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The role of IL-4 gene polymorphism in HCV-related hepatocellular carcinoma in Egyptian patients

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Abstract

Background: Interleukin-4 (IL-4), a pleiotropic anti-inflammatory cytokine, is produced mainly by activated T helper 2 (Th2). Hepatocellular carcinoma (HCC) is a typical inflammation-related cancer. Alterations influencing IL-4 expression may disturb immune response and may be associated with HCC risk. We aimed to verify role of IL4 gene polymorphism (*IL-4-589C/T (rs2243250)*) in HCV-related hepatocellular carcinoma in Egyptian patients. *IL-4-589C/T (rs2243250)* polymorphism was examined in 50 patients with HCC on top of HCV, 40 patients with HCV-induced liver cirrhosis, and 30 healthy controls using the polymerase chain reaction- restriction fragment length polymorphism method.

Results: Overall IL-4 gene polymorphism (*IL-4-589C/T (rs2243250)*) showed significant difference between hepatocellular carcinoma group versus liver cirrhosis and healthy control groups. *TT* homozygous genotype was more prevalent in HCC group (24%) versus (5%) in liver cirrhosis and (3.3%) in control. *TT* homozygous genotype had 10 times more risk of hepatocellular carcinoma versus healthy control group and 6.33 times more risk versus cirrhotic patients group (p value = 0.018 and 0.016 respectively).

Conclusion: *IL-4-589C/T (rs2243250)* polymorphism, *TT* homozygous genetic model, may be a risk factor in HCV-related HCC in Egyptian patients.

Keywords: Interleukin-4 (IL-4), Hepatocellular carcinoma (HCC), Hepatitis C virus (HCV)

Background

Hepatocellular carcinoma (HCC) is one of the main causes of cancer-related mortality. In Egypt, it is considered a major health problem being the fourth common malignancy [1]. The increased incidence of HCC cases in Egypt is related to the high prevalence of hepatitis C virus (HCV) infection [2].

HCC is diagnosed at late stages in most cases. This limits the treatment options especially it is refractory to the available chemotherapeutic drugs. So, current available therapies are effective only to small group of patients [3].

HCC is considered an inflammation-related cancer initiated by different etiological factors such as hepatitis virus, alcohol, and non-alcoholic steatohepatitis [4]. 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 [5].

IL-4 is a short four α-helix bundle glycoprotein secreted mainly by activated T helper 2 (Th2) cells. In addition, it is secreted from other immune cells within the lymphoid lineage compartment as type 2 innate lymphoid cells (ILC2s) and myeloid lineage compartment as basophils and mast cells. In its turn IL-4 delineates, Th2 phenotype [6]. It was reported that Th2 dominance might provoke carcinogenesis in chronic HCV patients [7].

Together with IL-13, which have 25% sequence similarity with IL-4 and encoded by adjacent genes that share several *cis*-acting regulatory regions, receptor subunits, and signaling molecules, they are classic inducers of M2,

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an alternatively activated macrophage [8, 9] which was reported to promote HCC development [10, 11].

Therefore, in this study we aimed to study the contribution of promoter polymorphism of *IL-4-589C/T (rs2243250)* to the susceptibility of HCV-related HCC.

Methods

Study participants

A total of 90 Egyptian chronic HCV-infected patients were recruited in the current study from the Hepatology and Gastroenterology Department, National Liver Institute, Menoufia University. After history taking, clinical evaluation, laboratory, and radiological investigations, patients were classified into 2 groups; (cirrhosis group 40 cirrhotic patients) with no radiological evidence of hepatocellular carcinoma and (HCC group 50 HCC patients) (arterial enhancement and washout in venous or delayed phase). In addition, 30 healthy age and sex matched individuals (control group) were enrolled as control group.

Patients with other coexistent morbidity such as HBV infection, autoimmune hepatitis, non-alcoholic steatohepatitis, and genetic metabolic diseases were excluded from the study. The protocol was approved by the local ethical committee of National Liver Institute and informed consent was taken before the study.

Routine investigations

Sera were subjected to routine laboratory study including liver and renal function tests performed on 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's instructions. Hepatitis B surface antigen (HBsAg), anti-hepatitis B core antibody (HBcAb), anti-hepatitis C antibodies (HCV Abs), and serum alpha fetoprotein were detected by Cobas e411 immunoassay analyzer (Roche diagnostics-Mannhein, Germany). Complete blood count was measured using Sysmex XT-1800i (Sysmex Corporation, Kobe, Japan) and HCV RNA level which was assessed by COBAS AmpliPrep/COBAS TaqMan (Roche Diagnostics Ltd., Germany) with detection limit of 15 IU/ml.

Study of *IL-4 rs2243250 C/T* polymorphism by PCR-RFLP

Genomic DNA was extracted from EDTA treated blood samples using Quick-gDNA™MiniPrep Genomic DNA Purification Kit (Zymo Research, USA). PCR amplification of the 195 base pair (bp) stretch within the promoter region of IL-4 gene was performed followed by Ava II restriction enzyme digestion (Biolabs, Inc., England) as was previously described [12]. The primers used were 5′-TAAACTTGGGAGAACATGGT-3′forward and 5′-TGGGGAAAGATAGAGTAATA-3′ reverse. PCR reaction mixture consisted of 1 μl of each of primers, 12.5 μl of MyTaq Red PCR master mix (Bioline USA Inc., USA), 5.5 μl of nuclease-free water and 5 μl of extracted DNA.

Amplification was performed in Perkin Elmer Gene Amp PCR System 2400 Thermal Cycler in the following conditions; 95 °C for 5 min, followed by 35 cycles, of 95 °C for 45 s, annealing at 50 °C for 45 s, 72 °C for 45 s and final extension at 72 °C for 10 min.

The genotyping analysis of the 195 bp amplicon 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 177 and 18 bp fragments were scored as *CC* genotypes, those with single 195 bp fragment as *TT* genotype and 195, 177, 18 bp fragments as *CT* genotype (Fig. 1).

Statistical methods

Results were statistically analyzed by using statistical package of social sciences (SPSS 22.0, IBM/SPSS Inc., Chicago, IL, USA). 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 equivalents Kruskal-Wallis or Mann-Whitney test were applied upon violation. Dunn-Sidak used as a post hoc test for multiple pairwise comparisons after significant Kruskal-Wallis test. Chi-square (χ^2) test was used to compare allele frequency and genotype distribution of IL-4 gene 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 level < 0.05.

Result

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

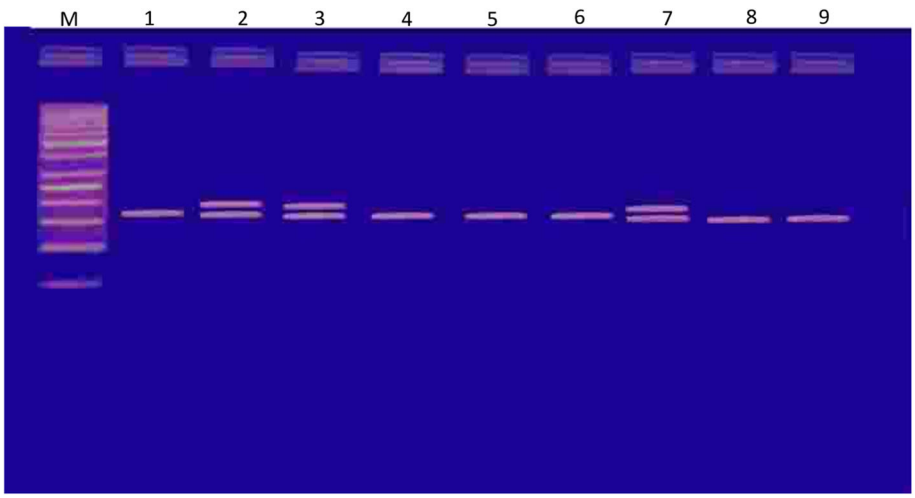

Fig. 1 Genotyping analysis of IL-4-589C/T polymorphism using PCR-RFLP technique. PCR-RFLP assay for analyzing IL-4-589C/T polymorphism. PCR product was digested by AVA II restriction enzyme and visualized on 3% agarose gel. M: marker (50 bp ladder). Lanes 1, 4, 5, 6, 8, and 9 show CC homozygous (177 bp band, 18 bp is not seen); lanes 2, 3, and 7 show CT heterozygous (195 and 177 bp bands)

more prevalent than female gender (36%). On the other hand, comparing liver and renal function tests between studied groups showed highly statistical significance difference between studied groups. HCC group had statistically significant elevated alanine aminotransferase (ALT), aspartate aminotransferase (AST), total and direct bilirubin, urea, and creatinine and significant decreased albumin level compared to control group (p < 0.001). On the other hand, there was no statistical significance between HCC and cirrhotic patients.

Genotype polymorphism and risk of hepatocellular carcinoma

Studying genotype distribution and allele frequency of IL-4 promotor polymorphism *(-589C/T)* among different groups showed that there was increase of *TT* genotype

Table 1 Studying demographic data, liver, and renal profile among studied groups

	Control (n = 30)	Cirrhosis (n = 40)	HCC (n = 50)	p value
Age (years) Median (range)	50.00 (40.00–59.00)	52.5 (43.00–66.00)	52.5 (42.00–67.00)	**0.066**
Gender Male (n, %) Female (n, %)	16 (53.3) 14 (46.7)	22 (55.0) 18 (45.0)	32 (64.0) 18 (36.0)	**0.562**
ALT (U/L) Median (range)	17.00 (9.0–36.0)	48.50 (16.0–146.0)	55.50 (20.0–102.0)	**< 0.001** [a,b]
AST (U/L) Median (range)	19.00 (9.0–36.0)	60.50 (16.0–146.0)	62.50 (20.0–102.0)	**< 0.001** [a,b]
Total bilirubin (mg/dL) Median (range)	0.50 (0.10–0.80)	1.19 (0.45–4.18)	1.50 (0.73–3.63)	**< 0.001** [a,b]
Direct bilirubin (mg/dL) Median (range)	0.10 (0.07–0.23)	0.38 (0.09–3.08)	0.62 (0.18–2.53)	**< 0.001** [a,b]
Albumin (g/dL) Median (range)	4.40 (3.50–5.10)	3.68 (2.20–4.90)	3.32 (2.20–4.52)	**< 0.001** [a,b]
Urea (mg/dL) Median (range)	27.50 (17.0–41.0)	37.50 (22.0–56.0)	38.00 (22.0–67.0)	**< 0.001** [a,b]
Creatinine (mg/dL) Median (range)	0.80 (0.50–1.20)	0.87 (0.58–1.50)	1.00 (0.50–1.50)	**0.001** [a,b]

ALT alanine aminotransferase, *AST* aspartate aminotransferase
Data are median and range in parenthesis, otherwise are n (%)
Significant p value < 0.05
[a]On comparing a control and cirrhotic patients
[b]Control and HCC patients
[c]Cirrhotic and HCC patients

(24%) in HCC group compared to cirrhotic patients group (5%) and control group (3.3%) with *T* allele predominance in HCC group (44%) compared to cirrhotic group (28.8%) and control group (26.7%).

The study revealed that *CC* genotype was more prevalent (50%) in healthy control and (47.5%) in cirrhotic patients than hepatocellular carcinoma group (36%). *CT* genotype frequency was increased in control, cirrhotic patients groups (46.7%, 47.5% respectively) compared to HCC group (40%) as shown in Table 2.

TT homozygous genotype was significantly increased in HCC group with OR (95% CI) 10 times more risk compared to control (p value = 0.018). *T* allele was associated with a significant increase of hepatocellular carcinoma with OR (95% CI) 2.16 more times risk (p value = 0.028). The *T* allele variant showed significant association with HCC risk in the recessive genetic model *(CC + CT* vs *TT)* (p = 0.025) not the dominant genetic model *(CC* vs *CT + TT)* (p = 0.218) as shown in Table 2.

Also, the homozygous *TT* genotype was more prevalent in HCC group compared to cirrhotic patients group with OR (95% CI) 6.33 times risk of hepatocellular carcinoma (p value = 0.016). Additionally, the *T* allele showed significant prevalence in HCC group with OR (95% CI) 1.95 more risk (p = 0.035). The *T* allele variant showed significant association with HCC risk in the recessive genetic model *(CC + CT* vs *TT)* (p = 0.013) not the dominant genetic model *(CC* vs *CT + TT)* (p = 0.271) as shown in Table 2.

On studying foci size and numbers in HCC group regarding the recessive genetic model of *-589C/T* SNP *(CC + CT* vs *TT)* (Table 3), we could not detect statistical significance difference between 2 groups.

Discussion

Chronic hepatitis C virus infection is considered a major risk factor for HCC development [13]. As an RNA virus, HCV does not 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 associated with chronic hepatitis [14], in addition to the direct effect of HCV viral products [15].

Interleukin-4 (IL-4), a pleiotropic anti-inflammatory cytokine, is involved in humoral and cell-mediated immunity [16]. It suppresses a number of pro-inflammatory cytokines and expands anti-inflammatory effect [17]; thus, it is suggested that any alterations that influence IL-4 expression or function may disturb immune responses increasing the liability to infections.

In addition, IL-4, together with other Th2 cytokines, induces M2 macrophage polarization. Traditionally, macrophages are classified into classically activated M1 phenotype and the alternatively activated M2 macrophages according to their responses to different microenvironmental stimuli [18].

M2-polarized macrophages are main components of tumor-infiltrating stromal cells. They are key factors participating in tumor progression. M2-polarized macrophages were also related to promote tumor growth, invasion, and metastasis by secreting certain chemokines growth factors, cytokines, and matrix metalloproteases [19].

IL-4 gene is located on chromosome 5q31 [17]. In the current study, we aimed to study the contribution of promoter polymorphism of *IL-4 -589C/T (rs2243250)* to the susceptibility of HCV-related HCC. It had been

Table 2 Genotypes distribution and allele frequencies of polymorphism *-589 C/T (rs2243250)* in control, cirrhotic, and HCC groups

polymorphism *-589 C/T (rs2243250)*	Control *n* = 30(%)	LC *n* = 40 (%)	HCC *n* = 50 (%)	*p* value	LC vs control		HCC vs. control		HCC vs. LC	
					OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*p* value
Genotypes				**0.044**						
CC	15 (50.0)	19 (47.5)	18 (36.0)		Ref	–	Ref	–	Ref	–
CT	14 (46.7)	19 (47.5)	20 (40.0)		1.07 (0.41–2.82)	0.889	1.19 (0.45–3.13)	0.724	1.11 (0.45–2.73)	0.818
TT	1 (3.3)	2 (5.0)	12 (24.0)		1.58 (0.13–19.12)	1.000	**10.0 (1.16–86.02)**	**0.018**	**6.33 (1.24–32.32)**	**0.016**
Dominant model				**0.391**						
CC	15 (50.0)	19 (47.5)	18 (36.0)		Ref	–	Ref	–	Ref	–
CT+TT	15 (50.0)	21 (52.5)	32 (64.0)		1.11 (0.43–2.85)	0.836	1.78 (0.71–4.46)	0.218	1.61 (0.69 – 3.76)	0.271
Recessive model				**0.008**						
CC+CT	29 (96.7)	38 (96.0)	38 (76.0)		Ref	–	Ref	–	Ref	–
TT	1 (3.3)	2 (5.0)	12 (24.0)		1.53 (0.13–17.66)	1.000	**9.16 (1.13–74.52)**	**0.025**	**6.00 (1.26–28.64)**	**0.013**
Alleles				**0.034**						
C	44 (73.3)	57 (71.3)	56 (56.0)		Ref	–	Ref	–	Ref	–
T	16 (26.7)	23 (28.8)	44 (44.0)		1.11 (0.52–2.35)	0.786	**2.16 (1.08–4.33)**	**0.028**	**1.95 (1.04–3.64)**	**0.035**

Data are *n* (%), *CI* confidence interval, significant *p* value < 0.05

Table 3 Studying Data are *n* (%). Significant *p* value < 0.05 size and number of foci in HCC group regarding recessive genetic model of *-589 C/T SNP ((CC + CT vs TT)*

Parameters	Genetic model for *-589 C/T* SNP (*CC + CT vs TT*)		*p* value
	CT + CC (*n* = 38)	*TT* (*n* = 12)	
	Number of foci [*n* (%)]		
Single	19 (50)	7 (58.3)	0.614
Multiple	19 (50)	5 (41.7)	
	Size of foci [*n* (%)]		
Small (≤ 2 cm)	10 (26.3)	2 (16.7)	0.834
Moderate (> 2–5 cm)	18 (47.4)	6 (50.0)	
Large (> 5 cm)	10 (26.3)	4 (33.3)	

Data are *n* (%). Significant *p* value < 0.05

stated the *T* allele of *IL-4 (-589C/T)* polymorphism is associated with an increased IL-4expression [20].

Our study showed that *TT* genotype was more prevalent in HCC group (24%) compared to control group (3.3%) and cirrhotic patient group (5%). *TT* homozygous genotype had 10 times more risk of hepatocellular carcinoma versus healthy control group and 6.33 times more risk versus cirrhotic patients group (*p* value = 0.018 and 0.016, respectively). The *T* allele frequency was increased in HCC group (44%) versus (26.7% and 28.8%, OR (95% CI) = 2.16 and 1.95) in control and cirrhotic patients group, respectively.

This was consistent with the study led by Khalil et al. among HCV-infected Egyptian patients, they reported that *IL-4-589 TT* genotype was significantly increased in HCV-infected patients (*p* = 0.001) with strong reduction in the control group (3.3%), the patients samples revealed only *CT* (82%) and *TT* (18%) genotypes [21].

Also, there was a study conducted by Yousif et al. among HBV-infected patients in Sudan. They noted that *CT* and *TT* genotypes were 73.2% and 19.6%, respectively among infected patients [22].

In addition, the meta-analysis study directed by Zheng et al. who reported significant association between the *IL4-589T* polymorphism and increased risk of liver diseases in Caucasian populations; however, this was not detected in Asian populations [23].

It is worth noting that in our study, the significant association of *IL4-589C/T* polymorphism among HCC patient group was related to *TT* genotype which was contrary to the *CC* genotype detected in chronic HBV-infected Chinese male patients in the study conducted by Lu et al. in 2014 [24].

It is proposed that there are differences in the IL-4 genetic background among different ethnic groups. As on studying genotype distribution and allele frequency in control group of our study, the *IL4–589 C* allele frequencies were (73.3%), which were similar to healthy Egyptian (61.7%) and healthy Caucasians results (86.3%) in the studies conducted by Khalil et al. in 2017 [21] and Zheng et al. in 2013 [23] respectively but higher than frequencies among healthy Chinese (17.4%) in the study conducted by Lu et al. [24], while the *T* allele frequencies accounted for (26.7%), which was similar to healthy Egyptian (38.3%) [21] and healthy Caucasians frequency results (13.7%) [23] but was lower than that of healthy Chinese (82.6%) [24]. Thus, the *T* allele may be the variant responsible for various diseases in Egyptian population, while the opposite is true for Chinese population. This suggests that distinct allele frequencies in the same polymorphism site may play different roles in the same disease. A larger-scale multicenter study is recommended to confirm this suggestion.

On the other hand, we studied size and number of foci lesions in HCC group in relation to recessive genetic model of *-589C/T* SNP (*CC + CT* vs *TT*), we did not find statistical significance between 2 groups.

Conclusion

Our study revealed that there was statistically significant association between the *IL-4-589C/T (rs2243250)* polymorphism (*TT* genotype) and HCV-related HCC. The *T* allele may be a variant responsible for the disease production among Egyptian population.

Abbreviations

IL-4: Interleukin-4; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; HBV: Hepatitis B virus; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma; Th2: T helper 2; ILC2s : Type 2 innate lymphoid cells; HBsAg: Hepatitis B surface antigen; AST : Aspartate aminotransferase; ALT: Alanine aminotransferase; HBsAg: Hepatitis B surface antigen; HCV Abs : Hepatitis C virus antibodies; CIs: Confidence intervals

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Authors' contributions

M-E A initiated the project, designed and implemented the study for application. MN analyzed the data, drafted and revised the paper. ME contributed to the data collection, analyzed the data, and revised the paper. EA analyzed the data, drafted and revised the paper. AN analyzed the data, drafted and revised the paper. All authors have read and approved the manuscript.

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Availability of data and materials
Data used to support the findings of this study are included within the article.

Ethics approval and consent to participate
The study was conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the institutional review board of National Liver Institute (NLI), Menoufia University, Egypt (approval no. 00198/2020). Written consents were filled and signed by all participants.

Consent for publication
Written informed consents were obtained from both patients and control. Patient involved in this study agree for publication of data.

Competing interests
The authors declare that they have no competing interests.

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