

SINGLE NUCLEOTIDE POLYMORPHISM IN CYTOKINES AND RISK OF HEPATOCELLULAR CARCINOMA IN EGYPTIAN PATIENTS

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Hepatocellular carcinoma (HCC), one of the most fatal malignancies, is particularly prevalent in Egypt; there are deficiencies in knowledge concerning HCC and its risk factors. Hepatitis C viral infections are highly prevalent in Egypt, pesticides are very commonly used, and diets are often contaminated by aflatoxin, especially in rural areas (Saleh *et al.*, 2015). Human hepatocellular carcinoma (HCC) is a heterogeneous disease, driven by different risk factors and presenting diverse clinicopathological features and outcomes (De Ponti *et al.*, 2015). HCC is one of the major malignant diseases in many healthcare systems. The growing number of new cases diagnosed each year is nearly equal to the number of deaths from this cancer (Galun *et al.*, 2015). Chronic low-grade inflammation alters both innate and adaptive immune responses; as a result tolerogenic environment is established in damaged organ. Up to date, incomplete understanding of HCC pathogenesis and the extend of biomarker variability among patients represent the major

obstacle for early diagnosis and for the choice of effective treatment (Kikodze *et al.*, 2015). The majority of hepatocellular carcinoma occurs over pre-existing chronic liver diseases that share cirrhosis as an endpoint. In the last decade, a strong association between lifestyle and hepatocellular carcinoma has become evident. Abundance of energy-rich food and sedentary lifestyles have caused metabolic conditions such as obesity and diabetes mellitus to become global epidemics (Saran *et al.*, 2016). Cytokines are humoral immunomodulatory proteins or glycoproteins which control or modulate the activities of target cells, generally those within the haematopoietic system. They act on target cells by binding to specific cytokine receptor ligands, initiating signal transduction and second messenger pathways within the target cell (Bidwell *et al.*, 1999). Recent advances in genetics have made it possible to study the role of cytokine gene polymorphisms in HCC development (Bunnapradist and Jordan, 2000). In recent years cytokines and their receptors have

been shown to be highly polymorphic. Polymorphisms in these genes have been associated with a number of immune diseases (Keen, 2002), and associated with high and low cytokine production and may modulate the magnitude of alloimmune responses during HCC (Hoffmann *et al.*, 2002; Ben-Ari *et al.*, 2004). Chronic hepatitis B virus (HBV) infection is one of the major causes of hepatocellular carcinoma (HCC), and the HBV X (HBx) gene plays a critical role in the molecular pathogenesis of HBV-related HCC (Zhu *et al.*, 2007). Cytokine gene single nucleotide polymorphisms (SNPs) are involved in the genesis and progression of hepatocellular carcinoma (HCC) (Bei *et al.*, 2014). Interleukin 10 (IL-10) is a pleiotropic cytokine designated as an immunosuppressive molecule, but may act as an immunostimulant factor in cancer development and progression (Hiroki *et al.*, 2015). The T helper cells (Th1 and Th2) CD4_T-cells differ in cytokine expression: Th1 cells produce interleukin (IL)-1 β , interferon gamma (IFN- γ) and TNF are generally referred to as proinflammatory, whereas Th2 cells express IL-4, -5, -6, -10 and -13, induce anti-inflammatory responses, This cytokine heterogeneity is not restricted to CD4_T-cells, as other cell types also contribute to the secretion of regulatory cytokines (Moran *et al.*, 2006). SGOT and SGPT are highly sensitive markers of liver damage due to various diseases or injury. Conversely, individuals developing chronic hepatitis C infection have minor elevations in their SGOT and SGPT levels, whereas, their liver is injured or dam-

aged substantially by the infection even leading to scarring (cirrhosis) from ongoing liver infection and inflammation (Hamza *et al.*, 2015).

We hypothesized that genetic variability in *IL-10*¹⁰⁸², *TNF- α* ³⁰⁸ and *IL-1 β* ⁵¹¹ Promoters Polymorphisms alleles may account for different susceptibility to HCC. Therefore, this study was designed to investigate the role of the *IL-10*¹⁰⁸², *TNF- α* ³⁰⁸ and *IL-1 β* ⁵¹¹ Promoters Polymorphisms in the progression of HCC infection in Egyptian patients.

MATERIAL AND METHODS

Study population

HCC participants for this study were Egyptian patients and consisted of 75 (55 males and 20 females), HCC patients were randomly selected from health screening program participants to exclude those with a history of cancer and other medical diseases and 75 cancer-free controls, who had no personal or family history of hepatocellular carcinoma, from May 2012 to March 2014, all patients were selected from the outpatient clinic, Internal Department Of Specialized Medical Hospital in Mansoura University. All HCC patients were diagnosed on the basis of histology or the combination of typical radiological findings of HCC, and underwent surgery in medical hospital in Mansoura University. The HBsAg and anti-hepatitis C virus (HCV) antibody were tested by microparticle enzyme immunoassays using commercial assay kits, which were used to determine the infec-

tion status of hepatitis B or hepatitis C. Clinical characteristics data as well as related risk factors, including gender, age, serum SGPT (ALT), SGOT (AST, albumin), Total bilirubin, family history of HCC and HBV, HCV serological markers, were summarized (Table 2).

Serologic testing

Serum levels of albumin, total bilirubin, direct bilirubin, alanine aminotransferase (ALT or SGPT), and aspartate aminotransferase (AST or SGOT) were tested. The diagnosis of cirrhosis was based on the presence of clinical manifestations of portal hypertension such as varices, encephalopathy, or ascites; Biochemical abnormalities including elevated serum bilirubin, serum albumin, or prothrombin; and obvious morphologic change of the liver detected by hepatic imaging such as ultrasonography.

HCC diagnosis

The diagnosis of HCC was made after reviewing images generated with several imaging modalities. Nodules larger than 1 cm found in the ultrasound screening of a cirrhotic liver were investigated further with either 4 phase multidetector computed tomography (CT) scan or dynamic contrast enhanced magnetic resonance imaging (MRI). If the appearances were typical of HCC, namely, hypervascular in the arterial phase with washout in the portal venous or delayed phase, the lesion was diagnosed as HCC. If the findings were not characteristic or the vascular profile was not typical, a se-

cond contrast enhanced study with the other imaging modality was performed or the lesion was biopsied. All patients were confirmed not to have other cancers in an initial screening examination.

Polymorphism genotyping

Genomic DNA was extracted from whole EDTA-treated peripheral blood using a QIAamp Blood Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. SNPs were analyzed in three cytokines (IL-1 β , TNF- α and IL-10) for genotype assignment. Amplification was performed in GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Singapore) with 100 ng of genomic DNA, 25 pmol of each primer, 200 μ M total dNTP, 1.5 mM MgCl₂, PCR buffer and 2.5 U Taq DNA polymerase (Promega, Madison, WI, USA). The following cycling conditions were used: 95°C for 5 min, followed by 35 cycles of 94°C for 60s, (55°C for TNF- α , IL-1 β and 60°C for IL-10) for 30s and 72°C for 60s, with a final extension at 72°C for 10 min. Amplification products (15 μ l) were digested with 2 units different restriction enzymes (New England Biolabs Ltd., Beijing, China) at 37°C for over 4 h to detect allele (Table 1), showed Primers, PCR sizes and restriction enzymes used for analyses the *IL-10*, *TNF- α* and *IL-1 β* gene polymorphisms and analyzed on a 2% agarose gel. DNA products were visualized by ethidium bromide staining. The cytokines showed two fragments (homozygous for the allele), while its homologue

were undigested and resulted in a single band (homozygous for allele). The presence of all three fragments defined heterozygotic individuals.

Statistical analysis

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. [40]. Qualitative data were described using number and percent. Quantitative data were described using range (minimum and maximum), mean, standard deviation and median. Comparison between different groups regarding categorical variables was tested using Chi-square test (Campbell, 2007). When more than 20% of the cells have expected count less than 5, correction for chi-square was conducted using Monte Carlo correction. The distributions of quantitative variables were tested for normality using Kolmogorov-Smirnov test, Shapiro-Wilk test and D'Agostino test, if it reveals normal data distribution, parametric tests was applied. If the data were abnormally distributed, non-parametric tests were used. For normally distributed data, comparison between two independent populations was done using independent t-test. For abnormally distributed data, comparison between two independent populations were done using Mann Whitney test. Odd ratio (OR) and 95% Confidence Interval were used to calculate the ratio of the odds of an event occurring in one patient group to the odds of it occurring in the control group. Significance of the obtained results was judged at the 5% level.

RESULTS AND DISCUSSION

Demographic and clinical information of patients and controls

The general demographic characteristics of the subjects and the most commonly enzymes used indicators of liver (hepatocellular) damage are the alanine aminotransferase (ALT) and aspartate aminotransferase (AST), formerly referred to as the SGPT and SGOT showed in Table (2). There were 75 HCC patients, 55 were men and 20 were women, with an age ranged from 40 to 70 years and 75 healthy controls, 49 were men and 26 were women, with an age ranged from 40 to 75 years, without any history of liver disease. There were no significant differences between HCC cases and cancer-free controls with regarded to gender and age distribution. Patients with HCC not only had lower serum albumin level ($P = 0.001$) but had high level in other enzymes in serum ($P = 0.001$) compare with control, No significant differences of gender and age were detected between the patients and controls. Significant differences observed of ALT enzyme ($Z=8.168^*$), AST enzyme ($Z=10.152^*$), Albumin ($Z=5.432^*$), Creatinin ($Z=5.003^*$), and Total bilirubin ($Z=7.031^*$), the patient and control groups were in accordance with Mann Whitney test. Table (2) shows the clinical information of HCC patients and controls, HCC Patients not only had low serum albumin level ($P = 0.001$) and ALT ($P = 0.037$), but also had high prothrombin ($P = 0.001$) and high AST.

Cytokine genotyping

Genotypic distributions of IL-1 β [single nucleotide polymorphisms (SNPs)], (TNF- α) and IL-10 for the patients group and control group separately, the observed genotype distribution in the controls did not differ from those expected from Mann Whitney equilibrium ($P > 0.05$). Table (3) shows the genotype and allele frequencies of IL-10, TNF- α and IL-1 β Promoters Polymorphisms in HCC Patients and Controls and the association between cytokine genotypes and risk of HCC. Using a chi-squared (χ^2) test, compared data showed the genotype and allele distribution at each of the cytokines HCC patients and controls. No significant differences of SNP genotype and allele distribution at 2 cytokines, IL-10¹⁰⁸² (G-A) and IL-1 β ⁵¹¹ (C-T) were observed between the patient and control groups. In contrast, significant differences of SNP genotype and allele distribution at the other cytokine TNF- α -308 G/A was detected. These were summarized as follows: the frequencies of 1082- G/A genotypes of IL-10 in our population are shown in Table (3). Among the HCC patients and controls studied, two carried the AA wild-type, 56 carried the GA genotype and 17 carried the GG genotype, The frequencies of 308- G/A genotypes of TNF- α are showed among the HCC patients and controls studied, 17 carried the AA wild-type, 44 carried the GA genotype and 14 carried the GG genotype and the frequencies of 511- C/T genotypes of IL-1 β are showed among the HCC patients and controls studied, 23 carried the TT wild-type, 44

carried the GA genotype and eight carried the GG genotype were detected, the frequency of T alleles in patients was 60% and 55.3% in controls for IL-1 β ⁵¹¹ (C-T) and the frequency of A alleles in patients was 40% and 46% in controls for IL-10¹⁰⁸² (G-A), so no significant differences of both genotype and allele frequencies were detected between the patient and control groups ($P < 0.05$). The A allele frequencies of the TNF- α promoter -308 G/A was 52% in patients and 12.7% in controls, polymorphisms in HCC patients showed a significant difference with control group. Furthermore, compared with GG and AA genotypes, the GA genotype at TNF- α ³⁰⁸ (G-A) locus was significantly associated with an increased risk of HCC (OR=6.769*, 95%, $P=0.001$). With respect to implication of its allele, compared with allele G, allele A is significantly associated with an increased risk of HCC (OR=7.469*, 95%, $P=0.001$).

Increasing the confirmation indicates the association of cytokines in hepatocarcinogenesis. Thus, various ways have been taken to elucidate changes in cytokine expression levels in patients with HCC, to assay immune response changes in HCC patients, the expression levels of pro- and anti-inflammatory We hypothesized that genetic variability in IL-10¹⁰⁸², TNF- α ³⁰⁸ and IL-1 β ⁵¹¹ Promoters Polymorphisms alleles may account for different susceptibility to HCC, may have an increased risk of HCC development. Although the associations we observed followed the hypothesized direction, they were very modest and did not reach statis-

tical significance with $IL-10^{1082}$ and $IL-1\beta^{511}$. However, the results showed a statistical significance with $TNF-\alpha^{308}$. Therefore, our study could not reach a definitive conclusion that $IL-10^{1082}$ and $IL-1\beta^{511}$ genetic polymorphisms play a significant role in HCC development among Egyptian patients, on the other hand the results showed the opposite with $TNF-\alpha^{308}$ with HCC patients. Interleukin-10 (IL-10) associated cytokine released by T cells, anti-inflammatory effects is normally highly expressed in hepatocytes, some studies suggest that increases in IL-10 cytokine correlate with HCC progression (He and Hu, 2001; Yang *et al.*, 2011). The ability of pro- and anti-inflammatory cytokine levels to predict patient outcome has been studied. IL-10 levels were significantly higher in HCC than in healthy individuals (Critelli *et al.*, 2015; Douglas *et al.*, 2008), and a multivariate analysis implied that IL-10 may be a predictor of the postresection outcome of HCC patients (He and Hu, 2001). IL-10 is a multifunctional anti-inflammatory cytokine that down regulates cell-mediated immune responses and cytotoxic inflammatory responses, whose effects are directed mainly against functions of mononuclear cells (Kockar *et al.*, 2012; Ye *et al.*, 2011). The polymorphisms in IL-10 have been extensively studied in HCC. The results are conflicting, the 1082 G/A polymorphism associated with reduced plasma levels (Abbas *et al.*, 2005), the association of IL-10 cytokine SNPs and the risk for developing HCC are less clear. A polymorphism of IL-10 in 1078 chronic HBV patients and HCC was strongly associated

with HCC and with an increased production of IL-10 (Liu *et al.*, 2015). The frequency of susceptibility increased in order from chronic hepatitis to liver cirrhosis and HCC among HBV patients (Zhu *et al.*, 2015). IL-10 polymorphisms (-1082G/A, -819C/T) were associated with reduced plasma IL-10 levels in a study of 77 patients with chronic HBV, and a separate study of 236 Japanese patients with HBV infection, no genetic difference was observed in IL-10 (-1082, -819, -592) (Renzulli and Golfieri, 2015; Heneghan *et al.*, 2003). Pro-inflammatory IL-1 β was shown to be elevated in HCC patients compared with healthy individuals (Huang *et al.*, 1999). IL-1 β has also been measured in HCC undergoing resection (Sato *et al.*, 1996). Higher IL-1 β was found in cirrhotic patients when compared with healthy individuals or preoperative patients and increased postoperatively (Sato *et al.*, 1996). The distribution of alleles may have accounted for the susceptibility of the Egyptian population to develop HCC. IL-1 β^{511} allele, which may be associated with high IL-1 β production in the liver, is a genetic marker for the development of HCC in chronic hepatitis B patients in Thai population (Hirankarn *et al.*, 2006). The IL-1 β^{511} genotype T/T was a significant risk factor for HCC. The C-511T-IL-1 β polymorphism was also examined in Thai patients with chronic HBV. In this cohort, the IL-1 β^{511} genotype C/C was found to be significantly higher in patients with HCC against healthy individuals (Popko *et al.*, 2008). $TNF-\alpha$ protein induces the expression of adhesion molecules, facilitating the inva-

sion of metastatic tumor cells (Bortolami *et al.*, 2002). TNF- α was high in HCC and metastases to the liver before operation compared with healthy individuals and increased postoperatively (Nakazaki, 1992; Bortolami *et al.*, 2002; Zhu *et al.*, 2005). TNF- α expression was elevated in HCC patients, the levels of the TNF- α were higher in HCC patients when compared with healthy individuals (Nakazaki, 1992; Bortolami *et al.*, 2002; Zekri *et al.*, 2005). An increased level of TNF- α was also shown to correlate with hepatic inflammation, necrosis, and hepatic failure (Nagai *et al.*, 2014). The level of this cytokine increased postoperatively and correlated with postoperative and hepatic failure (Kato *et al.*, 2003; Yang *et al.*, 2015). The TNF- α (-308) SNP in the promoter region of the gene, which includes TNF- α 1 (-308G) and TNF- α 2 (-308A) alleles, is associated with cancer susceptibility and induced expression of TNF- α . In a case study, 74 HCC and 89 healthy individuals were assayed for their -308 genotype (Popko *et al.*, 2008). Carriage of the TNF- α allele was associated with an increased risk (3.5 odds ratio) of HCC, the TNF- α allele was therefore a significant predictor of HCC (Akkiz *et al.*, 2009). In our study 75 HCC from Egypt cohort and 75 controls who were healthy individuals. As shown in Table (3); No statistical significance with *IL-10*¹⁰⁸², and *IL-1 β* ⁵¹¹ in HCC progression and TNF- α allele was associated with high increased risk of HCC was found in Egyptian patients. Consequently, the G/A polymorphism may play a role in altered *TNF- α* gene expression in patients. The G/G gen-

otype at -308 was found in all carriers with HCC but only in 78% of healthy individuals. The distribution in the HCC group was significantly different from the healthy group (Lertwittayapon *et al.*, 2012). Therefore, TNF- α may inhibit carcinogenesis through cytotoxic induction; however, under certain circumstances, this cytokine can promote this process. So the TNF- α polymorphism may therefore be a genetic marker for the development of hepatitis-related HCC.

SUMMARY

Polymorphisms in cytokine genes responsible for inflammatory and immune responses are associated with risk of hepatocellular carcinoma (HCC) in Egyptian population. HCC study had conducted on 75 HCC patients and 75 matched control subjects of Egyptian population. Genetic variants in the *IL-10*¹⁰⁸², TNF- α ³⁰⁸ and *IL-1 β* ⁵¹¹ genes were analyzed by SNP. The logistic regression method was used to analyze the data, relative to the putative high-activity genotypes; individual low-activity genotypes were associated with statistically non-significant increases in HCC risk. The genotypic frequencies in the cases were not similar to that of the controls, TNF- α ³⁰⁸ differences being statistically significant (P = 0.001). Using the GG genotype as the reference genotype, AA was significantly associated with increased risk of HCC (adjusted OR = 7.034, 95% CI, χ^2 = 54.399). Furthermore, we found A allele was significantly associated with increased risk of HCC, compared with G allele (χ^2 = 53.034, OR-95%

CI = 0.134 - 7.469). No such significant difference was found for cytokines (IL-1 β and IL-10). Conclusion: Our study showed that TNF- α ⁻³⁰⁸ G > A polymorphism was associated with increased HCC risk in Egypt population

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Table (1): Primers, PCR sizes and restriction enzymes used for analyses the *IL-10*¹⁰⁸², *TNF-α*³⁰⁸ and *IL-1β*⁵¹¹ Promoters Polymorphisms gene.

SNP	Primer sequences	Annealing temperature (°C)	Restriction enzymes	Amplification fragment (bp)	Genotype (bp)
<i>IL-10</i> ¹⁰⁸² (G→A)	Forward (F) : 5`-AGCAAACTCCTCGTCGCAAC -3` Reverse (R1): 5` -CCTATCCCTACTTCCCCC -3` specific for G Reverse (R2): 5` -CCTATCCCTACTTCCCCT -3` specific for A.	60°C	MnII	238 bp	A (238bp) G (136 bp + 102 bp)
<i>TNF-α</i> ³⁰⁸ (G→A)	Forward (F) : 5`- CTGCATCCCCGTCTTTCTCC- 3` Reverse (R1): 5`- ATAGGTTTTGAGGGGCATCG- 3` specific for G Reverse (R2): 5`- ATAGGTTTTGAGGGGCATCA -3` specific for A.	55°C	NcoI	147 bp	A (147) G (126 bp + 21 bp)
<i>IL-1β</i> ⁵¹¹ (C→T)	Forward (F) : 5`- TCTTTTCCCCTTTCCTTAACT-3` Reverse (R1): 5`- GAGAGACTCCCTTAGCACCTAGT-3`	55°C	AvaI	304 bp	T (304bp) C (190 bp + 114 bp)

Table (2): Demographic and clinical information of patients and controls.

Parameter	Healthy controls (n = 75)	HCC patients (n = 75)	P	Z
Gender (♂/♀)	49-26	55-20	0.288	
Age Mean ± SD.	40 – 75 54.35 ± 11.77	40 – 70 56.39 ± 9.23	0.239	
ALT (Median)	25.96 ± 10.48 (22.0)	74.11 ± 84.85 (60.0)	<0.001*	8.168*
AST (Median)	23.39 ± 9.92 (21.0)	95.72 ± 46.57 (86.0)	<0.001*	10.152*
Alb (Median)	3.79 ± 0.70 (3.60)	3.11 ± 1.0 (3.0)	<0.001*	5.432*
Creatinin (Median)	0.85 ± 0.26 (0.80)	1.50 ± 0.82 (1.20)	<0.001*	5.003*
T. billrub (Median)	0.78 ± 0.36 (0.85)	2.26 ± 2.10 (1.80)	<0.001*	7.031*
HCV	Negative	Negative		
HBV	Negative	Negative		

HCC, hepatocellular carcinoma;
AST, aspartate aminotransferase
Total bilirubin.

* Z: Z for Mann Whitney test

ALT, alanine aminotransferase;
Alb, albumin; T. billrub,

All data are presented as mean ± standard error (SE).

*: Statistically significant at $p \leq 0.05$

Table (3): The genotype and allele frequencies of *IL-10*¹⁰⁸², *TNF-α*³⁰⁸ and *IL-1β*⁵¹¹ promoters polymorphisms in HCC patients and controls.

Genotype	Controls (n = 75)		HCC patients (n = 75)		OR (95% CI)	P	χ ²
	No.	%	No.	%			
IL-10¹⁰⁸² (G-A)	No.	%	No.	%			
GG	11	14.7	17	22.7	1.705		
GA	59	78.7	56	74.7	0.799	0.266	2.564
AA	5	6.7	2	2.7	0.384		
Allele							
G	81	54.0	90	60.0	1.278	0.294	1.102
A	69	46.0	60	40.0	0.783		
TNF- α³⁰⁸ (G-A)	No.	%	No.	%	OR	P	χ ²
GG	59	78.7	14	18.7	0.062*		
GA	13	17.3	44	58.7	6.769*	<0.001*	54.399*
AA	3	4.0	17	22.7	7.034*		
Allele							
G	131	87.3	72	48.0	0.134*	<0.001*	53.034*
A	19	12.7	78	52.0	7.469*		
IL-1β⁵¹¹ (C-T)	No.	%	No.	%	OR	P	χ ²
CC	16	21.3	8	10.7	0.440		
CT	35	46.7	44	58.7	1.622	0.156	3.713
TT	24	32.0	23	30.7	0.940		
Allele							
C	67	44.7	60	40.0	0.826	0.413	0.669
T	83	55.3	90	60.0	1.211		

χ²: Chi square test

MC: Monte Carlo

*: Statistically significant at p ≤ 0.05