ISSN: 2230-9926

Available online at http://www.journalijdr.com

IJDR

International Journal of Development Research
Vol. 08, Issue, 12, pp.24678-24682, December, 2018

ORIGINAL RESEARCH ARTICLE OPEN ACCESS

CHEMICAL AND BIOCHEMICAL STUDIES OF RELATION OF TUMOR NECROSIS FACTOR GENE-POLYMORPHISM IN HEPATOCELLULAR CARCINOMA PATIENTS

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ARTICLE INFO

Article History:
Received 17th September, 2018
Received in revised form
12th October, 2018
Accepted 20th November, 2018
Published online 31st December, 2018

Key Words:
(TNF-α) Tumor necrosis factor-alpha 4,
HCC hepatocellular carcinoma,
HCV Hepatitis C virus.

ABSTRACT

Background: Tumor necrosis factor-alpha (TNF-α) encodes a proinflammatory cytokine that is secreted primarily by macrophages and plays critical roles in the pathogenesis of inflammatory autoimmune and malignant diseases. (TNF-α) expression may disturb immune response and may be associated with HCC risk. **Objectives:** To verify the role of Tumor necrosis factor-alpha gene −308G > A polymorphism in Hepatitis C virus related hepatocellular carcinoma in Egyptian patients. **Methods:** (TNF-α) −308 G > A polymorphismwas examined in 50 patients with HCV-related HCC, 40 patients with HCV-induced liver cirrhosis and 30 healthy controls, using the polymerase chain reaction- restriction fragment length polymorphism method. **Results:** Overall (TNF-α) −308 G > A gene polymorphism showed that AA genotype was more prevalent in HCC group (40%) compared to control group (6.7%), with OR (95% CI) 9.33 times risk of HCC (p value = 0.018) and cirrhotic patient group (17.5%), with OR (95% CI) 3.14 times risk (p value= 0.021). The A allele frequency was increased in HCC group (53%) versus (18.3% and 27.5 %, OR (95% CI) = 5.02 and 2.97) in control and cirrhotic patients group respectively. C**onclusion:** This studysuggests that (TNF-α) −308 G > A polymorphism, AA homozygous genetic model, may be a risk factor in HCV related HCC in Egyptian patients.

Citation: Aida Hamed Soliman, Ahmed Ebrahim, Olfat Mohamed Hendy, Heba Samy and Mohy Eldin Abd El Fattah. 2018. "Chemical and biochemical studies of relation of tumor necrosis factor gene-polymorphism in hepatocellular carcinoma Patients", *International Journal of Development Research*, 8, (12), 24678-24682.

INTRODUCTION

Hepatocellular carcinoma (HCC) is an aggressive human cancer with high fatality due to metastasis and recurrence after surgery (Bruix, 2004). In Egypt it is a major health problem representing the second most common cancer in men and the sixth most common cancer in women (GLOBOCAN, 2008).HCC is considered an example of inflammation-related cancer, as hepatitis B and C chronic inflammation is a major risk factor (El serag, 2012). Chronic inflammatory state is characterized by the continued expression of cytokines and recruitment of immune cells to the liver which appears to be necessary for the initiation and development of liver cancer (Berasain, 2009).

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TNF-α is a proinflammatory cytokine that affects the growth, differentiation, and survival of various cells. It is mainly produced by macrophages, in addition to other array of cells, including neutrophils, keratinocytes, mast cells, endothelial cells, neurons, NK cells, fibroblasts, T and B lymphocytes and tumor cells (Anderson, 2004).TNF-α has been reported to promote chronic inflammation-related carcinogenesis. TNF-α gene is located on chromosome 6p21.3 of the major histocompatibility complex classⅢregion. The most studied polymerphisms in TNF-α gene lies in the promoter region TNF-α (-1031T/C, -863 C/A, -857 C/T, -308 G/A and -238 G/A). These polymorphisms have been reported to modulate the production of TNF-α (Akkiz, 2009). TNF-α-308G/A is considered a critical risk factor for liver carcinogenesis (8), but TNF-α-238G/A was found to have a passive role in HCC risk (9). TNF-α-308GG genotype has been reported to be associated with adecrease of HCC incidence (10). On the other

hand, other studies reported higher risk of HCC in patients carrying TNF-α-308A allele (Akkiz, 2009).Therefore in this study we aimed to study the contribution of promoter polymorphism of TNF-α-308G/A to the susceptibility of HCV-related HCC.

Subjects and methods

Study participants: A total of 90 patients were recruited in this case-control study from Hepatology Department, National Liver Institute, Menoufia University. Thorough history taking , clinical evaluation , laboratory investigations in the form of liver profile, renal profile, complete blood count, Alfa fetoprotein(AFP) and radiological investigation in the form of triphasic CT abdomen, chest Xray, bone scan were done. They were further classified according to clinical examination, laboratory and radiological findings into 2 groups; Cirrhotic patients group (Group II) which included 40 cirrhotic patients with no radiological evidence of hepatocellular carcinoma and HCC group (Group III) (arterial enhancement and washout in venous or delayed phase is the imaging characterstics) which included 50 patients with HCC on top of HCV. In addition, 30 healthy age- sex matched individuals (Group I) were enrolled as control group. Patients with other coexistent liver disease as HBV infection, autoimmune hepatitis, non- alcoholic steatohepatitis, genetic metabolic diseases were excluded from the study. Protocol was approved by the local ethical committee of National Liver Institute and informed consent was taken from subjects before the study.

Blood collection and routine investigations: After assessment of the patients by clinical and radiological examination, venous blood samples were obtained from each participant and divided into three aliquots; a plain test tube vacutainer for separation of serum and two plastic tube vacutainer containing (K- EDTA) for complete blood count and genotyping. Sera were subjected to routine laboratory study including liver and renal function tests performedon Micro lab 300 auto analyzer (vital scientific, Netherlands) using Diamond Kits (Germany) for measuring urea, total and direct bilirubin; Human Kits (Germany) for measuring Aspartate Aminotransferase (AST), Alanine Aminotransferase(ALT) and creatinine and spinreact kits (Spain) for measuring albumin according to manufacturer instructions. HCV-Ab and serum alpha feto protein were detected by Cobas e411 immunoassay analyzer (Roche diagnostics- GmbH, D- 68305Mannhein, Germany). Complete blood count was measured using Sysmex XT- 1800i (Sysmex Corporation, Kobe 651-0073, Japan).

Study of TNF-α-308G/APolymorphism by PCR-RFLP: Genomic DNA was extracted from EDTA treated blood samples using Quick-gDNA™MiniPrepGenomic DNA Purification Kit(ZymoResearch,USA). PCR amplification of the 107 base pair (bp) stretch within the promoter region of TNF-αgene was performed followed by AvaII restriction enzyme digestion (Biolabs, Inc, England). The primers used were:5-AGGCAATAGGTTTTGAGGGCCAT-3forward 5-TCCTCCCTGCTCCGATTCCG-3(reverse). PCR reaction mixture consisted of 1µL of each of primers, 12.5µl of MyTaq Red PCR master mix (Bioline USA Inc, USA), 5.5µl of Water, nuclease- free and 5µl of extracted DNA. Amplification was performed in Perkin Elmer Gene Amp PCR System 2400 Thermal Cycler in the following cycle conditions; 95°C for 5 min, followed by 35 cycles, each cycle consisted of denaturation at 95°C for 45 seconds, annealing at 50°C for 45 seconds and extension at 72°C for 45 seconds. Final extension at 72°C for 10min was carried out. The genotyping analysis of the 195 bpamplicon was performed through digestion with Ava II restriction enzyme at 37°C for 20 min. Detection of the digested products was carried out by 3% agarose gel electrophoresis. PCR products with C at the polymorphic site were digested showing two fragments 177 and 18 bp, while those with T were not. Samples yielding 107 bp fragments were scored as GG genotype, those with 87 and 20 bp fragments as AA genotype, and 107, 87,20 bp as GA genotype.

Statistical methods

Results were statistically analyzed by using statistical package of social sciences (SPSS 22.0, IBM/SPSS Inc., Chicago, IL) Descriptive statistics were applied for summarizing clinical and demographical data as mean (X), standard deviation (SD), median (Med), and range or interquartile range (IQR) for quantitative data, and frequency with percentage (%) for qualitative data. Inferential statistics were conducted to test significant difference among the studied groups. For comparing continuous variables, ANOVA or Student's t test were used when normality and homogeneity assumptions were met, instead,their non-parametric equivalent sKruskal-Wallisor Mann-Whitney test were applied upon violation. The Chi-square (2) test was used to compare allele frequency and genotype distribution of TNF-α-308G/Agene polymorphism and to estimate the disease risk via odds ratio (OR) also Fisher's exact test was used when Chi-square assumptions were violated. Binary logistic regression analysis was applied to calculate the adjusted odds ratios (ORs) and 95% confidence intervals (CIs) after controlling for after age and gender status to exclude potential confounding effect. The P-values indicating the significance level was set at 0.05.

RESULTS

Characteristics of the study participants: Table (1) summarizes characteristics of the study population. There was no statistical difference concerning age and gender between different studied groups (p value >0.05), however, male gender in HCC group (64%) was more prevalent than female gender (36%). On the other hand, comparing liver and renal function tests between studied groups showed highly statistical significance between studied groups which was contributed to the statistical difference between control group and each of the cirrhotic and HCC group which was not the case on comparing between cirrhotic and HCC group (data not shown in table).

Genotype polymorphism and risk of hepatocellular carcinoma: Table 2 & 3 were studying genotype distribution and allele frequencies of TNF-α-308G/A among HCV- related HCC patients group compared to control group and cirrhotic patients group respectively. Table 2 revealed that the TNF-α −308 GG genotype distribution were (70%), which were similar to healthy Chinese result (96.4%), while the AA genotype distribution accounted for (6.7%), which was similar to healthy Chinese (0.4%)Thus, the A allele may be the variant responsible for various diseases in Egyptian population, which is similar to Chinese population.

Table 1. Study characteristics, Age, Gender, liver and renal profile between studied groups

Age (years)	**GIControl (n = 30)**	**GIICirrhosis (n = 40)**	**GIIIHCC (n = 50)**	**ANOA test**	***P-value***
Median (IQR)	50.00 (5.50)	52.5 (9.75)	52.5 (8.00)	F = 2.790	0.066 NS
Mean ± SD	50.37 ± 4.61	53.35 ± 5.88	52.86 ± 5.74		
Range (min-max)	40.00 - 59.00	43.00 - 66.00	42.00 - 67.00		
Gender				**Chi-Square test**	***P*-value**
Male [n, (%)]	16 (53.3)	22 (55.0)	32 (64.0)	χ^2= 1.152	0.562 ^{NS}
Female [n, (%)]	14 (46.7)	18 (45.0)	18 (36.0)		
Parameters				Kruskal-Wallis test	Pairwise comparisons [a]
ALT (U/L)				χ^2 =57.77	P_1< 0.001^{HS}
Median (IQR)	17.00 (8.25)	48.50 (31.50)	55.50 (28.25)	*P*-value	P_2< 0.001^{HS}
Mean ± SD	18.67 ± 6.46	54.05 ± 26.47	55.86 ± 21.74	<0.001^{HS}	P_3=0.893^{NS}
Range (min-max)	9.0 - 36.0	16.0 - 146.0	20.0 - 102.0		
AST (U/L)				χ^2 = 65.39	P_1< 0.001^{HS}
Median (IQR)	19.00 (4.50)	60.50 (35.75)	62.50 (31.00)	*P*-value	P_2< 0.001^{HS}
Mean ± SD	19.27 ± 4.04	65.70 ± 25.95	68.42 ± 33.29	<0.001^{HS}	P_3=1.000^{NS}
Range (min-max)	9.0 - 36.00	16.0 - 146.0	20.0 - 102.0		
Total bilirubin (mg/dL)				χ^2 = 59.52	P_1< 0.001^{HS}
Median (IQR)	0.50 (0.22)	1.19 (1.30)	1.50 (1.20)	*P*-value	P_2< 0.001^{HS}
Mean ± SD	0.52 ± 0.18	1.52 ± 0.96	1.71 ± 0.84	<0.001^{HS}	P_3 = 0.416^{NS}
Range (min-max)	0.10 - 0.80	0.45 - 4.18	0.73 - 3.63		
Direct bilirubin (mg/dL)				χ^2 = 59.35	P_1< 0.001^{HS}
Median (IQR)	0.10 (0.05)	0.38 (0.98)	0.62 (0.77)	*P*-value	P_2< 0.001^{HS}
Mean ± SD	0.12 ± 0.04	0.76 ± 0.78	0.89 ± 0.71	<0.001^{HS}	P_3 = 0.371^{NS}
Range (min-max)	0.07 - 0.23	0.09 - 3.08	0.18 - 2.53		
Albumin (g/dL)					
Median (IQR)	4.40 (0.51)	3.68 (1.04)	3.32 (1.10)	χ^2 =40.00	P_1< 0.001^{HS}
Mean ± SD	4.36 ± 0.39	3.56 ± 0.65	3.36 ± 0.64	*P*-value	P_2< 0.001^{HS}
Range (min-max)	3.50 - 5.10	2.20 - 4.90	2.20 - 4.52	<0.001^{HS}	P_3 = 0.478^{NS}
Urea (mg/dL)				χ^2 = 34.99	P_1< 0.001^{HS}
Median (IQR)	27.50 (8.00)	37.50 (14.25)	38.00 (10.50)	*P*- value<0.001^{HS}	P_2< 0.001^{HS}
Mean ± SD	27.43 ± 5.72	38.38 ± 9.28	39.30 ± 9.28		P_3 = 0.960^{NS}
Range (min-max)	17.0 - 41.0	22.0 - 56.0	22.0 - 67.0		
Creatinine (mg/dL)				χ^2 = 13.66	*P1* < 0.001^{HS}
Median (IQR)	0.80 (0.20)	0.87 (0.46)	1.00 (0.33)	*P*-value	*P2* < 0.001^{HS}
Mean ± SD	0.81 ± 0.17	0.94 ± 0.26	1.00 ± 0.21	=0.001^{HS}	*P3* = 0.231^{NS}
Range (min-max)	0.50 - 1.20	0.58 - 1.50	0.50 - 1.50		

-P1:P-value for the difference between control and cirrhosis group (GI vs. GII)
-P2:P-value for the difference between control and HCC group (GI vs. GIII)
-P3: P-value for the difference between cirrhosis and HCC group (GII vs. GIII)
a: Fisher's Exact test; b: Pearson Chi-Square test
%= percent of genotype or allele within group NS : Non significant at P-value ≥ 0.05
-S: Significant at P-value < 0.05 - HS: Highly significant at P-value < 0.01

Table 2. Comparison of genotype distribution and allele frequencies between control and HCC groups

Polymorphism of TNF-α −308 G > A	GIControl (n = 30)	GIIIHCC (n = 50)	OR (95% CI)	P-value
Genotypes [n (%)]				
GG	21 (70.0)	17 (34.0)	Ref.	
GA	7 (23.3)	13 (26.0)	2.29 (0.75-7.03)	0.142 ^{NS,a}
AA	2 (6.7)	20 (40.0)	12.35 (2.52-60.45)	< 0.001 $^{HS, a}$
Dominant model				
GG	21 (70.0)	17 (34.0)	Ref.	-
AA+GA	9 (30.0)	33 (66.0)	4.53 (1.71-12.02)	0.002 $^{HS, a}$
Recessive model				
GA+GG	28 (93.3)	30 (60.0)	Ref.	-
AA	2 (6.7)	20 (40.0)	9.33 (2.00-43.63)	0.001 $^{HS, a}$
Alleles[n (%)]				
G	49 (81.7)	47 (47.0)	Ref	-
A	11 (18.3)	53 (53.0)	5.02 (2.34-10.77)	<0.001 $^{HS, a}$

-[a]: Pearson Chi-Square test -[b]: Fisher's Exact test
- %= percent of genotype or allele within group-^{NS} : Non significant at *P*-value ≥ 0.05 -^{S}: Significant at *P*-value < 0.05
- ^{HS}: Highly significant at *P*-value < 0.01

Genotype have 10 times risk of hepatocellular carcinoma versus control group with significant difference (p value <0.05) which was more clear when adjusted OR (95% CI) was done which showed 17.8 more risk for TT genotype with more significant p value. On the other hand AA genotype was more prevalent in HCC group (40%) compared to control group (6.7%), with OR (95% CI) 9.33 times risk of HCC (p value = 0.018) and cirrhotic patient group (17.5%), with OR (95% CI) 3.14 times risk (p value= 0.021).

The A allele frequency was increased in HCC group (53%) versus (18.3% and 27.5 %, OR (95% CI) = 5.02 and 2.97) in control and cirrhotic patients group respectively (Table 3).Furthermore, Study of genetic model TNF-α-308G/ASNP (AA vs. GA+GG) regarding characteristics of focal lesions in HCC group (Table 4) revealed that showed no significant difference (P> 0.05).

Table 3. Comparison genotype distribution and allele frequencies between cirrhosis and HCC groups

Polymorphism of TNF-α −308 G > A	GII Cirrhosis (n = 40)	GIII HCC (n = 50)	OR (95% CI)	P-value
Genotypes [n (%)]				
GG	25 (62.5)	17 (34.0)	Ref.	-
GA	8 (20.0)	13 (26.0)	2.39 (0.82-7.00)	0.108 NS, a
AA	7 (17.5)	20 (40.0)	4.20 (1.46-12.11)	0.006 HS, a
Dominant model				
GG	25 (62.5)	17 (34.0)	Ref.	
AA+GA	15 (37.5)	33 (66.0)	3.24 (1.36-7.70)	0.007HS, a
Recessive model				
GA+GG	33 (82.5)	30 (60.0)	Ref.	
AA	7 (17.5)	20 (40.0)	3.14 (1.17-8.48)	0.021 S, a
Alleles[n (%)]				
G	58 (72.5)	47 (47.0)	Ref.	-
A	22 (27.5)	53 (53.0)	2.97 (1.59-5.57)	0.001 HS, a

-[a]: Pearson Chi-Square test - %= percent of genotype or allele within group
-[NS]: Non significant at *P*-value ≥ 0.05 -[S]: Significant at *P*-value < 0.05
- [HS]: Highly significant at *P*-value < 0.01

Table 4. Comparison between genotypes of -TNF-α −308 G > A polymorphism regarding characteristics of focal lesions in HCC group

Parameters	Genotypes of TNF-α −308 G > A Polymorphism in HCC			Test of significance	*P*-value
	AA (n=20)	GA (n=13)	GG (n=17)		
Number of foci [n (%)]					
Single	9 (45.0)	7 (53.8)	10 (58.8)	□□ = 0.73	0.695 NS, a
Multiple	11 (55.0)	6 (46.2)	7 (41.2)		
Size of foci [n (%)]					
Small (≤ 2 cm)	9 (45.0)	1 (7.7)	2(11.8)	□□ = 7.52	0.106 NS, b
Moderate (>2 -5 cm)	7 (35.0)	8 (61.5)	9 (52.9)		
Large (> 5 cm)	4 (20.0)	4 (30.8)	6 (35.3)		

-[a] : Pearson Chi-Square test -[b]: Fisher's Exact test
-[NS] : Non significant at *P*-value ≥ 0.05-[S]: Significant at *P*-value < 0.0
- [HS]: Highly significant at *P*-value < 0.01.

DISCUSSION

Hepatocellular carcinoma (HCC), a primary liver cancer, is the second most common cause of cancer mortality worldwide (Petrick, 2016). HCC arises almost exclusively in chronically inflamed livers triggered by various causes (Villanueva, 2015), where chronic inflammation is characterized by the continued expression of various cytokines and recruitment of many types immune cells to the liver. Chronic infection with hepatitis B virus and hepatitis C virus are considered major risk factors for HCC development (Goossens, 2015). HCV, an RNA virus, doesn`t integrate into the host genome, so pathogenesis of HCV-related HCC is proposed to involve generation of oxidative stress and increased expression of inflammatory cytokines process of chronic hepatitis (Liang, 2004), in addition to the direct role of HCV viral gene products (El-Nady, 2003). TNF-α is a proinflammatory cytokine that affects the growth, differentiation, and survival of various cells. It is mainly produced by macrophages, in addition to other array of cells, including neutrophils, keratinocytes, mast cells, endothelial cells, neurons, NK cells, fibroblasts, T and B lymphocytes and tumor cells (Anderson, 2004).TNF-α has been reported to promote chronic inflammation-related carcinogenesis. TNF-α gene is located on chromosome 6p21.3 of the major histocompatibility complex classⅢregion. The most studied polymerphisms in TNF-α gene lies in the promoter region TNF-α (-1031T/C, -863 C/A, -857 C/T, -308 G/A and -238 G/A). These polymorphisms have been reported to modulate the production of TNF-α (Akkiz, 2009). Our study showed that AA genotype was more prevalent in HCC group (40%) compared to control group (6.7%), with OR (95% CI) 9.33 times risk of HCC (p value = 0.018) and cirrhotic patient group (17.5%), with OR (95% CI) 3.14 times risk (p value= 0.021). The A allele frequency was increased in HCC group (53%) versus (18.3% and 27.5 %, OR (95% CI) = 5.02 and 2.97) in control and cirrhotic patients group respectively.This was consistent with the meta- analysis study conducted by Our results were consistent with the findings previously reported by Heneghan *et al*. and Ho *et al*. (2003), but different from Chen *et al* s study (2005). On studying genotype distribution and allele frequency in control group of our study, the TNF-α −308 GG genotype distribution were (70%), which were similar to healthy Chinese result (96.4%) (17), while the AA genotype distribution accounted for (6.7%), which was similar to healthy Chinese (0.4%)(17), Thus, the A allele may be the variant responsible for various diseases in Egyptian population, which is similar to Chinese population. Furthermore, The Characteristics of focal lesions in HCC group founded that (48%) of HCC patients had multiple foci and (52%) had single focal lesion ,while regarding foci size Half (48%) of HCC patients had moderate foci , (28%) had large foci and (24%) had small focal lesion, and with Comparison between genotypes in additive model (GG+GA vs AA) regarding characteristics of focal lesions in HCC group founded that no significant difference between genetic model (GG+GA vs AA) regarding characteristics of focal lesions in HCC group.

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