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THE TGF B1 509 C/T GENE POLYMORPHISM IN EGYPTIAN PATIENTS WITH CHRONIC HCV ANTIBODY POSITIVE PATIENTS AND ITS RELATION TO HCV VIRAL LOAD

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ABSTRACT

Background and aim: Hepatitis C virus is a significant health problem in Egypt as its prevalence is about of 14.9%. Many genetic factors influence the natural course of chronic hepatitis C. Transforming growth factor-β is a potent well-known suppressor of NK cells that inhibits IFN-γ and IL-12 production and blocks the proliferation and cytotoxicity of NK cells. The 509C/T mutation in the TGF-β1 gene is associated with promoter activity and with the natural clearance of HCV. Moreover, TGFβ1 is the strongest known inducer of fibrogenesis in the effectors cells of hepatic fibrosis. We aimed in this study to determine the TGF β1 509 C/T gene polymorphism by PCR- RFLP technique in chronic HCV patients and its relation to HCV viral load and the stage of hepatic fibrosis. **Patients and methods:** This study was conducted on 30 patients with +ve HCV, confirmed by the HCVAb presence by ELISA and by PCR and they were designed as group I. Group II: Included 20 healthy volunteers served as controls. Thorough clinical and laboratory assessments were done for all the participants in the study. PCR-RFLP for polymorphism of TGF β1509 was carried out for all subjects. Assessment of the stage of hepatic fibrosis was done for diseased group by analyzing results of Fibroscan for some patients and Actitest-Fibrotest for others. Informed consents were obtained. **Results:** There were a statistically significant relation between the TGF β1 509 T/C gene polymorphism in chronic HCV +ve patients and HCV viral load. The wild genotype CC and The C allele of TGF β1 509 was significantly higher in subjects with low HCV viral load compared with moderate to high viral load. There is no relation between the polymorphism of TGF β1 509 and the stage of

hepatic fibrosis. **Conclusion:** The wild genotype CC and The C allele of TGF β1 509 was significantly higher in subjects with low HCV viral load compared with moderate to high viral load. There is no relation between the polymorphism of TGF β1 509 and the stage of hepatic fibrosis.

Keywords: Hepatitis C virus-Transforming growth factor beta -Viral load -Hepatic fibrosis.

1-INTRODUCTION

The estimated global prevalence of Hepatitis C Virus (HCV) infection is 2.2%, corresponding to about 130 million HCV positive persons worldwide **(1).** Egypt has the highest prevalence of Hepatitis C (**2**). HCV prevalence in Egypt is estimated to be 14.9% in the year 2008 **(3).** Initial HCV infection is frequently asymptomatic and about 15-20% of infected patients experience natural clearance (**4**). Complications of chronic HCV infection occur in 20-30% of patients because of progressive liver fibrosis, leading to cirrhosis, liver failure, and hepatocellular carcinoma (HCC) (**5**). Liver fibrosis is a highly dynamic process in which multiple genes interact with environmental factors (**6**). A number of gene polymorphisms influence the progression of fibrosis in patients with chronic HCV infection (**5**). These genetic factors could explain the inter-individuals broad spectrum of responses to the same etiologic agent.

Transforming growth factor-β1 (TGF-β1) is one of the most dominant fibrogenic cytokines in hepatic fibrosis (**7**). In addition, it is a suppressor of natural killer (NK) cells that inhibits IFN-γ and IL-12 production and blocks the proliferation and cytotoxicity of NK cells (**8**). Insufficient production of cytokines could affect an individual's ability for virus clearance and prevention of chronic disease (**9)** A T/C transition at the -509 position of the promoter region of TGF-β1 gene is associated with a higher plasma concentration of TGF- β1 (**10**). High TGF-β1 producers might have more suppression of NK cells and may be less likely to resolve HCV infection **(11).** The aim of our study is to determine the TGF-β1 -509 C/T gene polymorphism in chronic HCV infected patients and its relation to HCV viral load and stage of hepatic fibrosis.

2-SUBJECTS AND METHODS

2.1-Study population

The study included 30 chronic HCV infected patients who were positive for HCV antibody for at least one year. They were recruited from the Tropical Clinic at Kasr Al-Ainy hospital,

Cairo University. The study was approved by the ethical committee of Kasr Al-Ainy School of Medicine, Cairo University and informed consent was obtained from patients. Patients receiving interferon therapy, positive for hepatitis B surface antigen, positive for anti-bilharzial antibody or co-infected with the human immunodeficiency virus were excluded from the study. Twenty age and sex matched healthy subjects were included in the study as a control group.

2.2-Methods

2.2.1-All Subjects in the study were subjected to the following

- **Full history taking:** Complaint, duration of the disease, history of hepatic complication, and history of any other chronic diseases.
- **Full clinical examination:** General and abdominal examination with especial emphasis on signs of liver affection.
- **Abdominal ultrasonography:**

US was performed to all patients. Comments were made on the size of the liver, smoothness of its surface, its texture, portal vein diameter, hepatic veins and presence of periportal fibrosis.

2.2.2-Hepatitis C Viral load

Determination of HCV viral load was done by quantitative RT-PCR for HCV RNA. Patients were classified according to their viral load into: low viral load: <600,000 IU/ml, moderate viral load: 600,000-800,000 IU/ml and high viral load: >800,000 IU/ml as described before **(12).**

2.2.3-Assessment of liver fibrosis

Hepatic fibrosis was assessed using Fibroscan (Echosens, Paris, France) as described before (**13)** The Fibroscan sends an elastic shear wave through the liver. The velocity of propagation of this wave is assessed as a measure of elasticity of liver tissue. Lower elasticity (fibrosis) causes the shear wave to move faster (**14**). Liver stiffness is expressed in Kilopascals and categorized into five scores (F0-F4). F0=no fibrosis, F1= minimal fibrosis, F2= moderate fibrosis, F3= severe fibrosis and F4=cirrhosis (**15**).

2.2.4-Analysis of TGF-β1 -509C/T Gene Polymorphism

Genomic DNA was extracted from whole blood using innuPREP Blood DNA Mini kit (Analytik Jena, Biometra, Germany). Genotyping was performed by polymerase chain

reaction-restriction fragment length polymorphism (PCR-RFLP) method according to **Tamizifar et al, (2007) (16)**. Reagents were provided by Bioflux, Japan. The primer sequences used were: Forward primer: 5'-CAGTAAATGTATGGGGTCGCAG-3' and Reverse primer: 5'- GGTGTCAGTGGGAGGAGGG-3'.

The PCR was carried out in thermal cycler (Hybaid, UK). Cycling conditions were; an initial denaturation at 94°C for 3 minutes followed by 35 cycles of amplification; denaturation at 94°C for 60 seconds., annealing at 61°C for 60 seconds and extension at 72°C for 60 seconds. In the last cycle, extension was prolonged to 5 minutes at 72°C. The PCR amplified product was digested using Eco81I restriction endonuclease enzyme (Fermentas, Thermo Scientific, USA). The restricted PCR products were electrophoresed through 2% ethidium bromide stained agarose gel, and visualized by ultraviolet light. Impaired cleavage occurred in the presence of the -509C/T polymorphism. Therefore, two fragments of 117bp and 36bp were detected in the presence of the C allele (wild type "CC"), only one 153bp band emerged when the T allele existed (homozygous "TT"), three bands of 153, 117 and 36bp were present in case of CT allele (heterozygous).

2-3-Statistical methods

Data were analyzed using SPSS win statistical package version 10. Numerical data were expressed as mean ±standard deviation (SD) and compared by student's t test. Qualitative data were expressed as frequency and percentage and compared by Chi-square test. Odds ratio (OR) with its 95% confidence interval (CI) were used for risk estimation. $p<0.05$ was considered significant.

3-RESULTS

The study included 30 chronic HCV infected patients (26 males and 4 females). The control group included 20 subjects (16 males and 4 females).

Table 1. compares patients and controls regarding demographic and laboratory data.

Quantitative RT-PCR for HCV RNA detection revealed that 17 patients (57%) had low viral load, 6 patients (20%) had moderate viral load and 7 patients (23%) had high viral load.

According to the results of Fibroscan, 13 patients (43.3%) had no or minimal hepatic fibrosis (F0-F1), 13 patients (43.3%) had moderate hepatic fibrosis (F2), 3 patients (10%) had severe hepatic fibrosis (F3) and 1 patient (3.3%) had cirrhosis (F4).

Table 2. shows the genotype frequency and allele frequency of TGF-β1 -509C/T gene polymorphism among patients and controls.

A statistically significant difference in genotype distribution could be detected between patients and controls (p=0.003), however the allele frequency among patients and controls was not different (p=0.08).

Patients carrying the wild homozygous genotype (CC) had a statistically significant lower viral load compared with those carrying the mutant T allele (CT-TT) (Table 3). The presence of the wild C allele is a predictor of HCV clearance (p=0.016, OR = 7.86, 95% CI = 1.31-47.04).

The degree of hepatic fibrosis among patients carrying the wild C allele is comparable to that of patients carrying the mutant T allele (Table 4).

TGF-β1 -509C/T gene polymorphism is not found to be a risk factor for the development of hepatic fibrosis (p=0.55, OR=1.57, 95%CI =0.36-6.87).

No statistically significant association could be detected between the degree of hepatic fibrosis and the HCV viral load (Table 4).

Table 1. Demographic and laboratory data of patients and controls.

Parameter	Patients n=30 (mean ±S.D)	Control n=20 (mean ±S.D)	p value
Age (years)	39.7± 8.60	39.10± 6.70	0.772
Hb (gm/dl)	11.26± 1.37	12.12±1.60	0.439
TLC(10^9/L)	6.06± 2.18	7.04± 2.40	0.142
Plt (10^9/L)	203.30± 181.60	170.90± 40.80	0.438
PC (%)	87.40± 9.63	91.70± 7.58	0.093
AST(IU/L)	53.03± 26.80	31.05± 7.92	<0.001
ALT (IU/L)	58.83± 28.62	32.10± 7.68	<0.001
Alk ph (IU/L)	101.43± 33.66	94.80± 23.59	0.452
Alb (gm/dl)	3.88± 0.69	4.49± 0.55	0.002
Bil (mg/dl)	1.03± 0.42	0.79± 0.20	0.010

Hb=Hemoglobin, TLC=Total leucocyte count, Plt= platelet, PC= prothrombin concentration, AST= Aspartate transaminase, ALT= Alanine transaminase, Alk ph= Alkaline phosphatase, Alb= Albumin, Bil= Bilirubin, p<0.05 is considered significant.

Table 2. TGF-β1 -509C/T genotype distribution and allele frequency among patients and controls

Gene polymorphism	Patients n=30	Controls n=20	p value
Wild type (CC)	12(40%)	0(0%)	0.003
Heterozygous(CT)	15(50%)	19(95%)	
Homozygous (TT)	3(10%)	1(5%)	
C allele	0.65	0.48	0.08
T allele	0.35	0.52	

Table 3. HCV viral load among patients with different TGF-β1 -509C/T genotypes

Gene polymorphism	High/Moderate viral load n=13	Low viral load n=17	p value
Wild type (CC)	2 (15.4%)	10 (58.8%)	p=0.04
Heterozygous (CT)	9 (69.2%)	6 (35.3%)	
Homozygous (TT)	2 (15.4%)	1 (5.9%)	
C allele	2 (15.4%)	10 (58.8%)	p=0.016
T allele	11 (84.6%)	7 (41.2%)	

Table 4. Genotype distribution and viral load among patients with different stages of hepatic fibrosis

	F0-F1 n=13	F2-F4 n=17	p value
Wild type (CC)	6 (46.2%)	6 (35.3%)	p=0.28
Heterozygous (CT)	7 (53.8%)	8 (47.1%)	
Homozygous (TT)	0 (0%)	3 (17.6%)	
High viral load	8 (61.5%)	9 (52.9%)	p=0.69
Moderate viral load	1 (7.7%)	5 (29.4%)	
Low viral load	4 (30.8%)	3 (17.6%)	

F0=no fibrosis, F1= minimal fibrosis, F2= moderate fibrosis, F3= severe fibrosis,F4=cirrhosis.

4-DISCUSSION

In this study, the -509T allele frequency among controls was 0.52. This frequency was higher than that detected among French Canadians (0.31) (**17**), Canadians (0.33) (**18**), French (0.35) (**19**), Iranians (0.36) (**16**), Italians (0.37) (**20**), Polish (0.40) (**21**) and Russian populations (0.42) (**22**).

Ethnic differences in the distribution of cytokine gene polymorphisms may explain ethnic differences in the outcome of HCV infection (**23**). Egyptian patients have high prevalence of

HCV, poor response to antiviral therapy, and an increased risk for the development of HCC (**24&25**).

In the current study, patients carrying the wild C allele of TGF- β1 -509C/T polymorphism had a significantly lower viral load. Few studies addressed the relation between TGF-β1 -509C/T polymorphism and HCV clearance. **Kimura et al., (2006)(11),** found that the -509C allele is associated with a higher clearance rate of HCV as well as with lower promoter activity leading to lower production of TGF-β1. These findings support the hypothesis that NK cells that are not inhibited by TGF-β1 may be protective in HCV infection (**26**). In a recent study, lack of TGF-β production by HCV-specific T cells during the acute phase of infection was found to be associated with HCV spontaneous clearance (**27**). In addition, polymorphisms associated with higher levels of TGF-β1 production are associated with viral persistence (**28**).

TGF-β1 has other non-NK cells functions. It has an important role in hepatic fibrogenesis (**29**). However, in the current study, TGF-β1-509C/T polymorphism was not found to be associated with the degree of hepatic fibrosis. In concordance with our result, **Suzuki et al., (2003)(7),** found no significant relationship between polymorphism at codon 10 of TGF-β1 gene and the development of progressive hepatic fibrosis in HCV infected Japanese patients. In contrast to these results, **Powell et al., (2000)(5),** detected a statistically significant relationship between inheritance of high TGF- β1 producing genotypes and the development of progressive hepatic fibrosis in chronic HCV infected patients. This inconsistency in results could be explained by the presence of other genetic factors that may affect the state of hepatic fibrosis. The pathogenesis of HCV induced liver fibrosis is not clearly understood. In some patients, the rate of fibrosis progression is fast and cirrhosis develops after 10 to 15 years, whereas in others, the rate of progression is negligible (**6).** A family history of liver disease is associated with developing cirrhosis at a younger age, suggesting a role for genetic susceptibility in fibrosis progression (**30**).

Polymorphisms in genes encoding cytokines involved in human fibrogenesis may regulate fibrosis progression in HCV infected patients. These cytokines include angiotensin II and TGF-β1 (**6).** Polymorphisms in the angiotensinogen gene (the angiotensin II precursor) as well as the TGF-β1 gene are major determinants of fibrosis progression in a series of patients with chronic HCV (**5**). Interestingly, patients having mutations in both genes progress more rapidly than those having only one polymorphism. The role of polymorphisms in genes

encoding pro-inflammatory cytokines is still unclear. While, **Yee et al., (2000)(31),** indicates that tumor necrosis factor promoter variants TNF2 (-238A) and TNF3 (-308A) conferred a 3.2-fold and 5.1-fold risk of cirrhosis respectively among patients with chronic HCV infection, another study has not confirmed these results (**5**). In addition, polymorphisms in the microsomal epoxide hydrolase gene, an enzyme involved in the metabolism of highly reactive epoxide intermediates, are associated with a more severe type of HCV-related liver disease (**32**).

In conclusion, TGF-β1 gene polymorphism may play a role in HCV clearance among Egyptian patients. There may not be a significant relationship between this polymorphism and development of hepatic fibrosis among HCV infected Egyptians. Large-scale studies are recommended to clarify the actual role of genetic factors in the development of liver fibrosis among Egyptian patients with chronic HCV infection.

5-CONCLUSION

TGF-β1 may play a role in HCV clearance among Egyptian patients. There may not be a significant relationship between TGF-β1 -509 C/T polymorphism and development of hepatic fibrosis among HCV infected Egyptians.

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