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RESEARCH ARTICLE

THE ASSOCIATION OF INTERLEUKIN-10 GENE POLYMORPHISMS WITH VISCERAL LEISHMANIASIS IN A SAMPLE OF IRAQI PATIENTS

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ABSTRACT

The study was designed to determine serum level of IL-10 in a sample of 44 Iraqi visceral leishmaniasis (VL) patients and 40 control subjects. In addition, single nucleotide polymorphisms (SNPs) of *IL10* gene were determined at three positions (*IL10*$_{-1082}$, *IL10*$_{-819}$ and*IL10*$_{-592}$).The results showed a significant increased serum level of IL-10 (40.02 ± 1.26 *vs*.18.60 ± 1.82pg/ml) in VL patients compared to controls, while analysis of genotypes and alleles of the three SNPs revealed no significant variations between VL patients and controls. Assessing the impact of these SNPs on 1L-10 serum level demonstrated that *IL10*$_{-1082}$ GG genotype showed the highest level of IL-10 in patients (45.73 ±3.15 pg/ml) compared to AA genotype (38.02± 1.48 pg/ml). For *IL10*$_{-819}$genotypes, they recorded approximated means of IL-10 level in patients. At *IL10*$_{-592}$, neither patients nor controls demonstrated a significant difference between the means of IL-10 in their genotypes.

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INTRODUCTION

In Iraq, as well as Afghanistan, Iran, Pakistan and Central Asia, VL is a zoonotic disease caused by *L. infantum* that especially infects infants and young children in the age group 1–4 years, and dogs are the predominant reservoir host in these regions (Pavli and Maltezou, 2010). The control of *Leishmania* infection is mainly mediated by a Th1-type (T helper 1) immune response, and experimental investigations in mouse models of leishmaniasis have documented an obvious dichotomy between Th1-mediated protection and Th2-mediated leishmaniasis susceptibility (Goto and Prianti, 2009). This dichotomy has been suggested to be influential during mouse *L. donovani* and *L. chagasi* disease, in which the curative Th1 responses are suppressed by IL-10 and TGF-β (Bankoti and Stager, 2012). It has also been observed that IL-10 blocked Th1 activation and a cytotoxic response by down-regulating the production of IL-12 and IFN-γ. Additionally, IL-10 inhibited macrophage activation and decreased these cells ability to kill *Leishmania* (Gautam *et al*., 2011). Bone marrow and lymph node cells from VL patients have also been demonstrated to simultaneously express IL-10 and IFN-γ transcripts, but after resolution of disease, the expression of IL-10 was decreased (Kumar and Nylén 2012).

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Studies of tissue cytokine mRNA expression revealed further a role for IL-10 in down-regulating the responses of CD4+ T-cell and IL-10 involvement in the pathology of VL (Mishra *et al*., 2015).

Studies have also examined the relationship between certain cytokine gene polymorphism and the susceptibility to and clinical severity of diseases, including VL (Hollegaard and Bidwell, 2006; Ulger *et al*., 2013). Accordingly, it has been suggested that susceptibility to leishmaniasis might be influenced by host genetic background and it is supposed that efficient activation of immune response, specifically the IFN-γ/IL-12 axis or IL-10 axis might play a key role in protection or progression of disease (Castellucci *et al*., 2012). Therefore, the present study aimed to determine the association between three SNPs of *IL10* gene and VL in Iraqi patients.

MATERIALS AND METHODS

Subjects

A total of 84 Iraqi Arab children (age range; 4 months to 12 years) were enrolled in the study. They were distributed as 44 visceral leishmaniasis (VL) patients and 40 apparently healthy controls. The patients were hospitalized cases and they were admitted to ten hospitals in Baghdad and Wasitduring the

period March 2013-February 2014. After a clinical examination of the patient by the medical staff at the hospitals, the serum was first screened for anti-VL antibodies by rapid immune-chromatographic strip test (Kalazar Detect™ Test kit: InBios International, USA), and if the test was positive the serum was further tested by indirect fluorescent antibody test (IFAT) for VL at the Central Public Health Laboratories.

Collection of Blood Samples

From each participating subject (patient and control), about 5 ml of venous blood were collected. The blood was distributed into two aliquots; the first was dispensed in a plain tube to collect serum, while the second aliquot was drawn in EDTA tube and stored at -20°C until DNA extraction. The serum was used for sero-diagnosis of VL and assessment of IL=10 level, while EDTA blood was used to extract DNA for the determination of *IL10* gene polymorphisms.

Serum Level of Cytokines

Serum level of IL-10 was determined by ELISA method using Abcam Cytokine kit (USA), which was designed for the quantitative measurement of IL-10in human sera, and instructions of manufacturer were followed.

Genomic DNA Extraction

Genomic DNA was extracted from the peripheral blood leukocytes (frozen EDTA blood samples) by Qiagen spin column technology (DNeasy blood kit: Qiagen, USA). The DNA concentration was measured by two methods. In the first, Nanodrop UV spectrophotometer was used; by which the optical density of DNA (2 µl) was measured at two wavelengths (260 and 280 nm).In most samples, DNA preparation gave A260/A280 ratio between 1.6 and 2.0, which was considered to be suitable for a further analysis in determining cytokine gene polymorphisms. The Nanodrop was also employed to assess the DNA concentration. After that, gel electrophoresis was used to confirm the existence of DNA in the samples (Kaur and Mehra, 2012).

Cytokine Gene Polymorphisms

The Cytokine CTS-PCR-SSP Tray Kit was used to determine the SNPs $IL10_{-1082,}$ $IL10_{-819}$ and$IL10_{-592}$. The PCR primers were prepared to identify alleles, genotypes. These primers were designed by the Department of Transplantation Immunology, University Clinic Heidelberg (Germany) according to the WHO international nomenclature committee of cytokines. The electrophoresis of PCR products was run for 20 minutes at 170 volts, and the patterns of observed bands of cytokine (alleles) were revealed according to internal control bands.

Statistical Analysis

Serum level of IL-10 was statistically analyzed using the computer programme SPSS (Statistical Package for Social Sciences) version 13. Their data were given as mean ± standard error (S.E.), and differences between means were assessed by ANOVA (Analysis of Variance), followed by LSD (Least Significant Difference) or Duncan test.. Allele frequencies of $IL10_{-1082,}$ $IL10_{-819}$ and$IL10_{-592}$SNPs were calculated by direct gene counting method, while significant departure from Hardy-Weinberg (H-W) equilibrium was assessed by Pearson's Chi-square test. Alleles and genotypes of SNPs were presented as percentage frequencies, and significant differences between their distributions in VL patients and controls were assessed by two-tailed Fisher's exact probability (P). In addition, relative risk (RR), etiological fraction (EF) and preventive fraction (PF) were also estimated to define the association between SNP alleles and genotypes with the disease (Ad'hiah, 1990). These estimations were calculated by using the WINPEPI computer programs for epidemiologists.

RESULTS

Serum Level of IL-10

Serum level of IL-10was significantly ($P \leq 0.001$) increased in VL patients compared to controls (40.02 ± 1.26 *vs*. 18.60 ± 1.82pg/ml).

Interleukin-10 Gene Polymorphisms

Hardy-Weinberg equilibrium analysis of the three investigated positions of *IL10* gene ($IL10_{-1082,}$ $IL10_{-819}$ and$IL10_{-592}$) revealed that their genotypes (GG, GA and AA; CC, CT and TT; and CC, CA and AA, respectively) were in a good agreement with the equilibrium, and no significant difference was observed between their observed and expected frequencies in VL patients and controls, with the exception of patients at position -819, in which a significant departure was observed. In addition, comparisons between patients to controls demonstrated no significant differences in the distribution of genotype and allele frequencies (Tables 1A, 1B, 2A, 2B, 3A and 3B).

Impact of$IL10_{-1082}$SNP on serum level of IL-10

The three genotypes of patients (GG, GA and AA) for $IL10_{-1082}$ showed a significant increased level of IL-10 ($P \leq 0.001$) compared to the corresponding genotypes in controls, while among each group (patients or controls), significant variations were observed. Among VL patients, The GG genotype recorded the highest level of IL-10 (45.73 ± 3.15pg/ml), but the difference was significant compared to AA genotype (38.02 ± 1.48pg/ml). In the case of controls, the AA genotype was observed with the highest mean (24.72 ± 3.04pg/ml), but the difference was significant compared to GA genotype (13.78 ± 1.74pg/ml) (Figure 1).

Impact of$IL10_{-819}$ SNP on serum level of IL-10

The three genotypes of VL patients (CC, CT and TT) for $IL10_{-819}$ showed a significant increased level of IL-10 ($P \leq 0.001$) compared to the corresponding genotypes in controls; however, these genotypes recorded approximated means in patients and no significant difference between them was observed. Among control, the TT genotype recorded the highest level of IL-10 (24.31 ± 3.95pg/ml), but the difference was significant compared to CT genotype (15.58 ± 2.86pg/ml) (Figure 2).

Table 1A. Observed numbers and percentage frequencies and Hardy-Weinberg (H-W) equilibrium of *IL10*$_{-1082}$genotypes and alleles in visceral leishmaniasis patients and controls

Groups			*IL10*$_{-1082}$Genotype or Allele					H-W P ≤
			GG	GA	AA	*G*	*A*	
Visceral Leishmaniasis Patients	Observed	No.	4	19	21	27	61	N.S.
(No.=44)		%	9.1	43.2	47.7	30.7	69.3	
	Expected	No.	4.1	18.7	21.2	Not Estimated		
		%	9.4	42.5	48.1			
Controls	Observed	No.	4	21	15	30	50	N.S.
(No. = 40)		%	10.0	52.5	37.5	37.5	62.5	
	Expected	No.	5.3	18.5	16.2	Not Estimated		
		%	13.1	46.2	40.7			

Table 1B. Statistical evaluations of associations between *IL10*$_{-1082}$genotypes or alleles and visceral leishmaniasis

IL10$_{-1082}$Genotype or Allele	Statistical Evaluations			
	Relative Risk	Etiological or Preventive Fraction	Fisher's Exact Probability	95% Confidence Intervals
GG	0.90	0.01	Not significant	0.21- 3.80
GA	0.69	0.16	Not significant	0.29- 1.61
AA	1.52	0.16	Not significant	0.64- 3.60
G	0.74	0.098	Not significant	0.39- 1.39
A	1.36	0.18	Not significant	0.72- 2.56

Table 2A. Observed numbers and percentage frequencies and Hardy-Weinberg (H-W) equilibrium of *IL10*$_{-819}$genotypes and alleles in visceral leishmaniasis patients and controls

Groups			*IL10*$_{-819}$Genotype or Allele					H-W P ≤
			CC	CT	TT	*C*	*T*	
Visceral Leishmaniasis Patients	Observed	No.	19	14	11	52	36	0.05
(No.=44)		%	43.2	31.8	25	59.1	40.9	
	Expected	No.	15.4	21.3	7.3	Not Estimated		
		%	34.9	48.4	16.7			
Controls	Observed	No.	19	15	6	53	27	N.S.
(No. = 40)		%	47.5	37.5	15	66.2	33.8	
	Expected	No.	17.6	17.9	4.5	Not Estimated		
		%	43.9	44.7	11.4			

Table 2B. Statistical evaluations of associations between *IL10*$_{-819}$genotypes or alleles and visceral leishmaniasis

IL10$_{-819}$Genotype or Allele	Statistical Evaluations			
	Relative Risk	Etiological or Preventive Fraction	Fisher's Exact Probability	95% Confidence Intervals
CC	0.84	0.08	Not significant	0.36- 1.97
CT	0.78	0.08	Not significant	0.32- 1.89
TT	1.89	0.12	Not significant	0.63- 5.62
C	0.74	0.18	Not significant	0.39- 1.37
T	1.36	0.11	Not significant	0.73- 2.54

Table 3A. Observed numbers and percentage frequencies and Hardy-Weinberg (H-W) equilibrium of *IL10*$_{-592}$genotypes and alleles in visceral leishmaniasis patients and controls

Groups			*IL10*$_{-592}$Genotype or Allele					H-W P ≤
			CC	CA	AA	*C*	*A*	
Visceral Leishmaniasis Patients	Observed	No.	18	20	6	56	32	N.S.
(No.=44)		%	40.9	45.5	13.6	63.6	36.4	
	Expected	No.	17.8	20.4	5.8	Not Estimated		
		%	40.5	46.3	13.2			
Controls	Observed	No.	20	16	4	56	24	N.S.
(No. = 40)		%	50.0	40.0	10.0	70.0	30.0	
	Expected	No.	19.6	16.8	3.6	Not Estimated		
		%	49.0	42.0	9.0			

Table 3B. Statistical evaluations of associations between *IL10*$_{-592}$genotypes or alleles and visceral leishmaniasis

IL10$_{-592}$Genotype or Allele	Statistical Evaluations			
	Relative Risk	Etiological or Preventive Fraction	Fisher's Exact Probability	95% Confidence Intervals
CC	0.69	0.15	Not significant	0.30- 1.62
CA	1.25	0.09	Not significant	0.53- 2.94
AA	1.42	0.04	Not significant	0.38- 5.36
C	0.75	0.18	Not significant	0.39- 1.43
A	1.33	0.09	Not significant	0.70- 2.53

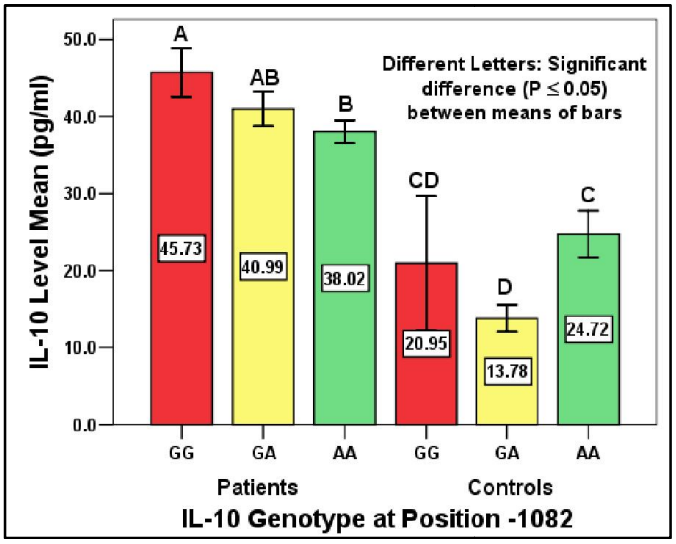

Figure 1. Serum level of IL-10 in visceral leishmaniasis patients and controls distributed by *IL10*$_{-1082}$ genotypes

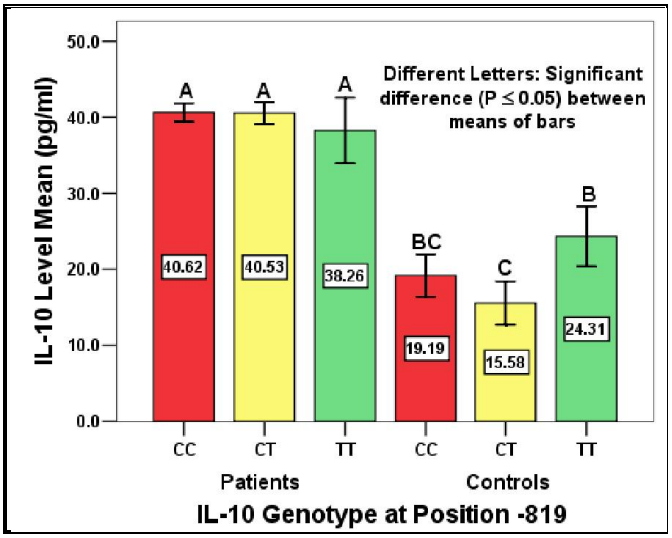

Figure 2. Serum level of IL-10 in visceral leishmaniasis patients and controls distributed by *IL10*$_{-819}$ genotypes

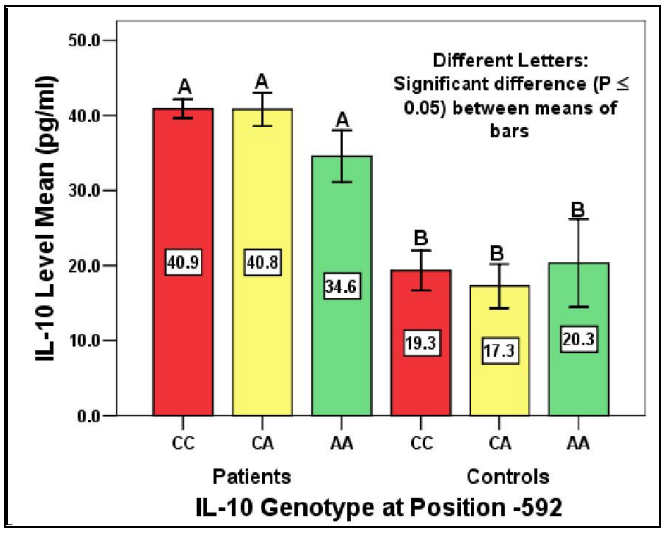

Figure 3. Serum level of IL-10 in visceral leishmaniasis patients and controls distributed by *IL10*$_{-592}$ genotypes

Impact of *IL10*$_{-592}$ SNP on serum level of IL-10

The three genotypes of VL patients (CC, CA and AA) for *IL10*$_{-592}$ showed a significant increased level of IL-10 ($P \leq 0.001$) compared to the corresponding genotypes in controls; however, neither patients nor controls demonstrated a significant difference between the means of IL-10 in their genotypes (Figure 3).

DISCUSSION

IL-10 is an important cytokine of Treg cells involved in the immune response to *Leishmania*, and it has been demonstrated that this cytokine can suppress Th1 immune responses with the consequent effect on macrophage activation (Couper *et al*., 2008). Interleukin-10 is encoded by a gene located on chromosome 1 at position 1q31.32. Three SNPs has been found on the promoter region of *IL10* gene; *IL10*$_{-1082}$ G/A, *IL10*$_{-819}$ C/T and *IL10*$_{-592}$ C/A. These SNPs exhibited a strong effect on the transcription of IL10 gene (Gambhir *et al*., 2009; Sofian*et al*., 2013). In the upstream promoter region of the *IL10* gene, the two linked bi-allelic SNPs at the -819 and -592 positions have also been involved in leishmaniasis. Genetic analysis of *IL10*$_{-819}$ in patients infected with *L. braziliensis* showed that the *C* allele was associated with increased lesions in the skin. Functional analysis of the polymorphism revealed that CC genotype was associated with increased levels of IL-10 than CT and TT genotypes. The authors demonstrated an important role for IL-10 in skin lesions in *L. braziliensis* infected patients, and identified circulating monocytes and Treg cells as principal sources of IL-10 in these patients (Salhi *et al*., 2008). A further study investigated the possible role of IL10 gene polymorphisms at three positions and development of Post-kala-azar dermal leishmaniasis (PKDL), but there was no evidence for an association between the three SNPs of *IL10* gene and susceptibility to PKDL in a Sudanese ethnic group (Masalit), although some evidence for haplotype association was observed (Farouk *et al*., 2010). The *IL10*$_{-819}$SNP was also evaluated in Iranian VL patients, and results revealed that such polymorphism was significantly associated with VL, and the genotype CT was significantly increased in patients. The data suggested that polymorphisms at the *IL10*$_{-819}$can influence VL susceptibility and CT might be considered as a risk genotype for the disease (Hajilooi *et al*., 2013).

In the present study, the three investigated SNPs in the promoter region of *IL10* gene have been suggested to be associated with alteration in the expression *IL10* gene and exhibited a strong effect on its transcription (Girnita *et al*., 2008). Furthermore, IL-10 is a key down-regulator of immune response against *Leishmania* through inhibiting Th1 functions (Couper *et al*., 2008), and the present study results were in agreement with such scope and demonstrated that IL-10 serum level was significantly increased during VL. However, genotyping for the three SNPs revealed that neither genotypes nor alleles of IL10 genes seemed to have an association with VL in Iraqi patients. Similarly, Al-Bashier, (2014) reported that there were no significant differences in the frequency of *IL10*$_{-1082}$ genotypes and alleles between Iraqi VL patients and controls. A further study from Sudan also did not find any association between *IL10*$_{-1082}$, *IL10*$_{-819}$ and *IL10*-$_{592}$ gene

polymorphisms and PKDL in Sudanese patients (Farouk *et al.*, 2010). In contrast, Two Iranian studies reported significant differences at positions *IL10*$_{-1082}$ (G/A) and *IL10*$_{-819}$ (C/T); in which the heterozygous genotype for the two positions was positively associated with VL, and the authors concluded that the two genotypes may influence VL susceptibility and considered them as a risk factor for the disease (Hajilooi *et al*., 2013; Hajilooi *et al*., 2014).

When the genotype impact on serum level of IL-10 was investigated, *IL10*$_{-1082}$ GG genotype was associated with higher serum level, while in Iranian VL patients; AG genotype recorded the highest level of IL-10 (Hajilooi *et al*., 2014). At the other two positions (*IL10*$_{-819}$ and *IL10*$_{-592}$), no significant variations were observed regarding the distribution of IL-10 serum level in their genotypes of patients; however, Salhi *et al*. (2008) reported a conflicting result, in which the CC genotype showed the highest level

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