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NRAMP1 gene polymorphisms and cutaneous leishmaniasis: An evaluation on host susceptibility and treatment outcome

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ABSTRACT

Background & objectives: Association between polymorphisms in the natural resistance associated macrophage protein 1 (*NRAMP1*) gene and susceptibility to cutaneous leishmaniasis (CL) has been demonstrated worldwide; however, the reported results were inconsistent. This study aimed to determine the association of *NRAMP1* variants with susceptibility to CL infection and patients' response to treatment in Isfahan province of Iran.

Methods: Peripheral blood samples were collected from 150 patients with CL and 136 healthy controls. The CL patients were treated with intralesional injection of meglumine antimoniate. The polymorphic variants at *NRAMP1* (A318V and D543N) were analyzed using PCR-RFLP. The chi-square test and Fisher's exact test were used to compare frequencies of alleles and genotypes of polymorphisms between patient and healthy control populations.

Results: There was a statistically significant difference in the D543N (rs17235409) polymorphism between the CL patients and healthy controls (p=0.008). However, no significant association was detected for A318V (rs201565523) polymorphism between groups (p=0.26). In addition, there was a lack of association between D543N and A318V genotypes with response to treatment (p=0.54 and p=0.31, respectively).

Interpretation & conclusion: The results indicated that genetic variations of D543N (rs17235409) might be associated with susceptibility to CL infection. These data may be used for detection of sensitive individuals and prevention of CL in endemic areas.

Key words A318V, *cutaneous,* D543N, Iran, leishmaniasis*, NRAMP1*

INTRODUCTION

Leishmaniasis is a group of parasitic diseases caused by protozoan parasites of the genus *Leishmania*, transmitted by the species of phlebotomine sandflies[1], with several disease manifestations ranging from cutaneous to visceral forms[2]. It is endemic in >80 countries of the world[3]. Cutaneous leishmaniasis (CL) is the most common disease phenotype in Iran[4]. It can not only develop to visceral or mucocutaneous forms, but can also cause destructive effects on mental health[5]. Hence, control and prevention of this parasitic disease is very essential.

Some studies in mice have shown that the stage of macrophage maturation involves resistance to infections with intracellular pathogens[6–7]. Host genetics might play an important role in microbicidal activity of macrophages and susceptibility to intracellular bacteria and parasites[8–10]. Natural resistance-associated macrophage protein 1 (*NRAMP1*) gene is a member of the solute carrier family 11 (proton-coupled divalent metal ion transporter), member A1 (*SLC11A1*)[9–12]. In humans, *NRAMP1* gene is located in the chromosome region 2q35, containing 16 exons[13]. In a resting macrophage, the protein encoded by *NRAMP1* is assembled into the membrane of late endosome; while following phagocytosis it is relocated to the membrane of phagosome[14]. This protein transfers divalent metal ions across the phagosomal membrane and might be a critical factor for resistance to some microbial infections. *NRAMP1* influences a variety of antimicrobial responses of a macrophage, including induction of radical oxygen and nitric oxide intermediates, production and activation of various pro-inflammatory cytokines (TNF-α and interleukin-1β)

and regulation of anti-inflammatory cytokine IL-10[15–16]. It has been shown that mutations in the *NRAMP1* gene result in a non-functional or unstable protein, resulting in an increased proliferation of parasites in the macrophage[17–18]. Macrophages from mice with a mutant NRAMP1 protein have deficiencies in antigen presentation, while the presence of a NRAMP1 protein improves the function of macrophages in susceptible mice to mycobacterium[19–20].

There is a correlation between *NRAMP1* mutations and susceptibility to a cluster of antigenetically unrelated intracellular pathogens including HIV[21], *Mycobacterium leprae*[22], *M. tuberculosis* and *Leishmania donovani*[9, 23–24]. Among all the known NRAMP1 polymorphisms, a number of variants are located in the coding regions, some of which are missense mutations while others are silent substitutions. The missense mutations are an aspartic acid-to-asparagine change at codon 543 in exon 15 (D543N polymorphism) and a single-base substitution resulting in an alanine-to-valine change at codon 318 in exon 9 (A318V polymorphism). These missense mutations are important because they may affect the function of NRAMP1 protein[18, 25]. Host genetic factors might play an important role in development of the clinical manifestations, especially in CL[26–29].

The most important hyper-endemic area of CL in Iran is the Isfahan province, where only a certain percentage of individuals bitten by an infected sandfly, develop CL. Also, reinfection has been observed in some individuals with a previous history of CL infection. These observations prompted us to investigate the impact of genetic factors on susceptibility to CL infection and responses to therapy in different patients. Due to the importance of *NRAMP1*, especially A318V (rs201565523) and D543N (rs17235409) polymorphisms in the control of intracellular infections, the associations of the mentioned polymorphisms with susceptibility to CL along with the host responses to treatment were investigated in this study.

MATERIAL & METHODS

Subjects and samples

The current study was performed in the Antimicrobial Research Center, Iran University of Medical Sciences, Tehran, Iran. University Ethics Committee approved the study and informed consent was obtained for all subjects. The samples were collected from Isfahan province, where CL is highly endemic. In order to exclude the effect of multiple ethnic groups, all samples were obtained from the same region (Isfahan province, Iran); also, all participants were the original inhabitants of Isfahan province. The clinical and demographic information were obtained through medical records. The patients were excluded if they suffered from other significant medical conditions such as cardiac, renal, malnutrition or liver disorders. Also pregnant and lactating women, children >5 yr of age, and patients with a positive history of systemic or local therapy for CL and any other diseases within the last six months were excluded.

The blood samples were collected from 150 CL patients (mean age 28.94 ± 11.78) who were referred to the Skin Disease and Leishmaniasis Research Center (Sedigheh Tahereh Center, Isfahan, Iran) during 2012–13. Blood samples of healthy controls (n=136) were collected from blood donors (mean age 35.40 ± 9.49) in Isfahan Blood Transfusion Service. Cutaneous leishmaniasis was confirmed in those with clinically apparent skin lesions based on direct smear and/or culture. *Leishmania major* and *L. tropica* were considered as the agents of CL in this center according to the epidemiological data. Since, *L. major* species of *Leishmania* were identified as a major diagnosed species, and to exclude the effect of different species, only patients infected with *L. major* were recruited in this study. In addition, only patients with a single lesion were considered in order to omit the effect of lesions number on the outcome.

All the included patients were treated with a weekly intralesional injection of meglumine antimoniate (Glucantime, Paris, France) at a dose of 20 mg/kg/day for six weeks. The patients were assessed at Weeks 0, 1, 2, 3, 4, 5 and 6 of treatment and thereafter monthly up to six months. At every visit, direct smear and/or culture were performed. Based on the results of the treatment, patients were classified as complete improvement (disappearance of induration in lesion with re-epithelialization and flattening), partial improvement (reduction in lesion size and induration with no sign of any epidermal crease in lesion), and no improvement (no clinical change in lesions and decrease in their size). The obtained treatment outcome, from the end of the follow-up time was considered as the final report.

DNA extraction

Whole blood sample was collected in tubes containing EDTA. The genomic DNA was extracted from white blood cells using Cinna pure DNA kit (Sinaclon Co, Tehran, Iran) according to the manufacturers' instructions and stored at −80°C.

Genotyping

Genotyping for the D543N *(*rs17235409) and A318V (rs201565523) polymorphisms was performed by PCR-RFLP with two primers described in Table 1. PCR reaction volume for both polymorphisms was 25 µl, including

Table 1. Sequence of primers and characteristics of polymorphisms

Name	Nucleotide and amino acid change	Primers, 5' to 3'	Restriction enzyme	Genotype	Fragment size
A 318 V	Ala (GCG) to Val (GTG) in exon 9	F: TCCTTGATCTTCGTAGTCTC R: GGCTTACAGGACATGAGTAC	BsoFl	TT CT CC	232 bp 232, 171 and 61 bp 171 and 61 bp
D 543 N	Asp (GAC) to Asn (AAC) in exon 15	F: GCATCTCCCCAATTCATGGT R: AACTGTCCCACTCTATCCTG	Avall	GG GA AA	126, 79 and 39 bp 126, 79, 39 and 205 bp 205 and 39 bp

12.5 µl master mix (Sinaclon Co, Tehran, Iran), 0.15 µg of genomic DNA and 0.4 µl (50 pmol/µl) each of the two primers (reverse and forward). After an initial incubation at 94 °C for 4 min, the temperature cycles (n=30) used were 94 °C for 60 s, 58 °C for 90 s and 72 °C for 40 s with a final extension step of 10 min at 72 °C. All PCR products were resolved by electrophoresis in a 2.5% agarose gel and stained with ethidium bromide. The PCR products were digested using restriction enzymes BsoFl (for A318V) and AvaII (for D543N) under conditions recommended by the enzyme supplier (Thermo Scientific Co, Lithuania). Restriction products were separated in a 12% polyacrylamide gel and stained with ethidium bromide. The fragment patterns are presented in Table 1.

Statistical analysis

Data were analyzed using the statistical software package SPSS 18.0, Chicago. The quantitative parameters were reported as mean ± SD. Associations of alleles and genotypes of polymorphisms with susceptibility to CL and treatment results were analyzed by χ^2-test and Fisher's exact test as per requirement. In order to test the Hardy-Weinberg equilibrium, all frequencies of various genotypes were compared using the χ^2-test. A *p*-value of < 0.05 was considered to be significant with 95% confidence intervals (CI).

RESULTS

Population characteristics

The characteristics of 286 subjects (150 patients and 136 controls) are summarized in Table 2. Cutaneous leishmaniasis patients included 71 (47.3%) men and 79 (52.7%) women with a mean age of 28.94 ± 11.78. There were no significant differences between the two groups with respect to age and sex (*p*=0.22 and 0.63, respectively).

D543N (rs17235409) and A318V (rs201565523) genotyping

Genotype and allelic frequencies of D543N (rs17235409) and A318V (rs201565523) gene polymor-

Table 2. Demographic data of the subjects

Variables	CL patients (n = 150)	Control group (n = 136)	*p*-value*
Sex			
Male	71 (47.3)	60 (44.11)	0.63
Female	79 (52.7)	76 (55.89)	
Age (yr) ± SD	28.94 ± 11.78	35.40 ± 9.49	0.22
Species of *Leishmania*			
L. major	150 (100)	0	–

CL—Cutaneous leishmaniasis. Figures in parentheses indicate percentages; *p*-value ≤ 0.05 means statistically significant.

phisms of the patients and controls are presented in Table 3. The genotype distributions were in concordance with Hardy-Weinberg equilibrium in each group. D543N (rs17235409) genotypes observed in the examined patients included GG in 115 (76.6%) and GA in 35 (23.4%) individuals, while no AA genotype was detected in control or the patients. The genotype distribution was significantly (*p*=0.008) different between patient and the control groups. In addition, G and A allele distributions for this variant were statistically significant between patient and the control groups (*p*=0.01, OR: 95%, CI: 2.263/1.207–4.243). Genotype frequencies observed for A318V (rs201565523) were CC in 144 patients (96%) and CT in six patients (4%) with no TT detection. The genotype distributions were not significantly different between patients and the healthy controls (*p*=0.26). Also, C and T allele distributions for this variant were not statistically significant between the two groups (*p*=0.63, OR/95% CI: 2.262/0.837–6.111).

Effect of gene polymorphism on treatment results

The relative effect of the studied variations on treatment result is shown in Table 4. Genotype distributions for D543N (rs17235409) were not significantly different (*p*=0.54) among patient categories, *i.e.* no improvement, partial improvement and complete improvement. The results showed that in no improvement patients, GG genotype was present in 35 (81.39%) patients, while GA geno-

Table 3. Frequency of alleles and genotypes of *NRAMP1* polymorphisms (A318V and D543N) in cutaneous leishmaniasis (CL) patient and the control groups

Variables	CL patients (n = 150)	Control (n = 136)	*p*-value*	OR (95% CI)
A318V				
Genotype (%)				
CC	144 (96)	125 (91.9)	0.26	
CT	6 (4)	10 (7.35)		
TT	0	1 (0.75)		
(TT+CT=1 and CC=2)				
TT + CT	6 (4)	11 (8.1)	0.21	2.112 (0.759–5.876)
CC	144 (96)	125 (91.9)		
Allele (%)				
C	294 (98)	260 (95.5)	0.63	2.262 (0.0.837–6.111)
T	6 (2)	12 (4.5)		
D543N				
Genotype (%)				
GG	115 (76.6)	121 (88.9)	0.008	
GA	35 (23.4)	15 (11.1)		
AA	0	0		
(AA+GA=1 and GG=2)				
AA+GA	35 (23.4)	15 (11.1)	0.008	2.455 (1.273–4.734)
GG	115 (76.6)	121 (88.9)		
Allele (%)				
A	35 (11.6)	15 (5.51)	0.01	2.263 (1.207–4.243)
G	265 (88.4)	257 (94.49)		

*p-value ≤0.05 means statistically significant.

Table 4. Distribution of D543N (rs17235409) and A318V (rs201565523) genotypes in complete improvement, partial improvement and no improvement patients

Variables	Complete improvement (n = 38)	Partial improvement (n = 69)	No improvement (n = 43)	*p*-value
A318V				
Genotype (%)				
CC	36 (94.7)	68 (98.5)	40 (93.02)	0.31
CT	2 (5.3)	1 (1.5)	3 (6.98)	
TT	0	0	0	
Allele (%)				
T	2 (2.6)	1 (0.72)	3 (3.48)	0.31
C	74 (97.4)	137 (99.28)	83 (96.52)	
D543N				
Genotype (%)				
GG	27 (71.05)	53 (76.81)	35 (81.39)	0.54
GA	11 (28.95)	16 (23.19)	8 (18.61)	
AA	0	0	0	
Allele (%)				
G	65 (85.5)	122 (88.40)	78 (90.7)	0.59
A	11 (14.5)	16 (11.6)	8 (9.3)	

type was present in only 8 (18.61%) patients. Genotype distributions in partial improvement patients were— GG in 53 (76.81%) patients and GA in 16 (23.19%) patients. AA genotype was not found in any of the subgroups. Also,

the genotype distributions for the A318V (rs201565523) among the patient categories were not significant (*p*=0.31). Among no improvement patients, the CC genotype was found in 40 (93.02%) and CT in 3 (6.98%) individuals. The CC and CT genotypes were identified in 68 (98.5%) and 1 (1.5%) of partial improvement patients, respectively. TT genotype was not found in any of the subgroups.

DISCUSSION

The protein encoded by *NRAMP1* gene, which is expressed in the macrophage, acts by transferring divalent metal ions across the phagosomal membrane and might be a critical factor for resistance to some microorganisms. Several polymorphisms of *NRAMP1* gene have been evaluated in clinical studies[21–24]. The majority of previous investigations have focused on the association between *NRAMP1* gene and diseases such as lung tuberculosis[19, 30], Crohn's disease[31], rheumatoid arthritis[32], chronic periodontitis[33], leprosy[34], visceral leishmaniasis[35–37], and CL[37–38]. However, to our knowledge, there are no published reports on the association between *NRAMP1* gene polymorphisms with response to treatment in the Iranian CL patients. Thus, the present study focused on the effects of D543N (rs17235409) and

A318V (rs201565523) polymorphisms on susceptibility to CL and the status of treatment outcome among susceptible individuals. The results revealed that the D543N (rs17235409) allele and genotype frequencies between patients and controls were statistically significant (*p*=0.008). The GA genotype was detected in 23.4% of the patients and in 11.1% of the controls. Moreover, frequency of allele A was more in patients than the controls (11.6 *vs* 5.51%). Although, Samaranayake *et al*[38], had reported a lack of association between the GA genotype and CL infection, however, the results of this study indicated that the GA genotype indeed favours susceptibility to CL. Such conflicting results have also been reported on the association between D543N polymorphism and certain other diseases. A number of studies have indicated significant differences in D543N allele and genotype distributions between patients and controls[33, 36, 39–42–43], however, there are some exceptions[30, 34–35, 37]. Indeed, D543N polymorphism by substituting the Asp (as negatively charged amino acid) with Asn (an uncharged residue) in the cytoplasmic carboxy-terminal domain could affect protein function and possibly alter the macrophage function. Therefore, its effect on susceptibility to CL is not unexpected.

As far as A318V (rs201565523) polymorphism is considered, no significant difference was detected in allele and genotype frequencies between the patients and controls. This finding is in agreement with the results of other studies[32, 37–38]. A318V polymorphism exchanges the Ala amino acid (with a small and hydrophobic R group) with a Val residue (a medium size and hydrophobic R group) in the extracellular region of the *NRAMP1*. The R groups in both amino acids are non-polar aliphatic with nearly similar chemical and physical properties. Thus, this finding along with previous reports support our hypothesis that Ala to Val substitution may not affect protein function.

The data on the response to treatment showed that D543N and A318V variants are not associated with patient's response towards the treatment provided. So, the presence of the Asn and Val residues at positions 543 and 318, respectively, of the NRAMP1 protein is not associated with resistance to treatment. Whereas, previous studies on cancer patients had reported a potential effect of *NRAMP1* on responses to BCG immunotherapy in patients with bladder cancer, wherein the patients at high risk of recurrence were those carrying the GA genotype[42]. However, Yang *et al*[32] in Korea reported that treated and untreated patients with rheumatoid arthritis had identical genotypes for *NRAMP1* polymorphisms. Also, Mehrotra *et al*[35], showed that treatment in VL patients with three

genotypes of 5' (GT)n polymorphisms had no effect on *NRAMP1* gene expression. Similarly in another study, no significant difference was observed between *NRAMP1* gene polymorphisms and recurrence time, muscle invasion frequency or disease-free survival in patients with non-muscle-invasive bladder cancer; nevertheless, a shorter time to tumor recurrence was observed in group with GG genotype of D543N polymorphisms[44]. Therefore, it is apparent that more studies with an emphasis on treatment type are required to determine the effect of *NRAMP1* polymorphisms on the outcome of treatments.

CONCLUSION

In conclusion, the results from this study showed that the inheritance of some genotypes is associated with polymorphisms of *NRAMP1* gene, such as the D543N and A318V, which appear to influence the function of NRAMP1 protein, and thus may be indicative of an association between host genetic factors and susceptibility to CL. However, in order to approve the role of *NRAMP1* polymorphisms in susceptibility to CL infection, more studies are needed to investigate the effects of gene polymorphisms on the expression and function of the protein. Further analysis is also required to reveal the mechanisms that may influence the progression of CL. These data will increase our knowledge on macrophage involving disorders such as autoimmune diseases and intracellular infections. The results of the current study might be useful in the control of CL infection in high risk individuals living in the endemic region of Isfahan.

Conflict of interest
The authors declare no conflict of interest.

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