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Original article

Interleukin-9: Investigating its possible role in liver cirrhosis progression and in tumour promotion of hepatocellular carcinoma in chronic hepatitis C virus infected patients; A single centre study

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ABSTRACT

Background: Cytokines play an essential role in the pathogenesis of hepatitis C virus (HCV) related cirrhosis and hepatocellular carcinoma (HCC). Interleukin-9 (IL-9) participates in inflammation, autoimmunity and tumor immunity. IL-9 was reported to affect liver fibrosis, severity and prognosis of HCV related diseases. **Aim of the study:** To assess the potential correlation of serum IL-9 level and its receptor with the progression and prognosis of patients having HCV related chronic liver disease and its probable association with HCC. **Methods:** Sixty-eight patients having HCV chronic liver disease and 20 apparently healthy controls (HCs) were enrolled in the study. The patients were grouped into 3 groups: 18 patients having compensated chronic liver disease (CCLD), 18 patients having decompensated chronic liver disease, and 32 patients having HCC. Serum IL-9 level was estimated by ELISA and IL-9 receptor (IL-9R) gene expression was assessed by RT-PCR in HCC group and HCs. **Results:** IL-9 level was significantly higher in patients in comparison to HCs and there was statistically significant difference between the 3 patients' groups ($p<0.001$). IL-9 was correlated with different laboratory and clinical parameters. At a cut-off value of >1600pg/ml, IL-9 had the ability to differentiate between CCLD group and HCC group with 75% sensitivity and 94.44% specificity. There was a highly significant statistical difference between HCC group and HCs in IL-9R gene expression ($p<0.001$). **Conclusion:** Serum IL-9 level is higher in chronic HCV infected patients than in HCs. IL-9R gene expression is higher among HCC patients than in HCs.

Introduction

Hepatitis C virus (HCV) causes chronic infection in more than 71 million people worldwide, with high morbidity and mortality rates [1]. Egypt has the highest prevalence of HCV in the world, reaching 14.7% [2]. Twenty to 30% of infected patients develop liver cirrhosis within 20 years after infection [1]. Of these, 1–8% will develop hepatocellular carcinoma (HCC) [3]. Hepatitis C virus infection is responsible for one-third of all HCC cases worldwide [4]. In Egypt, HCV accounts for 50% of HCC cases [5].

Although the administration of direct antiviral agents has led to a significant improvement

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in viral clearance in 90-95% of treated cases, compared to that elicited by peginterferon plus ribavirin combination therapy [1], there are other issues requiring new strategies in treatment, such as viral relapse, effectiveness among different genotypes and among different degrees of cirrhosis [6].

Despite being a non-cytopathic virus, the interaction between HCV and the host immune response elicits variable liver damage [7]. The early immunological events triggered by the virus leads to partial viral clearance and protective immunity, however the infiltrating T helper (Th) cells and T regulatory cells (Tregs) together with the cytokines produced, play prominent roles in liver inflammation and fibrosis [8,9].

A Th cell subset characterized by secreting large quantities of interleukin-9 (IL-9)- Th9 cells- has been defined. Naive T cells can differentiate into Th9 cells in the presence of IL-4 and TGF-β [10]. IL-9 is a member of the IL-2Rγc chain family [11]. IL-9 is secreted mainly by Th9 cells. Other cells include T cytotoxic 9 (Tc9) cells, type 2 innate lymphoid cells, mast cells, Th17 and Tregs [11-13].

IL-9 is a pleiotropic cytokine which regulates many cell types. It exerts either pro- or anti-inflammatory role through promoting the development of Th17 and Tregs, respectively. IL-9 can regulate the function of T cells, B cells, mast cells and airway epithelium by activating the signal transducers and activators of transcription (STAT) 1, STAT3 and STAT5 signalling pathways, which are involved in inflammatory, allergic and autoimmune diseases [11]. Meanwhile, IL-9 provides a protective role in immunity against intestinal parasites [14].

IL-9 has dual roles in tumour immunity. Being a lymphocyte growth factor, IL-9 promotes haematological malignancies, while in solid tumours, IL-9 may either inhibit or promote tumour development [11,15]. The mechanism of IL-9 in tumour immunity is not yet clear, requiring further research.

Considering the emerging role of IL-9 in inflammation and tumour immunity, together with the lack of information about its immunopathological role in HCV infection and HCC as one of its complications, we hereby aimed at assessing the potential correlation between the serum IL-9 level and the clinical characteristics, progression and prognosis of liver disease in different groups of chronically infected HCV patients, and to investigate the possible correlation of IL-9 level and its receptor expression with HCC.

Patients and Methods

A case control single-centre study was conducted on 68 chronically HCV infected patients attending the outpatient clinics of: Virology Unit, Faculty of Medicine Ain Shams Research Institute (MASRI), Internal Medicine, and Tropical Medicine, Faculty of Medicine, Ain Shams University Hospitals, Cairo, Egypt. Twenty apparently healthy subjects who tested negative for HCV antibody were enrolled as healthy controls (HCs). The study was approved by The Research Ethics Committee, Ain Shams University, Faculty of Medicine (FWA 00017585), and from MASRI. A written informed consent was obtained from each patient.

I- Patients

Sixty-eight patients were enrolled in the study and were divided into 3 groups: group 1: 18 patients having compensated chronic liver disease (CCLD) due to HCV, group 2: 18 patients having decompensated chronic liver disease (DCLD) and group 3: 32 patients suffering from HCC.

All patients were subjected to detailed history taking, thorough clinical examination and routine laboratory investigations for chronic viral hepatitis (liver functions testing, liver enzymes, complete blood count (CBC), HCV antibodies and quantitative RT- PCR for HCV for patients' group 1 and 2). Diagnosis of chronic HCV (CHC) was based on clinical, laboratory and sonographic findings of the liver. The degree of liver fibrosis was assessed by aspartate aminotransferase-to-platelet ratio index (APRI) [16] and fibrosis-4 index (FIB-4) [17]. The degree of liver cirrhosis was assessed according to Child–Pugh classification [18]. Diagnosis of HCC on top of HCV was performed according to the American Association for Study of Liver Diseases guidelines [19], based on radiological features. Tumour staging was performed according to Barcelona Clinic Liver Cancer (BCLC) [20] staging classification.

Exclusion criteria

Patients co-infected with other hepatic viruses e.g. HBV, or suffering from autoimmune diseases or immune deficiency disorders (including HIV infection), or having history of drug(s) intake that affects the immune system (e.g. corticosteroids, methotrexate) and alcoholics were excluded from the study.

II- Methods

Sample collection

Three ml of blood were collected from all subjects into tubes without additives for measuring serum IL-9 level

by ELISA. Another 3 ml of blood were collected from HCC patients' group and HCs into heparinized tubes for estimation of IL-9 receptor (IL-9R) gene expression.

Measurement of serum IL-9 level

Blood samples for estimation of serum IL-9 level were kept for 20 minutes at room temperature, then centrifuged for 10 minutes at 3000 rpm. Serum was aspirated and kept at -80^0 C till use. Serum IL-9 level was estimated using human IL-9 ELISA kit (Bioassay Technology Laboratory, Shanghai, China), as described by the manufacturer. Optical densities were read using a micro-plate reader (CLARIOstar®, BMG Labtech., Germany) within 10 min after adding the stop solution at 450 nm.

Estimating IL-9 receptor gene expression

IL-9R gene expression was measured in two groups: HCs and HCC patients' group.

Primers used (Bio-Rad's assays, USA):

1) For GAPDH (housekeeping gene) [10]:

Forward: 5′GGATTTGGTCGTATTGGG3′

Reverse: 5′GGAAGATGG TGATGGGATT3′

2) For IL-9R gene [21]:

Forward: 5′CGTGCCCTCTCCAGCGATGTTCT3′

Reverse: 5′GACGCGCTGGGCCACAA GTG3′

The peripheral blood mononuclear cells (PBMCs) were obtained from blood samples by density gradient centrifugation at 3000 rpm for 10 minutes. RNA was extracted from PBMCs using TRIZOL reagent (Invitrogen, USA), and reverse transcribed into cDNA using Biosystems® TaqMan® MicroRNA Reverse Transcription Kit (Thermofisher scientific, USA) according to the manufacturer's instructions. RT-PCR (Step One, Applied Biosystems, USA) was done using SYBR Green (Thermofisher scientific, USA). An initial denaturation step for 5 minutes at 94◦C was done, followed by a cycling 3 steps procedure (for 40 cycles): denaturation at 94◦ C for 30 seconds, annealing at 60◦ C for 30 seconds, and extension at 72◦C for 60 seconds. Specificity was verified by melt curve analysis run automatically. IL-9R gene expression level was normalized to the housekeeping gene transcripts level. Gene expression was quantified by the $2^{-\Delta\Delta CT}$ value.

Statistical analysis

Descriptive statistics

1. Mean, Standard deviation (± SD) and range for parametric numerical data, while Median and Interquartile range (IQR) for non-parametric numerical data.
2. Frequency and percentage of non-numerical data.

Analytical statistics

1. Student t test was used to assess the statistical significance of the difference between two study group means.
2. Mann Whitney test (U test) was used to assess the statistical significance of the difference of a non-parametric variable between two study groups.
3. Chi-Square test was used to examine the relationship between two qualitative variables.
4. ANOVA test was used to assess the statistical significance of the difference between more than two study group means.
5. Post Hoc Test is used for comparisons of all possible pairs of group means.
6. Correlation analysis (using Pearson's method): To assess the strength of association between two quantitative variables. The correlation coefficient denoted symbolically "r" defines the strength (magnitude) and direction (positive or negative) of the linear relationship between two variables.
7. Correlation analysis (using Spearman's method): is a non-parametric measure of rank correlation (statistical dependence between the rankings of two variables). The correlation coefficient denoted symbolically "r_s" defines the strength (magnitude) and direction (positive or negative) of the relationship between two variables.
8. Pairwise analysis by log rank test, when comparing sensitivities and specificities of different indices.

Results

The demographic, clinical and laboratory data of the patients together with tumour staging in HCC group are illustrated in **table (1)**. Tumour sizes ranged from 1 to 14 cm. Regarding the HCs, they were 13 males and 7 females with mean age of 52 years (±7.6 SD), ranging from 38 to 67 years old.

Serum IL-9 level in patients' groups and HCs

Serum IL-9 level was significantly higher in patients in comparison to HCs ($p<0.001$). Moreover, there was high statistically significant difference in serum IL-9 levels among the 3 different patients' group ($p<0.001$) **(Table 2)**. The mean values of serum IL-9 levels among patients' groups and HCs are illustrated in **table (2)**.

Correlation of serum IL-9 level with different laboratory and clinical parameters

As demonstrated in **table (3)**, in CCLD group, serum IL-9 level was positively correlated with aspartate transaminase (AST) (*p=0.01*), and negatively correlated with hemoglobin level (*p=0.011*). In HCC group, IL-9 level was positively correlated with AST (*p<0.001*), bilirubin level (*p<0.001*), INR (*p=0.004*), Child-Pugh score (*p<0.001*), tumour stage, size and number (*p<0.001*), while was negatively correlated with serum albumin level (*p<0.001*), hemoglobin level (*p<0.001*) and platelets count (*p<0.001*). Serum IL-9 level was positively correlated with APRI and FIB4 indices in the 3 patients' groups.

Cut-off value of serum IL-9 level for predicting HCC

From the receiver operating characteristic (ROC) curve, a cut-off value of >1600pg/ml for serum IL-9 level had the ability to differentiate between CCLD group and HCC group with sensitivity of 75%, specificity of 94.44%, positive predictive value (PPV) of 96% and negative predictive value (NPV) of 68% (AUC= 0.925, 95% CI, 0.815 to 0.981, *p value<0.0001*) **(Figure 1)**. When comparing the diagnostic performance of this cut-off value with APRI and FIB-4 indices, there was a significant statistical difference in the sensitivity and specificity of IL-9 cut off value of 1600pg when compared to that of APRI and FIB-4 scores (*p = 0.013* and *0.001*, respectively) **(Table 4)**

IL-9R gene expression in HCs and HCC patients' group

There was a highly significant statistical difference in IL-9R gene expression between HCC group and HCs (*p<0.001*) **(Table 5)**. IL-9R gene was 41 times more expressed in HCC group than in HCs. In HCC group, there was a positive correlation between serum IL-9 level and IL-9R gene expression (*p<0.001*), while in HCs there was no correlation between serum IL-9 level and IL-9R gene expression.

Correlation of IL-9R gene expression with different laboratory and clinical parameters

In HCC group, IL-9R gene expression was positively correlated with bilirubin level (*p=0.008*), INR (*p=0.012*), alfa fetoprotein (*p<0.001*), Child-Pugh score (*p<0.001*), APRI and FIB4 indices (*p<0.001*), BCLC staging (*p=0.001*), tumour size (p=0.017) and tumour number (*p=0.042*), while it was negatively correlated with serum albumin level (*p=0.002*) and platelets count (*p=0.003*) **(Table 6)**.

Table 1. Demographic, clinical and laboratory data of the patients' group.

		HCC patients' group	CCLD group	DCLD group	Chi square test		
		Number (%)	Number (%)	Number (%)	Value	*P value*	Sig.
Sex	**Male**	23 (71.9)	9 (50)	10 (55.6)	X2 = 2.73	*0.255*	NS
	Female	9 (28.1)	9 (50)	8 (44.4)			
Child-Pugh score	**A**	18 (56.25)	18 (100)	0 (0)	X2 = 39.17	*<0.001*	S
	B	11 (34.4)	0 (0)	6 (33.3)			
	C	3 (9.4)	0 (0)	12			
		Mean (SD)	**Mean (SD)**	**Mean (SD)**	**ANOVA test**		
Age (years)		53.94 (6.5)	49.33 (7.3)	57.11 (7.4)	F = 5.74	*0.005*	S
AST (IU/L)		72.44 (24.9)	47.89 (20.6)	56.17 (19.9)	F = 7.52	*0.001*	S
ALT (IU/L)		54.69 (20.3)	50.89 (22.3)	32.17 (12.7)	F = 8.13	*0.001*	S
Bilirubin (mg/dL)		3.25 (3.6)	1.02 (0.5)	4.99 (3.4)	F = 7.8	*0.001*	S
Serum albumin (g/dL)		2.93 (0.99)	3.78 (0.5)	2.40 (0.6)	F = 13.75	*<0.001*	S
INR		1.52 (0.55)	1.23 (0.22)	1.98 (0.37)	F = 13.19	*<0.001*	S
Hemoglobin (g/dL)		11.36 (1.7)	12.22 (1.5)	11.48 (1.2)	F = 1.89	*0.160*	NS
Total leucocytic count ($x10^3/mm^3$)		6.76 (2.3)	7.14 (2.1)	6.41 (2.4)	F = 0.46	*0.630*	NS

Platelets count ($x10^3/mm^3$)	109.28 (67.9)	112.50 (25.7)	118.56 (28.3)	F = 0.19	*0.826*	NS
Serum creatinine (mg/dl)	0.91 (0.3)	0.91 (0.3)	1.06 (0.4)	F = 1.32	*0.275*	NS
Alpha fetoprotein (ng/ml)	3164.24 (17028.9)	7.24 (3.5)	40.91 (37.1)	F = 0.6	*0.550*	NS
APRI	1.88 (1.5)	1.06 (0.7)	1.66 (0.6)	F = 2.92	*0.061*	NS
FIB 4	6.89 (4.7)	3.41 (2.2)	4.91 (1.2)	F = 6.09	*0.004*	S
		Median (IQR)	**Median (IQR**	**Mann Whitney test**		
HCV quantitative PCR (IU/ml)		1610850 (211944–2623279)	2237109 (1098359–3148981)	U = 109	*0.097*	NS

		N (%)
BCLC staging	0	7 (21.9)
	A	5 (15.6)
	B	10 (31.3)
	D	10 (31.3)
Tumour number	1	10 (31.3)
	2	7 (21.9)
	3	2 (6.3)
	Multiple	13 (40.6)

Abbreviations: ALT: Alanine transaminase, APRI: aminotransferase-to-platelet ratio index, AST: aspartate transaminase, BCLC: Barcelona Clinic Liver Cancer, CCLD: Compensated chronic liver disease, DCLD: decompensated chronic liver disease, FIB-4: fibrosis-4 index, HCC: hepatocellular carcinoma, HCV: hepatitis C virus, INR: international normalized ratio, IQR: interquartile range, SD: standard deviation, Sig: significance, S: significant, NS: non-significant.

Table 2. Serum IL-9 level among patients' group and healthy controls.

Serum IL-9 level (pg/ml)	**Healthy controls** (No.=20)	**Patients** (No.=68)	**T test***		**CCLD group**	**DCLD group**	**HCC patients' group**	**ANOVA test****	
Range (pg/ml)	350-2800	500-4200	**Value**	***P value***	500-2100	2100-4200	1100-4200	**Value**	***P value***
Mean (SD)	629 (593.1)	2377.94 (1095.8)	t=24.6	***<0.001***	1163.9 (358.4)	2986.1 (538.2)	2718.8 (1084.2)	F=27.5	***<0.001***

*Student t test was used to assess the statistical significance of the difference in serum IL-9 level between the healthy control group and the patients' group, ** while ANOVA test was used to assess the statistical significance of the difference between the three different patients' groups and by performing post hoc test a significant difference was observed between CCLD and HCC groups and between CCLD and DCLD groups. Abbreviations: CCLD: Compensated chronic liver disease, DCLD: decompensated chronic liver disease, HCC: hepatocellular carcinoma, SD: Standard deviation.

Table 3. Correlation of serum IL-9 level with different laboratory and clinical parameters and degree of fibrosis in the 3 patients' groups.

Group	IL-9 (pg/ml)					
	HCC N=32		CCLD group N= 18		DCLD group N= 18	
	r	*p value*	r	*p value*	r	*p value*
AST (IU/L)	**0.604**	***<0.001***	**0.589**	***0.010***	0.304	*0.221*
ALT (IU/L)	0.333	*0.062*	-0.406	*0.095*	-0.380	*0.120*
Bilirubin (mg/dL)	**0.698**	***<0.001***	0.202	*0.422*	0.235	*0.347*
Serum albumin (g/dL)	**-0.717**	***<0.001***	0.315	*0.203*	-0.379	*0.121*
INR	**0.498**	***0.004***	-0.143	*0.572*	0.111	*0.662*
Hemoglobin (g/dL)	**-0.621**	***<0.001***	**0.582**	***0.011***	-0.211	*0.401*
Total leucocytic count ($x10^3/mm^3$)	**0.011**	***0.952***	**0.029**	***0.911***	-0.367	*0.134*
Platelets count ($x10^3/mm^3$)	**-0.717**	***<0.001***	-0.199	*0.428*	-0.308	*0.213*
Serum creatinine (mg/dL)	-0.026	*0.888*	-0.220	*0.381*	-0.254	*0.310*
Alpha fetoprotein (ng/ml)	0.252	*0.164*	-0.102	*0.687*	0.124	*0.624*
Child-Pugh score	**0.893**	***<0.001***	**-0.043**	***0.867***	0.331	*0.179*
APRI	**0.649**	***<0.001***	**0.778**	***<0.001***	**0.743**	***<0.001***
FIB 4	**0.717**	***<0.001***	**0.912**	***<0.001***	**0.943**	***<0.001***
HCV PCR (IU/L)			-0.222	*0.377*	0.298	*0.230*
BCLC	**0.900**	***<0.001***				
Tumour size	**0.659**	***<0.001***				
Tumour number	**0.589**	***<0.001***				

Abbreviations: ALT: Alanine transaminase, APRI: aminotransferase-to-platelet ratio index, AST: aspartate transaminase, BCLC: Barcelona Clinic Liver Cancer staging classification, CCLD: Compensated chronic liver disease, DCLD: decompensated chronic liver disease, FIB-4: fibrosis-4 index, HCC: hepatocellular carcinoma, HCV: hepatitis C virus, INR: international normalized ratio, SD: standard deviation

Table 4. Diagnostic performance of IL-9 cut-off value (1600 pg) in comparison to APRI and FIB-4 indices.

	Sensitivity (%)	**Specificity (%)**	**PPV (%)**	**NPV (%)**	**Accuracy (%)**	**Log rank test**	
APRI	50	77.8	80	46.7	60	APRI vs IL-9	FIB4 vs IL-9
FIB4	78.1	55.6	75.8	58.8	70	*P = 0.013*	*P = 0.001*
IL-9	75	94.4	96	68	82		

Table 5. IL-9R gene expression in HCs and HCC patients' group.

	Healthy controls	HCC patients' group	t test	
	Mean (SD)	Mean (SD)	value	*p value*
$2^{\wedge-\Delta\Delta Ct}$ (RT-PCR for IL-9R gene)	1.12 (0.5)	41.49 (18.2)	t= 27.29	***<0.001***

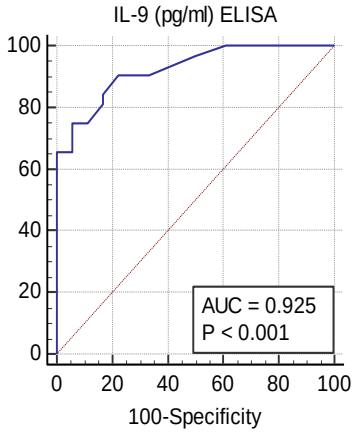
Abbreviations: HCC: hepatocellular carcinoma, SD: standard deviation

Table 6. Correlation of IL-9R gene expression with different laboratory and clinical parameters in HCC group.

Group	$2^{\wedge-\Delta\Delta Ct}$ (RT-PCR for IL-9R gene)	
	HCC (N=32)	
	r	*p value*
AST (IU/L)	0.253	*0.163*
ALT(IU/L)	-0.122	*0.505*
Bilirubin (mg/dL)	**0.460**	***0.008***
Serum albumin (g/dL)	**-0.534**	***0.002***
INR	**0.434**	***0.013***
Hemoglobin (g/dL)	-0.333	*0.063*
Total leucocytic count ($x10^3/mm^3$)	-0.095	*0.606*
Platelets count ($x10^3/mm^3$)	**-0.504**	***0.003***
Serum creatinine (mg/dL)	0.100	*0.587*
Alpha feto protein (ng/ml)	**0.646**	***<0.001***
Child-Pugh score	**0.608**	***<0.001***
APRI	**0.701**	***<0.001***
FIB 4	**0.561**	***0.001***
BCLC	**0.571**	***0.001***
Tumour size	**0.419**	***0.017***
Tumour number	**0.361**	***0.042***

Abbreviations: ALT: Alanine transaminase, APRI: aminotransferase-to-platelet ratio index, AST: aspartate transaminase, BCLC: Barcelona Clinic Liver Cancer staging classification, FIB-4: fibrosis-4 index, INR: international normalized ratio

Figure 1. Receiver operating characteristic (ROC) curve of serum IL-9 level to differentiate between compensated liver disease and hepatocellular carcinoma patient group (evaluated by Youden's J Statistical equation) (AUC: area under the curve).



Discussion

Although the role of Th9/IL-9 has been described in many immunopathological conditions, yet the role of this T cell subset and its characteristic cytokine, IL-9, in HCV infection is not fully investigated. We analysed the correlation between the serum IL-9 level with different lab parameters, degree of liver fibrosis and degree of liver cell failure and observed that there were more correlations in the HCC group (AST, bilirubin level, INR, Child-Pugh score, serum albumin level, hemoglobin level and platelets count), while in the DCLD group, there were no correlations between serum IL-9 level and laboratory and clinical parameters except for APRI and FIB-4 indices. We did not find a correlation between serum IL-9 level and the viral load in the studied groups. In CCLD group, serum IL-9 level was positively correlated with AST.

Interestingly, APRI and FIB-4 indices were positively correlated with serum IL-9 level in the 3 patients' groups, i.e. serum IL-9 level positively correlates with the degree of liver fibrosis in different conditions of HCV related diseases. Although APRI and FIB-4 indices are non-invasive and widely used in assessing the degree of fibrosis, yet their use has the risk of overestimating the fibrosis stage due to the impact of inflammatory activity on transaminases [22]. As we found that serum IL-9 level positively correlates with both indices, may be adding serum IL-9 level to these scoring indices may enhance their accuracy and sensitivity in estimating the degree of fibrosis, since the treatment duration and the post-treatment prognosis depend on the fibrosis stage, especially in cirrhotic patients or in advanced fibrosis [23].

Our results suggest that IL-9 may be associated with the immunopathogenesis of liver

disease in HCV infection, and with the progression to HCC development. IL-9 was also reported to be significantly elevated in other viral infections, e.g. zika virus, respiratory syncytial virus and chronic HBV infection [24-26]. Moreover, it was reported that elevated IL-9 level was predictive for HCV treatment failure with pegylated interferon-alpha plus ribavirin regimen, while viral clearance was associated with low IL-9 levels [27].

In agreement with our findings, **Ali et al.** [28] reported that they observed a positive correlation between Th9 cell frequency and the severity of HCV-related diseases and a direct association with its complications, denoted by a positive correlation with clinical parameters such as liver enzymes and Child–Pugh scores. However, the authors reported a positive correlation between Th9 cells frequency and HCV viral load [28].

A study on the role of IL-9 in hepatic fibrosis was carried on a mouse model of liver fibrosis. The study revealed that anti IL-9 antibody reduced the hepatic inflammation and fibrosis, with a decreased splenic Th9 percentage. Anti-IL-9 antibody treatment also reduced the plasma level of the inflammatory cytokines, TGF-β 1, IL-6, IL-4, IL-17 A and TNF-α, suggesting that IL-9 may regulate the inflammatory responses to liver fibrosis. The authors noticed that the percentages of IL-9 secreting Th9 cells were significantly elevated in both patients and mice with liver fibrosis, suggesting that the microenvironment induced by liver fibrosis favors Th9 proliferation and IL-9 secretion [29].

Regarding the tumorigenic role of IL-9, **Tan et al.** [30] demonstrated a tumour enhancing role of IL-9 secreting Th9. The authors reported that serum IL-9 level and circulating Th9 cells frequency were higher among HCC patients in comparison to HCs. Moreover, Th9 cells were significantly higher in tumour and peritumour tissues than in unaffected liver tissues, and the higher the frequency of Th9 in the HCC patient, the shorter the disease-free survival time after tumour resection, concluding that IL-9 promoted tumour progression. The authors reported that Th9 cells caused a significant elevation in chemokine ligand 20 (CCL20) in tumour cells, which is associated with HCC poor prognosis, and elevation in STAT3 mRNA levels, which targets CCL20 gene and these elevations were opposed by neutralizing IL-9. CCL20 induces epithelial-mesenchymal transition-like changes in HCC cells and enhances the metastasis [31]. Other supporting studies demonstrated that high IL-9 level activates STAT3 in tumour cells, promoting tumour proliferation [32,33].

Since CCLD group is a target group for HCC surveillance, due to the probability of receiving radical therapies [34], we investigated the cut-off value of serum IL-9 level that can differentiate between CCLD and HCC. We found that a cut off value of 1600pg/ml had a sensitivity of 75% and specificity of 94.44%. These results suggest a possible role for IL-9 as a prognostic marker for the development of HCC in CCLD patients and raises the question whether anti IL-9 could be a strategy in treating HCC.

In our study, we found that IL-9R gene expression was significantly higher among HCC group in comparison to HCs and was correlated with serum IL-9 level. IL-9R gene expression was also correlated with different laboratory and clinical parameters. IL-9R expression was positively corelated with tumour staging, number and size of the tumour. In agreement with our findings, **Ali et al.** [28] reported similar results, where they found that IL-9R was significantly elevated in all HCV infected patients' groups in their study.

IL-9R was reported to be associated with some autoimmune diseases, such as systemic lupus erythematosus and psoriasis, and malignancy, such as various human leukaemia and Hodgkin lymphomas [35-37]. In HCC cells, IL-9R overexpression can inhibit cell apoptosis, promote cell proliferation, and increase the invasiveness, mostly through the participation of STAT3, matrix metallopeptidase-9 proteins and vascular endothelial growth factor. IL9-R was also reported to be an independent predictor for HCC [10,36,38,39].

In conclusion, our study observed that IL-9 and its receptor are correlated with HCV related liver cell failure and progression to HCC, thus may represent a helpful predictor for HCV related complications. The findings suggest that IL-9 and IL-9R may be useful prognostic factors for identifying HCC in patients with high risk. Anti-IL-9 may be a potential novel strategy in treating HCV related complications. Being positively correlated with APRI and FIB-4 indices, adding serum IL-9 level to these scoring systems may enhance their accuracy in determining the degree of hepatic fibrosis.

Contributors

All authors have made substantial contributions to the design of the study. Sample collection, clinical examination and clinical diagnosis were performed by Dr/Heba I. Aly, Dr/Manar M.

Salah and Dr/Heba A. Faheem. The molecular and serological tests were performed by Dr/Amira E. Abdelhamid and Dr/Shimaa A. Abdel Salam. Data analysis and interpretation were contributed to all the authors. Drafting the article was performed by Dr/Amira E. Abdelhamid and Dr/Manar M. Salah. Revising the draft critically for important intellectual and scientific content was carried out by all the authors. All the authors provided final approval of the version to be published.

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