



## Is the Polymorphism at Position -1082 of IL-10 Gene Associated with Visceral Leishmaniasis?

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(Received 14 Feb 2014; accepted 16 May 2014)

### Abstract

**Background:** Immune responses play critical roles in the leishmaniasis eradication. IL-10 is a key regulator of immune responses, and the polymorphisms within its promoter region are associated with alteration in its expression. Therefore, this study was designed to examine the correlation between polymorphism at the -1082 position of the IL-10 gene and visceral leishmaniasis (VL).

**Methods:** The IL-10 -1082 polymorphism and anti-*Leishmania* antibody titration were examined in 110 patients with clinical presentation of VL and seropositive for the *Leishmania* (group 1), 74 seropositive patients but without clinical presentation (group 2) and 113 healthy controls (group 3) using the PCR-RFLP and immunofluorescence techniques, respectively.

**Results:** The polymorphism at IL-10 -1082 (A/G) position was significantly associated with VL and A/G genotype was significantly higher in VL patients when compared to the groups 2 and 3 ( $P < 0.001$ ). However, the results demonstrated that the A and G alleles were not associated with VL ( $P = 0.263$ ).

**Conclusions:** Previous investigations have shown that the polymorphism at the -1082 position of the IL-10 gene can influence its expression and also it has been proved that IL-10 level was increased during VL. Our results suggest that the A/G genotype may be considered as a risk factor for VL.

**Keywords:** IL-10, Polymorphism, Visceral leishmaniasis

### Introduction

Several species of *Leishmania* are responsible for leishmaniasis (1) with several clinical presentations including cutaneous, mucocutaneous and visceral leishmaniasis (VL) (2). Although, it has been reported that VL is sporadic in Iran, but in north-western and southern areas of Iran, the disease is endemic (3) and approximately 100–300 new cases of VL are detected annually (3).

Impaired immune responses against the *Leishmania* parasite is the main reason for distribution of the parasite and production of VL form, also known as Kala Azar (4). Thus, it seems that the host immune responses against the leishmaniasis determine the infection outcome (5). IL-10, as the main anti-inflammatory cytokine, plays important roles in the regulation/suppression of immune responses, es-

pecially during chronic infections including VL (6, 7). Serum levels of IL-10 were increased in VL (8, 9), hence, it can be hypothesized that the factors, such as genetic variations, which lead to alteration in IL-10 expression, can affect the outcome of leishmaniasis. The association of a single nucleotide polymorphism (SNP) within promoter region at -1082 position of the IL-10 gene (rs1800870) with altered IL-10 expression has been reported by investigators (10). Therefore, it can be suggested that the IL-10 -1082 polymorphism may be associated with chronic infections including VL. Therefore, this study was designed to evaluate the IL-10 -1082 polymorphism in the seropositive VL patients in comparison to seropositive and negative healthy controls.

## Material and Methods

### Subjects

In this cross-sectional study, the participants were divided to three groups including; patients with clinical presentation of VL and seropositive for the *Leishmania* (group 1 consisting of 110 patients), seropositive patients but without clinical presentation (group 2 consisting of 74 patients) and, healthy controls (group 3 consisting of 113 people). Table 1 presents the demographic data of the defined groups including mean age and sex ratios. VL was diagnosed by an expert specialist according to medical history, clinical presentations including; severe anemia, fever, cough, diarrhea, vomiting, fatigue, weakness, and appetite loss and finally laboratory findings. All of the participants in the present study were selected during 2004 to 2011 from Azar City (North-West of Iran) where the *L. infantum* is endemic (11, 12). An informed consent form was filled out by the participants prior to sample collection and the Ethical Committee of the Hamadan University of Medical Sciences has approved the study protocol.

### DNA extraction

DNA was extracted using a commercial kit from Bioneer company (South Korea) according to the manufacture's instruction.

### Polymorphism detection

The PCR-RFLP technique was used for evaluation of the IL-10 -1082 gene polymorphism. PCR was performed in a final volume of 50  $\mu$ L containing; 2  $\mu$ L of Taq DNA polymerase buffer (10x), 5 units of Taq DNA polymerase, 1  $\mu$ L of each dNTP (dATP, dCTP, dGTP and dTTP in 10 mM concentration), 4  $\mu$ L of each primer (25 ng/ $\mu$ L), 5  $\mu$ L of 1.5 mM MgCl<sub>2</sub>, 2.5  $\mu$ L of extracted DNA and sterile double distilled DNase free water. The forward primer sequence was 5'-GACAA-CACTACTAAGGCTCCTTTGGGA-3' and the reverse primer sequence was 5'-TGAG-CAAACTGAGGCACAGAAAT-3'. The following program was used for PCR amplifications: one cycle 95°C for 5 min (denaturation) and then 35 cycles of 30 sec at 95°C, 53°C for 30 sec and 72°C for 40 sec using a thermal cycler (Bioneer, South Korea). The *Bs*II restriction enzyme (Fermentase, Finland) was used to digest the amplicon (315 bp). Upon digestion of the PCR product with *Bs*II, in the case of G allele, resulted into three fragments of 25, 37 and 253 bp and in the case of A allele led to 37 and 278 bp. The products were analysed by electrophoresis on a 2.5% ethidium bromide pretreated agarose gel (Cinnaclon, Iran).

### Immunofluorescence assay

The titration of anti-*Leishmania* antibody was examined using a commercial kit (Qiagen, USA) according to the manufacturer's guidelines. Briefly, the serum samples were diluted to 1/20, 1/40, 1/80, 1/160 and 1/320 and the positive indirect fluorescent antibody test at each diluents was considered as the titration of anti-*Leishmania* antibody. The cutoff of the test has been calculated as follow: Mean titration of positive sample + mean titration of negative sample /2.

### Statistical Analysis

Hardy-Weinberg equilibrium analysis was used to confirm the validity of the raw data. SPSS statistical software (version 18, SPSS, Chicago, IL, USA) was used for data analysis and a *P* value of < 0.05 was considered significant. The association of SNP with disease and the calculation of odds

ratios (ORs), with 95% confidence intervals (CIs) was evaluated using  $\chi^2$  analysis. One-Way ANOVA test was used to analysis the differences of quantitative data between groups.

The genotype distributions between the VL patients were compared to participants without VL but seropositive and also to healthy controls by a logistic regression model controlling for age and sex. Akaike information criterion (AIC) was estimated for three analysis models to select the appropriate model for this study using stepwise model selection (SAS) (13).

## Results

IL-10 -1082 polymorphism was significantly associated with VL ( $P < 0.001$ ). The results demonstrated that the IL-10 -1082 A/G genotype was significantly increased in group 1 when compared to either group 2 or 3 (Table 2 and 3).

The statistical analysis revealed that the prevalence of IL-10 -1082 A and G alleles were not significantly differ among the groups ( $P = 0.263$ ) (Table 2 and 3).

The results also showed that the VL patients (group 1) ( $P = 0.783$ ) and participants in group 2 ( $P = 0.065$ ) carrying various IL-10 -1082 genotypes did not differ regarding the titration of anti-*Leishmania* antibodies (Table 4). The statistical analysis also demonstrated that the differences between the groups regarding the mean age and sex ratio were not significant (Table 1).

In order to show alternative effects of the variants among the four models including co-dominant, dominant, recessive and additive, a model analysis was performed.  $P$ -values and ORs of genotypes for various models are presented in Table 3. The results revealed that the best fit model was the co-dominant model based on AIC values favor.

**Table 1:** Demographic data of study population at entry

Group	Group 1	Group 2	Group 3	P-value
Age (yr)	20.52 ± 1.67	21.73 ± 1.93	22.54 ± 1.19	0.624
Sex				0.723
Male	60 (54.5)	40 (54.1)	56 (49.6)	
Female	50 (45.5)	34 (45.9)	57 (50.4)	

Group1: patients who are seropositive, Group2: seropositive healthy controls, Group3: seronegative healthy controls  
Data are presented as mean ± SEM or n (%).

**Table 2:** The prevalence of the A/A, A/G and G/G genotype as well as A and G alleles within IL-10 -1082 position in the study population

Genotypes	No. (%) of variant genotype			P-value
	Group 1 (n=110)	Group 2 (n=74)	Group 3 (n=113)	
G/G	8 (7.3)	11 (14.9)	24 (21.2)	< 0.001
A/G	102 (92.7)	55 (74.3)	87 (77.0)	
A/A	0	8 (10.8)	2 (1.8)	
Alleles				
A	102 (46.4)	71 (48.0)	91 (40.3)	0.263
G	118 (53.6)	77 (52)	135 (59.7)	

Group1: patients who are seropositive, Group2: seropositive healthy controls, Group3: seronegative healthy controls.

**Table 3:** Polymorphism association with for four different models including Full genotype, Dominant, Recessive, and Multiplicative (for alleles)

Model	Genotype	Group 1 n (%)	Group 2 n (%)	Group 3 n (%)	P-value	Group 1 vs. Group 2		Group 1 vs. Group 3	
						OR (95% CI)	AIC	OR (95% CI)	AIC
Full genotype	G/G	8. (7.3)	11. (14.9)	24. (21.2)	< 0.001	1.00	235.22	1.00	302.81
	A/G	102. (92.7)	55. (74.3)	87. (77.0)		2.55 (0.97-6.71)		3.52 (1.50-8.23)	
	A/A*	0	8. (10.8)	2. (1.8)		*-		*-	
Dominant	G/G	8. (7.3)	11. (14.9)	24. (21.2)	0.012	1.00	249.30	1.00	303.89
	A/G+A/A	102. (92.7)	63. (85.1)	89. (78.8)		2.23 (0.85-5.83)		3.44 (1.47-8.04)	
Recessive	G/G+A/ G	110. (100)	66. (89.2)	111. (98.2)	< 0.001	1.00	-	1.00	-
	A/A*	0	8. (10.8)	2. (1.8)		*-		*-	
Multiplicative	G	53. (48.2)	27. (36.5)	44. (38.9)	0.214	1.00	499.89	1.00	620.52
	A	57. (51.8)	47. (63.5)	69. (61.1)		0.94 (0.62-1.42)		1.29 (0.88-1.87)	

Group1: patients who are seropositive, Group2: seropositive healthy controls, Group3: seronegative healthy controls \*Computing OR (95% CI) was not possible because of small sample size.

**Table 4:** The titration of anti-leishmania antibody in group 1 and 2 carrying various IL-10 -1082 genotypes

	Anti- <i>Leishmania</i> antibody titration	
	Group 1	Group 2
IL-10 -1082 A/A genotype	2/9±0/2	2/75 ± 0.16
IL-10 -1082 A/G genotype	3/17 ± 0.05	3.1 ± 0.17
IL-10 --1082 G/G genotype	3/1 ± 0.12	3.00 ± 0.46
P value	P= 0.78	P= 0.06

## Discussion

It has been documented that IL-10 up-regulation in response to infectious diseases, including leishmaniasis, leads to impaired immune responses and hence, induces chronic forms of the diseases (6). Moreover, previous studies demonstrated that IL-10 plays crucial roles in the suppression of immune responses against *Leishmania* (8). Accordingly, Bhattacharya and colleagues reported that serum levels of IL-10 were increased in VL patients (9). Elevated serum levels of IL-10 in Indian VL patients were reported by Ganguly and colleagues (14). Saha et al. also showed that IL-10 results in susceptibility to VL and post-kala-azar dermal leishmaniasis (15). Interestingly, IL-10 receptor blockade immunotherapy also led to enhanced immune responses against VL (16). Furthermore, several studies revealed that treatment

with anti-*Leishmania* drugs resulted in decreased serum (17-19) and mRNA (20-22) levels of IL-10. These data proposed that IL-10 as a part of immune responses participates in the pathogenesis of *Leishmania* and its inappropriate production may be a risk factor for clearance of the parasite.

Another study demonstrated that IL-10 -1082 A/G genotype creates new binding sites for nuclear transcription factors and resulted in up-regulation of IL-10 in patients suffering from cutaneous leishmaniasis (23).

IL-10 -1082 A/G genotype was significantly increased in group 1 in comparison to groups 2 or 3. Thus, it may be concluded that the higher prevalence of IL-10 -1082 A/G genotype in Iranian VL patients may be considered as a risk factor which may lead to impaired immune responses against *Leishmania*. There are not yet adequate studies to describe why the IL-10 -1082 A/G genotype may

be responsible for increased expression of IL-10 or why individuals with the A/G genotype would produce more IL-10 than persons with A/A or G/G. It may be hypothesized that a heterozygotic patient may carry epigenetic events which result in increased the efficiency of the promoter but this cannot currently be identified. However, previous investigations reported that epigenetic factors such as micro-RNAs are able to alter cytokine expressions including IL-10 (24). For instance, let-7c, as a miRNA, can regulate IL-10 expression in an in vitro condition (24). Additionally, previous studies showed that there are several polymorphisms within promoter region of IL-10 which may alter IL-10 expression (25), hence, it seems that several genetic and epigenetic factors are involved in the regulation of IL-10 expression and evaluation of their status in VL patients can be considered for future investigations.

To the best of our knowledge, the current study is the first investigation which evaluated IL-10 -1082 gene polymorphism in VL patients in comparison to healthy controls. Our previous study on another polymorphism within -819 position of IL-10 gene also revealed that this polymorphism was significantly associated with VL (26). Another study on IL-10 -819 polymorphism have shown that this polymorphism was not associated with development of post kala-azar dermal leishmaniasis (27). De Jesus also revealed that the IL-10 -819 polymorphism was not associated with cutaneous outcome (28).

## Conclusion

Based on these studies and also the present study, it may be hypothesized that the IL-10 -1082 and -819 polymorphism can be associated with VL incidence in Iranian population and IL-10 -1082 A/G genotype can be considered as a risk factor for VL. Our results also demonstrated that the VL patients as well as participants in group 2 with various IL-10 -1082 genotypes did not differ regarding anti-*Leishmania* antibodies; hence, it appears that the -1082 polymorphism in IL-10 gene is not associated with production of anti-*Leishmania* antibody.

## Ethical considerations

Ethical issues (Including plagiarism, Informed Consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

## Acknowledgments

This project was supported by a grant from the Hamadan University of Medical Sciences. The authors declare that there is no conflict of interests.

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