

## Candidate gene study of susceptibility to cutaneous leishmaniasis in Sri Lanka

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### Summary

**OBJECTIVES** To investigate the association between selected single nucleotide polymorphisms (SNPs) in *TNF*, *LTA* and *SLC11A1* genes and risk of endemic cutaneous leishmaniasis (CL) in Sri Lanka through a case–control disease association study.

**METHODS** An anonymized DNA resource representative of the Sri Lankan population was genotyped initially to establish baseline parameters. This was followed up by genotyping 200 patients and 200 matched controls. Published or modified PCR/RFLP methods were employed for genotyping.

**RESULTS** Comparison of the different ethnic groups showed the distribution of alleles of *LTA* +252 A>G to differ significantly in Tamils and Moors when compared with Sinhalese. The differences seen at allele level were also reflected in the haplotypes defined by these SNPs at the *TNF* locus. The case–control analysis did not show an association between the SNPs or the haplotypes investigated and CL. The distribution of these variant alleles in other populations, where they are positively associated with leishmaniasis, differed significantly from the Sri Lankan study cohort.

**CONCLUSIONS** The selected polymorphisms do not predispose to CL in the Sri Lankan population. The study of extended haplotypes at these loci using a sufficiently powered sample collection would elaborate the findings of this study. In the face of an evolving disease pattern in the country with other forms of leishmaniasis now being reported, prevalence of polymorphisms predisposing to these forms calls for heightened surveillance and preparedness.

**keywords** cutaneous leishmaniasis, genetic susceptibility, *TNF* locus, *SLC11A1*, Sri Lanka

### Introduction

Leishmaniasis is a group of parasitic diseases of global proportions with 12 million people affected and an estimated two million new cases occurring every year (WHO 2009). The localized cutaneous form of leishmaniasis (CL) is endemic in Sri Lanka. The parasite in the country has been identified as *Leishmania donovani* MON 37 (Karunaweera *et al.* 2003). This species commonly causes visceral leishmaniasis (VL) in many Asian and East African foci. More than 2000 patients have been diagnosed since 2001 at the Department of Parasitology, Faculty of Medicine, Colombo alone, which is a tertiary referral centre for diagnosis of leishmaniasis in Sri Lanka. Even though not potentially fatal or debilitating as the visceral or the mucocutaneous forms, cutaneous leishmaniasis (CL) can be personally and socially disruptive (Herwaldt 1999) and has been recognized in its own right as a disease entity requiring intense control measures (WHO 2007).

As with many other parasitic diseases, the clinical manifestations of leishmaniasis are a result of the interplay

between parasite, host and environmental factors (Akilov *et al.* 2007). The finding that the three species of *Leishmania* responsible for different disease phenotypes in fact shows very little genetic diversity (Peacock *et al.* 2007) which further highlights the contribution of host factors in determining the outcome of infection. In Sri Lanka, our previous epidemiological findings that point towards clustering of the condition among Sinhalese suggest the possibility of genetically determined host factors contributing to susceptibility to CL (Samaranyake *et al.* 2008).

The variability in the immune response and the ensuing pathology confer susceptibility to leishmaniasis and also contribute to its varied clinical outcomes (Blackwell 1999; Handman *et al.* 2005). Thus, genes implicated in the immune responses have been the focus of many studies examining host susceptibility to leishmaniasis. Three candidate genes: tumour necrosis factor (*TNF*; previously called tumour necrosis factor alpha), lymphotoxin alpha (*LTA*; previously called tumour necrosis factor beta) and solute carrier family 11A member 1 (*SLC11A1*; previously called NRAMP1) – which have received such particular

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attention were selected by us for our candidate gene disease association studies.

T helper 1-(Th1) and Th2-type immune responses with their associated profile of cytokines have been associated with resistance or susceptibility to leishmaniasis respectively (Roberts *et al.* 2000). TNF, a Th1-type cytokine with a broad spectrum of biological effects, is one of the initial factors to be secreted in the body's defense response against infection and inflammation. Its role in activating macrophages is especially important in controlling infections with *Leishmania*; where the parasite is adapted to survive in the hostile environment of the lysosomal compartment of macrophages (Murphy *et al.* 2007). However, high levels of activity of TNF, as defined by an exaggerated and over-aggressive response, have been implicated in clinical manifestations of leishmaniasis as well as their severity (Castes *et al.* 1993). A single nucleotide polymorphism (SNP) in the promoter region of *TNF* (-308G>A) has been widely linked to the magnitude of this secretory response and confers susceptibility to mucocutaneous and visceral forms of leishmaniasis (Cabrera *et al.* 1995; Blackwell 1999; Karplus *et al.* 2002).

*LTA* which lies in tandem with *TNF* in HLA class111 region of chromosome 6 encodes lymphotoxin alpha, a proinflammatory cytokine with a similar but distinct role to that of TNF. A similar correlation has been made between the +252A>G SNP in intron 1 of *LTA* which predisposes to leishmaniasis, with the variant allele being associated with elevated cytokine levels (Messer *et al.* 1991). Furthermore, various studies have suggested that polymorphisms in genes encoding these two cytokines mutually influence their production (Pociot *et al.* 1993; Bouma *et al.* 1996). These two SNPs define four haplotypes and those constituting one or both variant alleles which have been significantly associated with different manifestations of CL in some populations (Cabrera *et al.* 1995).

*SLC11A1* is considered to play a key role in innate host resistance to intra macrophage pathogens. It encodes a proton/divalent cation (Fe<sup>2+</sup>, Zn<sup>2+</sup> and Mn<sup>2+</sup>) antiporter which localizes to the late endosomal/lysosomal compartment of the macrophage. Its functionality is associated with an enhanced activity of proinflammatory immune pathways, including the formation of nitric oxide (NO) via transcriptional stimulation of inducible nitric oxide synthase (iNOS) expression. Several polymorphisms of this gene in both 5' and 3' regions have been implicated in VL. In Sudan, the 274C>T polymorphism in exon 3 and 469+14G>C polymorphism in intron 4 were associated with susceptibility to VL caused by *L. donovani* (Mohamed *et al.* 2003) -the same species causing CL in Sri Lanka. We hypothesized that some of these candidate

markers may also predispose patients to the cutaneous form of the disease, caused by a dermatropic variant of the same parasite species.

There is only limited data on genetic variants predisposing to CL (Lara *et al.* 1991; Cabrera *et al.* 1995; Kamali-Sarvestani *et al.* 2006). Furthermore, there are no reports of such studies in South Asian populations. The objectives of this study therefore were: (i) to establish frequencies of the variant alleles of the *TNF* -308G>A, *LTA* +252 A>G and *SLC11A1* 274 C>T, *SLC11A1* 469+14G>C, *SLC11A1* D543N polymorphisms in the three major ethnic groups in the Sri Lankan population; - Sinhalese, Sri Lankan Tamils, and Moors (ii) to establish the frequencies of the haplotypes defined by these SNPs in the three major ethnic groups in the Sri Lankan population (iii) to carry out a case-control study to examine the association of these SNPs and the haplotypes defined by them with CL in the Sri Lankan population.

## Materials and methods

### Subjects

These investigations constituted an initial population genetic study, followed by a case-control study. Samples for the population genetic study were from an anonymized population-based sample collection maintained in the Human Genetics Unit of the Faculty of Medicine, University of Colombo (FMUC). This collection had been made for studies of this nature with the approval of the Ethics Review Committee of FMUC (Dissanayake *et al.* 2009). Thirty each of Sinhalese, Sri Lankan Tamil and Moor samples (50% men) were randomly selected from this collection and genotyped.

Subjects for the case-control studies were recruited prospectively according to a protocol approved by the Ethics Review Committee of FMUC. All subjects gave written informed consent to participate in the study. Cutaneous leishmaniasis was confirmed in those with clinically suggestive skin lesions by direct smear and/or culture.

The initial case-control collection consisted of 200 patients with CL and 200 controls matched for age, sex, ethnicity and area of residence. A detailed discussion of recruitment of this cohort and their phenotypic features has already been published (Samaranyake *et al.* 2008).

### Genotyping

DNA was extracted from peripheral venous blood using the Wizard genomic DNA purification kit (Promega Corp,

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USA). Genotyping was performed using PCR followed by restriction digestion, as described previously (Liu *et al.* 1995; Wattavidanage *et al.* 1999) or by using modified PCR primers to introduce restriction sites. Detailed genotyping methods are provided as supplementary information (Table S1).

**Statistical analysis**

The Chi-squared test was used to test the genotypes at each polymorphic locus for Hardy–Weinberg equilibrium (HWE) in the three populations and in cases and controls separately; and to compare differences in allele frequencies between cases and controls. Estimation of haplotype frequencies, measurement of pairwise linkage disequilibrium ( $D'$  and  $r^2$ ) and hypothesis testing were carried out using Haploview (Barrett *et al.* 2005). Rare haplotypes occurring at combined frequencies of <0.05 were omitted from the analysis. All reported  $P$ -values are two tailed. QUANTO version 1.2.3 (Gauderman & Morrison 2007) was used for power calculations assuming an additive model for the variant alleles.

**Results**

Tables 1 & 2 show the results of the population genetic study. The genotype and variant allele frequencies of the SNPs in the candidate genes are in Table 1. All SNPs were in HWE. The distribution of alleles of *LTA* +252 A>G in Tamils and Moors differed significantly from that of Sinhalese.

The haplotypes defined by the two SNPs in the *TNF* and *LTA* genes were considered together as the two genes are located next to each other on chromosome 6. The differences seen at SNP level were also reflected in the haplotypes defined by these SNPs. The haplotypes on chromosome 2 defined by the SNPs in the *SLC11A1* gene were considered separately. The results are in Table 2.

A summary of the demographic and phenotypic features of the initial collection of 200 cases with CL is given in Table 3. The number of samples which had to be excluded from the analysis because of the inability to determine genotype did not exceed 1% (4/400) for any of the SNPs. All polymorphisms in cases and controls were in HWE.

**Table 1** Genotype and variant allele frequencies of polymorphisms in the *TNF*, *LTA* and *SLC11A1* genes in the Sri Lankan population

Genotype/Variant allele	Sinhalese (S)	Tamils (T)	Moors (M)	Significance ( $P$ )		
	N (%)	N (%)	N (%)	SvsT	SvsM	TvsM
<i>TNF</i> G-308A (rs1800629)						
GG	17 (56.7)	13 (43.3)	19 (63.3)	0.10	0.82	0.07
GA	13 (43.3)	13 (43.3)	10 (33.3)			
AA	0 (0)	4 (13.3)	1 (3.3)			
rs1800629A	13 (21.7)	21 (35.0)	12 (20.0)			
<i>LTA</i> A252G (rs909253)						
GG	0 (0)	2 (6.7)	2 (6.7)	0.02	0.01	0.85
GA	10 (33.3)	17 (56.7)	18 (60.0)			
AA	20 (66.7)	11 (36.7)	10 (33.3)			
rs909253G	10 (16.7)	21 (35.0)	22 (36.7)			
<i>SLC</i> C274 T (rs2276631)						
CC	23 (76.7)	25 (86.2)	23 (76.7)	0.34	0.89	0.27
CT	7 (23.3)	4 (13.8)	6 (20.0)			
TT	0 (0.0)	0 (0.0)	1 (3.3)			
rs2276631T	7 (11.7)	4 (6.9)	8 (13.3)			
<i>SLC</i> G469 + 14C (rs3731865)						
GG	26 (92.9)	24 (80.0)	24 (80.0)	0.14	0.17	0.90
GC	2 (7.1)	6 (20.0)	6 (20.0)			
CC	0 (0.0)	0 (0.0)	0 (0.0)			
rs3731865C	2 (3.6)	6 (10.0)	6 (10.0)			
<i>SLC</i> D543N (rs17235409)						
GG	27 (93.1)	25 (86.2)	25 (83.3)	0.40	0.28	0.81
GA	2 (6.9)	4 (13.8)	5 (16.7)			
AA	0 (0.0)	0 (0.0)	0 (0.0)			
rs17235409A	2 (3.4)	4 (6.9)	5 (8.3)			

**Table 2** Haplotypes defined by SNPs on chromosome 6 (of *LTA* and *TNF*) and on chromosome 2 (of *SLC11A1*) in the Sri Lankan population

Locus/Haplotype	Frequency			Significance ( <i>P</i> -value)				
	Sinhalese (S)	Tamils (T)	Moors(M)	SvT	SvM	TvM		
<i>LTA</i> 252								
<i>TNF</i> -308								
A	G	0.69	0.47	0.58	0.01	0.17	0.29	
A	A	0.14	0.18	0.05	0.58	0.13	0.06	
G	G	0.09	0.18	0.22	0.16	0.04	0.62	
G	A	0.08	0.17	0.15	0.11	0.27	0.75	
<i>SLC11A1</i>	<i>SLC11A1</i>	<i>SLC11A1</i>						
274	469+14	1703						
C	G	G	0.83	0.79	0.74	0.58	0.20	0.50
T	G	G	0.11	0.03	0.07	0.10	0.62	0.24
C	G	A	0.02	0.06	0.07	0.17	NA	0.78
C	C	G	0.01	0.07	0.05	NA	NA	0.63

NA indicates those haplotypes with a combined frequency of <5.0%. These were excluded from the analysis.

**Table 3** Summary of demographic and phenotypic features of patients with CL

Characteristic	No. and (%) of patients
Gender	
Male	143 (71.5)
Female	57 (28.5)
Age group (years)	
0–9	3 (1.5)
10–19	28 (14.0)
20–29	48 (24.0)
30–39	58 (29.0)
40–49	27 (13.5)
50–59	15 (7.5)
60–69	14 (7.0)
70–79	6 (3.0)
80–89	1 (0.5)
Ethnic group	
Sinhalese	200 (100.0)
No. of lesions	
Single	180 (90.0)
Multiple (1–4)	20 (10.0)
Multiple affected family members	11 (5.5)

The results of the case–control analysis at single SNP level are in Table 4. All SNPs were in HWE. None of the SNPs or haplotypes (results not shown) was associated with CL.

In an attempt to understand the differences in results obtained in this study and previously published studies, we compared the allele frequencies and haplotype frequencies we had observed with those reported in previous studies, where a significant association for these polymorphisms with leishmaniasis was documented (Table 5). All variant

alleles of *TNF*, *LTA* and *SLC11A1* and most haplotype frequencies (results not shown) at the *TNF* locus in these populations differed significantly from those of Sinhalese. A haplotype comparison at *SLC11A1* was not possible as the relevant frequencies in the Sudanese have not been reported (Mohamed *et al.* 2003).

### Discussion

In this study, we set out to investigate the association of selected genes with susceptibility to CL, using SNPs and haplotypes defined by these SNPs as genetic markers. The analysis of these genetic markers in the cases and controls was preceded by a population genetic study to establish the background population frequencies.

*TNF* has been associated with many infectious and inflammatory conditions. Similarly, *SLC11A1* has been associated with mycobacterial diseases such as tuberculosis (Shaw *et al.* 1997), leprosy (Abel *et al.* 1998) and also a range of auto immune diseases (Rodríguez *et al.* 2002, Esposito *et al.* 1998, Kotze *et al.* 2001). Thus, the baseline data on the frequency of the variations in these genes in the Sri Lankan population that we report here would help in designing genetic association studies of sufficient power for these conditions in the Sri Lankan population.

The population genetic study showed significant differences in allele and haplotype distribution at the *TNF* locus among the main ethnic groups which constitute the Sri Lankan population, with Sinhalese differing significantly from Tamils and Moors. This finding was interesting in view of our observation during recruitment of cases, where the condition was confined almost exclusively to Sinhalese (Samaranyake *et al.* 2008).

**Table 4** Genotype and variant allele frequencies of *TNF*, *LTA* and *SLC11A1* polymorphisms among cases and controls and their association with CL

SNP/Gene	Genotype/Variant allele	Cases N (%)	Controls N (%)	OR	95% CI	P-value
rs1800629 <i>TNF</i>	GG	121 (60.5)	114 (57.0)	1.00		
	GA	67 (33.5)	73 (36.5)	0.865	0.569–1.314	0.5
	AA	10 (5.0)	11 (5.5)	0.856	0.35–2.094	0.73
rs909253 <i>LTA</i>	rs1800629A	87 (21.96)	95 (23.98)			
	AA	97 (48.5)	89 (44.5)	1.00		
	GA	91 (45.5)	97 (48.5)	0.861	0.574–1.291	0.47
rs2276631 <i>SLC11A1</i>	GG	11 (5.5)	14 (7.0)	0.721	0.31–1.671	0.44
	rs909253G	113 (28.3)	125 (31.25)			
	CC	151 (75.5)	147 (73.5)	1.00		
rs3731865 <i>SLC11A1</i>	CT	42 (21.0)	48 (24.0)	0.852	0.531–1.366	0.51
	TT	5 (2.5)	4 (2.0)	1.217	0.320–4.621	0.77
	rs2276631T	52 (13.3)	56 (14.5)			
rs17235409 <i>SLC11A1</i>	GG	175 (87.5)	176 (88.0)	1.00		
	GC	22 (11.0)	22 (11.0)	1.006	0.537–1.882	0.97
	CC	0 (0.0)	0 (0.0)			
rs17235409 <i>SLC11A1</i>	rs3731865T	22 (5.6)	22 (5.6)			
	GG	164 (82.0)	168 (84.0)	1.00		
	GA	34 (17.0)	27 (13.5)	1.29	0.745–2.234	0.36
	AA	1 (0.5)	1 (0.5)	1.024	0.064–16.515	0.97
	rs17235409A	36 (9.2)	29 (7.3)			

Gene	Allele	Population (phenotype)			Significance ( <i>P</i> -value)
		Sinhalese (CL)	Venezuelans (MCL)	Sudanese (VL)	
<i>TNF</i>	rs1800629A	21.7	7.0		<0.05
<i>LTA</i>	rs909253G	16.7	69.0		<0.05
<i>SLC11A1</i>	rs2276631T	11.7		79.9	<0.05
	rs3731865C	3.6		12.0	0.018
	rs17235409A	3.4		88.0	<0.05

**Table 5** Distribution (%) of variant alleles predisposing to leishmaniasis in other study populations

MCL, muco cutaneous leishmaniasis; VL, visceral leishmaniasis.

However, the case–control analysis provided no evidence for an association of the SNPs and haplotypes defined by these SNPs with predisposition to CL. To the best of our knowledge, this is one of the largest studies on the association of genes with CL to be conducted and the first such study to be conducted in the South Asian region. In this study, recruitment of patients was subjected to stringent criteria to avoid population stratification as mentioned previously. The sample size of this case–control study provided 80% power to detect a genotype relative risk between 1.5 and 2.1 depending on variant allele frequencies, at a significance level of 0.05. The power to detect smaller genetic effects, which may be the case in a multifactorial disease of this nature, is limited by sample

size. This may explain our finding of lack of predisposition of the selected markers and CL. Conversely, considering polymorphisms at the *TNF* locus, our results are in agreement with other studies (Cabrera *et al.* 1995; Kamali-Sarvestani *et al.* 2006) where no association was detected between these SNPs and CL. Interestingly, the studies which found significant associations of *TNF* locus with muco cutaneous leishmaniasis (Cabrera *et al.* 1995) caused by *Leishmania braziliensis* or symptomatic VL caused by *Leishmania chagasi* (Karplus *et al.* 2002) had 80% power to detect a genotype relative risk of 6.8 or 2.1 respectively, depending on variant allele frequencies assuming a baseline population risk of 50% and an additive model of inheritance. These observations, taken together with the

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observation that frequencies of variations in the candidate genes in Sinhalese differed significantly from other populations with positive associations, support the conclusion that these variations are not associated with CL in the Sinhalese.

We noted several interesting trends in our dataset even though statistically significant conclusions could not be drawn. The variant alleles of *TNF* and *LTA* which has been associated with susceptibility to CL in other studies (–308A and +252G respectively) showed a negative risk when the heterozygous and the rare homozygous states of patients were compared against the controls (OR<1). Further, in *SLC11A1* 469+14G>C, the risk was almost equivalent (OR = 1.006) in the heterozygous state.

The previous observations reiterate the fact that the final clinical outcome in leishmaniasis is dependent upon the diversity of the human host, parasite and less well defined other local factors. The contradictory results regarding the contribution of genotypes and alleles highlight the variable genetic effect across populations as well as the possibility of other polymorphisms in linkage disequilibrium which influence the outcome of disease. Thus, the importance of focusing on complete genes defined by their haplotypes rather than on individual polymorphisms in assessing genetic risk needs to be emphasized. Possible differences in the underlying mechanisms of pathophysiology should also be considered in interpreting these differences in findings. Furthermore, investigating the polymorphisms of a cytokine and association of disease in a population will not be complete without an understanding of the polymorphisms in the genes coding for its receptors as demonstrated at the TNF cluster (Hajeer & Hutchinson 2000).

In conclusion, the data of our study showed that the selected polymorphisms did not influence susceptibility to CL in the Sri Lankan population. The study of extended haplotypes at these loci using a sufficiently powered sample collection would elaborate the findings of this study. The allele and haplotype frequencies reported here would be useful in accurate design of such an investigation. The recent reports from Sri Lanka of patients with mucosal localization as well as the visceral form of disease acquired locally indicate an evolving pattern of disease. The prevalence of polymorphisms linked to susceptibility of these forms as demonstrated by this study, calls for heightened measures of surveillance and preparedness in the island.

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Table S1.** PCR conditions used for determination of the selected gene polymorphisms.

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