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RESEARCH ARTICLE

THE POLYMORPHISM OF TUMOR NECROSIS FACTOR ALPHA AND HEPATITIS C VIRUS RELATED HCC.

Mona Salah[1], Bothina Madkou[2], Rabab Fouad[2], Faiza El-Essawy[2], khaledTawfeek[2], Tamer Mahmoud El-baz[3] and Ayman Mohamed Abdel Aziz[4].

1. Department of Clinical and Chemical Pathology, Kasr Al Aini,-Cairo University, Cairo-Egypt.
2. Department of Hematology, Theodor Bilharz Research Institute, Cairo-Egypt.
3. Department of Tropical Medicine, Kasr Al Aini-Cairo University, Cairo-Egypt.
4. Department of Tropical Medicine, Theodor Bilharz Research Institute, Cairo-Egypt.

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Abstract

Background: Liver cancer is the second leading cause of cancer death worldwide. Hepatocellular carcinoma (HCC) is the most common type of primary malignant liver tumor.
Hepatitis C virus (HCV) infection is a major cause of chronic liver disease, cirrhosis, and hepatocellular carcinoma throughout the world. Hepatitis C virus (HCV) infection is a major public health burden in Egypt.
Proinflammatory cytokines as TNF-α were reported to be elevated among hepatitis patients. TNF-α has been implicated in liver development and regeneration but may also contribute to the pathogenesis of liver-related diseases such as cirhossis, fibrosis, and cancer.
Aim of the work: The aim of the present study is to determine the genotypes and alleles frequencies of TNF-α 308 G/A polymorphisms among some of the Egyptian patients with chronic HCV infection and healthy individuals to assess whether these genes are involved in chronic HCV susceptibility and/or HCC development.
Material and methods: A total of 55 HCV, 60 HCC and 50 healthy subjects were enrolled in the study for evaluation. Genomic DNA was extracted from the peripheral blood and genotyping was performed using Real Time polymerase chain reaction (RT- PCR) methodology.
Results: G/A, G/G and A/A frequencies in HCV patients were 20.0%%, 63.6%%, 16.4%% respectively, furthermore the frequencies were 10.0%, 90.0%, 00.0% in HCC patients and 8.0%, 88.0%, 4.0% in the control group respectively. The frequencies of G and A alleles in HCV patients were 73.7%, 26.3% and in HCC patients were 95.0%, 5.0% while in the control group the frequencies were 92.0%, 8.0% respectively. There was A significant difference between the studied groups regarding G/A, G/G, A/A frequencies. There was a significant difference in allele frequencies between patients with HCV and control groups (p=0.01). Moreover, there was highly significant difference in allele frequencies between HCC and HCV groups (p=0.001). While

Corresponding Author:- Mona Salah.
Address:- Department of Clinical and Chemical Pathology, Kasr Al Aini,-Cairo University, Cairo-Egypt.

there was no significant difference in allele frequencies between HCC and control groups (p=0.2).
Conclusion: The present study showed that TNF-α 308 G /A polymorphism was associated with increased HCC risk in Egyptian HCV infected population.

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Introduction:-

Liver cancer is the second leading cause of cancer death worldwide, with hepatocellular carcinoma (HCC) being the most common type of primary malignant liver tumour **[1].**

In Egypt, liver cancer forms 1.68% of the total malignancies. HCC constitutes 70.48% of all liver tumors among Egyptians **[2].**

HCC incidence has doubled in Egypt in the past 10 years, which could be attributed to the high prevalence of hepatitis C virus (HCV) and hepatitis B virus (HBV), although HBV rates have declined after the introduction of the vaccine in 1992 **[3].** In the Egyptian population, up to 90% of HCC cases were attributed to HCV infection **[4].**

The Egyptian Demographic Health Survey was conducted in 2015 on a large nationally representative sample. It estimated HCV prevalence among the 15–59 years age group to be 10%, Egypt has the highest HCV prevalence in the world **[5].**

Many Cytokines have been implicated in liver development, regeneration and also contribute to the pathogenesis of liver-related diseases such as cirhossis, fibrosis, and cancer **[6].**

HCV-specific T cells mediate ongoing viral clearance and liver injury by either directly killing infected hepatocytes or releasing pro-inflammatory cytokines, such as IFN-γ and Tumor necrosis factor-α (TNF-α) **[7].**

TNFα is multifunctional cytokine that helps to regulate different pathways involved in inflammation, immunity, apoptosis, antiapoptosis, and tumorigenesis **[8].**

Tumor necrosis factor-α (TNF α), which is encoded by the *TNFA* gene located on chromosome 6p21.3 **[9].**

Six diallelic polymorphisms in the α promoter, which are thought to affect TNF α production, have been reported and occur at positions 1031, 863, 857, 376,308, and 238 **[8].**

Single Nucleotide polymorphisms (SNPs) within the TNF α gene have the potential to cause structural changes within regulatory sites that could affect function or regulation of TNF-α production. The best documented example of this is the SNP at position –308 of the TNF α gene promoter, which involves the substitution of guanine (G) for adenine (A) and the creation of two alleles (–308G and –308A) and three genotypes GG, GA and AA **[10].**

Although some studies have investigated the associations between *TNFA* gene –308G/A polymorphisms and HCC, the results are conflicting **[11].**

The aim of the present study is to determine the genotypes and alleles frequencies of TNF-α −308 G > A polymorphisms among some of the Egyptian patients with chronic HCV infection (HCC and non-HCC) and healthy individuals to assess whether these genes are involved in chronic HCV susceptibility and/or HCC development.

Subjects and methods:-

Study population and demographic information:-

Our study was conducted in the Tropical department at Theodor Bilharz Research institute (TBRI) where 165 subjects were enrolled and divided into 3 groups; group A involved 55 patients diagnosed with HCV while group B constituted 60 patients with established diagnosis of HCC on top of HCV. Lastly; group C involved 50 healthy age and sex matched individuals who served as a control group. Exclusion criteria were made to eliminate those with HBV comorbidity, schistosomiasis, alcohol consumption or antiviral therapy from the study. An informed consent

was obtained from patients who participated in the study. Moreover, the procedures used were approved by TBRI ethics committee according to Helsinki Declaration.

Group A "HCV patients" was subdivided into 26(46.7%) males and 29(53.3%) females and their age ranged from 34 to 67 years (mean± SD= 44.3 ±13.9). in group B "HCC patients" there were 42 (70%) males versus 18 (30%) females with age ranged from 48 to 60 years (Mean± SD= 46.8±15.9). furthermore, group C included 40 (80%) males and 10 (20%) females with their age ranged from 32 to 57 years (Mean±SD= 46.7±13.3).

Serological markers:-
HCV IgG antibodies as well as HBsAg measurement were assessed using enzyme-linked immunosorbent assay (ELISA).

Genomic DNA extraction:-
Genomic DNA was obtained using the QIAamp DNA Mini Kit (Qiagen; catalog No.: 51104). 5ml peripheral venous whole blood was collected in a sterile vacuum tube containing EDTA for genomic DNA extraction by means of standard protocol using proteinase K. Lysis of red blood cells was done 3 times using lysis buffer. Afterwards, Sodium dodecyl sulfate (SDS) 10% and 10 ul proteinase K in the presence of guanidine HCL were added to treat the remaining white cells for a short incubation time (10 minutes at 56 C) in order to inactivate all nucleases. Cellular nucleic acids then bind to a special glass fibers pre-packed in high pure purification filter tube and a series of "wash and spin" steps were executed using 500 ul Buffer AW1 and 500 ul Buffer AW2 for getting rid of PCR impurities. Finally, elution buffer (200 ul Buffer AE) was added and incubation was done for 1 min at 15-25 C to release the nucleic acid from the glass fiber.

TNF-α genotyping:-
TNF-α G/A gene polymorphism **(rs1800629)** was detected using Taq Man SNP genotyping assay. This assay consists of a single, ready to use tube that contain two sequence specific primers for amplifying the polymorphism of interest together with two allele-specific Taq Man minor groove binder (MGB) probes for detecting the alleles for the specific polymorphism of interest. Each probe has a reporter dye; VIC dye is linked to the 5' end of allele A probe while FAM dye is linked to the 5' end of allele G probe. Each PCR reaction contained 2.5 ul of diluted DNA (5 ng/ul), 12.5 ul of 2× TaqMan Universal PCR Master Mix, 1.25 ul of 20× TaqMan SNP Genotyping Assay Mix and 8.75ul of Distilled water (DW). This PCR reaction was carried out in a thermal cycler using ABI 7500, with the following program shown in table (1).

Table 1:- PCR amplification run:

Pre-Read Run		Amplification (RQ-PCR program)			Post-Read Run	
1 Cycle		Temperature	1 Cycle	Cycles	1 Cycle	
60 °C	1 min.	95°C	10 min	X1	60 °C	1 min
		95°C	15 sec	X40		
		60°C	60°C			

Finally, a threshold is set at 0.1 for analysis; using ABI Prism "genetic analyzer". This comprises quantization of the amplified PCR product (DNA fragments) as well as determining the size of the fragments by comparing them to fragments contained in a size standard.

Statistical analysis:-
The data was analyzed using Microsoft Excel 2010 and statistical program for social science (SPSS version 22.0) for windows (SPSS IBM., Chicago, IL). Continuous normally distributed variables were represented as mean±SD with 95% confidence interval, in addition to using the alleles and genotype frequencies and percentage for Categorical and non-parametric variables; p value of less than 0.05 was considered statistically significant. The Student's t test was performed to compare the means of normally distributed variables between groups. ANOVA followed by Tukey-Kramer as a post-hoc test in multi groups, $\chi 2$ test or Fisher's exact test were used to determine the distribution of categorical variables between groups.

Results:-

In our study we evaluated *TNF-α* G/A gene polymorphism (rs1800629) in 55 HCV patients and 60 HCC patients together with 50 control subjects using RT- PCR, the study has been carried out in Cairo, Egypt at the tropical medicine department of TBRI, the study was approved by TBRI ethics committee. Our results showed that there was a significant difference in allele frequencies between patients with HCV and control groups (p=0.01). Moreover, there was highly significant difference in allele frequencies between HCC and HCV groups (p=0.001). While there was no significant difference in allele frequencies between HCC and control groups (p=0.2).

The G/G genotype was more frequent among HCC patients (90.0%) in comparison with control subjects (88.0%) and HCV patients (63.6%) while the frequency of A/A genotype was low among HCV patients (16.4%), HCC patients (00.0%) and controls (4.0%). In addition, G allele was remarkably frequent among HCC patients (95.0%) compared to HCV patients (73.7%) and the healthy individuals (92.0%), on the other hand C allele frequency was low in all the studied groups Data are presented in table (2) and figure 1.

Table 2:-Frequency of *TNF-α* Allele and genotype among all the studies groups.

TNF polymorphism		Groups			Total	P. value		
		Control N=50	HCV N=55	HCC N=60		Control & HCV	Control & HCC	HCV & HCC
TNF polymorphism	Hetero G/A	4(8.0%)	11(20.0%)	6(10.0%)	21	0.01*	0.2	0.001**
	Homo G/G	44(88.0%)	35(63.6%)	54(90.0%)	133			
	Homo A/A	2(4.0%)	9(16.4%)	0(0.0%)	11			
Total	G allele	92(92.0%)	81(73.7%)	114(95.0%)	287			
	A allele	8(8.0%)	29(26.3%)	6(5.0%)	43			

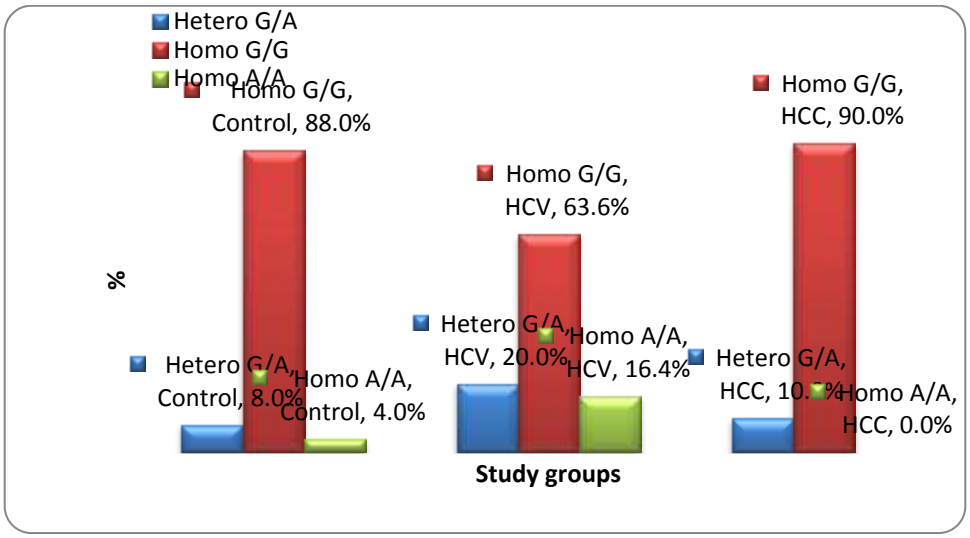

Figure:- Frequency of TNF- *α* Allele and genotype among the studied group.

Discussion:-

Hepatocellular carcinoma is the second leading cause of cancer related death worldwide, with hepatocellular carcinoma (HCC) being the most common type of primary malignant liver tumor, with a typically poor prognosis **[1].** Early detection of patients with HCC gives better prognosis as HCC tends to grow slowly and stay confined to the liver **[12].**

According to the World health organization (WHO) records, 3% of the world's population has been estimated to be infected with HCV and currently more than 170 million people are HCV chronic carriers **[13].**

HCV-induced HCC could now be established through many epidemiological studies revealed that many tumors occur in association with chronic infectious diseases, and it is also known that persistent inflammation increases the risk and accelerates the development of cancer **[14].**

Cytokines, as the products of host response to inflammation, play an important role in the defense against viral infections. However, in HCV infection they may play a prominent role in liver damage **[15].**

Tumor necrosis factor-alpha (TNF-α) gene encodes a pro-inflammatory cytokine that is secreted primarily by macrophages and plays critical roles in the pathogenesis of inflammatory autoimmune and malignant diseases. The TNF-α protein induces the expression of adhesion molecules, facilitating the invasion of metastatic tumor cells **[16].**

TNF-α, has an integral part of inflammation in chronic hepatitis C infection. Hepatocyte damage elicits an inflammatory response through activation of tissue macrophage Kupffer cells. These activated cells release an array of cytokines, including tumor necrosis factor (TNF) α, transforming growth factor-β, platelet-derived growth factor, and other factors that act on hepatic stellate cells that contribute to fibrogenesis **[17].**

Among antiviral cytokines, TNF- α plays a pivotal role in host immune response to HBV/HCV infection. Circulating TNF- α level increases during HBV and HCV infection **[18].**

An increased TNF- α level correlates with the severity of hepatic inflammation, fibrosis, and tissue injury **[18].**Persistent immune-mediated hepatic injury can initiate the process of fibrosis, cirrhosis and HCC **[19].**

Polymorphism in the human TNF-α promoter at –308 involves the substitution of adenosine for guanine in the uncommon alleles. The presence of guanine defines the common variant TNF1 allele, and the presence of adenine defines the less common variant TNF2 allele **[8].**

TNF- α-308G>A polymorphism has been reported to alter the risk of several types of cancers, such as breast cancer, lung cancer, non-Hodgkin lymphoma and breast cancer **[20].** But the role of TNF- α-308G>A polymorphism in the pathogenesis of HCV infection and risk of HCC development is controversial **[21].**

The aim of the present work was to study polymorphism (TNF α – 308 G/A), which is located in the promoter region of the TNF-α gene in patients with chronic HCV infection, patients with HCC on top of chronic HCV infection and healthy individuals, being an important cytokine in generating an immune response against infection with hepatitis C virus (HCV). It aimed also to investigate the role of this polymorphism with the progression of HCV patients to HCC.

This study was done through assessing 55 HCV patients, 60 HCC patients from those admitted to the tropical department at Theodor Bilharz Research Institute in addition to 50 healthy controls using RT-PCR.

We detected that there was a significant difference in allele frequencies between patients with HCV and control groups (p=0.01). Moreover, there was highly significant difference in allele frequencies between HCC and HCV groups (p=0.001). While there was no significant difference in allele frequencies between HCC and control groups (p=0.2).

Our results are in agreement ***Jeng et al., (2007)*** who found that presence of the TNF-α 308.2 and chronic hepatitis B virus/hepatitis C virus infection are independent risk factors for HCC. Presence of the TNF-α 308.2 allele correlates with disease severity and hepatic fibrosis, which may contribute to a higher risk for HCC **[22].**

Also ***Feng et al., (2014)*** found that TNF-α −308 G > A polymorphism was associated with increased HCC risk in a Han Chinese population **[20]**.

In addition a metanalysis study indicated that TNF-α −308 G > A polymorphism is significantly associated with increased susceptibility to HCC. However,to confirm this finding more studies are needed to be proved **[23].**

But on the other ***Romero-Gomez et al., (2004),*** who found no association between TNF α–308 polymorphisms and the severity of fibrosis in HCC **[24].** Also ***Sun et al, (2015)*** found that the *TNF*-308 G/A polymorphisms are not associated with hepatocellular carcinoma risk among Asians **[25].**

Also in contrast to our results ***Constantini et al.,(2002)*** who made a study about TNF, Interleukin 1and interleukin 10 polymorphisms and their relation to viral clearance found that there is no significant correlation between degree of liver fibrosis and viral clearance and any of the cytokine genotypes tested and he suggested focusing on other candidate genes [25] .

In conclusion, the present study showed that TNF-α −308 G /A polymorphism was associated with increased HCC risk in Egyptian population. Further prospective studies on large and different ethnic populations will be necessary to confirm our findings and elucidate the underlying molecular mechanism for the development of HCC.

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