

## Original Research Article

# Impact of IFN- $\gamma$ (+874T/A) and IL-10 (-1082G/A) on the Susceptibility to Visceral Leishmaniasis

Nada M. T. Al-Bashier\*

Al-Nahrain University/College of Medicine/ Medical Research Unit, Baghdad, Iraq

\*Corresponding author

## ABSTRACT

### Keywords

Visceral leishmaniasis; IL-10 (-1082G/A); IFN- $\gamma$  (+874T/A); SNP; ARMS-PCR.

Visceral leishmaniasis (VL) is a worldwide parasitic disease in which host immune response plays a critical role in the course of infection. Single nucleotide polymorphism (SNP) in IL-10 and IFN- $\gamma$  genes can influence the whole immune response and then the susceptibility of the host to this disease. This study aimed to investigate the association of two SNPs (IFN- $\gamma$  (+874T/A) and IL-10 (-1082G/A)) with incidence of VL in Iraqi patients. Sixty-seven patients with visceral leishmaniasis (VL) and 40 healthy control subjects were investigated for IL-10 (-1082G/A) and IFN- $\gamma$  (+874T/A) SNPs by Amplification Refractory Mutation System-Polymerase Chain Reaction (ARMS-PCR) method followed by agarose gel electrophoresis. Heterozygous genotype (AT) and allele A of IFN- $\gamma$  (+874T/A) have significant association with VL (OR=0.201, 95%CI=0.041-0.988 and OR=2.547, 95%CI=1.294-5.013 respectively), whereas neither mutant variant (heterozygous AG and homozygous GG) nor mutant allele (G) of IL-10 (-1082G/A) seem to have an association with this disease. IFN- $\gamma$  (+874T/A) but not IL-10 (-1082G/A) may have an association with the VL.

## Introduction

Leishmaniasis is a parasitic disease caused by hemoflagellate protozoan belonging to the genus *Leishmania*, of which nearly 24 species are pathogenic to human (1). The visceral form, kala-azar, is a major public health disaster with an estimated 200 million people worldwide at a risk, and an annual incidence of 500000 cases (2).

In order for *Leishmania* to develop a successful host-parasite relationship, the

parasite must evade both innate and adaptive immune response (3). Protective immune response against *L. donovani* is dependent on an IL-12-driven type 1 response and IFN- $\gamma$  production which results in induction of parasite killing by macrophages primarily via the production of reactive nitrogen and oxygen intermediate. On the other hand, IL-10 seems to represent the main macrophage-deactivating cytokine(3), and hence it aids in the progression of the disease.

Interferon- $\gamma$  is a Th1 cytokine involved in the activation of cellular immunity, particularly cytotoxic CD8+ T cells (4). Many studies demonstrated an association between the SNP in the first intron (+874A/T) of the IFN- $\gamma$  gene with different diseases such as pulmonary tuberculosis (5) and cutaneous leishmaniasis.

Interleukin 10 (IL-10) is a pleiotropic and potent immune-regulatory Th<sub>2</sub> cytokine that inhibits the production of pro-inflammatory cytokines by inhibition of Th<sub>1</sub> lymphocytes and stimulation of B and Th<sub>2</sub> lymphocytes. It is produced mainly by activated macrophages, dendritic cells, and B-lymphocytes.

Three single nucleotide polymorphisms (SNPs) in the IL-10 promoter region at position -819 (C $\square$ T), -592(C $\square$ A), and -1082(G $\square$ A) have been reported to influence the *IL-10* gene expression and to be associated with different diseases. Indeed, -1082(G) variant is associated with increased the production of IL-10 from T cells and monocytes. To our knowledge, there is no previous study highlighted the impact of polymorphisms in IFN- $\gamma$  and IL-10 genes on the parasitic diseases in Iraq. So this study aimed to investigate the association of certain SNPs in these genes with the VL.

## Materials and Methods

### Subjects

A total of sixty-seven ( $\square$ 1-7 years old, mean 4 $\pm$ 1.12; 41 males and 26 females) patients with VL were enrolled in this study from Al-Kadhimiya Teaching Hospital, and Central Pediatric Teaching Hospital/Baghdad during the period from

October, 2009 to March, 2012. Other 40 genetically unrelated, apparently healthy ( $\square$ 1-9 years old, mean 5 $\pm$ 2.11; 27 males and 13 females) children who reside in the same geographical area and visited these hospitals for routine vaccinations or for non-infectious cases such as fractures and anomalies were recruited as control subjects.

From each participant, 3 ml of venous blood were collected in EDTA tubes and kept at -20C until be used. A structural questionnaire was used to elicit information on epidemiological factors such as age, sex, and residency from the parents of the participants.

### DNA extraction and genotyping

DNA was extracted from each blood sample using ready kit (gSYNC<sup>TM</sup> DNA Mini Kit Whole Blood Protocol/ Geneaid/ Korea) according to the manufacturer's instructions. ARMS-PCR was used for amplification and genotyping of both SNPs. For IFN- $\gamma$  (+874T/A), allele T was amplified with forward sense primer T 5'-TTCTTACAACACAAAATCAAATCT-3', allele specific sense primer A 5'-TTCTTACAACACAAAATCAAATCA-3', and common antisense primer 5'-TCAACAAAGCTGATACTCCA-3'.

Amplification yielded 263-bp by PCR product. Human growth factor hormone primers were included in each PCR reaction as internal control with forward primer: 5'-GCCTTCCCAACCATTCCCTTA-3' and reverse primer 5'-TCACGGATTTCTGTTGTTTC-3' with 408-bp PCR product

For IL-10 (-1082G/A), allele A was amplified with forward sense primer A(5'-AACACTACTAAGGCTTCTTTGGGTA-

3'); while allele G was amplified with the primer G (5'-AACACTACTAA GGCTTCTTTGGGTG-3'). The common antisense primer (5'-GTAAGCTTCTG TCGCTGGTGGAGTC-3') in both reactions. These reactions amplify allele specific sequence of 161 bp of the promoter region of IL-10. Internal control primers amplifying a 796-bp fragment from the third intron of the HLA-DRB1 gene were included in each reaction. The forward primer: 5'-TGCCAAGTGGAGCACCCAA-3', and the reverse primer: 5'-GCATCTTGCTCTGTGCAGAT-3'. Ten  $\mu$ L template DNA from each sample and primers (5  $\mu$ L from each) were added to each master-mix tube (50  $\mu$ L PCR master-mix, Bioneer/Korea). The cycling conditions for IFN- $\gamma$  (+874T/A) were as follows:  $\therefore$  an initial denaturation at 95  $^{\circ}$ C for 1min, followed by 10 cycles of 95  $^{\circ}$ C for 15s, 62  $^{\circ}$ C for 50s and 72  $^{\circ}$ C for 40s. Then 20 cycles of 95  $^{\circ}$ C for 20s, 56  $^{\circ}$ C for 50s and 72  $^{\circ}$ C for 50s. The final extension step was at 72  $^{\circ}$ C for 5min, whereas, those for IL-10 (-1082G/A) included an initial denaturation at 95  $^{\circ}$ C for 5min, followed by 35 cycles at 95  $^{\circ}$ C for 30s, 63  $^{\circ}$ C for 30s and 72  $^{\circ}$ C for 30s. The final extension step was at 72  $^{\circ}$ C for 5min.

The amplification products were separated by electrophoresis on a 1.5% agarose gel stained with ethidium bromide. The gel was visualized under a UV transilluminator with a 100-base pair ladder.

### Statistical Analysis

The Statistical Package for the Social sciences version 14.0 (SPSS Inc., Chicago, USA) was used for statistical analysis. Chi-square test was used to test the deviation from Hardy-Weinberg Equilibrium (HWE) of SNPs by

comparing the observed and expected frequencies. The association between genotype and risk of VL was estimated by calculation of Odds ratio (OR) with 95% confidence interval (95%CI) using logistic regression. Statistical significance was set at a  $p$  value  $\leq$  0.05.

### Results and Discussion

Chi-square test revealed that alleles' distribution in both SNPs either in VL patients or control subjects is within Hardy-Weinberg equilibrium.

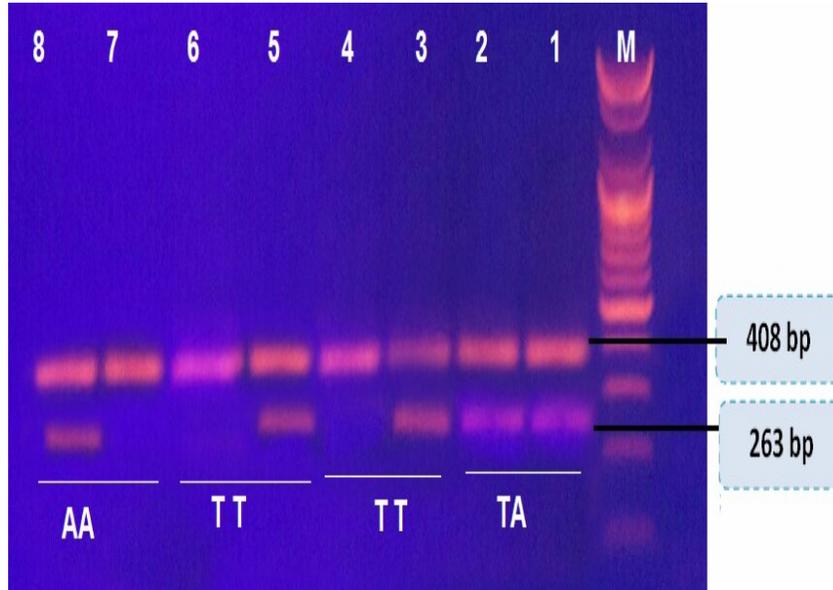
#### IFN- $\gamma$ (+874A/T)

Genotype and allele frequencies of IFN- $\gamma$ (+874A/T) SNP in VL patients and healthy control subjects are shown in table 1 and figure 1. For VL patients, the frequencies of TT, AT, and AA genotypes were 46.27%, 37.31%, and 16.41% respectively, compared with 70%, 25%, and 5% respectively in the control subjects with heterozygous genotype (AT) has significant association with the incidence of VL (OR=4.968, 95%CI= 1.012-24.38,  $p$ = 0.048). The frequencies of allele T and A were 64.92% and 35.07% respectively among VL patients and 82.5% and 17.5% respectively among control subjects with significant difference (OR= 2.547, 95%CI=1.294-5.013,  $p$ = 0.007).

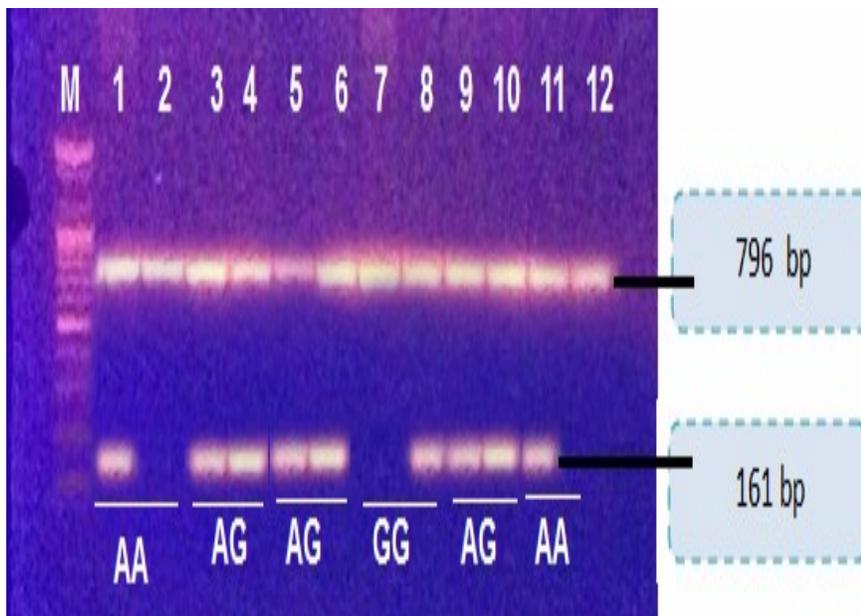
#### IL-10 (-1082 G/A)

Like IFN- $\gamma$ (+874A/T), this SNP had three genotypes: AA, AG, and GG with frequencies of 31.34%, 43.38%, and 25.37% respectively among VL patients, and 30%, 55%, and 15% respectively among control subjects. Despite these differences in the frequencies, logistic

**Figure.1** Gel electrophoresis visualized under UV light of IFN- $\gamma$  (+874T/A) after ARMS-PCR. M: 100 bp DNA marker. Lane 1,3,5, and 7: allele T is represented by the presence of a 263 bp PCR fragment. Lane 2,4,6, and 8: allele A is represented by the presence of a 263 bp PCR fragment. Each two lanes represent one sample. Lane 1-8 a 408 bp of PCR product of *Human growth factor hormone* gene (internal control).



**Figure.2** Gel electrophoresis visualized under UV light of IL-10 (-1082 G/A) after ARMS-PCR. Lane 1: 100 bp DNA marker. In lane 1,3,5,7,9, and11, allele A is represented by the presence of 161 bp PCR fragment. In lane 2,4,6,8,10, and 12, allele G is represented by the presence of 161 bp PCR fragment. Each two lanes represent one sample. Lane 1-12: 796 bp PCR fragment of third intron of the *HLA-DRB1* gene (internal control).



**Table.1** Distribution of genotypes and allele frequencies of IFN- $\gamma$  (+874A/T) and IL-10 (-1082 G/A) promoter polymorphisms in VL patients and control subjects

Variables	Cases N=67	Control N=40	P-value	OR(95%CI)
<b>IFN-<math>\gamma</math>(+874A/T)</b>				
TT	31 (46.27%)	28 (70%)	0.05	1.0
TA	25 (37.31%)	10 (25%)	0.048	4.968(1.012-24.38)
AA	11 (16.41%)	2 (5%)	0.074	2.258(0.924-5.520)
<b>Alleles</b>				
T	87 (64.92%)	66(82.5%)		1.0
A	47(35.07%)	14 (17.5%)	0.007	2.547(1.294-5.013)
<b>IL-10 (-1082 G/A)</b>				
AA	21 (31.34%)	12 (30%)	0.377	1.0
AG	29 (43.28%)	22 (55%)	0.537	0.753(0.306-1.853)
GG	17 (25.37%)	6 (15%)	0.42	1.619(0.502-5.217)
<b>Alleles</b>				
A	71 (52.99%)	46 (57.5%)		1.0
G	63 (47.01%)	34 (42.5%)	0.521	1.2 (0.687-2.098)

regression test revealed no significant differences. Similarly, the distribution of A and G allele (52.99% and 47.01% respectively among VL patients, and 57.5% and 42.5% respectively among control subjects) showed no significant difference (OR=1.2, 95%CI=0.687-2.098, p=0.521) (table 1 and figure 2).

Human VL presents as a spectrum of clinical manifestations from a self-controlled infection to a progressive disease depending on many factors. The level of immune response that the host exposes is, of course, one of the most important factors that determine the sequelae of such infection. In active VL, the immune system is highly activated and produce both the macrophage-activating cytokines (IFN- $\gamma$  and TNF- $\alpha$ ) and the macrophage-deactivating cytokines (IL-10 and TGF- $\beta$ ) (11). Plasma levels of IFN- $\gamma$  are influenced by numerous endogenous factors beside IFN- $\gamma$  gene activity. SNPs in the first intron of this gene (especially +874A/T) were shown to affect the transcription of the gene therein, and T

and A alleles of this SNP are associated with high and low production of IFN- $\gamma$  respectively (12). Results of this study indicated an increase of 2.54-fold risk for those individuals carrying mutant allele (A) compared to those carrying the wild type allele (T). This can be attributed to the feebleness of cell-mediated immune response among A allele carriers due to less IFN- $\gamma$  production.

This result is in accordance with many previous studies. Al-mayah and Chalooob (13) have found significant association between A allele carriers and the susceptibility to chronic hepatitis B viral infection in Iraqi patients. Similarly, such association was shown with pulmonary tuberculosis (5), and brucellosis (14), all of which are intracellular microorganisms and induced cell-mediated immune response. Accordingly this polymorphism may serve as a valuable marker for characterization of a group of patients at increased risk for intracellular microorganisms such as *Leishmania*.

Many studies indicated the suppressive role of IL-10 to immune response via blocking Th<sub>1</sub> activation and subsequently down regulation of IL-12 and IFN- $\gamma$  production (3). It is because this immunosuppressive property, it has been postulated that IL-10 can contribute to escape *leishmania* from immune surveillance and favor infection.

Like IFN- $\gamma$  +874A/T, IL-10-1082A/G SNP influences the plasma level of the cytokine, where the mutant allele (G) is considered to be associated with high production of IL-10 from peripheral mononuclear cells (15). However, our study did not confirm such notion as there was no significant association between G allele and susceptibility to VL. This is because that not only the amount of IL-10 is important, but also the timing and the cell population producing it (16). Furthermore, many studies have challenged the notion that IL-10 has always immunosuppressive activity. Systemic administration of IL-10 induced an effective, specific, and long-lived immune response against established tumor in mice (17), which indicates immune-regulatory rather than immune-suppressive effect of this cytokine. From the present study it can be concluded that IFN- $\gamma$  +874A variant but not IL-10-1082G variant may be a risk factor for VL.

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