A common *SELL* gene haplotype associated with Cutaneous Leishmaniasis

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Abstract
Allele, genotype and haplotype frequency of three single nucleotide polymorphisms in SELL gene (rs2205849 (-642C>T), rs2229569 (676C>T) and rs1131498 (616C>T)) were examined in a group of patients with cutaneous leishmaniasis (68 patients) in comparison with 70 apparently healthy subjects. Binary logistic regression and Chi square were used for calculation of odds ratio. None of these polymorphisms were associated with the incidence of cutaneous leishmaniasis.

However, haplotype analysis revealed that the haplotype block CCT from the three polymorphisms respectively was significantly associated with increase of the susceptibility to the disease (OR= 4.25, 95%CI=1.85-9.81). There was a strong linkage disequilibrium between these polymorphisms.

Keywords: Cutaneous leishmaniasis, *SELL* gene polymorphism, haplotype, linkage disequilibrium.

Introduction

Leishmaniasis, caused by a protozoan Leishmania, is a zoonotic disease transmitted by phlebotomine sandflies. It has a worldwide distribution affecting about 12 million people mostly in tropical and subtropical countries. As such, it is considered the ninth most prevalent infectious disease[1]. In human, leishmaniasis is characterized by a wide spectrum of disease phenotypes ranging from a symptomatic, self-healing cutaneous leishmaniasis (CL) to severe mucosal, life-threatening visceral leishmaniasis (VL)[2]. Approximately 220000 cases are thought to be infected with CL yearly worldwide[3]. In Iraq, CL is endemic and caused by *L. tropica* and *L. major*. The reported annual incidence was 8300 to 16,500 cases[4].

The incidence of CL varies considerably even among individuals who are age- and sex-matched and live under the same environmental conditions which reflect the crucial role of genetic factors. Human *SELL* gene (OMIM, 153240) is located on chromosome 1 (1q23-q25) and consists of ten exons and nine introns spanning a region of ~21.0 kb[5].

L-selectin is a type I transmembrane glycoprotein molecule belonging to the selectin family which is mainly expressed by leukocytes and endothelial cells and plays a major role in the initial phases of leucocytes adhesion to the endothelium[6]. This protein is involved in the recruitment of leukocytes on the activated vessel wall during inflammation and immune response7. On cytotoxic T-lymphocyte (CTL), L-selectin is expressed when antigen primed CD8 T-cells leave the lymph node. In such case, this molecule drives CD8 T-cells toward infected organs. Upregulation of L-selectin expression on activated CD8 T-cells will further increases the activity of these cells without noticeable evidence of changing in cytokine secretion or clonal expansion[8]. Some studies have demonstrated that L-selectin expression can augment the signaling of T-cell receptor, signifying some roles beyond just trafficking[9].

Single nucleotide polymorphism (SNP) in *SELL* gene whether in promoter or in exons can influence the quantity and/or quality of the expressed L-selectin which influences the immune response and eventually the susceptibility to CL. A large number of studied have addressed the role of different SNPs in *SELL* gene in non-infectious disease like sickle cell anemia, acute coronary syndrome, myocardial infarction[10-13]. However, very few studies investigated this role in infectious diseases. Thus, this study aimed to explore the possible association of three SNPs in *SELL* gene with incidence of CL among a sample of Iraqi patients.

Material and Methods

The study population: Based on clinical, epidemiological and parasitological criteria, 68 patients with active lesion(s) less than 6 months old and the presence of the Leishman Donovan bodies in their lesion smear were included in the present study. Those patients were attending the dermatological clinical of Baqubah Teaching Hospital/ Iraq from October 2017 to March 2018. Other age- and sex-matched family-unrelated 70 subjects who were attending the same hospital accompanying some patients were clinically examined for CL. After the confirmation of negative results, those subjects were considered as control group.

Patients or controls with a history of autoimmune diseases were excluded from the study. A consent form (written in local language, Arabic) explaining the aims of the study was obtained from each participant. Demographic and clinical data were obtained either by direct interview or from patient's records.

DNA extraction and genotyping: Genomic DNA was extracted from peripheral leukocyte using a ready commercial kit (gSYNCTM DNA Mini Kit Whole Blood Protocol/ Geneaid/ Korea). The manufacturer's instructions

were followed precisely. Three SNPs in L-selectin gene were investigated in this study. These were rs2205849, rs1131498 and rs2229569. Restriction fragment length polymorphism polymerase chain reaction (RFLP-PCR) was used for genotyping of the three SNPs.

The primers, restriction enzymes and fragment length for each SNP are listed in table 1. First, *SELL* gene amplification of the corresponding fragment of each SNP was achieved with conventional PCR. The reaction was performed in 25 µL of reaction mixture containing 2 µL of DNA template, 200 µM of each deoxynucleotide triphosphate (dNTP), 1.5–2.5 mM $MgCl_2$, 1 µL of each primer and 0.6-unit Taq DNA polymerase (Bioneer/ Korea). The volume was adjusted to 25 µL with deionized water. The reaction was amplified by a thermocycler PCR system (Hybaid/ UK). The PCR conditions involved an initial denaturation for 6 min at 95^0C followed by 35 cycles each with denaturation for 45 sec at 94^0C, annealing for 45 sec at 58-61^0C and an elongation for 45 seconds at 72^0C. Finally, there was a final elongation for 7 minutes at 72^0C.

The PCR products under 2% gel electrophoresis stained with ethidium bromide. The results were read under UV transluminator. PCR products were digested with 5 U of the *PflM I, Alu I* and *Hph* I (New England BioLabs, USA) enzymes for rs2205849, rs1131498 and rs2229569 respectively. The final volume of the reaction was 15µL and digested at 37^0C/1 h. The digested products were analyzed by gel electrophoresis 2%.

Statistical Analysis: Statistical evaluation was carried out using the Statistical package for the Social sciences (SPSS version 25.0) for windows. Numerical variables were expressed as mean ± standard deviation (SD) and analyzed with Student t-test. Dichotomous variables were expressed as numbers and percentages and analyzed with Chi-square. The deviation of different genotypes from Hardy-Weinberg Equilibrium (HWE) was calculated online using https://www.easycalculation.com/health/hardy-weinberg-equilibrium-calculator.php websites.

Binary logistic regression was used to calculate odds ratio (OR) and the corresponding 95% confidence intervals (CI) in order to assess the association between different polymorphisms with CL risk. SHEs is software was used for analysis of haplotype frequency and linkage disequilibrium (LD)[14]. All reported p-values were two-tailed. A $p< 0.05$ was accepted as the level of significant.

Results

Demographic characteristics: Table 2 shows the demographic and clinical characteristics of the study population. Mean age of patients and controls was 29.32±11.4 years and 31.18±12.9 years respectively with no significant difference. Likewise, there was no significant difference between the two groups in gender, residence or BMI.

In patients, the number of lesions ranged from 1 to 5. More than half of patients had more than 3 lesions at presentation. The most common type of lesions were dry lesions which affected 32 patients (47.09%) followed by wet lesions (32.34%) and then mixed lesions (20%).

Gene amplification and genotyping: Restriction fragment length polymorphism was used for genotyping of the three SNPs in *SELL* gene. According to this technique, rs2205849 appeared in three genotypes in patients and controls: these were TT, CT and CC. The same genotypes were also reported for rs1131498. Finally, the genotypes for rs2229569 were CC, CT and TT. The distribution of different genotypes of the three SNPs was in good agreement with Hardy Weinberg equilibrium (Table 3).

The frequency of TT, CT and CC genotypes in patients was 61.67%, 33.82% and 4.41% respectively compared to 52.86%, 37.14% and 10% respectively in controls with no significant differences. At allelic level, T allele was slightly more frequent in patients than controls (78.68% versus 71.43%), however, the difference was not significant. Almost similar pattern of distribution for genotypes and alleles was encountered for rs1131498 and rs2229569. The wild type allele and genotype were more frequent among patients while the mutant allele and genotype were more frequent among controls; however, the differences were not significant.

Table 1
Primers, restriction enzymes and fragment length of three SNPs in *SELL* gene

Polymorphism	Primer sequence 5' to 3'	$T_{annealing}$, C^o	Restriction enzyme	Fragment length (bp)
rs2205849	F: CCTCCTTCACTCATTGTTG R: AGGTACTTGTAGGCTCCC	59	*PfM I*	CC:445 CT:445+345+100 TT: 445+345
rs1131498	F: TTACCTAAGAAGAAGCAAAGA R: ATGGAGAATGTGTAGAAATCA	61	*Alu I*	AA:115 AG:115+74+41 GG: 74+41
rs2229569	F: TGATTCAGTGTGAGCCTTTG R: CTTGACAGGTTGGTTCTG	58	*Hph I*	CC:186 CT:186+141+45 TT:141+45

Table 2
Demographic and clinical characteristics of the study population

Variables	Patients (68)	Controls (70)	P-value
Age, years (mean±SD)	29.32±11.4	31.18±12.9	0.711
Gender, No (%)			
Male	44(64.71%)	39(55.71%)	0.178
Female	24(35.29%)	31(44.29%)	
Residence, No (%)			
Rural	56(82.5%)	48(68.57%)	0.431
Urban	12(17.65%)	22(31.43%)	
BMI, kg/m^2, mean±SD	26.72±4.78	23.91±8.23	0.196
Number of lesion, No (%)			
≤ 3	29(42.65%)		
>3	39(57.35%)		
Type of lesion, No (%)			
Dry	32(47.09%)		
Wet	22(32.34%)		
Mixed	14(20.58%)		

Table 3
The frequency of different genotypes and allele of *SELL* gene polymorphisms in patients with leishmaniasis and healthy controls.

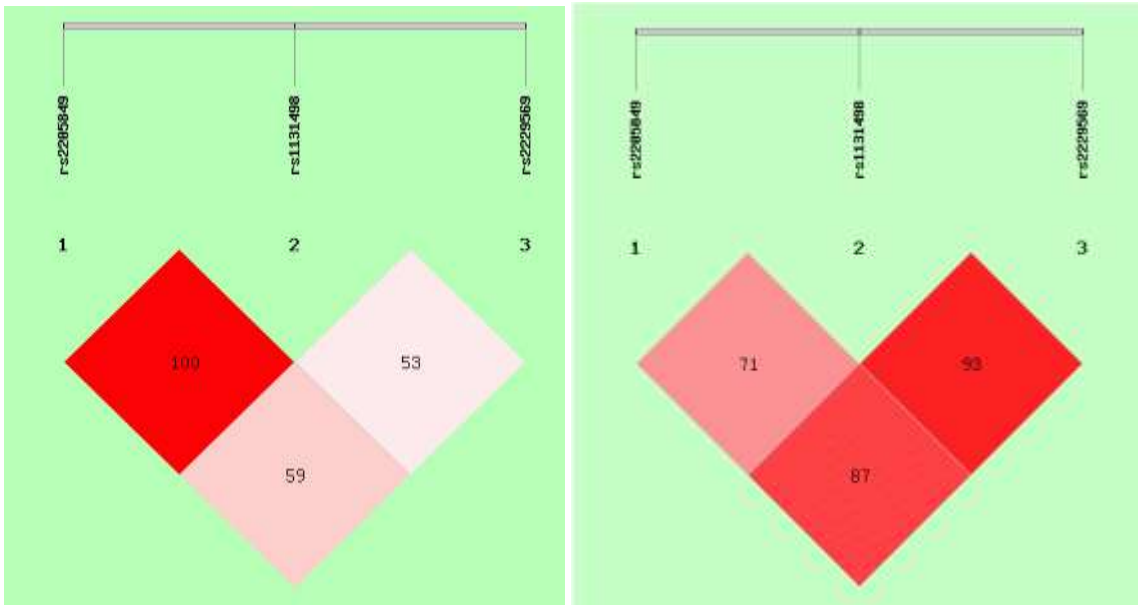
Variants	Cases (68)	Controls (70)	*P*-value	OR (95%CI)
rs2205849				
Genotypes				
TT	42(61.67%)	37(52.86%)	0.412	1.0 Reference
CT	23(33.82%)	26(37.14%)	0.614	0.83(0.41-1.7)
CC	3(4.41%)	7(10%)	0.191	0.39(0.09-1.61)
HWE	0.947	0.451		
Alleles				
T	107(78.68%)	100(71.43%)	0.146	1.0 Reference
C	29(21.32%)	40(28.57%)		0.68(0.39-1.18)
rs1131498				
Genotypes				
TT	51(76.47%)	46(65.71%)	0.263	1.0 Reference
TC	16(25%)	19(27.14%)	0.487	0.76(0.35-1.65)
CC	1(1.47%)	5(7.14%)	0.124	0.18(0.02-1.6)
HWE	0.84	0.146		
Alleles				
C	118(86.76%)	111(79.29%)	0.098	1.0 Reference
T	18(13.24%)	29(20.71%)		0.58(0.31-1.11)
rs2229569				
Genotypes				
CC	26(38.24%)	29(41.43%)	0.447	1.0 Reference
CT	37(54.41%)	32(45.71%)	0.483	1.29(0.63-2.62)
TT	5(7.35%)	9(12.86%)	0.44	0.62(0.18-2.09)
HWE	0.094	0.97		
Alleles				
C	89(65.44%)	90(64.29%)	0.841	1.0 Reference
T	47(34.56%)	50(35.71%)		2.95(0.58-1.56)

Haplotype analysis: Table 4 shows the most frequent five haplotype blocks for the three polymorphisms in the *SELL* gene. Among these blocks, only TTC was significantly more frequent among patients than controls (20.1% versus 7.83%, p<0.001). The CCC block was also more frequent in patients than controls (4.3% versus 1.0%); however, the difference was not significant (p= 0.09).

Analysis of linkage disequilibrium: The results of LD analysis are presented in figure 1. LD plot was constructed using genotype data from CL patients and controls. The three SNPs, rs2205849, rs1131498 and rs2229569 of *SELL* gene are in strong LD (the measure D' is very close to 1) especially in control group suggesting that major alleles of these variants are likely to be inherited together.

Table 4
Haplotype blocks of *SELL* polymorphism in *Leishmania* patients and healthy controls.

Haplotype blocks	Frequency in cases	Frequency in controls	p-value	OR (95%CI)
CCC	5.96 (4.3%)	1.46 (1.0%)	1.0	Reference
CCT	13.04 (9.4%)	20.13(14.4%)	0.205	0.62(0.29-1.3)
CTT	10.9 (7.4%)	16.63(11.9%)	0.204	0.59(0.26-1.34)
TTC	79.23 (57.4%)	86.76(62%)	0.436	0.83(0.51-1.34)
TTT	27.77 (20.1%)	7.83 (5.6%)	<0.001	4.25(1.85-9.81)



(A) **(B)**

Figure 1: Linkage disequilibrium plot for *SELL* variants (A) in cases and (B) controls. LD plot was built using combined genotype data and displayed as pairwise D' values multiplied by 100.

Discussion

The present study aimed to investigate the possible role of three SNPs in *SELL* gene in the susceptibility to CL. The results indicated that none of the included SNPs had a significant association.

For rs2205849 (-642C>T) polymorphism, it is located in the 5'-flanking sequence and accordingly it probably affects gene transcription through increase or decrease of the binding of the transcription factors. However, most available studies reported no significant association of this SNP with different pathologies. Alkhateeb et al[15] explored the role of some SNPs in the genes of adhesion molecules (including rs2205849 in *SELL* gene) in the recurrent aphthous stomatitis among Jordanian population while rs5361 SNP in *E-selectin* gene was associated with increase the risk of this disease. rs2205849 had no role.

In another study including 523 patients with systemic lupus erythematosus, Russel et al[16] reported no association between -642C>T polymorphism and the susceptibility to this immune disorder. However, in one study, the minor allele (allele T) was found to have a protection role against acute coronary syndrome (ACS)[11].

The functional SNP rs2229569 (Pro226Ser or 676C>T, also known as P213S or 725C>T) is located in exon 6 of *SELL* gene corresponding the domain 1 of L-selectin. Thus, it is hypothetically involved in the interaction between leukocytes and endothelium[17]. It involves the substitution of proline with serine amino acid. The results from studies regarding this polymorphism were very conflicting. In a local study, Saed et al[18] reported that allele T (encodes for serine) could increase the risk for type 2 diabetes mellitus (T2DM).

Similarly, in a recent Romanian study, Toma et al[10] found that TT genotype may represent a risk for colo-rectal cancer whereas the CC genotype may have a protective role. In contrast, T allele has been shown to have a protective role against ACS in the Mexican study[11]. On the other extreme, a British study showed no significant association between different genotypes and alleles of this SNP with the complications of sickle cell anemia[19].

The most frequently investigated SNP in *SELL* gene in previous studies was rs1131498 which is located in exon 7 of this gene. It is also known as Phe206Leu or 616C>T. It is position in the EGF-like domain which may cause altered ligand binding. In one study from Poland, the protective role of the C allele (encodes for phenylalanine) with respect to T1DM was demonstrated[20]. Likewise, an association between this allele and a higher concentration of *SELL* compared to carriers of the wild-type allele was demonstrated[15].

In a recent study among Iranian patients with tuberculosis (TB), Eini et al[21] found no significant relationship between this SNP and the incidence of TB. However, a previous study reported a significant association between C allele with the risk for brucellosis[22]. Another Iranian study which is similar to the current study but on VL revealed no significant association of this polymorphism with the incidence of VL[23].

The most interesting result in the current study was the significant association of TTC haplotype with the CL (OR= 4.25,95%CI=1.85-9.81, p<0.001), which implies that carriers of this haplotype have 4.25 times risk for infection with CL than those carrying CCC haplotype under the same environmental circumstances. These results suggest the possible role of each individual polymorphism, but may be because the small sample size, the individual effect of each SNP did not appear. When the three SNPs were considered collectively by haplotype analysis, their effect appeared significantly.

Two inferences follow from this result. First, it can be assumed that less L-selectin expression and/or effectivity is associated with TTC haplotypes according to the results of the previous studies that explored the association of each individual SNP with soluble L-selectin[12,16]. Secondly, this haplotype will associate with some defect in immune response against CL. In this regard, it was found that the monocyte recruitment to the skin and lymph nodes and subsequent differentiation into dendritic cells (DCs) is essential for immunity to CL. Monocytes migration through the dermal venules was found to be dependent on the interaction between L-selectin on these cells and peripheral node addressin (PNAd) on the epithelial cells of the venules[10].

When anti-L-selectin monoclonal antibodies were used, a complete blockade of monocyte migration happened[24]. Thus, the down-regulation of L-selection expression, or any structural defect in L-selectin can significantly influence monocyte migration with a subsequent impediment of immune response and increase susceptibility to CL.

Conclusion

Interestingly, there was almost strong LD between the three SNPs both in patients and controls with mean that the minor alleles of these SNPs pass simultaneously to the offspring. Clinically, this gives these SNPs another importance in that CCT carriers are not only themselves susceptible to CL, but their progenies also.

To sum up, these data indicate the non-significant association of the SNPs rs2205849, rs1131498 and rs2229569 separately with CL. However, the haplotype TTC from them can significantly increase the individual susceptibility to CL especially if there is a strong LD between them.

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