RFLP) analysis. DNA is amplified by PCR. The PCR products are then digested with specific restriction enzymes and analyzed by agarose gel electrophoresis.

**Table (1) Comparison between CHCV patients and controls as regard TNF-alpha-238 polymorphism.**

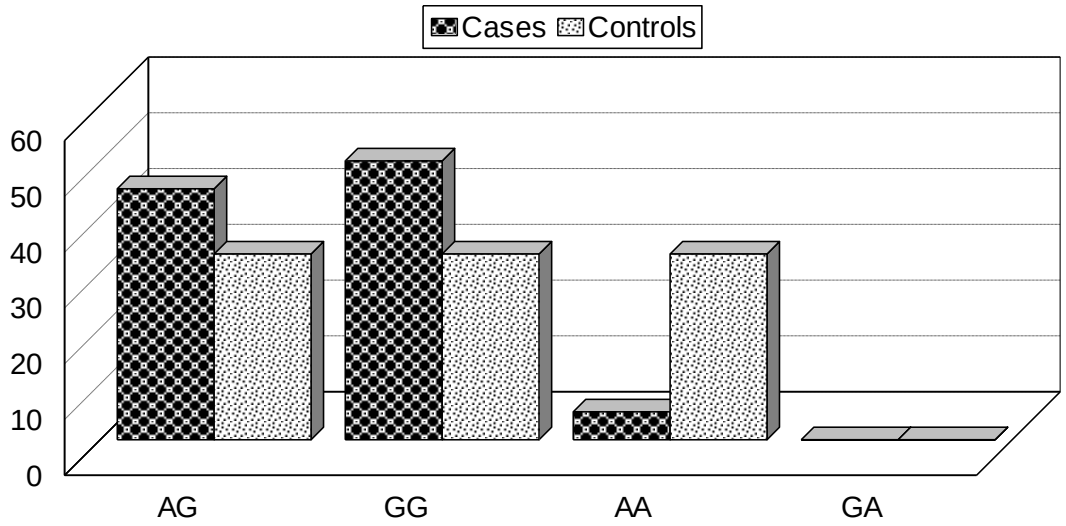
| Variables | Cases N=40 | Controls N=15 | $X^2$ | P |
|---|---|---|---|---|
| **AG** | 0 | 0 | 3.1 | >0.05 NS |
| **GG** | 21(52.5%) | 11(73.3%) | | |
| **AA** | 6(15%) | 0 | | |
| **GA** | 13(32.5%) | 4(26.7%) | | |

This table shows no statistically significant difference between both groups as regard different alleles by using chi-square test

**Table (2) Comparison between CHCV patients and controls as regard TNF-alpha-308 polymorphism.**

| Variables | Cases N=40 | Controls N=15 | $X^2$ | P |
|---|---|---|---|---|
| **AG** | 18(45%) | 5(33.3%) | **7.9** | **<0.05 S** |
| **GG** | 20(50%) | 5(33.3%) | | |
| **AA** | 2(5%) | 5(33.3%) | | |
| **GA** | 0 | 0 | | |

**This table shows that AG was more frequent among cases together with GG , while AA was more frequent among controls with statistically significant difference in between both groups by using chi-square test.**




**Graph (1): Illustrates the variants polymorphism in CHCV paients and controls as regarding TNF-alpha gene promoter region at -308 alleles genotypes.**

**Table(3): Distribution of the studied CHCV patients as regard biopsy results.**

| Variables | No 40 | % |
|---|---|---|
| **A1F1** | 25 | **62.5%** |
| **A1F2** | 6 | 15% |
| **A1F3** | 1 | 2.5% |
| **A2F1** | 1 | 2.5% |
| **A2F2** | 3 | 7.5% |
| **A2F3** | 3 | 7.5% |
| **A3F2** | 1 | 2.5% |

This table shows that the majority of cases had A1F1 Biopsy results and A1F2, while A3F2 was found among 2.5% of the studied cases.

**Table (4) Distribution of the studied CHCV cases as regard response to therapy**

| Variables | No | % |
|---|---|---|
| **Non responder** | 5 | 12.5% |
| **Responder** | 35 | 87.5% |

This table shows that 87.5 % of the studied cases had response to IFN therapy, while 12.5 % of studied cases had no response to therapy.

**Table (5): Comparison between responders and non responders to interferon and ribavirin therapy as regarding TNF-alpha -238 polymorphism after 6 months of the completion of a drug treatment.**

| Variables | Response | | $X^2$ | P |
|---|---|---|---|---|
| | No | Yes | | |
| **AG** | | | 1.9 | >0.05 NS |
| **GG** | 4(80%) | 17(48.6%) | | |
| **AA** | 0 | 6(17.1%) | | |
| **GA** | 1(20%) | 12(34.3%) | | |

This table shows no statistically significant relation between response to interferon therapy and TNF-alpha-238 alleles genotypes by using chi-square test.

**Table (6) Comparison between responders and non responders to interferon and ribavirin therapy as regarding TNF-alpha -308 polymorhism after 6 months of the completion of a drug treatment.**

| Variables | Response | | $X^2$ | P |
|---|---|---|---|---|
| | No | Yes | | |
| **AG** | 3(60%) | 15(42.9%) | 0.9 | >0.05 NS |
| **GG** | 2(40%) | 18(51.4%) | | |
| **AA** | 0 | 2(5.7%) | | |
| **GA** | 0 | 0 | | |

This table shows no statistically significant relation between response to interferon therapy and TNF-alpha–308 allelels genotypes by using chi-square test.

**Table (7): Distribution of the studied CHCV patients as regard PCR (polymerase chain reaction) before starting interferon therapy.**

| Variables | PCR |
|---|---|
| **Mean±SD** | 1367855±3271795 |
| **Range** | 150000-2000000 |

**Table (8): Comparison between responders to interferon therapy and non responders as regarding PCR .**

| Variables | PCR | | Z | P |
|---|---|---|---|---|
| | Mean | ± SD | | |
| **Non responder** | 707429 | 496494 | 0.4 | >0.05 NS |
| **Responders** | 14625858 | 348940 | | |

There is no significant difference between both groups as regard PCR by using Mann Whitney test.

**Table (9): Distribution of the studied CHCV patients and controls as regarding serum TNF-alpha.**

| Variables | Mean ± SD | Range |
|---|---|---|
| TNF-alpha (cases) | 79.3 ± 31 | 25.6 – 158.9 |
| TNF-alpha (controls) | 52 ± 1.2 | 50.7 - 55 |

This table shows that serum TNF-alpha was 79.3 in patients and was 52 in the controls.

**Table (10): Comparison between CHCV patients and controls as regarding serum TNF-alpha.**

| Variables | Cases N = 100 | Controls N = 20 | t | P |
|---|---|---|---|---|
| TNF- alpha | 79.3 ± 31 | 52 ± 1.2 | 3.8 | < 0.001 HS |

This table shows that serum TNF-alpha, was higher among cases as compared to controls with satistically highly significant difference in between by using unpaired t-test.

**Discussion**

Tumour necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ), which are prime and maintain antigen-specific cellular immunity (17,18) and are important in defense against viruses. Control of HCV replication may depend on effective Th lymphocyte activation (19,20). There is also an enhanced Th2 response during chronic HCV infection, which may partly be responsible for the persistence of HCV infection.

TNF-alpha -308 AG and AA genotypes were significantly associated with susceptibility to hepatitis C infection and responde to pegylated interferon-alpha and ribavirin therapy. Cytokines play a key role in the regulation of immune responses. In hepatitis C virus infection, the production of abnormal cytokine levels appears to contribute in the progression of the disease, viral persistence, and affects response to therapy. Cytokine genes polymorphisms located within the coding / regulatory regions have been shown to affect the overall expression and secretion of cytokines (21). A number of genes both within and outside the major histocompatibility complex (MHC) able to influence the nature and magnitude of the immune response may also play a role in clearance of hepatitis C virus. Polymorphisms in the tumour necrosis factor alpha (TNF-α) promoter gene sequence and the interleukin-10 (IL-10) promoter have come under scrutiny as potential candidates (22-24,6,25-27).

In the present study, we found that TNF-α -238 polymorphism show that there's no statistically significant difference between CHCV patients and controls as regarding different alleles. Hohler et al. (23) showed that at position 238, allele2 (A;TNF 238.2) has been reported to be associated with certain autoimmune and infectious diseases. Tumor necrosis factor alpha promoter polymorphism at position -238 is associated

with chronic active hepatitis C virus infection, these results were against our results.

In the present work, we found that TNF-α -308 AG = 45 % was more frequent among CHCV patients together with GG = 50 % while AA = 33.3 % was more frequent among controls with statistically significant difference in between. Abbas et al. (28) discovered that the frequencies of different dimorphic polymorphisms based on single nucleotide substitution were TNF-α -308 AG = 95 %, G/C = 5 %, these results are in agreements with our results.

In our study, we observed that GA transition in the TNF-α promoter regions -238 and -308 polymorphism. Chia-Yen et al. (29) explained that the G-A transition in the TNF-α promoter region at position -308 and -238 were determined in patients with chronic hepatitis C virus (HCV) infection. Patients received combination therapy with high-dose interferon (IFN) and ribavirin for 24 weeks.

Fargion et al. (10) found that polymorphisms in the promoter of the tumour necrosis factor alpha gene have been reported to affect the transcription rate and the release of this cytokine, all these finding were correlated with our results.

We found in our results that the majority of CHCV cases biopsy results was A1F1 = (62.5 %), A1F2 = (15 %), while A3F2 was found among (2.5 %) of the studied cases. Abbas et al. (28) showed that there's no significant differences in HAI were noted among polymorphisms of other cytokines including (TNF-α), these finding was in agreement with our results.

A correlation between baseline TNF-α levels and histologic grading score of hepatitis. The maximal capacity of cytokine production varies between individuals and may correlate with polymorphism in cytokine gene promoters (30).

Yee et al. (6) postulated that polymorphism in the TNF-α promoter appear to be associated with variability in the histological severity of chronic hepatitis C infection.

Romer-Gomez et al. (31) explained that the presence of -238 TNF A/G was associated with (53.8 %) advanced, (31.4 %) mild fibrosis. The combination of TNF-α -238 A/G and the presence of allele 3 in Caucasian Spanish patients is conductive to progression to pre-cirrhotic or cirrhotic stages of the disease, these finding were against our results.

We found in our results that 87.5 % of the studied CHCV patients had response to interferon therapy, and there's no statistically significant relation between response to interferon and ribavirin therapy as regarding TNF-α -238, or – 308 polymorphism after 6 months of the completion of the IFN treatment. Abbas et al. (28) approved that

Sustained virological response to the treatment was not influenced by the cytokine polymorphism.

Yee et al. (27) reported that there was no correlation between -308 TNF-α promoter polymorphisms and the response to combination therapy with interferon and ribavirin in patients with chronic HCV infection, these finding are in agreement with our finding.

Chia-Yen et al. (29) postulated that the -308 and -238 TNF-α promoter polymorphism have on response to combination therapy with high –dose IFN-α and ribavirin, these finding were against our results for -238 but in correlation with -308 TNF. Tam et al. (32) found that ribavirn has shown to enhance antiviral type [1] cytokine expression, including that of TNF-α, and to suppresses type [2] cytokine expression in human T cells.

Moreover, the cause of viral persistence during HCV infection may be the development of a weak antiviral immune response to the viral antigens, with corresponding inability to eradicate infected cells or sensitivity of the virus to such cytokines or insufficient production of cytokines (33).

Our results revealed that there's no significant difference between responders and non responders to IFN therapy as regarding the quantitative PCR before beginning therapy by using Mann Whitney test.

Chia-Yen et al. (29) explained that the TNF-α -308.2 allele was independently associated with an SVR, particularly in patients with HCV genotype 1b infection and > 200,000 Iu of HCV RNA / mL in serum, these results are in agreement with our results.

In the present study, we found that serum (TNF-α) was higher among CHCV patients as compared to controls with statistically highly significant difference in between. Knobler and Schattner (13) showed that the activation of the TNF-α system has a pivotal role in the inflammatory process of CHCV and TNF-α levels correlate with the degree of inflammation, these finding is in agreement with our results.

## References


1-**William R (2006).** Global challenges in liver disease. Hepatology., 44: 521- 526.

**2-WWW. Who.int/immunization/ topics/ hepatitis_c/en/.**

**3-Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R**, **etalic (2001).** Peginterferon alfa-2b plus ribavirin compared with interferon alpha-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomized trial. Lancet., 358: 958- 965.

**4-Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL, etalic (2002).** Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med, 347: 975-982.

**5-Lauer GM and Walker BD, (2001).** Hepatitis C virus infection. New Engl J Med., 345: 41-52.

**6-Yee LJ, Tang T, Herrera J, Kaslow RA, Van Leeuwen DJ, (2000).** Tumor necrosis factor gene polymorphisms in patients with cirrhosis from chronic hepatitis C virus infection. Genes Immunity ; 1: 386- 390.

**7-Larrea E, Garcia N, Qian C, Civeira MP , and Prieto J , (1996)**. Tumor Necrosis Factor alpha gene expression and the response to interferon in chronic hepatitis C. Hepatology; 23 : 210-217.

**8-Liorent L, Richard-Patin Y, Alcocer-Castillejos N, Ruiz-Soto R, Mercado MA, Orozco H, Gamboa-Dominguez A, Alcocer-Varela J, (1996).** Cytokine gene expression in cirrhotic and non-cirrhotic human liver. J Hepatol ; 24: 555- 563.

**9-Bataller R, North KE, Brenner DA, (2003).** Genetic polymorphisms and the progression of liver fibrosis : a critical appraisal. Hepatology; 37: 493-503.

**10-Fargion S, Valenti L, Dongiovanni P, Fracanzani AL, (2004)**. TNF alpha promoter polymorphisms. Methods Mol Med; 98: 47- 58.

**11-Wilson AG , De-Vries M, Pociot F, Di-Giovine FS, Van-der-putte LBA, and Duff GW, (1993).** An allelic polymorphism within the human tumor necrosis factor a promoter region is strongly associated with HLAA1, B8 and DR3 alleles. JEXP Med; 177: 557-560.

**12-D'Alfonso S, and Richardi PM, (1994).** Apolymorphic variation in a putative regulation box of the TNF-α promoter region. Immuno genesis; 39: 150-154.

**13-Knobler H, and Schattner A, (2005).** TNF-α, chronic hepatitis C and diabetes: a novel triad. QJM: An International Journal of Medicine; 98 (1): 1-6.

**14-Wilson AG, Giovine DFS, Blakemore AL, Duff GW, (1992).** Single base polymorphism in human tumour factor-alpha (TNF alpha) gene detectable by Ncol restriction of PCR product. Hum Mol Genet 1(5): 353.

**15-Hedayati M, Sharifi K, Rostami F, Daneshpour MS, Yeganeh MZ, Azizi F, (2012).** Association between TNF-alpha promoter G-308A and G-238A polymorphisms and obesity. Mol Biol Rep 39: 825-829.

**16-Miller MC, Ph.D, Knapp RG (1992).**Clinical Epidemiology and Biostatistics, published by Williams, Wilkins, Maryland: 3rd edition.

**17-Biron CA, (1994).** Cytokines in the generation of immune responses to, and resolution of virus infection. Curr Opin Immunol; 6: 530- 538.

**18-Tough DF, Borrow P, Sprent J,(1996).**Induction of bystander T cell proliferation by viruses and type 1 interferon in vivo. Science ; 272: 1947- 1950.

**19-Cramp ME, Carucci P, Rossol S, Chokshi S, Maertens G, Williams R, Naoumov NV, (1999).** Hepatitis C virus (HCV) specific immune responses in anti-HCV positive patients without hepatitis C viraemia. Gut ; 44: 424- 429.

**20-Chang KM, Thimme R, Melpolder JJ, Oldach D, Pemberton J, Moorhead-Loudis J, McHutchison JG, Alter HJ, Chisari FV, (2001)**. Differential CD4(+) and CD8(+) T-cell responsiveness in hepatitis C virus infection. Hepatology ; 33: 267- 276.

**21-Pasha HF, Radwan MI ,Hagrass HA , Tantawy EA , Emara MH , (2013).** Cytokines genes polymorphisms in chronic hepatitis C :

Impact on susceptibility to infection and response to therapy. Cytokine; 61 (2): 478- 484.

**22-Thio CL , Thomas DL , Carrington M , (2000).** Chronic viral hepatitis and the human genome. Hepatology ; 31: 819-827.

**23-Hohler T, Kruger A, Gerken G, Schneider PM, Meyer-Zum-Buchenfelde K , Rittner C , (1998).** Tumour necrosis factor-alpha promoter polymorphism at position-238 is associated with chronic active hepatitis C virus infection. J Med Virol ; 54; 173-177.

**24-Asti M, Martinetti M , Zavaglia C , etalic, (1999).** Human Leucocyte antigen class II and class III alleles and severity of hepatitis C virus – related chronic liver disease. Hepatology ; 29: 1272-1279.

**25-Edwards-Smith CJ , Johnsson JR , Purdie DM , Bansal A , Shorthouse C , Powell EE , (1999).** Interleukin-10 promoter polymorphism predicts initial response of hepatitis C virus to interferon alpha. Hepatology ; 30: 526 – 578.

**26-Powell EE , Edwards-Smith CJ , Hay JL , etal, (2000)**. Host Factors influence disease progression in chronic hepatitis C. Hepatology; 31: 828-833.

**27-Yee LJ, Tang J, Gibson AW, Kimberly R, Van Leeuwen DJ, Kaslow RA, (2001).** Interleukin 10 polymorphisms as predictors of sustained response in antiviral therapy for chronic hepatitis C infection. Hepatology; 33: 708- 712.

**28-Abbas Z, Moatter T, Hussainy A, Jafriw, (2005)**. Effect of cytokine gene polymorphism on histological activity index, viral load and response to treatment in patients with chronic hepatitis C genotype 3. World J Gastroenterol 11, (42): 6656-6661.

**29-Chia-Yen Dai, Wan-Long Chang, Wen-Yu Chang, Shinn-Cherng Chen, Li-Po Lee, Ming-Yen Hsieh, Nai-Jen Hou, Zu-Yau Lin, Jee-Fu Huang, Ming-Yuh Hsieh, Liang-Yen Wang and Ming-Lung Yu, (2011).** Tumor Necrosis Factor-α Promoter Polymorphism at Position –308 Predicts Response to Combination Therapy in Hepatitis C virus Infection. Journal Of Infectious Diseases; 193: 98- 101.

**30-Fabris C, Soardo G, Falleti E, Toniutto P, Vitulli D, Federico E, Forno DM, Mattiuzzo M, Gonano F, Pirisi M, (1998).** Relationship among hepatic inflammatory changes, circulating levels of cytokines and response to IFN-alpha in chronic hepatitis C, J Interferon cytokine Res; 18: 705- 709.

**31-Romero-Gomez M, Montes-Cano MA, Otero-Femandez HA, Torres B, Sanchez-Mufioz D, Aguile F, Barroso N, Gomez-Izauierda L, Castellano-Megias V, Nuner-Roldan A, Aguilar Reiena J, and Gonzalez-Escriband MF, (2004).** The Solute carrier family 11 member 1 ( SLC11 A1) Promoter gene polymorphisms and Fibrosis progression in chronic hepatitis C. Gut; 53 (3) : 446- 450.

**32-Tam RC, Pai B, Bard J, etalic (1999).** Ribavirin polarizes human T cell responses towards a type 1 cytokine profile. J Hepatol; 30: 376- 382.

**33-Cerny A, Chisari FV, (1999).** Pathogenesis of chronic hepatitis C: immunological Features of hepatic injury and viral persistence. Hepatology; 30: 595- 601.