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IFNG and IFNGR1 gene polymorphisms and susceptibility to post kala-azar dermal leishmaniasis in Sudan

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Abstract

Post kala-azar dermal leishmanaisis (PKDL) in Sudan is associated with elevated interferon-γ. To study interferon-γ pathways in PKDL, we genotyped 80 trios from the Masalit ethnic group for polymorphisms at −470 ins/delTT, -270T/C, -56T/C and +95T/C in IFNGR1, and at −179G/A and +874T/A in IFNG. No associations occurred at IFNG. Global association with haplotypes comprising all 4 markers at IFNGR1 (χ^2_{10df} = 21.97, P=0.015) was observed, associated with a significant (χ^2_{1df}=4.54, P=0.033) bias in transmission of the haplotype insTT T T T, and less (χ^2_{1df}=5.59, P=0.018) than expected transmission of insTT C C C. When compared with data on malaria associations from The Gambia, the results suggest a complex pattern of haplotypic variation at the IFNGR1 promoter locus associated with different infectious disease in African populations that reflect the complex roles of IFN-γ in parasite killing versus inflammation and pathogenesis.

Keywords

PKDL; leishmaniasis; association; IFNGR1

In Sudan more than 50% of cases successfully treated for visceral symptoms caused by *Leishmania donovani* go on to develop post kala-azar dermal leishmaniasis (PKDL).1 Interferon- γ (IFN-γ) is a key T helper 1 and natural killer cell cytokine. Its activates macrophages to kill parasites,2 exerting its effects through its receptor IFNGR1. IFNγ is present in PKDL lesions, and PKDL is associated with elevated antigen-specific IFNγ T cell responses.3,4

IFNG and IFNGR1, located on chromosomes 12q15 and 6q23.3, are important candidate genes for PKDL. Rare mutations in the coding region of *IFNGR1* are the cause of Mendelian inheritance of susceptibility to non-tuberculous mycobacteria in consanguineous families.5-7 Compound heterozygosity for rare alleles at *IFNGR1* was also associated with disseminated infection with non-tuberculous mycobacteria, and impaired cellular response to IFN-γ, in a child born to nonconsanguinous parents 8. In mapping this deficiency, a CA

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Electronic Database Information
1. Stata Version 8.0 (http://www.stata.com/)
2. GenAssoc package (http://www-gene.cimr.cam.ac.uk/clayton/software/stata/)
3. TRANSMIT program (http://www.gene.cimr.cam.ac.uk/clayton/software)

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repeat in intron 5 of *IFNGR1* was identified and designated FA1 8. We found FA1 was linked and associated specifically with PKDL, and not visceral leishmaniasis (VL) *per se*, in the Masalit ethnic group in Sudan 9.

To understand natural population variation at *IFNGR1*, sequence analysis of the promoter locus between -1400 and +100 of the translational start site was undertaken in 140 Gambian chromosomes.10 This identified 3 T-to-C single nucleotide polymorphisms (SNPs) at +95, -56 and -270 nucleotides, and a TT deletion at -470/-471 nucleotides. Variant alleles at the -56 bp (T/C alleles at frequency 0.5) and +95 bp SNPs (C allele 0.49) were common, and heterozygotes for the -56 bp SNP were protected against cerebral malaria and death from cerebral malaria.10 Variant alleles at -270 (C allele 0.02) and -470 (delTT allele 0.11) were less frequent in the Gambia. Nevertheless, for the Mandinka ethnic group, the double deletion at -470 bp was protective against severe malaria. No associations were observed for any of these polymorphisms with pulmonary tuberculosis caused by *Mycobacterium tuberculosis* in the Gambia,11 or at -56 T/C in Croatians.12

Two possible functional polymorphisms occur at *IFNG*. The +874 A/T SNP in intron 1 lies within a nuclear factor κB binding site, and the variant T allele is in complete linkage disequilibrium (LD) with a CA repeat allele associated with high IFN-γ production.13 A -179 G/A SNP in the proximal promoter also controls gene transcription.14

The aim of the present study was to determine whether further information on the association between PKDL and the IFN-γ pathway could be obtained by genotyping additional SNPs at *IFNGR1*, as well as the putative function-associated SNPs at *IFNG*. Samples were collected from two adjacent villages, Um Salala and El-Rugab, in the highly endemic area of eastern Sudan. All individuals were from the Masalit tribe, which is highly susceptible to VL and PKDL. Details of the site and diagnostic criteria for PKDL were described previously.9 Ethical approval for this study was from the Local Research Ethics Committee of the University of Khartoum. DNA from buccal swabs from 80 trios was genotyped for the -470 ins/del, -270 T/C, -56 T/C (rs2234711) and +95 (rs7749390) SNPs at *IFNGR1*, and the -179 G/A (rs2069709) and +874 (rs2430561) SNPs at *IFNG*. Tests using genetically independent family members confirmed that all four *IFNGR1* polymorphisms, and the two *IFNG* gene SNPs, were in HWE ($P>0.05$). As determined for Gambian populations (0.49 and 0.56, respectively),10 the frequencies of the minor allele in *IFNGR1* +95C and *IFNGR1* −56C were high (0.45 and 0.46, respectively). Frequencies for minor alleles -270C and -470delTT were 0.05 and 0.03 in the Masalit, compared to 0.02 and 0.11 in Mandinka Gambians. Eleven haplotypes that were present in the Masalit (Table 1), 5 of which were not reported at frequencies >1% for the Mandinka.10 Of interest is the presence in the Masalit of two haplotypes, insTT T C T at 16% and insTT T T C at 16%, that were present at 3% and 2% in the Mandinka.10 Minor allele frequencies at *IFNG* -179G/A and +874A/T were 0.06 and 0.1 in the Masalit.

Single marker transmission disequilibrium tests carried out using TRANSMIT (Table 2) showed a significant bias in transmission of alleles at *IFNGR1* -270T/C ($\chi^2_{1df}=6.98$, $P=0.0087$) from heterozygous parents to PKDL offspring, but not for other *IFNGR1* or *IFNG* (data not shown) markers. Conditional logistic regression showed that PKDL was associated with the common T allele at *IFNGR1*-270 (odds ratio 3.67; 95% CI 1.02-13.45; $P=0.045$), while the minor C allele was associated with protection (odds ratio 0.27; 95% CI 0.08-0.98; $P=0.046$). Considering the complex haplotypic structure observed for the 4 promoter locus polymorphisms in African populations, we also looked for haplotype associations using TRANSMIT (Table 2). Haplotype analysis using all 4 markers showed a significant ($P=0.015$) global χ^2 (21.97) on 10df, associated with a significant ($\chi^2_{1df}=4.54$, $P=0.033$) bias in transmission of haplotype insTT T T T but not in transmission of the

haplotypes insTT T C C or insTT T C T. This suggests involvement of a functional variant in PKDL susceptibility that is in LD with the -56 bp SNP (underlined), even though single marker analysis did not show a significant association with PKDL disease. The global haplotype association for all 4 markers was also associated with significantly (χ^2_{1df}=5.59, *P*=0.033) less than expected transmission of haplotype insTT C C T that is at low frequency (2%) in the Masalit. Significant associations (Table 2) for 3 marker (e.g. insTT C C; insTT C T) and 2 marker (e.g. C C and C T for -270/-56) haplotypes were also associated with less than expected transmission of haplotypes carrying the -270 C allele (underlined), i.e. independently of variation at the -56 bp SNP. The involvement of more than one etiological variant across the *IFNGR1* promoter locus in determining susceptibility to, or protection against, PKDL in the Masalit population is consistent with data showing that the -56 bp SNP is in LD with the -470 bp SNP (D'=0.73) but not the -270 bp SNP (D'=0.13) or the +95 bp SNP (D'=0.22), and that the -270 bp SNP is not in LD with the +95 bp SNP (D'=0.26).

IFN-γ provides first line defense against infectious diseases by activating macrophages to kill intracellular pathogens. IFN-γ is also part of an inflammatory response that, if exaggerated, can be associated with adverse pathology. In VL, IFN-γ production is low at diagnosis and increases with treatment and clinical cure from visceral symptoms in all patients.4 However, peripheral blood mononuclear cells from patients that go on to develop PKDL show elevated proliferative and IFN-γ responses to *Leishmania* antigen compared to patients that do not, with no differences in IL-4, IL-5 or IL-10.4 Our study shows that this elevated IFN-γ response in PKDL patients does not appear to be associated with polymorphisms at the *IFNG* gene, despite studies showing high IFN-γ associated with the +874 bp T allele and low IFN-γ with the A/A genotype.13 Failure to detect associations with PKDL could be due to low frequency for variant alleles at IFNG SNPs in the Masalit.

PKDL lesions contain parasites and antigen, and an inflammatory infiltrate comprising macrophages, lymphocytes, and plasma cells.3 IL-10 is the most prominent cytokine in lesions, but IFN-γ is always present and IL-4 occurs in most lesions.3 One reason for failure of IFN-γ to activate macrophages to kill *Leishmania* in lesions could be inhibitory IL-10. Another, possibly related to the action of IL-10, could be failure to adequately express IFNGR1 due to regulatory polymorphisms in the gene. Previously9 we demonstrated association between different alleles at the non functional FA1 *IFNGR1* microsatellite8 and susceptibility to, or protection from, PKDL. Using a larger sample of PKDL trios we genotyped the four promoter locus polymorphisms at *IFNGR1*, some or all of which may regulate *IFNGR1* expression. These were originally identified in Gambians,10 where the rare variant allele -470 delTT was associated with all severe malaria in the Mandinka ethnic group, while heterozygosity at the -56 T/C SNP was associated with protection from cerebral malaria. In our study we found no direct association between PKDL and either -470 bp or -56 bp polymorphisms. However, markedly different frequencies of certain haplotypes across the four promoter locus polymorphisms were observed when comparing the Masalit from Sudan with the Mandinka from Gambia. In particular, we found two haplotypes, insTT T T C and insTT T C T that were rare (2% and 3%, respectively) in the Mandinka were more common (both 16%) in the Masalit. Whereas the common haplotype insTT T T T was significantly associated with PKDL, insTT T T C T and insTT T C C showed no bias in transmission to PKDL patients, suggesting that a functional variant in LD with the -56 bp SNP (at position 3 in these haplotype) might contribute to susceptibility to PKDL even though we found no direct association with the -56 bp SNP itself. In addition, we found that the rare -270 C allele, and haplotypes that contained this variant, were associated with protection from PKDL independently of variation at the -56 bp SNP. This suggests that a complex pattern of haplotypic variants exists in different African ethnic groups that may relate to differing exposures and susceptibilities to infectious disease. In this respect it is

interesting that the Masalit have a larger range of haplotypes involving the -270 C allele, that are protective for PKDL, compared to the Mandinka.

At present there is little data to determine whether these promoter locus variants are functional in determining IFNGR1 expression. Although experimental studies15 show differences in luciferase reporter gene activity associated with variants at -56 T/C, with lower activity for the C allele, our studies suggest that this SNP itself does not itself contribute to PKDL but could be in LD with a functional SNP on different haplotypes. Malaria is endemic in the region of Sudan where the present work on PKDL took place, but transmission is highly seasonal and the incidence of severe malaria is low. In the Masalit, the -56 bp C and T alleles are both common, which could relate to heterozygous advantage against cerebral malaria shown for this SNP10. However, Jüliger and coworkers15 also failed to find a direct association between this SNP and either mild or severe malaria in Gabonese children, suggesting that the earlier association with protection from cerebral malaria10 might also be due to haplotypic variation between Gambians and Gabonese, and a functional variant in LD with this SNP rather than -56 T/C itself. Further experimental analysis will be important in elucidating the role of regulatory polymorphisms at *IFNGR1* in determining susceptibility to these important clinical manifestations of protozoan parasitic diseases throughout Africa.

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Table 1
Estimated haplotype probabilities at the *IFNGR1* promoter

Haplotype identifier	*Haplotype*	*Estimated haplotype probabilities*	
		Masalit, Sudan	*Mandinka, Gambia*
1	insTT T T T	0.33	0.47
2	insTT T C T	0.16	0.03
3	insTT T C C	0.23	0.37
4	insTT T T C	0.16	0.02
5	delTT T C C	0.004	0.08
6	insTT C C C	0.03	0.02
7	insTT C T T	0.025	0
8	insTT C T C	0.01	0
9	insTT C C T	0.02	0
10	delTT T T C	0.02	0
11	delTT T C T	0.011	0

DNA from buccal swabs from 80 trios was amplified using Multiple Displacement Amplification (Molecular Staging, USA; now supplied by Qiagen). The -470 ins/del, -270 and +95 (rs7749390) SNPs at *IFNGR1*, and the +874 SNP (rs2430561) at *IFNG*, were genotyped by Amplification Refractory Mutational System PCR using primers and probes as described.10,13 Restriction fragment length polymorphisms were used to genotype the *IFNGR1* -56 T/C SNP (rs2234711) and the *IFNG* -179 G/A SNP (rs2069709). PCR primers were: *IFNG* -179 forward 5′-ATC AAT GTG CTT TGT GAA TGA A-3′ and reverse 5′-CCG AGA GAA TTA AGC CAA AGA -3′ ; *IFNGR1* -56 forward 5′ - TCA GGC TCC AAG ACA ACC AGG -3′ and reverse 5′-CCA CGG AGC CCC AGT CTC G-3′. PCR products digested with *Ava11* (*IFNG* −179) and Bts1 (*IFNGR1* −56) were visualised on 1.5% agarose gels. Tests for deviation from Hardy Weinberg equilibrium (HWE) for genotype data, and LD (D' values range 0 for no LD to 1 for full LD) between markers, were performed within STATA v8.0 (Electronic Database Information 1) using the GenAssoc package available from (Electronic Database Information 2). Haplotype probabilities were estimated on the basis of maximum likelihood calculations performed within the TRANSMIT program (Electronic Database Information 3), using data from the Masalit families with PKDL from Sudan, and as previously reported10 from Mandinka families with severe malaria from Gambia. Only haplotypes with an estimated probability of >1% were reported for the Mandinka. Markers are shown in chromosomal order (i.e. *IFNGR1* -470, -270, -56 and +95).

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Table 2
Haplotype association testing using TRANSMIT

Haplotype groups	*IFNGR1 polymorphisms*			
	-470ins/del	-270 T/C	-56 T/C	+95 T/C
4 markers	χ^2 = 21.97; df=10; **p=0.015**			
3 markers	χ^2 = 8.34; df=5; p=0.14			
3 markers		χ^2 = 16.86; df=7; **p=0.018**		
2 markers	χ^2 = 8.30; df=2; **p=0.016**			
2 markers		χ^2 = 6.62; df=3; p=0.085		
2 markers			χ^2 = 10.58; df=3; **p=0.014**	
1 marker	χ^2 = 0.94; df=1; p=0.33	χ^2 = 6.57; df=1; **p=0.010**	χ^2 = 0.01; df=1; p=0.91	χ^2 = 0.27; df=1; p=0.60

Family based transmission disequilibrium tests for association were performed using TRANSMIT (available at Electronic Database Information 3) which allows for missing parental data and the analysis of extended haplotypes. Global χ^2 statistics ar shown for associations between single markers or all possible *IFNGR1* haplotypes for the 4 polymorphisms -470, -270, -56 and +95 and PKDL in the Masalit ethnic group in Sudan.