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Original Article

The Association of Human Leucocyte Antigen (HLA) Class I and II Genes with Cutaneous and Visceral Leishmaniasis in Iranian Patients: A Preliminary Case-Control Study

Mitra Eimanzadeh¹, *Mehdi Mohebali^{1,2}, Morteza Zarrabi³, Abbas Rahimi Foroushani⁴,
Mohammad Kazemi³, Homa Hajjaran¹, Zabih Zarei¹, Zahra Kakooei¹, *Behnaz Akhondi¹

1. Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

2. Center for Research of Endemic Parasites of Iran (CREPI), Tehran University of Medical Sciences, Tehran, Iran

3. Royan Stem Cell Technology, Cord Blood Bank, Tehran, Iran

4. Department of Epidemiology and Biostatistics, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

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*Correspondence Email:

mohebali@tums.ac.ir
b-akhondi@tums.ac.ir

Abstract

Background: Leishmaniasis is currently considered a re-emerging or emerging infection based on the geographic region. The outcome of leishmaniasis vastly depends on *Leishmania*-host interaction. This preliminary study aimed to show the association of human leukocyte antigen (HLA) class I and II genes with healed and non-healed cutaneous leishmaniasis (CL), and symptomatic and asymptomatic visceral leishmaniasis (VL) compared with control groups in Iran.

Methods: Ninety-five people, including 31 patients versus 64 individuals in the control group, were enrolled. Among them, 20 patients had confirmed CL based on amastigote observation, 10 had improved CL and 10 non-healed CL. Eleven patients were suffering from confirmed VL based on direct agglutination test (Five asymptomatic and six symptomatic VL cases). Besides, they were residents in an endemic area of VL in the northwest of Iran. To select a control group, it was ensured that they had no history of leishmaniasis. Peripheral blood samples were collected from each patient. After DNA extraction, HLA typing was conducted using polymerase chain reaction - sequence-specific priming (PCR-SSP). Subsequently, data were statistically analyzed by SPSS.

Results: There was a statistical relationship between the presence of HLA-A26 and CL, healed CL and the existence of the B38 allele, C1 allele and symptomatic VL, as well as B1.4 allele and asymptomatic VL ($P < 0.05$).

Conclusion: This primary finding indicates that several HLA genes have a potential role in the susceptibility of Iranian people to CL and VL.



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Introduction

L*eishmania* spp. are protozoa parasites that can be transmitted by the sand fly, causing human leishmaniasis infection (1). Disorders associated with lethal visceral leishmaniasis (VL), affect internal organs, particularly the spleen, liver, bone marrow, and lymphatic nodes. Leishmaniasis is commonly found in Africa, Latin America, Asia, the Middle East, and the Mediterranean (2,3). The major forms of leishmaniasis are diffuse (anergic) cutaneous leishmaniasis (DCL), localized cutaneous leishmaniasis (LCL), in which the chronic metastatic lesions and the disruption of mucosal membrane occur as well as VL, also known as Kala Azar, which is the most lethal and the severest type of leishmaniasis (4). Most VL cases occur in five countries of India, Bangladesh, Ethiopia, Sudan, and Brazil. This infection is zoonotic, and canine spp. is considered the main reservoir; however, humans are the only known hosts for *L. donovani* (5). Leishmaniasis is also considered an emerging infection among travelers, making its diagnosis and treatment. This a challenging task since clinicians in the non-endemic regions are not fully familiar with the symptoms (6).

The mechanism of action in the mammalian host after *Leishmania* infection includes the activation of cellular immunity and the subsequent activation of macrophages. Despite the strong activation of humoral immunity, antibodies are not significantly protective against *Leishmania* spp. Anti-*Leishmania* reactions by innate (neutrophils, macrophages, and dendritic cells) and acquired (T cells, in particular, CD4+T cells) immunity are significantly important (7).

The human leukocyte antigen (HLA) system (the major histocompatibility complex [MHC] in humans) is an important constituent of the immune system. It is considered a contributory genetic factor for leishmaniasis (8). This system is governed by genes located on chro-

mosome 6 and is responsible for encoding cell surface molecules specialized in presenting antigenic peptides to CD4+ and CD8+ T cells. CD4+ T cells are considered the main source of interferon- γ (IFN- γ) production, which can control *Leishmania* replication at the early stages of infection. In contrast, CD8+ T cells are involved in IFN- γ production and Th1 differentiation (9). Therefore, alterations in HLA class I and II due to the variability of *Leishmania* antigens can affect the susceptibility of humans to leishmaniasis. Reports from Southern America greatly inspire the role of HLA genes in CL. These reports suggest the role of several HLA-I (HLA-B) and HLA-II (HLA-DQ) alleles in the susceptibility or resistance of patients to CL (10).

Identification of human genetic factors involved in the susceptibility of patients to leishmaniasis, can help us understand the complex pathogenicity of *Leishmania* and the host immunity molecular pathways and can help us develop epitope-based drugs or vaccines. The present study aimed at the identification of distinct HLA-I and HLA-II alleles involved in the susceptibility or resistance to VL and CL infections in Iranian populations.

Materials and Methods

This study was approved by the Tehran University of Medical Sciences Ethical Committee (IR.TUMS.SPH.REC.1399.210).

This case-control study was conducted on 31 leishmaniasis patients in March-October 2020. Blood samples were collected from 20 CL patients referring to the leishmaniasis laboratory, the School of Public Health, Tehran University of Medical Sciences, and 11 VL patients residing in northwest Iran. Written consent was taken from all patients. For CL samples, parasitological evaluation was based on observing amastigotes' indirect smear. The CL patients were categorized as healed (those

who showed CL infection for the first time, those who had self-healed CL, and those who recovered from CL after treatment) and non-healed (those with infection recurrence, treatment failure, and drug resistance) cases. Direct agglutination testing (DAT) was carried out to diagnose VL infection. Accordingly, VL patients were categorized as symptomatic (DAT titer at 1:3200 and above) and asymptomatic (DAT titer at \leq 1:800) cases (11-13). The Control group included those living in the same area as the patients (preferably from family members or relatives). Exclusion criteria for the control group included a history of CL infection (14).

DNA extraction

For DNA extraction, 5 ml blood samples were collected from patients and the control group in EDTA-containing tubes. Then, the Royan Institute extracted genomic DNA using BAG Kit (for HLA type 1/ Cat n: 930E1 and HLA type 2 (Cat n: 031F1).

HLA typing

PCR sequence-specific priming (PCR-SSP) was carried out at the Royan Institute to determine the HLA-I and HLA-II alleles. In this technique, rather than using restriction enzymes, longer and more specific primers are used for each allele of polymorphisms. After running PCR products on 2.0% agarose gel, bands were visualized under UV illuminator, and alleles were assigned using the SSP UniMatch software (v 5.1). The frequencies of HLA-A and B were assessed by direct counting (15).

Statistical analysis

For statistical analysis, the SPSS software v. 24 (IBM Corp., Armonk, NY, USA) was used. The Fischer Exact Test was used to determine the association between alleles and leishmaniasis, and multinomial logistic regression analysis was used to investigate the simultaneous effect of factors on the association between alleles and the disease.

Results

Clinical characteristics of the patients

Twenty CL patients were recorded, among which 10 cases showed healed CL. Sixty percent (n=12) of cases demonstrated individual dried ulcers without hand pain in the last three months before referring to the clinic, and only 30% (n=6) of cases showed multiple ulcers. In patients with non-healed CL, 10 cases were recorded; all of them have multiple ulcers, especially on their faces and noses. Only 20% (n=4) of cases had the infection for <5 years, and 40% (n=8) had a history of leishmaniasis with treatment failure. Eleven VL cases were recorded from northwest Iran, considered an endemic region for Kala Azar. Among these cases, five patients showed asymptomatic VL infection, while 6 demonstrated symptomatic VL infection. Symptoms in the latter group included fever, hepatosplenomegaly, and anemia. The demographic features of the patients are summarized in Table 1.

Distribution of HLA alleles

All HLA-I and HLA-II alleles detected in healed and non-healed CL and symptomatic and asymptomatic VL cases are summarized in Table 2 and 3. The most common alleles in CL patients and the control group were HLA-B3, HLA-DQ6, and HLA-DQ5. Also, HLA-C7, HLA-DQ3, and HLA-A24 were the most common alleles in the patient group, while HLA-C17, -C2, -B56, -B53, -B50, and -B47, -B.1.1, -A68, -A33, -A31, -A30, and -A23 were not present in these patients. No significant association was observed between the age and sex of the patients with infection type (P -value>0.05). According to the multinomial logistic regression results, CL susceptibility in patients with A26 alleles was significantly greater than in those who did not carry this allele (P <0.05). Statistical analysis indicated a significant association between the infection type and the presence of C1 allele (at CI: 95%). In addition, susceptibility to symptomatic VL

was significantly higher in those carrying the C1 allele. The results of the Fischer Exact Test indicated a significant association between infection type and the presence of B1.4 alleles ($P < 0.05$). The multinomial logistic regression

results indicate a strong relationship between the presence of B1.4 alleles and susceptibility to asymptomatic VL (Tables 2 and 3).

Table 1: Summary of demographic features of patients with cutaneous and visceral leishmaniasis

Characteristic	Number of patients	Age group (yr)	Gender	
			Male	Female
Cutaneous leishmaniasis (healed)	1	10-19	9	1
	2	20-29		
	1	30-39		
	2	40-49		
	3	50-59		
Cutaneous leishmaniasis (non-healed)	3	20-29	5	5
	0	30-39		
	4	40-49		
	3	50-59		
Visceral leishmaniasis (asymptomatic)	5	0-9	3	3
	1	10-19		
Visceral leishmaniasis (symptomatic)	5	0-9	1	4
	16	0-9		
Healthy (control group)	22	10-19	38	26
	15	20-29		
	6	30-39		
	5	40-49		

Table 2: HLA class I and class II allele distribution in patients with localized cutaneous leishmaniasis and controls (20 patients and 54 controls)

Allele	Allele frequency		Total	P value*
	Patient	Control		
	Healed	Non-healed		
HLA-A				
A1	4	3	10	.257
A2	2	3	18	.843
A3	3	2	19	.781
A11	1	3	18	.160
A23	0	0	1	1.00
A24	4	4	12	.273
A26	4	0	3	.011
A29	1	0	4	1.00
A30	0	0	1	1.00
A31	0	0	2	.168
A32	1	0	5	.879
A33	0	0	5	1.00
A68	0	0	6	.485
A69	0	1	0	.271
HLA-B				
B1.1	0	0	7	.548
B1.3	1	1	11	.704
B1.4	2	3	10	.735

B1.7	0	2	11	13	.543
B1.8	0	1	0	1	.282
B1.9	1	1	0	2	.282
B1.10	2	1	1	4	.061
B1.11	4	2	12	18	.650
B1.12	1	0	0	1	.282
B1.13	2	2	8	12	.657
B1.14	1	1	4	6	1.00
B1.15	1	2	15	18	.316
B1.16	1	3	9	13	.614
B3	6	4	36	46	.273
B4	3	4	20	27	1.00
B5	4	6	24	34	.755
B7	2	1	5	8	.711
B8	0	1	5	6	1.00
B13	0	1	3	4	.728
B14	0	1	6	7	.822
B15	1	2	1	4	.057
B18	2	1	9	12	1.00
B27	1	0	1	2	.470
B35	3	0	19	22	.052
B37	1	0	1	2	.470
B38	3	0	1	1	.013
B40	0	1	3	4	.728
B41	1	0	2	3	.621
B44	1	0	5	6	1.00
B47	0	0	1	1	1.00
B49	0	2	2	4	.168
B50	0	0	5	5	.719
B51	1	3	14	18	.181
B52	0	1	11	12	.365
B53	0	0	1	1	1.00
B55	1	1	3	5	.410
B56	0	0	1	1	1.00
B58	1	1	3	5	.410
HLA-C					
C1	0	1	4	5	1.00
C2	0	0	3	3	1.00
C3	3	1	2	6	.054
C4	4	0	21	25	.064
C5	0	0	2	2	1.00
C6	3	1	8	12	.697
C7	2	6	12	20	.119
C8	0	1	6	7	.828
C12	3	2	21	26	.678
C14	0	3	5	8	.146
C15	1	2	11	14	.339
C16	2	1	3	6	.197
C17	0	0	2	2	1.00
HLA- DQ2	2	3	16	21	.967
HLA- DQ3	6	5	19	30	.493
HLA- DQ4	0	1	1	2	.479
HLA- DQ5	4	3	22	29	.622
HLA- DQ6	4	2	22	28	.267

* Fisher Exact Test

Table 3: HLA class I and class II allele distribution in patients with visceral leishmaniasis (symptomatic and asymptomatic) and controls (11 patients and 64 controls)

<i>Allele</i>	<i>Allele frequency</i>		<i>Control</i>	<i>Total</i>	<i>P value*</i>
	<i>Patient Symptomatic</i>	<i>Patient Asymptomatic</i>			
HLA-A					
A1	0	1	10	11	.643
A2	1	2	24	27	.176
A3	3	0	22	23	.260
A11	1	0	21	22	.419
A23	0	0	1	1	1.00
A24	0	2	13	15	.245
A26	0	1	5	6	.628
A29	1	0	4	5	.554
A30	1	1	3	5	.153
A31	0	1	3	4	.476
A32	0	0	5	5	.266
A33	1	0	6	7	.460
A68	1	1	6	8	.329
HLA-B^a					
B1.1	1	0	9	10	1.00
B1.3	2	0	14	16	1.00
B1.4	0	1	11	12	.014
B1.7	1	1	13	15	.364
B1.8	0	0	0	0	
B1.9	0	1	1	2	.270
B1.10	0	0	1	1	1.00
B1.11	2	2	13	17	.543
B1.12	2	0	5	7	.149
B1.13	2	1	10	13	.235
B1.14	0	0	4	4	1.00
B1.15	0	0	15	15	.104
B1.16	0	0	10	10	.799
B3	5	3	46	54	.084
B4	1	5	24	30	.220
B5	0	1	25	26	.130
B7	1	0	5	6	.689
B8	0	0	5	5	1.00
B13	1	0	3	4	.276
B14	0	0	6	6	1.00
B15	0	1	2	3	.381
B18	1	0	9	10	1.00
B27	0	1	1	2	.270
B35	0	1	19	20	.356
B37	0	0	1	1	1.00
B38	0	0	3	3	1.00
B40	0	0	3	3	1.00
B41	0	1	2	3	.208
B44	1	1	6	8	.329
B46	1	0	3	4	.476
B47	0	0	1	1	1.00
B49	0	0	2	2	1.00
B50	0	0	5	5	1.00

B51	0	3	21	24	.192
B52	0	0	11	11	.108
B53	0	0	1	1	.143
B55	2	0	4	6	.112
B56	0	0	1	1	1.00
B58	1	0	4	5	.343
HLA-C ^a					
C1	3	0	8	11	.029
C2	0	0	3	3	1.00
C3	1	0	3	4	.384
C4	0	2	21	23	.567
C5	0	1	2	21	.423
C6	1	0	8	9	.638
C7	2	0	13	15	.496
C8	0	0	6	6	1.00
C12	1	0	24	25	.114
C14	0	1	6	7	.460
C15	1	4	13	18	.109
C16	0	0	7	7	1.00
C17	0	1	2	3	.208
DQ2	1	1	19	21	.618
DQ3	3	3	24	30	.120
DQ4	0	1	1	2	.270
DQ5	1	0	25	26	.277
DQ6	3	1	27	31	.103

* Fisher Exact Test

Discussion

Leishmaniasis is currently considered a re-emerging or emerging infection based on the geographic region. Leishmaniasis in patients with suppressed immunity, such as AIDS, is considered a lethal infection. Observational evidence suggests the contribution of host-parasite interaction, the host immune system, and genetic factors in the outcome of the disease. Therefore, this study aimed to investigate the association between HLA-I and HLA-II alleles with healed and non-healed CL, as well as symptomatic and asymptomatic VL (16).

According to the distribution of HLA alleles, 8 HLA-A, 34 HLA-B, 10 HLA-C, and 5 HLA-DQ alleles were indicated in CL patients; whereas 11 HLA-A, 23 HLA-B, 10 HLA-C, and 5 HLA-DQ alleles were detected in VL patients. Samaranayake et al. (2) indicated the presence of 11 HLA-A, 23 HLA-B, 10 HLA-C, and 5 HLA-DQ alleles in CL patients.

Interestingly, despite the lower frequency of the patients in the current study compared to another study (2), the distribution of alleles was similar, and HLA-B was the most frequent allele. Despite the lower number of patients compared to our study and another study (2) similar frequencies of HLA alleles were reported. In another study from Iran, 500 healthy patients were subjected to HLA typing, and according to their results, 14 HLA-A, 24 HLA-B, and 10 HLA-DRB1 alleles were indicated (17). However, more investigations on larger populations are required to indicate the exact similarity between the distributions of HLA alleles in different countries.

The results of the current study indicated the association between the presence of the HLA-A26 allele and human susceptibility to CL. Few studies have established an association between HLA and CL susceptibility. For instance, Barbier et al. compared the distribution of HLA-A, HLA-B, and HLA-C alleles in

CL patients and the control group. Their results indicated a significant reduction in the frequency of the HLA-Cw7 allele among CL patients (p-value=0.01) (18).

Besides, several other investigations have indicated the role of Cw7, A28, Bw22, DQw8, and DR2 in susceptibility to CL (19-21). In 2017, Dikhit et al. (22) investigated the ability of HLA-A2 peptides to bind HLA-A02. They studied 29 healed VL, three acute VL patients, and 24 healthy controls. They collected 10 ml blood samples from these cases in heparinized tubes and investigated two HLA-A02 peptides, including LLATTVSGL (P1) and LMTNG-PLPV (P3). The capability of these peptides to induce protective immunity in the studied groups was evaluated. Their results indicated the significantly high frequency of CD8+ T cells against the selected peptides. In addition, the inductions of peripheral mononuclear (PMN) cells with unique epitopes contribute to protective immunity. The results showed that IFN- γ production acted in a CD8+ T-dependent manner, suggesting the immunoprophylactic function of these epitopes in an MHC class I-dependent manner (22). Differences between the type of alleles associated with CL susceptibility in the current study and previous studies can be attributed to the low frequency of samples in the present study and other variables such as ethnicity and parasite spp. In addition, our study demonstrated a significant association between the presence of the HLA-B*38 allele and susceptibility to healed CL. However, there was no significant association between the presence of HLA alleles and non-healed CL (23).

The occurrence of non-healed CL can be attributed to factors such as genes involved in the process of ulcer healed, as indicated in the study of Castelluci et al. who showed the association between *FLI1* (Friend leukemia virus integration 1) polymorphism and CL caused by *L. braziliensis*. Mapping chromosome 9 in mice (human chromosome q2411) introduced *Fli1* as a new candidate affecting resistance to *L. major* infection and ulcer

healed (24). Several studies have reported the contribution of anti- and pro-inflammatory response genes polymorphism (TNF, SLC11A1, CXCR1, IL-6, IL-10, MCP1) in the modulation of human *L. braziliensis* infection (21, 25, 26).

Recently, the involvement of HLA polymorphism in VL susceptibility or resistance has been indicated (27). We investigated the potential relationship between asymptomatic VL and HLA classes I/II in the present study. To the best of our knowledge, there are no previous investigations on the association between HLA and symptomatic and asymptomatic VL in the endemic region of Northwest Iran.

Our results demonstrate a strong association between the presence of the HLA-C1 allele and susceptibility to asymptomatic VL infection. A study from India (27) indicated HLA-II alleles, particularly HLA-DRB1, as a genetic risk factor for VL. In addition, sequence-based haplotype analysis indicated the association between HLA-14 and HLA-DRB1.13 alleles and VL susceptibility, while HLA-DRB1.15 was associated with VL resistance (27). However, in our study, no relationship was established between HLA-II alleles and VL susceptibility. In contrast, HLA-I, including HLA-A, HLA-B, and HLA-C, were associated with susceptibility to symptomatic and asymptomatic VL. Also, the study of Faghiri et al. demonstrated a significant association between the presence of HLA-A26 and VL susceptibility (28). According to these results, the association between HLA loci and VL susceptibility or resistance may vary depending on the geographic region. Unlike our study, Fakiola et al. indicated that HLA-II, including HLA-DQA1 and HLA-DRB1, were significantly associated with VL susceptibility in India and Brazil (29).

These discrepancies may be attributed to the differences in sample data. Whether VL infection remains asymptomatic or symptomatic depends greatly on the complex association between genetic factors, host immune re-

sponses, *Leishmania* spp., and socioeconomic factors. Nevertheless, alleles implicated in symptomatic and asymptomatic VL should be considered important risk factors. Further studies are required to confirm the association between these alleles and VL infection; however, the provision of health facilities could protect children carrying these alleles against *Leishmania* vectors. Overall, discrepancies in the results can be attributed to several factors, including small sample sizes, a limited number of studies, different HLA allele distributions in different countries/regions, and different species of *Leishmania* spp. Therefore, different antigens and host immune responses.

Conclusion

HLA-A26, HLA-B38, HLA-C1, and HLA-B1.4 of HLA-I alleles have been suggested to be involved in susceptibility to leishmaniasis, confirming the role of HLA in these infections. These results highlight the importance of patient screening in endemic regions for identifying host factors involved in susceptibility or resistance to leishmaniasis. This can be employed to develop effective vaccines for endemic areas.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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