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Original Article

Interleukin-18 and its gene single nucleotide polymorphisms (SNPs) influence chronic hepatitis C progression

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Abstract

Introduction: Interleukin-18 (IL-18) is a pro-inflammatory cytokine that is induced by hepatitis C virus (HCV) infection. Inter-individual variations of IL-18 gene expression may alter HCV-associated liver injury. Variable single nucleotide polymorphisms (SNPs) have been detected within IL-18 gene sequence. Quantitative assessment of IL-18 plasma level and detection of genotype frequencies of 2 functional polymorphisms of its gene (-607 C/A and -137 G/C) were done to assess their impact on the severity of chronic hepatitis C (CHC).

Methodology: Cases group (I) comprised 110 treatment naïve CHC Egyptian patients (78 Males and 32 Females, mean age = 40.7 ± 11.8 years) who underwent routine laboratory investigations. Assessment of plasma level of IL-18 was done by enzyme-linked immunosorbent assay (ELISA), detection of IL-18 gene polymorphisms at positions -607 C/A and -137 G/C by polymerase chain reaction sequence specific polymorphism (PCR-SSP) analysis and Liver biopsy with METAVIR scoring were done. The control group (II) comprised 90 healthy participants.

Results: Plasma levels of IL-18 were significantly higher in cases than the control group. We found a statistically highly significant ($p < 0.001$) positive correlation between IL-18 plasma level and both METAVIR necro-inflammatory grade and fibrosis stage. The A/A allele at -607 position was significantly more frequent ($p < 0.05$) in patients with $F \leq 1$.

Conclusions: Higher IL-18 plasma levels are found in CHC patients and positively correlate with the severity of liver disease. The presence of A/A allele at -607 position of IL-18 gene promoter is associated with milder liver disease.

Key words: Hepatitis C virus; interleukin 18; IL-18 single nucleotide polymorphism, liver fibrosis.

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Introduction

Hepatitis C virus (HCV) is a major etiology of chronic liver disease worldwide. It has been regarded as an epidemic in the Egyptian population with an overall anti-HCV antibody prevalence of 14.7% and the number of Egyptians estimated to be chronically infected with HCV is 9.8%. The main genotype in Egypt is genotype 4 which is responsible for > 90% of infections, with the remaining due to genotype-1 [1,2]. The natural history of hepatitis C infection is quite variable. In the majority of cases, neither the innate immune response nor the adaptive immunity can clear the virus and the infection turns to be chronic. This ineffective inflammatory response drives fibrogenesis and cirrhosis development [3]. Host genetics controlling his immune responses play a critical role in determining HCV infection outcome [4].

Interleukin-18 (IL-18), originally named IFN-gamma inducing factor, is a pro-inflammatory cytokine synthesized by Kupffer cells and activated macrophages [5]. IL-18 is involved in either Th1 or Th2 immune responses according to the inflammatory milieu. In the presence of IL-12, IL-18 induces interferon-gamma expression, promoting the Th1-mediated immune response. In the absence of IL-12, IL-18 stimulates Th2 immune responses [4]. IL-18 increases the susceptibility of liver endothelial cells to undergo apoptosis [6]. IL-18 plays a role in fighting viral infections e.g. herpes simplex, vaccinia virus and HIV [7]. IL-18 inhibits hepatitis B virus (HBV) replication in transgenic mice [8]. In chronic hepatitis

C (CHC) other than genotype-4, and cirrhosis, plasma IL-18 was reported to be elevated and was reduced when HCV was resolved (in patients with resolved HCV) [6].

Cytokine genes are polymorphic at specific sites, and some of these mutations have been associated with inter-individual variations of cytokine expression. Hence, genetic inter-individual variations of IL-18 expression may represent a risk factor for HCV-associated liver injury [9]. The aim of the present study was to detect genotype frequencies of two functional polymorphisms of the IL-18 gene (-607 C/A and -137 G/C) and to assess their impact on the severity of CHC (reflected by the liver biopsy findings). Quantitative assessment of IL-18 plasma level was also considered and its relation to disease severity was studied.

Methodology

This cross-sectional study, was conducted on 200 adult Egyptian participants divided into 2 groups: Group I (cases group) comprised 110 consecutive, treatment naïve patients with CHC (HCV-Ab positive and HCV-RNA-PCR positive). Group II (control group) comprised 90 age and sex matched apparently healthy participants with negative viral markers and normal liver enzymes. All were attending the Hepatology, Gastroenterology and Infectious Diseases Department at Benha University Hospitals and the Microbiology and Immunology Department at Faculty of Medicine, Benha University, Egypt during the period from January 2012 to February 2014. The study protocol was approved by the Ethical Scientific Committee of Benha Faculty of Medicine and its University Hospitals. An informed medical consent was obtained from all participants. The study was performed in compliance with the ethics principles of the 1975 Declaration of Helsinki and its later amendments with good clinical practice (GCP) guidelines. The exclusion criteria of the study were: age less than 18 or more than 60 years, pregnant females, patients with other causes of chronic hepatitis (alcoholic, autoimmune hepatitis, metabolic disorders, etc.), patients tested positive for other hepato-trophic viruses e.g. HBV, decompensated cirrhosis and those who refused signing the written medical consent.

All patients were subjected to detailed history taking, thorough clinical examination, routine laboratory investigations including: Complete Blood Count, Liver biochemical tests (ALT, AST, Alkaline phosphatase, Bilirubin (total and direct), serum albumin and Prothrombin time), serum creatinine, HCV antibody and HBS-Ag by Diasorin enzyme linked immunosorbent assay (ELISA) -Analyzer ETI-Max 3000 (DiaSorin Austria GmbH, Wien, Austria), quantitative HCV-RNA by real time polymerase chain reaction (PCR) (Taqman probe) ABI 7900 (Thermo Fisher Scientific, Waltham, USA), anti- Schistosoma antibody by Falcon assay screening test (FAST) ELISA (Becton Dickinson Labware, Lincoln Park, USA).

Assessment of plasma level of IL-18 by (ELISA Kit of Medical and Biological laboratories, Nagoya, Japan), detection of IL-18 gene polymorphisms by PCR sequence specific polymorphism (PCR-SSP) analysis. Abdominal ultrasonography by LOGIQ P6 PRO SET and finally liver biopsy for histopathological grading and staging applying the METAVIR scoring system in an attempt to assess disease severity considering A0 as no activity, A1 mild activity, A2 moderate activity and A3 as severe activity, while F0 as no fibrosis, F1 as mild fibrosis, significant fibrosis as $F \geq 2$, advanced fibrosis $F \geq 3$ and F4 denoting established cirrhosis [10].

Genotyping of IL-18 promoter region at -607 and -137 positions with polymerase chain reaction-sequence specific polymorphism (PCR-SSP) analysis

A- Genomic DNA extraction

Genomic DNA was extracted from the whole blood using Gene JET TM genomic DNA purification kit (Thermo Fisher Scientific Inc, Waltham, MA USA) according to the manufacturer's instructions. The

Table 1. Sequence specific primers for C/A and G/C alleles and their PCR product sizes for positions -607 and -137.

	Primer sequence	Product size
Primers for C/A allele at position -607		
Common reverse primer	5'-TAACCTCATTCAGGACTTCC-3'	
Sequence-specific forward primer 1	5'- GTTGCAGAAAGTGTAAAAATTATTAC-3'	196
Sequence-specific forward primer2	5'- GTTGCAGAAAGTGTAAAAATTATTAA-3'	196
Control forward primer	5'- CTTTGCTATCATTCCAGGAA-3'	301
Primers for G/C allele at position-137		
Common reverse primer	5'- AGGAGGGCAAAATG CACTGG-3'	
Sequence-specific forward primer 1	5' - CCCCAACTTTTACGGAAGAAAAG-3'	261
Sequence-specific forward primer2	5' - CCCCAACTTTTACGGAAGAAAAC-3'	261
Control forward primer	5'- CCAATAGGACTGATTAT TCCGCA-3'	**446**

extracted DNA concentration was detected through measurement by UV spectrophotometer. Readings were taken at wave lengths of 260 and 280 nm according to that reported by Al Husseini *et al*. [11]. The extracted DNA concentration was ranged from 20-30 ng DNA/µL. Purified DNA samples were stored at -20ºC till used in the amplification step.

B- DNA amplification

It was done as described by Manohar *et al.* [12].

We performed for each polymorphism; 3 separate reaction tubes; one for the wild type and one for the mutant type and a third one for the control and each was run on agarose gel for visualization separately (Table 1).

C- DNA detection

PCR products were visualized using 2% agarose gel electrophoresis stained by ethidium bromide and visualized by UV.

Assessment of IL-18 in plasma

Plasma samples were separated and stored at -20ºc till used for detection of IL-18 using Human IL-18 ELISA kit (Medical and Biological laboratories, Nagoya, Japan) as described by the manufacturer.

Statistical methodology

The clinical and laboratory data were recorded on an "investigation report form". These data were tabulated, coded then analyzed using the computer program SPSS (Statistical package for social science) version 16 (SPSS Inc., Chicago, IL, USA). Categorical data were presented as number and percentages, using Chi square test (χ^2) as the test of significance, risk association was assessed by OR and the corresponding 95% CI. Continuous variables were expressed as mean and standard deviation, Student's t-test and ANOVA (F) were used. The accepted level of significance in this work was stated at 0.05 ($P < 0.05$ was considered significant).

Results

This cross-sectional study was conducted on 200 participants 143 males (71.5%) and 57 females (28.5%). Group I included 110 CHC patients (78 males and 32 females, whose mean age was 40.7 ± 11.8 years). Group II included 90 apparently healthy participants (65 males and 25 females with mean age 38.6 ± 11.4years) who were age and sex matching the cases group. The demographic and laboratory features of the 2 groups were summarized in Table 2.

Table 2. Demographic and laboratory features of the studied groups.

Variable (Mean ± SD)	Cases (N = 110)	Controls (N = 90)	p-value
Age (years)	40.7 ± 11.8	38.6 ± 11.4	0.21
Male : Female	78:32	65:25	0.84
Hemoglobin (gm/dL)	10.97 ± 0.99	13.1 ± 1.00	**0.0001**
Total leucocyte count $\times 10^3$	4.3 ± 1.25	6.2 ± 1.27	**0.0001**
Platelet count$\times 10^3$	185.4 ± 63.9	199.2± 42.1	**0.08**
Alanine transaminase (u/L)	53.4 ± 10.01	29.7 ± 5.3	**0.0001**
Aspartate transaminase (u/L)	67.6 ± 11.7	35.9 ± 3.9	**0.0001**
Serum albumin (gm/dL)	4.12 ± 0.77	4.74 ± 0.54	**0.0001**
Serum creatinine (mg/dL)	0.95 ± 0.26	0.93 ± 0.25	0.21
Serum interleukin-18 level (pg/mL)	168.7 ± 51.2	81.6 ± 16.5	**0.001**
HCV-RNA PCR ($\times 10^5$)	5.7 ± 5.6	--	
Anti-Schistosoma antibody positive	11 (10%)	--	
Anti-Schistosoma antibody negative	99 (90%)	--	
METAVIR score			
Necro-inflammatory grade (A)			
A0	30 (27.3 %)		
A1	47 (42.7 %)		
A2	14 (12.7 %)		
A3	19 (17.3 %)		
Fibrosis stage (F)			
F0	30 (27.3 %)		
F1	27 (24.5 %)		
F2	22 (20.0 %)		
F3	12 (10.9 %)		
F4	19 (17.3 %)		

Table 3. Distribution of IL-18 genotypes and alleles frequencies among the studied cases and control groups.

Genotypes		HCV patients (N* = 110) N (%)	Controls (N = 90) N (%)	OR	95% Confidence interval	P-value
IL-18 -607 C/A	CC	30 (27.3)	24 (26.7)	1.03	0.55-1.93	0.92
	CA	53 (48.2)	46 (51.1)	0.89	0.51-1.55	0.68
	AA	27 (24.5)	20 (22.2)	1.14	0.59-2.2	0.69
	C allele	113 (51.4)	94 (52.2)	0.97	0.65-1.43	0.86
	A allele	107 (48.6)	86 (47.8)	1.04	0.7-1.54	0.86
IL-18 -137 G/C	GG	53 (48.2)	37 (41.1)	1.33	0.76-2.34	0.31
	GC	42 (38.2)	39 (43.3)	0.81	0.46-1.42	0.46
	CC	15 (13.6)	14 (15.3)	0.86	0.39-1.88	0.71
	G allele	148 (67.3)	113 (62.8)	1.22	0.81-1.84	0.35
	C allele	72 (32.7)	67 (37.2)	0.82	0.54-1.24	0.35

*N = number.

Plasma levels of IL-18 were significantly higher ($p < 0.001$) in cases than the control group (Table 2). There was no statistically significant difference in the frequencies distribution of all the alleles at -607 and -137 positions between cases and control groups, (Table 3). We found that there was a statistically highly significant positive correlation between IL-18 plasma level and severity of liver disease as regards both METAVIR necro-inflammatory grade (A) and fibrosis stage (F) ($p < 0.001$). On the other hand, Table 4 shows no significant difference in the alleles and genotypes of the 2 studied SNPs distributions among the different METAVIR necro-inflammatory grades or fibrosis stages. The A/A allele at -607 position was significantly more frequent ($p < 0.05$) in patients with no and mild fibrosis ($F \leq 1$), while other alleles at both -607 and -137 positions showed non-significant difference compared to patients with significant ($F \geq 2$) fibrosis. There was no statistically significant difference in distribution of alleles of IL-18 genotypes -607 and -137 in the studied cases as regards serum HCV-RNA viral load.

Table 4. Distribution of the studied cases according to METAVIR necro-inflammatory grade and fibrosis stage in IL-18 genotype -607 and genotype -137.

		Gene -607			Gene -137			Total
		CC	CA	AA	GG	GC	CC	
METAVIR activity grade (A)								
A0	Number	**9**	**13**	**8**	**16**	**11**	**3**	**30**
	% within gene	30%	24.5%	29.6%	30.2%	26.2%	20%	27.3%
A1	Number	12	26	9	23	18	6	47
	% within gene	40%	49.1%	33.3%	43.4%	42.9%	40%	42.7%
A2	Number	3	7	4	6	5	3	14
	% within gene	10%	13.2%	14.8%	11.3%	11.9%	20%	12.7%
A3	Number	6	7	6	8	8	3	19
	% within gene	20%	13.2%	22.2%	15.1%	19%	20%	17.3%
METAVIR fibrosis stage(F)								
F0	Number	9	13	**8**	16	11	3	30
	% within gene	30%	24.5%	**29.6%**	30.2%	26.2%	20%	27.3%
F1	Number	8	15	**4**	14	9	4	27
	% within gene	26.7%	28.3%	**14.8%**	26.4%	21.4%	26.7%	24.5%
F2	Number	4	12	**6**	9	10	3	22
	% within gene	13.3%	22.6%	**22.2%**	17%	23.8%	20%	20%
F3	Number	3	6	3	6	4	2	12
	% within gene	10%	11.3%	11.1%	11.3%	9.5%	13.3%	10.9%
F4	Number	6	7	6	8	8	3	19
	% within gene	20%	13.2%	22.2%	15.1%	19%	20%	17.3%
Total		**30**	**53**	**27**	**53**	**42**	**15**	**110**

P- value = 0.89 in Fibrotic stage of gene 607 and in activity grade P- value = 0.85; P- value = 0.99 in Fibrotic stage of gene 137 and in activity grade P- value = 0.96.

Table 5. Frequency distribution of IL-18 polymorphism alleles at -607 and -137 positions in the studied cases among anti-Schistosoma antibodies positive and negative cases.

Anti-Schistosoma antibodies		gene -607			gene -137			Total
		CC	CA	AA	GG	GC	CC	
Negative	Number	25	53	21	49	35	15	99
	% within gene	83.3%	100%	77.8%	92.5%	83.3%	100%	90%
Positive	Number	5	0	6	4	7	0	11
	% within gene	16.7%	0%	22.2%	7.5%	16.7%	0%	10%
Total		**30**	**53**	**27**	**53**	**42**	**15**	**110**
p-value			**(0.003)**			**(0.13)**		

When we studied the distribution of IL-18 polymorphism alleles at -607 and -137 positions among anti-Schistosoma Ab positive and negative cases, there was a highly significant difference in the distribution of the -607 polymorphism being significantly lower in Anti-Schistosoma Ab positive cases. There was no significant difference in the distribution of the -137 SNP (Table 5).

Discussion

It is well established that about 20% of patients infected with HCV will progress to cirrhosis within 20 years from infection, further resulting in an estimated annual risk of 3–7% of hepatocellular carcinoma (HCC) [13]. Indeed, HCV is not directly cytopathic, and the mechanisms by which it causes liver injury are not well established. Immune response that is essentially conducted by cytokines may play an important role in the pathogenesis of HCV infection [14].

The IL-18 gene is located on chromosome 11 q22.2–q22.3 and a variety of SNPs have been detected within IL-18 gene sequence. Several of these polymorphisms, especially those located in the promoter region, may be associated with differential levels of gene transcription. Giedraitis *et al*., (2001) [8] described two SNPs at positions -607 C/A and -137 G/C which consist, respectively, in a change from cytosine to adenine, and from guanine to cytosine. Both disrupt transcription factors binding sites, and at least decrease the level of IL-18 mRNA [15].

In the present study, plasma levels of IL-18 were significantly higher ($P < 0.05$) in the studied cases than the control group, which comes in agreement with EI Kady *et al*. [16], who reported that patients infected with HCV had significantly higher plasma levels of IL-18 than healthy controls and they added that the presence of schistosomiasis in Egyptian patients with CHC may down-regulate the stimulatory effect of HCV on IL-18. A recent meta-analysis by Niu and colleagues [17] also found a significant difference in the plasma level of IL-18 between CHC patients and controls despite the genotypic difference in the studied population, being mainly genotype- 4 in the current study.

The current study also revealed that there was no statistically significant difference ($p > 0.05$) in the frequencies of all the alleles at -607 and -137 positions between the cases and control groups. This observation was in agreement with that found by Manohar and his colleagues [12], who observed the same findings. However, they studied 204 Indian patients included only 11 patients with HCV genotype 4 while the predominant genotype was 3 (unlike ours) and to a lesser extent genotypes 1 and 2.

The results of the present study revealed that there was a statistically highly significant ($p < 0.001$) positive correlation between IL-18 plasma level and severity of liver disease as regards both METAVIR necro-inflammatory grade and fibrosis stage. This positive correlation between IL-18 and METAVIR necro-inflammatory grade and stage may be due to the continued secretion of TH1 cytokines, including IL- 18, in response to the HCV-related liver injury resulting in fibrosis and ultimately cirrhosis. These results come in agreement with Bouzgarrou and colleagues [18], who assumed that their study was the first to offer data about IL- 18 gene polymorphisms in HCV- related liver disease and carried out it on 81 Tunisian patients with CHC, genotype 1b (including 20 patients with HCC) and 82 controls. They told that the mean value of plasma IL-18 was significantly higher in patients than in controls. Again, Bouzgarrou *et al*. [19] confirmed this correlation. The positive correlation between high levels of Th 1 cytokines, including IL-18, secreted in response to HCV-related liver injury, and an increased necro-inflammatory activity, liver fibrosis or cirrhosis was also reported by many previous studies [20-23].

The present study showed no statistically significant difference in distribution of alleles of IL-18 genotypes -607 and -137 in the studied cases as regards serum HCV-RNA viral load. This observation comes in agreement with that found by previous studies [4,24,25].

In the present study, the studied cases were divided according to the METAVIR fibrosis stage (that reflects severity and advancement of hepatitis C) and allele frequencies were studied and compared in patients with different stages. Hence, when patients with no- and mild- fibrosis (F0andF1) were compared to those with significant fibrosis ($F \geq 2$), the A/A allele at -607 position was significantly more frequent ($p < 0.05$) in patients with no- and mild- fibrosis, while other alleles at both -607 and -137 positions showed non-significant difference between both groups. It was evident that the presence A/A allele at -607 position of IL-18 gene promoter was associated with milder liver disease. This comes in agreement with Bouzgarrou and colleagues. [18], who concluded that the carriage of at least one C allele at position -607 (CC + CA) was associated with a higher risk of progressive fibrosis and cirrhosis; and with Manohar *et al*. [21], who divided their studied patients into two groups on the basis of histological activity index (HAI ≤ 5 or >5) and hepatic fibrosis score (≤ 2 or > 2) and told that the A/A allele at -607 position was more frequent in patients with mild liver disease than with severe one and associated its presence with milder HCV presentation. This association was not observed with the presence of A/C or C/C allele at -607 position as well as with G/G, G/C and C/C alleles at -137 position.

The findings of Cheikhrouhou and colleagues [26] support our results. They concluded that the A allele or the A/A genotype could play a positive role in the immune regulatory system of patients with CHC and would protect against the development and progression of the disease. Bouzgarrou *et al.* [19], when assessed the combined effect of pro- and anti-inflammatory cytokine gene polymorphisms on susceptibility to HCV- related liver cirrhosis, also come in agreement with the current study results. They considered IL-18 -607 C/C and C/A as high risk genotype (beside the other assumed high risk genotypes in their study) and they finally believed that it could be possible to define a specific genetic profile associated with the highest risk for cirrhosis development and therefore identify those patients who require a more aggressive therapeutic management. Moreover, An *et al*. [4] attributed the ability to clear HCV infection to the presence of IL-18 -607A/A and their study highlighted the critical role of promoter polymorphisms in the IL-18 gene in resolving HCV infection. A meta-analysis by Yang and Liu, revealed that IL-18 promoter region − 137G/C polymorphism was associated with CHC infections, but they found no evidence to indicate an association between − 607C/A polymorphism and CHC [27].

Conclusion

In conclusion; this cross-sectional study shows the association of plasma IL-18 and its (-607 C/A and -137 G/C) SNPs with CHC in a different ethnic population where genotype 4 is the most prevalent and its interaction with a common parasitic infection in developing countries like schistosomiasis. The study revealed that plasma IL-18 levels were significantly higher in CHC patients than healthy controls and have a significant positive correlation with the severity of liver disease. There was no difference in the frequencies of alleles distribution at -607 and -137 positions between cases and controls or among the different necro-inflammatory grades or fibrosis stages or serum HCV-RNA viral load values. The distribution of the -607 polymorphism was significantly lower in patients with previous exposure to schistosomiasis

Authors Contribution

All authors have contributed significantly to finish this work. Design of the study: Ebada Mohamed Said; performance of management: Abeer Ahmed Aboelazm, Rania Zakaria Aamer, Mai Hamed Kamel; acquisition of the data: Mohamed Sanad Rashed; analysis of the data: Mohamed Sanad Rashed; interpretation of the data and drafting the article: Mohamed Said Soliman, Ebada Mohamed Said; article revision: Hend Ibrahim Shousha, Mohamed Sanad Rashed, Mai Hamed Kamel, Abeer Ahmed Aboelazm; Rania Zakaria Aamer and Fatma Mohamed Abdelsalam; final approval of the version: Fatma Mohamed Abdelsalam; article's submission: Hend Ibrahim Shousha.

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