

Interleukin-12B gene polymorphism and visceral Leishmaniasis in Iraqi patients

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Abstract

The present study aimed to determine the role of *IL12₋₁₁₈₈* genetic polymorphism in susceptibility or resistant to visceral Leishmaniasis (VL) by studying single nucleotide polymorphism (SNP) in the gene of *IL12B* at position -1188 in 44 Iraqi VL patients and 40 healthy controls. IL-12 serum level showed no significant difference between patients and controls (5.33 ± 3.26 vs. 2.17 ± 0.36 pg/ml). In addition, SNP analysis revealed that neither genotypes nor alleles of *IL12B₋₁₁₈₈* revealed a significant variation between VL patients and controls. To determine the impact of *IL12B₋₁₁₈₈* genotypes on IL-12 serum level, VL patients and controls were distributed according to their serum level in the three genotypes of this cytokine. It was found that *IL12₋₁₁₈₈* CC genotype demonstrated a significant increased level of IL-12 (26.16 ± 19.76 pg/ml) in patients compared to CA and AA genotype (1.35 ± 0.35 pg/ml and 1.48 ± 0.23 pg/ml respectively) of patients.

Keywords: Visceral leishmaniasis, Interleukin-12, Single nucleotide polymorphism

1. Introduction

Leishmaniasis is one of the vector-borne diseases caused by obligate protozoan parasites of the genus *Leishmania*. They are transmitted by different species of sand flies belong the genus *Phlebotomine* as extracellular flagellated promastigotes that replicate as intracellular parasite (aflagellate amastigotes) in mononuclear cells of mammalian hosts (Bates 2007). The disease ranges from asymptomatic self-healing infection to localized lesions in the skin, but can also develop into a progressive life-threatening visceral form of disease (Griensven & Diro 2012). It is one of the important parasitic diseases, affecting mainly low social class peoples in developing countries, and is more prevalent and endemic in the tropical and subtropical regions of Africa, Asia, the Mediterranean, Southern Europe (old world) and South and Central America (new world) (Alvar *et al.* 2012).

The protective immunity against *L. donovani* has been suggested to be dependent on IL-12-driven Th1 response and production of IFN- γ ; thus an induction of parasite killing can result by macrophages primarily via reactive nitrogen and oxygen intermediates production (Shio *et al.* 2012). A further effect of IL-12 was associated with a decrease of spontaneous or antigen-induced peripheral blood mononuclear cell (PBMC) apoptosis in VL patients, and using IL-12 in combination with *Leishmania* antigens *in vitro* restored PBMC proliferation in VL patients more efficiently than the use of anti-IL-4 or anti-IL-10 monoclonal antibodies (Singh *et al.* 2012).

A number of studies have investigated the role of genetic polymorphisms at candidate immune response genes that are associated with pro- and anti-inflammatory responses (i.e. cytokine genes) in regulating the clinical outcome of leishmaniasis in human (Hajilooi *et al.* 2013). The *IL12B₋₁₁₈₈* SNP has been shown to influence the production of IL-12 or protein expression, and it has also been associated with diseases that are Th1-mediated immune responses (Liu *et al.* 2012). This polymorphism has not been investigated in VL, while in CL, there has been one study that showed no significant variation between the *IL12B₋₁₁₈₈* genotypes or alleles in Brazilian patients (de Jesus Fernandes Covas *et al.* 2013).

2. Materials and Methods

2.1 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 Wasit during 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.

2.2 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-12 level, while EDTA blood was used to extract DNA for the determination of *IL12B* gene polymorphism.

2.3 Serum Level of IL-12

Serum level of IL-12 was determined by ELISA method using Abcam Cytokine kit (USA), which was designed for the quantitative measurement of IL-12 in human sera, and instructions of manufacturer were followed.

2.4 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 μ l) 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 & Mehra 2012).

2.5 Cytokine Gene Polymorphisms

The Cytokine CTS-PCR-SSP Tray Kit was used to determine the SNP *IL12B*₋₁₁₈₈. 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.

2.6 Statistical Analysis

Serum level of IL-12 was statistically analyzed using the computer programme SPSS (Statistical Package for Social Sciences) version 13. Their data were given as mean \pm 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 *IL2*₋₁₁₈₈ 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 the SNP 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 the SNP alleles and genotypes with the disease (Ad'hiah 1990). These estimations were calculated by using the WINPEPI computer programs for epidemiologists.

3. Results

3.1 Serum Level of Cytokines

Serum level of IL-12 was increased in VL patients compared to controls (5.33 ± 3.26 vs. 2.17 ± 0.36 pg/ml), but the difference failed to attend a significant level ($P > 0.05$).

3.2 Interlukin-12B Gene Polymorphisms

Genetic polymorphism of *IL12B* gene was investigated at the position -1188 (*IL12B*₋₁₁₈₈), which was presented with three genotypes (CC, CA and AA). A significant difference ($P \leq 0.05$) was noticed between the observed and expected *IL12B*₋₁₁₈₈ genotype frequencies (i.e. departure from H-W equilibrium) in VL patients, while no such departure was observed in controls. In addition, no significant variation between VL patients and controls was observed in these genotype or allele frequencies (Tables 1 and 2).

Table 1. Observed numbers and percentage frequencies and Hardy-Weinberg (H-W) equilibrium of *IL12B*₋₁₁₈₈ genotypes and alleles in visceral leishmaniasis patients and controls.

Groups			<i>IL12B</i> ₋₁₁₈₈ Genotype or Allele					H-W P \leq
			CC	CA	AA	C	A	
Visceral Leishmaniasis Patients (No.=44)	Observed	No.	7	13	24	27	61	0.05
		%	15.9	29.5	54.6	30.7	69.3	
	Expected	No.	4.2	18.7	21.1	Not Estimated		
		%	9.4	42.5	48.1			
Controls (No. = 40)	Observed	No.	3	10	27	16	64	N.S.
		%	7.5	25.0	67.5	20	80	
	Expected	No.	1.6	12.8	25.6	Not Estimated		
		%	4.0	32.0	64.0			

Table 2. Statistical evaluations of associations between *IL12B*₋₁₁₈₈ genotypes or alleles and visceral leishmaniasis.

<i>IL12B</i> ₋₁₁₈₈ Genotype or Allele	Statistical Evaluations			
	Relative Risk	Etiological or Preventive Fraction	Fisher's Exact Probability	95% Confidence Intervals
CC	2.33	0.09	Not significant	0.57- 9.56
CA	1.26	0.06	Not significant	0.48- 3.26
AA	0.58	0.29	Not significant	0.24- 1.39
C	1.77	0.13	Not significant	0.87- 3.59
A	0.56	0.34	Not significant	0.28- 1.14

3.3 Impact *IL12B*₋₁₁₈₈ SNP on serum level of IL-12

The *IL-12*₋₁₁₈₈CC genotype in VL patients recorded the highest mean of IL-12 (26.16 ± 19.76 pg/ml), and the difference was significant compared to all other genotypes in patients or controls (Figure 1).

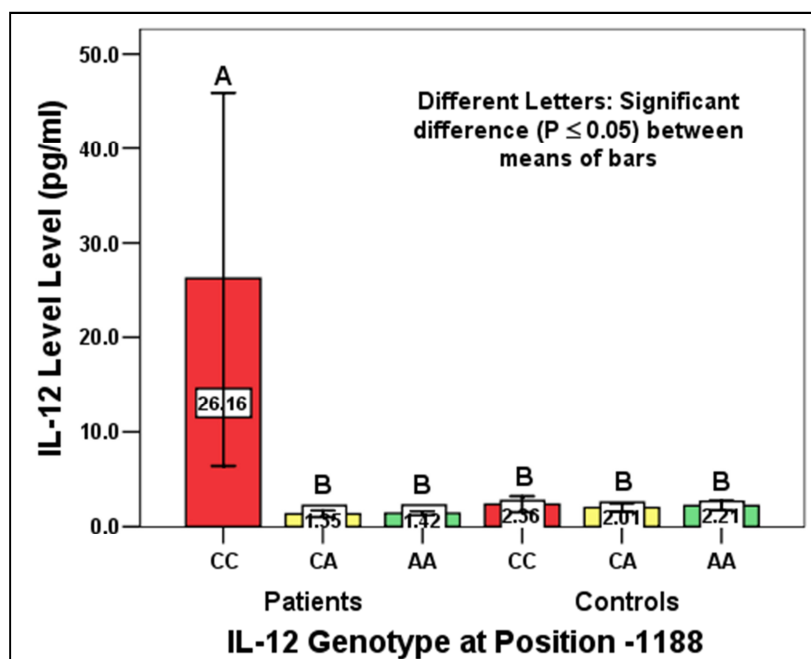


Figure 1. Serum level of IL-12 in visceral leishmaniasis patients and controls distributed by *IL12*₋₁₁₈₈ genotypes.

4. Discussion

Immune responses of the Th1 are characterized by IFN- γ and IL-2, which is the signature cytokine of Th1 immune response that act on macrophages, NK cells and B cells, and such effects are considered critical for Leishmania infection control (Bhattacharya & Ali 2013). The resistance in VL involves CD4+ and CD8+ T cells when Th1 responses exert their protective effects through synthesizing IL-2, IFN- γ and IL-12. In contrast, Th2 responses and through their cytokines (IL-4 and IL-13) are associated with susceptibility or persistence of infection, in addition to the role of IL-10, which is linked to Treg cell activation. Therefore, the cellular immune response in VL patient has Th1 responses that tend to limit the disease, while Th2 and Treg response are associated with disease progression (Kurkjian *et al.* 2006; Costa *et al.* 2012; Kamil *et al.* 2013). Caldas *et al.* (2005) confirmed further that the circulating levels of IL-12 and IFN- γ were elevated in VL patients, and such elevation was correlated with the active stage of disease.

Single nucleotide polymorphisms have been reported to control cytokine gene expression, and exacerbated cytokine response in some individuals might be genetically controlled (Medina *et al.* 2011). Further data suggested that cytokine SNPs can be considered as candidate markers of susceptibility and severity of VL, and can also influence disease resistance (Stanley & Engwerda 2007; de Jesus Fernandes Covas *et al.* 2013).

Interleukin-12 is a heterodimeric cytokine with a molecular weight of 70 kDa that is composed of two disulfide linked polypeptide chains: IL-12p35 (35 kDa) and IL-12p40 (40 kDa), which are encoded by *IL12A* and *IL12B* genes, respectively, but many studies have focused mainly on 3'UTR, at position -1188 A/C (*IL12B*₋₁₁₈₈) of *IL12B* gene, which is located on chromosome 5 at position 5p31-33.2 (Liu *et al.* 2012). IL-12 functions as the main physiological inducer of IFN- γ by activating T cells and promoting Th1-type CD4+ T cell

differentiation, and therefore plays an important role in inducing cell-mediated protection in response to Leishmania infection (Cummings *et al.* 2010). In this study, despite of the associated increased risk for VL with *IL12B*-1188 genotype CC (RR = 2.33) and C allele (RR = 1.77) that may shape a positive association in Iraqi patients, the differences failed to attend any significant level. de Jesus Fernandes Covas *et al.* (2013) also did not find a significant association between genotypes and alleles of *IL12B*-1188 and Mucocutaneous leishmaniasis (MCL) in Brazilian patients. However, the present study pointed out that *IL12B*-1188 CC genotype recorded the highest level of IL-12 in VL patients; an observation that may highlight the role of such SNP in pathogenesis of VL, because the other two genotypes (CA and AA) were observed with very low serum level of IL-12 compared to CC genotype, and both genotypes accounted for about 85% of VL cases. Therefore, most VL cases actually were presented with low IL-12 level, and this may explain the progression of disease in these cases, because immunity against *Leishmania* has almost been regarded to be dependent on IL-12-driven Th1 response (McCall *et al.* 2013). However, further investigations are certainly required to confirm these findings.

5. Conclusion

Accordingly, the presented findings promoted to reach the gene polymorphisms of *IL12B*-1188 might have no role in VL susceptibility, or a protection against disease development, but in contrast the *IL12*-1188 CC SNP could be involved in disease pathogenesis through influencing the amount of cytokine released in the patients; although it could not prevent the disease development.

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