JRHS
Journal of Research in Health Sciences
journal homepage: www.umsha.ac.ir/jrhs

Original Article

Evaluation of IFN-γ Polymorphism in Visceral Leishmaniasis

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ARTICLE INFORMATION

Article history:
Received: *24 August 2013*
Revised: *21 December 2013*
Accepted: *02 February 2014*
Available online: *15 February 2014*

Keywords:
Visceral leishmaniasis
Interferon-gamma
Polymorphism
ARMS-PCR

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ABSTRACT

Background: Leishmaniasis is a tropical disease that is endemic in some areas of Iran, including East Azerbaijan. IFN-γ is one of the cytokines that triggers cell-mediated immunity, thus initiating elimination of the infection. This case-control study was performed to investigate the association between the polymorphism of the IFN-γ gene at the +874A/T locus and visceral leishmaniasis (VL).

Methods: In this study conducted during 2012-2013, 267 participants were selected from individuals living in an endemic area of VL. Subjects were divided into three groups; 86 patients with VL, 82 seropositive individuals without any history of leishmaniasis, and 99 seronegative healthy controls. Genotyping of the IFN-γ +874A/T polymorphism was carried out using an Amplification Refractory Mutation System-PCR (ARMS-PCR).

Results: The frequency of the +874A allele in the patient group (75.5%) was higher than in the seropositive individuals (54%). The highest frequency of the +874T/T genotype was observed in seropositive individuals, while the patient group had the lowest frequency (34.1% vs. 24.5%). However, these differences were not significant.

Conclusion: There was no significant association between IFN-γ +874A/T polymorphism and VL.

Citation: Maghsood AH, Jadideslam G, Fallah M, Bazmani A. Evaluation of IFN-γ Polymorphism in Visceral Leishmaniasis. J Res Health Sci. 2014; 14(2): 136-139.

Introduction

The *Leishmania* organism is a member of the order Kinetoplastida and the family Trypanosomatidae. The parasite needs two different hosts, a vertebrate and an insect, in order to complete its lifecycle. The parasite is seen in amastigote form in vertebrate hosts, and contains a visible rod-shaped kinetoplast. Amastigotes are often seen in the intracellular fluids, especially in macrophages, where they can survive and reproduce by binary fission[1].

At the cellular level, leishmaniasis is established by attacking of the parasite to macrophages, prevention of killing mechanisms, intracellular replication of parasite, and the spread of the organisms[2].

Leishmaniasis is endemic in 88 countries around the world, and nearly 350 million people live in high risk areas. Official figures show that visceral leishmaniasis (VL), also known as kala-azar, is responsible for 50,000 deaths every year, and more than 90% of these occur in Bangladesh, India, Nepal, Sudan, Ethiopia and Brazil[3].

The question posed is, why only a small proportion of infected persons develop the disease? VL is almost always fatal if it is left untreated, while the mortality rate is 10% even when treated[4].

Resistance to the infection is associated with a type 1 T-helper cell response (Th-1), which produces cytokines such as; IFN-γ, IL-2 and IL-12. These cytokines trigger cell-mediated immunity to eliminate the infection. Individuals that provide a strong Th-1 response may kill the parasites and have temporarily positive serum antibody titers.

In some individuals the disease occurs as a result of a type 2 T-helper response (Th-2), with production of IL-4, IL-5, IL-6, IL-9 and IL-10, which leads to the stimulation of B-cell proliferation and antibody response. Produced antibodies are not effective and may even be harmful[5].

The IFN-γ gene polymorphism at position +874 increases susceptibility to post-kala-azar dermal leishmaniasis (PKDL), chronic cutaneous leishmaniasis, Bacille Calmette-Guérin (BCG) adenitis, intrauterine Hepatitis B Virus (HBV), tuberculosis, pulmonary tuberculosis, chronic myelogenous leukemia, systemic lupus erythematosus, IgA nephropathy, atopy, brucellosis, and type 1 and type 2 diabetes[6-21]. However, this polymorphism has had no effect on susceptibility to asthma, American tegumentary leishmaniasis, Alzheimer's disease, latent infection of HBV and multiple sclerosis[22-27].

This case-control study was planned to determine the relationship between IFN-γ +874A/T polymorphism and VL.

Methods

This comparative case-control study was conducted during 2012-2013 in the East Azerbaijan Province, in the north-

west of Iran. Three groups of individuals were recruited, with the study group comprised of 86 clinically and paraclinically confirmed VL patients. The seropositive and seronegative groups were comprised of 82, and 99 individuals, respectively; these subjects had no symptoms, or confirmed history of VL and cutaneous leishmaniasis, and they were selected from the patients' relatives. The demographic characteristics of the study population are shown in Table 1.

Table 1: Demographic characteristics of study population

	Patients				Seropositive				Seronegative				Total	
	Male (n=51)		Female (n=35)		Male (n=49)		Female (n=33)		Male (n=61)		Female (n=38)			
Age group (yr)	N	%	N	%	N	%	N	%	N	%	N	%	N	%
<2	39	76.4	28	80.0	39	79.5	26	78.7	45	73.7	28	73.6	205	76.7
2-5	6	11.7	3	8.5	6	12.2	4	12.1	7	11.4	5	13.1	31	11.6
6-10	5	9.8	3	8.5	3	6.1	2	6.0	7	11.4	4	10.5	24	8.9
>10	1	1.9	1	2.8	1	2.0	1	3.0	2	3.2	1	2.6	7	2.6

An indirect fluorescent antibody (IFA) test was performed to screen and allocate the seropositive and seronegative groups. The titers of ≥1:160 were considered as positive in the IFA, and those of ≤1:20 titer as seronegative.

DNA extraction was performed using the commercial kit (Pakgen-Yakhteh Kit, Iran) based on kit instruction. The resulting DNAs were stored at -20 ºC until they were required.

The proposal was approved by the Ethics Committee of the Vice-chancellor of Research and Technology, Hamadan University of Medical Sciences. All participants signed a research consent form.

Detection of the IFN-γ +874A/T polymorphism was carried out by an Amplification Refractory Mutation System-PCR (ARMS-PCR) which has been widely used for single nucleotide mutations in genomes. Forward and reverse primers used in this study were sense +874T: 5'TTC TTA CAA CAC AAA ATC AAA TCT3', sense +874A: 5'TTC TTA CAA CAC AAA ATC AAA TCA3' and antisense (common): 5'TCA ACA AAG CTG ATA CTC CA3' amplifying a 262 bp fragment. Internal control primers were BGR1: 5'ACA CAA CTG TGT TCA CTA GC3' and BGR2: 5'CAA CTT CAT CCA CGT TCA CC3' amplifying a 110 bp product of human Hb sequence.

PCR reactions were conducted in 20 µl, and each 100 µl of PCR reaction contained 10 µl of 10X reaction buffer, $MgCl_2$ 3.5 mM, each primer 20 pM, internal control primers 10 pM, dNTP mix 0.2 mM, 7 units Taq DNA polymerase, and 1 µg genomic DNA template.

The PCR reaction was carried out in a thermal cycler pc818 (Astec, Japan) under the following conditions: 1 cycle of primary denaturation at 94 ºC for 2 min, followed by 5 cycles of denaturation at 94 ºC for 20 sec, annealing at 64 ºC for 40 sec, extension at 72 ºC for 70 sec, proceeded by 25 cycles: 94 ºC for 20 sec, 57 ºC for 40 sec, 72 ºC for 40 sec, and a final extension cycle of 72 ºC for 3 min.

Electrophoresis of the PCR products was performed using 10 V/cm DC on a 1.5% agarose gel and scanned under a UV transilluminator using 7 µl/dl safe stain (CinnaGen, Iran) (Figures 1&2). The size of the PCR product was 262 bp and that of the internal control was 110 bp. The sizes of the amplicons were determined using a 50-bp ladder (Fermentas, SM 0373). Genotype frequencies in the three groups were compared by a chi-square and Phi-divergence test multivariable logistic regression, to investigate the effect of IFN-γ +874A/T polymorphism on VL.

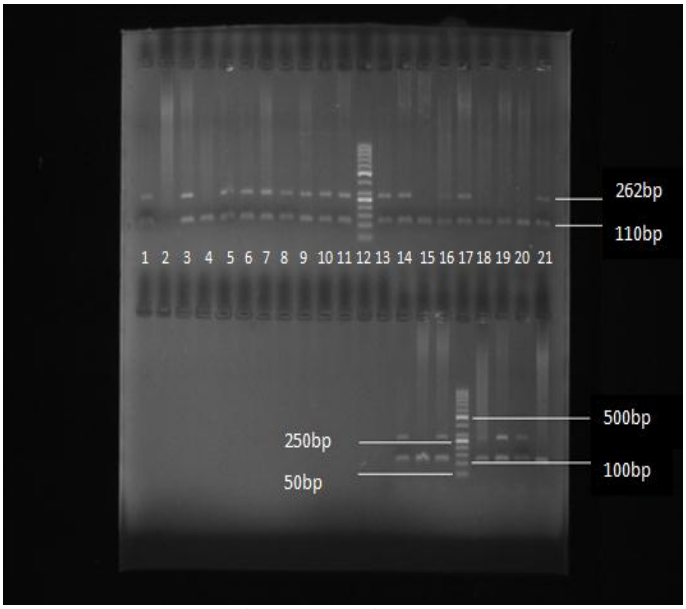

Figure 1: Electrophoresis of PCR product for +874A allele: (12) ladder, (2, 4, 15 and 18-20) without A allele, (1, 3, 5-11, 13, 14, 16, 17 and 21) with A allele

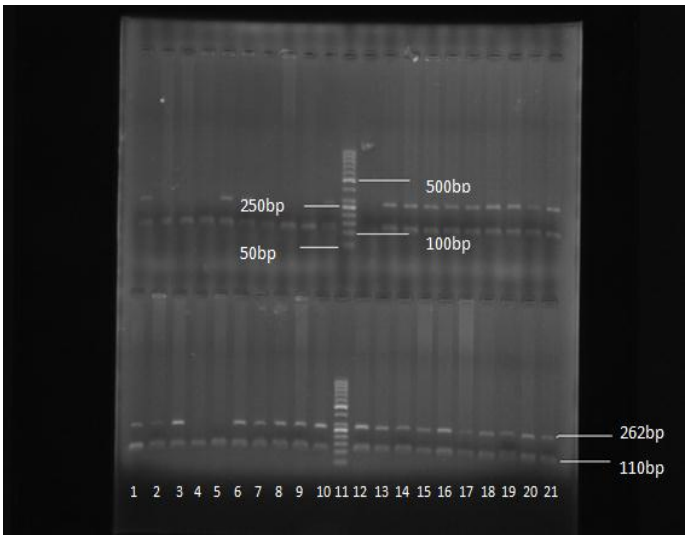

Figure 2: Electrophoresis of PCR product for +874T allele: (11) ladder (4 and 5) without T allele, others with T allele

All analysis was performed using SPSS (version 16) statistical analysis software. *P*-value less than 0.05 was considered as significant.

Results

The interferon gamma polymorphism at position +874 was evaluated in the three groups, and it was found that the +874A allele frequency was higher in the VL patients (75.5%) than in the seropositive subjects (65.9%). The fre-

quency of the wild allele (+874A) was 73.7% in the seronegative healthy controls. The frequency of the mutant allele (+874T) in the patient, seronegative, and seropositive groups was 63.9%, 67.7% and 62.2%, respectively.

The heterozygote genotype +874A/T was the most common among all the subjects (36.7%), and the +874T/T genotype had the lowest frequency in the total population (28.1%). The highest frequency of the T/T genotype (34.1%) was observed in the seropositive individuals, while the VL patients had the lowest frequency of this genotype (24.5%) (Figure 3). However, using statistical analysis, none of these differences were significant.

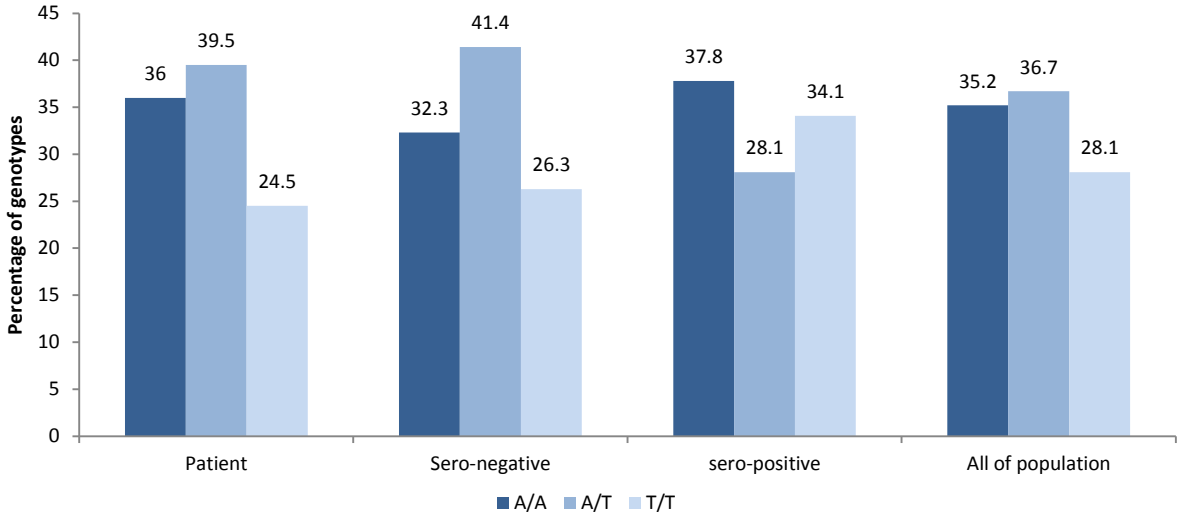

Figure 3: Percentages of +874A/T genotypes in the three groups

Discussion

Gamma interferon has immunomodulatory effects on immune function and in many cases where inflammation occurs, secretion of this cytokine increases. Cytokine gene polymorphisms may influence the secretion of cytokines and the disease process. Immunomodulatory gene polymorphisms can be effective on sensitivity or resistance to a particular disease, and the presence of a particular cytokine genotype can be the cause of one of these two modes.

In this study, the highest frequency of the +874T/T genotype was seen in the seropositive group. This genotype is associated with maximum production of gamma interferon, and this may explain the finding that in spite of exposure to a *Leishmania* infectious agent, these subjects did not show any signs or symptoms of Kala-azar. However, this difference was not statistically significant.

A number of studies were not able to find any significant relationship between polymorphisms of interferon-gamma at the +874A/T position and asthma[23,28], Alzheimer's disease[24], multiple sclerosis[25], occult HBV infection[26], and American tegumentary leishmaniasis[27]. On the other hand, a significant relationship was observed in studies performed regarding the association between this polymorphism and susceptibility to chronic myelogenous leukemia[6], tuberculosis[7,14,20], pulmonary tuberculosis[8], intrauterine Hepatitis B Virus infection[9], atopy [10], chronic cutaneous leishmaniasis [11], type 2 diabetes[12], visceral leishmaniasis[13], IgA nephropathy[15], BCG adenitis[16], type 1 diabetes[17,] brucellosis[18], PKDL[19] and systemic lupus erythematosus[21].

These differences in the relationship between interferon-gamma polymorphism and the aforementioned diseases could be related to the various ethnic groups or perhaps to a combination of factors which may be associated with different variables, for example IL-12, IL-18, etc. Further research is required to investigate the relationship between different immunological factors and their effects on various ethnic groups.

Conclusions

The frequency of A/T alleles at position +874 on the IFN-γ gene are close to each other. Moreover, the highest frequency of +874T/T genotype (associated with high production of interferon-gamma[22]) was observed in the seropositive healthy group despite exposure to *Leishmania infantum*, although this difference was not statistically significant.

Acknowledgments

This work was part of MSc thesis of Gholamreza Jadideslam which was approved by the Vice-chancellor of Research and Technology, Hamadan University of Medical Sciences, which is hereby gratefully acknowledged. Some parts of this work were conducted in the Tabriz Infectious and Tropical Diseases Research Center, Tabriz University of Medical Sciences, Tabriz, Iran. We would like to thank them for their kind cooperation. We also thank all staff of the Health Houses of East Azerbaijan Province for their kind assistance in collecting samples.

Conflict of interest statement

The authors have no conflict of interest.

Funding

This study was supported financially by Vice-chancellor of Research and Technology, Hamadan University of Medical Sciences.

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