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ARTICLE *in* PUBLIC HEALTH GENOMICS · AUGUST 2010

Impact Factor: 2.21 · DOI: 10.1159/000272457 · Source: PubMed

Original Paper

Public Health Genomics

Public Health Genomics 2010;13:362–367
DOI: 10.1159/000272457

Received: February 2, 2009
Accepted after revision: August 4, 2009
Published online: December 29, 2009

Interleukin 10 Gene Polymorphisms and Development of Post Kala-Azar Dermal Leishmaniasis in a Selected Sudanese Population

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Key Words
Genetic association · Interleukin 10 · Leishmaniasis · Post kala-azar dermal leishmaniasis

Abstract
Background: Post kala-azar dermal leishmaniasis (PKDL) is a cutaneous form of disease that develops at variable times after individuals have received treatment for clinical visceral leishmaniasis (VL). The study aimed to investigate the possible role of interleukin 10 (IL-10) and development of PKDL. ***Methods:*** 77 families composed of 41 complete case-parent trios and 36 case-parent pairs from the Masalit ethnic group were genotyped for 3 *IL10* promoter polymorphisms: -1082A/G, -819C/T and -592C/A. ***Results:*** Single point analysis using the transmission disequilibrium test showed no evidence of association between any of these *IL10* promoter single nucleotide polymorphisms (SNPs) and development of PKDL. Haplotype analysis performed using TRANSMIT showed borderline significance between PKDL and the haplotype AA across -592C/A and -1082A/G (p = 0.053). Haplotypes GCC (0.33) and ATA (0.30) were the common haplotypes in this Sudanese population. Allele frequencies for the 3 SNPs differed significantly in Sudan compared to other African (Gambian, Malawian, YRI) populations. ***Conclusion:*** There is no evidence for an association between 3 SNPs in the *IL10* gene promoter and susceptibility to PKDL in the Masalit ethnic group in Sudan, although some evidence for haplotype association was observed.

In Sudan post kala-azar dermal leishmaniasis (PKDL) is a known complication of visceral leishmaniasis (VL) caused by *Leishmania donovani* and occurs in some patients after treatment and cure of VL. PKDL occurs with a frequency of 56–62%, usually after a latent phase from months to years [1, 2]. Clinical characterization of the disease was previously described [3]. Experimental studies indicate that interleukin 10 (IL-10) plays an important regulatory role in the progression of VL. IL-10 was the most prominent cytokine in PKDL lesions [4] with high levels also observed in plasma [5]. However, interferon-γ (IFN-γ) was also seen in all lesions [4] and was detected in keratinocytes and/or sweat glands of patients who developed PKDL. In response to leishmanial antigens, peripheral blood mononuclear cells from most Sudanese PKDL patients proliferate and produce IFN-γ and IL-10 [4, 5]. Silva et al. (1992) postulated that the presence of IL-10 in the skin lesions could block the action of IFN-γ,

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rather than inhibit its production [6]. In mice, IL-10 was found to play an important role in parasite persistence as sterile cure was detected in IL-10 deficient mice on a resistant genetic background [7]. Recently IL-10 was found to be strongly associated with development of ulcerated skin lesions in *L. braziliensis* infection in humans, supported by data showing genetic associations for single nucleotide polymorphisms (SNPs) across the *IL10* gene and evidence that the -819C/T SNP in the *IL10* promoter plays a functional role in determining binding of nuclear transcription factors and levels of *IL10* gene expression [8].

In the present study we investigated the *IL10* promoter polymorphisms -1082A/G, -819C/T, and -592C/A. The importance of *IL10* three selected polymorphisms has been documented in several disease processes by independent studies. The -592C/A SNP lies within negative regulatory elements in the promoter [9]. It has been shown that the -592C/A polymorphism is associated with diminished IL10 production [10]. Different studies reported the functional role of -1082A/G polymorphisms. Turner et al. found that lymphocytes with the GG genotype responded with significantly enhanced IL-10 secretion compared to those with the AA genotype [11]. Another study showed high IL-10 plasma levels was associated with the -1082A allele in the Dutch population [12]. The -1082A allele was able to confer an increase in transcriptional activity of the *IL-10* promoter [13]. The study presented here looked for association between these SNPs and the development of PKDL.

Material and Methods

Ethical approval for this study was obtained from the Institute of Endemic Diseases, University of Khartoum Ethical Committee. The study was conducted in the region of the Rahad River in Eastern Sudan where VL and PKDL are endemic. Samples were collected from individuals belonging to the Masalit tribe who are highly susceptible to VL and PKDL [14–16]. Trios with PKDL were ascertained from epidemiological and medical records of the Institute of Endemic Diseases. Diagnosis was made on the basis of clinical, parasitological and serological criteria as described previously [14, 17]. Epidemiological and demographic details relating to the study site are also described in detail elsewhere [14]. DNA was extracted from buccal swap samples and successfully genotyped for 77 families that composed of 41 complete case-parent trios and 36 case-parent pairs (i.e. one parent missing). Amplification refractory mutation system (ARMS-PCR) was employed to genotype the -1082A/G (rs1800896) and -819C/T (rs1800871) using primers previously described [18] with specific sequence for both alleles, wild and mutant type and common primer. The -1082A/G primers were: common 5′-cagtgccaactgagaatttgg-3′; mutant type 5′-ctactaaggcttctttgggag-3′; and wild type 5′-actactaaggcttctttgggaa-3′ The -819C/T primers were: common 5′-aggatgtgttccaggctcct-3′; mutant type 5′-acccttgtacaggtgatgtaat-3′; and wild type 5′-cccttgtacaggtgatgtaac-3′. Human growth factor hormone primers were included in each PCR reaction as internal control: forward primer 5′-gccttcccaaccattccctta-3′ and reverse primer 5′-tcacggatttctgttgttttc-3′. ARMS PCR to detect the 2 polymorphisms (-819C/T) and (-1082A/G) was carried out in a total volume of 10.5 μl composed of; 1× Bioline PCR reaction buffer, 2 mM $MgCl_2$, 10 mM dNTPs, 1 unit Taq polymerase, 10 μM of each oligonucleotide, 0.5 μM of internal control primers and 5 μl of dd H_2O.

A touch down program was used as follows: denaturation at 96°C for 1 min, 9 cycles contain denaturation at 96°C for 35 s, annealing at 63°C for 45 s and elongation at 72°C for 35 s, 27 cycles of denaturation at 96°C for 25 s, annealing at 59°C for 50 s and elongation at 72°C for 40 s. Finally 9 cycles comprising denaturation at 72°C for 5 min, annealing at 55°C for 1 min and elongation at 72°C for 90 s were carried out. The PCR product was run in 2% agarose gel stained with ethidium bromide, visualized and photographed by gel documentation system. Genotype scoring was based on the presence or absence of target bands.

A restriction fragment length polymorphism (PCR-RFLP) was used to genotype the -592C/A (rs1800872) SNP, using forward primer 5′-ataaaatagagacggtaggg-3′ and reverse primer 5′-ggctaaatatcctcaaagtt-3′. The PCR reaction was carried out in a total volume 21.2 μl composed of 1× Bioline PCR reaction buffer, 2 mM $MgCl_2$, 10 mM dNTPs, 1 unit Taq polymerase, 10 μM of each primers and 8.3 μl of dd H_2O. The PCR conditions were: denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 30 s, elongation at 72°C for 45 s and final extension at 72°C for 10 min. The PCR product was digested by *Rsa*I restriction enzyme as for 3 h at 37°C. The digested products were run in 2.5% agarose gel, stained with bromide, visualized and photographed by gel documentation system.

Deviation from Hardy-Weinberg Equilibrium (HWE) was determined using a set of unrelated individuals from the families (parents), excluding any with PKDL. Tests for HWE were performed within STATA 9.1 (http://www.stata.com/) using the GenAssoc package (http://www-gene.cimr.cam.ac.uk/clayton/software/stata/). Tests for linkage disequilibrium (LD) between SNPs were performed using Haploview v3.32 (www.broad.mit.edu/mpg/haploview/). Single point and haplotype association tests (haplotype-based score tests) were performed using the transmission disequilibrium test (TDT) in the program TRANSMIT (http://www-gene.cimr.cam.ac.uk/clayton/software/) which takes account of missing parental genotype data. Genotype and allele frequencies in our study population were compared with other African groups, Mandinka, Wolf and Jola groups from Gambia [19], Bantu from Karonga district in Malawian [20], and the YRI HapMap data.

Results

Representative genotyping data for all 3 SNPs are shown in figures 1–3. For the -592C/A SNP, a PCR product of 269 bp is obtained (fig. 1). Digestion with *Rsa*I restriction enzyme results in 2 bands for the mutant allele

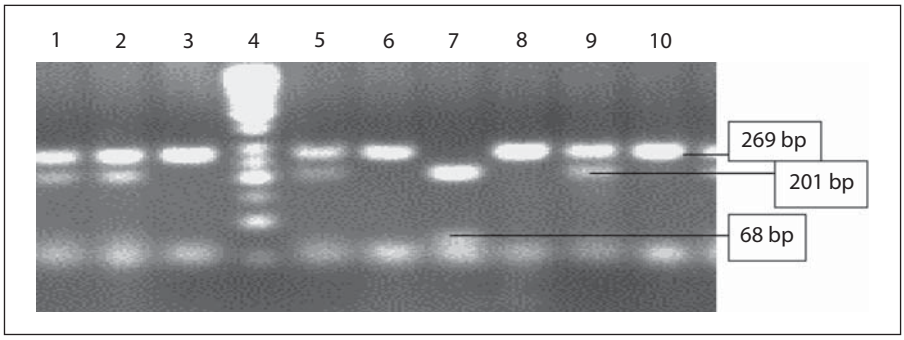

Fig. 1. -592C/A genotyping using PCR-RLFP. Lanes 1, 2, 5, 9: heterozygous; lanes 3, 6, 8, 10: homozygous for wild type allele (269 bp); lane 7: homozygous for mutant allele (201 bp and 68 bp). Lane 4 contains the 50 bp DNA molecular weight marker.

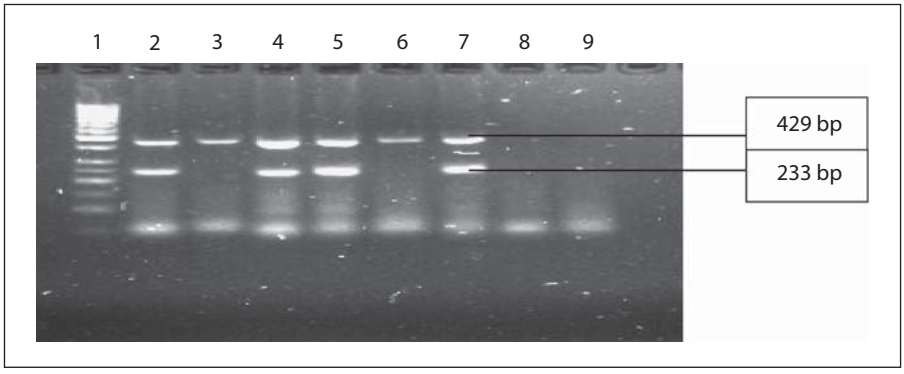

Fig. 2. -819C/T genotyping using ARMS-PCR. Lane 1: 50 bp DNA molecular weight marker. Lanes 2, 4, 6 and 8: allele C is represented by the presence of a 233 bp PCR fragment. Lanes 3, 5, 6 and 9: allele T is represented by the presence of a 233 bp PCR fragment. Each two lanes represent one sample: lanes 2 and 3, homozygous (CC); lanes 4 and 5, heterozygous (CT); lanes 6 and 7, homozygous (TT); lanes 8 and 9, negative controls. Lanes 2–7: a 429 bp represents the amplified product of the Human Growth Factor Hormone gene (internal control).

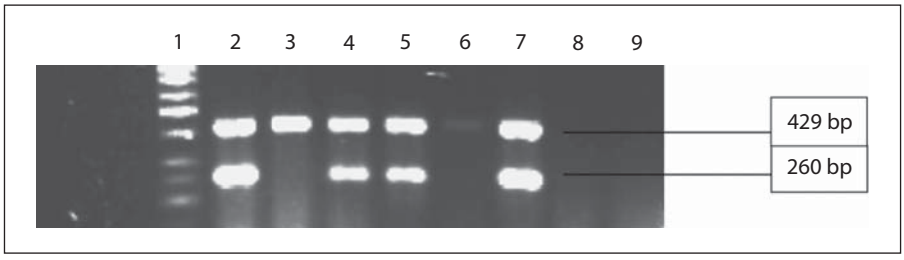

Fig. 3. -1082A/G genotyping using ARMS-PCR. Lane 1: 50 bp DNA molecular weight marker. Lanes 2, 4, 6 and 8: allele A is represented by the presence of a 260 bp PCR fragment. Lanes 3, 5, 6 and 9: allele G is represented by the presence of a 260 bp PCR fragment. Each two lanes represent one sample: lanes 2 and 3, homozygous (AA); lanes 4 and 5, heterozygous (AG); lanes 6 and 7, homozygous (GG); lanes 8 and 9, negative controls. Lanes 2–7, a 429 bp represents the amplified product of the Human Growth Factor Hormone gene (internal control).

equivalent to 201 bp and 68 bp, while the normal allele remains uncut (269 bp). The ARMS-PCR size bands from of *IL10* -819C/T and *IL10* -1082A/G were 233 bp and 260 bp respectively, while the internal control results in 429 bp (fig. 2, 3). All genotypes of the target *IL10* polymorphisms were in Hardy-Weinberg equilibrium ($p > 0.05$). Frequencies for -1082A, -819C and -592C alleles in our study population were 48%, 47% and 50%, respectively. There was no evidence for strong LD between different SNP pairs (D' range 0.47–0.63; r^2 range 0.20–0.35).

The single point TDT carried out in TRANSMIT showed no evidence of association between promoter SNPs -1082A/G, -819C/T and -592C/A and development of PKDL $p > 0.05$ (table 1). The haplotype-based score test in TRANSMIT showed borderline significance ($p = 0.05$) for association between PKDL and the over-transmitted haplotype AA across the -1082A/G and -592C/A SNPs (table 1; global $\chi^2 = 7.69$; df = 3; $p = 0.05$). In this population in Sudan, the GCC (33%) and ATA (30%) haplotypes were more frequent than other haplotypes across the 3 promoter SNPs (fig. 4). Whereas this Sudan population differed in allele frequency at the -1082A/G SNP compared to Gambian [21], Malawian [20] and the HapMap YRI population (fig. 5), for the -819C/T and -592C/A SNPs it was the Malawian population that was at variance with Sudan and the other African populations.

Discussion

VL is an important health problem in the Sudan, but remains largely a neglected disease. There is neither effective drug nor vaccines available. PKDL patients were suspected to be a source for VL infection in Sudan. The

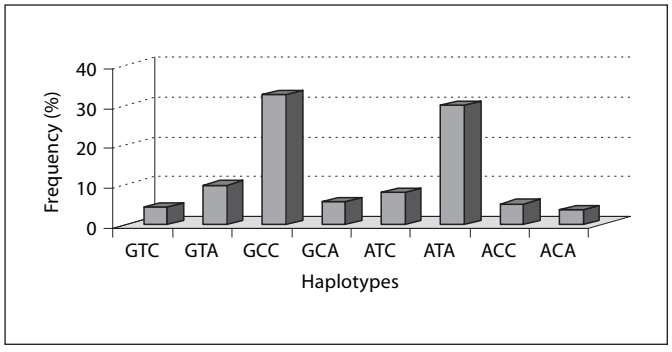

Fig. 4. Haplotype frequencies (-1082A/G, -819C/T and -592C/A) for the *IL10* gene polymorphisms in Sudan.

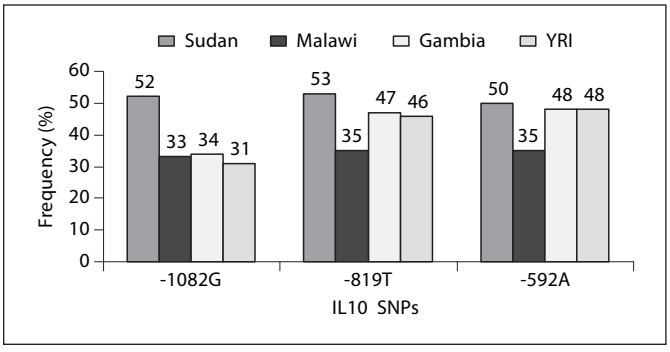

Fig. 5. Allele frequencies for the -1082G, -819T and -592A alleles in different African populations including the HapMap YRI population.

Table 1. Results of association analyses

Haplotype groups	*IL10* polymorphisms		
	-1082A/G	-819C/T	-592C/A
3 markers	$\chi^2 = 11.18$; df = 7; p = 0.13		
2 markers	$\chi^2 = 0.85$; df = 3; p = 0.84		
2 markers		$\chi^2 = 3.09$; df = 3; p = 0.38	
2 markers	$\chi^2 = 7.69$; df = 3; p = **0.05**		$\chi^2 = 7.69$; df = 3; p = **0.05**
1 marker	$\chi^2 = 0.23$; df = 1; p = 0.63	$\chi^2 = 0.24$; df = 1; p = 0.62	$\chi^2 = 28$; df = 1; p = 0.60

Grey shading indicates the combinations of markers used in the haplotypes tested.

factors that determine development or not of PKDL are not well understood, but a *Leishmania*-specific cellular immune response seems to play a fundamental role in the final control of infection. This study may help to identify provide biomarkers for future disease surveillance and indicate possible avenues for immunotherapeutic intervention in clinical disease.

IL-10 and IFN-γ mRNA are observed concurrently in lesions from patients with post-kala-azar dermal leishmaniasis (PKDL) that indicates the production of IL-10 during *L. donovani* infection, and suggests a role for this cytokine in the regulation of immune responsiveness during VL [22]. In spite of the role of IL-10 in parasite persistence and its expected function in disease promotion, there were no single point associations between the 3 target polymorphisms and development of PKDL in the Masalit population studied here. Borderline association (global $\chi^2 = 7.69$; df = 3; nominal p = 0.05) was observed between PKDL and haplotypes for the 2 outer markers -1082A/G and -592C/A. This could indicate that other functional SNPs in this region could be functional variants for an association with PKDL, or that there is a complex association that relies on interaction between different functional SNPs across the region. However, in the current study this association is not robust to correction for multiple testing and a larger sample size would be needed to confirm such an association. In a recent study of cutaneous leishmaniasis in Brazil, Salhi et al. found that production of IL-10 was strongly associated with development of ulcerated skin lesions in *L. braziliensis* infection [8]. Along with several SNPs within the *IL10* gene, they found single point associations with promoter SNPs -819C/T and -592C/A that were robust to correction for multiple testing, but not with promoter SNP -1082A/G. Functional studies demonstrated that the -819C/T SNP in the *IL10* promoter plays a functional role in determining binding of nuclear transcription factors and levels of *IL10* gene expression. In other studies the -1082G allele has been shown to be associ-

ated with higher IL-10 levels than the -1082A allele [11], but this could relate to LD across the region with other functional SNPs. In the Masalit tribe LD was not strong across the 3 SNPs, in contrast to data from Spanish, Caucasian and Asians where high LD was detected. Complete LD between -592C/A and -819C/T have been observed in Asians [23, 24]. Interestingly our study population showed higher frequency of the -1082G allele than other African groups. In our study there was no measurement for cytokines to compare different genotypes with IL-10 level.

The 2 most frequent haplotypes in the Masalit population were GCC and ATA (fig. 4). Although not statistically significant, the ATA haplotype was over-transmitted to PKDL patients in our study population. In previous studies, the ATA haplotype was found to be associated with high plasma levels of IL-10 in Finish population [25, 26]. In another study a slight increase in *IL10* transcriptional activity of the GCC haplotypes was observed [27]. The presence of other haplotypes, even in lower frequency, may be effective to differentiate between populations. We found the GTA haplotype to be 10% in Masalit which was previously described in Caucasian and Brazilian with 1% frequency [28]. The haplotype GTC, ATC and ACA were found in Masalit with frequencies 4.3%, 8% and 6% respectively, while the same haplotypes were respectively 1%, 4% and 1% in Caucasian and were absent in Brazilians. In contrast the ACC frequency, however, was 5% in Masalit and higher in Caucasian 29% [28] and Japanese 28% [29]. In this study the presence of rare haplotypes, in our population may indicate genetic heterogeneity of functional importance.

In conclusion there is no single point evidence for an association between 3 SNPs in the *IL10* gene promoter and susceptibility to PKDL in the Masalit ethnic group in Sudan, although some evidence for haplotype association was observed. High frequencies of the previously identified high IL10 producing haplotypes GCC and ATA were present in the Masalit. Further work using larger samples size and a wider range of SNPs may help to elucidate the role of IL-10 in PKDL development.

Acknowledgments

We thank the people from Um Salala, El-Rugab and Ali Babiker for their participation in this work. This work was funded by The Wellcome Trust.

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Erratum

In the article by De Marco 'Views on Personalized Medicine: Do the Attitudes of African American and White Prescription Drug Consumers Differ?' (Public Health Genomics 2010;13:276–283) the co-authors have been erroneously left out. Below all authors are listed:

Molly De Marco [a] Sam Cykert [b] Nettie Coad [c] Kay Doost [c] Jennifer Schaal [c] Brandolyn White [d] Deborah Young [d] Malika Roman Isler [a] Giselle Corbie-Smith [e]

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